Genetic diversity in *Puccinia striiformis* Westend. f.sp. *tritici* revealed by pathogen genome-specific repetitive sequence

Wei-xing Shan, Shou-yi Chen, Zhen-sheng Kang, Li-ren Wu, and Zhen-qi Li

Abstract: DNA fingerprinting was used to examine genetic variation in populations of *Puccinia striiformis* Westend. f.sp. *tritici*, an obligate fungus that causes wheat stripe rust, using as a probe a moderately repetitive DNA sequence PSR331 that shows species specificity in the genome of this pathogen. One hundred and sixty isolates sampled from six provinces throughout China were examined for genetic variation over 26 putative genetic loci defined by PSR331 and the restriction enzyme *Bgl*II. Because of the dikaryotic nature of this fungus, DNA fingerprints can not differentiate heterozygotes from homozygotes. We refer to the PSR DNA fingerprints as phenotypes rather than genotypes. Phenotypic diversity analysis revealed a high level of genetic variation. A total of 97 phenotypes was detected among 160 isolates. Phenotypic diversity varied among regions, ranging from 0.3742 in Shaanxi to 0.9380 in Gansu, as calculated with the normalized Shannon's index. Genetic subdivision analysis revealed a low level of genetic differentiation ($G_{ST} = 0.0084$) among regions (Gansu, Henan, Shaanxi, Sichuan, and Yunnan provinces) as well as within regions (Gansu and Sichuan provinces). This, together with the detection of the same phenotypes among regions, provided the molecular evidence for gene flow in *P. striiformis* f.sp. *tritici*. The results support conclusions from virulence surveys that Tianshui of southern Gansu is probably the most important "hotspot" area with respect to the potential to generate and maintain virulence variation. DNA polymorphism analysis also detected potential hotspot areas in addition to southern Gansu. This may result in more difficulties in management of genetic variation and thus the potential virulence variation in *P. striiformis* f.sp. *tritici* as well as providing opportunities for searching disease resistance factors.

Key words: genetic diversity, *Puccinia striiformis*, DNA fingerprinting, virulence variation.

Résumé : Les auteurs ont utilisé les empreintes d'ADN pour examiner la variation génétique dans des populations de *Puccinia striiformis* f.sp. *tritici*, un champignon parasite obligatoire qui cause la rouille striée du blé, en utilisant comme sonde la séquence d'ADN modérément répétitive PSR331, laquelle montre la spécificité des espèces dans la génome de ce champignon pathogène. Ils ont examiné 160 isolats provenant de six provinces dans l'ensemble de la Chine pour observer la variation génétique portant sur 26 lieux génétiques putatifs définis par le PSR331 et l'enzyme de restriction *Bgl*II. À cause de la nature dicaryotique de ce champignon, les empreintes d'ADN ne peuvent pas différencier les hétérozygotes des homozygotes. Les auteurs réfèrent aux empreintes d'ADN PSR comme des phénotypes plutôt que des génotypes. L'analyse de la diversité phénotypique révèle une forte variation génétique. Un total de 97 phénotypes ont été décelés parmi les 160 isolats. La diversité phénotypique varie selon les régions, allant de 0,3742 dans le Shaanxi à 0,9380 dans le Gansu, telle que calculée avec l'index normalisé de Shannon. L'analyse des subdivisions révèle une faible différenciation génétique ($G_{ST} = 0{,}0084$) entre les régions (provinces de Gansu, Henan, Shaanxi, Sichuan, et Yunnan), aussi bien qu'à l'intérieur des régions (provinces de Gansu et de Sichuan). Ceci, en plus de la détection des mêmes génotypes entre les régions, fournit la preuve moléculaire d'un flux de gènes chez le *P. striiformis* f.sp. *tritici*. Les résultats supportent les conclusions des observations sur la virulence, à l'effet que Tianshui, du sud de la province de Gansu, est probablement la région la plus critique par rapport au potentiel de génération et de maintien de la variation de virulence. L'analyse du polymorphisme de l'ADN met également en lumière des zones critiques en plus de celle du sud du Gansu. Ceci pourrait accroître les difficultés pour aménager la variation génétique et conséquemment la variation du potentiel de virulence chez le *P. striiformis* f.sp. *tritici*, en plus de présenter des opportunités pour détecter des facteurs de résistance à la maladie.

Mots clés : diversité génétique, *Puccinia striiformis*, empreintes ADN, variation de la virulence.

[Traduit par la rédaction]

Received May 8, 1997.

W.-X. Shan,[1] Z.-S. Kang, and Z.-Q. Li. Department of Plant Protection, Northwestern Agricultural University, Yangling, Shaanxi 712100, P.R. China.
S.-Y. Chen.[2] Institute of Genetics, Chinese Academy of Sciences, Beijing 100101, P.R. China.
L.-R. Wu. Institute of Plant Protection, Chinese Academy of Agricultural Sciences, Beijing 100094, P.R. China.

[1] Present address: Department of Plant Pathology, University of California, 1 Shields Avenue, Davis, CA 95616, U.S.A.
[2] Author to whom all correspondence should be addressed.

Introduction

Puccinia striiformis Westend. f.sp. *tritici*, an obligate fungus that causes stripe rust of wheat, has a worldwide distribution and is considered the major production problem in temperate regions (Saari and Prescott 1985), particularly in China (Li and Shang 1989). This fungus caused 6×10^9 and 3.2×10^9 kg losses of wheat in China in 1950 and 1964, respectively (Li and Shang 1989). In the late 1980s, the occurrence of race 29 that overcame resistance gene Yr 9 in cultivar Lovrin 13 rendered more than 1500 wheat cultivars susceptible prior to their release (Li and Shang 1989). Since the first demonstration of Mendelian inheritance in wheat of resistance genes against the stripe rust fungus (Biffen 1905), breeding for and deployment of new resistant cultivars have been the preferred means for managing plant diseases, and considerable effort has been directed towards understanding genetic resistance. This is the current strategy for management of wheat stripe rust in China. However, the deployment of resistance genes has been hampered by the ability of *P. striifromis* f.sp. *tritici* to develop new races that overcome such genes. Although up to 31 physiological races have been designated (Shan et al. 1995; Wang et al. 1995), it is known that there are a number of uncharacterized pathotypes, as detected by 17 wheat differential cultivars, and more pathotypes are expected to be detected when sample-sizes are increased.

