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EST-SSR based diversity analysis of *Phytophthora infestans* in different isolates collected from Gansu Province, China

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Abstract

Effective molecular markers are required to characterize the genetic diversity of plant pathogens. In this study, we used the software SSRIT searched 12,581 *Phytophthora infestans* ESTs in EST databases. Among these ESTs, there were 388 sequences of containing-SSR, in which 123 trinucleotide repeats are the most abundant type accounting for 31.7% of the total identified. Eleven pairs of primers were designed and synthesized, and genomic DNA of *Phytophthora infestans* was extracted, with 63 isolates of *Phytophthora infestans* used for polymerase chain reaction amplification, nine pairs of primers (81.8%) amplified polymorphic SSR bands. The 63 isolates of *Phytophthora infestans* were divided into 20 genotypes by cluster analysis. The results suggest that using EST-derived SSRs the *Phytophthora infestans* in Gansu are of rich genetic diversity.

Keywords: late blight, genetic diversity, genomes, molecular markers.
Abbreviations: EST, expressed sequence tags; SSR, Simple sequence repeats.

Introduction

Late blight caused by *Phytophthora infestans* (Mont.) de Bary is a destructive foliar disease in potato (*Solanum tuberosum* L.) crops worldwide. In severe cases, the disease can cause total loss of an entire crop field. In the 1840's, the disease was famous for the destruction of the potato crops in some European countries, resulting in famine and death of over a million people (Agrios, 2005). Today, the disease occurs wherever the host crops are grown and epidemics often occur under favorable, conducive environments. In recent years, for example, the blight disease causes severe damage to potato crops in Gansu province of northwestern China (Cheng, 2011), largely because the growing areas of potato crops have been expanded to the areas with cool spring with the climate in favor of the disease infection. *Phytophthora infestans* is heterothallic oomycete which produces asexual spores called sporangia. While it also produces sexual spores or oospores when both A1 and A2 mating types are present and paired (Jiang, 2006; Li, 1997). Although both A1 and A2 mating types must infect the same plant or tuber in order to produce oospores, the control of *P.infestans* is still difficult because of an increased genetic diversity and appearance of A2 mating type of the disease (Fry and Goodwin, 1997; Wang, 2010). An effective management and accurate forecast of the *P. infestans* outbreak depend on several factors, including a) population genetics and diversity, b) response of the pathogen to environmental conditions, and c) host resistance and fungicide sensitivity, among others. Therefore, it is important to explore the level of genetic diversity of *P. infestans* using high sensitive molecular markers and to establish the possible linkages to phenotypic diversity of *P. infestans* which determined using bio-assay (Tóth, 2000).

Simple sequence repeats (SSR) are powerful markers for the characterization and evaluation of genetic diversity. Also, the markers are used for the evolution of the phylogenetic relationship within and between species and populations (Ayers, 1997; May, 1997; Feng, 2002; Han, 2004; Barve, 2001). SSR markers are tandemly repeated motifs containing 1 to 6 nucleotide bases found in the nucleic genomes of all eukaryotic organisms and are often abundant and evenly dispersed (Tautz, 1984). They are highly polymorphic, multiallelic and codominant. Studies have shown that SSR markers are more efficient in detecting genetic diversity than RFLP and RAPD (Zhu, 2004; Sirjusingh, 2001). Several SSR markers have been derived from some databases publically available, and some of those markers have been used in the Expressed Sequence Tags (ESTs) analysis of plant pathogens such as *P. infestans*, *P. sojae*, *Sclerotinia sclerotiorum* and *Magnaporthe grisea* (Wang, 2003; Wang, 2007; Zhu, 2003, 2004); however, further research is required to better understand the EST-SSR marker system for analysis of genetic variation of *P. infenstans* populations. Therefore, the objective of this study was to develop SSR markers to analyze the genetic diversity of *P. infestans* populations in the semiarid potato growing areas of northwest China.

