Genetic Differentiation of the Wheat Leaf Rust Fungus *Puccinia triticina* in Pakistan and Genetic Relationship to Other Worldwide Populations

J. A. Kolmer, J. I. Mirza, M. Imtiaz, and S. J. A. Shah

First author: United States Department of Agriculture–Agricultural Research Service, Cereal Disease Laboratory, St. Paul, MN 55108; second author: Pakistan Agricultural Research Council, Crop Disease Institute, Sunny Bank Muree, Pakistan; third author: International Maize and Wheat Improvement Center, CSI Building, NARC, Park Road, Islamabad 44000, Pakistan; and fourth author: Nuclear Institute for Food and Agriculture, Plant Protection Division, G. T. Road, Peshawar, Pakistan.

Accepted for publication 16 February 2017.

ABSTRACT

Collections of *Puccinia triticina*, the wheat leaf rust pathogen, were obtained from Pakistan in 2008, 2010, 2011, 2013, and 2014. Collections were also obtained from Bhutan in 2013. Single uredinial isolates were derived and tested for virulence phenotype to 20 lines of Thatcher wheat that differ for single leaf rust resistance genes, and for molecular genotype with 23 simple-sequence repeat (SSR) primers. Twenty-four virulence phenotypes were described among the 89 isolates tested for virulence. None of the isolates had virulence to Thatcher lines with *Lr9*, *Lr24*, or *Lr18*. Virulence to most of the other Thatcher lines was over 50%. The two most common virulence phenotypes, FHPSQ and KHPQQ, had virulence to

Lr16, *Lr17*, and *Lr26*. Twenty-seven SSR genotypes were found among the 38 isolates tested for molecular variation. The SSR genotypes had high levels of observed heterozygosity and significant correlation with virulence phenotype, which indicated clonal reproduction. Cluster analysis and principal component plots indicated three groups of SSR genotypes that also varied significantly for virulence. Isolates with MBDSS and MCDSS virulence phenotypes from Pakistan and Bhutan were highly related for SSR genotype and virulence to isolates from Turkey, Europe, Central Asia, the Middle East, North America and South America, indicating the possible migration of the rust fungus between continental regions.

Leaf rust, caused by *Puccinia triticina* Erikss., is an important disease of wheat (*Triticum aestivum* L.) on a worldwide basis (Roelfs et al. 1992). The leaf rust disease can be most severe in continental areas such as the Great Plains of North America (Kolmer and Hughes 2013) and South Asia (Nagarajan and Joshi 1985), where wheat matures in warm to hot summer temperatures. Worldwide populations of the leaf rust fungus are highly variable, with large numbers of races or virulence phenotypes in addition to a high diversity of molecular variation (Ordoñez and Kolmer 2009). The high degree of virulence variation in *P. triticina* has made breeding for stable and durable leaf rust resistance in wheat difficult to achieve (Kolmer 2013).

In Pakistan, leaf rust occurs on an annual basis throughout the wheat-growing regions of the country (Saari and Prescott 1985), causing regular yield losses. Leaf rust can survive on wheat during the summer in the mountainous western region and then spreads to the wheat-growing regions of the Indus basin in Sindh and Punjab provinces (Nagarajan and Joshi 1985). Susceptible alternate hosts for *P. triticina* are not known to be present in Pakistan; therefore, for survival and dispersal from year to year, it is dependent on the clonal urediniospore stage. Leaf rust is found throughout the wheat-growing areas of the country, and is most common in the southern and central regions. In 1978, a severe leaf rust epidemic caused a nationwide 10% yield loss (Hussain et al. 1980). Pakistan is the world's eighth leading wheat-producing country, with 25 million metric tons harvested annually. White-seeded spring wheat cultivars, often selected from advanced lines in the International Maize and

Wheat Improvement Center breeding programs, are grown in Pakistan (CIMMYT 2016).

Urediniospores of cereal rust fungi can be spread over thousands of kilometers. In North America, urediniospores of *P. triticina* are carried from the southern winter wheat region to the northern spring wheat region by southerly winds in the spring and early summer. The same virulence phenotypes can be found in the southern and northern wheat production areas (Kolmer and Hughes 2015). Numerous phenotypes of *P. triticina* and *P. striiformis*, the wheat stripe rust fungus, from foreign sources have been introduced to Australia and South Africa (Kolmer 2005). New phenotypes of *P. striiformis* have recently been introduced to Australia, North America, and Europe (Hovmøller et al. 2011, Hovmøller et al. 2016). In the 1990s, *P. striiformis* races with virulence to *Yr9* moved out of East Africa across the Arabian peninsula and into south Asia, including Pakistan (Singh et al. 2004). The introduction and establishment of introduced virulence phenotypes may also affect populations of *P. triticina* worldwide. Kolmer (2001) hypothesized that a group of *P. triticina* phenotypes with virulence to leaf rust resistance gene *Lr17* was introduced to the United States and Canada in the mid-1990s. In the current study, collections of *P. triticina* from Pakistan and Bhutan were characterized for virulence using a standard set of host differential lines and also for variation at simple-sequence repeat (SSR) loci with a standard set of primer pairs. The objectives were to characterize the overall levels of virulence and molecular diversity and to determine whether distinct groups of isolates based on virulence phenotype and SSR genotype were present in Pakistan and Bhutan. The isolates from Pakistan and Bhutan were also compared for virulence and molecular genotype with a worldwide collection of *P. triticina* in order to determine their relatedness to *P. triticina* groups in other continental regions and to provide further insight on the movement and diversity of this important wheat pathogen.

