Molecular polymorphism and virulence phenotypes of the wheat leaf rust fungus *Puccinia triticina* in Canada

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Abstract: Sixty-nine *Puccinia triticina* isolates from the eastern region of Ontario and Quebec, the prairie region of Manitoba and Saskatchewan, and Alberta were tested for virulence to 22 near-isogenic Thatcher wheat lines in seedling tests and the Thatcher line with *Lr22b* in adult plant tests. The isolates were also tested for amplified fragment length polymorphism (AFLP) using 10 specific amplification primer pairs. Thirty-seven distinct virulence phenotypes were identified using the Thatcher lines and 69 molecular phenotypes were identified with 164 AFLP markers. The correlation of isolate grouping based on virulence phenotypes and AFLP phenotypes was 0.53. Almost all isolates from Manitoba and Saskatchewan with virulence to *Lr17* had AFLP phenotypes that differed significantly from isolates in the same region that were avirulent to *Lr17*. This indicated that isolates with virulence to *Lr17* are most likely a recent introduction to the prairie region. The presence of distinct groups of isolates based on virulence and AFLP variation provides evidence that a number of different *P. triticina* phenotypes have been introduced to North America.

Key words: *Puccinia recondita* f.sp. *tritici*, AFLP, specific virulence.

Résumé : Les auteurs ont déterminé la virulence de 69 isolats du *Puccinia triticina* provenant de la région de l'est, Ontario et Québec, de la région des prairies, Manitoba et Saskatchewan, et de l'Alberta; les essais de virulence ont été effectués sur 22 lignées de blé Thatcher presqu'isogéniques au stade plantule, et sur lignée Thatcher avec *Lr22b* sur plantes adultes. Ces isolats ont également été caractérisés par amplification du polymorphisme de la longueur des fragments (AFLP) d'ADN, en utilisant 10 paires d'amorces d'amplification spécifiques. On reconnaît 37 phénotypes distincts de virulence en utilisant les lignées Thatcher, et 69 phénotypes moléculaires avec les marqueurs AFLP 164. La corrélation de regroupement des isolats, basée sur les phénotypes de virulence et les phénotypes AFLP, est de 0,53. Presque tous les isolats du Manitoba et de la Saskatchewan avec une virulence pour *Lr17* ont des phénotypes qui diffèrent significativement d'isolats de la même région, qui sont avirulents pour *Lr17*. Ceci indique que les isolats avec virulence pour *Lr17* constituent très probablement une introduction récente dans la région des prairies. La présence de groupes distincts d'isolats, basés sur la virulence et la variation AFLP, fournit une preuve que plusieurs phénotypes différents du *P. triticina* ont été introduits en Amérique du Nord.

Mots clés : *Puccinia recondita* f.sp. *tritici*, AFLP, virulence spécifique.

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Introduction

Leaf rust of wheat (*Triticum aestivum* Thell.) caused by *Puccinia triticina* is found nearly everywhere that wheat is grown in North America and throughout the world (Samborski 1985). The degree of yield losses in wheat caused by leaf rust infection varies depending on the resistance of the wheat cultivar and the stage of crop development when initial infections occur (Chester 1946). Wheat breeding programs throughout the world have devoted considerable resources to the development of wheats that are genetically resistant to the leaf rust fungus (Roelfs et al. 1992).

Long-lasting or durable resistance in wheat to leaf rust has been difficult to achieve owing to the highly variable nature of *P. triticina* populations and the speed at which these populations adapt to resistance genes present in wheat cultivars. To date, over 45 leaf rust resistance genes in wheat have been characterized (McIntosh et al. 1995). Samborski and Dyck (1976) determined that avirulence genes in *P. triticina* and resistance genes in wheat interact in a gene-for-gene relationship. The widespread use of leaf rust resistant wheat cultivars places strong selection pressure on the *P. triticina* population for isolates with virulence to these resistance genes. Cereal rust populations have been subjected to "man guided evolution" (Johnson 1961) since modern plant breeding began in the early 1900s. Isolates of *P. triticina* with virulence to specific leaf rust resistance genes in wheat can increase in a regional population from 0 to >50% within 2 years (Kolmer 1999*b*). As a result, wheat

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cultivars that were highly resistant when initially released often suffer high leaf rust infections and subsequent yield losses within a few years.

Puccinia triticina reproduces in North America by the asexual production of dikaryotic urediniospores. Isolated reports of the sexual cycle on species of the alternate host *Thalictrum* in North America have been reported (Levine and Hildreth 1957); however, there is no direct evidence that sexual reproduction is important in maintaining variation in this fungus. Yet even in the absence of sexual recombination, *P. triticina* is well suited for maintaining populations that are genetically variable for virulence specificities. Mutation to virulence for specific resistance genes in wheat is an inevitable recurrent event in *P. triticina* populations, since the populations are extremely large. Wheat cultivars grown in a single region are often heterogeneous for specific leaf rust resistance genes. The differential selection imposed by the presence of various resistance genes helps to maintain a diversity of *P. triticina* virulence phenotypes. Since *P. triticina* is dikaryotic, heterozygosity is an additional source of genetic variation. Isolates of *P. triticina* that are heterozygous at virulence loci (Kolmer 1992*b*) would need only a single mutation to gain virulence to a specific resistance gene.

In North America, leaf rust infections are found on wheat in Texas and the southeastern states in late fall and winter and progress northward on wheat through the Great Plains and Ohio Valley regions by wind-dispersed urediniospores throughout the spring and summer months. Eventually, the leaf rust infection reaches the prairie provinces of Manitoba and Saskatchewan and the eastern provinces of Ontario and Quebec in Canada. Populations of *P. triticina* have been characterized for virulence phenotypes on a yearly basis in Canada since 1931 (Kolmer 1989*b*, 1991, 1999*b*). From the beginning of the surveys until the mid-1940s, the most common *P. triticina* virulence phenotypes in eastern Canada were also found in the prairies of Manitoba and Saskatchewan. These populations diverged for virulence phenotype owing to the effect of selection by resistant hosts in the prairie region and the continued cultivation of susceptible wheats in eastern Canada. Distinct populations of *P. triticina* virulence phenotypes are currently found in different geographical regions of Canada (Kolmer 1992*a*, 1998, 1999*a*, 1999*b*) and the United States (Leonard et al. 1992). *Puccinia triticina* infections survive the winter on the leaf rust susceptible winter wheats that are commonly grown in Ontario and Quebec, providing a local source of inoculum. Urediniospores can also migrate to eastern Canada from adjacent regions in the United States. The *P. triticina* populations in the prairies and eastern regions of Canada have differed for predominant virulence phenotypes owing to the differences in inoculum sources and resistance genes that have been used. The leaf rust population in Alberta and British Columbia may be epidemiologically isolated from other *P. triticina* populations in Canada and have been subjected to host selection by the wheats grown in this region and also from wheats grown in the adjacent region of the United States.

