Differentiation of Molecular Genotypes and Virulence Phenotypes of *Puccinia triticina* from Common Wheat in North America

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ABSTRACT

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Wheat leaf rust caused by *Puccinia triticina* is widely distributed in the wheat growing regions of the United States and Canada, and is subject to selection for virulence phenotype by leaf rust resistance genes in wheat cultivars. The objective of this study was to determine the number of genetically differentiated groups of *P. triticina* that are currently present in North America. In total, 148 isolates of *P. triticina* from the 1980s to 2005 were collected from wheat-growing regions of the United States and Canada and tested for virulence on 20 lines of wheat with single genes for leaf rust resistance and for molecular genotype with 23 simple sequence repeat (SSR) markers. In total, 91 virulence phenotypes and 65 SSR genotypes were found. After removal of isolates with identical virulence and SSR genotypes, 125 isolates were included for further analysis. Bayesian cluster analysis indicated five different groups of isolates based on SSR genotypes that also differed for virulence to leaf rust resistance genes *Lr2a, Lr2c, Lr3bg, Lr17,* and *Lr28*. Isolates avirulent to *Lr14a* and *Lr20* that have increased since 2003 had SSR genotypes identical or similar to older isolates in one of the five groups, indicating that these isolates were derived by mutation from the previously existing population of *P. triticina*. The representative collection of *P. triticina* isolates had characteristics consistent with an asexual dikaryotic population of genetically differentiated groups of SSR genotypes with high levels of heterozygosity and disequilibrium within which stepwise mutation at avirulence or virulence loci regularly occurs.

Wheat leaf rust caused by *Puccinia triticina* Erikss. is the most common and widely distributed disease of wheat (*Triticum aestivum* L.) in North America and worldwide (33). The geographic range of *Thalictrum speciossissimum* L., the alternate host of *P. triticina*, and the origin of wheat overlap in southwest Asia (8), which is where *P. triticina* likely originated (39). Arthur (3) indicated that leaf rust of wheat was introduced to North America from Eurasia. Susceptible alternate hosts for *P. triticina* are not native in North America and *P. triticina* is not found on the native wild grasses, being restricted almost exclusively to wheat (4,28). In the absence of a suitable alternate host, *P. triticina* reproduces in North America by the asexual production of urediniospores on wheat.

Leaf rust is found on soft red winter wheat cultivars that are grown in the southern and eastern states in the United States: soft white winter cultivars that are grown in New York, Michigan, and Ontario; hard red winter wheat cultivars that are grown in the southern-mid Great Plains states of the United States; and hard red spring wheat cultivars grown in the northern Great Plains states and the provinces of western Canada. Leaf rust is also found to a lesser extent on the soft white spring and winter wheat cultivars grown in the Pacific Northwest and the hard red spring cultivars in California. The wheat classes differ for leaf rust resistance genes (*Lr*); thus, different races or virulence phenotypes of *P. triticina* are found in the various regions of the United States and Canada. In recent years, many new phenotypes with virulence to *Lr17*, *Lr24*, and *Lr41* have increased due to selection by hard red winter wheat cultivars in the southern Great Plains while phenotypes with virulence to *Lr11*and *Lr26* are most common in the southeastern states where soft red winter wheat cultivars are grown (22). Virulence surveys of *P. triticina* have been conducted in the United States since 1926 (15) and Canada since 1931 (14); thus, changes in virulence phenotypes can be tracked over time and the selective effects of *Lr* genes in the wheat cultivars on the *P. triticina* population can be assessed. (21–23).

Studies using various types of molecular markers combined with virulence have provided further insight into the population biology of *P. triticina.* Previous studies with random amplified polymorphic DNA (RAPD) (20) and amplified fragment length polymorphisim (AFLP) (19) markers determined that distinct groups of *P. triticina* related for molecular polymorphism and virulence phenotype were present in Canada in the early and mid-1990s. The RAPD markers easily distinguished between isolates in different groups; however, there was very little polymorphism within the groups. The AFLP markers easily distinguished among isolates between and within groups of *P. triticina* virulence phenotypes; however, because AFLP and RAPD markers are dominantly expressed, it was not possible to distinguish between heterozygous and homozygous genotypes in either of these studies.

Introductions of new *P. triticina* virulence phenotypes have occurred in recent years. In 1984, a leaf rust phenotype with virulence to *Lr16*, *Lr27*, and *Lr31* and unique isozyme variation was first detected in Australia (30). In 2004, all of the leaf rust phenotypes identified in Australia were derived from this introduction. In 1996, phenotypes of *P. triticina* with virulence to resistance genes *Lr17, Lr3bg*, and *LrB* and avirulence to *Lr28* were found in large numbers for the first time in the Great Plains of the United States and Canada. Based on AFLP markers and virulence, Kolmer (19) speculated that these virulence phenotypes were a recent introduction to North America. In 2001, phenotypes of *P. triticina* with virulence to previously resistant durum wheat cultivars were first found in Mexico (35). Isolates of *P. triticina* col-

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lected from durum wheat in Europe and South America were very similar or identical to the Mexican isolates for virulence and simple sequence repeat (SSR) genotypes (29) that indicated a possible intercontinental migration of leaf rust. Since 2003, *P. triticina* isolates that are avirulent to *Lr14a* and *Lr20* have been detected in the Great Plains of the United States (21). Because isolates with avirulence to these two genes had not been present for over 40 years in this region (16), Kolmer et al. (21) speculated that these virulence phenotypes might have been recently introduced to the United States.

The overall objective of this study was to determine the number of distinct groups of *P. triticina* from common wheat that are currently present in North America. With the emergence of many new virulence phenotypes in recent years, a specific objective was to determine whether any new groups of *P. triticina* have been recently introduced. Further objectives included using recently developed SSR markers (9,38) to characterize *P. triticina* isolates in North America for heterozygosity, allelic diversity, and multilocus disequilibrium in order to confirm the clonal characteristics of wheat leaf rust in North America, and to determine whether the pattern of variation of virulence phenotypes and SSR genotypes between and within the different groups of *P. triticina* could be attributed to single-step mutations.

MATERIALS AND METHODS

***Puccinia triticina* isolates.** In total, 145 *P. triticina* uredinial isolates from infected leaves of common wheat and 3 isolates from infected *Aegilops cylindrica* L. (jointed goatgrass) leaves from North America were used (Table 1). Ninety of the isolates were collected in 2004 and 2005 during the annual surveys of virulence in *P. triticina* conducted by the United States Department of Agriculture–Agricultural Research Service (USDA-ARS) Cereal Disease Laboratory in the wheat-growing regions of the Great Plains, Ohio Valley, Gulf Coast, and southeastern states of the United States. The isolates were chosen based on their virulence phenotypes as recorded in the surveys (USDA-ARS Cereal Disease Laboratory website). Single-uredinial isolates were obtained from all of the collections, following previously described methods (22). Fifty-eight isolates from collections from the United States and Canada (17,19) from 1979 to 2003 were also used; these included the historical isolates race 1 (collected in 1954), race 9 (1959), race 5 (1959), and race 15 (1964). Isolates from Canada were also chosen on the basis of virulence phenotypes in previous studies (17,19). Two isolates that were collected from durum wheat in Mexico in 2004 were also included for comparison of SSR genotypes with the isolates from common wheat. The two isolates from durum wheat in Mexico are representative of a group of isolates from Europe, South America, and Mexico that were all nearly identical for SSR genotype and virulence phenotype (29). All isolates were stored as urediniospores at either –80°C in liquid nitrogen or in vacuum tubes at 4°C. The virulence of the isolates were not altered over time with any storage method.

