Virulence and Molecular Polymorphism in *Puccinia recondita* f. sp. *tritici* in Canada

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ABSTRACT

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Sixty-four representative single-uredinial isolates of *Puccinia recondita* f. sp. *tritici* collected from wheat in the eastern (Ontario and Quebec), prairie (Manitoba and Saskatchewan), and Pacific (Alberta and British Columbia) regions of Canada were analyzed for virulence polymorphism on 19 near-isogenic wheat differential lines and for randomly amplified polymorphic DNA (RAPD) using 10 arbitrary decamer primers. Thirty-seven phenotypes of *P. r. tritici* were distinguished by the 19 host differential lines. Fifteen molecular phenotypes were distinguished by the 10 primers in the RAPD analysis. Forty-five unique phenotypes of *P. r. tritici* were distinguished by combined virulence and RAPD data. Molecular variation was greatest between isolates of different virulence phenotypes. There was some molecular variation among isolates with identical virulences. Virulence and RAPD data were used separately and combined in cluster analyses. Two major clusters were distinguished with the combined data sets. The first cluster consisted of isolates virulent or avirulent to both resistance genes *Lr2a* and *Lr2c*, and the second cluster consisted of isolates avirulent to *Lr2a* and virulent to *Lr2c*. The molecular polymorphisms were more effective in distinguishing between the major clusters of *P. r. tritici* compared to the virulence polymorphisms. However, virulence polymorphisms were more effective in distinguishing between isolates within the major clusters compared to the molecular polymorphisms. There was a correlation of 0.58 between the virulence and molecular dissimilarity matrices. The cluster analyses indicated a general relationship between virulence and molecular polymorphism in *P. r. tritici* in Canada. There are currently two major groups of *P. r. tritici* in Canada. One group consists of isolates found in the prairie and eastern regions. Isolates in this group have limited molecular polymorphisms and consist of many virulence phenotypes that are either virulent or avirulent to both *Lr2a* and *Lr2c*. Isolates in the second group are found in eastern Canada and in the Pacific region. These isolates are avirulent to *Lr2a* and virulent to *Lr2c* and have molecular phenotypes distinct from isolates in the first group. The molecular data support conclusions from virulence survey data that distinct geographic populations of *P. r. tritici* exist in Canada and that new virulences arise and increase in the prairie population by mutations from preexisting phenotypes.

Additional keywords: host selection, physiologic specialization, *Triticum aestivum*, wheat leaf rust.

Genetic variation in populations of *Puccinia recondita* Roberge ex Desmaz. f. sp. *tritici* Eriks. & E. Henn., the causal agent of wheat leaf rust, has been studied at the Agriculture and Agri-Food Canada Research Centre in Winnipeg, Manitoba, since 1931 (11,13,25,27). The yearly surveys of physiologic specialization of *P. r. tritici* have provided a continuous record of information concerning the epidemiology and evolution of different populations of the wheat leaf rust fungus in Canada. Until the late 1960s, single-uredinial isolates of *P. r. tritici* were characterized for virulence on eight differential cultivars. Dyck and Samborski (5,29) developed a series of near-isogenic lines of the wheat cultivar Thatcher that differed by single leaf rust resistance genes. The Thatcher lines have been used to characterize virulence in *P. r. tritici* populations in Canada (16) and throughout the world (6,7,22,30). Use of near-isogenic differential lines has made possible the distinction of *P. r. tritici* isolates that differ by a single virulence (29).

Samborski and Dyck (5,28,29) also demonstrated a gene-for-gene relationship in the *P. r. tritici–Triticum aestivum* L. pathosystem. For most loci in the pathogen, virulence/avirulence was inherited as a single gene that corresponded to a specific resistance gene in the near-isogenic host lines. The expression of avirulence and resistance in this gene-for-gene system varied from completely dominant to completely recessive (17). Since *P. r. tritici* is dikaryotic, isolates that express intermediate avirulent infection types are often heterozygous at virulence loci (17). High levels of virulence heterozygosity have been described in *P. r. tritici* populations in Canada (15).

Distinct populations of *P. r. tritici* as defined by virulence on the near-isogenic differential lines are currently found in the eastern (Ontario and Quebec), prairie (Manitoba and Saskatchewan), and Pacific (Alberta and British Columbia) regions of Canada (11,12,14). Phenotypes virulent or avirulent to both resistance genes *Lr2a* and *Lr2c* are found in the prairie and eastern regions, while phenotypes avirulent to *Lr2a* and virulent to *Lr2c* are found in the eastern region. Kolmer (13) hypothesized that the *P. r. tritici* populations in the eastern and prairie regions were similar prior to the use of leaf rust-resistant cultivars in the prairie region. The current distinct populations may have resulted from the selective effects of host resistance. In recent years, *P. r. tritici* virulence phenotypes have changed very rapidly in the prairie region due to the introduction of winter wheat cultivars in the United States with resistance genes *Lr11, Lr24,* and *Lr26* (10,12,16).

Virulence markers that describe genetic variation in plant pathogen populations are obviously important because they provide direct information concerning the effects of host selection and the potential effectiveness of resistance genes. However, virulence markers may represent only a small portion of the total genetic variation present in the population. In plant pathogenic fungi, biochemical markers, such as isozymes (2,3,20), or molecular markers, such as restriction fragment length polymorphisms (1,9,

19) and randomly amplified polymorphic DNA (RAPD) (4,32), are generally assumed to be independent of host selection. These markers can be used in plant pathogens to assess genetic variation that is not directly affected by host selection. As an example, isolates of a pathogen may have highly different virulence phenotypes yet have identical molecular phenotypes, indicating that the isolates may have diverged by host selection from a common ancestral genotype. Virulence and molecular markers combined should provide further insight into the genetic variation and structure of plant-pathogen populations.

The objective of this study was to examine the relationship between virulence and molecular polymorphism in representative isolates of *P. r. tritici* from Canada. We evaluated the degree of molecular variation between isolates collected from different geographic populations of *P. r. tritici* in Canada. The isolates were evaluated for virulence on an expanded series of near-isogenic differential lines and were assessed for molecular variation with the RAPD method. The isolates were grouped with cluster analyses, using the virulence and molecular data separately and combined.

MATERIALS AND METHODS

***P. r. tritici* isolates.** Fifty-nine single-uredinial isolates of *P. r. tritici* collected from wheat were selected from the 1989, 1992, and 1993 (12,16) virulence surveys (Table 1). Also included were four isolates collected prior to 1965 and an isolate originally derived from a sexual population of *P. r. tritici* (15). The isolates from the virulence surveys were representative of the predominant virulence phenotypes found during those years, based on infection types to the 12 near-isogenic differentials in the *Prt* nomenclature (21). The most common virulence phenotypes were represented by more than one isolate. One isolate each was used for the less frequent virulence phenotypes. Single-uredinial isolation and characterization for virulence in the surveys were as described previously (12). 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 of the pots to prevent cross-contamination of the isolates. Urediniospores were collected into size 00 gelatin capsules with a cyclone spore collector and stored at −70 C.