Puccinia triticina populations in North America have also been characterized for variation other than virulence markers. Kolmer et al. (1995) tested 64 isolates for randomly amplified polymorphic DNA (RAPD). Fifteen different RAPD phenotypes of the fungus were distinguished using 10 polymorphic DNA bands among 64 isolates. However, there were 37 virulence phenotypes of the fungus as determined by 19 near-isogenic differential lines of wheat. The RAPD markers grouped the *P. triticina* virulence phenotypes in a consistent manner; however, there was relatively little molecular polymorphism compared with the number of virulence phenotypes. In the Manitoba and Saskatchewan population, many isolates had identical RAPD phenotypes yet differed for virulence phenotype. RAPD markers have been useful for characterizing variation between regional groups of *P. triticina* virulence phenotypes but less so for detecting variation between closely related virulence phenotypes within or between regions.

In 1996 virulence phenotypes of *P. triticina* with virulence to *Lr17* increased rapidly in the prairie region of Canada (Kolmer 1998). By 1998 nearly 60% of isolates had virulence to *Lr17*. These isolates were likely selected by the winter wheat cultivar Jagger, which has *Lr17* and is widely grown in Kansas. There are a number of possibilities concerning the origin of isolates with virulence to *Lr17*. The first is that these isolates were derived by mutation and selection from the pre-existing *P. triticina* population in the prairie region. This would seem plausible since isolates in the prairie region were highly diverse for virulence phenotype even though there was very little RAPD variation among isolates in this population (Kolmer et al. 1995). Previous evidence had supported this hypothesis because isolates with virulence to *Lr17* differed from isolates avirulent to *Lr17* by only one RAPD band (Kolmer and Liu 2000). A second possibility is that isolates with virulence to *Lr17* arose by somatic recombination from the pre-existing virulence phenotypes. Park et al. (1999) attributed the origin of a new virulence phenotype in Australia to somatic recombination between isolates in two different groups of virulence phenotypes. In this case, it would be expected that isolates derived from somatic recombination would have virulence and molecular characteristics similar to at least two other groups of isolates in North America. An additional possibility is that isolates virulent to *Lr17* were introduced to the prairie region from a different part of North America. In this case, it might be expected that recently introduced *P. triticina* virulence phenotypes would be distinct with regard to molecular variation compared with isolates in the pre-existing prairie region population.

The objective of this research was to examine the molecular genetic diversity of *P. triticina* populations in Canada in greater detail using the amplified fragment length polymorphism (AFLP) technique (Vos et al. 1995). The AFLP technique has the potential to detect greater amounts of molecular variation in *P. triticina* compared with the RAPD method since a larger part of the genome is sampled in each experiment. In this study the objectives were to determine if the increased amount of variation generated by the AFLP technique could be used to detect molecular variation in isolates of different virulence phenotypes within and between geographical regions and also between isolates that had the same virulence phenotype. The last objective was to determine, by comparison of AFLP phenotypes, if isolates with virulence to *Lr17* arose by mutation and selection from the pre-existing *P. triticina* population in Manitoba and Saskatchewan, by somatic recombination, or by introduction of

these isolates to the prairie region from a different part of North America.

Materials and methods

Puccinia triticina isolates

Sixty-five single-uredinial isolates of *P. triticina* were selected from the 1996 and 1997 virulence surveys (Kolmer 1998, 1999*a*) in Canada. The selected isolates were representative of the predominant virulence phenotypes found in Canada for those years. The most common virulence phenotypes were represented by more than one isolate. The less frequent virulence phenotypes were represented by a single isolate. Single-uredinial isolation and characterization for virulence in the surveys were as previously described. Also included for historical perspective were four isolates collected prior to 1965. The isolates were increased on seedlings of the wheat cultivar Little Club, which had been treated with maleic hydrazide to enhance spore production, in 10-cm pots. A plastic cylinder was placed over each pot to prevent cross contamination of the isolates. Urediniospores from each isolate were increased and germinated for DNA extraction as previously described (Kolmer et al. 1995).

Determination of virulence phenotypes

All single-uredinial isolates were tested for virulence on seedling plants of Thatcher wheat lines that are near-isogenic for the leaf rust resistance genes *Lr1*-RL 6003, *Lr2a*-RL 6000, *Lr2c*-RL 6047, *Lr3*-RL 6002, *Lr3bg*-RL 6042, *Lr3ka*-RL 6007, *Lr9*-RL 6010, *Lr10*-RL 6004, *Lr11*-RL 6053, *Lr14a*-RL 6013, *Lr14b*-RL 6006, *Lr15*-RL 6052, *Lr16*-RL 6005, *Lr17*-RL 6008, *Lr18*-RL 6009, *Lr20*-RL 6092, *Lr23*-RL 6012, *Lr24*-RL 6064, *Lr26*-RL 6078, *Lr28*-RL 6079, *Lr30*-RL 6049, and *LrB*-RL 6051. Thatcher-RL 6161 was included as a susceptible control in seedling tests. Inoculation, incubation, and greenhouse conditions were as previously described (Long and Kolmer 1989). Infection types 0 (no fungal sporulation) to 2^+ (relatively little sporulation) were classified as avirulent, and infection types 3 (abundant sporulation) and 4 (very abundant sporulation) were classified as virulent. Virulence phenotypes of the isolates were described with a hexadecimal-based system (Long and Kolmer 1989). The first three letters correspond to the avirulent–virulent infection types on the three sets of four differentials in the wheat leaf rust nomenclature: *Lr1, Lr2a, Lr2c, Lr3; Lr9, Lr16, Lr24, Lr26;* and *Lr3ka, Lr11, Lr17, Lr30*, respectively. The fourth letter describes avirulent or virulent infection types on differentials with genes *LrB, Lr3bg, Lr10,* and *Lr14a*. The fifth letter describes infection types on differentials with genes *Lr14b, Lr15, Lr18,* and *Lr20*. The sixth letter describes infection types on differentials with genes *Lr23* and *Lr28*, with two imaginary differentials always set to avirulent. Isolates were also tested for virulence to adult plants of Thatcher, which has the adult plant resistance gene *Lr22b.*

Determination of AFLP phenotypes

Urediniospores collected from infected susceptible wheat seedlings were germinated on the surface of a buffer solution. Mats of germinated spores were collected, and mats of the individual *P. triticina* isolates were ground with sterilized sand in liquid N_2. The procedures for DNA isolation were essentially the same as previously described (Kolmer et al. 1995) except that a phenol–chloroform extraction followed the incubation with CTAB buffer. DNA concentration was determined by spectrophotometer readings at A_{260} and A_{280}. The DNA solution of each isolate was diluted to 50 ng/μL.

