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Population Genetic Analysis of *Phytophthora parasitica* From Tobacco in Chongqing, Southwestern China

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Abstract

Tobacco black shank, caused by *Phytophthora parasitica*, is one of the most notorious tobacco diseases and causes huge economic losses worldwide. Understanding the genetic variation of *P. parasitica* populations is essential to the development of disease control measures. In this research, 210 simple sequence repeat (SSR) markers for *P. parasitica* were identified, 10 of which were polymorphic among nine reference strains. We further performed population genetic analysis of 245 *P. parasitica* isolates randomly collected from tobacco fields in Chongqing for mating type, molecular variation at 14 SSR loci (four of which were identified previously), and sensitivity to the fungicide metalaxyl. The results showed that the A2 mating type was dominant and no A1 mating type isolate was discovered. SSR genotyping distinguished 245 *P. parasitica* isolates into 46 genotypes, four of which were dominant in the population. Low genotypic diversity and excess heterozygosity were common in nearly all of the populations from Chongqing. Population analysis showed that no differentiation existed among different populations. All isolates tested were highly sensitive to metalaxyl. Taken together, our results showed that the *P. parasitica* populations from tobacco fields in Chongqing belonged to a clonal lineage and were highly sensitive to metalaxyl.

Keywords: simple sequence repeat, *Phytophthora parasitica*, pathogen diversity, metalaxyl sensitivity

Phytophthora parasitica Dastur (syn. *P. nicotianae* Breda de Haan), one of the most notorious oomycete plant pathogens, was first discovered as the pathogen triggering citrus root rot and gummosis and tobacco black shank (Kamoun et al. 2015). *P. parasitica* is a typical soilborne pathogen with a broad host spectrum comprising more than 255 plant genera in 90 families (Cline et al. 2008). Tobacco black shank caused by *P. parasitica* is one of main limiting factors for tobacco production worldwide, including in Chongqing, a mountainous region in Southwestern China.

Because oomycete pathogens are phylogenetically distant from fungi, they are insensitive to most fungicides. The acylalanine fungicide metalaxyl is a member of the few chemical agents used for control of diseases caused by *Phytophthora* spp. However, resistant strains were discovered soon after its use in several *Phytophthora* species. For example, metalaxyl-resistant *P. parasitica* isolates from burley tobacco were reported in Korea (Kang 2000). However, no resistant *P. parasitica* isolates have been found in Southwestern China, including in Guizhou (Wang et al. 2013) and Chongqing (Qian 2011).

P. parasitica can reproduce sexually and asexually, and the oospores resulting from sexual reproduction have been observed under laboratory conditions (Ko 1978). Coexistence of A1 and A2 mating type isolates allows the completion of the sexual cycle. Sexual reproduction leads to gene recombination in the progeny, which can incorporate favorable mutations and eliminate harmful ones (Crow 1994), and may result in better adaptability to the variable environment. However, the mating type distribution of *P. parasitica* populations from tobacco fields around the world was reported, which indicated that mostly only one mating type is found in the field (Gallup et al. 2006). Both A1 and A2 mating types were found in Virginia but A2 was dominant (96%) (Parkunan et al. 2010). The A2 mating type was reported to be dominant in Anhui (Ma and Gao 2006) and Yunnan (Wang et al. 1997), China, whereas the A2 and A0 isolates (which do not produce oospores with either A1 or A2 mating type strains) coexisted in equal proportion and no A1 isolates were found in Chongqing (Sun 2009). However, Qian (2011) reported that A2 isolates were dominant in Chongqing, suggesting that the mating type distribution in Chongqing may have undergone a shift in the past 10 years.

Molecular genetic markers make it possible to track and trace individual genotypes and to improve understanding of pathogen population diversity (Parker et al. 1998). Simple sequence repeat (SSR) markers are one of the most useful molecular markers in population genetic analysis and have the following advantages: good reproducibility, codominance, low DNA template limitation, and low technical difficulty. SSR markers have been widely used in population analyses of *Phytophthora* pathogens, such as *P. infestans* (Flier et al. 2003; Grünwald et al. 2001; Li et al. 2012, 2013; Montarry et al. 2010; Montes et al. 2016; Tian et al. 2015a, b, 2016), *P. cinnamomi* (Pagliaccia et al. 2013), and *P. ramorum* (Eyre et al. 2013; Ivors et al. 2006). SSR markers have also been used for genotyping and population analysis of *P. parasitica* (Biasi et al. 2015, 2016; Li et al. 2017). Because of these advantages, SSR markers were used in this research to analyze the *P. parasitica* population from Chongqing.

Investigating the population structure and genetic diversity of *P. parasitica* populations facilitates a better understanding of the life cycle, primary infection sources, and evolution of the pathogen, which are important for developing effective disease management practices. *P. parasitica* populations in Chongqing have not yet been studied in depth. The aim of this research is to examine the genetic diversity of *P. parasitica* populations in tobacco fields in

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Chongqing, including mating type, metalaxyl sensitivity, and SSR maker-based determination of genetic diversity and mode of reproduction of the pathogen.

