Genetics

Relationship Between Virulence Variation
and DNA Polymorphism in *Puccinia striiformis*

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ABSTRACT

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One-hundred, fifteen single-urediospore isolates from 23 collections (five isolates per collection) of *Puccinia striiformis* f. sp. *tritici* (stripe rust of wheat) selected to represent different regions in North America and different virulence patterns were analyzed for variation in virulence and DNA polymorphism. Virulence characterization was based on the expression of infection types on 15 wheat cultivars used to differentiate races of *P. striiformis* in North America. Of the 23 collections, 20 had uniformly different virulence patterns, i.e., each of the 20 collections was a different race, and all five isolates of each collection had the same virulence pattern. Two collections consisted of isolates with two virulence patterns, and one collection consisted of isolates with three virulence patterns. Of the 26 virulence patterns (races), four (designated as races CDL-47, CDL-48, CDL-49, and CDL-50) had not been detected previously. DNA polymorphism was determined by random amplified polymorphic DNA (RAPD) assay. Random primers were used to amplify DNA extracted from the 115 isolates. Of 107 RAPD bands that were amplified with 11 random primers, 47% were polymorphic. DNA polymorphism was detected among races and among single-spore isolates within races. Race

CDL-21, originally collected from triticale in California, was different from other races in 13% of the RAPD bands and had only 67% similarity with other races. All other races were more closely related (about 80% similarity). Based on cluster analyses, the 115 isolates were placed in six virulence and five RAPD groups. The correlation coefficient between the virulence and RAPD similarity matrices was low (0.20). Cluster analysis based on RAPD data separated isolates virulent on stripe rust-resistance gene *Yr1* from those avirulent on *Yr1*. Virulence groups were highly associated with epidemic regions. RAPD groups generally were not associated with geographic regions. The low association between virulence and RAPD patterns indicates that DNA polymorphisms are independent of virulence, and selection for virulence by growing hosts with race-specific resistance plays a major role in determining the race structure of the pathogen.

Additional keywords: evolutionary relationship, host-parasite interactions, polymerase chain reaction, yellow rust of wheat.

Stripe rust (yellow rust), caused by *Puccinia striiformis* Westend. f. sp. *tritici*, is one of the most important diseases of wheat in the world (31,33). In North America, the disease is especially destructive in the western United States and is sometimes destructive in the south-central United States (20). Use of resistant cultivars is the major method to control the disease. However, cultivars with race-specific resistance often become susceptible within a few years after their release because of the rapid evolution of new virulent races of the pathogen.

Breeding for resistance depends on information about the pathogenicity of races. Germ plasm and breeding lines are evaluated for resistance by inoculation with a specific race, mixture of races, or exposure of plants in field plots to naturally occurring inoculum. Breeding for race-specific resistance is sometimes unpredictable because existing differential cultivars may not detect virulences. Cultivars with high-temperature, adult-plant resistance to stripe rust have been more durable, but they may not provide protection at early growth stages and cool temperatures. Furthermore, identification of high-temperature, adult-plant resistance requires knowledge of race-specific resistance. Understanding the

relationships among the races and how virulence evolves is useful for developing strategies for managing the disease, as well as for developing new cultivars.

In North America, stripe rust distribution, prevalence, and severity are monitored by trap-plots consisting of differential cultivars as well as other cultivars planted at various sites and by surveying wheat fields. Virulence of the pathogen population is determined based on trap-plot data and by testing rust collections on a set of wheat differential cultivars (cultivars that have different genes for resistance) under controlled conditions (18). Evolutionary relationships among the races have been postulated based on the chronological appearance, geographical distribution, and virulence of the races (18,20). However, genetic interpretation of the relationship among races of *P. striiformis* has been limited because of the absence of the pycnial and aecial stages and a paucity of genetic markers for the pathogen.

Newton et al (26) used isozyme and double-stranded RNA (dsRNA) to study the relationships of formae speciales and races of *P. striiformis* in England. They reported that *P. s. tritici* (wheat stripe rust) and *P. s. hordei* (barley stripe rust) have different isozyme and dsRNA patterns, but they could not find any differences related to races of *P. striiformis*. Using dsRNA phenotypes, Dickinson and Pryor (7) and Dickinson et al (8) found that *P. striiformis* was different from *P. graminis, P. menthae, P. recondita, P. sorghi,* and *Melampsora lini.* They reported limited variations among isolates of *P. striiformis* from Australia, but the variations were not correlated with races. Using DNA sequence analysis of the internal transcribed spacer region of the ribosomal DNA, Zambino et al (36) also differentiated *P. striiformis* from other rust species and reported their interspecific relationships. Genetic variations among races of *P. striiformis* at the DNA level have not been reported.

The random amplified polymorphic DNA (RAPD) assay developed by Williams et al (35) and Welsh and McClelland (34) has been useful for analyzing genomic variations. The technique uses short oligonucleotides (about 10 bases long) as random primers to amplify genomic DNA in a polymerase chain reaction (PCR). A main advantage of the technique is that it does not require cloned DNA fragments as hybridization probes or sequence information for the synthesis of primers (35). The RAPD technique has been used for genome mapping of tomato (13), conifers (2), lettuce (27), *Brassica* species (11,28), *Neurospora crassa* (35), and *Magnaporthe grisea* (16) and to study genetic variation in *Leptosphaeria maculans* (9), *Fusarium solani* f. sp. *cucurbitae* (6), *Erysiphe graminis* f. sp. *hordei* (24), and *Colletotrichum graminicola* (10).

Smith et al (32) were able to distinguish *P. s. hordei* from *P. hordei* by the RAPD assay. Using the RAPD assay, we differentiated *P. graminis, P. recondita,* and *P. striiformis* of wheat and observed genetic variations among isolates within each species (X. M. Chen, R. F. Line, and H. Leung, *unpublished data*). Our results suggested that the RAPD assay might be useful for determining relationships among races of *P. striiformis.* The objectives of this research were to study the genetic heterogeneity of working collections of *P. striiformis* in terms of virulence and DNA polymorphism, to determine the associations of molecular phenotypes and virulence types, and to expand our understanding of the evolutionary relationships of the races in North America.

MATERIALS AND METHODS

Isolation of single spores and analysis of virulence. Urediospores of 23 North American collections of *P. s. tritici* (Table 1) were selected based on geographic region, year when first detected, and virulence group (race classification) (20). Five single-spore isolates were obtained from each of the 23 collections. Using a microscope, a single spore was transferred with a fine glass needle from a slide to a leaf of a susceptible wheat cultivar. The inoculated seedlings were placed in a dew chamber at 10 C for 24 h and then in isolation booths within a growth chamber as described by Chen and Line (3,4). To prevent mixing of isolates, spores of each isolate were increased on susceptible plants in

isolation booths and, when possible, on cultivars that were susceptible to the specific race but resistant to other races. Isolates that were virulent on common cultivars were increased in separate facilities at a different time. Urediospores used for the virulence tests and DNA extraction were collected and stored in a desiccator at about 4 C until used.