The materials and protocols for the restrictions, pre-amplification, and selective amplifications were adapted from the AFLP™ Analysis System II (Gibco BRL-Life Technologies, Gaithersburg, Md.). For the first restriction digest, 2.0 μL of 10× React 1 buffer, 0.50 μL of *Mse*I (5 U/μL), 12.50 μL of ddH_2O, and 5.0 μL of *P. triticina* DNA were incubated at 37°C for 2 h. For the second digest, 8.0 μL of 10× React 3 buffer, 0.25 μL of *Eco*RI (10 U/μL), and 51.75 μL of ddH_2O were added to the first restriction mixture. The total restriction mixture was incubated at 37°C for 2 h. The two DNA single strands for both the M and E adapters were combined and then annealed at 65, 37, and 22°C for 10 min at each temperature. For the ligation, 16 μL of 5× ligation buffer, 1.0 μL of 50 pmol annealed M-adapter, 1.0 μL of 5 pmol annealed E-adapter, 1.0 μL of T4 DNA liagase, 21.0 μL of ddH_2O, and 40 μL of restricted DNA mixture were combined and incubated at 22°C for 2 h. The restricted and ligated DNA mixture (80 μL) was then diluted with 160 μL of ddH_2O to a concentration of 0.52 ng/μL. For the pre-amplification, 2.5 μL of 10× PCR buffer and $MgCl_2$, 2.0 μL of dNTPs, 1.0 μL of E primer (30 ng/μL), 1.0 μL of M + C primer (30 ng/μL), 0.20 μL of *Taq* polymerase (5 U/μL), 13.5 μL of ddH_2O, and 4.8 μL of restricted-ligated DNA were combined. The pre-amplification mixtures were placed in an MJ thermocycler (MJ Research, Watertown, Mass.) for 20 cycles of 94°C for 30 s, 56°C for 60 s, and 72°C for 60 s. The pre-amplification products (25 μL) were diluted with 225 μL of ddH_2O. For the selective amplification, 2.0 μL of 10× PCR buffer and $MgCl_2$, 1.6 μL of dNTPs, 0.2 μL of *Taq* polymerase, 2.0 μL of *Eco*RI primer (2.5 ng/μL), 2.0 μL of *Mse*I primer (15 ng/μL), 10.2 μL of ddH_2O, and 2.0 μL of pre-amplification product were combined. The *Eco*RI primer had two selective bases, and the *Mse*I primer had three selective bases. The selective amplification mixtures were cycled at 94°C for 60 s, 65°C for 60 s, and 72°C for 90 s for 10 cycles with decreasing temperature of 1°C for each cycle for the middle step. The mixtures were then cycled for 94°C for 30 s, 56°C for 30 s, and 72°C for 60 s for 23 cycles. Sequencing dye (20 μL) was added to each selective amplification product followed by denaturation at 90°C for 10 min. From each selective amplification product, 7.5 μL was loaded on a 5.0% denaturing polyacrylamide gel cast in a Sequi-Gen GT® nucleic acid electrophoresis cell (BIO-RAD Life Science, Mississauga, Ont.) with 1× TBE buffer. One lane of pGEM marker was also loaded with each gel. The gels were pre-run for 1 h and run at a constant 85 W (approximately 2000 V and 45 mA) for 2–3 h. The DNA bands in the gels were developed using the Silver Sequence™ DNA Sequencing system (Promega, Madison, Wisc.). After development, the gels were scored as plus or minus for polymorphic DNA bands using a light box.

The ten primer pairs (Table 1) used in this study were selected from screening 20 primer pairs for DNA polymorphism on 45 *P. triticina* isolates from different regions of the world. The ten pairs chosen had the highest number of repeatable, easily scored polymorphic DNA bands. DNA polymorphism generated by the 10 primer pairs was confirmed twice in separate gels using different specific amplification mixtures.

Data analysis

The 69 *P. triticina* isolates were analyzed individually and on a group basis for virulence and molecular polymorphism. From results of previous studies, isolates were placed into five groups based on virulence to genes *Lr2a, Lr2c, Lr17,* and *Lr22b.* Groupings of North American *P. triticina* isolates based on virulences to these genes generally correlated with groupings based on RAPD phenotypes (Kolmer et al. 1995; Kolmer and Liu 2000).

The average number of virulence differences within and between pairs of isolates in the different groups was determined using the simple distance coefficient:

$$d/n$$

where d is the number of virulence differences between paired isolates and n is the total number of virulences.

Table 1. Sequences, number of polymorphic DNA bands, and correlation of DNA polymorphism with virulence polymorphism of selective amplification primer pairs used in the amplified fragment length polymorphism analysis of *Puccinia triticina* isolates from Canada.

Selective primer pair	*Eco*RI primer	*Mse*I primer	Number of polymorphic bands	Correlation with virulence polymorphism[a]
1	TA	CTC	15	0.44
2	AG	CTG	12	0.48
3	AG	CAA	17	0.39
4	TA	CAA	18	0.40
5	AG	CAG	11	0.35
6	AC	CAA	15	0.35
7	AC	CAC	21	0.18
8	AA	CAA	22	0.47
9	TC	CTA	12	0.20
10	AA	CAC	21	0.41
Total			164	0.53

[a]Dissimilarity matrices of AFLP polymorphism generated by the selective primer pairs were correlated with the dissimilarity matrix generated by the virulence polymorphism using MXCOMP in NTSYS -pc 1.8.

The average AFLP difference within and between pairs of isolates in the different groups was determined using the complement of the Dice coefficient (Sneath and Sokal 1973):

$$1 - (2a/2a + b + c)$$

where the *a*'s are the bands present in isolates i and j, *b* is the band present in isolate i but absent in isolate j, and *c* is the band present in isolate j but absent in isolate i.

Separate two-dimensional principal coordinate plots of the individual isolates based on virulence and AFLP phenotype were developed using NTSYS-pc version 1.8 (Exeter Software, Setauket, N.Y.). The two-dimensional eigenvectors for individual isolates were derived by transforming the symmetric dissimilarity matrices with DCENTER. The double centred matrices were then used with EIGEN to calculate the eignevectors. MXCOMP was used to determine degree of correlation between matrices.

The matrices of the average virulence and AFLP differences between the five groups of isolates were used to construct dendrograms using SAHN clustering in the UPGMA program in NTSYS-pc. COPH was used to derive cophenetic value matrices from the dendrograms, which were then correlated with the original dissimilarity matrices. Three-dimensional principal coordinate plots of the differences between isolate groups were also constructed using the DCENTER and EIGEN programs.

Results

Virulence phenotypes

Thirty-seven virulence phenotypes were distinguished among the 69 isolates by avirulent–virulent infection types to 22 Thatcher near-isogenic lines (Table 2). Forty-two isolates that were collected throughout Canada were placed into a single group designated here as group 1. These isolates were all avirulent or virulent to both resistance genes *Lr2a* and *Lr2c*, avirulent to *Lr17*, and virulent to *Lr22b*. Almost all of the group 1 isolates were also avirulent to genes *LrB* and *Lr3bg* and virulent to *Lr28*. Phenotypes with virulence to either *Lr11*, *Lr3ka*, *Lr24*, or *Lr26* (KFBFRQ, MBGFRQ, MBRFRQ, MBRFTQ, MDBFRQ, MDMFRQ, MDQFRQ, MDRFRQ, TBGFRQ, TDGFRQ (Table 2)) have been common in Manitoba and Saskatchewan since 1993. Of these phenotypes, MBRFRQ has been a predominant virulence phenotype in the prairie region that has also been found in eastern Canada and Alberta. Virulence phenotypes in group 1 with virulence to *Lr16* (MJBFRQ, TGBFRQ, THBFRQ, TJBFRQ, TKBFRQ) have been found mostly in Manitoba since 1996. Other phenotypes in this group (BBBCRB, CHBFGQ, MBBTRQ) were found throughout Canada in the 1950s and 1960s (Kolmer 1989*b*). There were 20 virulence phenotypes in the 42 group 1 isolates.

