Molecular and virulence diversity and linkage disequilibria in asexual and sexual populations of the wheat leaf rust fungus, *Puccinia recondita*

J.Q. Liu and J.A. Kolmer

Abstract: An asexual field population and a sexually derived population of the wheat leaf rust fungus, *Puccinia recondita*, were examined and compared for diversity and linkage disequilibria between virulence and molecular phenotypes. Isolates in both populations were tested for virulence to 20 Thatcher wheat lines near-isogenic for leaf rust resistance genes, and for random amplified polymorphic DNA (RAPD) variation using 10 DNA primers. In the asexual field population, 36 virulence phenotypes and 14 RAPD phenotypes were identified in 43 isolates. In the sexual population, 87 virulence phenotypes and 79 RAPD phenotypes were identified in 104 isolates. Linkage disequilibria was less in the sexual population compared to the asexual field population. Virulence-RAPD phenotype pairs (110 in total) were directly compared between the two populations for association. In the asexual population, 39 virulence-RAPD phenotype pairs were associated ($P < 0.05$), compared with 18 pairs in the sexual population. Linkage was not evident, as some residual disequilibria remained between virulence and RAPD phenotypes. In the asexual population 18 RAPD phenotype pairs were associated, compared with 9 pairs in the sexual population. The sexual population was also tested for RAPD variation with an additional six primers. In the sexual population, amplification sites of four different primers were tightly linked which indicated a chromosomal segment in *P. recondita* may not readily undergo recombination. Disequilibria between virulence and RAPD phenotypes in field populations of *P. recondita* in Canada is maintained by asexual reproduction.

Key words: *Puccinia recondita*, molecular diversity, virulence diversity, linkage disequilibria, wheat leaf rust.

Résumé : Deux populations de l'agent pathogène de la rouille des feuilles chez le blé, *Puccinia recondita*, l'une asexuée provenant du champ et l'autre dérivée par reproduction sexuée, ont été examinées et comparées quant à leur diversité et au déséquilibre de linkage entre la virulence et des phénotypes moléculaires. Des isolats des deux populations ont été testées pour leur virulence sur 20 lignées quasi-isogéniques du blé Thatcher portant divers gènes de résistance à la rouille des feuilles. En parallèle, la variation moléculaire a été examinée chez ces isolats à l'aide de la technique RAPD (ADN polymorphe amplifié au hasard) en utilisant dix amorces. Chez la population asexuée provenant du champ, 36 phénotypes de virulence et 14 phénotypes RAPD ont été identifiés parmi 43 isolats. Chez la population sexuée, 87 phénotypes de virulence et 79 phénotypes RAPD ont été observés parmi 104 isolats. Les déséquilibres de linkage étaient moins nombreux chez la population sexuée que chez la population asexuée. Des paires de phénotypes virulence-RAPD (110 au total) ont été comparées directement entre les deux populations afin de déceler des associations. Chez la population asexuée, 39 paires phénotypiques étaient associées ($P < 0{,}05$) alors que 18 paires étaient associées chez la population sexuée. Le linkage n'était pas évident car il demeurait des déséquilibres résiduels entre les phénotypes de virulence et RAPD. Chez la population asexuée, 18 paires de phénotypes RAPD étaient associés alors que neuf paires RAPD l'étaient chez la population sexuée. La variation au sein de la population sexuée a été examinée à l'aide de six amorces additionnelles. Chez la population sexuée, les sites d'amplification pour quatre amorces étaient étroitement liés ce qui indique qu'un segment chromosomique du *P. recondita* pourrait ne pas faire l'objet de beaucoup de recombinaison. Les déséquilibres de linkage entre les phénotypes de virulence et RAPD chez les populations de champ du *P. recondita* sont maintenues par reproduction asexuée.

Mots clés : *Puccinia recondita*, diversité moléculaire, diversité de virulence, déséquilibre de linkage, rouille des feuilles chez le blé.

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Introduction

Leaf rust, caused by the fungus *Puccinia recondita* f. sp. Roberge ex. Desmaz *tritici*, is a disease of wheat (*Triticum aestivum* L.) that occurs throughout North America and worldwide. In North America *P. recondita* reproduces on wheat by asexual reproduction of dikaryotic urediniospores. The sexual phase of the fungus has never been important epidemiologically since a susceptible alternate host such as *Thalictrum speciosissimum*, does not exist in Canada or the United States. The native *Thalictrum* spp. in North America are resistant to *P. recondita* f. sp. *tritici* (Samborski 1985). Isolated reports of aeciospores pathogenic to wheat that originated from pycnial infections on *Thalictrum* spp. native to North America have been noted (Levine and Hildreth 1957). Sexual reproduction may potentially contribute to the generation of new genotypes in this fungus, however previous virulence survey data and molecular phenotype data (Kolmer et al. 1995) have not indicated that sexual recombination is a significant source of variation in *P. recondita* in North America.

