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Full Length Research Paper

Low genetic diversity of *Phytophthora infestans* population in potato in north China

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Late blight, caused by *Phytophthora infestans* is the most important disease of potato (*Solanum tuberosum*). This study reveals the genetic diversity of *P. infestans* population in north China. A total of 134 strains of *P. infestans* were isolated from different agricultural fields in Hebei, Liaoning, Jinlin and Heilongjiang Provinces in north China. The genetic variation among these strains were analyzed using 15 'simple-sequence repeat' (SSR) markers. The results show that forty different SSR genotypes and an average of 3.8 (range 2 to 9) alleles per locus were found. Low genetic diversity (Shannon's diversity index = 0.26) was found among these 134 strains from four provinces, revealing the presence of clonal populations of the pathogen in this region. The average heterozygosity was 0.162, indicating the low level of genetic variations of *P. infestans* populations. There was no correlation between population genetic diversity of *P. infestans* and geographical origin. These results provided a foundation for making integrated control measures in the future.

Key words: *Phytophthora infestans*, population genetics, simple-sequence repeat (SSR), potato late blight.

INTRODUCTION

Phytophthora infestans (Mont.) de Bary, causing the most important disease of potato (late blight), has been widely studied for its severe economic impact on agriculture. Since 1990, the annual economic losses caused by the disease worldwide have been about 170 billion US dollars (Haverkort et al., 2008) and about 40 billion US dollars in China (Jiang et al., 2012). Characteristic of the pathogen is strong adaptability and fast physiological variation. Especially, the A2 mating type, a novel mating type, allowed *P. infestans* to reproduce sexually and increase the adaptability of the organism (Brurberg et al., 2011). The A2 mating type, which appears in many potato production areas of the world, and some new physiological races of *P. infestans* with stronger pathogenicity to potato have been reported in many countries, resulting in more difficult control of potato late blight (Fry, 2008). It suggested that in comparison with asexual reproduction, sexual reproduction of *P. infestans,* is more likely to play important roles in increasing the physiological variations and adaptability, as well as resulting to the emergence of more virulent and pesticide resistant races in the near future (Hermansen et al., 2000; Flier et al., 2007; Widmark et al., 2007; Guo, 2009). Therefore, many researchers are concern with the compositions and changes in population genetic structure of *P. infestans*, especially the relationship between genotypes of *P. infestans* and their occurrences as well as prevalence of the disease, and the report of Fry and Goodwin (1997), Mahuku et al. (2000) and Day et al. (2004) have shown changes of *P. infestans* genotypes which could cause an epidemic outbreak of potato late blight.

Recent genetic structure analyses of various populations of *P. infestans* has been performed using molecular markers such as isoenzyme, mtDNA, random amplified polymorphic DNA (RAPD), restriction fragement length polymorphism (RFLP), amplified fragment length polymorphism (AFLP) and SSR (Cooke and Lees, 2004; Cárdenas et al., 2011; Brurberg et al., 2011). Among them, SSR (simple sequence repeat) markers which are ideal single-locus co-dominant markers characterized by a high degree of length polymorphism, which have high variability and dense distribution throughout the genome

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could improve comparison between *P.* infestans in different laboratories and countries, and their potential for monitoring the genetic variations of *P. infestans* was recently demonstrated worldwide (Knapova and Gisi, 2002; Lees et al., 2006; Cárdenas et al., 2011). Yang et al. (2008) using two SSR loci (Pi4B and Pi4G) analyzed genotypic diversity of *P. infestans* in Yunnan Provence of south China, and found that the genetic diversity of *P. infestans* populations in middle multiple cropping zones is high and significantly different from that in south winter planting zones. Genetic diversity of *P. infestans*, in north of China (Hebei and Heilongjiang province), was significantly different from that in Yunnan and Fujian in Southern China (Yao et al., 2009; Li et al., 2009). The results of Gou et al. (2009) showed that all isolates from Inner Mongolia in northern China had the same SSR genotype. However, the genetic diversity of *P. infestans* from Liaoning and Jilin provinces was not investigated by the earlier researchers. And little work that used large set of markers to identify multilocus genotypes and elucidate the genetic diversity of *P. infestans* in north China has been done. In this experiment, 15 loci were used to investigate the genetic diversity of 134 *P. infestans* strains isolated from all important potato growing regions in north China. This result reveals the genetic variation of *P. infestans* in the north of China and provides a foundation for making integrated pest control measures.