Genetic variation in populations of *P. striiformis* f.sp. *tritici* has been studied in China as a joint program since the early 1950s. The yearly surveys of physiological specialization of *P. striiformis* f.sp. *tritici* have resulted in the accumulation of much information concerning the epidemiology and evolution of races of this pathogen in China (Li and Shang 1989; Wang et al. 1995). Virulence markers, as defined by virulence of pathogen isolates on the host differentials, have been extensively used for describing genetic variation in populations of plant pathogens. These markers are obviously important, because they provide direct information concerning the effects of host selection and the potential effectiveness of resistance genes. Virulence markers, however, may represent only a small portion of the total genetic variation present in the population. In plant pathogenic fungi, virulence is considered to be controlled by relatively few loci (Flor 1971; Michelmore and Hulbert 1987), and may give no indication of the extent of genetic variation either between races or between independent collections of a race. It is not known whether the ability of the pathogen to overcome resistant cultivars reflects shifts in the frequency of formerly rare pathotypes, the generation of genetic changes to new virulence forms, or a combination of both phenomena. Therefore, detailed genetic information on population structure is essential for understanding mechanisms involved in virulence variation and dynamics of the pathogen, and for developing more effective strategies for management of wheat stripe rust.

Our knowledge of population genetics of plant pathogenic fungi has accumulated rapidly in recent years owing largely to the development of effective molecular genetic marker systems (Fry et al. 1992; Leung et al. 1993; Levy et al. 1993; McDermott and McDonald 1993; Wolf and McDermott 1994; McDonald et al. 1995; Goodwin 1997). For obligate pathogens such as rust fungi, however, our knowledge of population biology is based almost exclusively on virulence surveys, despite much effort given to the development of biochemical (e.g., isozyme) (Burdon and Roelfs 1985; Newton et al. 1985; Michelmore and Hulbert 1987; McCain et al. 1992; Roelfs et al. 1997), and molecular (e.g., double-stranded RNA and DNA) (Newton et al. 1985; Dickinson et al. 1990; Chen et al. 1993; Zhang et al. 1994; Kolmer et al. 1995; McDonald et al. 1995; Shan et al. 1995, 1996, 1997) markers. The double-stranded RNA and isozyme markers were demonstrated to be of little value for differentiating *P. striiformis* f.sp. *tritici* isolates (Newton et al. 1985; Zhang et al. 1994), while the random amplified polymorphic DNA (RAPD) markers from any given primer were simply not numerous enough to define isolate-specific phenotypes (Chen et al. 1993, 1995; Shan et al. 1995). Therefore, numerous polymorphic markers are necessary to distinguish *P. striiformis* f.sp. *tritici* isolates unambiguously and to assess the level of genetic diversity accurately.

The development of DNA fingerprinting probes that detect numerous restriction fragment length polymorphisms (RFLPs) within the genome of *P. striiformis* makes it possible for more detailed analysis of *P. striiformis* f.sp. *tritici* isolates. In a previous report, we identified a family of moderately repetitive DNA sequences (PSR sequences) specific to the genome of *P. striiformis* (Shan et al. 1997). Among the four PSR sequences, PSR331 was homologous to 33 *Bgl*II fragments ranging in size from 1.6 to 20 kilobases (kb). Using PSR331 as a probe, almost all of the 16 Chinese reference strains of *P. striiformis* f.sp. *tritici* were distinctly resolved (Shan et al. 1996). Thus, probe PSR331 provides genetic markers that probably span a large part of the *P. striiformis* genome, and PSR331 DNA fingerprinting is expected to be a powerful tool for epidemiology and population biology purposes of *P. striiformis* f.sp. *tritici*, an obligate pathogen that has significant asexual reproduction.

In the present investigation, we attempted to gain preliminary insight into the genetic diversity within and among *P. striiformis* f.sp. *tritici* populations in China using a combination of the DNA fingerprinting probe PSR331 and the restriction enzyme *Bgl*II.

Materials and methods

Sample collection and culture of rust isolates

One hundred and sixty *P. striiformis* f.sp. *tritici* isolates from wheat in six provinces throughout China were collected in virulence surveys from 1993 to 1995. The sampling sites are shown in Fig. 1. During the very early stage of rust development, single-lesion samples were collected randomly from wheat plants in commercial wheat fields, as well as in nurseries used for evaluating stripe rust resistance and monitoring the occurrence of new virulence genes. The single-lesion, and in most cases, single-pustule samples, were dried with paper towels, then delivered to the greenhouses in Beijing, Chendu, Lanzhou, and Yangling for characterization. This caused many samples to die, because of long delivery times during the hot season. The sample leaves were incubated under wet conditions (in Petri dishes, 100% humidity) to activate urediospores. The urediospores from single or a few pustules were dispersed onto seedlings of wheat cultivar Mixian 169, which is susceptible to all known races of *P. striiformis* f.sp. *tritici* and is routinely used for maintaining and multiplying fungal isolates (Wang et al. 1986). The urediospores were collected for routine virulence characterization, while the diseased leaves were used for DNA analysis. Pure urediospores or pathogen-infected wheat leaves were frozen in liquid nitrogen and stored at –70°C for DNA

extraction. The reference strain of race 29, which was used as a standard in this study, is widely used for evaluating resistance in wheat germplasm materials, and is routinely maintained in the Cereal Rusts Laboratory, Institute of Plant Protection, Chinese Academy of Agricultural Sciences, Beijing.

DNA isolation

Genomic DNA was extracted directly from urediospores as described previously (Shan et al. 1997). With this procedure, approximately 2 μg of total DNA can be obtained from 20 mg of urediospores, which is sufficient for restriction analysis. For extracting DNA from rust-infected wheat leaves, a procedure based on alkali lysis was used. Briefly, 0.5–1.0 g of diseased wheat leaves (Minxian169) were ground to a fine powder in liquid nitrogen. The ground material was dispersed in 6 mL of extraction buffer (100 mM Tris-HCl (pH 8.0), 50 mM EDTA (pH 8.0), 500 mM NaCl; 1% PVP-360 (w/v); 2% SDS (w/v); 0.1% 2-mercaptoethanol (v/v)). The mixture was incubated at 65°C for 30 min with occasional gentle shaking. Then, 2 mL of 5 M potassium acetate (pH 6.5) was added to each tube, mixed, and incubated in ice-water for 5 min. Eight millilitres of chloroform was added to the mixture, gently mixed, and centrifuged at 4°C for 15 min (12 000 × g). The supernatant was transferred into clean tubes containing 6 mL of isopropanol and incubated at 4°C to precipitate nucleic acids. The DNA pellet was washed with 70% ethanol, air-dried, and dissolved in 400 μL TE (10 mM Tris-HCl, 1 mM EDTA (pH 8.0)) and incubated with 2 μL RNAse (10 mg/mL) at 37°C for 30 min or at 4°C overnight.