Virulence genes in *P. recondita* interact with host resistance genes in a gene-for-gene relationship (Samborski and Dyck 1976; Kolmer and Dyck 1994). Specificity of virulence, and the use of many different resistance genes in North American wheats has resulted in a *P. recondita* population that is highly diverse for physiologic races or virulence phenotypes. In Canada 40–50 virulence phenotypes of *P. recondita* are detected annually (Kolmer and Liu 1997*a*) by examination of isolates for infection type on 20 lines of Thatcher wheat that are near-isogenic for different resistance genes.

A field population of representative *P. recondita* isolates from Canada was previously examined for virulence and molecular polymorphism (Kolmer et al. 1995). The asexual field *P. recondita* population was highly structured with distinct clusters of isolates. Individual isolates within each cluster were more closely related to each other for virulence and random amplified polymorphic DNA (RAPD) variation compared to isolates in other clusters. The virulence and molecular polymorphism was not evenly distributed in the population as there were strong associations between certain groups of virulence phenotypes with certain groups of molecular phenotypes. In general, isolates typically found only in eastern Canada had virulence and RAPD phenotypes that were distinct from isolates collected in western Canada. The objective of this study was to determine if the associations or disequilibria between virulence and molecular polymorphism that characterised populations of *P. recondita* in Canada, were due to linkage between the virulence genes and RAPD phenotypes or due to asexual reproduction of this cereal rust in North America. In this study we compared linkage disequilibria between virulences and RAPD phenotypes in the asexual field population of *P. recondita* (Kolmer et al. 1995) with virulence and RAPD phenotypes in a sexually derived population of the fungus.

Materials and methods

Single-uredinial isolates (104 in total) of *P. recondita* derived from randomly mated pycnia were examined in this study. The sexual population was developed by bulking 5 mg of urediniospores from each of 47 single-uredinial *P. recondita* isolates that were collected in eastern Canada in 1987 (Kolmer 1988) and inoculating the bulked collection onto adult wheat plants to produce teliospores. The teliospores were germinated over young leaves of *Thalictrum speciosissimum* L., a susceptible alternate host of *P. recondita* f. sp. *tritici*. Pycnia on the same leaves were randomly intermated, and 1–3 single-uredinial isolates were derived from each fertilized aecium. The aeciospore-derived isolates were evaluated for virulence phenotype on 12 near-isogenic Thatcher wheat lines that differed for leaf rust resistance genes. Details of teliospore germination, mating of pycnial infections, and virulence phenotypes of single-uredinial isolates in the sexual population have been described previously (Kolmer 1992*a*, 1992*b*).

Starting in 1994, 72 single-uredinial isolates originally derived from 44 aecial clusters from the sexual population were recovered from vacuum-dried storage and re-purified by single-uredinial isolation. In addition, 32 single-uredinial isolates were developed from a bulk collection of the aeciospore-derived isolates that had been stored at –70°C. Methods for single-uredinial isolation, inoculation, incubation, and urediniospore increase were as previously described (Kolmer et al. 1995). All isolates from the sexual population were evaluated for virulence to the Thatcher lines (RL #) with genes: *Lr1*–RL6003, *Lr2a*–RL6000, *Lr2c*–RL6047, *Lr3*–RL6002, *Lr9*–RL6010, *Lr16*–RL6005, *Lr24*–RL6064, *Lr26*–RL6078, *Lr3ka*–RL6007, *Lr11*–RL6053, *Lr17*–RL6008, *Lr30*–RL6049, *LrB*–RL6051, *Lr3bg*–RL6042, *Lr10*–RL6004, *Lr14a*–RL6013, *Lr14b*–RL6006, *Lr15*–RL6052, *Lr18*–RL6009, *Lr19*–RL6040, *Lr20*–RL6092, *Lr21*–RL6043, *Lr23*–RL6012, *Lr25*–RL6084, *Lr28*–RL6079, *Lr29*–RL6080, and *Lr32*–RL6086. Thatcher (RL6061) was also included in the differential sets as a susceptible control. An isolate was classified as avirulent if it produced infection types 0 to 2^+ (small faint hypersensitive flecks to small-moderate uredinia with chlorosis) and virulent if it had infection types of 3 to 4 (moderate to large uredinia without chlorosis) on the differential lines. The isolates were all either virulent or avirulent on differential lines with *Lr9*, *Lr19*, *Lr20*, *Lr21*, *Lr25*, *Lr29*, and *Lr32*. Infection types for these lines were not included in the data analysis. The isolates were given a virulence phenotype designation based on virulent or avirulent infection types to 20 Thatcher differential lines.