Virulence phenotypes. Urediniospores from each isolate were used to inoculate 7-day-old seedlings of wheat cv. Thatcher (CI 10003) that had been treated with a maleic hydrazide solution (≈0.01 g dissolved in 30 ml of H_2O per pot) to enhance spore production. Each pot (3.5 by 3.5 cm filled with vermiculite) of 10 to 20 seedlings was sprayed with 0.25 ml of a suspension of urediniospores in Soltrol 170 (Phillips Petroleum, Bartlesville, OK) mineral oil. After drying for 1 h, inoculated plants were placed in a dew chamber overnight at 18°C. The plants were then placed in individual plastic isolation chambers in a greenhouse where temperatures varied between 18 and 25°C daily with at least 8 h of natural light plus supplemental greenhouse lighting. After 12 to 15 days, a cyclone spore collector was used to collect urediniospores from plants for each isolate.

To confirm virulence phenotypes of the *P. triticina* isolates, five sets of four Thatcher near-isogenic lines of wheat, each carrying one leaf rust resistance gene, were used: set 1, *Lr1* (isogenic line RL6003), *Lr2a* (RL6000), *Lr2c* (RL6047), and *Lr3* (RL6002); set 2, *Lr9* (RL6010), *Lr16* (RL6005), *Lr24* (RL 6064), and *Lr26* (6078); set 3, *Lr3ka* (RL6007), *Lr11* (RL6053), *Lr17* (RL6008), and *Lr30* (RL6049); set 4, *LrB* (RL6047), *Lr10* (RL6004), *Lr14a* (RL6013), and *Lr18* (RL6009); and set 5, *Lr3bg* (RL6042), *Lr14b* (RL6006), *Lr20* (RL 6092), and *Lr28* (RL6079). Seedlings of Thatcher were included as a susceptible control for comparison of infection types. Urediniospores of each isolate (1 to 2 mg) were mixed with 0.25 ml of oil and atomized onto 7- to 8-day-old sets of Thatcher differentials that were planted in groups of four in 3.5-by-3.5-cm pots filled with vermiculite. The inoculated differential sets were placed in a mist chamber overnight. After incubation, the differential sets were removed from the mist chamber, allowed to dry for 1 h, and then placed in a greenhouse at 18 to 25°C with at least 8 h of natural and supplemental lighting. Virulence phenotypes were determined 10 to 12 days after inoculation for each isolate on each Thatcher differential line using the scale described by Long and Kolmer (26). Infection types 0 to 2^+ (immune response to moderate uredinia with chlorosis and necrosis) 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 given a five-letter code based on virulence or avirulence to the 20 near-isogenic Thatcher lines adapted from the nomenclature used by Long and Kolmer (27).

Molecular genotypes. DNA was extracted from 25 to 30 mg of urediniospores of each isolate by first grinding the spores with 25 mg of glass beads in a Savant FastPrep shaker (FP120; Holbrook, NY) for 20 s, and then using an OmniPrep extraction kit (GenoTech, St. Louis) according to instructions. In all, 10 to 20 ng of DNA was used for each polymerase chain reaction (PCR) amplification.

Twenty-three SSR microsatellite primer pairs developed from genomic libraries were used to characterize the collection: PtSSR 3, PtSSR 13, PtSSR 50, PtSSR 55, PtSSR 61, PtSSR 68-1, PtSSR 76, PtSSR 91, PtSSR 92, PtSSR 151A, PtSSR 152, PtSSR 154, PtSSR 158, PtSSR 161, PtSSR 164, PtSSR 173, PtSSR 184, PtSSR 186 (38), RB 1, RB 8, RB 11, RB 26, and RB 35 (9). Amplification and electrophoresis were carried out as previously described (24). Allele sizes in base pairs were scored visually for each primer pair by using a LI-COR (Lincoln, NE) 4200 or 4300 DNA sequencer that was calibrated with IRDye 700 molecular weight size standards. DNA bands generated by each primer pair were compared with the allele sizes in the initial characterization of the SSR primers (9,38) and also with other *P. triticina* isolates previously characterized using the same set of SSR primers (24,29). Separate DNA samples of isolates included in both previous studies (24,29) and in the current study as controls had the same SSR genotypes.

Data analysis. The program Structure v.2 was used to define groups of isolates based on SSR genotype data using the algorithm developed by Pritchard et al. (32). A burnin period of 100,000 and 100,000 iterations of Markov Chain Monte Carlo were used with the admixture model. The program uses multilocus genotype data to assign individuals to groups based on allele frequencies at the examined loci using a Bayesian model-based clustering method. Although Structure v.2 was developed for use with sexual populations, *P. triticina* SSR genotypes from an asexual population were previously grouped with this program in a manner that was nearly identical to grouping based on unrooted neighbor joining trees (24).

The total number of SSR genotypes and virulence phenotypes was calculated with GenClone (2). Linkage disequilibrium across all SSR loci was calculated with the index of association (I_A), and also with a measure corrected for the number of loci, $\bar{r}_D$, using

MultiLocus v1.3 software (1). Tests of departure from random mating for both indices were done with 1,000 randomizations of the data set.

Averages of single-locus statistics for the isolates in the SSR groups—number of alleles, number of effective alleles, Shannon's information index (I), observed heterozygosity (H_O), expected heterozygosity (H_e), and fixation index (F_{IS})—were calculated with GenAlEx6 (11). Genetic differentiation via the AMOVA with 999 permutations of the data set was calculated for the SSR genotypes with R_{ST} that assumes a stepwise mutation model and by F_{ST} that assumes the infinite alleles model. An analogous measure developed for binary data, Φ_{PT}, was used to calculate dif-

TABLE 1. Isolates of *Puccinia triticina*, virulence phenotype, and North American (NA) group based on assignment of simple sequence repeat genotypes with Structure v.2