Virulence patterns of the single-spore isolates were determined on 15 differential wheat cultivars (Table 1) as described by Line et al and Line and Qayoum (19,20). The virulence tests were repeated at least twice to confirm the consistency of the virulence patterns. Conditions for growing plants before and after inoculation and the method of recording infection types are described by Chen and Line (3,4).

DNA extraction and amplification. DNA was extracted using a modification of the cetyltrimethylammonium bromide (CTAB) procedure described by Murray and Thompson (25). Most molecular studies with fungi have utilized DNA extracted from mycelium (6,9,10,14,29). *P. striiformis* is an obligate parasite and is difficult to grow on artificial media; therefore, DNA was extracted directly from urediospores. For each isolate, 20 mg of urediospores was mixed with sterile sand and ground into a fine powder with a mortar and pestle. The powder was transferred into a 1.5-ml microfuge tube and dispersed in 1 ml of extraction buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 100 mM EDTA). After mixing thoroughly and adding 0.06 ml of 20% sodium dodecyl sulphate (final concentration 1%), the mixture was gently shaken for 1 h at about 20 C, mixed with 0.15 ml of 5 M NaCl (final concentration 0.8 M) and 0.13 ml of CTAB/NaCl solution (10% CTAB in 0.7 M NaCl, final concentration 1%), and kept at 65 C for 20 min. The mixture was divided into two 1.5-ml microfuge tubes and extracted with chloroform/isoamyl alcohol (24:1). The top aqueous phase was transferred to a clean tube, and 0.6 volumes (about 360 μl) of cold isopropanol was added. After 20 min of incubation at 4 C, the solution was centrifuged for 10 min at 10,000 rpm at 20 C to precipitate the nucleic acid. The pellet was rinsed twice with cold 70% ethanol, dried in a vacuum, and dissolved in 0.5 ml of TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8.0). One microliter of ribonuclease at 10 mg/ml was added (final concentration 20 μg/ml) and kept at 4 C overnight to completely digest the RNA. The DNA was reprecipitated, rinsed with cold 70% ethanol, dried, and dissolved in 40 μl of TE. The DNA was quantified by the minigel method (23). An average of 0.2 μg of DNA was obtained from 20 mg of urediospores. After quantification, the DNA was dissolved in 200 μl of TE and kept at −20 C for later use. A working DNA solution was made by diluting the stock DNA solution to about 0.1 ng/μl.

Each amplification reaction was performed in a 13-μl volume consisting of 0.2 mM each of dATP, dCTP, dGTP, and TTP (Sigma Chemical Co., St. Louis, MO); 2 mM MgCl$_2$; 0.3 units of *Taq* DNA Polymerase (Promega, Madison, WI); 2–4 μM primer, depending on specific primers; 0.2 ng of DNA template; 1.25 μl of 10× *Taq* polymerase buffer (Promega); and sterile water added to a final volume of 13 μl. Sterile distilled H$_2$O was used in place of the DNA template as a control to ensure that there was no contamination. The solution was overlaid with mineral oil. Amplification was carried out in a Perkin-Elmer model 480 thermal cycler programmed for 10 min at 94 C for initial denaturation and 40 cycles that consisted of 1 min at 94 C, 2 min at 37 C, and 2.5 min at 72 C, followed by a final 10-min extension at 72 C. The fastest ramp time was used for temperature transition.

After amplification, 5 μl of the solution for each sample was electrophoresed in a 1.5% agarose gel in 0.5× TBE buffer (0.089 M Tris-borate, 0.089 M boric acid, and 0.002 M EDTA). A 1-kb DNA ladder (0.15 μg) (Gibco BRL, Bethesda, MD) was used to estimate the size of each amplified DNA fragment. The gel was run for 90 min at 100 volts, stained with ethidium bromide (0.5 μg/ml) for 30 min, and photographed under ultraviolet light. Ninety-six random primers (Operon Technologies, Alameda, CA) were screened with three isolates of *P. striiformis.* Eleven of the primers that yielded consistent banding patterns (Table 2) were

selected for analyzing the 115 single-spore isolates. The test with each primer was repeated at least twice to ensure the consistency of each RAPD band.

Analysis of data. Dendrograms were constructed based on the virulence and RAPD data, using the Numerical Taxonomy System for personal computer (NTSYS-pc), version 1.70 (30). Only bands repeatable in at least two experiments with the same primer conducted at different times were used in the phenetic analysis. The presence or absence of an RAPD band was considered an alternative character and was coded as 1 and 0, respectively. Simi-larly, for analysis, virulence was coded as 1 and avirulence as 0. A similarity matrix based on simple matching was generated by the SIMQUAL program of NTSYS-pc (version 1.70; 30). Cluster analysis was done with the unweighted pair group arithmetic mean method (UPGMA) in the SAHN program of NTSYS-pc (version 1.70; 30). The dendrogram with the best fit to the similarity matrix based on cophenetic values (COPH) and MXCOMP, a matrix comparison program of NTSYS-pc, was chosen. Correlation of the RAPD and virulence data was determined by comparison of the two similarity matrices by MXCOMP.

TABLE 1. Geographic origin, virulence pattern on 15 North American differential cultivars, race designation, and random amplified polymorphic DNA (RAPD) group (RG) of 115 single-urediospore isolates of *Puccinia striiformis* f. sp. *tritici* from 23 collections