Materials and Methods

Sampling and isolation of *P. parasitica*. Diseased tobacco samples were collected from six counties in Chongqing and were regarded as independent populations (Wulong, Qianjiang, and Youyang in 2014 and Wulong, Qianjiang, Fengjie, Wushan, and Pengshui in 2015, hereafter referred to as WL2014, QJ2014, YY2014, WL2015, QJ2015, FJ2015, WS2015, and PS2015, respectively). Pathogen isolation and purification were performed in the laboratory. The typical disc-like pith tissues were placed on a 5% CA agar plate (50 ml of carrot extract, 0.1 g of $CaCO_3$, 0.01 g of β-sitosterol, and 8 g of agar/liter) with the proper antibiotics (100 μg/ml of ampicillin, 10 μg/ml of rifampicin, and 9 μg/ml of nystatin) directly. For those without the typical disc-like pith tissues, a small piece of pith tissue of new developing lesions was surface disinfected in 75% ethanol for 30 s, rinsed in sterilized water three times, and placed on 5% CA agar plates with the proper antibiotics (100 μg/ml of ampicillin, 10 μg/ml of rifampicin, and 9 μg/ml of nystatin). All isolates were incubated at 23°C in the dark and identified as *P. parasitica* based on morphological characters. Mature cultures of isolates were covered with sterile liquid paraffin and stored at 16°C as previously described (Onions 1971).

Mating type determination. Mating types were determined by pairing unknown isolates with known A1 and A2 mating type strains of *P. parasitica* Pp007 and Pp018, respectively, on 10% V8 juice agar plates with 0.02 g/liter of β-sitosterol. After incubation at 23°C in the dark for about 10 days, oospores were surveyed under a microscope. Isolates were also paired with themselves to examine whether they were self-fertile.

DNA extraction. *P. parasitica* mycelium was harvested after incubation in 5% CA broth in the dark at 23°C for 4 days. Liquid nitrogen freeze-dried mycelium was ground to a fine powder. Genomic DNA of *P. parasitica* was extracted following the protocol as previously described (Goodwin et al. 1992; Tian et al. 2015a) with minor modifications. Briefly, the mycelium powder was dissolved thoroughly in the DNA extraction buffer (7 M of urea, 350 mM of NaCl, 4 mM of EDTA, 50 mM of Tris-HCl, pH 8.0, 1% Na-lauryl sarcosine, and 5% Tris-saturated phenol, pH 8.0) and extracted with Tris-saturated phenol, pH 8.0, and chloroform. The DNA was precipitated with a half volume of 7.5 M of NH_4OAC and 1.5 volumes of isopropanol and was then washed in 75% ethanol twice. The air-dried DNA pellet was resuspended in ddH_2O with 0.1 mg/ml of RNase A and the DNA solution was incubated at 37°C for 30 min. Then the DNA was again extracted with phenol and chloroform and precipitated with NH_4OAC and isopropanol as above. Finally, the air-dried DNA pellet was dissolved in 30 μL of ddH_2O and stored at −80°C for later use.

Development of SSR markers. To identify SSR markers suitable for *P. parasitica* population genetic analyses, the publicly available *P. parasitica* reference genome INRA-310 version 2.0 (GenBank assembly accession number GCA_000247585.2) was used to scan for candidate SSR loci (2 to 6 bp per motif) with SSR Locator version 1 (da Maia et al. 2008). The parameters were set as 2 mers (×10), 3 mers (×7), 4 mers (×5), 5 mers (×4), and 6 mers (×4), respectively. Only perfect repeats were allowed during this search. More than 300 SSR loci were identified and 210 SSRs with the longest length were chosen. The primers flanking the selected SSR loci were designed with Primer3 (Untergasser et al. 2012). All of the primers designed were examined for polymorphism among nine reference *P. parasitica* strains (Supplementary Table S1) by PCR amplification followed by polyacrylamide gel electrophoresis (PAGE). In order to obtain enough SSR markers polymorphic in Chongqing populations, the candidates (polymorphic among the reference strains) from the last 145 primers (SSR66 to SSR210) were also examined for polymorphism among a subset of Chongqing isolates (90 randomly selected isolates). Those primers that showed high polymorphisms among reference strains (for the first 65 primers) or showed polymorphisms in Chongqing isolates (for the last 145 primers) were chosen for further analysis. To confirm the size of PCR products of different *P. parasitica* strains, selected PCR products amplified with high-fidelity DNA polymerase Pfu (Promega) were linked into T-vector pMD19 (simple) (Takara) and sequenced. Polymorphism information content (PIC) was calculated as:

$$\mathrm{PIC} = 1 - \sum_{\mathrm{i}}^{\mathrm{k}} P_{\mathrm{i}}^2$$

(Anderson et al. 1993; Botstein et al. 1980), where P_i is the frequency of the *i*th allele in the examined *P. parasitica* strains and k is the total number of alleles for each SSR locus.

Population analysis of *P. parasitica* using SSR markers. The obtained *P. parasitica* isolates were analyzed with 14 SSR markers, among which 10 were developed in this study and four were developed in previous reports (Biasi et al. 2015; Li et al. 2017). The size of the PCR products of different representative genotypes was confirmed as described above.