MATERIALS AND METHODS

Collections of *P. triticina* were obtained from common wheat in research plots and fields in Pakistan in 2008, 2010, 2011, 2013, and

Corresponding author: J. A. Kolmer; E-mail address: Jim.Kolmer@ars.usda.gov

*The e-Xtra logo stands for “electronic extra” and indicates that one supplementary table is published online.

This article is in the public domain and not copyrightable. It may be freely reprinted with customary crediting of the source. The American Phytopathological Society, 2017.

2014. In 2008, 2010, and 2011, *P. tritricina* uredinia were isolated from collections that had been made for *P. graminis* f. sp. *tritici*, the wheat stem rust fungus. *P. tritricina* was obtained from two collections in 2008, four collections in 2010, and seven collections from three locations in 2011. In 2013, collections of *P. tritricina* were made from 17 locations in Punjab, Sindh, Khyber Pakhtunkhwa, Azad Jammu, and Kashmir provinces. In 2014, seven collections were made from Punjab, Azad Jammu, and Sindh provinces. In 2013, seven collections of *P. tritricina* were obtained from Bhutan.

Urediniospores from the collections were vacuumed into size 00 gel capsules, using small cyclone spore collectors (Browder 1971). The gel capsules were filled with 0.25 ml of Soltrol (Philips Petroleum) nonphytotoxic paraffinic oil; then, the oil-spore mixture was atomized onto a single small pot with 10 to 15 seedlings (7 to 8 days old) of 'Little Club' wheat. The Little Club seedlings had been treated with a solution of maleic hydrazide (approximately 30 ml/pot of a solution of 1 g per 3.0 liters) at coleoptile emergence. Isolation and increase of two to three single uredinial isolates from each collection from the inoculated Little Club seedlings were as previously described (Kolmer and Hughes 2015) for collections of *P. tritricina* in the annual U.S. virulence survey. Urediniospores of each single-uredinial isolate were inoculated to a 7- to 8-day-old differential set of 20 lines of 'Thatcher' wheat that are near-isogenic for leaf rust resistance genes. Five sets of four differentials were used. Set 1 was lines with genes *Lr1* (isogenic line RL6003), *Lr2a* (RL6000), *Lr2c* (RL6047), and *Lr3* (RL6002); set 2 was *Lr9* (RL6010), *Lr16* (RL6005), *Lr24* (RL 6064), and *Lr26* (6078); set 3 was *Lr3ka* (RL6007), *Lr11* (RL6053), *Lr17* (RL6008), and *Lr30* (RL6049); set 4 was *LrB* (RL6047), *Lr10* (RL6004), *Lr14a* (RL6013), and *Lr18* (RL6009); and set 5 was *Lr3bg* (RL6042), *Lr14b* (RL6006), *Lr20* (RL6092), and *Lr28* (RL6079). Thatcher was included as a susceptible control. Infection types 0 to 2+ (immune response to moderate uredinia with necrosis or chlorosis) were classified as avirulent and infection types 3 to 4 (moderate to large uredinia without chlorosis or necrosis) were classified as virulent. Each isolate was assigned a five-letter designation based on the hexadecimal code adapted for virulence nomenclature of *P. tritricina* in North America (Long and Kolmer 1989). In total, 89 single uredinial isolates were tested for virulence phenotype.

DNA was extracted from the single uredinial isolates using the OmniPrep (G-BioSciences) extraction kit. At least two isolates of each virulence phenotype were tested for molecular genotype. Isolates with the same phenotype from different locations in the same year were also tested. Twenty-three microsatellite pairs (Ordóñez and Kolmer 2009) developed from genomic libraries (Duan et al. 2003; Szabo and Kolmer 2007) were used to determine the molecular genotypes of 43 isolates. Polymerase chain reaction (PCR) amplifications were carried out as previously described (Szabo and Kolmer 2007). Allele sizes in base pairs were scored visually for each primer pair by using a LI-COR 4200 or 4300 DNA sequencer that was calibrated with IRDye 700 molecular weight size standards. DNA bands generated by each primer pair were standardized with other *P. tritricina* isolates previously characterized using the same set of SSR primers.