Determination of virulence phenotypes. The isolates were retested for virulence on the 12 Thatcher near-isogenic lines in the *Prt* nomenclature with genes *Lr1*, RL 6003; *Lr2a*, RL 6000; *Lr2c*, RL 6047; *Lr3*, RL 6002; *Lr3ka*, RL 6007; *Lr9*, RL 6010; *Lr11*, RL 6053; *Lr16*, RL 6005; *Lr17*, RL 6008; *Lr24*, RL 6064; *Lr26*, RL 6078; and *Lr30*, RL 6049. The isolates also were tested on the near-isogenic lines with genes *LrB*, RL 6047; *Lr3bg*, RL 6042; *Lr10*, RL 6004; *Lr14a*, RL 6013; *Lr14b*, RL 6006; *Lr15*, RL 6006; *Lr18*, RL 6009; *Lr19*, RL 6040; *Lr20*, RL 6092; *Lr21*, RL 6043; *Lr25*, RL 6084; *Lr28*, RL 6079; *Lr29*, RL 6080; and *Lr32*, RL 5494-1. Thatcher (RL 6101) also was included as a susceptible control. Inoculation, incubation, and greenhouse conditions were as described previously (12). Infection types 0–2+ were classified as avirulent, and infection types 3–4 were classified as virulent. Virulence phenotypes of the isolates (Table 1) were described with an expanded version of the *Prt* nomenclature. The first three letters correspond to the three sets of four differentials in the *Prt* nomenclature (21). The fourth letter describes the virulence/avirulence on differentials *LrB, Lr3bg, Lr10,* and *Lr14a*. The fifth letter corresponds to the virulence/avirulence on differentials with *Lr15, Lr18,* and *Lr28*. An imaginary fourth differential in the fifth set was always avirulent. The isolates were all either virulent or avirulent on the remaining differentials. Virulence/avirulence on these differentials was not included in the cluster analysis.

Determination of molecular phenotypes. The *P. r. tritici* isolates (2 mg of urediniospores) were each inoculated on Little Club seedlings grown in eight 10-cm pots. Each set of Little Clubs was kept in a separate greenhouse compartment or had plastic cylinders placed over the pots to prevent cross-contamination. Urediniospores were collected 2 wk after inoculation by tapping the infected seedlings over cellophane paper in a Plexiglas chamber that was flushed out with water in between collection of different isolates. In 16-cm^2 glass baking dishes, 150 mg of freshly collected urediniospores of each isolate was spread over a 200-ml gramicidin solution (ddH_2O with 1.2 mg of gramicidin D [Sigma Chemical Co., St. Louis] dissolved in 0.25 ml of absolute ethanol). A nonanol solution (1.5 μl of nonanol/ml of acetone in ddH_2O) was added to the bottom of an 8-cm glass petri dish. The petri dish bottoms were placed in the baking dishes, supported at a level above the urediniospore suspension. The baking dishes were tightly covered with plastic food wrap. After 6 h, the germinated urediniospores were collected by skimming off the mycelial mat with an 8- × 2-cm piece of hard rubber. The mats were wrapped in aluminum foil, snap frozen in liquid N_2 and stored at −70 C.

For DNA extraction (8), the mats were retrieved and ground to a fine powder in liquid N_2 with a small amount of acid-washed sand using a precooled mortar and pestle. The ground material was placed in a 30-ml Nalge tube with 6 ml of lysis buffer (150 mM NaCl, 50 mM EDTA, 10 mM Tris, pH 7.4), 30 μg of proteinase K, and 0.1 volume of 20% sodium dodecyl sulfate. Tubes were incubated at 65 C for 30 min, with occasional gentle rocking, and were spun in a clinical centrifuge for 10 min. The supernatant was decanted into clean Nalge tubes, and 0.54 g of NaCl was added to each tube. Cetyltri-methylammonium bromide (CTAB) buffer (10% CTAB, 500 mM Tris, 100 mM EDTA) at 0.1 volume was added, and the tubes were incubated at 65 C for 10 min with occasional rocking. The solutions were cooled to 20 C, and an equal volume of 24:1 chloroform/isoamyl alcohol was added. The emulsions were mixed and centrifuged for 10 min. The aqueous layers were removed and extracted a second time with chloroform/isoamyl alcohol. The aqueous phases were placed in clean tubes and 2.5 volumes of cold 95% ethanol was added. The DNA-ethanol solutions were kept at −20 C overnight and centrifuged for 10 min. The ethanol was decanted, the pellets were dried and resuspended in 1 ml of sterile ddH_2O, and 10 μl of a RNase solution (Promega, Madison, WI) at 10 mg/ml was added. The solutions were incubated for 1 h at 37 C, and an equal volume of buffer-saturated phenol/chloroform/isoamyl alcohol (25:24:1) was added. The tubes were centrifuged for 10 min, and the aqueous layers removed and extracted with chloroform/isoamyl alcohol. After the second extraction, 0.5 volume of 7.5 M NH_4-acetate and 2.5 volumes of 95% ethanol were added. The tubes were kept overnight at −20 C, followed by centrifugation for 10 min. The ethanol was decanted, the pellets washed with 70% ethanol, centrifuged, and the ethanol decanted. The pellets were dried and resuspended in 500 μl of sterile ddH_2O. DNA concentration was determined with a Varian DMS 100 UV visible spectrophotometer (Varian Techtron Pty Limited, Mulgrave, Victoria, Australia). Usually 500–1,200 μg of DNA was obtained from 150 mg of fresh urediniospores. A working DNA preparation at 10 ng/μl was made for use in the RAPD reactions, and stored at 4 C. The concentrated DNA stocks were stored at −20 C in ethanol solutions.

In the RAPD reactions, 2.5 μl of 10× *Taq* polymerase reaction buffer (Promega), 1.5 μl of 25 mM $MgCl_2$, 2.0 μl of a 2.5 mM stock each of dATP, dCTP, dGTP, and dTTP, 0.25 μl of *Taq* polymerase (5 units/μl) (Promega), 2 μl of *P. r. tritici* DNA (10 ng/μl), and 0.5 μl of primer (0.05 μg/μl) were added to 16.25 μl of sterile ddH_2O in 0.5-ml microcentrifuge tubes. Paraffin oil (25 μl) was added as an overlay to each tube. A Thermolyne thermocycler (Barnstead/Thermolyne Corp., Dubuque, IA) and an MJ thermocycler (MJ Research Inc., Watertown, MA), programmed at 94 C for 3 min; followed by 40 cycles of 1 min at 94 C, 2 min at 36 C, and 2 min at 72 C; followed by 10 min at 72 C, were used for DNA amplification. The fastest ramp speed was used. Identical results between the two machines were obtained for all *P. r. tritici* DNA preparations with the selected primers.

Five microliters of a bromophenol blue solution (24) was added to each reaction tube after amplification. One-half of each reaction (12.5 μl) was electrophoresed in 1.4% agarose gels (Promega) with 1× Tris-acetate EDTA (24) running buffer. Lambda DNA

TABLE 1. Isolate number, *Prt*[a] code, virulence, molecular phenotype, and origin of single-uredinial isolates of *Puccinia recondita* f. sp. *tritici* collected in Canada