Approximately 30 mg of freshly collected urediniospores from each isolate in the sexual population were spread over 20 mL of a gramicidin D solution (ddH_2O with 120 µg of gramicidin D, Sigma Chemical Co., St. Louis, Mo., dissolved in 25 µL of absolute ethanol) in 9.5 cm dia. petrie dishes. Ten microlitres of a nonanol solution (1.5 µL nonanol/mL of acetone in 19 mL of ddH_2O) were added to each petrie dish in order to stimulate spore germination. Filter paper soaked in the nonanol solution was placed in the petrie dish lid. The covered petrie dishes with the spore suspensions were kept at room temperature for 6 h, after which the germinated urediniospores were skimmed off as a single mat with a metal spatula. The mats were blotted on paper towels, placed in 1.5 mL microcentrifuge tubes, snap frozen in liquid N_2, and stored at –70°C.

For DNA extraction the frozen mats were ground in the microcentrifuge tubes to a fine slurry with the addition of approximately 3 mg of acid washed sand. Extraction buffer (600 µL of 1 M Tris/HCL, pH 8.7; 0.5 M EDTA, pH 8; 5 M NaCl; 10% CTAB composed of 10 g CTAB, 500 mM Tris/HCl, and 100 mM EDTA) and proteinase K (3 µL at 10 mg/mL TE, ph 7.6) were added to each tube. The tubes were vortexed briefly and 3 µL of 20% SDS added before the tubes were incubated at 65°C for 2 h. The tubes were inverted by hand approximately every 20 min. After incubation an equal volume of 24:1 chloroform – isoamyl alcohol was added, and the tubes were gently shaken for 20 min. The emulsions were centrifuged (13 000 × *g*) for 5 min and the aqueous layer was

Fig. 1. DNA banding patterns generated by simple sequence repeat primers CRC25, CRC28, CRC29, and CRC32 with *Puccinia recondita* isolates derived from a sexual population. Lambda DNA digested with *Hin*dIII and *Eco*RI is the molecular weight standard. The primer sequences are given in the Materials and methods section.

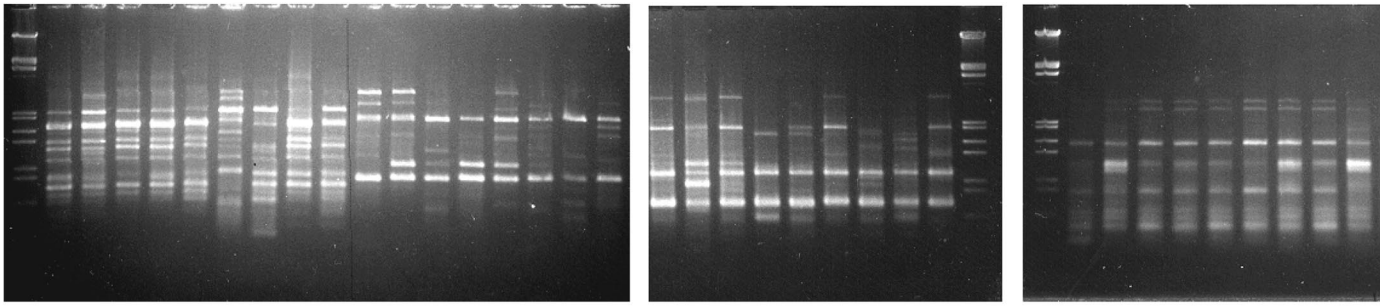

Table 1. Frequency (%) of isolates virulent to wheat lines with specific leaf rust resistance genes in an asexual field population and a sexual population of *Puccinia recondita* f. sp. *tritici*.[a]

Resistance gene (*Lr*)	Field population	Sexual population
Lr1	72	83
Lr2a	37	36
Lr2c	58	60
Lr3	88	94
Lr16	2	3
Lr24	30	19
Lr26	26	4
Lr3ka	26	57
Lr11	40	44
Lr17	9	34
Lr30	21	35
LrB	19	40
Lr3bg	14	29
Lr10	91	88
Lr14a	81	31
Lr14b	100	90
Lr15	95	96
Lr18	12	40
Lr28	93	91

[a]There were 43 isolates in the field population and 104 isolates in the sexual population.

placed in a clean tube. Isopropanol (0.6 volume) was added to each tube and the DNA was precipitated after centrifugation for 5 min. The DNA pellets were washed twice with 70% ethanol, dried, and resuspended in 300 μL of sterile ddH_2O. DNA preparations were treated with RNase (Promega, Madison, Wis.) and re-precipitated as previously described (Kolmer et al. 1995). DNA concentrations were determined with a spectrophotometer, and working solutions of 10 ng/μL were made and kept at 4°C. The concentrated DNA stocks were dried and kept at –20°C.