Isolate genotypes were determined by using molecular weights of alleles at the 23 SSR loci. The data were clone corrected, such that isolates with the same SSR genotype and virulence phenotype were not considered further, leaving 38 isolates for analysis. GENODIVE v2.021b (Meirmans and van Tienderen 2004) was used to determine the number of SSR genotypes total. The SSR genotypes were examined for similarity using a two-dimensional principal coordinate (PCoA) plot in GENALEX 6.502 (Peakall and Smouse 2006, 2012). Genetic distance was calculated in GENALEX for codominant data between each pair of isolates in an approach based on allelic dosage, as described by Smouse and Peakall (1999).

The number of SSR groups was determined by clustering of genotypes in the PCoA plots. The number of SSR groups was also determined using k-means clustering implemented in GENODIVE. Euclidean distances were calculated from within individuals allele frequencies, with simulated annealing using 60,000 steps and 60,000

random starts. The number of groups was based on the Calinski and Harabasz pseudo *F* statistic. SSR genotype groups were characterized by the average number of alleles per locus, average effective number of alleles per locus, and Shannon's gene diversity, as determined with GENALEX. Observed and expected values of gene diversity and inbreeding values were also calculated in GENALEX. Nei's corrected genetic diversity was calculated using GENODIVE and compared with diversity expected under random mating with 999 randomizations of the original dataset. Genetic differentiation of SSR genotypes between groups was calculated via the analysis of molecular variance (AMOVA) in GENALEX with 999 permutations of the data set using F_{ST} .

Each isolate was designated with a 20-digit binary number based on virulence or avirulence. Differentiation of virulence phenotypes between SSR groups was calculated in GENALEX via AMOVA with Φ_{PT} , an analogous measure used for haploids. Significant differences in frequency of virulence to differential lines in the SSR groups was determined with Fisher's exact test (Steel and Torrie 1980). The SSR genotypes and virulence phenotypes were tested for correlation using a Mantel test in GENALEX.

The SSR genotypes of the 38 isolates from Pakistan and Bhutan were compared with genotypes of isolates in a worldwide collection of 831 *P. tritricina* isolates (J. Kolmer, unpublished data) from North America, South America, Europe, the Middle East, Russia, China, Central Asia, South Africa, and New Zealand. SSR groups from Pakistan were compared with 35 worldwide groups using Nei's genetic distance and for genetic differentiation with F_{ST} in GENALEX.

RESULTS

In total, 24 virulence phenotypes were found among the collections from Bhutan in 2013 and from Pakistan from 2008 to 2014 (Table 1). Phenotype TCJSS, with virulence to *Lr1*, *Lr2a*, *Lr2c*, *Lr3*, *Lr26*, *Lr11*, *Lr17*, *LrB*, *Lr10*, *Lr14a*, *Lr3bg*, *Lr14b*, and *Lr20*, comprised 8 of the 13 isolates from Bhutan in 2013. FHPSQ, with virulence to *Lr2c*, *Lr3*, *Lr16*, *Lr26*, *Lr3ka*, *Lr17*, *Lr30*, *LrB*, *Lr10*, *Lr14a*, *Lr3b*, and *Lr14b*, was the most common phenotype over the 5 years in Pakistan at 23.6%, and was the most common phenotype in 2013 at 51.3%. KHPQQ, with virulence to *Lr2a*, *Lr2c*, *Lr3*, *Lr16*, *Lr26*, *Lr3ka*, *Lr17*, *Lr30*, *LrB*, *Lr10*, *Lr3bg*, and *Lr14b*, the second most common phenotype overall at 12.4%, was the only phenotype identified in 2008 and was 50% of isolates in 2010. PBMQQ, with virulence to *Lr1*, *Lr2c*, *Lr3*, *Lr3ka*, *Lr30*, *LrB*, *Lr10*, *Lr3bg*, and *Lr14b*, the third most common phenotype at 11.2% overall, was at 23.1% of isolates in 2013 and was identified as one isolate in 2011. No other phenotypes were found at >10% frequency overall. Many of the phenotypes were found only one time in a single year. Only five virulence phenotypes were found in more than 1 year. None of the phenotypes had virulence to Thatcher lines with *Lr9*, *Lr18*, or *Lr24* (Supplementary Table S1). Virulence to lines with *Lr11* and *Lr28* occurred in <10% of isolates overall. Less than 50% of isolates had virulence to lines with *Lr2a* and *Lr20*. Virulence to all other differential lines was >50%.

Thirty-eight isolates representing 23 virulence phenotypes were genotyped at 23 SSR loci. The single isolate with the KCMQQ phenotype was not genotyped due to poor amplification of primer products. In total, 27 SSR genotypes were described among 38 tested isolates (Table 2). The SSR genotypes were placed into three clusters based on the pseudo *F* statistic in the k-means clustering procedure. The six genotypes from Bhutan clustered strongly with those from Pakistan and were grouped with Pakistan genotypes. There were 17 isolates in Pakistan group 1 (PK-1), with 10 SSR genotypes and eight virulence phenotypes. PK-2 had 12 isolates with eight SSR genotypes and 12 virulence phenotypes. PK-3 had nine isolates, each with a different SSR genotype, and three virulence phenotypes. In PK-2, there were five isolates with phenotypes MBDSS or MCDSS. In PK-3, there were six SSR genotypes with phenotype FHPSQ. The SSR genotypes also fell into three discrete groups based on the PCoA plot (Fig. 1A). One isolate (13PAK401-MBMSQ) did not cluster strongly

with any group in the PCoA plot and was placed in PK-1 based on the k-means clustering. The three PK SSR groups were significantly differentiated, with an overall F_{ST} of 0.27 at $P < 0.001$. All three pairs of PK groups were significantly differentiated at $P < 0.001$.