Isolates in group 2 were collected from eastern Canada, Alberta, and Saskatchewan. These isolates were all avirulent to *Lr2a*, virulent to *Lr2c*, avirulent to *Lr17*, and virulent to *Lr22b*. Eight virulence phenotypes were distinguished among the 10 isolates in group 2. PBLNRQ has been the most common phenotype in Ontario since 1987 (Kolmer 1999*b*). NBBNPQ has been found on a regular basis in Alberta. One isolate of FDMDTQ was found in 1997 in Saskatchewan. Isolates of this phenotype or other phenotypes in group 2 are rarely found in the prairie region of Manitoba and Saskatchewan. Isolates in group 3 were collected from Alberta, Saskatchewan, and Manitoba. These isolates were composed of five virulence phenotypes that were all avirulent to *Lr2a* and *Lr2c*, virulent to *Lr17*, and virulent to *Lr22b*. Almost all of these isolates were also virulent to *LrB*, *Lr3bg*, and avirulent to *Lr28*. MBDTRL and MCDTRL have been the most common phenotypes in Manitoba and Saskatchwan with virulence to *Lr17*. Group 4 consisted of two isolates with virulence phenotype PBDQHQ, which is avirulent to *Lr2a*, virulent to *Lr2c*, virulent to *Lr17*, and virulent to *Lr22b*. One isolate of PBDQHQ came from Alberta and the other from Manitoba. PBDQHQ has been collected in previous years from Alberta and was found in Manitoba only in 1996. Isolates in group 5 were avirulent to *Lr22b*, the adult plant resistance gene in Thatcher. Phenotypes SBGGRG and TDTRGG were collected from Quebec and Saskatchewan, respectively, in 1996, and SBDDRG was collected from British Columbia in 1959.

The 37 virulence phenotypes were graphed using two-dimensional principal coordinates (Fig. 1A). The first two dimensions accounted for 65% of the variation. With two exceptions, the virulence phenotypes in group 1 were placed together within one region of the graph between –0.14 and –0.02 for coordinate 1 and between 0.16 and –0.18 for coor-

Table 2. Isolates of *Puccinia triticina* from Canada used in study of amplified fragment length polymorphism.

Virulence phenotype[a]	Virulences (*Lr* genes)	Region of collection[b]	Isolate group	Number of isolates
BBBCRB	*14a, 14b, 15, 20*	BC	1	1
BCBFTQ	*26, 10, 14a, 14b, 15, 18, 20, 23, 28*	PQ	1	1
CHBFRG	*3, 16, 26, 10, 14a, 14b, 15, 20, 28*	MB	1	1
KFBFRQ	*2a, 2c, 3, 24, 26, 10, 14a, 14b, 15, 20, 23, 28*	SK	1	1
MBBTRQ	*1, 3, B, 3bg, 10, 14a, 14b, 15, 20, 23, 28*	MB	1	1
MBGFRQ	*1, 3, 11, 10, 14a, 14b, 15, 20, 23, 28*	AB, ON	1	2
MBRFRQ	*1, 3, 3ka, 11, 30, 10, 14a, 14b, 15, 20, 23, 28*	AB, SK, MB, ON, PQ	1	7
MBRFTQ	*1, 3, 3ka, 11, 30, 10, 14a, 14b, 15, 18, 20, 23, 28*	PQ	1	1
MDBFRQ	*1, 3, 24, 10, 14a, 14b, 15, 20, 23, 28*	SK, MB	1	2
MDMFRQ	*1, 3, 24, 3ka, 30, 10, 14a, 14b, 15, 20, 23, 28*	MB	1	1
MDQFRQ	*1, 3, 24, 3ka, 11, 10, 14a, 14b, 15, 20, 23, 28*	MB	1	1
MDRFRQ	*1, 3, 24, 3ka, 11, 30, 10, 14a, 15, 20, 23, 28*	SK	1	3
MJBFRQ	*1, 3, 16, 24, 10, 14a, 14b, 15, 20, 23, 28*	MB	1	3
TDGFRQ	*1, 2a, 2c, 3, 24, 11, 10, 14a, 14b, 15, 20, 23, 28*	SK	1	1
TGBFRQ	*1, 2a, 2c, 3, 16, 10, 14a, 14b, 15, 20, 23, 28*	SK, MB	1	6
THBFRQ	*1, 2a, 2c, 3, 16, 26, 10, 14a, 14b, 15, 20, 23, 28*	MB	1	3
TJBFRQ	*1, 2a, 2c, 3, 16, 24, 10, 14a, 14b, 15, 20, 23, 28*	PQ	1	1
TKBFRQ	*1, 2a, 2c, 3, 16, 24, 26, 10, 14a, 14b, 15, 20, 23,28*	MB, ON, PQ	1	4
TLGKTQ	*1, 2a, 2c, 3, 9, 11, 3bg, 10, 14a, 14b, 15, 18, 20, 23, 28*	ON	1	1
TBGFRQ	*1, 2a, 2c, 3, 11, 10, 14a, 14b, 15, 20, 23, 28*	SK	1	1
DBBNML	*2c, B, 10, 14b, 20, 23*	PQ	2	1
FCMPRQ	*2c, 3, 26, 3ka, 30, B, 10, 14a, 14b, 15, 20, 23, 28*	PQ	2	1
FDMDTQ	*2c, 3, 24, 3ka, 30, 10, 14b, 15, 18, 20, 23, 28*	SK	2	1
NBBNPQ	*1, 2c, B, 10, 14b, 18, 20, 23, 28*	AB	2	2
PBLNRQ	*1, 2c, 3, 3ka, B, 10, 14b, 15, 20, 23, 28*	ON	2	1
PBMSRQ	*1, 2c, 3, 3ka, 30, B, 3bg, 10, 14b, 15, 20, 23, 28*	ON	2	1
PBRSRQ	*1, 2c, 3, 3ka, 11, 30, B, 3bg, 10, 14b, 15, 20, 23, 28*	ON	2	1
PBRSTQ	*1, 2c, 3, 3ka, 11, 30, B, 3bg, 10, 14b, 15, 18, 20, 23, 28*	ON	2	2
CCDPRL	*3, 26, 17, B, 10, 14a, 14b, 15, 20, 23*	AB	3	1
MBDTRL	*1, 3, 17, B, 3bg, 10, 14a, 14b, 15, 20, 23*	SK, MB	3	6
MCDTRL	*1, 3, 26, 17, B, 3bg, 10, 14a, 14b, 15, 20, 23*	SK, MB	3	3
MGDTRL	*1, 3, 16, 17, B, 3bg, 10, 14a, 14b, 15, 20, 23*	MB	3	1
MHDTRL	*1, 3, 16, 26, 17, B, 3bg, 10, 14a, 14b, 15, 20, 23*	MB	3	1
PBDQHQ	*1, 2c, 3, 17, B, 3bg, 15, 20, 23, 28*	AB, MB	4	2
SBGGRG	*1, 2c, 2a, 11, 3bg, 14b, 15, 20, 28*	PQ	5	1
SBDDRG	*1, 2a, 2c, 17, 10, 14b, 15, 20, 28*	BC	5	1
TDTRGG	*1, 2a, 2c, 3, 24, 3ka, 11, 17, 30, B, 3bg, 14a, 15, 28*	SK	5	1