Isolates in the sexual population were examined for molecular polymorphism using 16 arbitrary DNA primers. The first 10 primers (402, 450, 489, 490, 517, 519, 521, 531, 538, and 556) were 10-base primers obtained from the Oligonucleotide Synthesis Laboratory at the University of British Columbia, Vancouver, B.C. and were designated as primer set #1. Sequences for these primers have been published previously (Kolmer et al. 1995). Primers OPQ9 (5′-GGCTAACCGA-3′) and OPR2 (5′-CACAGCTGCC-3′) obtained from Operon Technologies, Alameda, Cal. were also used. Conditions and procedures for the DNA amplifications, agarose gel electrophoresis, visualization, and recording of DNA banding patterns generated by the 12 decamer primers were the same as previously described (Kolmer et al. 1995). Four simple sequence repeat (SSR) primers with degenerate 5′ ends were also used to characterize RAPD polymorphism in the sexual population. SSR primers CRC25 5′-DVH$[ACA]_5$ -3′; CRC28 5′- HVH $[GCT]_5$- 3′; CRC29 5′-BDB[CCA]5–3′; and CRC32 5′-DBD$[AAC]_5$ -3′ synthesized by Gibco Life Technologies were used. In the degenerate 5′ ends B = C,G,T, but not A; D = A,G,T, but not C; H = A,C,T, but not G; and V = A,C,G, but not T. Amplification conditions for the four SSR primers were the same as for the 10-base primers, however the annealing temperature was 50°C. Conditions and procedures for agarose gel electrophoresis, visualization, and recording of DNA banding patterns generated by the SSR primers were identical to those used for the 10-base primers (Kolmer et al. 1995). For all primers only the major polymorphic DNA bands were considered. All isolates were tested at least twice with each primer to confirm the DNA banding patterns. For each primer the most common banding pattern was designated as type 1, and the next most common as type 2, etc. The primers OPQ9, OPR2, the four SSR primers, and the ten primers in primer set #1 were designated as primer set #2. Photographs of DNA banding patterns generated by eight of the primers in set #1 were published previously (Kolmer et al. 1995). DNA banding patterns generated by the four SSR primers with isolates in the sexual population are shown in Fig. 1. Each *P. recondita* isolate in the sexual population was assigned a 16 digit molecular phenotype based on the molecular polymorphism generated by the 16 primers.

An asexual field population of *P. recondita* isolates collected in Canada was previously characterized for virulence and RAPD variation (Kolmer et al. 1995). The field population consisted of 60 single-uredinial isolates collected from wheat in 1989, 1992, 1993, and four isolates collected prior to 1965. The isolates from 1989–1993 were representative of the predominant virulence phenotypes found in those years in the eastern (Ontario and Quebec) and western (Manitoba and Saskatchewan) regions of Canada. The field population was analysed for virulence polymorphism on 20 Thatcher differential lines and for RAPD variation using the 10 decamer primers in primer set #1. Forty-three isolates each with a unique virulence-RAPD phenotype were included for comparison with the sexual population.

Virulence and RAPD phenotypes in the field and sexual populations were sorted according to frequency of occurrence. Diversity of virulence and molecular phenotypes was calculated using the Shannon index (Groth and Roelfs 1987). Significance of differences in Shannon indexes between the asexual field population and the sexual population were determined with a *t*-test (Poole 1974) Frequencies of virulence to resistance genes and frequencies of

Table 2. Frequency (%) distribution of randomly amplified polymorphic DNA (RAPD) phenotypes generated by DNA primers in an asexual field population and a sexual population of *Puccinia recondita* f. sp. *tritici*.[a]

	RAPD phenotype										
	Field population					Sexual population					
Primer	1	2	3	4	5	1	2	3	4	5	6
402	82	18				63	36				
450	58	42				87	12	1[b]			
489	58	42				88	12				
490	65	35				87	12	1[b]			
517	77	23				61	39				
519	79	14	5	2[b]		35	39	13	8	4	1[b]
521	79	21				43	57				
531	67	19	7	5		52	39	6	3[b]		
538	79	21				82	18				
556	96	2	2[b]			64	24	12			
CRC25	—[c]	—				58	25	11	7		
CRC28	—	—				41	57	2[b]			
CRC29	—	—				38	62				
CRC32	—	—				84	16				
OPQ9	—	—				87	13				
OPR2	—	—				58	42				

[a]There were 43 isolates in the field population and 104 isolates in the sexual population.
[b]Rare phenotypes were not included in contingency table analysis.
[c]The field population was not tested with these primers.

