# AFLP fingerprinting of Chinese epidemic strains of *Puccinia striiformis* f. sp. *tritici* *

ZHENG Wenming (郑文明)[1,4], LIU Feng (刘 峰)[2], KANG Zhensheng (康振生)[1], CHEN Shouyi (陈受宜)[2**], LI Zhenqi (李振岐)[1] and WU Liren (吴立人)[3]

1. Department of Plant Protection, Northwestern Agricultural University, Yangling 712100, China
2. Institute of Genetics, Chinese Academy of Sciences, Beijing 100101, China
3. Institute of Plant Protection, Chinese Academy of Agricultural Science, Beijing 100094, China
4. Bioengineering College, Henan Agricultural University, Zhengzhou 450002, China

Received December 15, 2000; revised January 8, 2001

**Abstract** Amplified fragment length polymorphism (AFLP) was used to fingerprint the epidemic strains CY25, CY27, CY28, CY29, CY30, CY31, Hy3, Hy7, Sy13 and a mutant strain WV-4 of *P. striiformis* f. sp. *tritici*, the pathogen of wheat stripe rust. The results showed that (i) genetic diversity existed in the pathogen populations, and based on it a dendrogram of these strains was constructed by unweighted pair-group mean average to demonstrate the relationships of the tested strains; (ii) no significant correlation between virulence of the pathogens and the polymorphism of DNA fingerprints was found; (iii) AFLP fingerprints showed higher polymorphism than that of the virulence variation; (iv) several new pathotypes identified might evolve independently of the reference strains identified before.

**Keywords:** ***Puccinia striiformis*, AFLP, DNA fingerprinting, cluster analysis, evolution relationship.**

Wheat stripe rust is one of the most destructive epidemic diseases of wheat in China. Although the degree of the disease has been controlled recently by the application of resistant varieties of wheat, the resistance breakdown of major varieties still has been endangering the wheat production in China. It has been realized that the virulence development is one of the important factors for losing the resistance and causing the disease. The appearance and increase of new races usually are the prelude of resistance breakdown of the dominant varieties[1,2]. However, the mechanism for the rust fungus to overcome the host resistance has not been defined yet. Both the evolutionary relationships among the races and the molecular mechanism of virulence variation have not been known clearly. The traditional virulence analysis for the races is very important, because it can provide the direct information about effect of host selectivity and efficiency of resistant genes, but the virulence markers from this method may represent only a small portion of the total genetic variation present in the population. In fact, the reference race of *P. striiformis* f. sp. *tritici* was identified according to its pathogenicity

* Project supported by the National Natural Science Foundation of China (Grant No. 39770486) and National '973' Project (Grant No. G200016201).

** Corresponding author, E-mail: sychen@igtp.ac.cn.

on host differentials at seedling stage, which is not a natural classification unit in biology and cannot reflect the consanguinity relationship among rust fungal strains. The former research results also indicated that the putative evolutionary relation among rust fungal races based on virulence characteristics might not truthfully reflect the mechanism of pathogen virulence and systematic evolution[3].

Amplified fragment length polymorphism (AFLP) is one of the most effective molecular markers so far[4,5], and has been widely used for gene localization, genetic mapping and genetic diversity analysis[6~8]. We attempted in this study to utilize AFLP to investigate the DNA polymorphism of Chinese epidemic races and 3 new pathotypes of *P. striiformis* f. sp. *tritici* showing a stable tendency to rise in the recent years, and compare the genetic diversity of the tested strains with their virulence, therefore to provide some information on epidemiology of *P. striiformis* f. sp. *tritici* and to make strategy for resistant varieties breeding and deployment.

## 1 Materials and methods

### 1.1 Strains

The origins and their virulence patterns of the tested *P. striiformis* f. sp. *tritici* on 17 differential wheat cultivars are listed in Table 1. Among them WV-4 is an ultraviolet mutant strain from wild strain CY29-1. All tested strains were from single-spore isolations. The methods of strain inoculation and identification were described in refs. [1,9]. All single-spore strains were reproduced in Mingxian169 wheat, which was susceptible to all known races of *P. striiformis* f. sp. *tritici*. Urediospores were collected from the infected seedlings. Pure urediospores were frozen in liquid nitrogen and stored at −70℃ for DNA extraction.