Isolate no.	Designation	Virulence code[a]	NA group	Assigned probability
1	Race 1	BBBDJ	1	0.90
2	04NE 346-2	BBBDJ	1	0.92
3	04 NE 477-1	BBBDJ	1	0.69
4	05 ND 465	BBBDJ	1	0.84
5	84 PA-1	PBDSP	1	0.94
6	84 MN 126B	PGDGP	1	0.91
7	99 NC	LBBKK	1	0.96
8	90 PA 542	LBGKK	1	0.83
9	CAN 247-2-97	DBBQK	1	0.92
10	05 OH 215	TCTBS	1	0.92
11	03 VA 190	TCTDL	1	0.97
12	04 NC 170	TCTDN	1	0.96
13	84 VA	PBJSF	2	0.55
14	84 PA-2	PBJSK	2	0.48
15	CAN 38-2-96	PBDQF	2	0.52
16	CAN 175-1-97	NBBQT	2	0.97
17	93 WA-1	NBBRK	2	0.96
18	CAN 6-98-23	NBBRK	2	0.97
19	82 MN 116-1	NBGKK	2	0.95
20	04 NY 629-1	NBGTK	2	0.90
21	CAN 1-194	FBLBT	2	0.98
22	04 GA 8803	FBMTK	2	0.98
23	CAN 136-1-96	FBMTK	2	0.98
24	CAN 252 2-97	FCMNK	2	0.98
25	01 AL 21	FCMTK	2	0.98
26	82 TX 2	FLLLK	2	0.98
27	91 MI 432	PBLQK	2	0.98
28	CAN 134-3-96	PBLQK	2	0.97
29	CAN 232-2-97	PBLQK	2	0.98
30	MI 91-2	PBMGK	2	0.98
31	87 NY 676-2	PBMGK	2	0.98
32	CAN 127-2-98	PBMNK	2	0.98
33	CAN 235-1-97	PBRHK	2	0.98
34	CAN 104-2-96	PBRRT	2	0.98
35	94 LA 101	PNMRK	2	0.98
36	CAN 215-1-97	CCDJJ	2	0.55
37	82 PA-1	NBJDK	2	0.66
38	32-1 SP5 CAN	MBDSS	3	0.97
39	04 TX 32	MBDSS	3	0.98
40	05 MN 389	MBDSS	3	0.98
41	05 LA 144	MBTSS	3	0.98
42	00 SD 520	MCDSS	3	0.98
43	03 CA 360-2	MCDSS	3	0.98
44	04 ND 447	MCJSS	3	0.98
45	04 ND 477	MCPSS	3	0.97
46	04 TX 08	MFPSS	3	0.91
47	04 OH 613-1	MFPSS	3	0.97
48	05 TX 85	MFPSS	3	0.98
49	03 OH 237	MHDSS	3	0.96
50	04 TX 78	MLDSS	3	0.96
51	04 KS 21	MLDSS	3	0.97
52	04 KS 256	MLDSS	3	0.91
53	05 TX 55	MLDSS	3	0.98
54	04 TX 67	TBDSS	3	0.98
55	04 MN 486	TBDSS	3	0.98
56	05 SD 371	TBDSS	3	0.98
57	04 KS 213	TCPSS	3	0.96
58	04 TX 86	TDFSS	3	0.53
59	CAN 71-2-96	TDTDM	4	0.98
60	04 MN 364	SBDBK	4	0.98
61	05 OK 194	SBDBK	4	0.98
62	05 MN 365	SBDBT	4	0.98
63	Race 9	SBDGK	4	0.98
64	05 WA 203	PBDST	5	0.38
65	04 MN 388	TBBGH	5	0.97
66	04 TX 68-1	TDBGH	5	0.97
67	04 TX 72	TDBGH	5	0.97
68	05 SD 408	TDBGH	5	0.97
69	05 LA 146	TDGGH	5	0.97
70	04 TX 68-2	TFBGH	5	0.97
71	05 TX 32	TFBGH	5	0.97
72	04 MN 485	TGBGH	5	0.97
73	04 ND 429	THBGR	5	097
74	04 OH 300	TJBGH	5	0.97
75	04 MN 408-1	TBBJK	5	0.97
76	05 SD 306	TBBJK	5	0.98
77	05 ND 499	TBBJT	5	0.98
78	79 TX	TBDFT	5	0.97
79	04 TX 92	TBRJK	5	0.98
80	04 TX 104	TCBJK	5	0.98
81	04 MS 202-2	TCBJT	5	0.97
82	99 NC	TCRJK	5	0.98
83	05 VA 375	TCRKF	5	0.98
84	05 AL 77	TCRKK	5	0.98
85	05 AL 91	TCRKK	5	0.98
86	82 MN	TDBJK	5	0.88
87	04 TX 24	TDBJK	5	0.97
88	05 SD 304	TDRJK	5	0.98
89	05 SD 305	TFBJT	5	0.98
90	04 ND 520-1	TGBJK	5	0.98
91	04 KS 236	TGBJK	5	0.98
92	04 TX 97	THBJK	5	0.98
93	05 NE 311	THBJK	5	0.98
94	05 KS 277	TJBJK	5	0.98
95	CAN 258-1-97	TKBJK	5	0.98
96	00 SC 218	TLGFT	5	0.88
97	04 TX 47	TMGJK	5	0.83
98	05 TX 29	TMQJK	5	0.98
99	04 TX 99	TNRJK	5	0.98
100	05 TX 27	TNRJK	5	0.98
101	04 TX 22	TLGJK	5	0.98
102	99 ND 588	THBJK	5	0.98
103	04 CA 641-1	TFBJK	5	0.87
104	84 MN 526-1	KBBJK	5	0.97
105	84 MN 526-2	KBBJK	5	0.97
106	92 218-A	KGBJK	5	0.98
107	05 TX 15	THBJH	5	0.97
108	05 OK 165	TJBJH	5	0.98
109	05 SD 416	TDBDH	5	0.98
110	Race 5	MBBJT	5	0.97
111	99 NC 23-1B	MBGDK	5	0.97
112	99 ND 16-2B	MBRJK	5	0.98
113	CAN 203-2-97	MBRJK	5	0.98
114	CAN 253-3-97	MBRKK	5	0.98
115	04 TX 50	MBRKK	5	0.98
116	04 NE 343	MCRKK	5	0.98
117	95 WA 475	MDBJK	5	0.94
118	05 ID 332	MDBJK	5	0.98
119	05 AR 21	MFGJK	5	0.97
120	05 AL 76	MFGKK	5	0.97
121	CAN 49-2	MFMJK	5	0.93
122	97 NE 406	MJBJK	5	0.97
123	CAN 31-1-96	CBDDK	5	0.97
124	Race 15	CBBJK	5	0.70
125	04 OK 232	TBRKK	5	0.83

[a] Five-letter code indicates virulence or avirulence to 20 Thatcher wheat isolines with different leaf rust resistance genes as adapted from Long and Kolmer (26).

ferentiation of the virulence phenotypes in the SSR groups. Pairwise values of R_{ST}, F_{ST}, and Φ_{PT} were calculated via AMOVA among SSR groups that were defined by Structure, and a Mantel correlation test between matrices of R_{ST}, F_{ST}, and Φ_{PT} was done using GenAlEx6, with 999 permutations of the data set. R_{ST} pairwise values between SSR groups were also plotted as an unrooted tree with PHYLIP 3.6 (11) using the NEIGHBOR clustering option.