Collection	Region[a]	Single-spore isolate	Virulence on differential cultivars[b]	CDL race[c]	Virulence group	RAPD group 50 bands	RAPD group 107 bands
1	1	1-1	1,2	1	VG 1	RG 2	RG 5
		1-2 to 1-5	1,2	1	VG 1	RG 2	RG 2
2	1	2-2,2-5	1,3	3	VG 2	RG 3	RG 3
		2-1,2-3,2-4	1,3	3	VG 2	RG 3	RG 5
3	1	3-1,3-2,3-4,3-5	1,3,4	5	VG 2	RG 3	RG 3
		3-3	1,3,4	5	VG 2	RG 5	RG 5
4	1	4-1,4-2	1,6,8,12	6	VG 6	RG 3	RG 3
		4-3	1,6,8,12	6	VG 6	RG 5	RG 5
		4-4	1,6,8,12,13	47	VG 6	RG 3	RG 3
		4-5	1,6,8,12,13,14	48	VG 6	RG 5	RG 5
5	1	5-1 to 5-3,5-5	1,3,5	7	VG 2	RG 3	RG 3
		5-4	1,3,5	7	VG 2	RG 5	RG 5
6	3	6-1 to 6-5	1,3,9	8	VG 3	RG 3	RG 3
7	1	7-1,7-3,7-4	1,8,12	14	VG 6	RG 3	RG 3
		7-2	1,8,12	14	VG 6	RG 5	RG 3
		7-5	1,8,12	14	VG 6	RG 5	RG 5
8	5	8-1 to 8-5	1,3,6,10	15	VG 5	RG 3	RG 3
9	1	9-1	1,3,9,11	16	VG 3	RG 5	RG 5
		9-2 to 9-5	1,3,9,11	16	VG 3	RG 3	RG 3
10	5	10-1,10-3 to 10-5	1,2,3,9,11	17	VG 2	RG 3	RG 2
		10-2	1,2,3,9,11	17	VG 3	RG 4	RG 4
11	1	11-1 to 11-5	1,6,8,10,12	20	VG 6	RG 3	RG 3
12	6	12-1 to 12-5	2	21	VG 1	RG 1	RG 1
13	1	13-1 to 13-4	1,3,12	22	VG 2	RG 3	RG 3
		13-5	1,3,12	22	VG 2	RG 5	RG 5
14	5	14-1,14-2,14-4	1,3,6,9,10	23	VG 5	RG 3	RG 3
		14-3,14-5	1,3,6,9,10	23	VG 5	RG 5	RG 5
15	1	15-1,15-2	1,3,6,8,9,10,12	25	VG 5	RG 2	RG 2
		15-3	1,3,6,8,9,10,12	25	VG 5	RG 2	RG 5
		15-4	1,3,6,8,9,10,12	25	VG 5	RG 5	RG 5
		15-5	1,3,6,8,9,10,12	25	VG 5	RG 3	RG 3
16	1	16-1	1,3,12,13,15	45	VG 4	RG 4	RG 5
		16-2,16-3,16-5	1,3,12,13,15	45	VG 4	RG 4	RG 4
		16-4	1,3,12,13,15	45	VG 4	RG 3	RG 3
17	1	17-1 to 17-4	1,3,4,12	28	VG 2	RG 3	RG 3
		17-5	1,3,4,12	28	VG 2	RG 2	RG 3
18	1	18-1,18-2	1,3,4,5	29	VG 2	RG 2	RG 2
		18-3,18-4	1,3,4,5	29	VG 2	RG 3	RG 3
		18-5	1,3,4,5	29	VG 2	RG 3	RG 4
19	6	19-1	1,10	35	VG 1	RG 4	RG 4
		19-2 to 19-4	1,10	35	VG 1	RG 3	RG 3
		19-5	1,10	35	VG 1	RG 3	RG 5
20	5	20-1	1,3,6,8,9,10,11,12	37	VG 5	RG 3	RG 5
		20-2,20-3	1,3,6,8,9,10,11,12	37	VG 5	RG 3	RG 3
		20-4,20-5	1,3,6,9,10,11,12	46	VG 5	RG 3	RG 3
21	1	21-1	1,3,11	38	VG 3	RG 3	RG 3
		21-2	1,3,11	38	VG 3	RG 3	RG 4
		21-3 to 21-5	1,3,11,13	49	VG 3	RG 3	RG 3
22	1	22-1,22-4	1,3,4,5,14	50	VG 2	RG 3	RG 3
		22-2,22-5	1,3,4,5,14	50	VG 2	RG 4	RG 4
		22-3	1,3,4,5,14	50	VG 2	RG 3	RG 4
23	1	23-1 to 23-4	1,3,12,13,15	45	VG 4	RG 3	RG 3
		23-5	1,3,12,13,15	45	VG 4	RG 2	RG 2

[a] Region 1 = eastern Washington, northeastern Oregon, and northern Idaho of the United States and southern Alberta of Canada; region 3 = southern Idaho, southeastern Oregon, northern Nevada, northern Utah, and western Colorado; region 5 = western Washington; and region 6 = central California (20).
[b] Wheat differential cultivars: 1 = Lemhi; 2 = Chinese 166; 3 = Heines VII; 4 = Moro; 5 = Paha; 6 = Druchamp; 7 = Riebesel 47/51; 8 = Produra; 9 = Yamhill; 10 = Stephens; 11 = Lee; 12 = Fielder; 13 = Tyee; 14 = Tres; and 15 = Hyak (18).
[c] Cereal Disease Laboratory (CDL) race (20).

RESULTS

Virulence variation. The results of virulence tests are shown in Table 1. Twenty-six virulence patterns (races) were identified from the 23 collections. Twenty collections showed no isolate variation in virulence, i.e., all five isolates of each collection were the same race. Collections 16 and 23 had the same virulence pattern (race CDL-45; Cereal Disease Laboratory race [20]). Of the collections that had variation, collection 4 consisted of races CDL-6, CDL-47, and CDL-48; collection 20 consisted of races CDL-37 and CDL-46; and collection 21 consisted of races CDL-38 and CDL-49. Races CDL-47 to CDL-50 had not been identified previously.

Two slightly different virulence dendrograms were obtained using the FIND option in the SAHN program. The two dendrograms had identical goodness-of-fit to the similarity matrix (data not shown). The correlation coefficient (r) of the dendrograms to the similarity matrix was 0.74. Major clusters were similar in the two dendrograms with only minor differences in the arrangement of a few isolates within the clusters. One of the dendrograms is shown in Figure 1. The 115 isolates were clustered into six virulence groups (VGs), using 80% similarity as the cut-off point. Races in VG 1 (CDL-1, CDL-21, and CDL-35) had a narrow virulence spectrum; races CDL-1, CDL-21, and CDL-35 were virulent on differential cultivars Chinese 166 and Lemhi, Chinese 166, Lemhi and Stephens, respectively. Races in VG 2 (CDL-3, CDL-5, CDL-7, CDL-22, CDL-28, CDL-29, and CDL-

50) and VG 3 (CDL-8, CDL-16, CDL-17, CDL-38, and CDL-49) had common virulences on Lemhi and Heines VII. The two groups were more closely related (about 78% similarity) than were other groups. VG 3 differed from VG 2 in its virulence on Yamhill and/or Lee. VG 1, VG 2, and VG 3 had 74% similarity. VG 4 consisted of only one race (CDL-45), which was virulent on Lemhi, Heines VII, Fielder, Tyee, and Hyak and had about 69% similarity with VG 1, VG 2, and VG 3. VG 5 (CDL-15, CDL-23, CDL-25, CDL-37, and CDL-46) had common virulence on Lemhi, Heines VII, Druchamp, and Stephens. VG 6 (CDL-6, CDL-14, CDL-20, CDL-47, and CDL-48) had common virulence on Lemhi, Produra, and Fielder. VG 5 and VG 6 had about 70% similarity.