The mean number of SSR alleles at each locus in the three groups varied between 2.17 and 2.48 in the three groups, with an overall mean of 3.74 (Table 3). The mean effective number of alleles per locus within populations varied between 1.71 and 2.02, with an overall mean of 2.41. PK-3 had the lowest Shannon gene diversity at 0.49 and PK-2 had the highest gene diversity at 0.74, with an overall mean of 0.86. All three PK groups had significantly higher observed gene heterozygosity compared with expected heterozygosity, with significant negative values of F . The overall Nei's genotypic SSR diversity of 0.947 differed significantly ($P = 0.001$) from the expected diversity under random mating. The high levels of heterozygosity and deviation from random mating were indicators of clonal reproduction.

The *P. tritricina* phenotypes in the three PK SSR groups differed significantly for virulence to Thatcher wheat lines with *Lr2c*, *Lr16*, *Lr26*, *Lr3ka*, *Lr11*, *Lr17*, *Lr30*, *Lr3bg*, *Lr20*, and *Lr28* (Table 4). The overall Φ_{PT} for virulence variation between the PK SSR groups was 0.320, with $P < 0.001$. All three pairs of PK groups were significantly differentiated ($P < 0.001$). The Mantel correlation between virulence phenotype and SSR genotype was 0.42 ($P < 0.001$). The general relationship between virulence and SSR genotype was an additional indicator of clonal reproduction.

The three PK groups were tested for F_{ST} differentiation of SSR genotypes with a worldwide collection of *P. tritricina* genotype groups (Kolmer and Ordoñez 2007; Kolmer et al. 2011, 2012, 2015; Ordoñez and Kolmer 2009; Ordoñez et al. 2010). Isolates in PK-1 were not differentiated for SSR genotype from isolates in European group 7 (EU-7) ($P < 0.05$) that were mostly F— phenotypes from Turkey, Germany, Italy, Spain, and the United Kingdom. Isolates in PK-2 were not differentiated from isolates in Middle East group 1 (ME-1), EU-8, North American group 3 (NA-3), and South American group 3 (SA-3) ($P < 0.05$). Isolates in PK-3 were not differentiated from isolates in EU-2 ($P < 0.05$) that were mostly F— and P— phenotypes from Turkey. Isolate 10PAK3.1-PBPSQ in PK-1 had an SSR genotype identical to isolates ISR-4015-8-MBMQ from Israel and

TABLE 2. Simple-sequence repeat (SSR) group and SSR genotype designations of isolates of *Puccinia tritricina* from Bhutan and Pakistan

Isolate	Group	Genotype
13PAK17-1-CCPSL	1	1
10PAK4.1-KHPQQ	1	2
11PAK31.1-KHMQQ	1	3
10PAK3.1-PBPSQ	1	4
10PAK1.3-PGPSS	1	5
13PAK4-1-MBMSQ	1	17
BHU9-1-PCBSQ	1	22
BHU9-3-PCBSQ	1	23
10PAK5.1-KHPQQ	1	24
11PAK30.3-KHMQQ	1	24
10PAK1.2-PGPSS	1	25
10PAK3.2-FCPSS	1	25
10PAK3.3-PBPSQ	1	25
13PAK39-1-PBPQQ	1	25
13PAK49-1-PBMQQ	1	25
13PAK74-2-PGMSL	1	25
13PAK9-2-PBMQQ	1	25
14PAK1-3-BBBDH	2	9
13PAK40-MBDSS	2	10
11PA1.1-TCBJK	2	11
14PAK1-4-CBBDK	2	12
11PAK1.2-FCBQQ	2	15
14PAK85-PBLQT	2	16
13PAK13-1-MBDSS	2	26
14PAK51-3-MCDSS	2	26
BHU7-MCDSS	2	26
14PAK51-1-MBNSQ	2	27
14PAK51-2-MBDSS	2	27
14PAK53-MBNSQ	2	27
13PAK74-1-PHPSQ	3	6
13PAK69-2-FHPSQ	3	7
BHU4-3-TCJSS	3	8
BHU5-FHPSQ	3	13
13PAK13-2-FHPSQ	3	14
13PAK15-1-FHPSQ	3	18
13PAK6-1-FHPSQ	3	19
BHU6-2-TCJSS	3	20
13PAK16-1FHPSQ	3	21

TABLE 1. Frequency of virulence phenotypes of isolates of *Puccinia tritricina* collected from Bhutan in 2013 and Pakistan in 2008, 2010, 2011, 2013, and 2014 on 20 lines of Thatcher wheat near isogenic for leaf rust resistance genes