Isolate no.	*Prt* code	Virulence (*Lr* genes)[b]	Molecular phenotype[c]	Province of origin[d]	Year
1	BBBCL	14a, 15	1111111112	BC	1954
2	TBGFN	1, 2a, 2c, 3, 11, 10, 14a, 15, 28	1222111111	MB	1989
3	MFBFN	1, 3, 24, 26, 10, 14a, 15, 28	1222111311	SA	1989
4	MBGFN	1, 3, 11, 10, 14a, 15, 28	1111111111	ON	1989
5	PBLSN	1, 2c, 3, 3ka, B, 3bg, 10, 15, 28	2222222221	ON	1989
6	MDBFN	1, 3, 24, 10, 14a, 15, 28	1111111111	MB	1989
7	KBGFN	2a, 2c, 3, 11, 10, 14a, 15, 28	1221111111	MB	1989
8	FBMSN	2c, 3, 3ka, 30, B, 3bg, 10, 15, 28	2222222221	ON	1989
9	NBBNJ	1, 2c, B, 10, 18, 28	2221241221	BC	1989
10	SBDDN	1, 2a, 2c, 17, 10, 15, 28	1222132513	BC	1959
11	MBBKN	1, 3, 3bg, 10, 14a, 15, 28	1222111111	MB	1959
12	CHBFN	3, 16, 26, 10, 14a, 15, 28	1222111111	MB	1964
13	KBGFN	2a, 2c, 3, 11, 10, 14a, 15, 28	1111111111	SA	1992
14	KBGFN	2a, 2c, 3, 11, 10, 14a, 15, 28	1111111111	MB	1992
15	LBBFQ	1, 10, 14a, 15, 18	1111111111	PQ	1992
16	KDGFN	2a, 2c, 3, 24, 11, 10, 14a, 15, 28	1111111111	AB	1992
17	PBDJN	1, 2c, 3, 17, 3bg, 10, 15, 28	1222211121	BC	1992
18	TBBFN	1, 2a, 2c, 3, 10, 14a, 15, 28	1111111111	MB	1992
19	TDGFN	1, 2a, 2c, 3, 24, 11, 10, 14a, 15, 28	1111111111	AB	1992
20	TFBFN	1, 2a, 2c, 3, 24, 26, 10, 14a, 15, 28	1111111111	MB	1992
21	CBBCN	3, 14a, 15, 28	1222111111	PQ	1992
22	MBRFN	1, 3, 11, 3ka, 30, 10, 14a, 15, 28	1111111111	MB	1992
23	TDTFN	1, 2a, 2c, 3, 24, 3ka, 11, 17, 30, 10, 14a, 15, 28	1111112112	Aecial	1989
24	KBJFN	2a, 2c, 3, 11, 17, 10, 14a, 15, 28	1221111111	MB	1992
25	KDGFN	2a, 2c, 3, 24, 11, 10, 14a, 15, 28	1221111111	MB	1992
26	TFGFN	1, 2a, 2c, 3, 24, 26, 11, 10, 14a, 15, 28	1111111111	MB	1992
27	TBGFN	1, 2a, 2c, 3, 11, 10, 14a, 15, 28	1111111111	MB	1992
28	TFBFN	1, 2a, 2c, 3, 24, 26, 10, 14a, 15, 28	1111111111	AB	1992
29	MBBFN	1, 3, 10, 14a, 15, 28	1222111111	BC	1992
30	MBGCN	1, 3, 11, 14a, 15, 28	1111111111	ON	1992
31	PBLSN	1, 2c, 3, 3ka, B, 3bg, 10, 15, 28	2222222221	ON	1992
32	PBLSN	1, 2c, 3, 3ka, B, 3bg, 10, 15, 28	2222222221	PQ	1992
33	TDGFN	1, 2a, 2c, 3, 24, 11, 10, 14a, 15, 28	1111111111	AB	1992
34	TDGFN	1, 2a, 2c, 3, 24, 11, 10, 14a, 15, 28	1111111111	MB	1992
35	MFBFN	1, 3, 24, 26, 10, 14a, 15, 28	1111111111	MB	1992
36	KBGFN	2a, 2c, 3, 11, 10, 14a, 15, 28	1111111111	SA	1992
37	KBGFN	2a, 2c, 3, 11, 10, 14a, 15, 28	1111212111	MB	1992
38	KBGFN	2a, 2c, 3, 11, 10, 14a, 15, 28	1111111111	SA	1992
39	TBGFN	1, 2a, 2c, 3, 11, 10, 14a, 15, 28	1111111111	MB	1992
40	TBGFN	1, 2a, 2c, 3, 11, 10, 14a, 15, 28	1111111411	SA	1992
41	MFBFN	1, 3, 24, 26, 10, 14a, 15, 28	1111111111	MB	1992
42	MBGCN	1, 3, 11, 14a, 15, 28	1111111111	ON	1992
43	PBLSN	1, 2c, 3, 3ka, B, 3bg, 10, 15, 28	2222222221	ON	1992
44	TFBFN	1, 2a, 2c, 3, 24, 26, 10, 14a, 15, 28	1111111111	AB	1992
45	MCBFN	1, 3, 26, 10, 14, 15, 28	1222111111	BC	1992
46	TDGFN	1, 2a, 2c, 3, 24, 11, 10, 14a, 15, 28	1111111111	SA	1992
47	MBGFN	1, 3, 11, 10, 14a, 15, 28	1111111111	PQ	1992
48	MFBFN	1, 3, 24, 26, 10, 14a, 15, 28	1111111111	SA	1992
49	KFGFN	2a, 2c, 3, 11, 10, 14a, 15, 28	1111111111	MB	1993
50	MFMFN	1, 3, 24, 26, 3ka, 30, 10, 14a, 15, 28	1111111311	MB	1993
51	MFMFN	1, 3, 24, 26, 3ka, 30, 10, 14a, 15, 28	1111111111	MB	1993
52	PBMMS	1, 2c, 3, 3ka, 30, B, 14a, 15, 18, 28	2111222221	PQ	1993
53	MBRFN	1, 3, 3ka, 11, 30, 10, 14a, 15, 28	1111111111	MB	1993
54	MDMFN	1, 3, 24, 3ka, 30, 10, 14a, 15, 28	1111111311	MB	1993
55	MCRFN	1, 3, 26, 3ka, 11, 17, 30, 10, 14a, 15, 28	1222111111	MB	1993
56	KFBFN	2a, 2c, 3, 24, 26, 10, 14a, 15, 28	1111111111	MB	1993
57	FBMPQ	1, 2c, 3, 3ka, 30, B, 10, 14a, 15, 18	2222222221	PQ	1993
58	PBGSN	1, 2c, 3, 11, B, 3bg, 10, 15, 28	2111222221	ON	1993
59	MBQFN	1, 3, 3ka, 11, 10, 14a, 15, 28	1111111411	MB	1993
60	PNMSN	1, 2c, 3, 9, 24, 3ka, 30, B, 3bg, 10, 15, 28	2222222221	ON	1993
61	TFGFN	1, 2a, 2c, 3, 24, 26, 11, 10, 14a, 15, 28	1111111111	MB	1993
62	NBBNJ	1, 2c, B, 10, 18, 28	2111242221	AB	1993
63	MFMFN	1, 3, 24, 26, 10, 14a, 15, 28	1111111111	MB	1993
64	TDBFN	1, 2a, 2c, 3, 24, 10, 14a, 15, 28	1111111111	SA	1993

[a] *Prt* nomenclature designation (21).

[b] Isolates were evaluated for high or low infection type on Thatcher lines of wheat isogenic for leaf rust resistance genes: *Lr1, Lr2a, Lr2c, Lr3, Lr9, Lr16, Lr24, Lr26, Lr3ka, Lr11, Lr17, Lr30, LrB, Lr3bg, Lr10, Lr14a, Lr15, Lr18,* and *Lr28.*

[c] Isolates were tested for randomly amplified polymorphic DNA with decamer primers 402, 450, 489, 490, 517, 519, 521, 531, 538, and 556 obtained from the University of British Columbia biotechnology laboratories; 1 indicates most common DNA banding pattern, 2 indicates the second most common banding pattern, etc.

[d] PQ = Quebec, ON = Ontario, MB = Manitoba, SA = Saskatchewan, AB = Alberta, BC = British Columbia.

restricted with *Eco*R1 and *Hind*III (Promega) was included as a molecular weight marker. The RAPD products were electrophoresed at 72 V for 4 h. The gels were stained with ethidium bromide at 0.5 μg/ml for 30 min and destained for 1 h in dH_2O while being shaken gently. The RAPD products were visualized under ultraviolet light and photographed with type 57 Polaroid film (Polaroid Corp., Cambridge, MA).