A matrix of Jaccard coefficients derived from SSR allele differences of all pairs of isolates and a matrix of simple matching coefficients derived from virulence differences between isolates were generated in NTSys-pc 2.1 (Exeter Software, Seatauket, NY). These matrices were compared with the Mantel correlation coefficient to determine the correlation between SSR genotype and virulence phenotype. Unrooted neighbor-joining trees of individual *P. triticina* isolates based on SSR genotypes and virulence phenotypes were done with NTSYS-pc 2.1 with the distance matrices imported from GenAlex6.

RESULTS

Among the 148 isolates, 91 virulence phenotypes and 65 SSR genotypes were found. After clone correction by removal of isolates with identical virulence phenotypes and SSR genotypes, 125 isolates of *P. triticina* from the United States and Canada were used in the analysis. Results from Structure indicated that the optimal *k* value (number of groups) was five, based on the highest log *P* (X/K) of runs with K values of 1 to 8. Isolates were assigned to five North American (NA) groups based on the highest assignment probability. Isolates in the Structure-defined groups NA-3, NA-4, and NA-5 had average assignment probabilities of 0.98 and 0.95 (Table 2). Isolates in the groups NA-1 and NA-2 were more heterogeneous for SSR genotypes and had average probabilities of 0.90 and 0.88, respectively. NA-5 was the largest of the five groups and had the highest number of virulence phenotypes with 48 and SSR genotypes with 23. NA-4 was the smallest group and had four virulence phenotypes and three SSR genotypes.

The NA group designation and assigned probabilities of the individual isolates are listed in Table 1. The 10 recent isolates avirulent to *Lr14a* and *Lr20* (65-04 MN388 TBBGH, 66-04 TX68-1 TDBGH, 67-04 TX72 TDBGH, 70-04 TX68-2 TFBGH, 72-04 MN485 TGBGH, 73-04 ND 429 THBGR, 69-05 LA146 TDGGH, 71-05TX32 TFBGH, 74-04 OH300 TJBGH, and 68-05 SD408 TDDGH) were assigned to NA-5 with the probability of 0.97. The historical isolates 110-race 5 MBBJT and 124-race 15 CBBJK were also assigned to NA-5 with probabilities of 0.97 and 0.70, respectively. Isolate 64-05 WA203 PBDST was the only isolate with an assignment probability of <0.50 in NA-5. Isolate 58-04 TX 86 TDFSS was the only isolate assigned to NA-3 with a probability <0.90. Isolates 14-84 PA-2 PBJSK, 13-84VA PBJSF, 15-CAN 38-2-96 PBDFQ, and 36-CAN 215-1-97 CCDJJ in NA-2 had assignment probabilities of <0.60; all other isolates had probabilities of >0.90 in this group. The historical isolates 63-race 9 SBDGK and 1-race 1 BBBDJ had assignment probabilities of 0.98 and 0.90 to groups NA-4 and NA-1, respectively. Isolates 2-04 NE 346-2 BBBDJ, 4-05 ND 465 BBBDJ, and 8-90 PA 542 LBGKK had assignment probabilities <0.90 in NA-1.

The five groups of isolates varied for the average number of alleles per SSR locus from 3.13 in NA-1 to 1.43 in NA-4 (Table 3). NA-1 and NA-4 also had the highest and lowest number of effective alleles, respectively. Diversity of SSR alleles as measured by the Shannon information index was highest in NA-1 at 0.78 and lowest at 0.25 in NA-4. All five groups had expected values of heterozygosity (H_e) that were greater than the observed values (H_o). Linkage disequilibria between SSR loci for the entire collection of isolates was high, with an I_A of 5.05 and $\bar{r}_D$ of 0.237.

Values of average genetic differentiation of isolates based on R_{ST} (differentiation based on stepwise mutation) between the five NA groups plus the two isolates collected from durum wheat were used to plot the neighbor-joining dendrogram in Figure 1. All pairs of groups were significantly differentiated ($P < 0.05$) except for NA-1 and NA-5. The two durum leaf rust isolates in NA-6 were the most differentiated isolates. Of the isolates from common wheat, isolates in NA-3 had the largest average differentiation from isolates in NA-1, NA-2, NA-4, and NA-5. All groups were significantly differentiated based on F_{ST} (differentiation based on infinite alleles model); however, the plot had a star pattern and did not show any phylogeny between the different groups (not shown). The pairwise values of F_{ST} for the five groups from common wheat had a correlation of 0.28 with the R_{ST} values.

When the isolates were grouped based on regional wheat class, only isolates collected from the soft white winter and spring wheat regions were significantly differentiated from isolates collected from the hard red spring wheat and hard red winter wheat based on SSR genotypes (Table 4). All other pairs of collections were not significantly differentiated, indicating that most SSR genotypes of *P. triticina* are widely distributed in North America.

TABLE 2. Genotypic diversity for 23 simple sequence repeat (SSR) loci and virulence variation to 20 Thatcher lines in five groups of *Puccinia triticina* isolates from North America grouped by Structure v.2 based on SSR genotypes

	North American (NA) SSR group					
Parameters	NA-1	NA-2	NA-3	NA-4	NA-5	Total
No. of isolates	12	25	21	5	62	125
No. of virulence phenotypes	9	19	11	4	48	91
No. of SSR genotypes	12	16	11	3	23	65
Average Structure group probability	0.90	0.88	0.95	0.98	0.95	…

TABLE 3. Average of single-locus statistics of *Puccinia triticina* isolates from North America in groups defined by Structure v.2 clustering based on simple sequence repeat (SSR) genotypes

	North American (NA) SSR group[b]					
Parameters[a]	NA-1	NA-2	NA-3	NA-4	NA-5	NA means
No. of alleles	3.13 (0.35)	2.52 (0.18)	1.96 (0.16)	1.43 (0.14)	2.26 (0.191)	2.33 (0.102)
No. of effective alleles	2.20 (0.22)	2.04 (0.07)	1.69 ((0.10)	1.34 (0.11)	1.70 (0.116)	1.80 (0.060)
Shannon *I*	0.78 (0.089)	0.74 (0.04)	0.50 (0.07)	0.25 (0.08)	0.503 (0.076)	0.57 (0.04)
H_o	0.60 (0.07)	0.84 (0.05)	0.64 (0.10)	0.31 (0.10)	0.619 (0.098)	0.62 (0.04)
H_e	0.45 (0.05)	0.49 (0.02)	0.34 (0.09)	0.17 (0.05)	0.336 (0.051)	0.37 (0.02)
F_{IS}	–0.30 (0.05)	–0.69 (0.06)	–0.82 (0.07)	–0.82 (0.07)	–0.672 (0.084)	–0.57 (0.04)

[a] $I = -\Sigma p_i(lnp_i)$, where p_i = frequency of *i*th allele; H_o = observed heterozygosity; H_e = expected heterozygosity; and $F_{IS} = H_e - H_o/H_e$.
[b] Numbers in parentheses = standard error.