RAPD phenotypes were determined using PROC FREQ in SAS (SAS Institute, Cary, North Carolina). Associations between virulences and RAPD phenotypes were measured using the adjusted χ^2 (Sokal and Rohlf 1981) with PROC FREQ.

Results

The frequencies of isolates in both populations with virulence to the near-isogenic Thatcher lines are listed in Table 1. In the field population, virulences to lines with genes *Lr10*, *Lr14b*, *Lr15*, and *Lr28* were above 90%, and virulences to lines with *Lr16*, and *Lr17*, were less than 10%. Virulence frequencies to all other lines were between 10–90%. In the sexual population virulences to lines with genes *Lr3*, *Lr14b*, *Lr15*, and *Lr28* were greater or equal to 90%, and virulences to *Lr16* and *Lr26* were less than 5%. Virulence frequencies to all other lines in the sexual population was between 10–90%.

Each of the primers used in the characterization of RAPD phenotypes generated two common banding patterns that together usually accounted for over 90% of isolates in both populations (Table 2). For almost all primers in both populations, the two most common RAPD phenotypes separately were between 5–95% of isolates. In the field population, the two most common RAPD phenotypes generated by all primers except 531 accounted for over 90% of the isolates. In the field population RAPD phenotypes for individual primers that occurred at 2% or less were not included in the contingency table analysis. In the sexual population, the two most common RAPD phenotypes generated by primers 519, 556, and CRC25 accounted for 74, 88, and 83% of the isolates. The other primers used to characterize the sexual population generated two common RAPD phenotypes that together comprised over 90% of the isolates. In the sexual population RAPD phenotypes for individual primers that occurred at 1% or less were not included in the contingency table analysis.

The sexual population was more diverse for virulence and RAPD variation compared to the field population (Table 3). Thirty-six and 87 virulence phenotypes were distinguished in the field and sexual populations, respectively. The sexual population also had a significantly higher Shannon index of virulence diversity compared to the field population. In the field population only 14 RAPD phenotypes were distinguished using primer set #1, while there were 79 RAPD phenotypes in the sexual population. The sexual population also had a significantly higher Shannon index of RAPD diversity compared to the field population. The six additional primers used in primer set #2 generated a further 17 RAPD phenotypes in the sexual population.

For the contingency table analysis, virulences with frequencies between 10–90% of isolates in both populations (Table 1) were tested for association with RAPD phenotypes generated by primer set #1 or primer set #2. Virulences to *Lr3*, *Lr10*, *Lr14b*, *Lr15*, *Lr16*, *Lr17*, and *Lr26* were not included in the contingency table analysis. Virulence-RAPD phenotype pairs (110 in total) were tested using primer set #1 in the field and sexual populations. In the field population, 39 virulence-RAPD phenotype pairs were significantly associated ($P < 0.05$) (Table 4). Thirty of the associated pairs in the field population were not significantly associated in the sexual population. Nine of the associated pairs in the field population were also associated in the sexual population, while nine of the pairs that were not associated in the field population were significantly associated in the sexual population. There were a total of 18 RAPD phenotype pairs associated in the sexual population.

The observed vs. expected numbers of isolates in each phenotypic class for the virulence-RAPD phenotype pairs with significant association in the sexual population did not

Table 3. Virulence and random amplified polymorphic DNA (RAPD) phenotypic diversity in an asexual field population and a sexual population of *Puccinia recondita* f. sp. *tritici*.

	Field population	Sexual population
Number of isolates	43	104
Number of virulence phenotypes[a]	36	87
Shannon index of virulence diversity	3.48	4.38
Number of RAPD phenotypes: Primer set 1[b]	14	79
Number of RAPD phenotypes: Primer set 2[c]	—[d]	96
Shannon index of RAPD diversity: Primer set 1	2.09	4.29
Shannon index of RAPD diversity: Primer set 2	—	4.54

[a]As measured by virulent or avirulent infection types to Thatcher near-isogenic lines that differ for leaf rust resistance genes.
[b]Primer set 1: University of British Columbia primers 402, 450, 489, 490, 517, 5519, 521, 531, 538, and 556.
[c]Primer set 2: Primer set #1 plus primers CRC25, CRC28, CRC29, CRC32, OPQ9, and OPR2.
[d]The field population was not tested with primer set #2.

Table 4. Contingency table tests of association between virulence to specific leaf rust resistance genes and random amplified polymorphic DNA (RAPD) phenotypes generated by DNA primers in an asexual field population and a sexual population of *Puccinia recondita* f. sp. *tritici* as measured by the adjusted χ^2.