Considering that the large asexual reproductive ability and repetitive sample collection may influence the data analysis (Chen and McDonald 1996), we constructed a "clone-corrected data set" that included a representative isolate of each genotype in each population. This data set was used for the analysis of Weir and Cockerham's coefficient of differentiation θ among different populations, analysis of molecular variance (AMOVA), principal coordinate analysis (PCoA), and Nei's original measures of genetic distance. The full data set was used for calculating the genotype accumulation curve, genotypic diversity, and gene diversity analysis.

Genetic diversity was estimated by both genotypic diversity and gene diversity. Genotypic diversity is described by three indices: richness, diversity, and evenness. Given the difference among the sample number of each population, the genotypic richness was calculated based on the rarefaction curve (Grünwald et al. 2003). Genotypic diversity was estimated by $1 - \lambda$ and λ (Simpson's index lambda; Simpson 1949) is calculated as:

$$\lambda = \sum[n_{\mathrm{i}} \times (n_{\mathrm{i}} - 1)] / [N \times (N - 1)]$$

where n_i is the number of individuals of the *i*th genotypes and N is the sample size for each population. To reduce the influence of sample size, $1 - \lambda$ was corrected as:

$$1 - \lambda = 1 - N/(N - 1) \times \lambda$$

Genotypic evenness was quantified by E_5 (Grünwald et al. 2003), which is less dependent on the number of genotypes in a sample. All of the indices above were calculated with the R package Poppr (Kamvar et al. 2014). Gene diversity was estimated by analyzing the observed heterozygosity (H_O), unbiased expected heterozygosity (H_E), and fixation index (F_{IS}). H_E and H_O values were calculated by POPGENE version 1.31 (Yeh et al. 1997) and fixation index values were computed as:

$$F_{\mathrm{IS}} = 1 - H_{\mathrm{E}}/H_{\mathrm{O}}$$

AMOVA was performed using GenAlEx 6.5 (Peakall and Smouse 2006, 2012). Weir and Cockerham's coefficient of differentiation θ was calculated with MULTILOCUS version 1.3 (Agapow and Burt 2001). These two analyses were used to test the null hypothesis that no difference exists between populations. PCoA was performed using GenAlEx 6.5 (Peakall and Smouse 2006, 2012) to unravel whether there was any population structure among multilocus genotypes (MLGs). Nei's original measures of genetic distance were performed with POPGENE version 1.31 (Yeh et al. 1997). The genotype accumulation curve was generated with the R package Poppr to test whether the SSR markers had enough discrimination power to differentiate genotypes.

To reveal the genetic relationship among the SSR genotypes detected in Chongqing and the nine reference strains, an unrooted neighbor-joining tree using Bruvo's distance (Bruvo et al. 2004) was constructed with the R package Poppr and visualized in Mega 6.0 (Tamura et al. 2013).

Determination of sensitivity of *P. parasitica* isolates to metalaxyl. Metalaxyl sensitivity was estimated with half maximal effective

concentrations (EC_{50} values), which means the dose for 50% mycelial growth inhibition. A stock metalaxyl solution was made by dissolving 0.104 mg of 96% metalaxyl in 1 ml of acetone. The stock was diluted with ~40°C soluble 5% CA agar medium to prepare plates of different final metalaxyl concentrations: 0, 0.01, 0.10, 0.50, 1.00, 2.50, 5.00, and 10.00 μg/ml. Selected *P. parasitica* isolates were grown on a 5% CA agar medium plate for 3 days in the dark and 6-mm diameter mycelial plugs were made around the edge of colony. One mycelial plug was placed in the center of the 5% CA agar plate with different concentrations of metalaxyl and incubated at 23°C in the dark for 3 days. The colony diameter was measured and EC_{50} values were calculated. Based on the EC_{50} value, the isolates were regarded as resistant ($EC_{50} \geq 100$ μg/ml), intermediate ($100 > EC_{50} \geq 1$ μg/ml), or sensitive ($EC_{50} < 1$ μg/ml) (Wang et al. 2013).

Results

Identification of SSR markers. Based on the publicly available *P. parasitica* genome, 210 candidate loci were identified and selected for further analysis (Supplementary Table S2). Examination for polymorphism among nine *P. parasitica* strains isolated from different hosts or origins showed that 10 of 210 SSR markers were polymorphic (Table 1). All of the markers selected were distributed in different supercontigs (Table 1), suggesting their independent inheritance. Sequencing of the PCR products from representative genotypes

Table 1. Simple sequence repeat (SSR) markers developed for *Phytophthora parasitica*