Southern hybridization

Genomic DNA was digested with the restriction enzyme *Bgl*II according to the manufacturer's instructions, size fractionated on 1% agarose gels in 1× Tris borate EDTA (TBE) buffer, and capillary-blotted onto nylon membrane (Hybond-N +, Amersham Corp.) using 0.4 M NaOH as the transfer agent. For pure fungal genomic DNA isolated directly from urediospores, 1.5 μg was sufficient for RFLP analysis. For total DNA isolated from rust-infected wheat leaves, 5 μg of DNA mixture was sufficient to give a fingerprint pattern. *Bgl*II digested DNA of wheat leaves infected with a reference strain of race 29 was used as a reference.

DNA blots were prehybridized for 2 h at 65°C in 30 mL of hybridization solution (5 × SSC, 0.1% Sarkosyl (w/v), 0.1% Ficoll-400 (w/v), 0.6% SDS (w/v), 0.1% BSA (w/v), 0.15% dextran sulfate, and 100 μg/mL of denatured salmon sperm DNA). The subclone PSR331s3 (Shan et al. 1996), labeled with α-^{32}P-dCTP by the random-primer labeling method of Feinberg and Vogelstein (1983), was used as a hybridization probe. Hybridizations were carried out overnight at 65°C and the membranes washed at 65°C three times for 15 min each in 2 × SSC, 0.1% SDS; 1 × SSC, 0.1% SDS; and 0.5 × SSC, 0.1% SDS. The membranes were exposed to X-ray film (RX, Fuji Photo Co.) for 1–4 days at –70°C, with intensifying screens.

Data analysis

The DNA fingerprint pattern of the Southern blots was scored for each isolate on the basis of the presence (1) or absence (0) of hybridization bands regardless of the intensity of hybridization signals. Each hybridization band was assumed to represent a different putative genetic locus that is segregating plus or minus for the band. Because of the dikaryotic nature of this fungus, it is impossible to differentiate heterozygotes from homozygotes based on the PSR DNA fingerprint. We therefore referred to the PSR fingerprints as phenotypes rather than genotypes that are commonly used for haploid fungi. We are also unable to perform gene diversity analysis based on allele frequencies because of a lack of information on the genetics of the hybridization bands. Although the moderately repetitive sequences in most cases, if not all, are dispersed in the genome, this feature cannot exclude the possibility of co-segregation of some hybridization bands, as in the cases of E9 probe of *Erysiphe graminis* DC (Brown and Simpson 1994) and RG57 probe of *Phytophthora infestans* (Mont. de Bary) (Goodwin et al. 1992).

Table 1. Regions sampled for *Puccinia striiformis* f.sp. *tritici* from 1993 to 1995 and the occurrence of PSR phenotypes defined by PSR331/*Bgl*II in each location in China.

Geographic origin (Province and locale)	No.of isolates	PSR phenotypes[a]
Henan	8	1–7
Gansu		
Gangu	10	73–80
Gannan	2	89, 90
Lanzhou/Lingtao	4	34, 86–88
Tianshui	32	10, 34, 46, 49–72
Others	7	81–85, 89
Shaanxi		
Longxian/Yangling	2	36
Chenggu/Hanzhong	6	35, 36
Shandong	2	92, 93
Sichuan		
Mianyang	54	6, 8–26
Neijiang	8	37–44
Pingshan	2	36, 48
Yaan	2	45, 46
Yunjing	1	47
Wenjiang/Xindu	15	27–36
Yunnan		
Kunming	3	39, 96, 97
Yuxi	2	94, 95

[a]Designation of phenotypes is given in Table 2.

A multilocus phenotype was derived for each isolates and the isolates with the same PSR331 fingerprint pattern were considered to be identical phenotypes. The Shannon diversity index (Nei and Chesser 1983) was used to measure phenotypic diversity. Phenotypic diversity in each population was calculated as

$$[1] \quad M = \sum g_i \ln g_i$$

where g_i is the frequency of isolates with the ith phenotype in a population. Taking into consideration the biased estimation due to small sample sizes, the phenotype diversity was normalized, following the method of Sheldon (Stubbs 1985), as

$$[2] \quad M^* = M/\ln k$$

where k is the population size (number of isolates).

To measure genetic differentiation of *P. striiformis* f.sp. *tritici* populations, the total diversity was partitioned into two components: the proportion of total diversity that is due to differences within regions and among regions. The genetic differentiation was calculated, following Nei's method (1973), as