		Pakistan													
		Bhutan		2008		2010		2011		2013		2014		Total	
Race	Virulence	<i>n</i>	%	<i>n</i>	%	<i>n</i>	%	<i>n</i>	%	<i>n</i>	%	<i>n</i>	%	<i>n</i>	%
BBBDH	14a,14b,28	0	0	0	0	0	0	0	0	0	0	3	25	3	3.4
CBBDK	3,14a,14b,20,28	0	0	0	0	0	0	0	0	0	0	1	8.3	1	1.1
CCPSL	3,26,3ka,17,30,B,10,14a,3bg	0	0	0	0	0	0	0	0	1	2.6	0	0	1	1.1
FCBQQ	2c,3,26,B,10,3bg,14b	0	0	0	0	0	0	1	12.5	0	0	0	0	1	1.1
FCPSS	2c,3,26,3ka,17,30,B,10,14a,3bg,14b,20	0	0	0	0	1	8.3	0	0	0	0	0	0	1	1.1
FHPSQ	2c,3,16,26,3ka,17,30,B,10,14a,3bg,14b	1	7.7	0	0	0	0	0	0	20	51.3	0	0	21	23.6
KCMQQ	2a,2c,3,26,3ka,30,B,10,3bg,14b	0	0	0	0	0	0	1	12.5	0	0	0	0	1	1.1
KHMQQ	2a,2c,3,16,26,3ka,30,B,10,3bg,14b	0	0	0	0	0	0	4	50	0	0	0	0	4	4.5
KHPQQ	2a,2c,3,16,26,3ka,17,30,B,10,3bg,14b	0	0	5	100	6	50	0	0	0	0	0	0	11	12.4
MBDSS	1,3,17,B,10,14a,3bg,14b,20	0	0	0	0	0	0	0	0	3	7.7	3	25	6	6.7
MBMSQ	1,3,3ka,30,B,10,14a,3bg,14b	0	0	0	0	0	0	0	0	1	2.6	0	0	1	1.1
MBNSQ	1,3,3ka,17,B,10,14a,3bg,14b	0	0	0	0	0	0	0	0	0	0	1	8.3	1	1.1
MBNSS	1,3,3ka,17,B,10,14a,3bg,14b,20	0	0	0	0	0	0	0	0	0	0	1	8.3	1	1.1
MCDSS	1,3,26,17,B,10,14a,3bg,14b,20	2	15.4	0	0	0	0	0	0	1	2.6	1	8.3	4	4.5
PBLQT	1,2c,3,3ka,B,10,3bg,14b,20,28	0	0	0	0	0	0	0	0	0	0	2	16.7	2	2.2
PBMQQ	1,2c,3,3ka,30,B,10,3bg,14b	0	0	0	0	0	0	1	12.5	9	23.1	0	0	10	11.2
PBPQQ	1,2c,3,3ka,17,30,B,10,3bg,14b	0	0	0	0	0	0	0	0	1	2.6	0	0	1	1.1
PBPSQ	1,2c,3,3ka,17,30,B,10,14a,3bg,14b	0	0	0	0	2	16.7	0	0	0	0	0	0	2	2.2
PCBSQ	1,2c,3,26,B,10,14a,3bg,14b	2	15.4	0	0	0	0	0	0	0	0	0	0	2	2.2
PGMSL	1,2c,3,16,3ka,30,B,10,14a,3bg	0	0	0	0	0	0	0	0	2	5.1	0	0	2	2.2
PGPSS	1,2c,3,16,3ka,17,30,B,10,14a,3bg,14b,20	0	0	0	0	3	25	0	0	0	0	0	0	3	3.4
PHPSQ	1,2c,3,16,26,3ka,17,30,B,10,14a,3bg,14b	0	0	0	0	0	0	0	0	1	2.6	0	0	1	1.1
TCBJK	1,2a,2c,3,26,10,14a,14b,20,28	0	0	0	0	0	0	1	12.5	0	0	0	0	1	1.1
TCJSS	1,2a,2c,3,26,11,17,B,10,14a,3bg,14b,20	8	61.5	0	0	0	0	0	0	0	0	0	0	8	9
Total	...	13	...	5	...	12	...	8	...	39	...	12	...	89	...

EGY-116-A CGMQS, both in ME-2. Isolate 13PAK40-1-MBDSS in PK-2 had an SSR genotype identical to a number of isolates with MBDSS, MCDSS, and other closely related phenotypes from SA-3, ME-1, EU-8, and NA-3. Isolate 14PAK85-PBLQT in PK-2 had an SSR genotype identical to isolates 91MI-432-PBLQK and 91-2MI PBMGK from NA-2. Isolate 13PAK15-1-FHPSQ in PK-3 had an SSR genotype identical to isolates with FC—and FH—phenotypes from Turkey, Russia, and Kazakhstan in EU-2, Russia group 2 (RU-2), and Central Asia group 1 (CA-1), respectively.