Arbitrary decamer primers 400–599 were obtained from the University of British Columbia biotechnology laboratories in Vancouver, Canada. All 200 primers were initially screened for amplification of polymorphic major bands with *P. r. tritici* isolates 1–10 (Table 1). Primers that initially detected polymorphism were tested a second time on the same set of isolates. Only the major polymorphic bands were considered. Polymorphism among bands that amplified weakly was ignored. After at least two tests for repeatable polymorphism, the primers were tested on the entire set of DNA from the 64 *P. r. tritici* isolates. Ten primers (402, GGAAGGCTGT; 450, CGGAGAGCCC; 489, CGCACGCACA; 490, AGTCGACCTT; 517, GGTCGCAGCT; 519, ACCGGACACT; 521, CCGCCCCACT; 531, GCTCACTGTT; 538, TGACCTCTCC; and 556, ATGGATGACG) amplified repeatable major band polymorphism between the isolates. For each primer, the most common banding pattern was designated as type 1, the next most common was type 2, etc. Each *P. r. tritici* isolate was assigned a 10-digit molecular phenotype based on the banding pattern for each of the 10 primers (Table 1).

Data analysis. The virulence and molecular data were used in separate and combined cluster analyses. Only *P. r. tritici* isolates that had unique combinations of virulence and molecular phenotypes were included in the cluster analyses. As an example, because all four isolates with the virulence phenotype PBLSN had identical molecular phenotypes of 2222222221, only one of these isolates was included in the data analyses. Isolates with identical virulence phenotypes were included in the analyses if they had different molecular phenotypes.

Dissimilarity (number of marker differences) matrices for both markers separately and combined were derived for all possible pairs of isolates with a SAS (SAS Institute, Cary, NC) program. Dendrograms were derived from the dissimilarity matrices with the SAHN program in the numerical taxonomy system for personal computer (NTSYS-pc), version 1.8 (Exeter Software, Setauket NY). The unweighted pair group arithmetic mean method (UPGMA), the unweighted pair group centroid method (UPGMC), and the single-linkage clustering method were used. For each of the three clustering methods, a cophenetic value distance matrix was derived from the respective dendrograms with the COPH program in NTSYS-pc. The cophenetic value distance matrices for the three clustering methods were compared for level of correlation with the original distance matrix (31) with the MXCOMP program in NTSYS-pc. For the virulence and molecular data, separately and combined, the dendrograms from the UPGMA and UPGMC clustering methods had the highest level of correlation with the original distance matrices. The UPGMA dendrograms were chosen to illustrate the isolate groupings. Levels of dissimilarity were standardized in the dendrograms by dividing the number of differences by the total number of markers. The separate virulence and molecular dissimilarity matrices were compared for level of correlation using the MXCOMP program. A three-dimensional cluster representation, using the combined data sets, was derived with a SAS program that calculated the average distance between all isolates in all possible pair-wise comparisons of clusters.

RESULTS

Virulence variation. Thirty-seven virulence phenotypes were identified among the 64 single-uredinial isolates by the Thatcher near-isogenic differential lines (Table 1; Fig. 1). The *P. r. tritici* isolates were polymorphic for virulence on the 19 differential lines with resistance genes *Lr1, Lr2a, Lr2c, Lr3, Lr3bg, Lr3ka, Lr9, Lr10, Lr11, Lr14a, Lr15, Lr16, Lr17, Lr18, Lr24, Lr26, Lr28, Lr30,* and *LrB*. The tested isolates were all virulent on the differential lines with *Lr14b* and *Lr20* and were all avirulent to lines with *Lr19, Lr21, Lr25, Lr29,* and *Lr32*.

Cluster analysis of virulence phenotypes with UPGMA showed no separate groups of isolates at the 50% dissimilarity level (Fig. 1). The first separation of isolates occurred at 38% dissimilarity. Isolates in group A were all avirulent or virulent to both *Lr2a* and *Lr2c*. Isolates in group B, with one exception (10-SBDDN), were all avirulent to *Lr2a* and virulent to *Lr2c* (Fig. 1). No other distinct virulence associations characterized the two groups of isolates.

The dendrogram from the UPGMC clustering was very similar to the UPGMA dendrogram (UPGMC dendrogram not shown). With the UPGMC clustering, the isolates in group A of Figure 1 were the same; however, isolates 52-PBMMS and 57-FBMPQ were separated from the isolates in group B of Figure 1 at the 32% dissimilarity level. No separation of isolate groups could be readily observed in the dendrogram from the single-linkage clustering method (single-linkage dendrogram not shown). The cophenetic value distance matrices of the UPGMA, UPGMC, and single-linkage methods had correlations with the original virulence distance matrix of 0.79, 0.79, and 0.71, respectively.

Molecular variation. Ten primers were selected based on ability to reproducibly amplify major DNA bands that were polymorphic between the *P. r. tritici* isolates (Table 1; Fig. 2). Primers 402, 450, 489, 490, 517, 521, and 538 each distinguished two molecular phenotypes among the *P. r. tritici* isolates. Primer 519 detected four phenotypes; primer 531 detected five phenotypes; and primer 556 detected three phenotypes. The molecular phenotypes distinguished by each primer generally varied by only one band from the most common banding pattern. Primers 402, 517, 519, 521, 531, and 538 generally distinguished between isolates that were avirulent to *Lr2a* and virulent to *Lr2c*, versus isolates that were avirulent or virulent to both resistance genes. Polymorphism detected by primers 450, 489, 490, and 556 occurred in a less distinct pattern among the *P. r. tritici* isolates. The molecular phenotypes distinguished by primers 450 and 489 were identical, although the two primers generated polymorphic bands of different molecular weights (Table 1; Fig. 2). All type 2 phenotypes distinguished by primer 490 were also type 2 phenotypes as distinguished by primers 450 and 489 (Table 1; Fig. 2).

Molecular variation also was found between isolates that had identical infection types on the 19 differential lines. As an example, of the six isolates with virulence phenotype KBGFN, four were the molecular phenotype 1111111111, one was 1221111111, and one was 1111212111 (Table 1). Similarly, there were three molecular phenotypes for TBGFN, two molecular phenotypes for MFBFN, and two molecular phenotypes for KDGFN (Table 1). Polymorphism detected by primers 450, 489, and 490 accounted for most of the molecular variation with these virulence phenotypes.

The 10 primers amplified DNA polymorphism that distinguished 15 molecular phenotypes among the 64 *P. r. tritici* isolates (Table 1). UPGMA clustering determined that at 50% dissimilarity there were three groups of *P. r. tritici* isolates characterized by molecular variation (Fig. 3). Isolates in group A, with one exception (17-PBDJN) were either virulent or avirulent to both resistance genes *Lr2a* and *Lr2c*. Group B consisted of one isolate, 10-SBDDN, which was virulent to both *Lr2a* and *Lr2c*. Isolates in group C were all avirulent to *Lr2a* and virulent to *Lr2c*. The dendrograms from the UPGMC and single-linkage clustering methods grouped the isolates in the same manner as the UPGMA dendrogram in Figure 3. The cophenetic value distance matrices from the UPGMA, UPGMC, and single-linkage clustering, correlated with the original molecular distance matrix at 0.94, 0.94, and 0.92, respectively.

Virulence and molecular variation combined. The correlation between the virulence and molecular distance matrices was 0.58, which indicated some degree of relationship between the two types of genetic variation in the *P. r. tritici* population. When the virulence and molecular data were combined, 45 phenotypes were distinguished by a total of 29 genetic markers. From the UPGMA dendrogram (Fig. 4) at 50% dissimilarity, group A included all

isolates that were either avirulent or virulent to both *Lr2a* and *Lr2c* (except for isolate 17-PBDJN), and group B included all isolates that were avirulent to *Lr2a* and virulent to *Lr2c*. The dendrogram from the UPGMC clustering grouped the isolates in the same manner as the UPGMA dendrogram in Figure 4. The single-linkage clustering differed slightly, placing isolate 10-SBDDN outside of groups A and B in Figure 4 at 50% dissimilarity. The cophenetic value distance matrices from the UPGMA, UPGMC, and single-linkage clustering correlated with the original combined distance matrix at 0.92, 0.92, and 0.87, respectively.