SSR marker	SSR primer[a]	Scaffold[b]	Target size (bp)	Motif
SSR01[c]	F: CGGTATGGAGCCGAAATAGA R: GCCCTAAGTGGCTGATGAAG	Supercontig_2.9	187	ACTC
SSR08[c]	F: AACAATGCTGCTGTGAGTGC R: CTCACACCATTGGACACTGC	Supercontig_2.3	230	(AGGCAC)x-(CAAGCT)y
SSR25[c]	F: TTTGTCCTCAGATCCCAAGG R: GCCCACGTCCTCTAGATTCA	Supercontig_2.23	188	CTGA
SSR30[c]	F: ACGTCGGCTTCAACTCTGAT R: AACAGCCTATCAGATCGCGT	Supercontig_2.6	250	TCTG
SSR42[c]	F: GCTCAACAAGTCATACGGCA R: CGCGAGACCGTAGCTTTATC	Supercontig_2.113	213	CAG
SSR48[c]	F: GTGCAGTTGAGGCACAAAGA R: CGTGACCGTTAATTTGGCTT	Supercontig_2.5	247	TTAG
SSR122[c]	F: CATGGTTAAGAAAATGAGAACAATG R: TGATGTTTTTGGCTGGTTGA	Supercontig_2.25	123	AAGA
SSR141[c]	F: CTCAGTCAGTCAGCCACTCG R: TGTGTTGCACGCAAGTTTTT	Supercontig_2.49	250	CTCA
SSR166[c]	F: TAACGACTCGATCCCAGGTC R: TTTGTGGAAATTGAAATGCG	Supercontig_2.71	232	CA
SSR182[c]	F: TTGGTGTTCTCGTGTCTTGG R: TTGCGGTCAGAATTGACAAG	Supercontig_2.215	233	GTA
Pn251′[d]	F: CAGCTGAAGCGTAACGACAG R: ATAAGTGAGGGAGGGAGGGA	Supercontig_2.196	219	CTCA
P5′[d]	F: AACTTCGAGTCTCGTCACGG R: CTCCGAGGTCCAAATGTGAT[e]	Supercontig_2.21	227	TCTG
P15[f]	F: AGCTTCTGCAGTAACGGTAA R: CGATCAAAGATTACTGCAACT	Supercontig_2.7	110	GTA
P17[f]	F: GTCCTCAGGGATCAGCACAT R: TGGATATCGTTCCCGTTGTT	Supercontig_2.53	147	GTT

[a] F = forward and R = reverse.
[b] The scaffold is based on the *P. parasitica* INRA-310 genome sequence (version 2.0).
[c] Primers developed in this research.
[d] Pn251′ and P5′ were redesigned based on SSR loci Pn251 and P5 published previously (Li et al. 2017).
[e] Same as P5 reverse primer in Biasi et al. (2015).
[f] Primers reported in Biasi et al. (2015).

Table 2. Polymorphism analysis of new simple sequence repeat (SSR) markers among nine *Phytophthora parasitica* strains

SSR locus	Pp002	Pp007	Pp009	Pp014	Pp016	Pp018	Pp019	Pp023	Pp025	PIC[a]
SSR01	167/191[b]	175/183	179/195	179/179	179/179	183/187	183/183	155/195	159/183	0.81
SSR08	224/248	230/230	230/230	230/230	230/230	230/230	ND[c]	ND	218/230	0.47
SSR25	170/190	170/190	170/170	170/190	170/190	170/190	170/190	170/170	170/170	0.44
SSR30	246/246	238/250	242/242	238/250	238/250	242/250	242/250	242/242	242/242	0.69
SSR42	210/210	201/213	210/210	201/213	201/213	201/210	201/210	210/210	210/210	0.59
SSR48	181/185	185/189	177/189	185/189	185/189	181/185/189[d]	181/185/189	181/197	181/193/217	0.77
SSR122	131/131	119/123	127/127	119/123	119/123	119/119	119/119	127/131	147/147	0.75
SSR141	250/250	238/250	246/250	238/250	238/250	250/250	ND	250/250	250/250	0.40
SSR166	224/224	220/220	220/224	220/232	220/232	220/224	220/224	220/224	220/224	0.59
SSR182	216/216	216/216	204/204	216/216	216/216	216/216	216/216	216/216	216/216	0.20

[a] PIC = $1 - \sum_i^k P_i^2$, where P_i is the frequency of *i*th allele in nine reference strains.
[b] The allele length (in basepairs) for each locus in different isolates.
[c] ND = not determined.
[d] The presence of three alleles suggests that the strain may be of segmental polyploidy, which was also reported in Biasi et al. (2015).

revealed that the variation resulted from copy number differences of SSR motifs (Table 2). Results from PIC value calculation revealed that all of the selected SSR markers showed high polymorphism with PIC (>0.40, except SSR182) (Table 2).

Four reported SSR loci (Pn251, P17, P15, and P5) (Biasi et al. 2015; Li et al. 2017) were also used to analyze the *P. parasitica* populations from Chongqing, which were distributed in four supercontigs different from the SSR loci identified in this research (Table 1).

Isolation and mating type determination of *P. parasitica*. A total of 245 *P. parasitica* isolates were obtained from diseased tobacco plants in two consecutive years (Fig. 1). In 2014, 96 diseased tobacco plants were collected from three counties and 78 isolates were obtained, comprising 13, 38, and 27 isolates for Wulong, Qianjiang and Youyang, respectively. In 2015, 167 isolates were obtained from 186 diseased tobacco plants from five counties, comprising 26, 42, 24, 33, and 42 isolates for Wulong, Pengshui, Qianjiang, Fengjie, and Wushan, respectively (Fig. 1).