$$[3] \quad G_{ST} = 1 - H_S/H_T$$

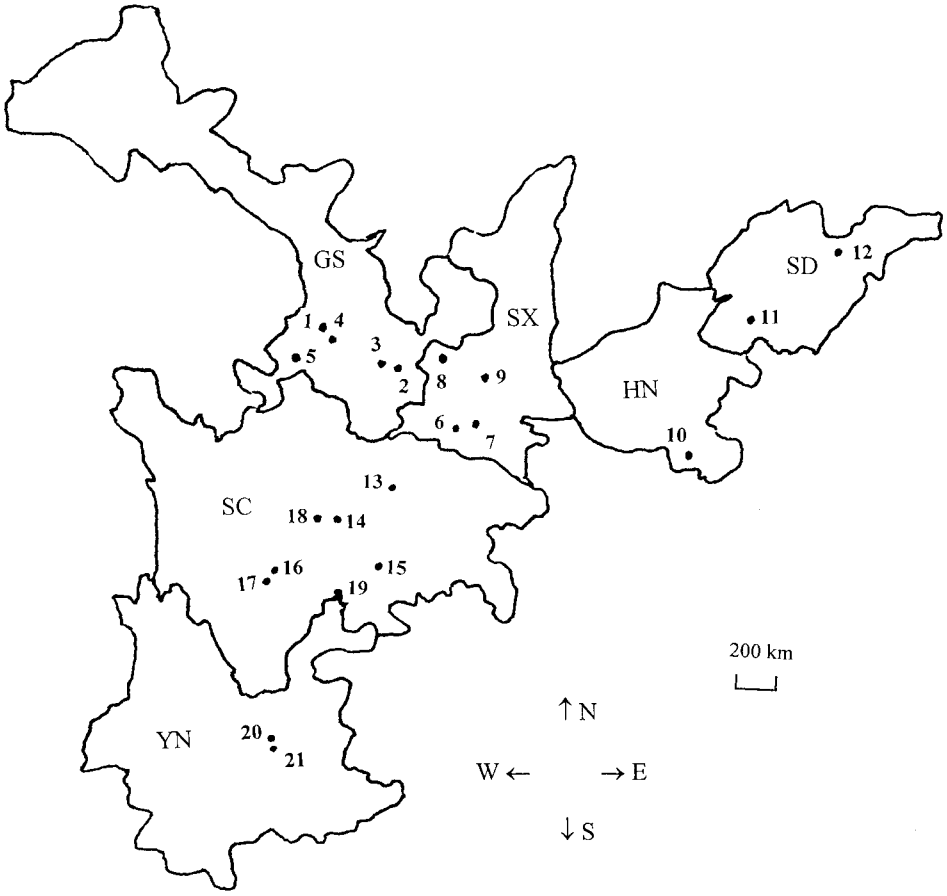
where H_T is the total phenotypic diversity of the whole populations throughout China, whereas H_S is the mean of phenotypic diversities of populations from different regions. Normalized Shannon's index for phenotypic diversity was used for population subdivision analysis.

Results

DNA fingerprints generated with PSR331

Because PSR sequences are specific to the genome of *P. striiformis*, it is possible to perform DNA fingerprinting with DNA

Fig. 1. Locations of sampling sites in six provinces of China where *Puccinia striiformis* f.sp. *tritici* isolates were collected from 1993 to 1995. 1, Lingtao, Gansu (GS); 2, Tianshui, Gansu; 3, Gangu, Gansu; 4, Lanzhou, Gansu; 5, Gannan, Gansu; 6, Hanzhong, Shaanxi (SX); 7, Chenggu, Shaanxi; 8, Longxian, Shaanxi; 9, Yangling, Shaanxi; 10, Xinyang, Henan (HN); 11, Jining, Shandong (SD); 12, Weifang, Shandong; 13, Mianyang, Sichuan (SC); 14, Wenjiang, Sichuan; 15, Neijiang, Sichuan; 16, Yaan, Sichuan; 17, Yunjing, Sichuan; 18, Xindu, Sichuan; 19, Pingshan, Sichuan; 20, Kunming, Yunnan (YN); 21, Yuxi, Yunnan.



mixtures isolated from rust-infected wheat leaves. With the methods described above, preliminary work showed that the fingerprint patterns were better than those of pure fungal genomic DNA. This is probably due to partial degradation of DNA resulting from inactivation of pure urediospores, which is a common problem during laboratory manipulations. Based on the intensity of hybridization signal, up to one third of total DNA from diseased wheat leaves was of fungal origin. Under our experimental conditions, PSR331s3 hybridized to 29 *Bgl*II fragments of genomic DNA ranging in size from 3 to 20 kb. Using as standards λ DNA digested with *Eco*RI and *Hind*III, and a reference strain of race 29, 26 of 29 fragments that were well resolved were scored to assess genetic variation of 160 isolates collected in six geographic regions throughout China (Table 1, Fig. 1). Ninety-seven phenotypes were identified among the 160 isolates (Table 2). Examples of the DNA fingerprints are shown in Fig. 2. The phenotypes were scored from largest to smallest hybridization bands.

Phenotypic diversity

The number of phenotypes varied among regions. In Mianyang of Sichuan Province, 20 phenotypes were detected among 54 isolates, whereas 27 phenotypes were found among 32 isolates sampled from Tianshui of Gansu province. In populations consisting of a small numbers of isolates, multiple phenotypes were also identified (Table 3). A total of 97 phenotypes, defined by PSR331 and *Bgl*II, were found among 160 isolates. Using Shannon's index (*M*), phenotypic diversity was very high for the pathogen population of Tianshui, Gansu province (3.2383), with 27 phenotypes among 32 samples, whereas in Shaanxi the M value was only 0.7781. Interestingly, seven of eight isolates from Henan province were unique phenotypes. Because of the biased measurement of phenotypic diversity caused by small sample sizes, a normalized Shannon's index (*M**) was adopted. For example, the *M* value for Mianyang population was 2.11, while the *M* value of the Gangu population was 2.0253. However, the normalized calculation suggested that the phenotypic diversity of the Gangu population ($M^* = 0.8795$) was higher than that of the Mianyang population ($M^* = 0.5290$). In general, the normalized Shannon's index for pathogen population varied within, as well as among, regions (Table 3).

Genetic differentiation of *P. striiformis* f.sp. *tritici* populations

The partitioning of genetic variation indicated that there was a low level of genetic differentiation among *P. striiformis* f.sp. *tritici* populations in China. The genetic differentiation (G_{ST})

Table 2. DNA fingerprint patterns defined by PSR331/*Bgl*II and the frequency of each phenotype among 160 isolates of *Puccinia striiformis* f.sp. *tritici* from China.

Phenotype	PSR fingerprint (Electrophoresis direction →)	Frequency[a]
1	11000110111111100110011 0111	1
2	11011100111110001010010011	1
3	11110110111111001100010111	1
4	11010110111111001100111111	2
5	11011111111111001010011011	1
6	11101101111111001101011111	5
7	11111110111110001011010111	1
8	10111110111100001111111010	27
9	11011100111100001110110011	1
10	11101111111111001011101111	3
11	11111110111100000010100011	2
12	11101111111110001011011111	1
13	01101010110011001010011011	1
14	11001100111111001010011111	1
15	11101110111110001010011111	1
16	11001111111110000010001110	1
17	11101110111111001110001111	2
18	11101111111110001110111111	2
19	11101111111111001110101010	1
20	11101111111110001110101111	2
21	11111111111110001110101010	1
22	11111111111111001110010111	1
23	11111110111111001110111011	1
24	10001111111110000011101111	1
25	11111101111111001110101011	1
26	11101111111111001111010111	1
27	11101111101110011100111111	1
28	11001101111110001010111111	1
29	11011111111110001110111111	3
30	11111100111111001111011011	2
31	11111111111100001110011011	1
32	10111110111111001010011111	1
33	11111101111110001110100111	1
34	10111111110111001001001111	4
35	11111111111110101110111011	4
36	11111101111110001011111011	8
37	10101101111111001111100001	1
38	10111101111111001111110100	1
39	11101101111110001010100010	2
40	11101101111111001101101110	1
41	10010111111110001011100000	1
42	10001101111110001010101110	1
43	11001101111111001110101010	1
44	10111101111110001111111010	1
45	11101100111111001010011111	1
46	11101111101110011111001111	2
47	10111101111111001110111111	1
48	11111101111111011111011011	1
49	10011101111110000111111010	1

Table 2. (*concluded*).