MATERIALS AND METHODS

Isolation of *P. infestans* strains

The 134 isolates used for population analysis were obtained from Hebei (44), Liaoning (15), Jilin (20) and Heilongjiang (55) provinces of north China from commercial fields of the major potato growing areas, during the summers of 2008 and 2009. For the samples, a single diseased potato leaflet was collected from each disease outbreak (more than three replicates per site) and placed in a paper bag, recording time and site of collection. The mycelia on the diseased spots of potato leaves from the fields were directly picked off with a sterilized toothpick on the rye sucrose agar (RSA) medium with antibiotics added (ampicillin 100 µg/ml, nystatin 100 µg/ml and rifampicin 50 µg/ml) and incubated at 18 to 22°C. After growth (about 5 to 7 days), the white mycelia of *P. infestans* were transferred to fresh RSA medium (without antibiotics) with a sterilized toothpick, and repeated several times to get the purified strains. The purified strains of *P. infestans* were stored on the RSA medium agar under 8 to 10°C and were transferred every 4 to 6 weeks.

DNA extraction and SSR analysis

DNA was extracted from mycelium using the method of CTAB (Goodwin et al., 1992). The 134 *P. infestans* DNA were amplified using polymerase chain reaction (PCR) with 15 primer pairs, including Pi4B, Pi4G, Pi02, Pi04, Pi16, Pi26, Pi33, Pi56, Pi63, Pi65, Pi66, Pi70, Pi89, G11 and D13 (Lees et al., 2006; Knapova and Gisi, 2002). The primers were obtained from Saibaisheng Biotechnological Company in Beijing. Microsatellite PCR amplifications were performed in 25 µl containing 50 ng genomic DNA, 2.5 µl tenfold reaction buffer, 0.28 µl dNTP (10 mmol/L), 1.25 µl each of forward and reverse primers (10 pmol/µl), 0.14 µl Taq DNA polymerase (5 U/µl) (dNTP, buffer and Taq DNA polymerase purchased from Takara company), and reaction tubes filled with deionized water. The PCR program was programmed as follows: denaturing at 95°C for 5 min, 40 s at 94°C, 40 s at 58°C and 20 s at 72°C, repeated for 33 cycles and then held for 10 min at 72°C.

SSR genotype and data analysis

SSR multilocus genotype classification and denomination of *P. infestans* strains were based on the banding pattern similarity at the locus of the fifteen markers Pi4B, Pi4G, Pi02, Pi04, Pi16, Pi26, Pi33, Pi56, Pi63, Pi65, Pi66, Pi70, Pi89, G11 and D13 from 134 isolates from north China, according to that described by Knapova and Gisi (2002) and Cárdenas et al. (2011), as indicated by genotype-I, genotype-II, genotype-III and so on.

The population genetic diversities of isolated strains were analyzed by using NTSYSpc version 2.1 software, UPGMA (unweighted pair-group method with arithmetic averages), and the dendrogram was constructed. POPGENE software was used to calculate the percentage of polymorphic loci (PPL), average number of alleles (A), effective number of alleles (AE), average heterozygosity (H) and Shannon's diversity index (I) of 15 microsatellite loci, as well as genetic distance and genetic similarity between populations (Yeh et al., 2000).

RESULTS

All the 15 SSR loci tested were polymorphic for the 134 *P. infestans* strains. A total of 57 alleles were detected at 15 SSR loci in these strains with a mean of 3.8 and ranging from 2 to 9 alleles per locus (Table 1). For locus Pi4B, the most frequent alleles were 206 and 218 bp. Allele 218 bp was considerably less frequent in Jilin than in the other three provinces (Table 1). While the allele 206 bp of locus Pi4B was noticeably more frequent in Jilin and Heilongjiang than in Hebei and Liaoning (Table 1). The most frequent alleles of loci Pi02 and Pi33 were much more common in Heilongjiang than that in other provinces. The average gene diversity of any single locus over all 4 provinces ranged from 0.04 to 0.53 (Table 1), and the average diversity for all loci was 0.26. The gene diversity for locus Pi33 was the highest (0.53) and locus Pi70 was the lowest (0.04). Forty different SSR genotypes were obtained from 134 strains of *P. infestans*, and genotype-III was dominant, accounting for 37%. The number of genotypes in Hebei, Heilongjiang, Jilin, and Liaoning was 18, 18, 8 and 6, respectively.