DISCUSSION

The *P. triticina* population in Pakistan was fairly diverse for both virulence phenotype and SSR genotype. Although there was not a systematic sampling of collections across the wheat-producing regions of Pakistan, except in 2013, a large number of different virulence phenotypes and SSR genotypes were still identified. If large-scale surveys were conducted on a regular basis as in North America (Kolmer and Hughes 2015), it is very likely that more phenotypes would have been detected. The limitations of the sampling in this study

were apparent because relatively few virulence phenotypes were detected in more than 1 year. Increased sampling would have likely detected more phenotypes across different years.

The *P. triticina* population in Pakistan is likely influenced by selection caused by leaf rust resistance genes in the commonly grown wheat cultivars. Rattu et al. (2010) postulated the presence of *Lr1*, *Lr3*, *Lr9*, *Lr10*, *Lr13*, *Lr14a*, *Lr16*, *Lr17*, *Lr23*, *Lr26*, and *Lr27+Lr31* in Pakistani wheat cultivars and breeding lines. Of the 76 entries, 36 had *Lr26*, carried on the 1B-1R wheat-rye translocation that also carries *Yr9* and *Sr31* (McIntosh et al. 1995). The two most common phenotypes identified in this study, FHPSQ and KHPQQ, were both virulent to *Lr3*, *Lr10*, *Lr16*, *Lr17*, and *Lr26*. The third most common phenotype, PBMQQ, was virulent to *Lr1*, *Lr3*, and *Lr10*. In another test (J. Kolmer, unpublished data), the genes *Lr1*, *Lr10*, *Lr16*, *Lr14a*, *Lr17*, *Lr24*, and *Lr26* were postulated to be present in recent Pakistani wheat cultivars and breeding lines.

There are very few previous reports of virulence in *P. triticina* populations in Pakistan. Nagarajan and Joshi (1985) listed races of *P. triticina* in Pakistan identified using the International Standard differentials, that were the equivalent of virulence phenotypes F—, S—, K—, T—, and P—. In the current study, S— phenotypes were not identified in Pakistan. Rattu et al. (2009) reported very high frequency (>80%) of virulence to many of the single-gene Thatcher lines in the *P. triticina* population in Pakistan from 2002 to 2004. Only Thatcher lines with *Lr9*, *Lr19*, and *Lr28* had <10% of isolates with virulence.

Three groups of SSR *P. triticina* genotypes were found in Pakistan. All three groups and the overall population showed strong characteristics of clonal reproduction. High levels of observed heterozygosity and a general correlation between SSR genotypes and virulence phenotypes were also found in other worldwide *P. triticina* populations

Fig. 1. A, Principal coordinates plot of isolates of *Puccinia triticina* from Bhutan and Pakistan in three groups of simple-sequence repeat (SSR) genotypes based on k-means clustering. B, Principal coordinates plot of virulence phenotypes of isolates of *P. triticina* from Bhutan and Pakistan in three groups of SSR genotypes based on k-means clustering.

TABLE 4. Frequency of virulence to leaf rust resistance (*Lr*) genes in groups of *Puccinia triticina* isolates from Pakistan grouped by simple-sequence repeat genotype

Gene	PK-1	PK-2	PK-3	P
<i>Lr1</i>	0.647	0.750	0.333	0.138
<i>Lr2a</i>	0.235	0.083	0.222	0.555
<i>Lr2c</i>	0.882	0.250	1.000	0.00
<i>Lr3</i>	1.000	0.917	1.000	0.329
<i>Lr9</i>	0.000	0.000	0.000	1
<i>Lr16</i>	0.412	0.000	0.778	0.001
<i>Lr24</i>	0.000	0.083	0.000	0.329
<i>Lr26</i>	0.471	0.250	1.000	0.002
<i>Lr3ka</i>	0.882	0.250	0.778	0.001
<i>Lr11</i>	0.000	0.000	0.222	0.033
<i>Lr17</i>	0.529	0.583	1.000	0.045
<i>Lr30</i>	0.882	0.000	0.778	0.00
<i>LrB</i>	1.000	0.750	1.000	1
<i>Lr10</i>	1.000	0.833	1.000	0.297
<i>Lr14a</i>	0.588	0.833	1.000	0.05
<i>Lr18</i>	0.000	0.000	0.000	1
<i>Lr3bg</i>	1.000	0.750	1.000	0.029
<i>Lr14a</i>	0.882	1.000	1.000	0.271
<i>Lr20</i>	0.176	0.750	0.222	0.004
<i>Lr28</i>	0.000	0.333	0.000	0.008
Number of isolates	17	12	9	...