Phenotype	PSR fingerprint (Electrophoresis direction →)	Frequency[a]
50	10111110111111001111011010	1
51	10101100111110001100100010	1
52	11001101111110001110110011	3
53	11001110111110001100100010	1
54	10111101101010111111110011	1
55	11011101101010111111010011	1
56	10001110111010001111100111	1
57	10111101110101000101111011	1
58	10111100111110001101110011	2
59	10111110110110001100111110	2
60	10110111111101001101110111	1
61	10011110101110001110111010	1
62	11110111111110110110111111	1
63	11100110111110000111111010	1
64	11111111111111001111111011	2
65	11101111101101001111101011	1
66	11101101101111001011110011	1
67	10111110110111001111111001	1
68	11101110111110011111101010	1
69	11111111111110001111111011	1
70	10101101111010001110010011	1
71	10111110111111001010111011	1
72	00001111111111001101001111	1
73	11110111101100001110111010	1
74	11111100111111001111110010	2
75	11111111111111001011111011	1
76	11101101111110001110110010	1
77	10011110101111001101111010	1
78	10001111110111001010001111	1
79	10000110111110001101011111	2
80	11110111111111001111111111	1
81	11110110111111001011111011	2
82	10011110111111001110011110	1
83	11011110111111001110011010	1
84	11011110111111001110111010	1
85	11111111111111001100001010	1
86	10111111111110001101101111	1
87	10001101110111001010000111	1
88	01110111111110001111111111	1
89	10000110111110001101011111	1
90	00101111011111001001001111	1
91	11111111111110001001010011	1
92	00111111101110011111111001	1
93	11111100101100001110111001	1
94	10111101111111001111100010	1
95	11011101111011011010111011	1
96	11011100111110001111011110	1
97	10011010111010011010110011	1

[a]Number of times found.

was merely 0.0084 among regions (Gansu, Sichuan, Henan, Shaanxi, and Yunnan provinces) based on DNA fingerprints of 160 isolates. The index of genetic differentiation was even lower among locations within regions, merely 0.0010 among Tianshui, Gangu and Lanzhou (including samples from Lingtao) of Gansu Province and 0.0394 among Mianyang, Neijiang and Wenjiang (including samples from Xindu) of Sichuan Province (Table 4).

Fig. 2. DNA fingerprints of *Puccinia striiformis* f.sp. *tritici* isolates defined by PSR331/*Bgl*II combination. Four phenotypes (6, 8, 37, and 38) can be observed on this autoradiograph. Phenotype designations were shown in parentheses (see Table 2 for details). M, λ DNA digested with *Hin*dIII and *Eco*RI; Ps29, DNA from reference race 29 infected wheat leaves, used as a standard.

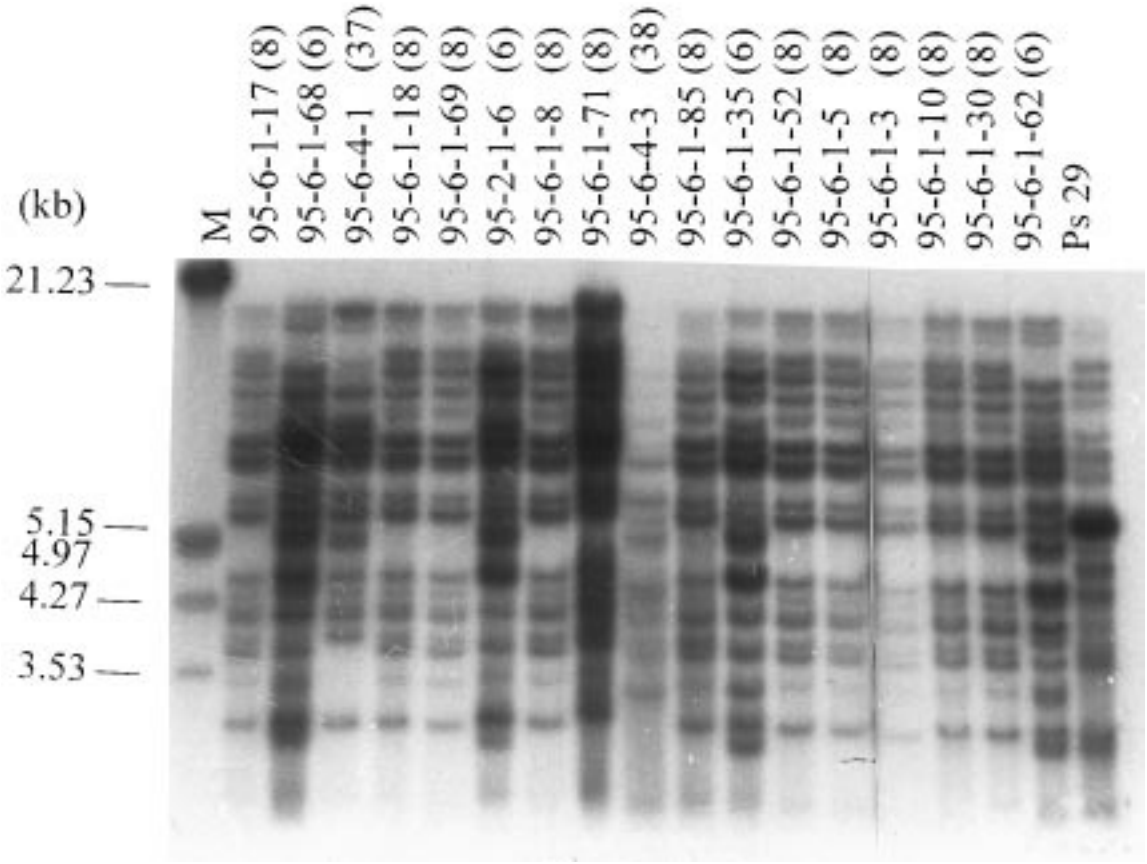


Table 3. Phenotypic diversities of *Puccinia striiformis* f.sp. *tritici* populations as measured by DNA fingerprinting over 26 putative genetic loci defined by PSR331/*Bgl*II.