The polymorphic loci of *P. infestans* strains in Hebei Province was 50, accounting for 68.49%, followed by Jilin with 40 polymorphic loci, accounting for 60.27%. In 2009, polymorphic loci of 134 *P. infestans* strains in 4 provinces was 58, accounting for 79.45%, and significantly higher than that in 2008 (48) (Table 2).

The highest number of alleles was in the *P. infestans* strains from Hebei Province (1.685), while the highest effective number of alleles was in the Jilin Province (1.318). The result shows that in Jilin, the number of alleles which played a role in *P. infestans* populations

Table 1. Allele frequencies for SSR markers in 134 *P. infestans* strains from the four provinces collected in 2008 and 2009.

SSR locus	Allele	Hebei	Liaoning	Jilin	Heilongjiang	2008	2009	Overall	Gene diversity
G11	156	0.02	0.00	0.13	0.03	0.01	0.05	0.04	0.26
	160	0.79	1.00	0.50	0.77	0.83	0.70	0.73	
	162	0.02	0.00	0.13	0.00	0.01	0.03	0.02	
	164	0.00	0.00	0.00	0.03	0.00	0.02	0.01	
	172	0.63	0.74	0.68	0.77	0.83	0.67	0.70	
	174	0.00	0.00	0.00	0.03	0.00	0.02	0.01	
	188	0.00	0.00	0.00	0.03	0.00	0.02	0.01	
	190	0.63	0.74	0.68	0.77	0.83	0.67	0.70	
	192	0.00	0.00	0.00	0.03	0.00	0.02	0.01	
Pi4B	206	0.70	0.74	1.00	1.00	0.83	0.80	0.81	0.22
	214	0.02	0.03	0.00	0.02	0.05	0.01	0.02	
	218	0.79	0.74	0.55	0.73	0.71	0.72	0.71	
	226	0.01	0.03	0.00	0.00	0.00	0.01	0.01	
	208	0.00	0.00	0.11	0.00	0.00	0.02	0.02	
	220	0.00	0.00	0.00	0.02	0.00	0.01	0.01	
Pi02	152	0.01	0.03	0.00	0.00	0.03	0.00	0.01	0.31
	162	0.52	0.63	0.61	1.00	0.62	0.68	0.67	
	164	0.07	0.03	0.05	0.00	0.01	0.04	0.03	
	166	0.05	0.03	0.03	0.00	0.06	0.01	0.02	
	180	0.52	0.63	0.41	1.00	0.62	0.63	0.62	
	182	0.12	0.07	0.19	0.00	0.08	0.07	0.07	
Pi4G	159	0.00	0.00	0.00	0.02	0.03	0.00	0.01	0.34
	161	0.48	0.48	0.61	0.43	0.38	0.51	0.47	
	163	0.10	0.11	0.19	0.18	0.16	0.14	0.14	
	177	0.07	0.03	0.00	0.06	0.00	0.07	0.03	
D13	108	0.79	1.00	0.50	1.00	0.83	0.76	0.77	0.23
	118	0.05	0.00	0.03	0.00	0.03	0.02	0.02	
	124	0.00	0.00	0.11	0.04	0.00	0.04	0.03	
	136	0.04	0.00	0.13	0.00	0.01	0.04	0.03	
Pi16	174	0.16	0.14	0.23	0.30	0.21	0.23	0.22	0.35
	176	0.70	0.74	0.68	0.77	0.83	0.70	0.73	
	178	1.00	1.00	1.00	1.00	1.00	1.00	1.00	
	182	1.00	1.00	1.00	0.87	1.00	0.90	0.91	
Pi26	179	0.00	0.00	0.11	0.00	0.00	0.02	0.02	0.31
	181	0.08	0.11	0.13	0.25	0.16	0.16	0.16	
	183	0.63	0.63	0.50	0.34	0.51	0.46	0.47	
	185	0.10	0.03	0.19	0.03	0.03	0.09	0.07	

Table 1. Continued.