Results

Distribution analysis of P. infestans SSR motifs in EST sequence

A total of 389 SSRs were detected from the 12,581 ESTs that were screened. Among all ESTs containing SSRs, 47 were

Table 1. The frequency of *phytophthora infestans* SSR in the EST.

Types	Numbers	The proportion of various types (%)
Binucleotide	47	12.1
Trinucleotide	123	31.6
Tetranucleotide	79	20.3
Pentanucleotide	33	8.5
Hexanucleotide	107	27.5
Total	389	100

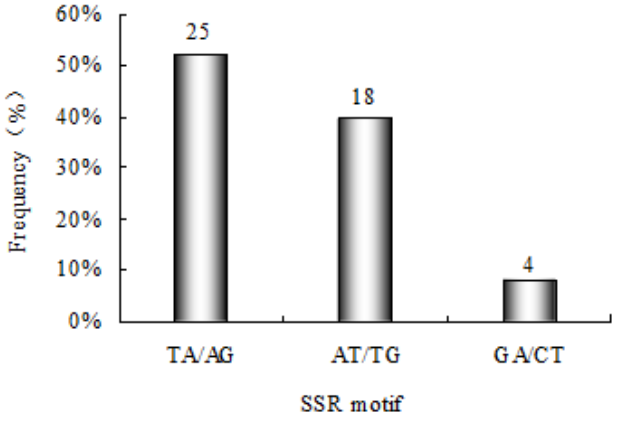

Fig 1. The frequency of 47 binucleotide in 389 EST-SSRs selected in the study, the vertical bars indicate the percent of every motif, the number on the bar is quantity of every motif (the same below).

Table 2. SSR primers used in the analysis.

Primer	EST	Repeat motif	Forward primer (F) &	Primer T	Expected
P1	AJ235744	$(GTCGG)_3$	F: ACCTATCTTTCCTGCTTGT	54	768
P2	GT866078	$(CTTCAC)_3$	F: GAGTAATCTTGGCTTTGGT	55	200
P3	GT866096	$(GAA)_5$	F: ATCTCACTTCTGCGGCTAT	54	198
P4	GT866059	$(GAA)_5$	F:ATCTCACTTCTGCGGCTAT	55	580
P5	GT866049	$(CAGCAA)_3$	F:TCCAGGGAACCCGATAACC	60	223
P6	FL483061	$(GCT)_5$	F: GAAGTTGTGGCGGAGGTTG	59	365
P7	GR294100	$(GAAGGC)_3$	F: CCTGAGACCTGCCTCCTTT	54	289
P8	GR294059	$(GAGG)_3$	F: ATGTCGGAGGCGGAAGAGT	61	232
P9	GR293985	$(GAAT)_3$	F: CCTGAGACCTGCCTCCTTT	56	310
P10	GR301044	$(AGCG)_3$	F: ATCGGTTGTCTGTGGTTTC	52	269
P11	GR301042	$(CAG)_3$	F: TACTCTACTCCCGAGAACCG	56	267

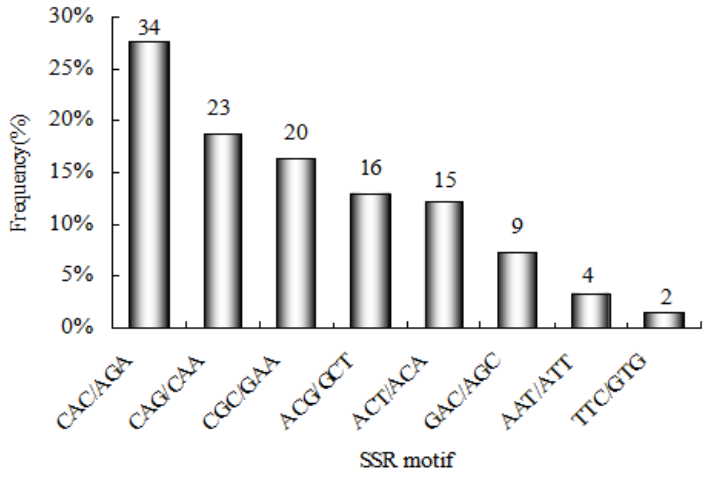

Fig 2. The frequency of123 trinucleotide in 389 EST-SSRs selected in the study.