Location	No. of isolates	No. of phenotypes	Phenotypic diversity	
			Shannon (*M*)	Normalized (*M**)
Henan	8	7	1.9062	0.9167
Gansu	55	44	3.7587	0.9380
Gangu	10	8	2.0253	0.8795
Lanzhou/Lingtao	4	4	1.3863	1.0000
Tianshui	32	27	3.2383	0.9329
Shaanxi	8	2	0.7781	0.3742
Sichuan	82	41	3.1521	0.7153
Mianyang	54	20	2.1100	0.5290
Neijiang	8	8	3.7587	1.0000
Wenjiang/Xindu	15	10	1.4415	0.5323
Yunnan	5	5	1.6094	1.0000
Total	160	97	4.0371	0.7955

Discussion

Since the late 1980s and early 1990s, the development of a variety of molecular genetic markers, notably RFLPs in mitochondrial DNA and nuclear DNA, and RAPD, has greatly facilitated our understanding of the population genetic structure of fungal plant pathogens (Fry et al. 1992; Leung et al. 1993; Levy et al. 1993; McDermott and McDonald 1993; Wolf and McDermott 1994; McDonald et al. 1995; Goodwin 1997). Although molecular genetic analysis of rust fungi has been reported (Chen et al. 1993; Zhang et al. 1994; Chen et al. 1995; Kolmer et al. 1995; Shan et al. 1995, 1996, 1997; Roelfs et al. 1997), surveys of large populations of rust fungi are difficult because of the difficulties in manipulating rust fungal cultures and in obtaining sufficient amounts of fungal genomic DNA

Table 4. Population subdivision analysis of the proportion of the total phenotypic diversity due to differentiation among sampling locations (G_{ST}) for different levels of subdivision.

Subpopulation	H_S	H_T	G_{ST}
Total, among regions[a]	0.7888	0.7955	0.0084
Gansu, among locations[b]	0.9375	0.9380	0.0010
Sichuan, among locations[c]	0.6871	0.7153	0.0394

[a]Based on analysis of 82, 55, 8, 8, and 5 isolates from Sichuan, Gansu, Henan, Shaanxi, and Yunnan, respectively.

[b]Based on 32, 10, and 4 isolates from Tianshui, Gangu, and Lanzhou, respectively. Two isolates from Lingtao were included in the Lanzhou collection.

[c]Based on 54, 15, and 8 isolates from Mianyang, Wenjiang, and Neijiang, respectively. Four isolates from Xindu were included in the Wenjiang collection.

for molecular analysis. In a previous report, we described a species-specific repetitive DNA sequence, PSR331, in the genome of *P. striiformis*, which because of its highly polymorphic nature, was anticipated to be useful for large-scale characterization of population structures of *P. striiformis* (Shan et al. 1996, 1997). It has also been possible to use PSR331 to detect genetic differences with DNA isolated from rust-infected wheat leaves without laboriously and tediously obtaining pure fungal DNA. DNA fingerprinting revealed that all of the 26 putative genetic loci defined by PSR331 and *Bgl*II were polymorphic among the 160 isolates tested. Compared with 18 polymorphic bands of 24 PSR331/*Bgl*II loci detected in a previous analysis of 16 reference strains of *P. striiformis* f.sp. *tritici* (Shan et al. 1996), this further indicates the potential of PSR331 for detecting genetic variation in *P. striiformis* f.sp. *tritici* populations.

Using PSR331s3 as a probe, a total of 97 phenotypes was identified among 160 isolates sampled throughout China. Phenotypic diversity analysis revealed a high level of genetic diversity of *P. striiformis* f.sp. *tritici* in China. The level of genetic diversity varied among regions, ranging from a low level of 0.3742 in Shaanxi to a high level of 0.9380 in Gansu, as calculated with the normalized Shannon's index.

Tianshui of Gansu Province was well known as the most important hotspot area with respect to the potential to generate new races of *P. striiformis* f.sp. *tritici* in China (Li and Shang 1989), a conclusion drawn from yearly virulence surveys. This conclusion is supported by our DNA fingerprinting analysis, which detected a high level of molecular genetic diversity in the pathogen population sampled from this area. In Tianshui, a cool mountain area that is most favorable for development of stripe rust epidemics, the rust fungus is able to both overwinter and oversummer, in contrast to most epidemic regions. This feature would enable the pathogen to generate, and most importantly, to maintain genetic variation, because the primary source of genetic variation, mutation, has more time to introduce new variants, and the genetic drift has more time to increase the frequencies of new alleles to detectable levels. Populations that have evolved over a long time at one location are expected to have more alleles than populations that moved into an area more recently. Such a complex genetic structure of pathogen populations suggests the potential of the pathogen to quickly adapt to diverse environments (Fry et al. 1992; Leung et al. 1993; Levy et al. 1993; McDermott and McDonald 1993; McDonald et al. 1995).

Most samples analyzed were from single or a few mixed pustules that were not necessarily derived from a single spore. Therefore, the phenotypic diversity may be either higher or lower than estimated because of the possibility of the presence of two or more phenotypes in one lesion. This was confirmed by analysis of several single-spore isolates, although in most cases the single-spore isolates are consistent with the single-pustule isolates from which they are derived. The preliminary results indicated that the presence of two or more phenotypes might be more common in isolates sampled from Gansu Province than the rest. For example, different phenotypes were detected in samples 94-7-6-13, 94-7-6-69, and 93-2-28 of Tianshui; 94-7-7-15 and 93-1-92 of Gangu; and 93-20-12 of Lanzhou. Thus, the phenotypic diversity in populations from Gansu may be more complex than determined here.

A higher level of genetic diversity was also detected in populations sampled from Mianyang and Neijiang of Sichuan province and Yunnan province. This suggested that the complexity of pathogen populations in these areas was comparable with that of Gansu and that populations of some other areas might also have high potential to generate new virulent races, i.e., to quickly break down newly introduced disease resistance genes.