	166	0.40	0.37	0.26	0.31	0.31	0.34	0.34	
Pi04	168	0.20	0.23	0.31	0.27	0.27	0.25	0.25	0.40
	170	1.00	1.00	1.00	1.00	1.00	1.00	1.00	
	203	0.03	0.03	0.13	0.03	0.05	0.05	0.05	
Pi33	206	0.74	0.74	0.50	0.77	0.70	0.70	0.70	0.53
	209	0.52	0.63	0.61	1.00	0.62	0.68	0.67	
	178	0.01	0.03	0.11	0.00	0.03	0.02	0.02	
Pi56	180	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.12
	190	0.00	0.00	0.05	0.00	0.00	0.01	0.01	
	148	0.01	0.03	0.00	0.00	0.03	0.00	0.01	
Pi63	151	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.15
	157	1.00	1.00	1.00	1.00	1.00	1.00	1.00	
Pi65	147	1.00	1.00	1.00	1.00	1.00	1.00	0.91	0.07
	151	0.01	0.00	0.13	0.02	0.00	0.04	0.02	
Pi66	153	0.85	1.00	1.00	1.00	0.83	1.00	0.91	0.20
	155	0.01	0.03	0.11	0.00	0.03	0.02	0.02	
Pi70	192	0.00	0.00	0.00	0.01	0.00	0.01	0.00	0.04
	195	1.00	1.00	1.00	1.00	1.00	1.00	1.00	
Pi89	130	0.52	0.63	0.55	0.60	0.58	0.56	0.57	0.46
	191	0.85	1.00	1.00	1.00	0.83	1.00	0.91	

Table 2. Analysis of genetic variation among 134 strains of *P. infestans* in potato.

Population	Polymorphic loci	PPL/%	A	AE	H	I
Hebei	50	68.49	1.685	1.263	0.158	0.247
Liaoning	40	54.79	1.548	1.212	0.132	0.209
Jilin	44	60.27	1.603	1.318	0.189	0.289
Heilongjiang	41	56.16	1.562	1.189	0.119	0.191
2008	48	65.75	1.657	1.244	0.152	0.241
2009	58	79.45	1.795	1.263	0.160	0.252
Total	65	89.04	1.890	1.264	0.162	0.260

PPL: The percentage of polymorphic loci; A: Average number of alleles; AE: effective number of alleles; H: Average heterozygosity; I: Shannon's diversity index.

was greater than that in Hebei Province. The average number of alleles for 4 provinces in 2009 was higher than that in 2008, but there were no significant differences in effective number of alleles between 2008 and 2009 (Table 2).

The levels of genetic variation (H) in the 4 provinces which ranked from highest to lowest was in the following order: Jilin (0.189), Hebei (0.158), Liaoning (0.132) and Heilongjiang (0.119). Additionally, 'H' was higher in 2009 (0.160) than in 2008 (0.152). The total average