binucleotide repeats, 123 were trinucleotide repeats, 79 were tetranucleotide repeats, 33 were pentanucleotide repeats, and 107 were hexanucleotide repeats, which accounted for 12.1%, 31.6%, 20.3%, 8.5% and 27.5% of the total SSRs respectively. The trinucleotide motifs were the most abundant type (Table 1). In the obtained SSR repeat units in binucleotide, the most abundant repeat motifs were TA/AG and AT/TC, with their total distribution of 44, accounting for 91.7% of all the repeat motifs in the binucleotide repeats (Fig. 1). Among all SSR trinucleotide motifs, CAC/AGA was the most superior repeat motifs, with the total distribution of 34, accounting for 27.6% of all the repeat motifs in the trinucleotide repeats (Fig. 2), In tetranucleotide, ATGG/CAAG was the most abundant with the total distribution being 20, which accounted for 25.3% of all the repeat motifs in the repeats (Fig 3). By contrast, in pentanucleotide, there were only two kinds of repeat motifs: TTCCT/AGAGC, CATCG/GTCGG (Fig. 4). In hexanucleotide repeats, the GGCAAG/CAAGGC had a total distribution of 31, accounting for 28.9% of all the repeat motifs (Fig. 5).

Primer efficiency

To select appropriate EST sequences containing SSRs，we designed 11 pairs of primers in this study (P1-P11 in Table 2). Under the given conditions with *P. infestans* strains' genomic DNA for PCR amplification, 2 pairs of primers failed to be the effectively amplified product, and 9 pairs of the primers (82%) were amplified SSR fragments. As an example, Fig. 6 showed that SSR amplification profiles from one isolate of *P. infestans* with 11 designed primer pairs. Among which, P1 and P4 yielded no bands, and P7, P8, P10, and P11 each gave rise to a single band. In contrast, P2, P3, P5, P6, and P9 produced polymorphic bands, with P5 giving the better quality of bands. All primer amplified products and the fragments obtained the right size ranging between 200 bp and 800 bp. The number of fragments of polymorphism was from 2 to 3.

Diversity of isolates of P. infestans from different collections

In order to reveal the genetic diversity of *P. infestans*, the DNAs of 63 isolates were amplified using primer P5 (Fig. 7). With a few exceptions (such as isolate #36, #63), all the other isolates evaluated in the study produced polymorphic bands. Using the unweighted pair group method, we separated the 63 isolates into 20 genotypic clusters at the level of 0.85 coefficient, and 8 genotypic clusters at the 0.69 coefficient (Fig. 8). The cluster analysis showed that there was a quite high genetic diversity among isolates, and the diversity was related to the environment where the isolates were collected. For example, the four isolates (#24, #25, #26, #27) collected from Wei-yuan county of Gansu Province were genetically distinct and clustered into four different groups, whereas the three other isolates (#21, #22, #23) collected from Longxi county of Gansu Province were within the same cluster as they had one genotype.

Discussion

EST database – effective in developing plant pathogenic SSR markers

With the development of biotechnology and of genomics programs, a large amount of bioinformation has been produced that provides a rich resource for the development of SSR markers. By analyzing the 12,581 ESTs of *P. infestans*, we found SSR loci exist in the 388 ESTs. To search SSR markers from DNA sequence database, EST has become the most rapid and effective method. This method has been used in the studies of other plant pathogens, for instance, 1,454 SSRs were identified from 28,197 *P. sojae* ESTs, 142 SSRs were identified from 2,185 *Cryphonectria parasitica* ESTs, and 4,112 SSRs were identified from 4,112 *Gibberella zeae* ESTs (Lin, 2008).