[a]*Prt* nomenclature according to Long and Kolmer (1989).
[b]AB, Alberta; BC, British Columbia; MB, Manitoba; ON, Ontario; PQ, Quebec; SK, Saskatchewan.

dinate 2. Virulence phenotypes MBBTRQ and BBBCRB had coordinates of 0.10, 0.01 and 0.07, –0.10, respectively, which separated these two isolates from the other group 1 isolates. Seven of the virulence phenotypes in group 2 were scattered through the graph between 0.15 and –0.02 for coordinate 1 and between 0.27 and –0.07 for coordinate 2. Virulence phenotype FDMDTQ at coordinates –0.25, 0.10 was separated from the other group 2 isolates. The five virulence phenotypes in group 3 were clustered together between 0.17 and 0.20 for coordinate 1 and between –0.13 and 0.001 for coordinate 2. The two isolates in group 4 with virulence phenotype PBDQHQ had the coordinates 0.22, 0.04, which placed these isolates close to the five virulence phenotypes in group 3. The three virulence phenotypes in group 5 were scattered throughout the graph. Phenotype SBDDRG was at coordinates 0.001, 0.28; phenotype SBGGRC was at coordinates –0.03, 0.04; and phenotype TDTRGG was at coordinates 0.00, –0.08.

Within the five groups of isolates, the weighted average difference for virulence was 0.21 ± 0.11 (simple distance coefficient), and between isolates among the groups the weighted average difference was 0.36. Phenotypes in group 3 and the three isolates in group 5 differed the most with an average of 0.41, while the phenotypes in group 3 and the two isolates in group 4 differed the least with an average of 0.26. In the UPGMA clustering in Fig. 2A, three of the isolate groups could be considered distinct for virulence phenotype. The phenotypes in group 1, the phenotypes in group 2, and the phenotypes in group 5 differed significantly for virulence variation with average branch points greater than the upper 95% confidence level of the mean within-group variation for virulence. The phenotypes in group 3 and the two

Fig. 1. Two-dimensional principal coordinate plots of 69 *Puccinia triticina* isolates from Canada. (A) Principal coordinates based on virulence differences to 22 Thatcher near-isogenic lines. (B) Principal coordinates based on differences in 164 digit AFLP phenotype. Group 1 isolates are avirulent or virulent to both resistance genes *Lr2a* and *Lr2c* and avirulent to *Lr17*. Group 2 isolates are avirulent to *Lr2a*, virulent to *Lr2c*, and avirulent to *Lr17*. Group 3 isolates are avirulent to resistance genes *Lr2a*, avirulent to *Lr2c*, and virulent to *Lr17*. Group 4 isolates are avirulent to *Lr2a*, virulent to *Lr2c*, and virulent to *Lr17*. Group 5 isolates are avirulent to *Lr22b*.

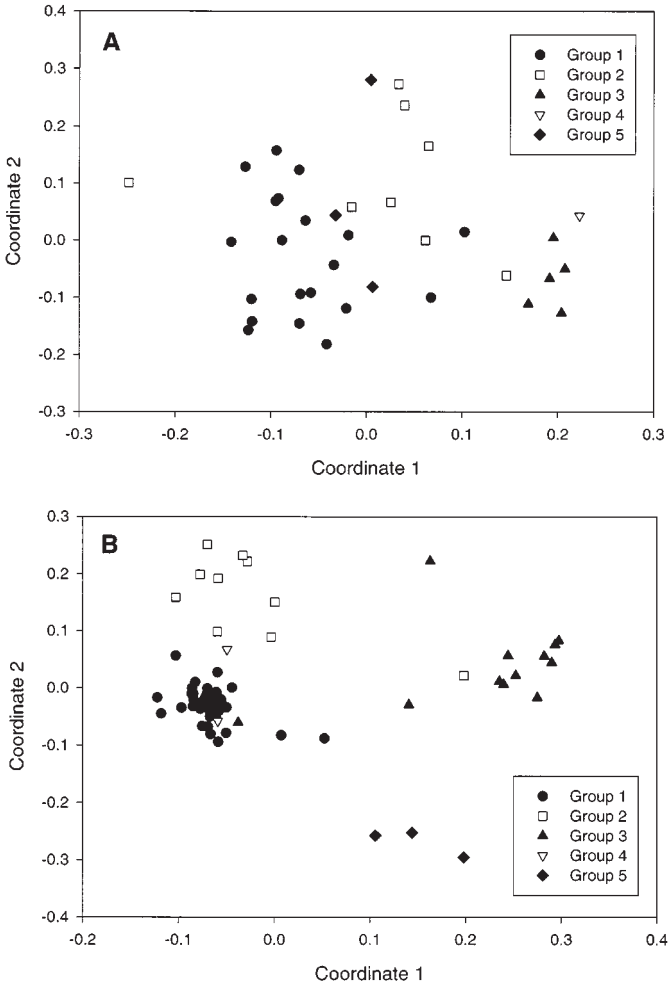
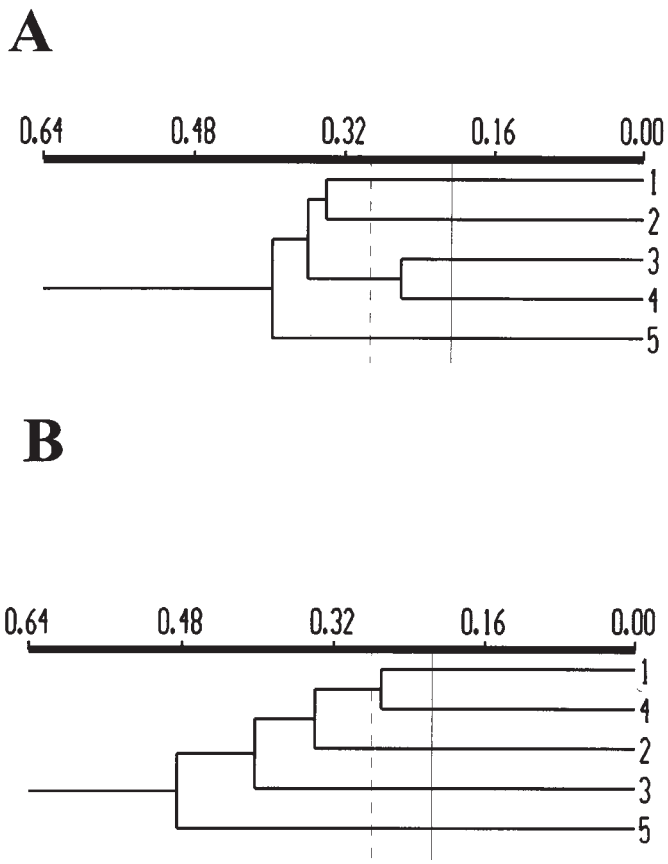