TABLE 3. Average of single-locus parameters of gene diversity of simple-sequence repeat genotype groups of *Puccinia triticina* isolates from Pakistan and Bhutan^a

Population	Alleles (<i>n</i>)	Effective (<i>n</i>)	Shannon <i>I</i>	<i>H_o</i>	<i>H_e</i>	<i>F_b</i> ^b
PK-1	2.48 (0.20)	1.87 (0.13)	0.62 (0.08)	0.66 (0.09)	0.40 (0.05)	−0.50 (0.11)
PK-2	2.78 (0.23)	2.02 (0.17)	0.74 (0.07)	0.63 (0.07)	0.44 (0.04)	−0.37 (0.08)
PK-3	2.17 (0.29)	1.71 (0.19)	0.49 (0.10)	0.41 (0.08)	0.29 (0.06)	−0.37 (0.06)
Total ^c	3.74 (0.43)	2.41 (0.28)	0.86 (0.10)	0.60 (0.06)	0.49 (0.05)	−0.21 (0.05)

^a Numbers in parentheses indicate standard error. Alleles = number of alleles, Effective = number of alleles, *H_o* = observed gene heterozygosity, and *H_e* = expected heterozygosity.

^b Significant at *P* < 0.05.

^c Total population.

(Kolmer et al. 2012; Ordoñez and Kolmer 2009). The *P. tritricina* population was still relatively diverse for virulence and molecular genotype, despite the lack of a susceptible alternate host in Pakistan. Mutation followed by selection for virulence is likely the main driver of diversity in the *P. tritricina* population. Migration from other regions may also contribute to the overall diversity because the SSR groups may represent different introductions of the leaf rust fungus into Pakistan. Isolates with MBDSS and MCDSS phenotypes and identical SSR genotypes have recently been found in North America, South America, Europe, Ethiopia, the Middle East, and Pakistan (J. Kolmer, unpublished data). This phenotype has rapidly spread to many of the wheat-producing regions since being detected in large numbers in North America in 1996. Genotypes in PK-3 may be a recent introduction because isolates in this group were the most related for SSR genotype, had the lowest level of gene diversity, and had only three different virulence phenotypes. The limited genetic variation in this group may reflect the limited amount of mutation at SSR loci that has occurred. Isolates in PK-1 and PK-2 were more diverse for SSR genotype and virulence phenotype, possibly reflecting different introductions of *P. tritricina* or a greater length of time for mutations to occur at SSR loci. The close genetic similarity of the PK groups with groups and individual isolates from Europe, Russia, Turkey, the Middle East, Central Asia, North America, and South America suggests that migration of leaf rust can occur between continental regions and is likely an important factor in the *P. tritricina* population in Pakistan.

ACKNOWLEDGMENTS

We thank K. Xiao and M. Hughes for excellent technical assistance; and A. Hafiz, M. Ijaz, F. Muhammad, I. A. Khalil, I. A. Shah, K. A. Khanzada, S. Ahmeed, S. Khatt, A. R. Rattu, K. Afridi, M. Fayyaz, A. Munir, A. M. Satti, and A. Hussain for collections of *P. tritricina* in Pakistan. Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