The relationships between different groups of *P. r. tritici* phenotypes are summarized in a three-dimensional cluster diagram (Fig. 5). At the 29% level of dissimilarity, the average distances between phenotypes in different clusters were all greater than the average distances between phenotypes within each cluster. All 35 phenotypes in cluster 1 were avirulent or virulent to both *Lr2a* and *Lr2c*. The six phenotypes in cluster 2 were all avirulent to *Lr2a* and virulent to *Lr2c*. The two phenotypes in cluster 3 were avirulent to *Lr2a* and *Lr3*, virulent to *Lr2c*, and differed for molecular phenotype with three primers. Cluster 4 consisted of isolate 17-PBDJN, and cluster 5 consisted of isolate 10-SBDDN.

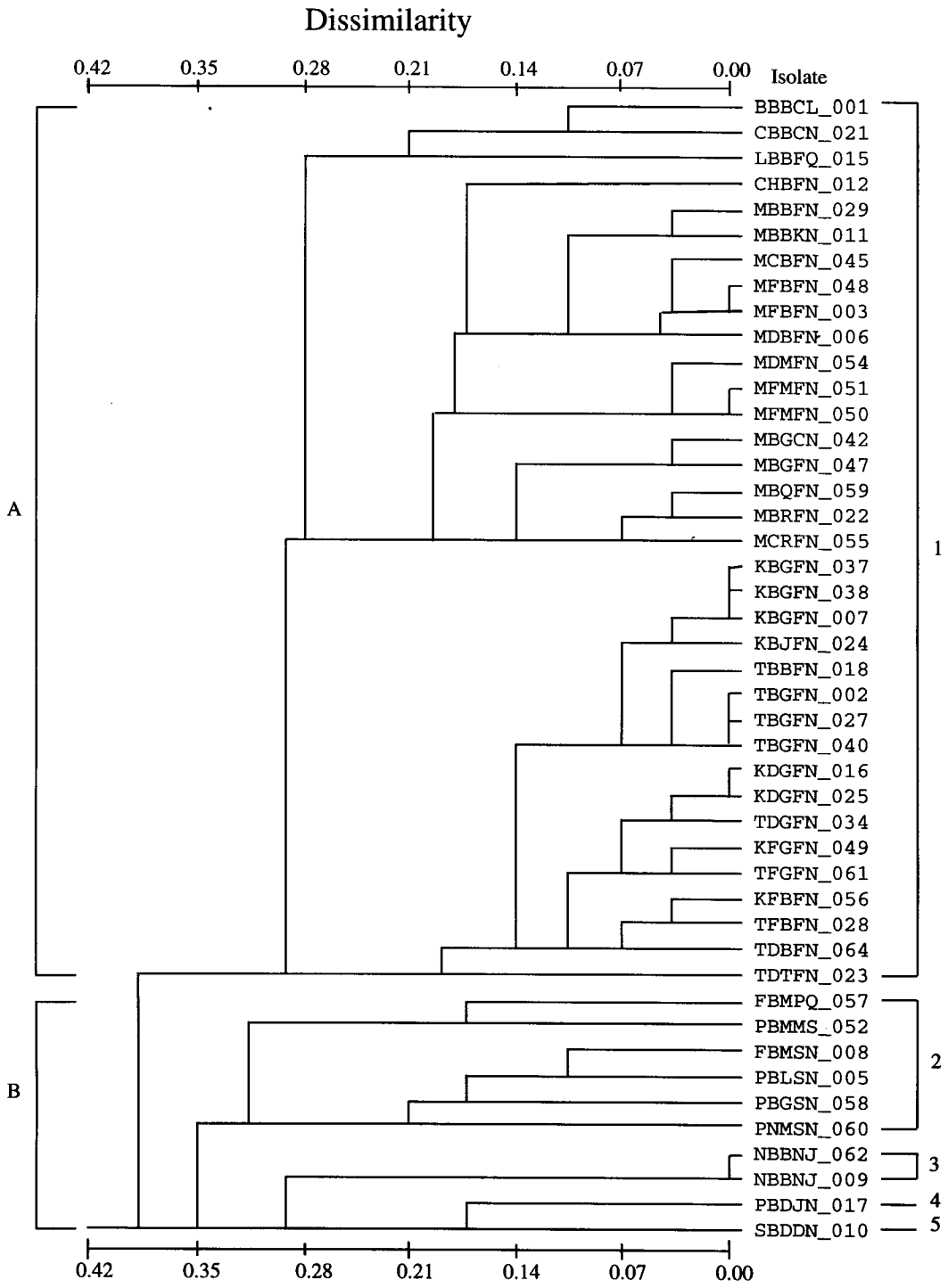

Fig. 1. Dendrogram of 45 *Puccinia recondita* f. sp. *tritici* single-uredinial isolates based on virulence/avirulence to 19 Thatcher wheat lines that differ by single leaf rust resistance genes. The unweighted pair group arithmetic mean method in the SAHN program of NTSYS-pc, version 1.8, was used. The left set of brackets indicates grouping of isolates based on virulence polymorphism; the right set of brackets indicates the equivalent grouping of the isolates in the three-dimensional diagram in Figure 5.

Fifty isolates were among the 35 phenotypes in cluster 1. Ten isolates were among the six phenotypes in cluster 2. Isolates in clusters 1 and 2 had the largest average distance, 15.11 (Fig. 5). The smallest average distances were between clusters 4 and 5 (9.0) and clusters 2 and 3 (9.5) (Fig. 5).

DISCUSSION

There are currently two major groups of *P. r. tritici* in Canada as determined by the combined analyses of virulence and molecular variation. One group, which is found on hard red spring wheat in the prairie provinces of Manitoba and Saskatchewan (11,14,16), consists of virulence phenotypes that are either virulent or avirulent to both resistance genes *Lr2a* and *Lr2c*. Isolates in this group also are found in the eastern provinces of Ontario and Quebec predominantly on spring wheat (14). The other major group consists of virulence phenotypes that are avirulent to *Lr2a* and virulent to *Lr2c*. These isolates are found almost exclusively in Ontario and Quebec (11,14,16), predominately on winter wheats, but also in British Columbia and Alberta.

Burdon and Roelfs (2) examined isozyme variation within and among virulence phenotypes of *P. r. tritici* isolates collected in the United States. Of 13 isozymes examined, polymorphism was found only at the *Est-2* locus. Isolates heterozygous at the *Est-2*