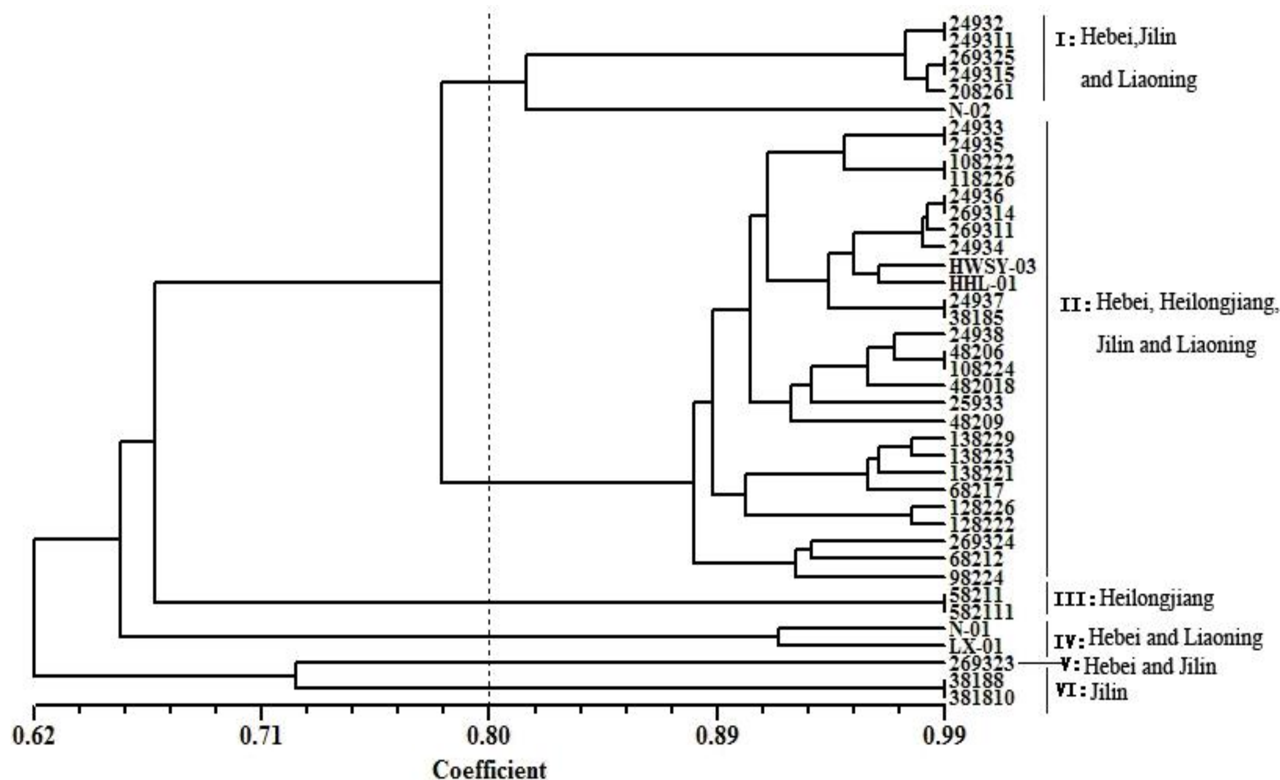

Figure 1. Dendrogram of 134 strains of *P. infestans* constructed using UPGMA method (some strains were omitted due to the genotype repeated).

heterozygosity was 0.162, indicating the low level of genetic variations of *P. infestans* populations in 4 provinces (Table 2). In addition, the order of average Shannon index was Jilin, Hebei, Liaoning and Heilongjiang Province. The Shannon index in 2009 was higher than that in 2008 (Table 2).

The 134 *P. infestans* strains from 4 provinces were clustered into six major clusters using UPGMA method (Figure 1). Cluster I contained 7 strains of Hebei, 1 strain of Jilin and 1 strain of Liaoning; cluster II contained Jilin (14 strains), Liaoning (13 strains), Hebei (34 strains) and Heilongjiang (52 strains); there were 3 strains from Heilongjiang Province that belonged to cluster III; while cluster IV contained only two strains, and 1 strain was from Hebei and 1 from Liaoning; cluster V consisted of 2 strains of Hebei and 1 strain of Jilin; cluster VI had only 2 strains from Jilin. These results indicated that there was no correlation between strains of P. infestans and geographical origins in the four provinces of northern China. Moreover, the 269323, 38188 and 381810 strains isolated from Hebei and Jilin, apparently separated out from other strains, at 0.62 (similarity coefficient) (Figure 1), indicating that the genetic distances of these three isolates were far from the others.

The software of POPGENE32 was used to calculate genetic distance and genetic similarity among populations of P. infestans in the 4 provinces. The genetic distance ranged from 0.005 to 0.028, and the minimum genetic distance was 0.005 between Hebei and Liaoning, and the maximum genetic distance was 0.028 between Heilongjiang and Jilin. The results show very low differentiation among these 4 provinces (Table 3).