LITERATURE CITED

- Browder, L. E. 1971. Pathogenic Specialization in Cereal Rust Fungi, Especially *Puccinia recondita* f. sp. *tritici*: Concepts, Methods of Study, and Application. U.S. Dep. Agric. Tech. Bull. No. 1432.
- CIMMYT. 2016. Wheat Atlas. Pakistan wheat varieties. <http://wheatatlas.org/country/PAK>
- Duan, X., Enjalbert, J., Vautrin, D., Solignac, C., and Giraud, T. 2003. Isolation of 12 microsatellite loci, using an enrichment protocol, in the phytopathogenic fungus *Puccinia tritricina*. Mol. Ecol. Notes 3:65-67.
- Hovmöller, M. S., Sørensen, C. K., Walker, S., and Justesen, A. F. 2011. Diversity of *Puccinia striiformis* on cereals and grasses. Annu. Rev. Phytopathol. 49:197-217.
- Hovmöller, M. S., Walter, S., Bayles, R. A., Hubbard, A., Flath, K., Sommerfeld, N., Leconte, M., Czembor, P., Rodríguez-Algaba, J., Thach, T., Hansen, J. G., Lassen, P., Justesen, A. F., and de Vallaville-Pope, C. 2016. Replacement of the European wheat yellow rust population by new races from the centre of diversity in the near-Himalayan region. Plant Pathol. 65: 402-411.
- Hussain, M., Hassan, S. F., and Kirmani, M. A. S. 1980. Virulences in *Puccinia recondita* Rob. Ex. Desm. f. sp. *tritici* in Pakistan during 1978 and 1979. Pages 179-184 in: Proc. Fifth Eur. Mediterr. Cereal Rusts Conf. Bari, Italy.
- Kolmer, J. 2013. Leaf rust of wheat: Pathogen biology, variation and host resistance. Forests 4:70-84.
- Kolmer, J. A. 2001. Molecular polymorphism and virulence phenotypes of the wheat leaf rust fungus *Puccinia tritricina* in Canada. Can. J. Bot. 79:917-926.
- Kolmer, J. A. 2005. Tracking wheat rust on a continental scale. Curr. Opin. Plant Biol. 8:441-449.
- Kolmer, J. A., Hanzalova, A., Goyeau, H., Bayles, R., and Morgounov, A. 2012. Genetic differentiation of the wheat leaf rust fungus *Puccinia tritricina* in Europe. Plant Pathol. 62:21-31.
- Kolmer, J. A., and Hughes, M. E. 2013. Physiological specialization of *Puccinia tritricina* on wheat in the United States in 2011. Plant Dis. 97: 1103-1108.
- Kolmer, J. A., and Hughes, M. E. 2015. Physiologic specialization of *Puccinia tritricina* on wheat in the United States in 2013. Plant Dis. 99:1261-1267.
- Kolmer, J. A., Kabdulova, M. G., Mustafina, M. A., Zhemchuzhina, N. S., and Dubovoy, V. 2015. Russian populations of *Puccinia tritricina* in distant regions are not differentiated for virulence and molecular genotype. Plant Pathol. 64:328-336.
- Kolmer, J. A., and Ordoñez, M. E. 2007. Genetic differentiation of *Puccinia tritricina* populations in Central Asia and the Caucasus. Phytopathology 97:1141-1149.
- Kolmer, J. A., Ordoñez, M. E., Manisterski, J., and Anikster, Y. 2011. Genetic differentiation of *Puccinia tritricina* populations in the Middle East and genetic similarity with populations in Central Asia. Phytopathology 101: 870-877.
- Long, D. L., and Kolmer, J. A. 1989. A North American system of nomenclature for *Puccinia recondita* f. sp. *tritici*. Phytopathology 79:525-529.
- McIntosh, R. A., Wellings, C. R., and Park, R. F. 1995. Wheat Rusts: An Atlas of Resistance Genes. CSIRO Australia, Kluwer Academic Publishers, Dordrecht, The Netherlands.
- Meirmans, P. G., and van Tienderen, P. H. 2004. GENOTYPE and GENODIVE: Two programs for the analysis of genetic diversity of asexual organisms. Mol. Ecol. Notes 4:792-794.
- Nagarajan, S., and Joshi, L. M. 1985. Epidemiology in the Indian Subcontinent. Pages 371-402 in: The Cereal Rusts, Vol. II. Diseases, Distribution, Epidemiology and Control. A. P. Roelfs and W. R. Bushnell, eds. Academic Press, Orlando, FL.
- Ordoñez, M. E., German, S. E., and Kolmer, J. A. 2010. Genetic differentiation within the *Puccinia tritricina* population in South America and comparison with the North American population suggests common ancestry and intercontinental migration. Phytopathology 100:376-383.
- Ordoñez, M. E., and Kolmer, J. A. 2009. Differentiation of molecular genotypes and virulence phenotypes of *Puccinia tritricina* from common wheat in North America. Phytopathology 99:750-758.
- Peakall, R., and Smouse, P. E. 2006. GENALEX 6: Genetic analysis in Excel. Population genetic software for teaching and research. Mol. Ecol. Notes 6:288-295.
- Peakall, R., and Smouse, P. E. 2012. GENALEX 6: Genetic analysis in Excel. Population genetic software for teaching and research: An update. Bioinformatics 28:2537-2539.
- Rattu, A. R., Ahmad, I., Fayyaz, I., Akhtar, M. A., Haque, I. U., Zakria, M., and Afzal, S. N. 2009. Virulence analysis of *Puccinia tritricina* cause of leaf rust of wheat. Pak. J. Bot. 41:1957-1964.
- Rattu, A. R., Ahmad, I., Singh, R. P., Fayyaz, M., Mirza, J. I., Khanzada, K. A., and Haque, M. I. 2010. Resistance to *Puccinia tritricina* in some Pakistani wheats. Pak. J. Bot. 42:2719-2735.
- Roelfs, A. P., Singh, R. P., and Saari, E. E. 1992. Rust Diseases of Wheat: Concepts and Methods of Disease Management. CIMMYT, Mexico.
- Saari, E. E., and Prescott, J. M. 1985. World distribution in relation to economic losses. Pages 259-298 in: The Cereal Rusts, Vol. II. Diseases, Distribution, Epidemiology and Control. A. P. Roelfs and W. R. Bushnell, eds. Academic Press, Orlando, FL.
- Singh, R. P., William, H. M., Huerto-Espino, J., and Rosewarne, G. 2004. Wheat rust in Asia: Meeting the challenges with old and new technologies. Pages 1-13 in: Proc. 4th Int. Crop Sci. Congress, Brisbane, Australia.
- Smouse, P. E., and Peakall, R. 1999. Spatial autocorrelation analysis of individual multiallele and multilocus genetic structure. Heredity 82:561-573.
- Steel, R. D., and Torrie, J. H. 1980. Principles and Procedures of Statistics, 2nd ed. McGraw-Hill Book Co., New York.
- Szabo, L. S., and Kolmer, J. A. 2007. Development of simple sequence repeat markers for the plant pathogenic rust fungus *Puccinia tritricina*. Mol. Ecol. Notes 7:708-710.