Fig. 2. Dendrograms of 69 *Puccinia triticina* isolates from Canada in five groups. The unweighted pair group arithmetic mean method in the SAHN program of NTSYS-pc version 1.8 was used. The solid vertical line indicates the average variation within populations, the dotted line indicates the upper 95% confidence limit of the average within population variation. (A) Dendrogram of simple distance differences between groups based on virulence to 22 Thatcher near-isogenic lines. (B) Dendrogram of (1-Dice coefficient) differences between groups based on 164 digit AFLP phenotype. Group 1 isolates are avirulent or virulent to both resistance genes *Lr2a* and *Lr2c*, and avirulent to *Lr17*. Group 2 isolates are avirulent to *Lr2a*, virulent to *Lr2c*, and avirulent to *Lr17*. Group 3 isolates are avirulent to resistance gene *Lr2a*, avirulent to resistance gene *Lr2c*, and virulent to *Lr17*. Group 4 isolates are avirulent to *Lr2a*, virulent to *Lr2c*, and virulent to *Lr17*. Group 5 isolates are avirulent to *Lr22b*.



isolates in group 4 did not differ significantly for virulence variation, but when combined together they differed significantly from the other three groups. The cophenetic correlation of the dendrogram with the original data matrix was 0.74. In the three-dimensional principal coordinate representation of the average differences between groups of isolates, the phenotypes in group 1, the phenotypes in group 2, and the phenotypes in group 5 were spaced apart in the first two dimensions. The phenotypes in group 3 were closely grouped to the two isolates in group 4 in the first two dimensions and differed mostly in the third dimension (Fig. 3A). The first three dimensions accounted for 97% of the variation.

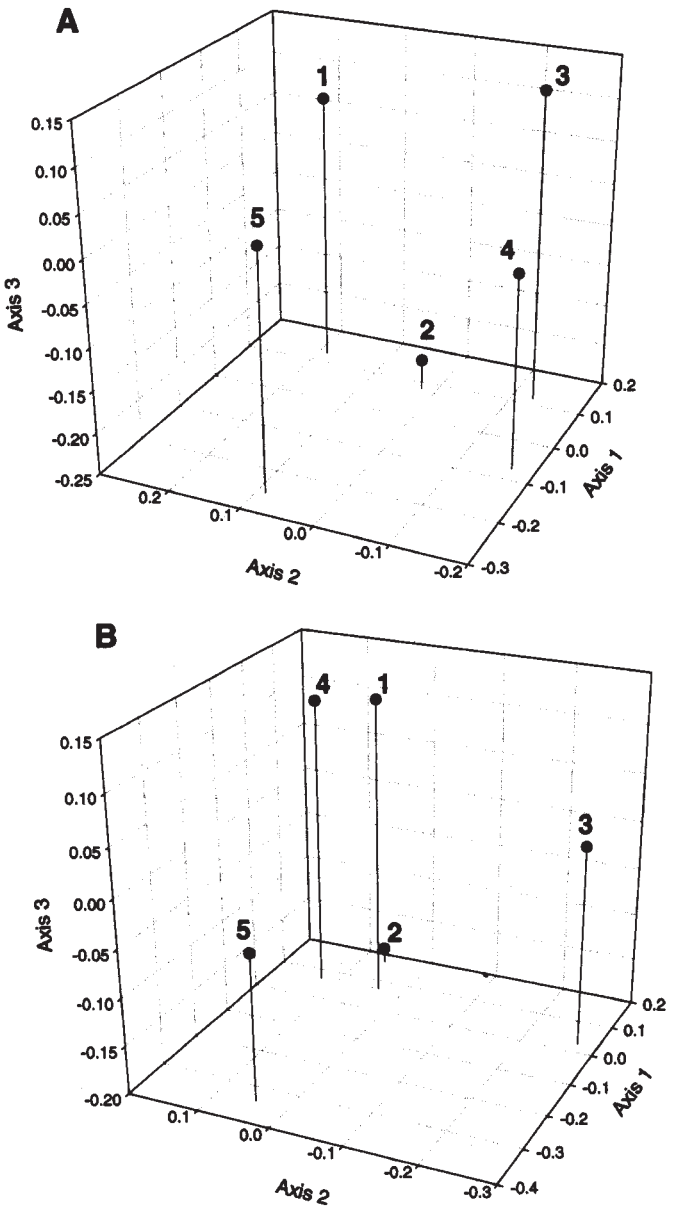
AFLP phenotypes

After selective amplification, the ten primer pairs individually produced between 11 and 22 polymorphic DNA bands among the 69 *P. triticina* isolates, with a total of 164 polymorphic bands (Table 1). All of the isolates had a unique AFLP phenotype; isolates with identical virulence phenotypes differed for AFLP phenotypes. Distance matrices (1 – Dice coefficient) of all pairs of AFLP phenotypes generated by the individual selective primer pairs were compared with the virulence distance matrix. Correlation of matrices based on AFLP phenotypes with the virulence phenotypes varied from 0.18 for primer pair 7 to 0.47 for primer pair 8. The highest AFLP-virulence correlation of 0.53 was obtained when the matrix based on the complete AFLP phenotype of 164 bands was compared with the virulence phenotype matrix.

The 69 isolates were clustered by AFLP phenotypes using two-dimensional principal coordinates (Fig. 1B). The first two dimensions accounted for 51% of the variation. Most isolates in group 1 were clustered between –0.12 and –0.04 for coordinate 1 and between –0.09 and 0.06 for coordinate 2. Two isolates with virulence phenotype TJBFRQ and TKBFRQ were slightly outside of other group 1 isolates with coordinates of 0.01, –0.08; and 0.05, –0.09, respectively. Nine of the group 2 isolates graphed between –0.10 and 0.00 for coordinate 1 and between 0.08 and 0.25 for coordinate 2. One group 2 isolate with virulence phenotype PBRSTQ had an AFLP phenotype that placed it close to the group 3 isolates at coordinates 0.20, 0.02. All but one of the group 3 isolates had distinct AFLP phenotypes compared with the isolates in the other groups. Nine of the group 3 isolates had AFLP phenotypes that graphed between 0.23 and 0.30 for coordinate 1 and between –0.02 and 0.08 for coordinate 2. Two other group 3 isolates with virulence phenotypes CCDPRL and MCDTRL had coordinates of 0.16, 0.22 and 0.14, –0.03, respectively, which were slightly outside of the other group 3 isolates. One group 3 isolate designated as virulence phenotype MBDTRL-43 had an AFLP phenotype very close to the group 1 isolates with coordinates of –0.04, –0.06. The two isolates in group 4 had AFLP phenotypes that graphed at coordinates –0.05, 0.07 and –0.06, –0.06, which placed these isolates close to the isolates in groups 1 and 2. The three isolates in group 5 had AFLP phenotypes that graphed at coordinates 0.14, –0.25; 0.11, –0.26; and 0.20, –0.30, which separated these isolates from the other groups.