All *P. parasitica* isolates were examined for mating type. The results showed that no A1 mating type or self-fertile isolates were detected and nearly all isolates (99.6%) were of the A2 mating type, except one isolated in 2015 was of the A0 mating type (Fig. 1).

SSR genotyping of *P. parasitica* populations. A total of 46 multilocus SSR genotypes were detected from 245 *P. parasitica* isolates sampled in two consecutive years (78 and 167 isolates for 2014 and 2015, respectively) based on 14 SSR markers (nine of which were polymorphic in the *P. parasitica* population from Chongqing) (Supplementary Table S3). The genotype accumulation curve showed that these nine polymorphic SSR markers had enough discrimination power to differentiate genotypes (Fig. 2). Eight genotypes were unique in 2014 and 34 were unique in 2015. There were four shared identical SSR genotypes (MLG1, MLG2, MLG5, and MLG7) in both years (Fig. 3).

Four SSR genotypes (MLG1, MLG2, MLG3, and MLG4) were dominant in *P. parasitica* populations, with a frequency of 20.82, 17.14, 11.43, and 10.61%, respectively. Forty genotypes occurred at a very low frequency (<5%), 27 of which were singletons. Apart from WL2015 and PS2015, all of the other populations were dominated by single genotypes, with MLG1 being dominant in WL2014 and YY2014, MLG2 being dominant in Qianjiang in both years, MLG3 being dominant in WS2015, and MLG4 being dominant in FJ2015 (Fig. 3).

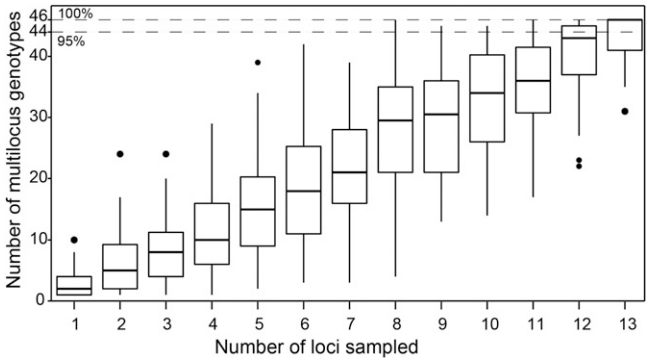

Fig. 2. Genotype accumulation curve for the *Phytophthora parasitica* population from Chongqing.

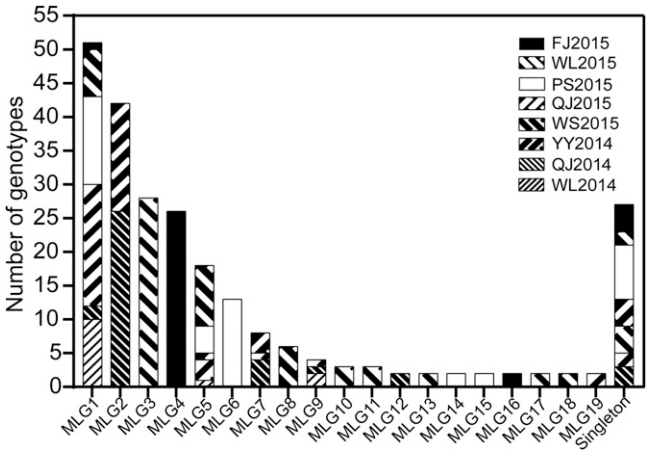

Fig. 3. Genotype distribution of *Phytophthora parasitica* populations in Chongqing. MLG1 to MLG19 indicate the 19 simple sequence repeat (SSR) multilocus genotypes detected more than once. 2014 and 2015 indicate the years of sample collection from the following: FJ = Fengjie, WL = Wulong, PS = Pengshui, QJ = Qianjiang, WS = Wushan, and YY = Youyang.

