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Genetic diversity among asexual and sexual progenies of *Phytophthora capsici* detected with ISSR markers

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Abstract: The population structure of *Phytophthora capsici* among asexual and sexual progenies was analyzed using ISSR. Thirty asexual progenies of one parent and three sexual populations were assayed for genetic diversity using 5 ISSR primers and DNA from 120 offspring of *P. capsici* was amplified. In total, 71 reproducible ISSR fragments were obtained, of which 100% were polymorphic, revealing high polymorphism among the isolates. Among them, the percentages of polymorphism of sexual and asexual progeny isolates were 100.00 and 77.46%, respectively. Genetic similarity coefficients among all the isolates ranged from 0.54 to 0.73. The sexual offspring population showed much more variability than the asexual offspring population with 76.26% variability attributed to diversity within populations as compared with 23.74% among populations. This research reveals that the sexual progeny population of *P. capsici* contributes more genetic diversity than that of asexual progeny population.

Keywords: asexual offspring population; ISSR-PCR; pepper phytophthora blight; sexual offspring population

The oomycete plant pathogen, *Phytophthora capsici* Leonian, is distributed globally and is becoming a critical threat to vegetable yield and quality (Leonian 1922; Erwin & Ribeiro 1996; Yin et al. 2012). *P. capsici* can infect both the vegetative and reproductive organs of many vegetable crops, especially species of the Solanaceae and Cucurbitaceae families (Babadoost 2000; Lamour et al. 2012). At the time of infection, resulting symptoms include damping off, blight, and root or fruit rot depending on the developmental stage of the plant (Hausbeck & Lamour 2004). Crop rotation, soil amendments, grafting, biological protection and chemical control methods have been widely used to control this disease (Gilardi et al. 2013; Granke et al. 2012; Ristaino & Johnston 1999). Although zoospores of *P. capsici* may live in fields for a short time, its thick-walled oospores which could survive under unfavorable conditions play an important role in infecting the hosts and limiting the effectiveness of control strategies (Do et al. 2012).

Most *Phytophthora* species are capable of reproducing asexually and sexually. As for *P. capsici*, asexual reproduction is completed by zoospores while sexual reproduction occur through an interaction between A1 and A2 mating types which form oospores to over winter (Hausbeck & Lamour 2004). Oospores germinate either directly through the formation of a germ tube or indirectly sporangium formation after a period of dormancy. The pathogen also reproduces asexually through sporangia that germinate indirectly and release motile zoospores with biflagellates on plant surfaces or in saturated soil. Zoospores move

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easily under saturated conditions and infect roots or aboveground portions of plants (Ristaino & Johnston 1999). Offspring produced by these two different reproductive models in different regions have shown abundant genetic variation among *P. capsici* isolates, allowing the pathogen to easily adapt to all kinds of selection pressure.

Some studies have demonstrated that asexual and sexual modes of reproduction promote the population structure and diversity of *P. capsic*i (Dunn et al. 2010; Hulvey et al. 2011). The population genetic structure reflects the evolutionary history of the plant pathogen; therefore, the research about the population structure is of great significance to understand the occurrence and epidemic of the disease, providing a valuable reference for breeding disease-resistant varieties (McDonald & Linde 2002). However, the genetic diversity of *P. capsici* offspring produced by asexual and sexual reproduction has not been reported.

Currently, the best strategy for determining the genetic diversity of the plant pathogen is based on DNA molecular markers (Piotrowskaa et al. 2016). Research on the genetic diversity of *P. capsici* began in the late 1980s. The use of random amplified polymorphic DNA, simple sequence repeat, amplified fragment length polymorphism, inter simple sequence repeat (ISSR) and other marker systems revealed abundant genetic diversity of the pathogen worldwide (Archana et al. 2014; Rezinciuca et al. 2014; Tucker et al. 2015; Kashyap et al. 2016). Among available markers, the ISSR technique has the advantages of low cost, simple operation and reproducibility, producing large amounts of genetic information compared to other DNA-based markers (Rampersad 2013). We assumed that the sexual stage and clonal reproduction for propagation and survival in populations of *P. capsici*. The aim of this study was to investigate the genetic diversity of *P. capsici* isolates obtained from offspring of asexually and sexually reproducing populations based on ISSR markers and elucidate the influence of different methods on the epidemic regularity of the disease. Our expected results suggest significant variation is produced following sexual and asexual reproduction and that sexual reproduction may drive the overall diversity. Understanding the genetic structure of *P. capsici* among offspring from different reproductive modes will contribute to estimating the occurrence and prevalence of pepper phytophthora blight and will assist in control strategy for pepper growers.