Interestingly, multiple phenotypes were detected in the samples from Henan. It is known that the stripe rust is unable to oversummer in the most important wheat production areas such as Shaanxi, Henan, and Shandong provinces in China. This means that the pathogen cannot complete its life cycle in these areas. Each year, the rust epidemic is initiated in the spring from diseased wheat plants that were infected in the last fall by pathogen inoculum from areas where the pathogen can survive in the summer. Yearly virulence surveys suggested that stripe rust in Gansu Province is critically important to the next year's wheat production in the wheat belt that are hundreds of kilometres apart. However, the rust doesn't cause a big problem for wheat production in the areas that presumed to provide primary inoculum to the wheat belt (Li and Shang 1989). The multiple phenotypes characteristic of the pathogen population in Henan suggests that stripe rust is initiated by mixed inoculum from diverse areas, or from genetically complex populations produced in single or few areas.

Population subdivision analysis revealed a low level of genetic differentiation among geographic regions, with a differentiation index of merely 0.1049 among Gansu, Sichuan, Henan, Shaanxi, and Yunnan Provinces. With respect to different locations within regions, the genetic differentiation was even lower (the G_{ST} values were 0.0338 and 0.0691 in Sichuan and Gansu, respectively). In addition to the low level of genetic differentiation among regions revealed by phenotypic diversity analysis, certain single phenotypes were identified in different populations. For example, isolate 94-7-6-37 of Gansu (Tianshui) shared the same phenotype with isolate 95-6-1-22 of Sichuan (Mianyang), isolate 95-6-4-4 of Sichuan (Neijiang) was identical to isolate 95-15-3-6 of Yunnan (Kunming), isolate 95-2-1-6 of Henan (Xinyang) was identical to isolate 95-6-1-68 of Sichuan (Mianyang), isolates 94-s-18 (Pingshan) and 94-s-19 (Xindu) of Sichuan were identical to isolate 94-s-10 of Shaanxi. These data provide direct molecular evidence for gene flow in *P. striiformis* f.sp. *tritici*. Based on

virulence surveys, it was predicted that pathogen long-distance migration is common in *P. striiformis* f.sp. *tritici* in China, with southern Gansu (particularly the Tianshui area) as the possible origin of new virulent races (Li and Shang 1989). New virulent races were usually first found in Gansu province, about 2 or 3 years in advance to other epidemic regions (Li and Shang 1989). However, virulence markers are subjected to strong selection and are not suitable for evaluation of genetic differentiation of pathogen populations.

It is difficult to obtain more information concerning the extent and direction of gene flow based on the DNA fingerprinting results of the small number of isolates presented here. We found that some isolates sampled 1 or 2 years earlier in Gansu shared the same phenotypes with those collected in other regions. For example, the isolates 94-7-6-37s of Tianshui (Gansu) and 95-6-1-22 of Mianyang (Sichuan), isolates 93-23-40s2 of Lingtao and 93-2-99s1 of Tianshui (Gansu), and 94-6-3-2 of Wenjiang (Sichuan) shared identical DNA fingerprint patterns, respectively. This is, however, not sufficient to indicate the direction of pathogen migration because of too few isolates and the lack of isolates sampled from different regions in different years. The majority of samples analyzed here were from 1995. Analysis of more isolates, in connection with the Joint Program for Virulence Survey, is expected to yield useful information about the extent and direction of gene flow. This will aid rational deployment of resistance genes. Analysis of more isolates will also facilitate the detection of potential hotspot areas. In connection with future characterization of interactions between wheat cultivars and rust populations defined by both virulence characters and PSR331 DNA fingerprints, this information is anticipated to facilitate breeding efforts for disease resistance against *P. striiformis* f.sp. *tritici*.

Molecular variation among isolates detected with PSR fingerprinting showed that *P. striiformis* f.sp. *tritici* is highly variable in terms of multiple phenotypes. The asexual urediospores are the only known means of reproduction; there is a lack of evidence for the presence of pycnial and aecial stages in *P. striiformis*. Therefore, there must be mechanisms that oppose genetic drift to maintain intrapopulation genetic variation in *P. striiformis* f.sp. *tritici*. Point mutations as well as heterokaryonsis were presumed the most important sources of new variation in virulence for *P. striiformis* (Li and Shang 1989). We do not know yet if there is a nuclear fusion and (or) recombination event, though the survey of both laboratory and field urediospores suggested that heterokaryonsis is common in *P. striiformis* f.sp. *tritici* (Kang et al. 1994). More recently, parasexual recombination was proposed as the mechanism responsible for generation of genetic variation that may allow for selection of virulence to occur (Chen et al. 1993). However, molecular mechanisms underlying the observed genetic variation are not yet clear. The high level of phenotype diversity in *P. striiformis* f.sp. *tritici* suggests that sexual production may be occurring in this fungus. This can be tested by measuring gametic disequilibrium using unlinked genetic markers and relatively large sample sizes. However, as the asexual urediospores are the only known means of reproduction, it is more likely that clones will be overrepresented in the sample collections. DNA fingerprinting with PSR331 will be useful to test these hypotheses by clone-correcting data set (McDonald et al. 1995).

Acknowledgments

We are grateful to Mr. Q. Meng for production of urediospores, Professor W. Yuan for providing some isolates, and three anonymous reviewers for critical evaluation of the manuscript. This work was supported by the Climbing Program of China and Plant Biotechnology Laboratory, Chinese Academy of Sciences.

References

Biffen, R.H. 1905. Mendel's law of inheritance and wheat breeding. J. Sci. **1**: 4–48.

Brown, J.K.M., and Simpson, C.G. 1994. Genetic analysis of DNA fingerprints and virulences in *Erysiphe graminis* f.sp. *hordei*. Curr. Genet. **26** : 172–178.

Burdon, J.J., and Roelfs, A.P. 1985. The effect of sexual and asexual reproduction on the isozyme structure of populations of *Puccinia graminis*. Phytopathology, **75**: 1068–1073.

Chen, X.M., Line, R.F., and Leung, H. 1993. Relationship between virulence variation and DNA polymorphism in *Puccinia striiformis*. Phytopathology, **83**: 1489–1497.