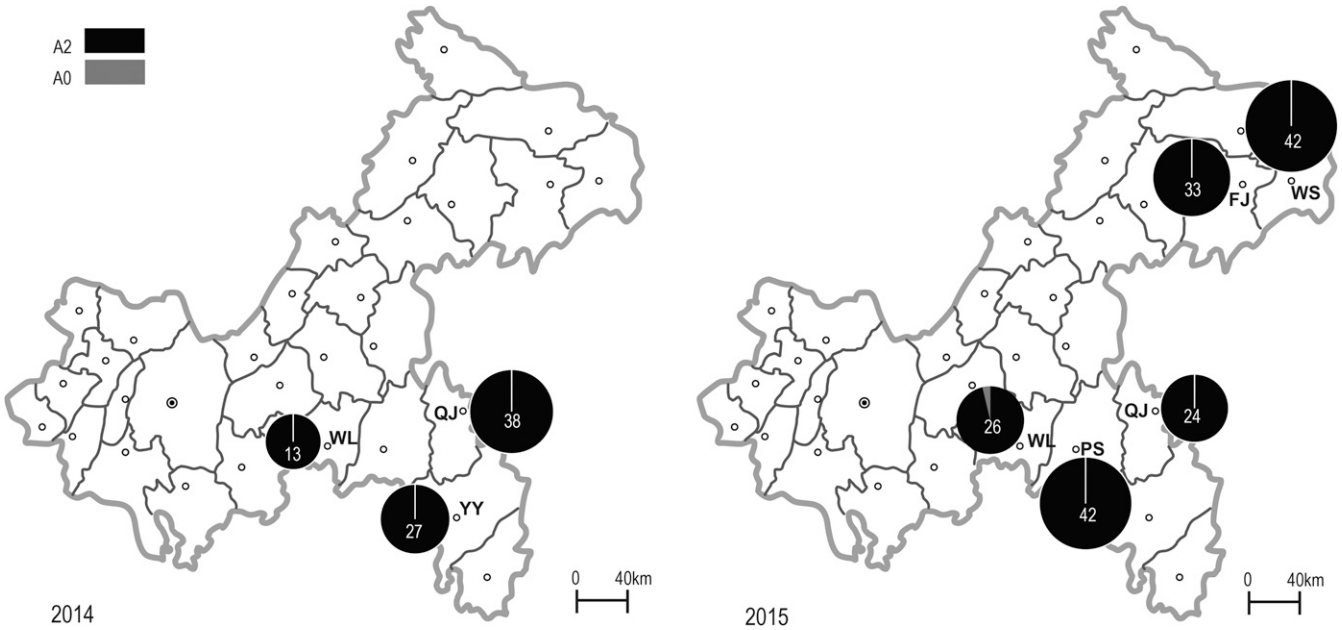

Fig. 1. Sampling locations and mating type distribution of *Phytophthora parasitica* populations from Chongqing. The total number of *P. parasitica* isolates for each region is marked in the center and the circle size reflects relative sample sizes from different regions. The obtained *P. parasitica* isolates were paired with known mating type strains of A1 (Pp007) and A2 (Pp018) on 10% V8 juice agar plates supplemented with 0.02 g/liter β-sitosterol. WL = Wulong, YY = Youyang, QJ = Qianjiang, FJ = Fengjie, WS = Wushan, and PS = Pengshui.

Genotypic and gene diversities. The genotypic richness among populations was similar, ranging from 3.00 to 6.32. The genotypic diversity index 1 − λ showed a low level of genotypic diversity in all of the populations (varying from 0.383 to 0.554) except that of WL2015 and PS2015. Genotypic evenness estimated with E_5 was not highly variable among all of the populations (ranging from 0.413 to 0.624) except that of WL2015. Compared with other populations, the PS2015 population showed higher evenness (i.e., 0.810) and the WL2015 population showed both higher diversity (i.e., 0.775) and evenness (i.e., 0.834). In all populations, the observed heterozygosity was higher than the expected heterozygosity, resulting in a negative fixation index F_{IS}. The F_{IS} values were not highly variable in all populations, varying from −0.920 to −0.833 (Table 3).

Genetic structure analysis. The AMOVA showed that the major variance was within populations (98%, estimated variance = 4.950), whereas the remaining variance (2%, estimated variance = 0.100, F_{st} value = 0.035, P = 0.099) was among populations. The estimation of Weir and Cockerham's coefficient of differentiation θ (ranging from −0.130 to 0.022) accepted the null hypothesis that there was no significant differentiation among populations in the same year, nor between populations from the same region in different years (Table 4). The Nei's genetic distance between populations ranged from 0.003 to 0.063 (Table 4). PCoA showed that there was no association between geographic regions and MLGs.

***P. parasitica* isolates from Chongqing were genetically different from the reference strains.** To reveal the genetic relationship between *P. parasitica* isolates from Chongqing and those from other regions, a neighbor-joining tree was constructed based on 10 shared SSR markers (SSR01, SSR08, SSR25, SSR30, SSR42, SSR48, SSR122, SSR141, SSR166, and SSR182) using Bruvo's distance. The results showed that all 46 MLGs from Chongqing were genetically similar to each other and formed a unique cluster. The isolates from Zhejiang Province, China, and Australia were genetically distant from that of Chongqing and formed the other cluster (Fig. 4).

***P. parasitica* isolates from Chongqing were sensitive to metalaxyl.** To examine sensitivity of *P. parasitica* isolates to fungicides, we randomly selected an isolate representing each multilocus SSR genotype (excluding MLG34 and MLG35, which did not grow well on the CA agar plates) and tested their tolerance to the commonly used fungicide metalaxyl. Results revealed by EC_{50} values showed that all tested isolates were sensitive to metalaxyl (EC_{50} < 1 μg/ml), with EC_{50} values ranging from 0.0520 to 0.3320 μg/ml (Supplementary Table S4).

Discussion

P. parasitica is a typical soilborne plant pathogen with a wide host range. The higher optimum growth temperature suggests that *P. parasitica* will gain more importance in the foreseeable future because of global warming (Kamoun et al. 2015). A better understanding of *P. parasitica* evolution and population dynamics will facilitate development of effective disease management strategies. In this study, we identified a new set of SSR markers and analyzed population structures of *P. parasitica* populations from tobacco fields from Chongqing.