Each of the virulence groups, except for VG 4, can be separated into two subgroups, using 88% similarity as a cut-off point. Races in VG 1A (CDL-1 and CDL-21) were virulent on Chinese 166. Race CDL-35 of VG 1B was avirulent on Chinese 166 but virulent on Stephens. Races in VG 2A (CDL-3, CDL-5, CDL-22, and CDL-28) were avirulent on Paha, and races in VG 2B (CDL-7, CDL-29, and CDL-50) were virulent on Paha. Races in VG 3A (CDL-8, CDL-16, and CDL-17) were virulent on Yamhill, and races in VG 3B (CDL-38 and CDL-49) were avirulent on Yamhill. Races in VG 5A (CDL-15, CDL-23, and CDL-46) were avirulent

TABLE 2. Number of scored and polymorphic RAPD bands produced by *Puccinia striiformis*, using primers from Operon Technologies (Alameda, CA)

Primer	Nucleotide sequence	Scored bands	Polymorphic bands[a]
OPA-02	5′-TGCCGAGCTG-3′	11	5
OPA-03	5′AGTCAGCCAC-3′	9	5
OPB-15	5′-GGAGGGTGGT-3′	10	6
OPB-17	5′-AGGGAACGAG-3′	6	5
OPC-02	5′-GTGAGGCGTC-3′	8	3
OPC-18	5′-TGAGTGGGTG-3′	5	2
OPD-18	5′-GAGAGCCAAC-3′	10	5
OPE-07	5′-AGATGCAGCC-3′	11	5
OPF-01	5′-ACGGATCCTG-3′	12	5
OPG-02	5′-GGCACTGAGG-3′	11	3
OPG-15	5′-ACTGGGACTC-3′	14	6
Total		107	50

[a] Bands with 10–90% frequency for 115 isolates and present or absent for all five isolates of a collection.
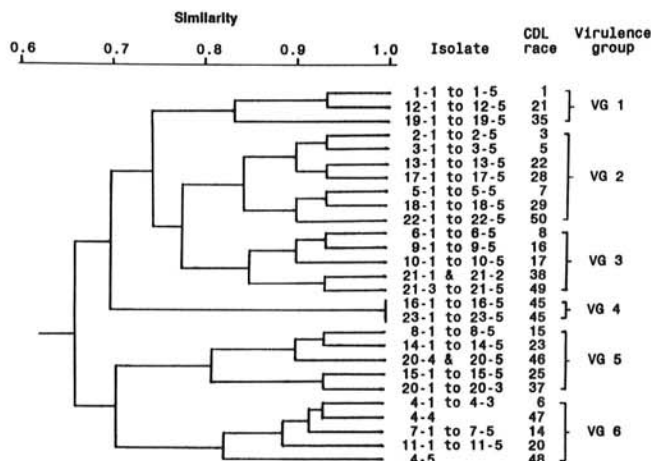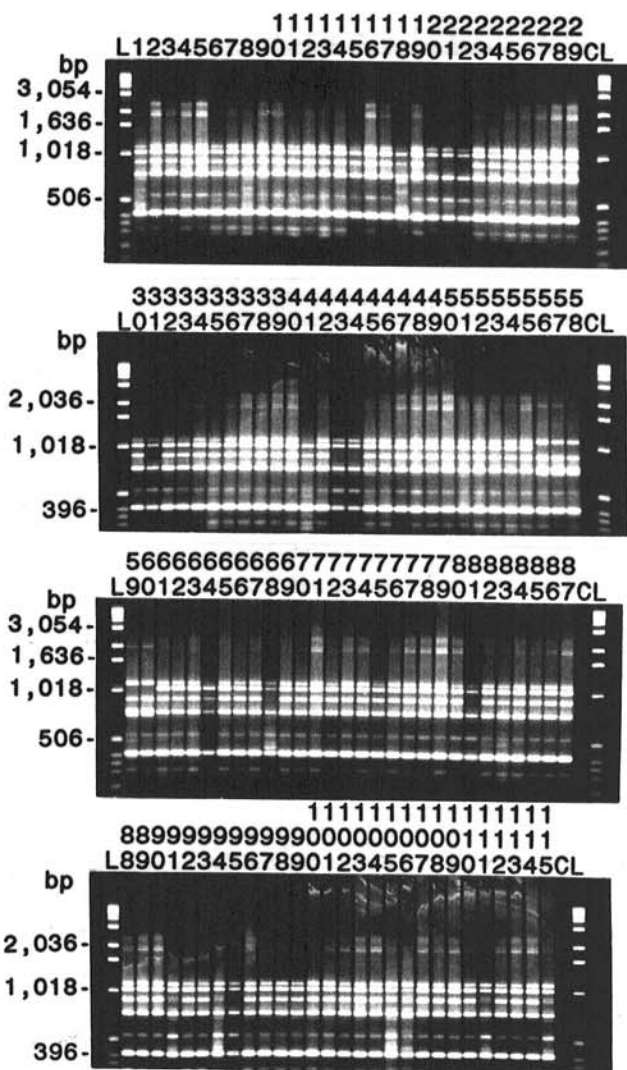

Fig. 1. Dendrogram of 115 single-spore isolates of *Puccinia striiformis* based on virulence and avirulence on 15 wheat differential genotypes listed in Table 1, using the unweighted pair group arithmetic mean (UPGMA) program of NTSYS-pc (version 1.70; 30).

Fig. 2. Random amplified DNA polymorphisms of 115 single-urediospore isolates (five isolates each of 23 collections) of *Puccinia striiformis* with primer OPG-02. Lanes marked 1 to 115 are single-urediospore isolates in the same order of collections and isolates in each collection listed in Table 1. Each group of five isolates (i.e., 1–5, 6–10, etc.) represents a collection. Lanes marked L are 1-kb ladder DNA, and those marked with C are controls with sterile water.

on Produra and Fielder, and races in VG 5B (CDL-25 and CDL-37) were virulent on Produra and Fielder. Races in VG 6A (CDL-6, CDL-47, CDL-14, and CDL-20) were avirulent on Tres, but race CDL-48 of VG 6B was virulent on Tres.

DNA polymorphism. Of the 107 reproducible RAPD bands obtained with 11 random primers, 23 were present in all isolates. RAPD bands with 10–90% frequency and the same in all five isolates within a collection were considered polymorphic markers. Based on these criteria, 50 of the 107 bands were polymorphic (Table 2). Figure 2 shows the DNA polymorphism among the 115 single-urediospore isolates that were amplified using primer OPG-02. Isolates 56–60 (the five isolates of race CDL-21) had a unique band of about 800 bp but lacked the 1,000-bp band present in all the other isolates. Variations in amplified DNA among races are shown in Figure 3, in which a representative isolate showing the most common RAPD pattern for each race was used. Figure 4 shows variation among races but uniformity within a single race at least for a single primer. Race CDL-21 was clearly different from the other two races. Of the 107 RAPD bands, 14 were uniquely present or absent for race CDL-21. Specific bands also were found for some other races. DNA polymorphism also was observed among isolates within some other races (Figs. 2 and 5).