The frequencies of isolates with virulence to the 20 Thatcher isolines in the five NA groups are listed in Table 5. All of the isolates in NA-1 were avirulent to *Lr9* and *Lr24* and all were virulent to *Lr20*. Isolates in NA-2 were distinct from the other groups because almost all of these were avirulent to *Lr2a* and virulent to *Lr2c*. Isolates in NA-3 were fixed at 100% for virulence to genes *LrB* and *Lr3bg* and for avirulence to *Lr28*. All isolates in NA-3 were also virulent to *Lr17,* in addition to the five isolates in NA-4. The frequency of isolates with virulence to *Lr17* and *Lr20* was lowest in NA-5, and all isolates in NA-5 were virulent to *Lr28*. The differing frequencies of virulence to leaf rust resistance genes in the NA groups was also reflected in the significant differentiation for virulence phenotypes with Φ_{PT} statistics between all groups as indicated in Table 6. The pairwise values of Φ_{PT} had a correlation of 0.49 with the R_{ST} values and 0.13 with the F_{ST} values for differentiation of groups based on SSR genotypes.

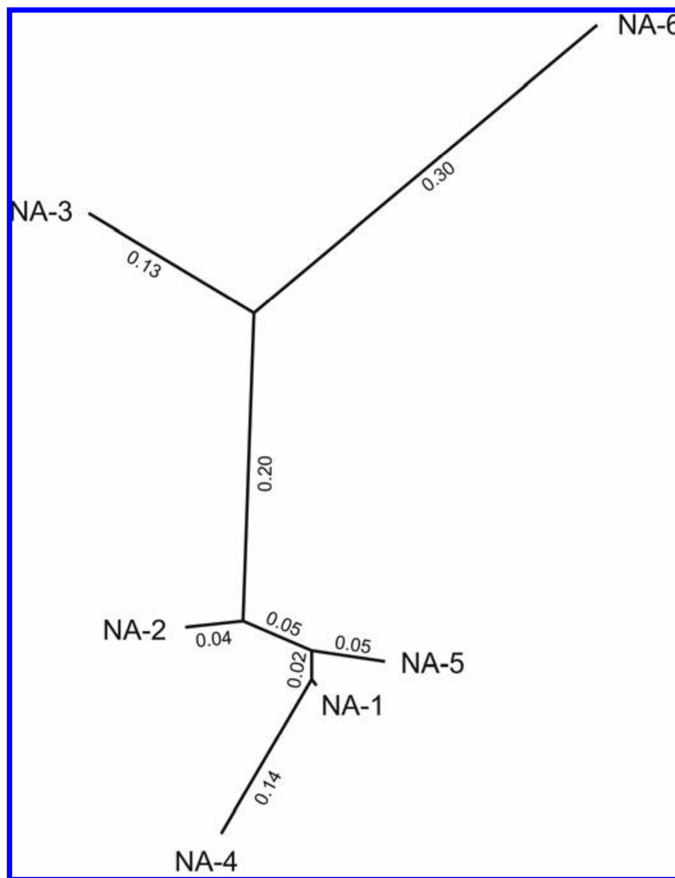

Fig. 1. Unrooted neighbor-joining dendrogram of values of R_{ST} differentiation between groups of *Puccinia triticina* simple sequence repeat genotypes defined by Structure v.2. Isolates in North American NA-1, NA-2, NA-3, NA-4, and NA-5 were collected from common wheat in the United States and Canada. Isolates in NA-6 were collected from durum wheat in Mexico. Values of R_{ST} as indicated for each branch were derived by 999 permutations of the data set in GenAlex 6 (31).

The matrix of Jaccard coefficients of SSR differences for all pairwise comparisons of the 125 isolates was compared with a matrix of simple matching coefficients for virulence differences among the isolates using the Mantel correlation coefficient. The virulence phenotypes had a moderate correlation with the SSR genotypes at 0.43 (P = 1.0). With a subset of the isolines *Lr2a, Lr2c, Lr17, Lr3bg,* and *Lr28* chosen based on differences in frequencies of virulence to these genes between NA groups, the virulence variation had a correlation of 0.34 (P = 1.0) with the SSR variation.

The SSR genotypes and virulence phenotypes of isolates in NA-3 and NA-5 were examined in neighbor-joining dendrograms to determine the number of molecular and virulence differences between all pairs of isolates within each group. In NA-3, all isolates differed by a single SSR allele to their nearest neighbor or had SSR genotypes identical with other isolates, except for two isolates (Fig. 2A). Isolates 04 KS213 TCPSS and 04 TX86 TDFSS differed for two SSR alleles. Only two virulence phenotypes in NA-3 differed from their nearest neighbor for more than one virulence (Fig. 2B). Isolates 04 KS213 TCPSS and 04 TX86 TDFSS differed for two virulences to their closest neighbors. In NA-5, all isolates had either identical SSR genotypes or differed by a single SSR allele from their closest neighbor, except for three isolates (Fig. 3A). Isolate 00 SC218 TLGFT differed by two SSR alleles from its closest neighbor; isolate race 15 CBBJK differed by three SSR alleles from it closest neighbor; and isolate 05 WA203 PBDST differed by six alleles to it closest neighbor. All virulence phenotypes in NA-5 differed from their closest neighbor by a single virulence, except for four pairs of isolates (Fig. 3B). Isolates 05 WA203 PBDST and race 5 MBBJT differed by three virulences, race 15 CBBJK and CAN 31-1-96 CBDDK

TABLE 5. Frequencies of virulence to leaf rust resistance genes of isolates of *Puccinia triticina* from North America in groups defined by Structure v.2 assignment based on simple sequence repeat (SSR) genotypes[a]

Gene	NA-1	NA-2	NA-3	NA-4	NA-5
Lr1	58.3	72.0	100.0	100.0	92.0
Lr2a	25.0	0.0	19.0	100.0	74.2
Lr2c	50.0	88.0	19.0	100.0	75.8
Lr3	41.7	76.0	100.0	20.0	100.0
Lr9	0.0	8.0	19.0	0.0	8.1
Lr16	8.3	0.0	4.8	0.0	22.6
Lr24	0.0	4.0	19.0	20.0	40.3
Lr26	25.0	12.0	42.9	0.0	32.3
Lr3ka	25.0	56.0	28.6	20.0	22.6
Lr11	33.3	28.0	9.5	20.0	35.5
Lr17	41.7	20.0	100.0	100.0	4.8
Lr30	25.0	40.0	33.3	20.0	24.2
LrB	25.0	72.0	100.0	0.0	1.6
Lr10	50.0	80.0	100.0	20.0	93.5
Lr14a	66.7	44.0	100.0	20.0	80.6
Lr18	25.0	40.0	0.0	0.0	16.1
Lr3bg	33.0	12.0	100.0	20.0	12.9
Lr14b	75.0	92.0	100.0	80.0	98.4
Lr20	100.0	100.0	100.0	80.0	77.4
Lr28	50.0	96.0	0.0	100.0	100.0

[a] Number of isolates in each group: NA-1 = 12, NA-2 = 25, NA-3 = 21, NA-4 = 5, and NA-5 = 62.