	Virulence to gene										
Primer	*Lr1*	*Lr2a*	*Lr2c*	*Lr24*	*Lr3ka*	*Lr11*	*Lr30*	*LrB*	*Lr3bg*	*Lr14a*	*Lr18*
402	[a]	f[b]	f		f			f s[c]	f	f s	f
450			s[d]					s			
489			s								
490		f	s			f			f	f	
517	s		f					f	f	f s	f
519		s			f s			f	f s	f s	s
521	s		f					f s	f	f s	
531	s	f	f	f	f	f s	f	f	f	f	f
538			f					f	f	f	f
556											
CRC25[e]		s	s					s		s	
CRC28											
CRC29											
CRC32		s	s					s			
OPQ9			s								
OPR2					s			s		s	

[a]Non-significant association ($P > 0.05$).
[b]Significant association ($P < 0.05$) in field population.
[c]Significant association ($P < 0.05$) in field and sexual population.
[d]Significant association ($P < 0.05$) in sexual population.
[e]The field population was not tested with primers CRC25, CRC28, CRC29, CRC32, OPQ9, and OPR2.

indicate linkage between the virulences and the RAPD phenotypes as the associations were weak. Some linkage disequilibria between the virulences and the RAPD phenotypes most likely remained after one generation of random mating. An additional generation of random mating most likely would eliminate the residual linkage disequilibria between these virulences and RAPD phenotypes. The observed vs. expected numbers of isolates of four virulence-RAPD phenotype pairs in linkage disequilibrium are shown in Table 5.

Forty-five pairs of RAPD phenotypes using primer set #1 were tested for association in the field and sexual populations. In the field population 18 pairs of RAPD phenotypes were significantly associated ($P < 0.05$) according to the adjusted χ^2 test (Table 6). In the sexual population, only nine pairs of RAPD phenotypes were significantly associated using primer set #1. RAPD phenotypes generated by primers 450, 489, and 490 were highly associated. In the field population, RAPD phenotypes generated by primer pair 450–489 were all 1,1 or 2,2; phenotypes generated by primer pairs 450–490 and 489–490 were almost all 1,1 or 2,2 with only a few 2,1. For these three primer pairs, there were very few recombinant phenotypes of 1,2 or 2,1 in the sexual population, which indicated linkage between certain amplification sites for primers 450, 489, and 490 (Table 7). The other four pairs of RAPD phenotypes that were significantly associated in the sexual population using primer set #1, did not appear to be linked according to the observed vs. expected numbers of RAPD phenotype classes. As in the case of the virulence-RAPD associations an additional generation of random mating would probably remove the residual disequilibria between these RAPD phenotypes. Using primer

Table 5. Observed and expected number of virulence phenotypes and random amplified polymorphic DNA (RAPD) phenotypes of four virulence and primer pairs in linkage disequilibria in a sexual population of *Puccinia recondita* f. sp. *tritici*.

Virulence: Primer pair	Avirulent (A); Virulent (V)	RAPD phenotype	Observed number	Expected number	Adjusted χ^2 (P value)
Lr2c–450	A	1	31	35.82	6.87 (0.01)
	A	2	10	5.17	
	V	1	59	54.17	
	V	2	3	7.82	
Lr14a–517	A	1	37	43.61	7.06 (0.01)
	A	2	35	28.38	
	V	1	26	19.38	
	V	2	6	12.61	
Lr3ka–OPR2	A	1	19	25.96	6.70 (0.01)
	A	2	26	19.03	
	V	1	41	34.03	
	V	2	18	24.96	
LrB–402	A	1	47	39.34	8.81 (0.00)
	A	2	15	22.65	
	V	1	19	26.65	
	V	2	23	15.34	

Table 6. Contingency table tests for association between random amplified polymorphic DNA (RAPD) phenotypes generated by DNA primers in an asexual field population and a sexual population of *Puccinia recondita* f. sp. *tritici* as measured by the adjusted χ^2.