When the 50 polymorphic RAPD bands were used for cluster analyses using the FIND option in UPGMA of the SAHN program, nine different dendrograms were obtained. These dendrograms were similar with only minor differences in the arrangement of a few isolates. Figure 6 shows the dendrogram that had the highest correlation coefficient ($r = 0.923$) to the similarity matrix. Isolates of races CDL-1 (isolates 1-1 to 1-5), CDL-3 (isolates 2-1 to 2-5), CDL-15 (isolates 8-1 to 8-5), CDL-20 (isolates 11-1 to 11-5), CDL-21 (isolates 12-1 to 12-5), CDL-37 (isolates 20-1 to 20-3), and CDL-46 (isolates 20-4 to 20-5) were relatively homogeneous, whereas the other races showed different degrees of heterogeneity. The 115 isolates can be classified into five RAPD groups (RGs), using 80% similarity as a cut-off point (Fig. 6A).

RG 1, consisting of the five isolates of race CDL-21, was homogeneous and distinct from other groups (about 40% similarity) (Fig. 6B). RG 2 (16 isolates) contained all five isolates of race CDL-1, four isolates of race CDL-17 (isolates 10-1 and 10-3 to 10-5), three isolates of race CDL-25 (isolates 15-1 to 15-3), two
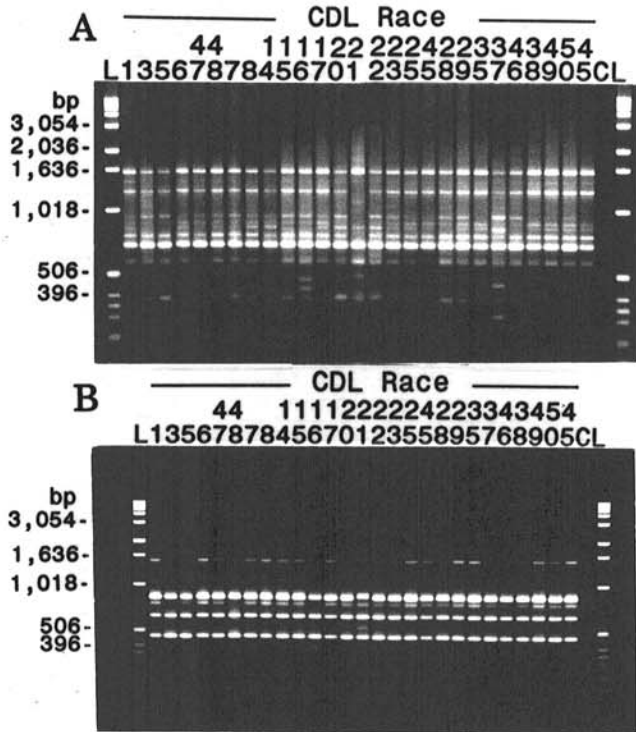

Fig. 3. Random amplified DNA polymorphisms of 27 isolates representing 26 races of *Puccinia striiformis* amplified with primers **A**, OPB-15 and **B**, OPC-02 showing differences among races. Lanes marked L are 1-kb ladder DNA, and those marked with C are controls with sterile water.
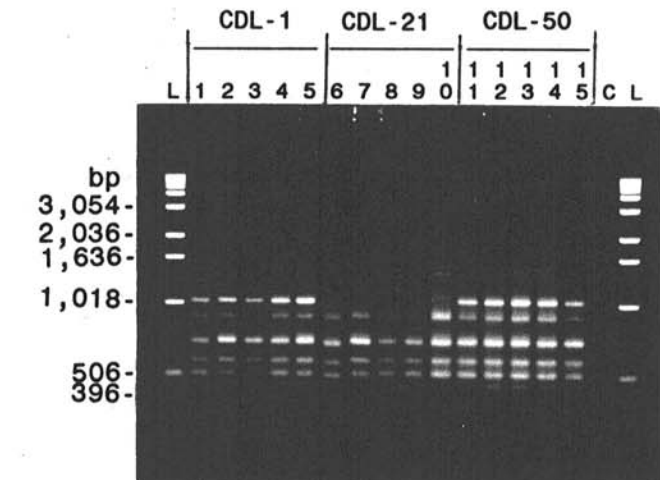

Fig. 4. Random amplified DNA polymorphisms of 15 isolates representing three races of *Puccinia striiformis* with primer OPA-03, showing differences among races. Lanes marked L are 1-kb ladder DNA, and the lane marked with C is the control with sterile water.
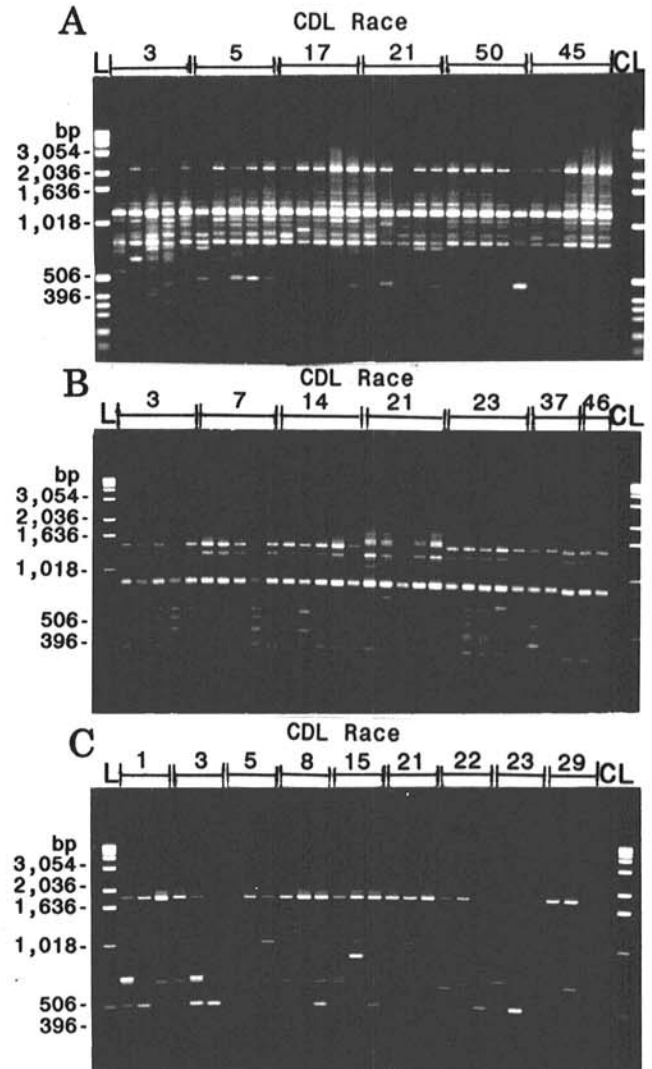