Table 1 Information on the *Puccinia striiformis* f. sp. *tritici* isolates

| Race or type[a)] | Number | Virulence on differentials[b)] | Source |
|---|---|---|---|
| CY25 | A | 1,2,3,4,5,6,7,8,9,11 | LPI[c)] |
| CY27 | B | 1,2,3,4,5,6,7,8,9,10,11 | LPI |
| CY28 | C | 1,2,3,4,5,6,7,8,9,11,16 | LCRD[d)] |
| CY29 | D | 1,2,3,4,5,6,7,8,9,11,12,16 | LPI |
| CY30 | E | 1,2,3,4,5,6,7,8,9,11,12,16,17 | LCRD |
| CY31 | F | 1,2,3,4,5,6,7,8,9,11,12,14,16,17 | LCRD |
| Hy3 | G | 1,2,3,4,5,6,7,8,9,11,12,13,14,16,17 | LCRD |
| Hy7 | H | 2,3,4,5,6,7,8,9,10,11,12,13,14,16,17 | LCRD |
| Sy13 | I | 1,2,3,4,6,7,8,9,10,11,14 | LCRD |
| WV-4 | J | 2,10,11 | LPI |

a) Designated by Laboratory of Cereal Rust Diseases, Institute of Plant Protection, Chinese Academy of Agricultural Science (CAAS).

b) Wheat differential cultivars: 1, Trigo eureka; 2, Fulhard; 3, Lutescens 128; 4, Metana; 5, Virglilio; 6, Abbondanza; 7, Early Preminum; 8, Funo; 9, Denmark No.1; 10, Jubilejina 2; 11, Fengchan 3; 12, Lovrin 13; 13, Kangyin 655; 14, Shuiyuan 11; 15, Zhong 4; 16, Lovrin 10; 17, Hybrid 46.

c) Laboratory of Plant Immunology, Northwestern Agricultural University.

d) Laboratory of Cereal Rust Disease, CAAS.

### 1.2 AFLP analysis

Genomic DNA was extracted from urediospores as described by Shan et al[10]. AFLP was

conducted according to the experimental protocol described in ref. [5]. After genomic DNA (100 ng) was digested with restriction endonuclease *Eco*R I and *Mse* I, digested fragments were ligated to *Eco*R I and *Mse* I adapters. The ligation products got in 1∶1 dilution and 5 μL diluted ligation products were used for preamplification. The preamplification primers had a selective base (A for *Eco*R I, C for *Mse* I). PCR was conducted for total 20 cycles with a protocol of 94℃ 30 s, 56℃ 60 s, 72℃ 60 s. The preamplification products were diluted and were used as the templates for selective AFLP amplification. 5′- and 3′- end primers had 3 selective bases respectively. Different primer combinations were used for the amplification (*Eco*R I + ACA/*Mse* I + CAA, *Eco*R I + ACG/*Mse* I + CAA and *Eco*R I + ACT/*Mse* I + CTG). The *Eco*R I primer was end-labeled with $^{33}$P-ATP.

PCR was performed as follows: first cycle included 94℃ 30 s, 65℃ 30 s, 72℃ 60 s, followed by annealing temperature descending 0.7℃ per cycle for 13 cycles down to 56℃, then another 23 cycles of amplification were performed under the conditions of 94℃ 30 s, 56℃ 30 s, 72℃ 60 s.

PCR products were resolved by polyacrylamide gel electrophoresis. The gel was then transferred to a filter paper, and exposed at −70℃ to an X-ray film. Band patterns were recorded manually.

### 1.3 Data analysis

The virulent (susceptible, S) and avirulent (resistant, R) strains were scored as 1 and 0 respectively. All AFLP bands that appeared of 20% ~ 90% frequencies in tested strains were considered as polymorphic bands[11], and the presence and absence of each polymorphic band were scored as 1 and 0, respectively. The cluster analysis was performed by unweighted pair-group mean average (UPGMA) method using computer software NTSYS as a statistical tool. The genetic similarity of tested strains was expressed as Nei's genetic distance[12]. The evolutionary dendrogram (unrooted tree) was constructed by DOLLOP method of PHYLIP software package[1].

## 2 Results

### 2.1 Genetic diversity and similarity of the epidemic races

The distinct AFLP fingerprints were resolved for each of 3 primer combinations of *Eco*R I + ACA/*Mse* I + CAA, *Eco*R I + ACG/*Mse* I + CAA and *Eco*R I + ACT/*Mse* I + CTG, and showed high polymorphism (Fig. 1). The result indicated that the tested epidemic races were rich in genetic variation at DNA level. A total of 135 bands were generated for 3 primer combinations, among which 112 bands were polymorphic that amounted to 76.19% of all bands. Each band was considered as a genetic locus for statistical analysis. The dendrogram of cluster analysis of the tested strains was constructed (Figure 2).