SSR markers are widely used in plant pathogen population genetic analysis. Based on the publicly available *P. parasitica* genome, several SSR markers were identified and used for *P. parasitica* population diversity analysis (Biasi et al. 2015, 2016; Li et al. 2017). This research was started before the first study was published in 2015. A low level of polymorphisms was identified using these SSR markers among 90 *P. parasitica* isolates from Chongqing tobacco fields, suggesting that the number of polymorphic SSR markers may not be sufficient for the genetic analysis. Therefore, we used both the markers published previously and newly identified in this research for the genetic analysis of *P. parasitica* populations from Chongqing. In this study, we performed a genome survey for development of SSR markers for *P. parasitica* population genetic research. We identified 210 SSR markers: 10 showed polymorphisms among nine *P. parasitica* strains isolated from different regions and different hosts. All of the SSR loci identified were distributed in different supercontigs, suggesting that they are not physically linked. Besides these 10 SSR markers used in this research, we also found 15 SSR markers that showed polymorphisms among nine reference strains with one or two clear bands during PAGE, which may also be candidate choices for other researchers to test in their

Table 3. Genotypic diversity and gene diversity analysis of *Phytophthora parasitica* populations from Chongqing in 2014 and 2015

Index[a]	WL2014[b]	QJ2014	YY2014	WS2015	QJ2015	PS2015	WL2015	FJ2015
N	13	38	27	42	24	42	26	33
NG_O	3	8	7	9	7	13	6	7
NG_E	3.00	4.34	4.54	4.66	4.63	6.32	4.75	3.61
1 − λ	0.410	0.526	0.553	0.552	0.554	0.810	0.775	0.383
E_5	0.617	0.460	0.494	0.441	0.494	0.624	0.834	0.413
H_O	0.714	0.712	0.712	0.711	0.714	0.711	0.714	0.714
H_E	0.379	0.373	0.375	0.375	0.383	0.386	0.390	0.372
F_{IS}[c]	−0.887	−0.908	−0.896	−0.895	−0.866	−0.844	−0.833	−0.920

[a] N = sample size, NG_O = number of observed multilocus genotypes, NG_E = number of expected multilocus genotype; E_5 = evenness of genotypes, H_O = the observed heterozygosity, H_E = Nei's expected heterozygosity (Nei 1973), and F_{IS} = fixation index.
[b] 2014 and 2015 indicate the years of sample collection from the following: WL = Wulong, QJ = Qianjiang, YY = Youyang, WS = Wushan, PS = Pengshui, and FJ = Fengjie.
[c] F_{IS} was calculated as follows: $F_{IS} = 1 - H_O/H_E$. 1 − λ was corrected for sample size and calculated as $1 - \lambda = 1 - N/(N-1) \times \lambda$.

Table 4. Estimation of Weir and Cockerham's coefficient of differentiation θ and Nei's genetic distance among *Phytophthora parasitica* populations from Chongqing[a]

Populations	WL2014[b]	QJ2014	YY2014	WS2015	QJ2015	PS2015	WL2015	FJ2015
WL2014	–	0.018	0.003	–	–	–	0.023	–
QJ2014	−0.096[ns]	–	0.014	–	0.007	–	–	–
YY2014	−0.130[ns]	−0.047[ns]	–	–	–	–	–	–
WS2015	–	–	–	–	0.062	0.048	0.063	0.039
QJ2015	–	−0.061[ns]	–	0.022[ns]	–	0.038	0.019	0.038
PS2015	–	–	–	0.022[ns]	−0.001[ns]	–	0.037	0.022
WL2015	−0.102[ns]	–	–	0.017[ns]	−0.054[ns]	−0.009[ns]	–	0.043
FJ2015	–	–	–	−0.008[ns]	−0.020[ns]	−0.024[ns]	−0.019[ns]	–

[a] Above the diagonal is Nei's genetic distance (Nei 1972), and below the diagonal is the θ value. Dashes indicate that the indices were not calculated.
[b] 2014 and 2015 indicate the years of sample collection from the following: WL = Wulong, QJ = Qianjiang, YY = Youyang, WS = Wushan, PS = Pengshui, and FJ = Fengjie. ns = P > 0.05 indicates no significant difference.

P. parasitica populations. In this research, the polymorphisms of candidate SSR loci were analyzed among nine *P. parasitica* reference strains. These strains are diverse in terms of host origin and geographic location, which is suggestive of a certain level of diversity required for the detection of polymorphism and the development of genetic markers. During analysis of *P. parasitica* populations with identified SSR markers, a genotype accumulation curve was constructed, which demonstrated that the markers we identified had enough discrimination power to differentiate genotypes in *P. parasitica* populations from Chongqing.