Fig. 5. Random amplified DNA polymorphisms of selected isolates showing variations within races of *Puccinia striiformis* with primers **A**, OPE-07; **B**, OPF-01; and **C**, OPG-15. Lanes marked L are 1-kb ladder DNA, and those marked C are controls with sterile water.

isolates of race CDL-29 (isolates 18-1 and 18-2), and one isolate each of races CDL-28 (isolate 17-5) and CDL-45 (isolate 23-5). RG 3, the largest group (75 isolates), contained the five isolates of races CDL-3, CDL-8 (isolates 6-1 to 6-5), CDL-15, and CDL-20 and five of the 10 isolates of CDL-45 (isolates 16-4 and 23-1 to 23-4); four isolates each of races CDL-5 (isolates 3-1, 3-2, 3-4, and 3-5), CDL-7 (isolates 5-1 to 5-3 and 5-5), CDL-16 (isolates 9-2 to 9-5), CDL-22 (isolates 13-1 to 13-4), CDL-28 (isolates 17-1 to 17-4), and CDL-35 (isolates 19-2 to 19-4); three isolates each of races CDL-6 (isolates 4-1, 4-2, and

4-4), CDL-14 (isolates 7-1, 7-3, and 7-4), CDL-23 (isolates 14-1, 14-2, and 14-4), CDL-29 (isolates 18-3 to 18-5), CDL-37, CDL-49 (isolates 21-3 to 21-5), and CDL-50 (isolates 22-1, 22-3, and 22-4); two isolates of races CDL-38 (isolates 21-1 and 21-2) and CDL-46 (isolates 20-4 and 20-5); and one isolate of race CDL-25 (isolate 15-5) (Fig. 6C). Only races CDL-1 (isolates 1-1 to 1-5), CDL-17 (isolates 10-1 to 10-5), and CDL-21 (isolates 12-1 to 12-5), virulent on Chinese 166 (*Yr*1), were not in RG 3. RG 4 (eight isolates) consisted of four isolates of race CDL-45 (isolates 16-1 to 16-3 and 16-5), two isolates of race
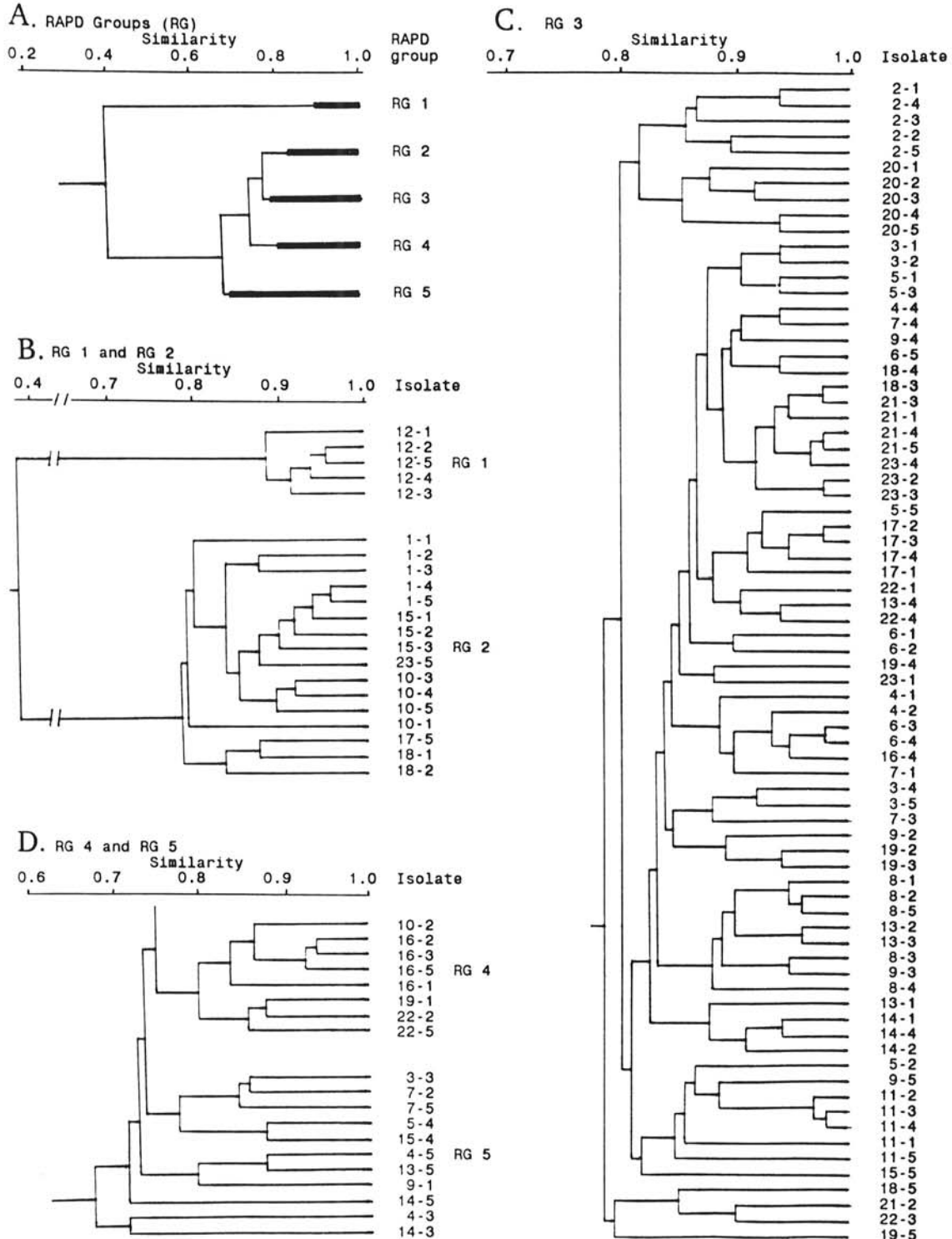

Fig. 6. Dendrogram of 115 single-spore isolates (five isolates each of 23 collections) of *Puccinia striiformis* based on 50 selected random amplified polymorphic DNA (RAPD) loci, using the unweighted pair group arithmetic mean (UPGMA) program of NTSYS-pc (version 1.70; 30), showing similarities **A**, among the five RAPD groups and **B, C, and D** within the groups. The thick bars indicate dissimilarities within groups. The race designation corresponding to the isolates is shown in Table 1.