The weighted average AFLP difference among isolates within the five groups was 0.21 ± 0.04, and the weighted average AFLP difference between isolates in different groups was 0.38. The isolates in group 3 and the isolates in group 5 differed the most for AFLP phenotype at an average of 0.49, while the isolates in group 1 and the two isolates in group 4 differed the least at 0.27. The group 3 isolates had the lowest within group AFLP variation of 0.17. In Fig. 2B, isolates in groups 2, 3, and 5 differed significantly for AFLP variation with average branch points greater than the upper 95% confidence level of the mean within group AFLP difference. The isolates in groups 1 and 4 did not differ significantly for AFLP variation but were significantly different when compared with the isolates in the other groups. The cophenetic correlation of the dendrogram with the data matrix was 0.95. In the three-dimensional principal coordinates representation of the average AFLP differences between isolates in different groups, isolates in groups 3 and 5 were widely spaced apart in the first two dimensions, while isolates in groups 1 and 4 were close together (Fig. 3B). Isolates in group 2 differed from those in groups 1 and 4 isolates mostly in the third dimension. The first three dimensions accounted for 90% of the variation.

Fig. 3. Three-dimensional principal coordinates of 69 *Puccinia triticina* isolates from Canada in five groups. (A) Principal coordinates based on average difference in virulence to 22 Thatcher near-isogenic lines. (B) Principal coordinates based on average differences (1-Dice coefficient) in 164 polymorphic DNA bands generated by the AFLP technique. Group 1 isolates are avirulent or virulent to resistance genes *Lr2a* and *Lr2c* and avirulent to *Lr17*. Group 2 isolates are avirulent to *Lr2a*, virulent to *Lr2c*, and avirulent to *Lr17*. Group 3 isolates are avirulent to resistance gene *Lr2a*, avirulent to resistance gene *Lr2c*, and virulent to *Lr17*. Group 4 isolates are avirulent to *Lr2a*, virulent to *Lr2c*, and virulent to *Lr17*. Group 5 isolates are avirulent to *Lr22b*.



Discussion

This study showed that isolates of *P. triticina* that were closely related or identical for virulence phenotype could be distinguished using the AFLP technique to detect molecular polymorphism. In this study, all isolates had unique AFLP phenotypes whereas in the previous study with RAPD markers there was limited molecular variation relative to the abundant virulence polymorphism (Kolmer et al. 1995). In the RAPD study there were 27 virulence phenotypes and only eight RAPD phenotypes among a group of 44 isolates that were equivalent to the group 1 isolates in this study. Isolates with different virulence phenotypes often had identical RAPD phenotypes, or differed by only one or two RAPD

bands. Of nine isolates from the eastern region, which were equivalent to isolates in group 2, there were six virulence phenotypes, with only two RAPD phenotypes that differed by two polymorphic DNA bands. The RAPD variation easily distinguished between isolates from different groups of virulence phenotypes; however, it was inadequate to distinguish among isolates within these groups. The AFLP technique was able to distinguish between *P. triticina* isolates within a group of isolates that were closely related or identical for virulence phenotype, as well as between isolates from different virulence phenotype groups.

In Manitoba and Saskatchewan, there are at least two major groups of isolates based on virulence and AFLP phenotypes. Almost all isolates in group 3 differed from isolates in group 1 for virulence to *LrB*, *Lr3bg*, *Lr28*, and *Lr17*, and also for AFLP variation. It is likely that most of the isolates in group 3 that have become common in Manitoba and Saskatchewan since 1996 were introduced to this region from another part of North America. Two other cereal rust pathogens have recently been introduced to new areas in North America. In the early 1990s, barley stripe rust (*Puccinia striiformis* f.sp. *hordei*) migrated from Mexico to the Pacific Northwest of the United States through Texas, Oklahoma, Colorado, and California (Chen et al. 1995). The *Puccinia graminis* (wheat stem rust) virulence phenotype QCCJ, which is virulent to the *Rpg1* resistance gene in cultivated barley, became prevalent in the Great Plains of North America in the early 1990s. Allozyme and RAPD phenotypes of QCCJ isolates compared with the previous *P. graminis* virulence phenotypes suggested that QCCJ was introduced to the Great Plains region from the Pacific Northwest (Roelfs et al. 1997; Fox et al. 1995). It is also possible that isolates of *P. triticina* with virulence and molecular characteristics of group 3 have been present in the Great Plains for a number of years but became common recently only owing to the selection of these isolates by the wheat cultivar Jagger and the migration of that inoculum into Canada. Previous to the release of Jagger, isolates with virulence to *Lr17* have been found at very low levels in the prairie population of *P. triticina* in Canada (Kolmer 1989*a*, 1993); however, these isolates were avirulent to resistance gene *LrB*.

If an introduction of *P. triticina* has occurred recently, the origin of the group 3 isolates with virulence to *LrB*, *Lr3bg*, and *Lr17*, and avirulence to *Lr28* is not obvious. A single isolate with virulence phenotype PBRSTQ in group 2 had an AFLP phenotype close to most of the group 3 isolates. However, PBRSTQ differs from most of the group 3 isolates by at least eight virulences, and since there is no evidence for simultaneous multiple mutations affecting virulence in *P. triticina*, it would be unlikely that the group 3 isolates were derived from any group 2 isolates. *Puccina triticina* isolates from Slovakia, Spain, Israel, and Great Britain with virulence to *LrB*, *Lr3bg*, and *Lr17*, and avirulence to *Lr28* have been described; however, none of these isolates had the same RAPD phenotypes as the group 3 isolates in Canada (Kolmer and Liu 2000). One isolate from Brazil had the same RAPD phenotype as the group 3 isolates from Canada but differed for virulence to *LrB* and *Lr28*. *Puccina triticina* isolates with virulence to *Lr17* have been used in Mexico at Centro Mejormiento Maize y Trigo (CIMMYT) for screening of bread wheats for leaf rust resistance; however, the RAPD and AFLP phenotypes of these isolates in relation to other North American isolates have not yet been determined.

A single group 3 isolate from Saskatchewan, MDBTRL-43, had an AFLP phenotype very close to the group 1 isolates, and shared virulences to genes *Lr17*, *LrB*, and *Lr3bg* with the PBDQHQ isolates in group 4. Isolates in group 1, the two PBDQHQ isolates, and MBDTRL-43 all had very similar AFLP phenotypes. MBDTRL-43 is avirulent to *Lr28* while PBDQHQ and all group 1 isolates except BBBCRB, which was collected from British Columbia in 1954, are virulent to *Lr28*. Somatic recombination between isolates in group 4 with virulence to *Lr17*, *LrB*, and *Lr3bg*, and isolates in group 1 with avirulence to *Lr28* may have resulted in an isolate with a MBDTRL virulence phenotype and an AFLP phenotype like isolates in groups 1 and 4. Avirulence to *Lr28* is very rare in *P. triticina* isolates from North America. However since phenotype BBBCRB is avirulent to *Lr28*, other phenotypes avirulent to *Lr28* and with an AFLP phenotype like the group 1 isolates may also exist in areas such as British Columbia, which has not been extensively sampled for wheat leaf rust in the annual virulence surveys. Another possibility is that somatic recombination between isolates that are both virulent to *Lr28*, may result in isolates avirulent to this gene if avirulence to *Lr28* is recessive. Most avirulence genes in *P. triticina* are incompletely dominant; however, Kolmer and Dyck (1994) showed that avirulence to *Lr30* is recessive. A final possibility concerning the origin of the MBDTRL-43 isolate is that multiple mutations affecting virulence to *Lr17*, *LrB*, *Lr3bg*, and *Lr28* occurred in isolates with group 1 AFLP phenotypes. However, there is little evidence for the occurrence of simultaneous multiple mutations in *P. triticina*, and avirulence to *Lr28* would have to be expressed in a recessive manner for this to occur.