TABLE 4. R_{ST} values of genetic differentiation of *Puccinia triticina* isolates from North America based on simple sequence repeat genotype grouped by regional wheat class where collected

		Wheat regions[a]			
No. of isolates	Wheat regions	Hard red spring	Hard red winter	Soft red winter	Soft white winter/spring
34	Hard red spring	0.00	…	…	…
40	Hard red winter	0.00 (0.29)	0.00	…	…
23	Soft red winter	0.00 (0.34)	0.01 (0.26)	0.00	…
28	Soft white winter/spring	0.05 (0.04)	0.06 (0.02)	0.02 (0.13)	0.00

[a] Numbers in parentheses = probability, significant differentiation if <0.05.

differed for two virulences, 79 TX TBDFT and 00SC 218 TLGFT differed by three virulences, and 05 OK165 TJBJH and 05 SD416 TDBDH differed by two virulences.

DISCUSSION

Based on SSR genotypes, there is evidence for at least four and possibly five distinct groups of *P. triticina* collected from common wheat in North America. Isolates in groups NA-1 and NA-5 were not significantly differentiated based on R_{ST} statistics yet were strongly separated in the Structure grouping, because isolates in NA-1, on average, had less than a 0.05 probability of belonging to NA-5. The five NA groups were also characterized by differences in avirulence or virulence to selected *Lr* genes. In recent years, isolates with virulence phenotypes found in NA-3 and NA-5 have composed >90% of the *P. triticina* population in the United States (21,22).

In a previous study with 64 *P. triticina* isolates from the early 1990s in Canada, (20) two major groups of isolates that differed for virulence and RAPD phenotypes were distinguished. In a later study with 69 isolates from the late 1990s in Canada (19), there were three large groups of isolates that differed for AFLP phenotype and virulence. In these studies, the major groups of isolates corresponded to the NA-2, NA-3, and NA-5 groups that were distinguished in the current study.

Although distinct groups of *P. triticina* isolates were found in North America, there was relatively little geographic differentiation of SSR genotypes. Virulence phenotypes of *P triticina* often originate in certain regions due to selection caused by wheat cultivars with host resistance genes. However, because *P. triticina* is wind dispersed, the predominant virulence phenotypes are often the same in the different regions (21,22). In this study, only the *P. triticina* SSR genotypes collected from soft white wheat cultivars were differentiated from isolates collected from other wheat regions, suggesting that some overwintering of leaf rust may occur on these wheat lines in the northeastern states and Pacific northwest region.

The SSR data provided strong evidence for clonality in this collection of *P. triticina* from North America. The high I_A value between SSR loci and the large amount of excess heterozygosity at SSR loci are consistent with populations that reproduce asexually (5,13). Excessive heterozygosity at SSR loci was also found in *P. triticina* populations from France (12) and Central Asia (24), which also reproduce asexually. In the absence of sexual recombination in the fungus, mutation at SSR loci and at loci that condition avirulence or virulence has generated a large amount of genetic variation.

The stepwise mutation model (R_{ST}) for differentiation of SSR genotypes clustered the groups more closely to the clustering of the same groups based on virulence phenotypes compared with the infinite alleles model (F_{ST}). This suggests that stepwise mutations have occurred within groups of isolates with the same or similar SSR genotypes and virulence phenotypes. Treatment of *P. triticina* isolates with chemical mutations has been shown to result in mutants with altered virulence to one or more leaf rust resistance genes (36). In the field populations of *P. triticina,* it would be expected that ultraviolet radiation would account for most mutations. The two isolates from durum wheat in Mexico were very distinct for SSR genotype compared with the isolates from common wheat. Ordoñez and Kolmer (29) previously found that isolates collected from durum wheat in Mexico and California were nearly identical for SSR genotype to other durum wheat isolates from Europe and South America, and highly

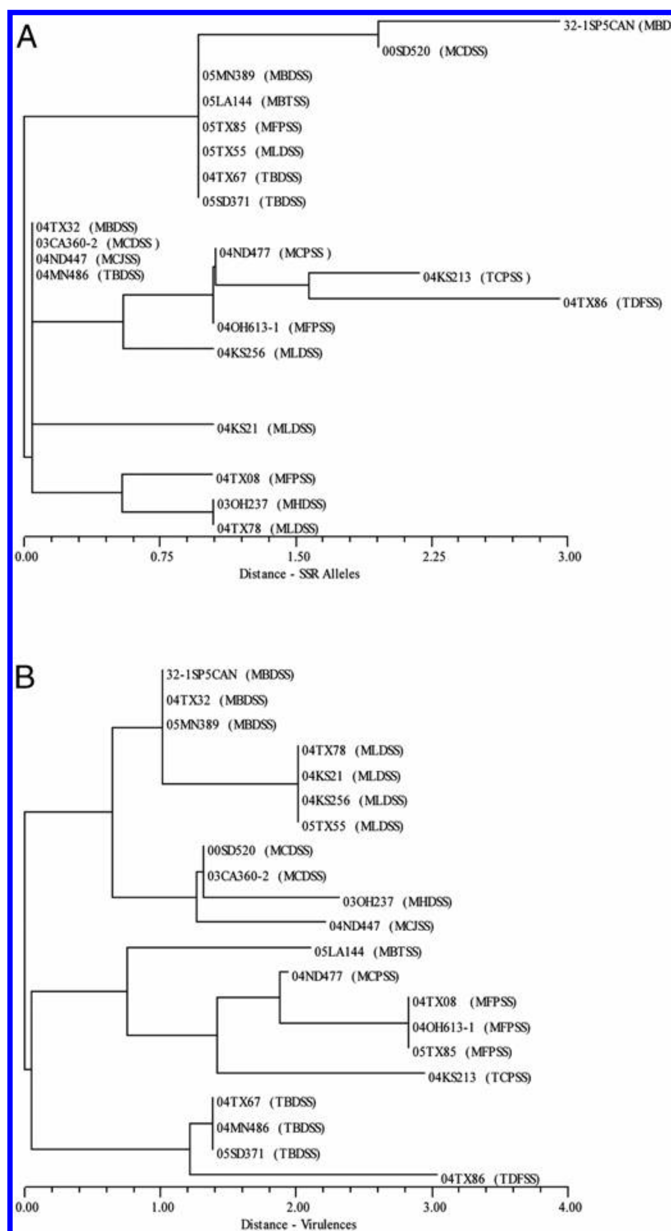

Fig. 2. Unrooted neighbor-joining dendrograms of *Puccinia triticina* isolates in simple sequence repeat (SSR) genotype group NA-3: **A,** SSR genotypes and **B,** virulence phenotypes.