CDL-50 (isolates 22-2 and 22-5), and one isolate each of races CDL-17 (isolate 10-2) and CDL-35 (isolate 19-1) (Fig. 6D). RG 5 (11 isolates) was the most heterogeneous group and contained two isolates each of races CDL-14 (isolates 7-2 and 7-5) and CDL-23 (isolates 14-3 and 14-5) and one isolate each of races CDL-5 (isolate 3-3), CDL-6 (isolate 4-3), CDL-7 (isolate 5-4), CDL-16 (isolate 9-1), CDL-22 (isolate 13-5), CDL-25 (isolate 15-4), and CDL-48 (isolate 4-5). When all 107 RAPD bands were used for cluster analyses, the five groups were separated at a cut-off point of 90% similarity. The similarities among the RGs and among isolates within each group were greater than those based on the 50 polymorphic bands. RG 1 was the same when both the 107 and the 50 RAPD bands were analyzed (Table 1). However, RG 1 had 67% similarity to other RG groups based on analysis of the 107 RAPD bands, a 67.5% increase compared to the 40% similarity based on 50 polymorphic RAPD bands. When the 107 RAPD bands were analyzed, RG 2 (13 isolates) and RG 3 (69 isolates) were smaller, and RG 4 (10 isolates) and RG 5 (18 isolates) were larger. RGs 2 and 3 had 88% similarity; RG 4 had 84% similarity with RGs 2 and 3; and RG 5 had 78% similarity with RGs 2, 3, and 4. Despite these differences, the general conclusions on the relationship among the isolates based on either the 107 or 50 RAPD bands are the same.

To determine the association of virulence and RAPD patterns, similarity matrices based on RAPD and virulence data were compared by correlation analysis. When the similarity matrix of virulence data was compared with that generated by the 107 RAPD bands, the correlation coefficient was 0.173. When the virulence matrix was compared with that generated by the 50 RAPD bands (Fig. 7), the correlation coefficient was 0.206. In both cases, the results indicate a low correlation between virulence and RAPD patterns.

DISCUSSION

Heterogeneity assessed by virulence. The results of this study show that the populations of *P. striiformis* in North America are highly heterogeneous. The 26 races identified represent more than 50% of the virulence patterns detected in North America (18). Virulence spectra of these races ranged from virulence on one differential cultivar to virulence on eight differential cultivars (Table 1). Stripe rust epidemics in North America occur most frequently in region 1 (eastern Washington, northeastern Oregon, and northern Idaho, the United States, and southern Alberta, Canada). Consequently, cultivars with diverse race-specific resistance genes have been released to this region. It is not surprising that great variation in virulence is observed in this region.

Among the 26 races identified in this study, four had not been reported previously. The identification of the new races was achieved with single-spore isolation and the current 15 differential cultivars. Single-spore isolation is not feasible in the routine iden-

tification of the virulence patterns of a large number of collections because of the time required. Some races could not be identified in the past, especially those that occurred in low frequencies and mixed with other races. Furthermore, because race identification depends on the host genotype, an early collection identified as a single race can sometimes be separated into more than one race when more differential cultivars are used. Therefore, natural variation in virulence of *P. striiformis* in North America is most likely greater than we observed.

The six virulence groups (VGs) obtained by cluster analysis show some relationship to epidemic regions. Line and Qayoum (20) defined seven epidemic regions of *P. striiformis* in North America based on geographic barriers, prevailing winds, and race distribution. Races in VG 1 (CDL-1, CDL-21, and CDL-35) had the narrowest virulence spectrum. Races CDL-21 and CDL-35 have been detected only in California (region 6). Race CDL-1 is an old race that has been used in region 1. Since 1968, it has occurred primarily in regions 5 (western Washington, the United States, and British Columbia, Canada) and 6 (central California) (20). All races in VGs 2 and 4 were first detected in region 1; races CDL-7, CDL-29, and CDL-50 in VG 2B, race CDL-28 in VG 2A, and race CDL-45 in VG 4 have been detected only in region 1. Except for race CDL-38, all of the races in VG 3 were first detected in region 5. All of the races in VG 3, except CDL-17, have been detected in region 1. Except for CDL-25, all races in VG 5 were first detected in region 5, and all races in VG 5 commonly occur in region 5. Races in VG 6 occur in region 1, but some have been detected in other regions. The six virulence groups determined by cluster analysis agree closely with the race relationships postulated by Line and Qayoum (20) based on the virulence, chronological appearance, and geographic distribution of the races.

Heterogeneity assessed by DNA polymorphisms. DNA polymorphisms were detected among races and isolates within races. Based on DNA polymorphism, some races, especially race CDL-21, are clearly different from others. RAPD markers are especially suitable for the analysis of obligate parasites such as *P. striiformis* because only nanograms of DNA are required for PCR amplification. The amount of DNA (about 200 ng) obtained directly from 20 mg of urediospores is insufficient for standard restriction fragment length polymorphic (RFLP) analysis but sufficient for hundreds of RAPD assays. The DNA polymorphisms detected by the RAPD assay were consistent in at least two tests conducted at different times. Although the inheritance of these bands cannot be confirmed by genetic analysis, the bands scored in this study can be used as molecular markers for future population studies.

The RAPD variations detected among races and isolates within races show that *P. striiformis* is highly variable. As Williams et al (35) discussed, nucleotide changes, deletions, and insertions in a priming region and/or an amplified DNA segment can result in DNA polymorphism that is detectable by RAPD assay. Point mutations are probably the most common source of new variation for *P. striiformis*. Because the pycnial and aecial stages have never been observed for *P. striiformis*, asexual urediospores are the only known means of reproduction. Somatic recombination has been demonstrated in *P. striiformis* (21,22). Detection of races with new combinations of existing virulences further suggests that somatic recombination can be frequent (20). In addition, a multinucleate condition has been reported for *P. striiformis* (5). Thus, somatic recombination and/or reassortment of genetically different nuclei could contribute to the observed DNA polymorphism. Finally, aneuploidy and chromosomal length polymorphism, which have occurred in many other fungi (12) including *P. graminis* (1), could give rise to the DNA polymorphism.

Association of RAPD and virulence patterns and evolutionary implications. We have defined five groups based on RAPD patterns (Fig. 6). Some of the RAPD groups show relationships with virulence. For example, the RAPD assay clearly separated race CDL-21 (isolates 12-1 to 12-5) from the other races. Race CDL-21 is avirulent on all of the differential cultivars, except Chinese 166, which has stripe rust resistance gene *Yr1* (3,4). It is the only race detected in North America that is avirulent on
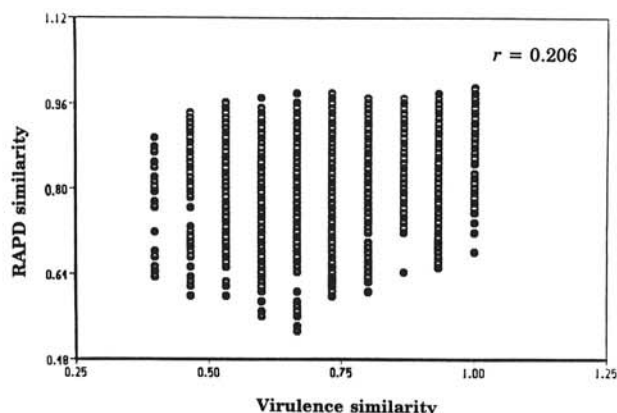

Fig. 7. Comparison of similarity matrices of virulence and 50 random amplified polymorphic DNA loci for the 115 single-urediospore isolates of *Puccinia striiformis*, using MXCOMP, a matrix comparison program of NTSYS-pc (version 1.7; 30).