The isolates with virulence phenotypes CHBFRG and MBBTRQ were collected from Manitoba previous to the early 1960s. These isolates are relatively avirulent compared with the present isolates from the prairie region. Three of the older isolates (BBBCRB, CHBFRG, and MBBTRQ) were closely related for AFLP phenotype with the group 1 isolates collected in the prairie region in 1996 and 1997. Isolates in Manitoba and Saskatchewan have changed for virulence phenotype in the last 30 years because of host selection (Kolmer 1989*b*, 1999*b*), yet have diverged relatively little for molecular variation from the older isolates. Since 1987 isolates in the prairie region have also been selected for virulence to resistance genes *Lr11*, *Lr3ka*, *Lr24*, *Lr26*, and *Lr16*, which have been used in wheats grown in the Great Plains region of the United States and Canada (Kolmer 1999*b*). Virulence to these resistance genes most likely arose by mutation and selection within a single group of *P. triticina* isolates that were closely related for AFLP phenotypes. Isolates in group 1 were found throughout Canada. These isolates are also found throughout the United States, where they overwinter in the southeastern states and southern plains states. Wind blown dispersal of group 1 isolates from overwintering sites in the United States during the spring and summer would account for their cosmopolitan occurrence in Canada.

The *P. triticina* isolates in group 2 were all avirulent to the resistance gene *Lr2a* and virulent to gene *Lr2c*, which distinguishes them from isolates in groups 1 and 3, which are ei-

ther avirulent or virulent to both *Lr2a* and *Lr2c*. Isolates in group 2 also had distinct AFLP phenotypes compared with all other groups of isolates. For the last 40 years, virulence phenotypes in group 2 have been found in Ontario and Quebec, and occasionally in Alberta or British Columbia as in the case of isolate NBBNPQ in 1997. A single isolate of FDMDTQ was found in Saskatchewan in 1997. Most virulence phenotypes in group 2 from eastern Canada likely originated from a localized population, where these isolates may overwinter.

The PBDQHQ virulence phenotype (group 4 isolates) has been collected in Alberta and British Columbia in recent years. A single isolate with this phenotype was also collected in Manitoba in 1996. Since isolates with this virulence phenotype are unusual in the prairie region, the occurrence of this isolate may be due to selection by the wheat cultivar Jagger for virulence to *Lr17*. The two isolates of *P. triticina* with virulence phenotype PBDQHQ had AFLP phenotypes very close to the isolates in group 1. However, these two isolates were more similar for virulence phenotype to the group 3 isolates. The AFLP data suggests that the group 4 isolates were derived from group 1 isolates. The PBDQHQ isolates may have diverged over a period of years for virulence phenotype from the isolates in group 1 by host selection of wheats grown in Alberta and British Columbia, or in the adjacent regions of the United States. In the previous study (Kolmer et al. 1995) a single isolate of PBDQHQ from British Columbia had a unique RAPD phenotype that clustered it separately from all other groups of isolates. Since PBDQHQ is avirulent to *Lr2a* and virulent to *Lr2c*, these two isolates are unique in that they have molecular phenotypes similar to isolates that are either virulent or avirulent to both resistance genes. All other isolates that were avirulent to *Lr2a* and virulent to *Lr2c* had distinct AFLP phenotypes compared to isolates avirulent or virulent to both genes. Isolates of *P. triticina* from western Europe and New Zealand that were avirulent to *Lr2a* and virulent to *Lr2c* had distinct RAPD phenotypes compared to isolates that were avirulent or virulent to both resistance genes (Kolmer and Liu 2000). Previous groupings of isolates in Canada based on virulence to *Lr2a* and *Lr2c* had a high correlation with isolate groupings based on RAPD phenotypes (Kolmer et al. 1995).

The isolates in group 5 with virulence phenotypes SBDDRG, SBGGRC, and TDTRGG are unique since they are avirulent to *Lr22b*, the adult plant gene in Thatcher. These three isolates also had distinct AFLP phenotypes compared with all other isolates used in this study. Isolates with virulence phenotype of Unified Numeration (UN) race 9, which was the original race designation of the SBDDRG isolate, were commonly found in eastern Canada and the prairie region from 1931–1944 (Kolmer 1991). The frequency of UN race 9 declined after the release of Thatcher in the mid-1930s and winter wheats with *Lr3* in the mid-1940s, since race 9 was avirulent to *Lr22b* and *Lr3*. Since the 1950s these isolates of race 9 have only rarely been isolated from wheat in Canada. Previous to the introduction of wheat cultivars with *Lr3* and *Lr22b*, isolates of UN race 9 would have constituted a major group of *P. triticina* in North America distinct for both AFLP and virulence background. In recent years isolates of UN race 9 have been collected from *Aegilops cylindrica* in the southern plains of the United States (Long et al. 1998). Since one of the group 5 isolates was collected from Quebec in 1996, the second from Saskatchewan in 1996, and the third from British Columbia in 1959, this supports the previous survey data (Kolmer 1991) that isolates avirulent to *Lr22b* were once widespread in North America. Isolates avirulent to *Lr22b* may currently survive only in areas or niches where there are leaf rust susceptible hosts, such as Quebec and British Columbia, or where there are other hosts such as *A. cylindrica.*

The results of this study and the previous RAPD study are in general agreement with the results obtained with other plant pathogens that have specialized physiologic forms and reproduce asexually. In populations of the rice blast fungus, *Magnaporthe grisea*, there was evidence for relationship between individual isolates in molecular groups and their virulence to differential cultivars or specific resistance genes in rice (Zeigler et al. 1995). Allozyme genotype and virulence phenotype had an almost exact correlation in populations of the wheat stem rust fungus, *P. graminis* in the United States (Burdon and Roelfs 1985).

The existence of groups of *P. triticina* isolates in Canada that differ for virulence and AFLP phenotypes suggests that the current populations in North America have most likely evolved from a number of introductions of the fungus that differed for molecular background. Since native *Thalictrum* spp. in North America are resistant to *P. triticina*, it is likely that *P. triticina* was introduced to North America with the establishment of wheat cultivation (Arthur 1934). Differentiation of *P. triticina* groups within North America has been influenced by the selective effects of resistant wheat cultivars grown in various regions of the continent (Kolmer 1989*b*, 1991). Susceptible wheats grown in the eastern region of Quebec and Ontario have maintained *P. triticina* virulence phenotypes that differ from those in the prairie region of Manitoba and Saskatchewan where leaf rust resistant wheats are grown. Asexual reproduction has maintained the distinctiveness of the eastern and prairie *P. triticina* populations in Canada (Kolmer 1999*b*), and also the association between virulence and molecular background (Liu and Kolmer 1998). Since molecular markers are not directly selected by the host resistance genes, differences in AFLP phenotype may more accurately represent among the current populations, the genetic relationships that existed between the original introductions of the fungus.

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