MATERIAL AND METHODS

***P. capsici* isolates and experiment design.** A *P. capsici* isolate with weak pathogenicity collected from Hexian, Anhui (HX2, A1 mating type) and a *P. capsici* isolate with strong pathogenicity collected from Fuyang, Anhui (FY2, A2 mating type) were selected as parents. The two parents were each obtained from a single zoospore and stored at 10 °C. Thirty zoospore progenies of HX2 ($HX2ZG_1$), self-oospore progenies of each parent ($HX2S_1$, $FY2S_1$), and hybrid oospore progenies of two parents (OG_1) were analyzed to examine the population structure

Production of zoospore progeny. Culture discs (F6 mm) cut from the edge of the colony of each parent were placed into a petri dish (F90 mm) with 15 mL of 10% V-8 broth consisting of original 10% vegetable juice (USA) and 0.1% $CaCO_3$. After incubating in darkness at 25 °C in darkness for 72 h, the broth was poured out, 15 mL of sterilized water was added and changed once every 12 h, and a large number of zoosporangia were produced after 24 hours (Chen et al. 2005). Then, the treated sporangia were refrigerated of 5 °C for 10–15 min, removed, and incubated at room temperature for 30 min to induce simultaneous release of numerous zoospores. About 150 μL of the spore suspension containing 200 zoospores were spread on a 2% water agar plate and incubated at 25 °C in darkness for 12 houra (Zheng 1997). During germination, a germ tube was generated, and 30 colonies originating from single zoospores were transferred to carrot agar plates under a microscope. Thirty asexual progenies were formed, and this was perfomed for parent HX2.

Production of oospore progeny. Hybrid progenies (OG_1) were formed from the cross of each parent with confrontation culture by growing on 10% V8 juice agar plates consisting of original 10% vegetable juice, 0.02% $CaCO_3$, and 2% agar as described previously with some modifications (Zheng 1997). Self progenies of each parent ($HX2S_1$, $FY2S_1$) were formed using indirect pairing with poly carbon film as described previously (Zheng 1997). All plates were maintained at 25 °C in darkness at least for 2 months (Hord & Ristaino 1991). Oospores were produced and induced to germinate according to the method described previously (You et al. 2001). Thirty hybrid progenies and 30 self progenies of each parent were obtained and used in ISSR analysis.

DNA extraction. All the parents and their progenies were first cultivated on carrot agar for 3 days,

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the edges of the colonies were cut, and the colonies were grown in Plich's liquid medium (0.5 g KH_2PO_4, 0.25 g $MgSO_4 \cdot 7H_2O$, 1 g asparagine, 1 mg thiamine, 0.5 g yeast extract, 25 g glucose, and 1 000 mL distilled water) to harvest the mycelia for DNA preparation. Total genomic DNA of each isolate was extracted according to the previously described methods (Li et al. 2012). All DNA samples were quantified using a spectrophotometer at 260 nm with a concentration of 50 ng/μL.

ISSR-PCR amplification. Next, 5 of 35 primers synthesized by Sangon Biotechnology Ltd. Co. (China), which produced clear and reproducible bands on 16 isolates, were selected for ISSR-PCR amplification. The amplification was performed in a reaction volume of 25 μL containing 2.5 μL 10 × PCR buffer, 200 μM dNTPs, 0.2 μM primer, 1.25 U Taq DNA polymerase, and 0.5 μL of template DNA. The thermal cycling program was set as described previously (Li et al. 2012). PCR amplification products were separated on 1.8% agarose gel stained with ethidium bromide at a voltage of 5 V/cm for 3 h and photographed under a UV transilluminator. Amplification was repeated 3 times, and sterilized water without template was used as a control.

Data analysis. Band profiles generated by ISSR-PCR amplification were scored and converted to a binary matrix on the basis of presence (1) or absence (0). Genetic parameters of each primer and progeny population, total genetic diversity (H_t), and genetic diversity within groups (H_s) were analyzed using POPGENE (version 1.3.1.) (Yeh et al. 1999). Nei's genetic similarity values, genetic differentiation coefficient (G_{ST}), genetic identity (*I*), and genetic distance (*D*) were determined for all pairwise combinations of progenies (Nei 1972; Slatkin & Barton 1989). Jaccard's similarity coefficients were calculated via NTSYS-pc (version 2.1) to generate a dendrogram using the unweighted pair-group method with arithmetic averages (UPGMA) (Rohlf 2000).

RESULTS

ISSR-PCR polymorphism. Five primers (P10: 5'-GAGAGAGAGAGAGAGATG-3'; UBC807: 5'-AGAGAGAGAGAGAGAGT-3; UBC816: 5'-CACACACACACACACACAT-3'; UBC835: 5'-AGAGAGAGAGAGAGAGYC-3'; UBC841: 5'-GAGAGAGAGAGAGAGAYC-3') that produced clear and reproducible bands on 16 isolates were selected for ISSR-PCR amplification. Properties of ISSR primers used for amplification of *P. capsici* isolates are shown in Table 1. An example of the banding profiles of DNA of progeny isolates using primer UBC835 is shown in Figure 1.