Sexual field population	Asexual field population															
	402	450	489	490	517	519	521	531	538	556	25[a]	28	29	32	Q9	R2
402	—	[b]			f[c]	f	f	f	f							
450		—	f	f												
489		s[c]	—	f												
490		s	s	—												
517					—	f	f	f	f							
519						—	f	f	f							
521	s				s	s	—	f	f							
531	s				s		s	—	f							
538									—							
556										—						
25	s				s	s	s	s			—					
28										s	s	—				
29										s	s	s	—			
32		s	s	s									s	—		
Q9		s	s	s						s		s	s	s	—	
R2	s				s	s	s	s			s	s	s			—

[a]The field population was not tested with primers CRC25, CRC28, CRC29, CRC32, OPQ9, and OPR2.
[b]Non-significant association ($P > 0.05$).
[c]Significant association ($P < 0.05$).

set #2 in the sexual population, RAPD phenotypes generated by primers OPQ9 and CRC32 were highly associated with each other and with RAPD phenotypes generated by primers 450, 489, and 490. The RAPD phenotypes generated by primers CRC28 and CRC29 also appeared to be linked (Table 7). Twenty pairs of RAPD phenotypes using primer set #2 were significantly associated ($P < 0.05$) (Table 6) but did not appear to be linked by inspection of the observed vs. expected numbers of isolates in the different RAPD phenotype classes. Recombination between RAPD phenotypes generated by primers 450, 489, 490, and OPQ9 averaged 2.8% (Table 7). Recombination between RAPD phenotypes generated by primer CRC32 with primers 450, 489, 490, and OPQ9 averaged 8.5%. Certain amplification sites for primers 450, 489, 490, and OPQ9 appeared to be tightly linked, and were more loosely linked with a site for primer CRC32.

Discussion

Recombination in the sexual *P. recondita* population eliminated most of the linkage disequilibria between virulences and RAPD phenotypes that were characteristic of the asex-

Table 7. Observed and expected numbers of random amplified polymorphic DNA (RAPD) phenotypes for primer pairs showing linkage in a sexual population of *Puccinia recondita* f. sp. *tritici* isolates.

Primer pair	RAPD phenotypes	Observed number	Expected number	Adjusted χ^2 (*P* value)	Percent recombination
450–489	1 1	89	78.64	77.59 (0.00)	1.9
	1 2	1	11.35		
	2 1	1	11.35		
	2 2	12	1.64		
450–490	1 1	88	78.52	68.34 (0.00)	2.8
	1 2	1	10.47		
	2 1	2	11.47		
	2 2	11	1.52		
450–CRC32	1 1	84	75.14	44.58 (0.00)	7.6
	1 2	6	14.85		
	2 1	2	10.85		
	2 2	11	2.14		
45–OPQ9	1 1	88	77.76	71.00 (0.00)	2.8
	1 2	2	12.23		
	2 1	1	11.23		
	2 2	12	1.76		
489–490	1 1	88	79.51	54.53 (0.00)	4.8
	1 2	2	10.48		
	2 2	10	1.5		
489–CRC32	1 1	85	76.12	45.09 (0.00)	7.6
	1 2	6	14.87		
	2 1	2	10.87		
	2 2	11	2.12		
489–OPQ9	1 1	89	78.75	71.23 (0.00)	2.8
	1 2	2	12.25		
	2 1	1	11.25		
	2 2	12	1.75		
490–CRC32	1 1	83	75.98	29.09 (0.00)	10.6
	1 2	8	15.01		
	2 1	3	10.01		
	2 2	9	1.98		
490–OPQ9	1 1	89	78.63	78.21 (0.00)	1.9
	1 2	2	12.36		
	2 1	0	10.38		
	2 2	12	1.63		
CRC32–OPQ9	1 1	84	75.29	40.70 (0.00)	8.7
	1 2	3	11.75		
	2 1	6	14.7		
	2 2	11	2.29		
CRC28–CRC29	1 1	38	16.01	79.35 (0.00)	4.8
	1 2	5	29.08		
	2 1	0	19.9		
	2 2	59	37.01		

ual field population. Most of the virulence-RAPD phenotype pairs and RAPD phenotype pairs that were associated in the asexual field population were not associated in the sexual population. The highly structured nature of the virulence and RAPD polymorphism in the field population resulted from the clonal reproduction of urediniospores. In the field population, isolates avirulent to *Lr2a* and virulent to *Lr2c* all had RAPD phenotypes that were distinct from isolates that were virulent or avirulent to both genes (Kolmer et al. 1995). This was the most characteristic virulence-RAPD phenotype association in the field population. The two distinct groups of isolates accounted for 90% of the field population. Polymorphisms generated by eight primers in set #1 were significantly associated with virulence to *Lr2a* or *Lr2c* in the field population, however in the sexual population only one RAPD phenotype was associated with virulence to *Lr2a*, and only RAPD phenotypes generated by the primers 450, 489, and 490 were associated with virulence to *Lr2c*.