Phytophthora infestans EST-SSR distribution characteristics

By analyzing a total of 12,581 *P. infestans* ESTs in this study, we found 5.2% of ESTs contained SSRs, of which trinucleotide was the most abundant type. Our findings on the frequency of *P. infestans*, the EST sequences containing SSRs, and 3 bp repeats type in SSR were in agreement with the results previously reported (Wang, 2010). The analysis on the frequency of repeats motifs showed that SSR repeats motifs type of (CAC/AGA) appeared most in *P. infestans*, which differed from the result of Wang et al. (Wang, 2010); this was probably due to the fact that the quantity of EST investigated in our study and the search SSR criteria were different from those reported in other studies. In our study, 81.8% the primers were capable of amplifying SSR fragments among 11 pairs primers excavated, with44.4% having a single fragment and 55.6% of the polymorphic bands being effectively amplification primers.

Application of SSR marker

EST-SSR markers derived from the coding region of gene transcription can be more conservative compared with genomic SSR markers, and the conservation characteristics often limit their polymorphism within species. ESTs containing SSRs and known genes typically have high homology, therefore, EST-SSR markers provide "absolute" tag for the functional genes, making it is possible to effectively identify alleles which determine the important phenotypes. Virulence differentiation exists in the plant pathogen. Existing molecular markers such as RFLP, RAPD and AFLP have been used to analyze genetic diversity within species, and molecular markers have been rarely used to identify physiological races of pathogens. Our study demonstrated that SSR markers provide a high possibility for the identification of the physiological races in plant pathogens such as *P. infestans*. Our study also showed that the latest EST database can be used to screen SSRs, which offers a convenient tool for the development of new types of *P. infestans* EST-SSR markers and other molecular research. The genetic cluster analysis showed that there was relatively high genetic variation among isolates in same place. These results are in full agreement with the bio-assay test. The SSR analysis indicated that *P. infenstans* isolates in the Gansu Province were diverse, with little or no shared genotypes among counties where the isolates were collected, those results may have been caused by quit different climate conditions in Gansu province, the climate condition in Weuyuan County is always cool and rainy, the higher RH could prolonged favorable condition for infectivity and reproduction more generation, this may be the reason why the isolates collected from this place have more polymorphic bands. In our studies the sample size analyzed was however not big enough to reflect the true diversity situation within the province, so necessitating a more structured survey to reveal the factual population structure of *P.infenstans* in the Province.

Materials and Methods

Testing strains and DNA extraction

A total of 63 *P.infestans* isolates used in this study were collected and isolated from central part of Gansu province in

Table 3. Information of 63 isolates of *P. infestans* collected from Gansu Province.