DISCUSSION

Brurberg et al. (2011) detected 49 alleles and identified 169 multilocus genotypes based on nine loci among 191 isolates from Denmark, Finland, Norway and Sweden. In our experiments, a total of 57 alleles were detected and 40 multilocus genotypes were identified using 15 SSR markers, including nine SSR markers which were used in the study of Brurberg et al. (2011) for 134 *P. infestans* strains from 4 provinces in north China. The results of Lees et al. (2006) showed that increasing the number of markers generally increases the number of genotypes. However, our result shows that the number of genotypes was 40 (using 15 SSR markers) in north China which was significantly smaller than 169 genotypes (using 9 SSR markers) in Northern Europe (Brurberg et al., 2011). This result indicated low levels of genotypic diversity in north China.

Li et al. (2009a) identified 72 alleles, based on 14 SSR markers in 60 *P. infestans* isolates from some areas in

Table 3. Genetic distance and population differentiation for *P. infestans* populations from Hebei, Liaoning, Jilin and Heilongjiang.

Population	Hebei	Jilin	Heilongjiang	Liaoning
Hebei	****	0.985	0.981	0.995[a]
Jilin	0.015	****	0.973	0.980
Heilongjiang	0.019	0.028	****	0.988
Liaoning	0.005[b]	0.020	0.012	****

[a] The higher the number above diagonal (marked by asterisks), the higher the genetic similarity. [b] The lower the number below diagonal, the smaller the genetic differences.

China, but did not identify SSR genotype. The diversity of alleles in the study of Li et al. (2009a) was significantly higher than that of our results because the study areas include some part of south China in which the genetic diversity is significantly higher than that in north China (Zhao et al., 2008; Li et al., 2009b). Of the 15 markers used in the experiment, G11 produced most of the nine bands. The range of allele sizes obtained in this study was similar to those reported by Lees et al. (2006) and Brurberg et al. (2011), confirming the validated SSR protocols for improved comparison between *P. infestans* isolates in different laboratories.

Among the four provinces, the number of genotypes in Hebei and Heilongjiang were larger than that in Jilin and Liaoning. This may be due to the fact that potato garden were larger and there was higher migration rate of seed potatoes among these areas (Goodwin and Drenth, 1997). Yao et al. (2009) used two loci (Pi4B and Pi4G) to analyze genetic diversity of *P. infestans*, the results showed that genetic diversity of isolates from Hebei province was mostly the same as that from Heilongjiang province. These results are entirely consistent with the findings in this study. Li et al. (2009) showed that genetic diversity of from Hebei Province was similar that that of *P. infestans* from Heilongjiang Province.

No particular genotypes dominated across the 4 provinces, but genotype-III dominated over other genotypes. This may be due to the fact that *P. infestans* population in northern China represents a clonal lineage. The results of Guo et al. (2009) suggest that all isolates from northern China had the same SSR genotype using two SSR markers to identify them. On the other hand, Brurberg et al. (2011) advanced the hypothesis that sexual reproduction contribute substantially to the genetic variations of *P. infestans*. However, the proportion of the A2 mating type of *P. infestans* in north China was very low. Zhang et al. (1996) first discovered 3 isolates of A2 mating type only in Inner Mongolia and Shanxi Provence of China which then decline with the occurrence of the A2 mating type (1.7%) in China (Li et al., 2009). So, there was a very low chance of sexual reproduction in the natural state, thus resulting in a low level of genetic diversity of *P. infestans* (Yang et al., 2004). These results agree with the data reported by Gotoh et al. (2005). In addition, our data showed that genetic diversity of *P. infestans* in 2009 was higher than that in 2008. This result may be associated with the scope and time of sampling.

In the potato growing area of north China, we found only small, insignificant differences in the genetic diversity of *P. infestans* among four provinces (Table 2). The small differences in genetic diversity found in different potato growing provinces may be due to gene recombination and mutation after the colonization by the pathogen in various production areas (Goodwin et al., 1994). Additionally, cluster analysis showed that there was no relationship between population genetic diversity of *P. infestans* and geographical origin. This result which is similar to that of a study in Canada (Mahuku et al., 2000), is probably due to geographical environment and climatic conditions of the potato producing areas in four provinces in northern China, which are similar to those found in Canada (Yang et al., 2008; Li et al., 2009).

In general, in order to better understand the occurrence of potato late blight and further predict the development of the disease, pest control personnel need to accurately and timely take hold of the population genetic structure and variation of *P. infestans* in China. This provided a scientific basis for developing effective control measures against late blight.

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