Lemhi (*Yr*Lem) (3,20). The separation of race CDL-21 from other races also appears to be related to geographic distribution and virulence on nonwheat hosts. Race CDL-21 was first detected on triticale (*Tritisecales*) in region 5 during 1978 and has never been found in other regions (20).

Races CDL-1 and CDL-17 were clustered in RG 2. Like race CDL-21 in RG 1, the two races are virulent on Chinese 166. However, other isolates that are not virulent on Chinese 166 are also in RG 2. RG 3 contains 65% of the isolates tested in the study. A distinctive feature of this group is avirulence on Chinese 166. In considering the relationships of the races and their chronological appearance, Line and Qayoum (20) separated the North American races into three groups: the first group virulent on Chinese 166; the second group virulent on Heines VII; and the third group avirulent on both Chinese 166 and Heines VII. Results of RAPD grouping generally support this hypothesis.

Some subclusters within RAPD groups also show a relationship with virulence. For example, collection 20 consisted of races CDL-37 and CDL-46. Isolates of race CDL-37 were virulent on Produra and isolates of race CDL-46 were avirulent on Produra (Table 1). The three isolates of race CDL-37 (20-1, 20-2, and 20-3) and the two isolates of race CDL-46 (20-4 and 20-5) were clustered together, and the two races were clustered next to each other in RG 3 (Fig. 6). These clusters reflect the difference between the two races as well as their common virulence, because races CDL-37 and CDL-46 are virulent on Lemhi, Heines VII, Druchamp, Yamhill, Stephens, Lee, and Fielder and race CDL-37 also is virulent on Produra.

The overall association between virulence and RAPD patterns was low, as shown by the comparison of the similarity matrices (Fig. 7). The low correlation appears to be due to the high degree of DNA polymorphism among isolates within many races. For example, isolates of collections 16 and 23 had identical virulence (race CDL-45), but most isolates of collection 16 were in RG 4, and most isolates of collection 23 were in RG 3 (Table 1; Fig. 6). The separation of the two collections by RAPD patterns is probably due to collection 16 being made in 1983 and collection 23 being made in 1990. Conversely, isolates of race CDL-21, which are distinctly different in RAPD patterns (Fig. 6), were grouped together with races CDL-1 and CDL-35 in VG 1 (Fig.1). The low correlation between groups determined by RAPD and virulence indicates that most of the observed DNA polymorphism is independent of virulence, which is under host selection.

The results of RAPD and virulence analyses of the 115 isolates allow postulation of evolutionary relationships between some of the races. The separation of race CDL-21 from the other races (Fig. 6) suggests that race CDL-21 may have a different origin. The results of isozyme assay of race CDL-21 and a few other races also support this hypothesis (20). Race CDL-21 may have evolved on rye or other grass species, because triticale is a hybrid of wheat and rye and CDL-21 was first detected in an area where stripe rust commonly occurs on wild *Hordeum* species. Though less aggressive on wheat (20), race CDL-21 is clearly a rust of wheat because it attacks several wheat cultivars. Based on RAPD data, races CDL-1 and CDL-17 were clustered together in RG 2. The two races are both virulent on Chinese 166 (*Yr1*) and occur in the same region. Race CDL-17 has a wider virulence spectrum than has race CDL-1 and was detected many years later than was race CDL-1 (20). Races CDL-17 and CDL-1 could have a common origin and have evolved divergently as a result of selections by different host genotypes. Collections 16 and 23 contain the same race, CDL-45 (Table 1; Fig. 1), however, they are in different RAPD groups (Fig. 6). The results suggest that the two populations of race CDL-45 have different genetic backgrounds and may have undergone convergent evolution through host selection.

The RAPD assay separated races that are virulent on Chinese 166 (CDL-1, CDL-17, and CDL-21) from the races that are avirulent on Chinese 166 and separated race CDL-21 (avirulent on Lemhi) from races that are virulent on Lemhi (Fig 6). The results suggest that the North American populations of *P. striiformis* may have at least three origins (one origin for race

CDL-21, one origin for all other races virulent on Chinese 166, and one origin for races virulent on Heines VII), which is in general agreement with Line and Qayoum's (20) postulations about race relationships based on virulence, chronological appearance, and geographic distribution. However, based on RAPD assay, race CDL-21 is not as closely related to race CDL-1 as virulence analysis indicates.

Because race CDL-37 has an additional virulence on Lee compared to race CDL-25, it was thought that race CDL-37 might have evolved from race CDL-25 (20). However, the isolates of race CDL-37 were clustered in RG 3 and most isolates of race CDL-25 were in RG 2. The results may be due to collections 15 (race CDL-25) and 20 (CDL-37) being from different regions (Table 1). Race CDL-37 (virulent on eight of 15 differentials) was clustered together with race CDL-3 (virulent on two of 15 differentials) in RG 3 (Fig. 6), indicating the two races have similar genetic backgrounds even though they differ in virulence. Therefore, it appears that the evolution of virulence is independent of the evolution of neutral DNA markers.

RG 5 is a heterogeneous group of isolates (Fig. 6) that cannot be accounted for by the known biological properties of these isolates. This interpretation is based on virulence and RAPD patterns that are known about these isolates. If more genetic, physiological, and biochemical traits were known, the isolates in RG 5 might be placed in more meaningful biological groups.

Plant pathologists have searched for the associations of various characteristics with virulence as a means to supplement the time-consuming task of testing the pathogen on differential cultivars or species. Leung et al (15) classified associations of molecular markers with virulence patterns for plant pathogens into three categories: perfect, partial, and no associations. Close associations between molecular markers and pathotypes have been reported for only a few pathogens. Using RFLP and RAPD markers, Koch et al (14) and Goodwin and Annis (9) reported the association of different DNA patterns with avirulent and virulent populations of *Leptosphaeria maculans*. Crowhurst et al (6) differentiated *Fusarium solani* f. sp. *cucurbitae* race 1 from race 2 by RAPD markers. Using a moderately repetitive DNA sequence as a probe, Levy et al (17) reported a close association between pathotypes and DNA lineages for *Magnaporthe grisea* isolates from the United States. In our study, we differentiated race CDL-21 from other races of *P. striiformis* with RAPD markers. However, the RAPD assay did not adequately differentiate other races. The low degree of association is expected considering the high degree of variation in virulence and the long-distance, airborne dissemination of *P. striiformis*. In general, the low association between virulence and RAPD patterns indicates that DNA polymorphisms are independent of virulence and that selection for virulence by growing hosts with race-specific resistance plays a major role in determining the race structure of the pathogen.

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