NO of isolate	Code of isolate	Collection places	Physiological race type
1	CT-4	Kangle, Caotan	1.4.6.7.11
2	CT-6	Kangle, Caotan	1.4.6.7.11
3	CT-7	Kangle, Caotan	4.6.7.11
4	CT-C	Kangle ,Caotan Hezhen ,	1.4.6.7.11
5	DT-2	Diaotan	1.4.6.7
6	DT-4	Hezhen, Diaotan	1.4.6.7
7	PZ-B	Dongxiang, Pingzhuang	1.4
8	PZ-1	Dongxiang, Pingzhuang	4.6.7.11
9	PZ-2	Dongxiang, Pingzhuang	4.7.11
10	PZ-3	Dongxiang, Pingzhuang	1.4.7.11
11	PZ-4	Dongxiang, Pingzhuang	1.4.6.7
12	PZ-11	Dongxiang, Pingzhuang	1.4.6.7
13	TQ-D	Linxia, Tuqiao	1.3.4.6.7
14	TQ-1	Linxia, Tuqiao	1.3.4.6.7
15	TQ-H	Linxia, Tuqiao	1.4
16	TJ-2	Anding,Tuanjie	1.4.6.7.10.11
17	TJ-3	Anding,Tuanjie	1.3.4.11
18	TJ-5	Anding,Tuanjie	4.6.7.11
19	TJ-6	Anding,Tuanjie	4.6.7.11
20	CZ-1	Longxi,Caizi	1.4.6.7.11
21	CZ-2	Longxi,Caizi	1.3.4.5.6.7.11
22	CZ-3	Longxi,Caizi	1.4.5.6.7.11
23	CZ-7	Longxi,Caizi	1.3.4.11
24	HC-2	Weiyuan,Huichuan	1.4.6.7.10.11
25	HC-3	Weiyuan,Huichuan	4.6.7.11
26	HC-5	Weiyuan,Huichuan	1.3.4
27	HC-11	Weiyuan,Huichuan	4.6.7.11
28	PY-3	Qingyang,Pengyuan	1.4.7.11
29	PY-5	Qingyang,Pengyuan	1.4
30	PY-7	Qingyang,Pengyuan	1.4.5.6.7.8.10.11
31	ZH-1	Zhuanglang, Zhenhe	1.4.6.7.10
32	ZH-2	Zhuanglang, Zhenhe	4.6.7.11
33	ZH-4	Zhuanglang, Zhenhe	4.6.7.11
34	QC-1	Yongdeng, Qinchuan	1.4.6.7.11
35	QC-3	Yongdeng, Qinchuan	1.4.6.7.11
36	QC-4	Yongdeng, Qinchuan	4.6.7.11
37	QC-5	Yongdeng, Qinchuan	1.3.4
38	TY-19	Lintao, Taoyang	4.6.7.11
39	TY-37	Lintao,Taoyang	4.6.7.11
40	XK-1	Lintao,Xiakou	4.6.7.11
41	XK-2	Lintao,Xiakou	4.6.7.11
42	XK-4	Lintao,Xiakou	1.4.6.7.10.11
43	XK-6	Lintao,Xiakou	4.6.7.11
44	XK-7	Lintao,Xiakou	4.6.7.11
45	WP-4	Qinan,Wangpu	1.4.7.11
46	WP-6	Qinan,Wangpu	1.4.6.7.11
47	WP-7	Qinan,Wangpu	4
48	WP-17	Qinan,Wangpu	4.6.7.11
49	WP-15	Qinan,Wangpu	1.3.4.5.6.7.8.10.11
50	WP-23	Qinan,Wangpu	1.4.6.7.10.11
51	GJ-4	Qinan,Guojia	1.4.6.7.10.11
52	GJ-5	Qinan,Guojia	1.4.6.7.10.11
53	GJ-12	Qinan,Guojia	1.4.6.7.11
54	GJ-13	Qinan,Guojia	1.4.6.7.11
55	GJ-16	Qinan,Guojia	1.3.4.5.6.7.8.10.11
56	XL-3	Yuzhong,Xinglongshan	3.4.6.7
57	XL-4	Yuzhong,Xinglongshan	1.3.4.6.7
58	XL-6	Yuzhong,Xinglongshan	1.4
59	YP-1	Yongjin,Pingyang	4
60	YP-2	Yongjin,Pingyang	1.4.6.7.10
61	YP-3	Yongjin,Pingyang	1.4.6.7.10
62	FX-7	Longxi,Fuxin	4.6.7.11
63	FX-9	Longxi,Fuxin	4.6.7.11

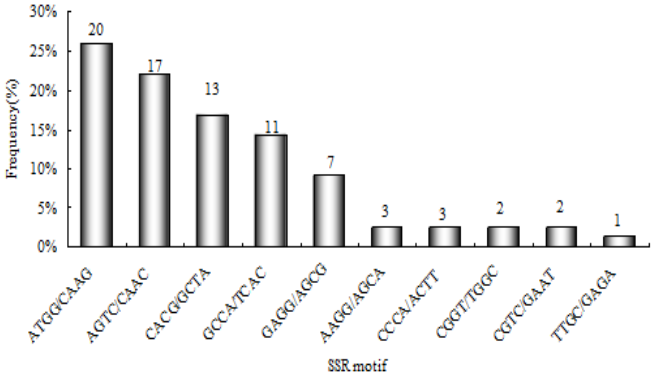

Fig 3. The frequency of 79 tetranucleotide in 389 EST-SSRs selected in the study.