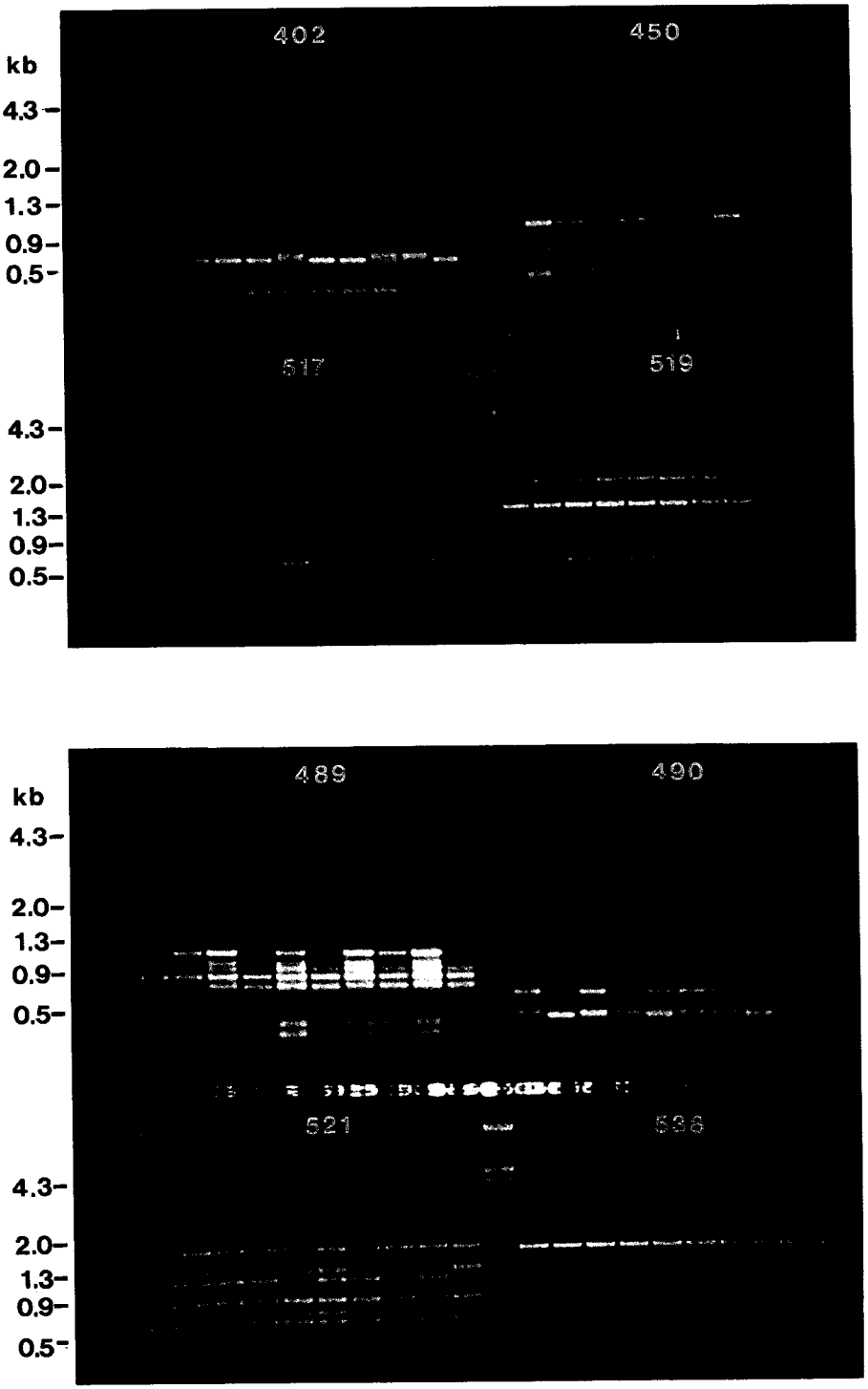

Fig. 2. Randomly amplified polymorphic DNA (RAPD) of *Puccinia recondita* f. sp. *tritici* single-uredinial isolates 1–10 with University of British Columbia biotechnology laboratory primers 402, 450, 517, 519, 489, 490, 521, and 538. RAPD with primers 531 and 556 are not shown. Lambda DNA restricted with *Eco*R1 and *Hin*dIII is the molecular weight marker to the left of each RAPD group.

locus were almost exclusively avirulent to *Lr2a* and virulent to *Lr2c*. Isolates homozygous at the *Est-2* locus were almost exclusively avirulent or virulent to both *Lr2a* and *Lr2c*. The limited isozyme variation in *P. r. tritici* in North America is in close agreement with the relationship between virulence and molecular variation observed in this study.

Dyck and Samborski (5) also recognized the importance of the virulence/avirulence relationship to *Lr2a* and *Lr2c* among *P. r. tritici* isolates. They found that isolates virulent to *Lr2a* were always virulent to *Lr2c* and were homozygous for the virulence allele *p2*. Isolates avirulent to both genes were either homozygous or heterozygous for avirulence allele *P2*. Isolates avirulent to *Lr2a* and virulent to *Lr2c* were heterozygous for *P2* and for another gene that inhibited the expression of avirulence to *Lr2a*. The molecular polymorphisms we observed that distinguished between the two major groups of isolates may be linked to the *p2* virulence locus or the inhibitor of avirulence. Another possibility is that the two groups of isolates originated from separate introductions that are genetically different at a number of loci distributed throughout the *P. r. tritici* genome.

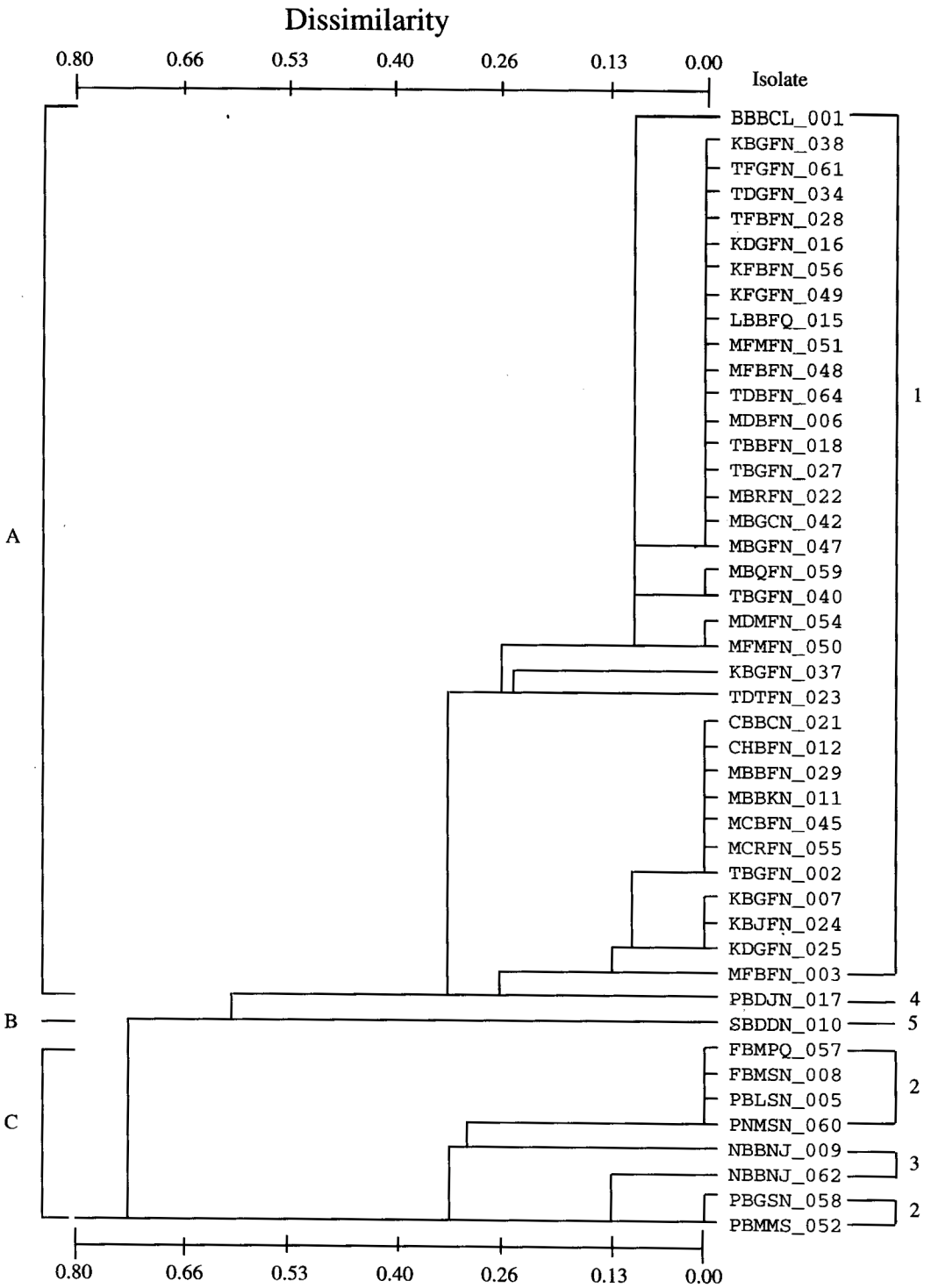

Fig. 3. Dendrogram of 45 *Puccinia recondita* f. sp. *tritici* single-uredinial isolates based on DNA banding patterns of randomly amplified polymorphic DNA with 10 random decamer primers. The unweighted pair group arithmetic mean method in the SAHN program of NTSYS-pc, version 1.8, was used. The left set of brackets indicates grouping of isolates based on molecular polymorphism; the right set of brackets indicates the equivalent grouping of the isolates in the three-dimensional diagram in Figure 5.