Our results indicated that all of the populations from the two consecutive years were genetically similar to each other. First, the estimation of Weir and Cockerham's coefficient of differentiation accepted the null hypothesis; namely, there was no significant difference among different populations in the same year or between populations of the same place from different years. Second, AMOVA results showed that the major variation was within populations and a low fraction of the variance was attributed to differences among populations. Third, genetic distance analysis showed that all SSR genotypes detected from Chongqing formed a unique cluster being distant from *P. parasitica* strains from Zhejiang Province, China, and Australia. Fourth, PCoA results showed that no clusters were formed, indicating no association between geographic locations and SSR genotype. Finally, the conclusion was also supported by low Nei's genetic distance values (<0.063) between all populations (Table 4).

The occurrence of oospores contributes to the genetic diversity of *Phytophthora* pathogens (Meng et al. 2014). As to *P. parasitica*, oospores can be produced by pairing A1 and A2 mating type strains under the laboratory condition (Ko 1978). In the sexually reproducing or recombining populations, the two mating types (A1 and A2) are expected to be in similar frequency (Milgroom 1996). However, in this study, 244 of 245 isolates were of the A2 mating type and no A1 mating type isolates were found. This is consistent with previous reports on the mating type structure of *P. parasitica* populations from tobacco worldwide. In the United States, Parkunan et al. (2010) found that the A2 mating type was dominant (94%) in Virginia and 6% belonged to the A1 mating type. Most isolates (56 of 59) were identified as the A2 mating type and three were of A1 mating type in Georgia (Li et al. 2017). In China, Peng et al. (2011) reported that all *P. parasitica* isolates from Guizhou were of the A2 mating type, which was also predominant in Anhui (Ma and Gao 2006) and Yunnan (Wang et al. 1997). The unequal distribution of mating types suggested that sexual reproduction was unlikely. For all populations analyzed in this research, the observed heterozygosity was higher than the expected heterozygosity, which gave a negative fixation index. In diploid organism, excess heterozygosity often results from extreme clonality (Balloux et al. 2003). Qu (2017) in our laboratory analyzed 34 isolates representing different MLGs from different populations and found that all of the isolates belonged to race 1, indicating that no physiological differentiation was detected in *P. parasitica* populations from Chongqing. Considering all of the above results, *P. parasitica* populations from Chongqing belonged to a clonal lineage and the limited variation might result from random mutation, not from sexual recombination.

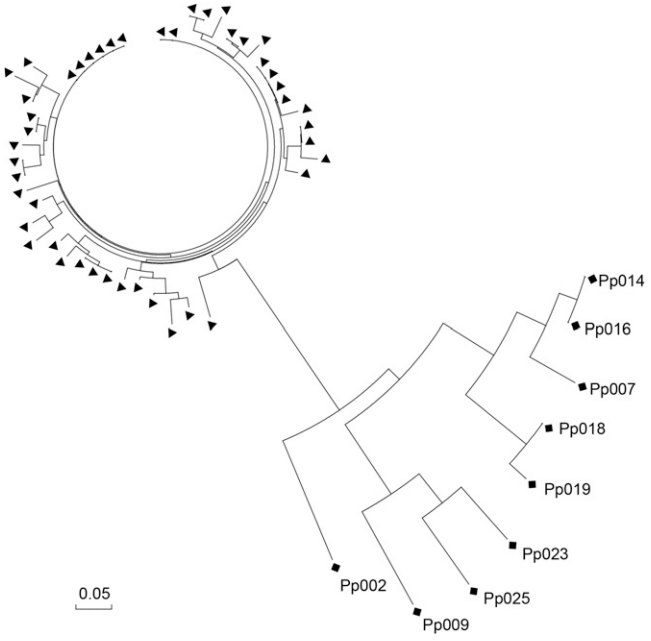

Fig. 4. Genetic relationship analysis of *Phytophthora parasitica* isolates from Chongqing and that of other regions examined. This analysis was done using the neighbor-joining method based on Bruvo's distance. Filled triangles indicate the 46 multilocus genotypes detected in the Chongqing population and filled squares indicate the nine reference strains (Pp002 to Pp025) from Zhejiang, China, and Australia.

Our results showed that *P. parasitica* isolates from Chongqing were metalaxyl sensitive. The EC_{50} values of *P. parasitica* isolates were all <1 μg/ml, indicating that *P. parasitica* isolates in Chongqing are very sensitive to metalaxyl and this fungicide should control the tobacco black shank. However, excessive use of metalaxyl may result in extensive development of resistant strains, so it is necessary to use this fungicide moderately and use appropriate fungicide resistance management strategies (van den Bosch et al. 2014). We also found a slight variation of EC_{50} values among isolates examined (from 0.0520 to 0.3320 μg/ml), which was also found in other regions (Csinos and Bertrand 1994; Morales-Rodríguez et al. 2014; van Jaarsveld et al. 2002); whether there is association between EC_{50} value variation and geographic locations remains to be investigated in future work.

In summary, 210 SSR loci were identified and 10 were detected to be new polymorphic SSR markers for *P. parasitica* population genetic analysis. *P. parasitica* populations from tobacco fields from Chongqing were determined systematically to be asexual, lacking differentiation among regions, highly sensitive to fungicide metalaxyl and genetically distant from that of other regions examined.

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