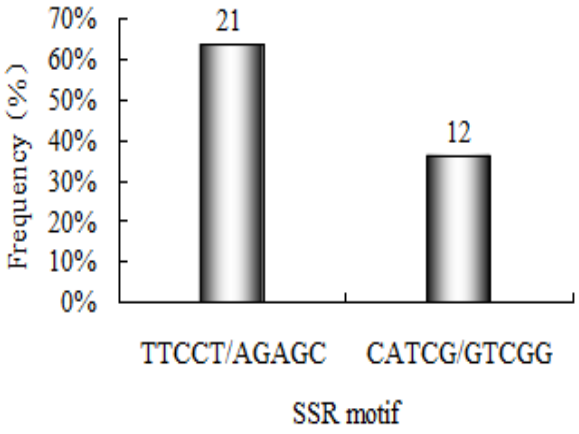

Fig 4. The frequency of 33 pentanucleotide in 389 EST-SSRs selected in the study.

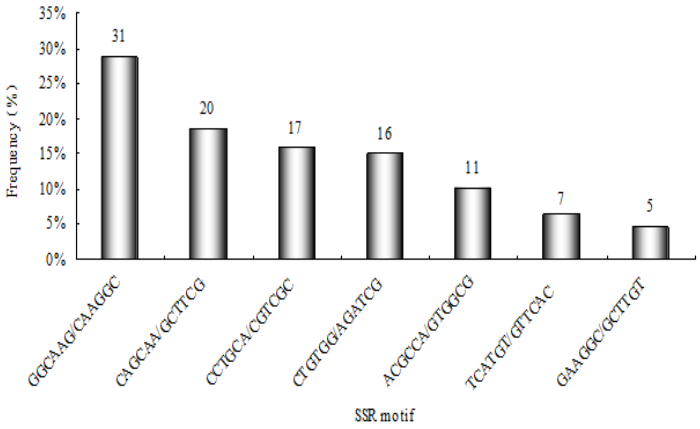

Fig 5. The frequency of 107 hexanucleotide in 389 EST-SSRs selected

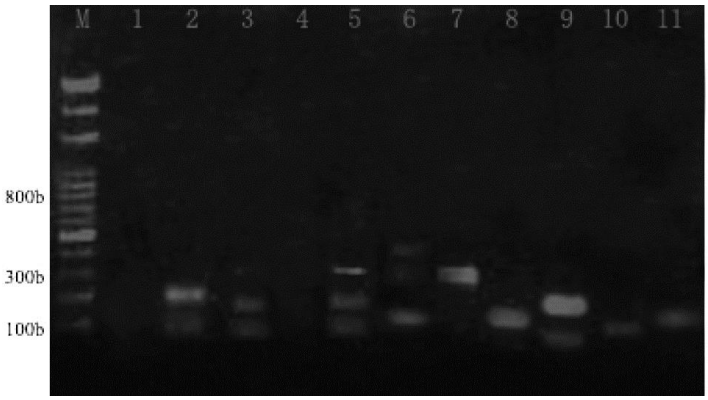

Fig 6. SSR amplification profiles from the isolate of *P. infestans* with 11 designed primers M: DNA Marker; 1: negative control, 2-11: design primer P1, P2, P3, P4, P5, P6, P7, P8, P9, P10

the growth season of 2009(Tab3.). Each isolates was yielded from single spore and cultured on rye medium at 16~18℃for 30 days in an incubator(model:GXZ-160A, Jiangnan, China). Subculture of each isolate was yield for further experiment in order to avoid contamination. Genomic DNA samples of 63 isolates were extracted following the protocol described by Wang (2007). Extracted DNA samples were diluted to 20 ng/μl (working concentration) in dd H_2O and stored at -20℃ for further analysis.