TABLE 6. Φ_{PT} values of genetic differentiation of virulence phenotypes of *Puccinia triticina* from North America in groups defined by Structure v.2 assignment based on simple sequence repeat (SSR) genotypes

	North American (NA) SSR group[a]				
	NA-1	NA-2	NA-3	NA-4	NA-5
NA-1	0	…	…	…	…
NA-2	0.10 (0.006)	0	…	…	…
NA-3	0.37 (0.001)	0.46 (0.001)	0	…	…
NA-4	0.17 (0.028)	0.33 (0.001)	0.59(0.001)	0	…
NA-5	0.22 (0.001)	0.25 (0.001)	0.50 (0.001)	0.30 (0.001)	0

[a] Numbers in parentheses = probability, significant differentiation if <0.05.

differentiated compared with genotypes from common wheat. It is highly unlikely that isolates from durum wheat were derived by mutation from common wheat isolates.

Isolates in the NA-5 group were collected between 1959 and 2005 throughout North America and are diverse for virulence phenotype. Mutation at avirulence loci followed by host selection for virulence has created this large group of virulence phenotypes that are highly related for SSR genotype. With nearly all isolates in NA-5 differing by no more than a single SSR allele from their closest neighbor, stepwise mutation is likely the main source of variation in *P. triticina* in North America. This group of isolates is long established in North America because isolates race 5 MBBJK and race 15 CBBJK, collected in 1959 and 1964, respectively, were placed in this group with the other isolates in NA-5 that were collected in the 1990s and from 2004 to 2005. A similar pattern of stepwise difference was found among AFLP phenotypes in isolates of *P. striiformis* in Australia (37). The isolates in NA-1 were collected throughout North America from 1954 to 2005 and are diverse for virulence phenotype but are rarely found in the annual surveys (21–23). Isolates in NA-1 are usually collected from susceptible wheat cultivars because most of these isolates are avirulent to many *Lr* genes. Because isolates in NA-1 and NA-5 were not significantly differentiated with R_{ST}, there may be some ancestral relationship between isolates in these two groups.

The virulence phenotypes that are avirulent to *Lr14a* and *Lr20* were included in the NA-5 group, which suggests that these relatively recent and unique virulence phenotypes were derived by mutation from other isolates in NA-5. Statler (36) noted in *P. triticina* that chemically induced mutations from virulence to avirulence to a number of leaf rust resistance genes also occurred in addition to the more common mutations of avirulence to virulence. A dominant gene that inhibits expression of avirulence in *P. triticina* to the *Lr2* locus has been characterized (10). Mutation at a regulatory locus that inhibits expression of avirulence may account for the loss of virulence to *Lr14b* and *Lr20* in these recently detected isolates. Alternatively, independent mutations may have occurred at both virulence loci. Neither *Lr14b* or *Lr20* are present in U.S. wheat cultivars; thus, mutations in *P. triticina* that affect virulence to these genes have no obvious selective consequence.

Isolates in the NA-3 group were first introduced to the United States and Canada in 1996 (19). Isolates in this group are distinct from those in NA-5 because they are all virulent to *Lr17, LrB* and *Lr3bg* and avirulent to *Lr28*. Hard red winter cultivars with *Lr17* have selected these isolates which have been wind dispersed across all wheat-growing regions in North America. This group was established with isolates that had virulence phenotypes MBDS and MCDS. Since 1996, a number of additional phenotypes with virulence in addition to *Lr17, Lr3bg*, and *LrB* have been detected (22,23). Mutations that affect virulence followed by host selection have subsequently produced new virulence phenotypes in NA-3. The high degree of similarity of isolates in NA-3 for SSR genotypes also indicates stepwise mutation, as