The cluster analysis showed that according to the genetic similarity based on AFLP data tested strains could be divided into two groups: one included CY28, CY30 and Sy13; another included CY25, CY27, CY29, CY31, Hy3, Hy7 and WV-4. The biggest values of genetic distance between the groups were 0.61, and between individuals within a group 0.51. Three new pathotypes Hy3, Hy7

1) Felsenstein, J. Phylogeny inference package, version 3.57, Department of Genetics, University of Washington.

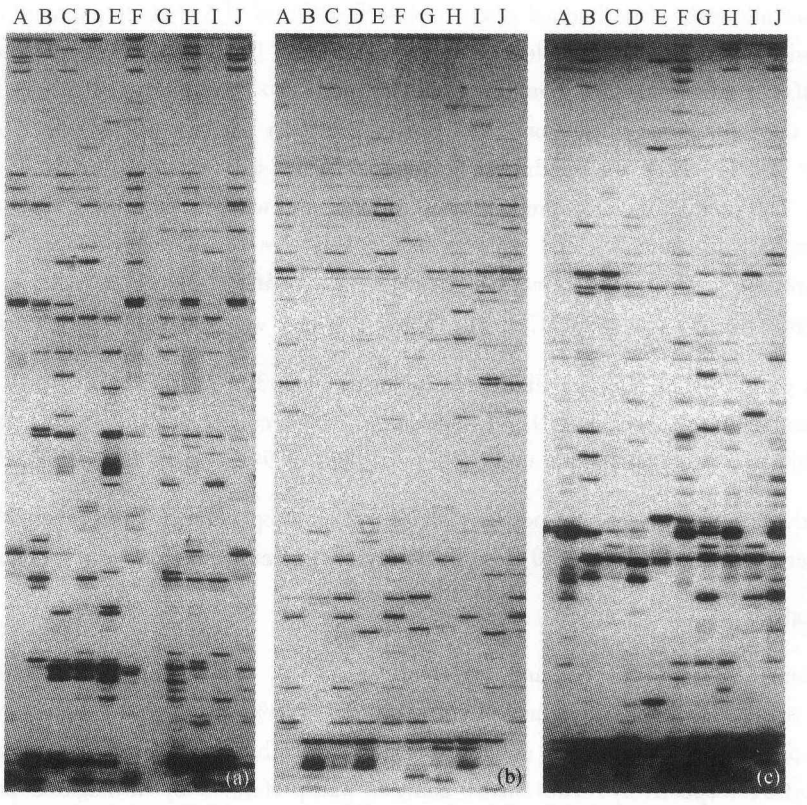


Fig. 1 AFLP profiles of tested stains. (a) *Eco*R I + ACA/*Mse* I + CAA primer combination, (b) *Eco*R I + ACG/*Mse* I + CAA primer combination, (c) *Eco*R I + ACT/*Mse* I + CTG primer combination.

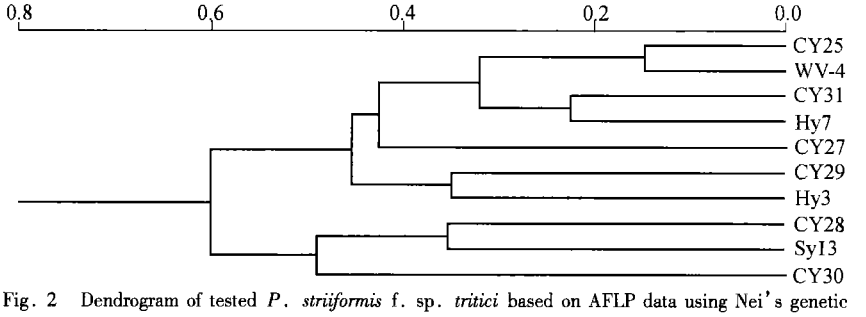


Fig. 2 Dendrogram of tested *P. striiformis* f. sp. *tritici* based on AFLP data using Nei's genetic distance and the UPGMA cluster method.

and Sy13 were the nearest to CY29, CY31 and CY28, with the genetic distances of 0.35, 0.23 and 0.36, respectively. They were closer to each other in origin. WV-4 isolate was most similar to CY25 on genetic background, whereas CY29-1, the wild type strain of mutant strain WV-4, and CY29 were different isolates but had the same virulence characteristics. This result suggested that the isolates that had the same virulence characteristics may probably have different genetic backgrounds.