***Phytophthora infestans* ESTs**

A total of 12,581 ESTs of *P. infestans* were found from the online database (http://www.ncbi.nlm.nih.gov/). Simple Sequence Repeat Identification Tool (SSRIT) provided by GRAMENE website (http://www.gramene.org/db/markers/ssrtool) was used for the SSR Screening and identification. The program was run online and the parameters were set for detection of bi-, tri-, tetra-, penta-, and hexanucleotide motif with a minimum of 8, 5, 4, 3, 3 repeats, respectively.

The design of primers

Comparison among the EST sequences containing SSRs for their homology allowed the removal of non-redundant ESTs. We selected the appropriate EST sequences that contain SSR for the primer design by using the Primer 5.0. The main parameters of the designed primer contained: primer length of 19-21bp; the size of PCR product 200-800bp; the annealing temperature in the range of 50-65°C, and the upper and lower anneal temperature discrepancy of not exceeding 2°C, G+C content of 40% to 70% (the optimum 50%). Primers were synthesized by Shanghai Biological Engineering Co. Ltd.

DNA amplification and detection of amplification products

A 25 ng of genomic DNA were amplified in a volume of 20 μl containing 2.0 μl 10 × buffer (1.75 mmol. L^{-1} Mg^{2+}), 1.0 μl (10 mmol. L^{-1}) dNTPs, forward primer and reverse primer 2.0 μl each (1.5μ mol. L^{-1}), and 1.0 μl (2 U)Taq DNA polymerase, with ddH_2O top to 20 μl. Reaction was carried out on the Thermal Cycler (Bio-Rad Co., USA). The condition started with a denaturation step of 2 min at 94°C, followed by 35 cycles of 94°C for 30 s, 63°C for 30 s, 72°C for 30 s and a final

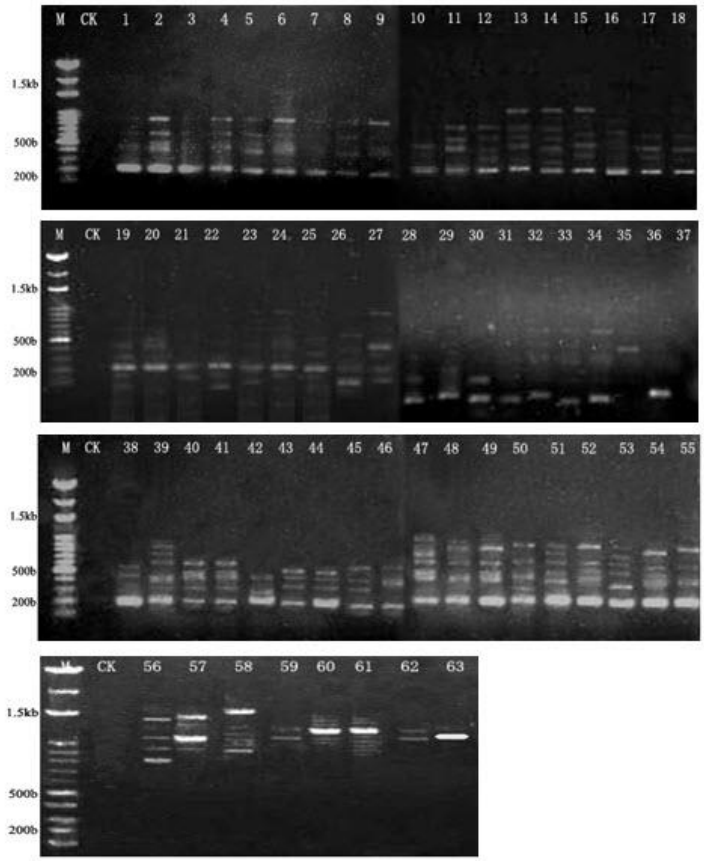

Fig.7 The PCR bands of 63 isolates, along with DNA marker (M) and the negative control (CK), with the amplification of primer P5, showing the genetic diversity of *P. infestans.* For example, isolate #51 has 6 polymorphic loci (i.e., 6 bands), whereas isolate #63 has only a single locus.