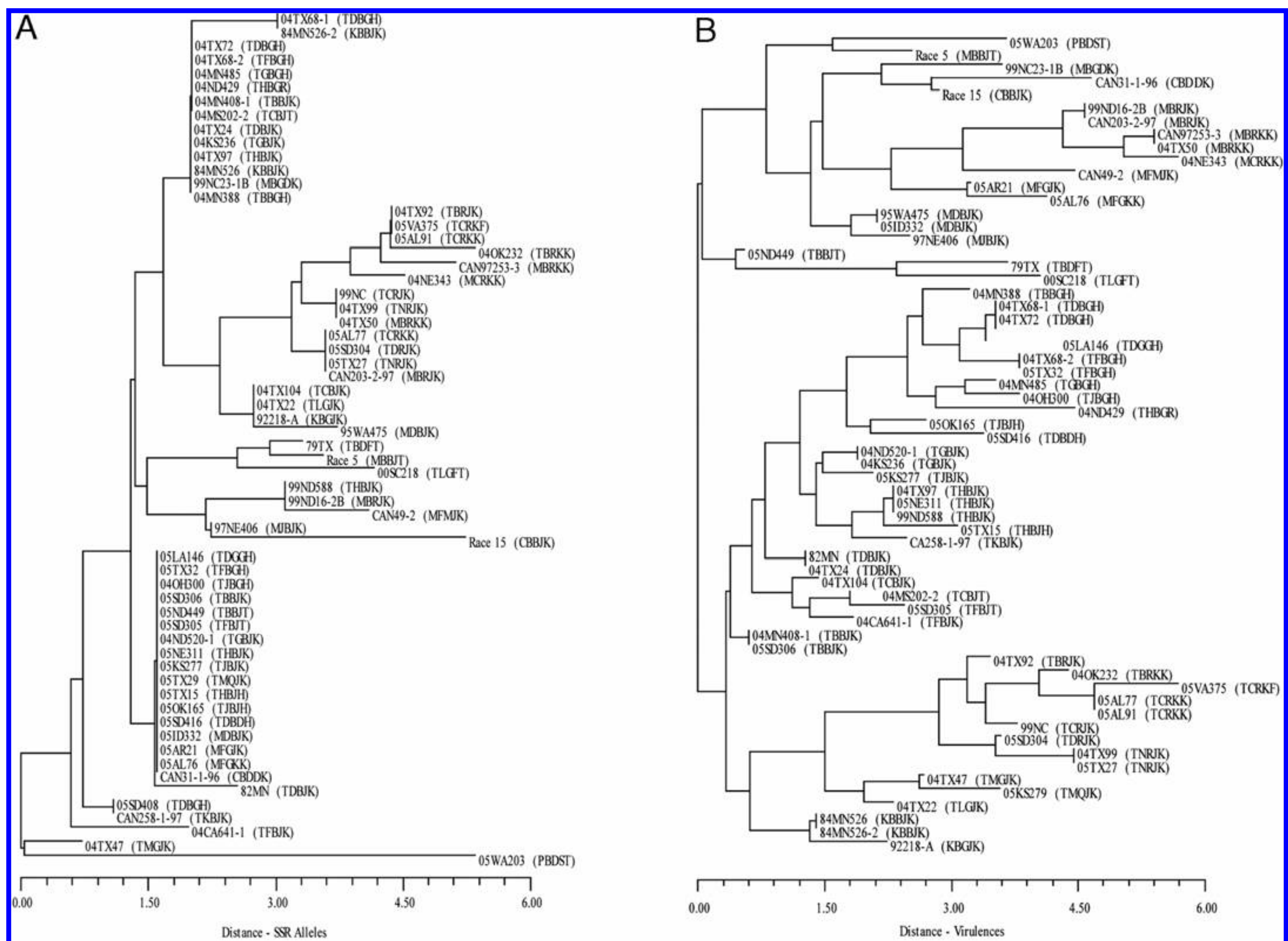

Fig. 3. Unrooted neighbor-joining tree dendrograms of *Puccinia triticina* isolates in simple sequence repeat (SSR) genotype group NA-5: **A,** SSR genotypes and **B,** virulence phenotypes.

does the single virulence difference between almost all pairs of nearest isolates. Isolates with virulence phenotypes identical to those in NA-3 were found in the mid 1990s in Mexico (R. Singh, *personal communication*) that is the likely origin of these isolates.

The isolates in NA-2 were primarily collected from the soft white winter wheat grown in Ontario, New York and Michigan and the soft white spring wheat in Alberta and Washington. Soft white winter and spring wheat cultivars are generally very susceptible to leaf rust, often lacking any effective *Lr* genes. Almost all of these isolates are avirulent to *Lr2a* and virulent to *Lr2c*. Isolates with this virulence combination are rarely found on the hard red wheat cultivars in the Great Plains region. Isolates avirulent to *Lr2a* and virulent to *Lr2c* were found on an annual basis in southern Ontario in the late 1980s and 1990s (18) and very likely overwinter in this region. Evidence for overwintering of isolates avirulent to *Lr2a* and virulent to *Lr2c* on soft red winter wheat lines in Pennsylvania was also previously described (34). Isolates that are avirulent to *Lr2a* and virulent to *Lr2c* may have some special adaptation to the soft white wheat and soft red wheat germplasm with regard to overwintering survival or other fitness attributes.

The isolates in NA-4 are likely derived from an introduction of *P. triticina* that was once very common in North America but has declined with the release of leaf rust-resistant spring and winter wheat. Four of the isolates in NA-4 (63-race 9 SBDBK, 60-04 MN364 SBDBK, 61-05 OK194 SBDK, and 62-05 MN365 SBDBT) are unique because these are virulent to *Lr1, Lr2a, Lr2c*, and *Lr17* and avirulent to *Lr3*. These virulence phenotypes are currently almost exclusively collected from *A. cylindricum* that grows as a weed in wheat fields in Kansas and Oklahoma. Isolates with the race 9 virulence phenotype were very common on wheat in Canada (16) and the United States (15) previous to the introduction of hard red winter wheat cultivars with *Lr3* in the early 1940s. Goatgrass may be a special host niche for these isolates because isolates with other virulence phenotypes are not collected from goatgrass. These four isolates and the other isolate in NA-4, 59-CAN 71-2-96 TDTDM, were also avirulent to the *Lr22b* adult plant resistance gene in Thatcher wheat (J. A. Kolmer, *unpublished data*).

In the absence of sexual recombination in *P. triticina* in North America, the clonal reproduction of urediniospores has resulted in nonrandom distribution of virulence among the different groups of SSR genotypes. Differences in virulence or avirulence to genes *Lr2a, Lr2c, Lr3bg, Lr17,* and *Lr28* were characteristic of the *P. triticina* SSR genotype groups in North America. Differences in virulence to these genes was also characteristic of the groups defined by RAPD (20) and AFLP (19) markers in the previous studies. In a population of *P. triticina* derived from sexually produced aeciospores (26), there was no association of virulence phenotypes with molecular markers. In asexual populations of *P. graminis* f. sp. *tritici* in the United States, there was a nonrandom distribution of virulence and isozyme markers (6); however, in *P. striiformis* in the United States which also reproduces asexually, there was little or no association between virulence and molecular markers (7). Association between molecular genotypes and virulence was also previously noted in asexual populations of *Magnaporthe grisea* in Colombia (25) and Arkansas (40).

The different SSR groups of *P. triticina* in North America have not been equally affected by host selection. The clearest example of this can be seen by comparing selection for virulence to different *Lr* genes in the NA-3 and NA-5 groups. Isolates in these two groups are subject to intense selection for virulence by leaf rust resistance genes in hard red winter and hard red spring wheat grown throughout the Great Plains. Virulence to *Lr17* increased in the southern Great Plains from <10% of all isolates in 1996 to >80% in 2001 due to selection by hard winter wheat cultivars with this gene (22,23). However, only 3 of the 62 isolates in NA-5 were virulent to *Lr17* whereas, in contrast, all of the isolates in NA-3 were virulent to this gene. In 2006 (22), nearly all of the isolates with virulence to *Lr17* had virulence phenotypes identical or similar to the isolates in NA-3. As another example, isolates with avirulence to *Lr14a* and *Lr20* were found only in NA-5 and were not found in NA-3. Evidently, short-term selective processes may not occur at equal rates at all loci across the different groups of *P. triticina*. Mutation followed by selection for virulence to certain leaf rust resistance genes may not occur to the same degree in different groups of *P. triticina* genotypes. Similar nonrandom associations between virulence to rice differentials and molecular lineage were also described in populations of *M. grisea* in the Philippines (41). In contrast with virulence to *Lr17*, isolates with virulence to *Lr24* have increased from <10% in 2001 to >70% in 2006 in the southern Great Plains (22,23) due to selection by hard red winter wheat cultivars with this gene, and isolates with virulence to *Lr24* occur in both NA-3 and NA-5.

The results of this study confirmed that the *P. triticina* population in North America has characteristics that are entirely consistent with a clonally reproducing population that comprises groups of isolates that differ for SSR genotypes and for virulence to certain resistance genes in wheat. Isolates within the different groups of *P. triticina* are highly related for SSR genotype and share characteristic virulences, with the pattern of variation indicating the single-step mutations that most commonly occur. The stability of different groups of isolates as defined over time with RAPD, AFLP, and, most recently, SSR markers indicates that nonselected molecular markers are quite stable. The nonrandom association of virulence phenotypes with SSR genotypes and the continued avirulence of some SSR genotypes to certain leaf rust resistance genes may offer a strategy to prolong the effectiveness of host resistance genes by combining two or more genes that individually provide resistance to different groups of *P. triticina*. A similar approach has been advocated for control of rice blast (41). Studies are currently underway to characterize *P. triticina* populations from other continents (24) in order to determine the genetic relationships between worldwide populations.

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