Chen, X.M., Line, R.F., and Leung, H. 1995. Virulence and polymorphic DNA relationships of *Puccinia striiformis* f.sp. *hordei* to other rusts. Phytopathology, **85**: 1335–1342.

Dickinson, M.J., Wellings, C.R., and Pryor, A. 1990. Variation in the double-stranded RNA phenotype between and within different rust species. Can. J. Bot. **68**: 599–604.

Feinberg, A.P., and Vogelstein, B. 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. **132**: 6–13.

Flor, H.H. 1971. Current status of gene-for-gene concept. Annu. Rev. Phytopathol. **9**: 275–296.

Fry, W.E., Goodwin, S.B., Matsuzak, J.M., Spielman, L.J., Milgroom, M.G., and Drenth, A. 1992. Population genetics and intercontinental migrations of *Phytophthora infestans*. Annu. Rev. Phytopathol. **30**: 107–129.

Goodwin, S.B. 1997. The population genetics of *Phytophthora*. Phytopathology, **87**: 462-473.

Goodwin, S.B., Drenth, A., and Fry, W.E. 1992. Cloning and genetic analyses of two highly polymorphic, moderately repetitive nuclear DNAs from *Phytophthora infestans*. Curr. Genet. **22**: 107–115.

Kang, Z.-S., Li, Z.-Q., and Shang, H.-S. 1994. On the screening of new heterokaryons of wheat stripe rust and its nucleal dissociation. Acta Phytopathol. Sin. **24**(2): 101–105.

Kolmer, J.A., Liu, J.Q., and Sies, M. 1995. Virulence and molecular polymorphism in *Puccinia recondita* f.sp.*tritici* in Canada. Phytopathology, **85**: 276–285.

Leung, H., Nelson, R.J., and Leach, J.E. 1993. Population structure of plant pathogenic fungi and bacteria. Adv. Plant Pathol. **10**: 157–205.

Levy, M., Correa-Victoria, F.J., Zeigler, R.S., Xu, S., and Hamer, J.E. 1993. Genetic diversity of the rice blast fungus in a disease nursery in Colombia. Phytopathology, **83**: 1427–1433.

Li, Z.-Q., and Shang, H.-S. 1989. Wheat rusts and their control. Shanghai Science and Technology Press, Shanghai, China.

McCain, J.W., Groth, J.V., and Roelfs, A.P. 1992. Inter- and intrapopulation isozyme variation in collections from sexually reproducing populations of the bean rust fungus, *Uromyces appendiculatus*. Mycologia, **84**: 329–340.

McDermott, J.M., and McDonald, B.A. 1993. Gene flow in plant pathosystems. Annu. Rev. Phytopathol. **31**: 353–373.

McDonald, B.A., Pettway, R.E., Chen, R.S., Boeger, J.M., and Martinez, J.P. 1995. The population genetics of *Septoria tritici* (telemorph *Mycosphaerella graminicola*). Can. J. Bot. **73**(Suppl.): S292–S301.

Michelmore, R.W., and Hulbert, S.H. 1987. Molecular markers for genetic analysis of phytopathogenic fungi. Annu. Rev. Phytopathol. **25**: 283–304.

Nei, M. 1973. Analysis of gene diversity in subdivided populations. Proc. Natl. Acad. Sci. U.S.A. **70**: 3321–3323.

Nei, M., and Chesser, R.K. 1983. Estimation of fixation indices and gene diversities. Ann. Hum. Genet. **47**: 253–259.

Newton, A.C., Caten, C.E., and Johnson, R. 1985. Variation for isozyme and double-stranded RNA among isolates of *Puccinia striiformis* and two other cereal rusts. Plant Pathol. **34**: 235–247.

Roelfs, A.P., McCallum, B., McVey, D.V., and Groth, J.V. 1997. Comparison of virulence and isozyme phenotypes of Pgt-QCCJ and Great Plains races of *Puccinia graminis* f.sp. *tritici*. Phytopathology, **87**: 910–914.

Saari, E.E., and Prescott, J.M. 1985. World distribution in relation to economic losses. *In* The cereal rusts. Vol. 2. Diseases, distribution, epidemiology, and control. *Edited by* A.P. Roelfs and W.R. Bushnell. Academic Press, Orlando, Fla. pp. 260–298.

Shan, W.-X., Chen, S.-Y., Wu, L.-R., and Li, Z.-Q. 1995. RAPD analysis of the epidemic races of *Puccinia striiformis* f.sp. *tritici* in China. Sci. Agric. Sin. **28**(5):1–7.

Shan, W.-X., Chen, S.-Y., Hui, D.-W., Wu, L.-R., and Li, Z.-Q. 1996. DNA fingerprinting of the reference strains of *Puccinia striiformis* f.sp. *tritici* in China. Chin. Sci. Bull. **41**(24): 2078–2082.

Shan, W.-X., Chen, S.-Y., Zhang, G.-Y., Wu, L.-R., and Li, Z.-Q. 1997. Identification of a family of dispersed, moderately repetitive DNA sequences in an obligate fungal plant pathogen *Puccinia striiformis*. Sci. China (Ser. B), **27**(3): 241–246.

Stubbs, R.W. 1985. Stripe rust. *In* The cereal rusts. Vol. 2. Diseases, distribution, epidemiology, and control. *Edited by* A.P. Roelfs and W.R. Bushnell. Academic Press, Orlando, Fla. pp. 61–101.

Wang, F.-L., Wu, L.-R., and Wan, A.M. 1995. Studies on virulence variation of wheat stripe rust population in China. Sci. Agric. Sin. **28**(1): 8–14.

Wang, K.N., Wu, L.R., Meng, Q.Y., Xie, S.X., Lu, D.Y., Yuan, W., Yu, X.R., Song, W.Z., Liu, S.J., Wang, J.Q., Yang, J.X., Li, Y.F., and Yang, S.C. 1986. On the physiologic specialization of stripe rust of wheat in China during 1975–1984. Acta Phytopathol. Sin. **16**(2): 79–85.

Wolf, M.S., and McDermott, J.M. 1994. Population genetics of plant pathogen interactions: the example of the *Erysiphe graminis* – *Hordeum vulgare* pathosystem. Annu. Rev. Phytopathol. **32**: 89–113.

Zhang, R., Dickinson, M.J., and Pryor, A. 1994. Double-stranded RNAs in the rust fungi. Annu. Rev. Phytopathol. **32**: 115–133.