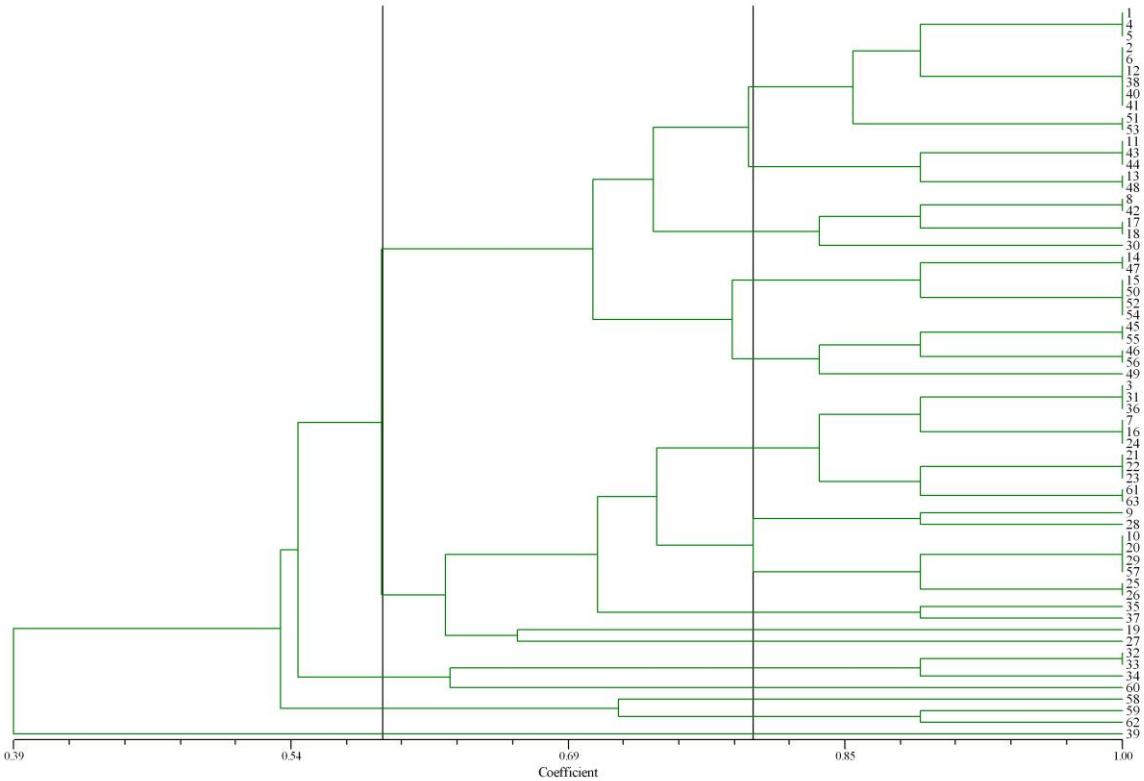

Fig 8. Dendrogram of 63 *P. infestans* isolates obtained from different places in the Gansu Province of China using unweighted pair group method cluster analysis (software NTSYSpc-2.10e). The various clusters are based on simple sequence repeats (SSR); those isolates with genetic similarity are included within the same cluster. The x axis shows the SM Coefficient 0.39-1.00. The vertical bars indicate groups of similar isolates.

step of 7 min at 72°C. Finally the amplified products stored at 4 °C. After amplification, the PCR products were examined by electrophoresis in 0.8% agarose-gel containing 0.5× Tris-borate-EDTA buffer (pH 8.0). Electrophoresis was conducted for 1.5 h at a constant power of 100 V. The DNA bands were visualized by EB staining. The products of PCR were detected and photographed using the Bio-Rad GEL imaging system.

Statistical analysis

The NTSYSpc statistical package, version 2.10e (Exeter Software, Setauket, NY), was used to perform unweighted pair group method cluster analysis (Rohlf, 1992).

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