Recombination also increased the diversity of molecular and virulence phenotypes in the sexual population compared

to the field population. Studies with other plant pathogens also showed greater phenotypic diversity in sexual populations, and a greater number of associations between virulences and molecular markers in asexual populations compared to populations that undergo some degree of sexual reproduction. Burdon and Roelfs (1985*a*, 1985*b*) compared virulence and isozyme variation in sexual and asexual populations of wheat stem rust, *Puccinia graminis*. Of 110 isolates from an asexual field population, 23 virulence phenotypes and 9 isozyme phenotypes were described, with an association between virulence and isozyme phenotypes. In 92 isolates derived from a sexual population of *P. graminis*, 61 virulence phenotypes and 80 isozyme phenotypes were described, with no association between virulences and isozymes. Peever and Milgroom (1994) examined disequilibria between RAPD phenotypes in five populations of *Pyrenophora teres*, the causal agent of barley net blotch. Of 49 pairs of RAPD phenotypes only 11 were associated, which provided evidence that most populations of *P. teres* undergo some degree of sexual reproduction. Of the RAPD marker pairs that were associated, most were from a *P. teres* population which was speculated to be predominantly asexual. Two pairs of RAPD phenotypes were associated in all five *P. teres* populations which indicated possible linkage between the two markers or selection for those phenotypes. Keller et al. (1997) examined populations of *Phaeosphaeria nodorum*, the causal agent of wheat glume blotch, in Europe and North America for restriction fragment length polymorphism (RFLP). Of 21 locus by locus comparisons made in populations from Switzerland and Oregon, only three pairs were significantly associated, supporting other evidence that sexual reproduction is important in the epidemiology of this plant pathogen. Milgroom (1996) has recently reviewed linkage disequilibria in plant pathogen populations.

Some weak associations between virulences and RAPD phenotypes were detected in the sexual *P. recondita* population, however this was most likely due to residual linkage disequilibria. Residual disequilibrium between pairs of unlinked virulences was also found previously in the sexual population (Kolmer 1992*b*). Strong associations between virulences and RAPD phenotypes with large adjusted χ^2 values were not detected, which indicated no linkage between these markers. This was not unexpected given the relatively few primers used in this study, and the estimated genome size of 90–94 megabase pairs for *P. recondita* f. sp. *tritici* (W.R. Bushnell, personal communication). In *Puccinia graminis*, 18 chromosomes were described (Boehm et al. 1992) and it is likely that *P. recondita* would have at least as many chromosomes and linkage groups. Use of a more powerful technique for identification of molecular polymorphism, such as amplified restriction fragment length polymorphism (AFLP) may help provide molecular markers linked to virulence genes. RAPD phenotypes generated by primers 450, 489, 490, and OPQ9 were tightly linked. It may be coincidental that certain amplification sites for these four primers were linked, or it may indicate that there is a chromosomal segment such as a translocation in *P. recondita* that does not readily undergo recombination.

Information on the genetic relationships between the RAPD phenotypes and virulences will aid in interpretation of population genetic data from other asexual field populations of *P. recondita*. The RAPD phenotypes we have used to date should be able to serve as neutral, unselected markers since none of them were linked to virulences. It should be possible to eliminate certain primers such as OPQ9 from further consideration since an amplification site generated by this primer is tightly linked to sites generated by three other primers. Isolates in other field collections that differ for RAPD phenotypes with primers 489 or OPQ9 have not been found (Kolmer and Liu 1997).

In conclusion, many of the associations between virulences and RAPD phenotypes that characterized the asexual field population were not evident in the sexual population. The asexual reproduction of the field population maintains associations of virulence and molecular polymorphism within distinct groups of *P. recondita* in North America. Long term associations between virulences have been characteristic of *P. recondita* populations in Canada (Kolmer 1989; Kolmer 1992*b*; Kolmer 1997). Associations between virulence genes have arisen by selection of phenotypes with virulence to combinations of resistance genes, linkage between virulence loci (Kolmer 1992*b*), and by chance. Since we did not find any evidence for linkage between virulence and RAPD phenotypes, and the RAPD phenotypes are presumably unselected, the characteristic associations between virulence and RAPD phenotypes in the asexual field population may be reflective of relationships between virulence alleles and RAPD phenotypes in the populations of leaf rust that were introduced to North America. Persistence of these associations in clonal lineages provides evidence that neither sexual reproduction nor parasexual recombination has occurred to any significant amount in the *P. recondita* f. sp. *tritici* population in North America. Introduction of various *P. recondita* populations followed by differential selection of phenotypes within and between populations by host resistance genes (Kolmer 1991) most likely accounts for the current population structure in North America. Virulence and RAPD variation in an international collection of *P. recondita* (Kolmer and Liu 1997*b*) from five continents is currently being examined to determine if the characteristic virulence-RAPD phenotype associations in the North American populations are also found in other *P. recondita* populations.

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