## 2.2 Comparison of AFLP results with virulence characteristics

According to the virulence of the tested strains on 17 differentials, the dendrogram of epidemic strains of *P. striiformis* f. sp. *tritici* was constructed by UPGMA cluster analysis (Figure 3).

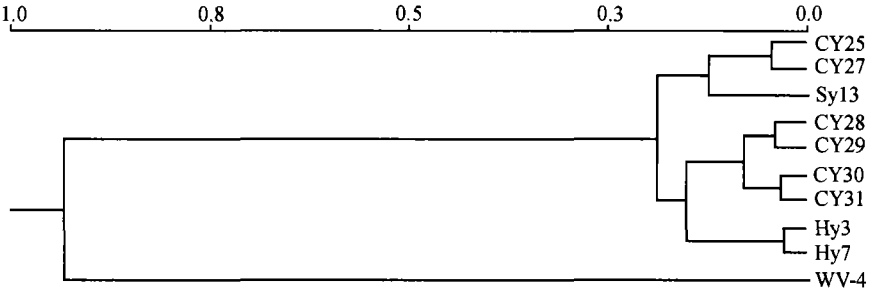


Fig. 3 Dendrogram of tested *P. striiformis* f. sp. *tritici* based on virulence data using Nei's genetic distance and UPGMA clustering.

By comparing the cluster result of virulence with AFLP data, it was found that the DNA polymorphism of the tested strains was not correlated with their virulence characteristic, and putative genetic relations among strains based on them were different. According to their virulence, CY28 was very close to CY29 in genetic distance, and CY30 to CY31, but their genetic relations based on AFLP analysis were distant owing to the different groups. Strain WV-4 was very distinct from the other strains on the virulence, but AFLP analysis showed that there was no great difference in the genetic backgrounds. Hence the deduction of evolutionary relation of strains is irrelevant to two different kinds of analysis. Furthermore, the genetic differences among the strains revealed by AFLP were obviously larger than those revealed by the virulence. Except for the mutant strain WV-4, the largest genetic distance based on the virulence between the tested strains was less than 0.3; however, the one based on AFLP reached more than 0.6. This result indicated that the genetic variations of the tested strains revealed by DNA polymorphism was larger than that revealed by traditional virulence analysis. Therefore, AFLP is more suitable to the genetic diversity analysis of *P. striiformis* f.sp. *tritici*.

### 2.3 Evolutionary relationship among the strains

According to AFLP data, the evolutionary relationship among the tested strains was established by DOLLOP tree building method, and is shown in Figure 4.

The strains at terminal nodes originate from some unknown strains at internal nodes. Their original progenity is unknown. Peripheral branches and interior branches reflect their evolutionary process. It was found from the molecular phylogenetic tree that new pathotypes Hy3 and Hy7 had the same interior nodes as CY29 and CY31 respectively; thus they are most likely evolved from those strains with the same origin.

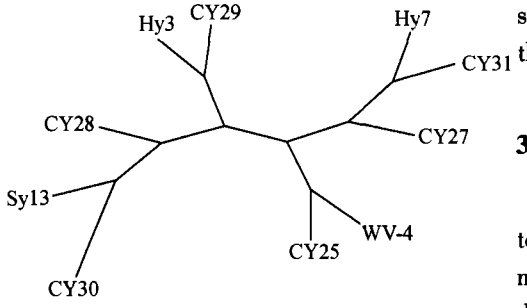


Fig. 4 Systematic evolution unrooted tree generated by DOLLOP.

## 3 Discussion

AFLP is a new molecular marker based on PCR technique which needs only a little of DNA materials[4] and is very suitable for biotrophs that are difficult to culture such as wheat rust fungus. It can supply more information and has great potential to the genetic analysis of obligate pathogens.

New dominant races are usually able to overcome the resistance of dominant varieties of wheat and make the disease epidemic. Now it is still unknown whether this "overcoming" reflects the generation of new virulence forms by the genetic changes, or the rise of frequency of original pathotypes, or a combination of both the phenomena. However, it is obvious that the populations with complicated genetic background are prone to overcome the host resistance. Hence an understanding of genetic background will be not only helpful to reducing the blindness of rust resistance breeding and increasing the rationality of resistant varieties deployment but also to maintaining the resistance of varieties. CY28, CY29, CY30 and CY31 have main dominant races since the 1990s, while Hy3, Hy7 and Sy13 are new pathotypes, and they have prevailed continually since 1996[13]. AFLP fingerprinting of these strains and clarifying of their genetic relations may give some useful clues to the mechanism of virulence variation.