Avirulence/virulence as defined on the 19 host differential lines distinguished a greater number of phenotypes compared to the molecular polymorphisms generated by the 10 primers in the RAPD reactions. However, UPGMA and UPGMC cluster analyses of the virulence markers did not separate groups at 50% dissimilarity, and a grouping of isolates could not be distinguished with the single-linkage clustering. Cluster analysis of virulence markers in *P. r. tritici* did not yield the distinct virulence clusters that have been observed in asexual populations of *P. g. tritici* (26). Although the *P. r. tritici* population in Canada is highly variable for virulence (10,12,16), there are often only 1–3 differences in virulence between phenotypes. One possible explanation is that the virulence phenotypes currently observed arose by mutation and subsequent host selection from relatively few original genotypes of *P. r. tritici* that were either introduced to North America or originated from native grasses. The molecular variation observed within virulence phenotypes leaves open the possibility that new virulence phenotypes arise by single virulence changes from different progenitor phenotypes. However, a greater number of isolates within each virulence phenotype needs to be examined for molecular polymorphism to address this issue.

Chen et al (4) examined the relationship between virulence and

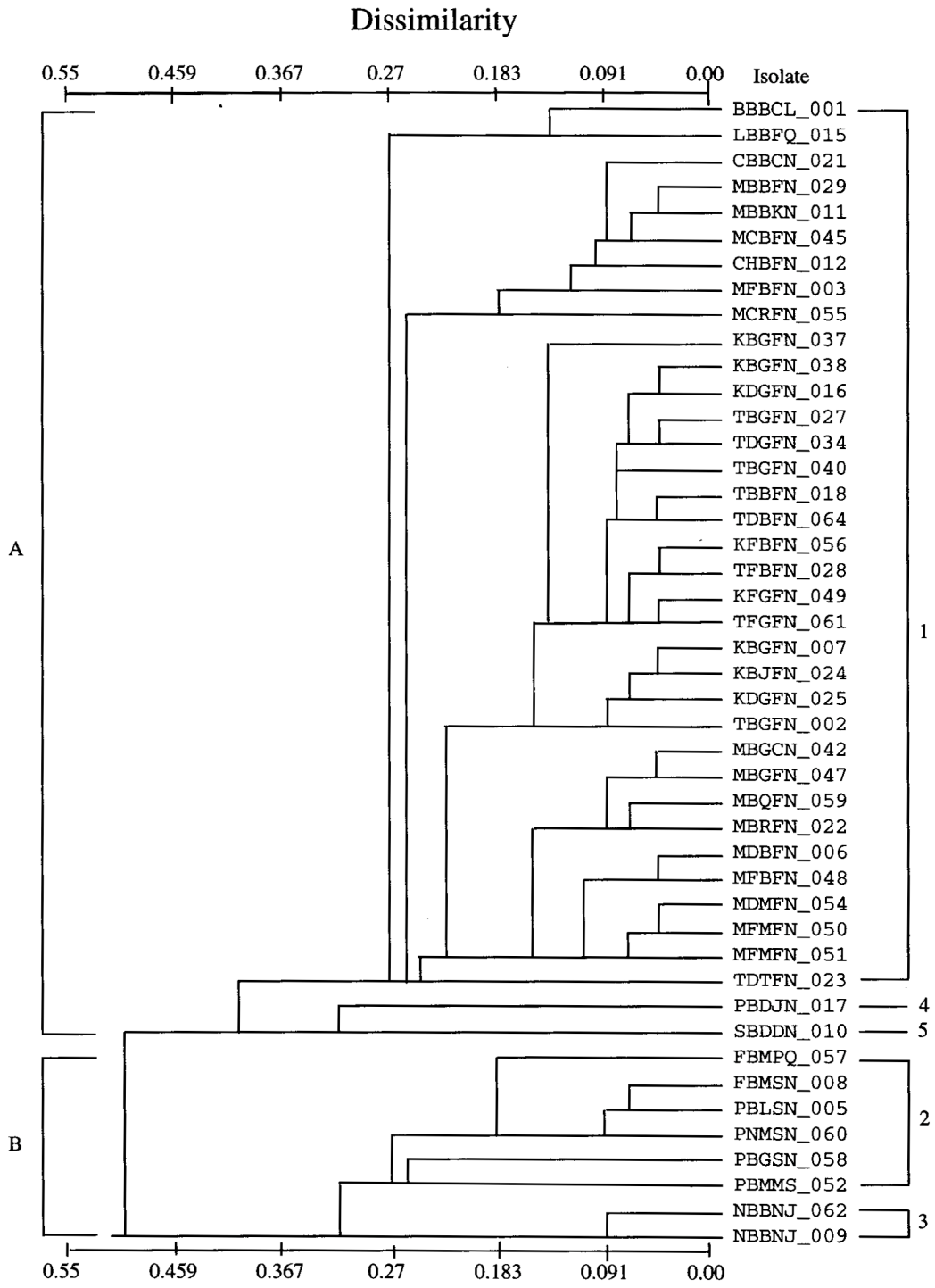

Fig. 4. Dendrogram of 45 *Puccinia recondita* f. sp. *tritici* single-uredinial isolates based on virulence/avirulence to 19 Thatcher wheat lines that differ by single leaf rust resistance genes and DNA banding patterns of randomly amplified polymorphic DNA with 10 random decamer primers. The unweighted pair group arithmetic mean method in the SAHN program of NTSYS-pc, version 1.8, was used. The left set of brackets indicates grouping of isolates based on virulence and molecular polymorphism; the right set of brackets indicates the equivalent grouping of the isolates in the three-dimensional diagram in Figure 5.

molecular variation in populations of the wheat stripe rust fungus, *P. striiformis*, collected in the Pacific Northwest of the United States. They found a high degree of molecular polymorphism among isolates that had the same virulence phenotype. With abundant molecular polymorphism within as well as between virulence phenotypes, there was a relatively low correlation of 0.20 between the virulence and molecular similarity matrices. Chen et al (4) concluded that the molecular polymorphism observed in *P. striiformis* was largely independent of host selection for virulence polymorphism.

Burdon and Roelfs (2,3) examined isozyme polymorphism among and between isolates of the wheat stem rust fungus, *P. graminis* f. sp. *tritici*, that were in different clusters as defined by their virulence phenotypes (26). They found that isolates with the same or similar virulence phenotype almost always had identical isozyme genotypes, whereas isolates in different virulence clusters showed different isozyme genotypes. For *P. g. tritici*, isozyme and virulence variation was highly correlated. Levy et al (19) examined collections of an asexual *Magnaporthe grisea* population from a heavily infected rice nursery in Colombia for DNA fingerprint variation and virulence on differential rice cultivars. They found that *M. grisea* isolates related on the basis of molecular variation were also closely related for virulence.