The accumulation of genetic variations in a population is beneficial to the survival and evolution of living organisms[14]. Wheat stripe rust fungus is a kind of incomplete fungus; its sexual stage has not been found. Gene exchange among its individuals would rarely occur, and individuals would seclude each other during reproduction. These characteristics apparently impede the generation of genetic variations. However, its population actually shows high genetic polymorphism in population analysis[11,15]. These variations would derive from heterokaryonsis, parasexuality or mutation[16]. It was even presumed that the sexual reproduction did exist[11,16]. Our research suggests that both virulence and DNA polymorphism of the epidemic strains of *P. striiformis* f. sp. *tritici* are of diversity, especially the latter; but these two are not correlated significantly, and putative genetic relations based on them are also not correlated. The virulence is often affected by some factors like the selection of host differentials, whereas the molecular characters are generally neutral and would reflect its genetic background truthfully. According to AFLP analysis results, we proposed preliminarily the evolutionary relations among the tested strains and constructed a systematic unrooted tree. It is noted that Hy3 and Hy7 have very close genetic relations with CY29 and CY31 respectively, which obviously does not coincide with their virulence characteristics. This perhaps implicates that the so-called new pathotypes would evolve independently of the reference races identified before.

**Acknowledgements** We are grateful to Zhang Jinsong, Zheng Xianwu, Ouyang Jian, Wei Guorong and all colleagues of 803 group in the Institute of Genetics, the Chinese Academy of Sciences, for their support.

## References

1 Li, Z. Q. et al. Wheat Rusts and Their Control. Shanghai: Shanghai Scientech Press, 1989.
2 Li, Z. Q. et al. The cause of resistance losing of wheat varieties to stripe rust in China and its solution. Scientia Agricultura Sinica, 1980(3): 72.
3 Shan, W. S. et al. DNA fingerprinting of reference strains of *Puccinia striiformis* f. sp. *trtici* in China. Chinese Science Bulletin, 1996(15): 1427.
4 Zebeau, M. et al. Selective restriction fragment amplification: a general method for DNA fingerprinting. European patent application number: 92402629.7. Publication number: 0534858 A1.
5 Vos, P. et al. AFLP: a new technique for DNA fingerprinting. Nucleic Acids Research, 1995, 23: 4407.
6 Russell J R, et al. Direct comparison of level of genetic variation among barley accession detected by RFLPs, AFLPs, SSRs and RAPDs. Theor. Appl. Genet., 1997, 95: 714.
7 Cato S. A. et al. Evaluation of AFLP for genetic mapping in *Pinus radiata* D. Don. Molecular Breeding, 1999, 5: 275.
8 Angiolillo A. et al. Olive genetic diversity assessed using amplified fragment length polymorphisms. Theor. Appl. Genet.,

1999, (98): 411.

9 Yang, H. A. et al. Analysis of pathogenicity and virulence factors of Chinese races of *Puccinia striiformis tritici*. Acta Phytopathologica Sinica, 1990, 20(3): 213.

10 Shan, W. X. et al. Identification of a moderate repeated DNA family of *Puccinia striiformis* f. sp. *tritici*. Science in China (C) (in Chinese), 1997, 27(3): 241.

11 Shan, W. X. et al. Genetic diversity in *Puccinia striiformis* Westend. f. sp. *tritici* revealed by pathogen genome-specific repetitive sequence. Can. J. Bot., 1998, (76): 587.

12 Nei, M. et al. Mathematical models for studying genetic variation in terms of restriction endonucleases. Proc. Natl. Acad. Sci. USA, 1979, (76): 5369.

13 Wan, A. M. et al., Physiologic specialization of stripe rust of wheat in China during 1991 ~ 1996. Acta Phytopathologica Sinica, 1999, 29(1): 15.

14 Zhang, Y. Biology Evolution (in Chinese). Beijing: Peking University Press, 1998.

15 Ingram, D. S. et al. The Advances in Plant Pathology, Vol. 6. New York: Academic Press, 1988.

16 Kang, Z. S. et al. Studies on nucleic situation of urediospore of *Puccinia striiformis* f. sp. *tritici*. Acta Phytopathologica Sinica, 1994, 24(1): 26.