In the relationship between virulence and molecular variation, the *P. r. tritici* population more closely resembled the *P. g. tritici* population compared to the *P. striiformis* population. In North America, the three wheat rusts reproduce by the clonal asexual propagation of urediniospores (an isolated sexual population of *P. g. tritici* has been found in the Pacific Northwest [26]). The relationship between the host selected virulence markers and the, presumably, unselected biochemical and molecular markers in *P. g. tritici* and *P. r. tritici* is consistent with expectations for clonally reproducing organisms, in which there is only a limited amount of parasexual recombination, if any. The low degree of correlation between virulence and molecular markers in *P. striiformis* might be explained by frequent parasexual recombination (4) that may allow for selection of virulence polymorphism to occur independently of molecular polymorphism. Another possibility is that virulence phenotypes of *P. striiformis* originate from multiple molecular backgrounds. Burdon and Roelfs (3) found that in the sexual population of *P. g. tritici* there was no association between isozyme alleles and virulences.

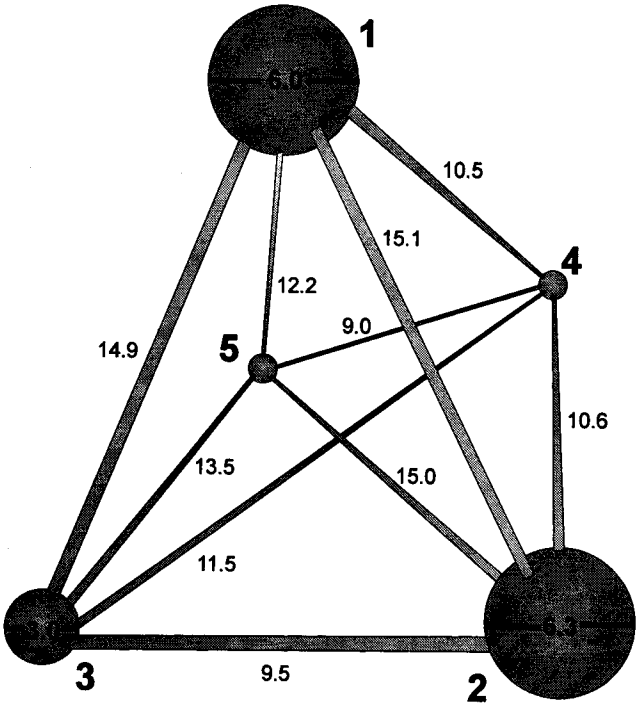

Fig. 5. Three-dimensional cluster diagram of *Puccinia recondita* f. sp. *tritici* single-uredinial isolates based on virulence/avirulence to 19 Thatcher wheat lines that differ by single leaf rust resistance genes and DNA banding patterns of randomly amplified polymorphic DNA with 10 random decamer primers. Numbers between clusters represent the average number of virulence and molecular differences between *P. r. tritici* isolates in each cluster. Numbers within clusters represent the average number of virulence and molecular differences between *P. r. tritici* isolates within the cluster. The 35 phenotypes in cluster 1 are virulent or avirulent to both leaf rust resistance genes *Lr2a* and *Lr2c*. The six phenotypes in cluster 2 are avirulent to *Lr2a* and virulent to *Lr2c*. The two isolates in cluster 3 have the virulence phenotype NBBNJ and differ for three DNA banding patterns. Cluster 4 has one isolate of virulence phenotype PBDJN, and cluster 5 has one isolate of virulence phenotype SBDDN.

Although two major groups with over 90% of the *P. r. tritici* isolates were distinguished in the present study, three virulence phenotypes were outside clusters 1 and 2 in Figure 5. The virulence phenotype of the two isolates in cluster 3 (NBBNJ) is detected regularly in British Columbia (10,12), occasionally in Ontario (16), and also in the northeastern United States (22). The virulence phenotype of the single isolate in cluster 4 (PBDSN) is collected regularly in British Columbia (10,12,16). These two virulence phenotypes may have diverged by host selection from other clusters in Figure 5 or may be remnants of other isolate groups. Collections of *P. r. tritici* from British Columbia and the Pacific Northwest of the United States often have virulence phenotypes that are not found in other parts of North America. Similarly, *P. r. tritici* virulence phenotypes from Ontario, Quebec (14), and the soft wheat area of the eastern United States (18,23) are usually not found in the Great Plains region of North America. Virulence and molecular analysis of an increased number of *P. r. tritici* collections from these areas may have distinguished other phenotypes that are isolated from the current two major clusters. The isolates that were chosen for this study were representative of the predominant *P. r. tritici* virulence phenotypes in Canada.

The present day arrangement of the two major clusters has undoubtedly been influenced by the effects of host selection on the *P. r. tritici* population in North America. Race 9, the original designation of the single isolate in cluster 5 (Fig. 5), 10-SBDDN, was one of the most common virulence phenotypes from 1931 to 1943 in the eastern and prairie populations of *P. r. tritici* in Canada (13,25). Race 9 virtually disappeared in both populations after the introduction of winter wheats with the resistance gene *Lr3*. In recent surveys, isolates with the race 9 virulence phenotype are generally collected only from goatgrass, *Aegilops cylindrica*, in the southern plains of the United States (23). Molecular analysis of race 9 phenotypes from 1931 to 1943 may have revealed an additional major cluster of *P. r. tritici* isolates. The race 9 isolate examined by Burdon and Roelfs (2) had the unique combination of virulence to both *Lr2a* and *Lr2c* and heterozygosity at the *Est-2* locus.

The common virulence phenotypes from 1931 to 1960 in the eastern and prairie populations of *P. r. tritici* in Canada (11) were races 1–3, 5, 6, and 9. With the exception of race 9, the present day equivalents of all of these races are found in either clusters 1 or 2 (Fig. 5). Isolates 1-BBBCL, equivalent to race 1; 11-MBBKN, equivalent to race 5; and 12-CHBFN, equivalent to race 2, were collected in the 1950s and are all in cluster 1. The common virulence phenotypes from 1931 to 1960 would most likely be found in either cluster 1 or 2, except for the race 9 phenotypes, which may have comprised an additional major cluster.

In the virulence surveys since 1960, phenotypes of *P. r. tritici* avirulent to *Lr2a* and virulent to *Lr2c* have been found in Ontario and Quebec, British Columbia and Alberta, and very rarely in Manitoba and Saskatchewan (10–12,16). Prior to 1960, these virulence phenotypes were found in both the eastern and the prairie provinces (13). The divergence of the eastern and prairie *P. r. tritici* populations can be attributed to the use of host resistance genes (13). Virulence and molecular analysis of the eastern and prairie populations during the early years of the survey (1931–1944) (25) would most likely have shown that clusters 1 and 2, and possibly cluster 5, in Figure 5 were composed of *P. r. tritici* virulence phenotypes common to both geographic populations.

Host selection for virulences to resistance genes *Lr11, Lr24, Lr26, Lr3ka,* and *Lr30* has occurred since 1986 in the prairie population of *P. r. tritici*. Prior to 1986, phenotypes with virulence to these genes were uncommon in the prairie population. In the 1993 virulence survey, all *P. r. tritici* isolates in Manitoba and Saskatchewan had virulence to at least one of these genes (J. A. Kolmer, *unpublished data*). The increase of virulence to these resistance genes occurred entirely among isolates within cluster A in Figure 3 and within cluster 1 in Figure 5. The present population of *P. r. tritici* in the Great Plains of North America is composed of many virulence phenotypes with very similar molecular backgrounds. At present, there is no evidence that immigration of virulence phenotypes with dissimilar molecular backgrounds from other regions of North America has occurred. The pattern of molecular polymorphism in *P. r. tritici* generally agrees with the evolutionary relationships postulated from long-term virulence surveys in North America (11,13,14,18).

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