A total of 71 ISSR bands were amplified with 5 primers in 120 progenies of *P. capsici*. Out of these bands, 71 were polymorphic, and the percentage of polymorphism was 100%. No single isolate or group of isolates from progeny contained all 71 markers. For each primer (Table 1), the annealing temperature ranged from 48 to 54 °C. The total number of bands ranged from 12 to 15, with an average of 14.2 bands per primer. Nei's (1972) gene diversity (*h*) values ranged from 0.274 to 0.423, and Shannon's information index (*Is*) ranged from 0.414 to 0.613, indicating that these 5 primers were appropriate for distinguishing diverse asexual and sexual progenies isolates of *P. capsici*.

Analysis of genetic structure among each asexual and sexual progeny population. Genetic characteristics of the asexual and sexual progeny isolates from the two parental isolates were analyzed (Table 2). The polymorphic bands of three populations from sexual progeny isolates were different. The percentages of polymorphism among self progeny from isolates

Table 1. Properties of ISSR primers used for amplification of *Phytophthora capsici* isolates in a study of genetic diversity in asexual and sexual progenies

Primer	Annealing temperature (°C)	Bands amplified	Polymorphic bands	Polymorphism (%)	Mean	
					h^a	I_s^b
P10	51	12	12	100.0	0.312	0.479
UBC807	52	15	15	100.0	0.327	0.483
UBC816	52	15	15	100.0	0.274	0.414
UBC835	50	15	15	100.0	0.335	0.506
UBC841	54	14	14	100.0	0.423	0.613

[a]Nei's (1972) gene diversity; [b]Shannon's information index, as a measure of gene diversity

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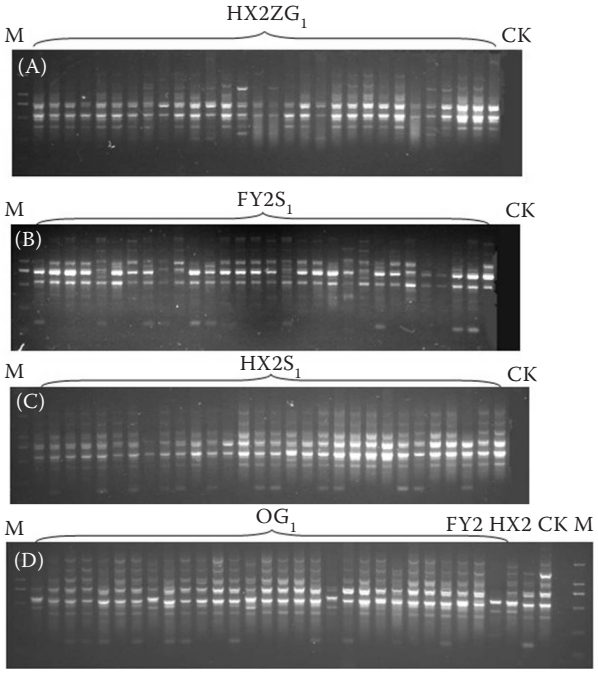

Figure 1. The genetic fingerprints of different groups of *Phytophthora capsici* isolates with primer UBC835

Lane M – DL 2 000 marker; CK – no DNA template; (A) $HX2ZG_1$: first zoospore generation of isolate HX2; (B) $HX2S_1$: the first selfed progeny of isolate HX2; (C) $FY2S_1$: the first selfed progeny of isolate FY2; (D) OG_1: the cross between isolate FY2 and HX2

FY2 and HX2 were 69.01 and 80.28%, respectively, and that of the hybrid progeny isolates from the two parents was highest at 95.77%. This was consistent with earlier studies that hybridization is an important part of the life cycle of *P. capsici* and that recombination has an important effect on the genetic structure of the population (Lamour & Hausbeck 2000, 2001). In general, the percentages of polymorphism of sexual and asexual progeny isolates were 100.00 and 77.46%, respectively.

Based on the POPGENE analysis, the *h* values of each progeny population ranged from 0.2104 to 0.3256 and were higher in the sexual progeny population (0.3395) than in the asexual progeny population (0.2264). Meanwhile, the *Is* values of each progeny population ranged from 0.3187 to 0.4867 and were higher in the sexual progeny population (0.5043) than in the asexual progeny population (0.3470).Therefore, the diversity of the sexual progeny population was significantly higher than that of the asexual progeny population.This explains why the sexual stage of the *P. capsici* life cycle plays an important role not only in survival but also in adapting to environmental stress such as fungicide and cold winter temperature (Lamour and Hausbeck 2002; Hu et al. 2013). The H_t of the four progeny populations was 0.3349, and the H_s was 0.2554, indicating that 76.26% of the genetic diversity was caused by the genetic differences of progenies.

Matrices of genetic distances and genetic identities (Nei's unbiased measures) among the four progeny populations were established. Genetic distance (*D*) and genetic identity (*I*) values were obtained from pairwise comparisons (Table 3). The results indicated that the highest genetic identity was between self progenies of isolate HX2 and hybrid progenies (0.9453), whereas the genetic identity between self progenies of isolate HX2 and isolate FY2 was the lowest (0.7995).

The values of G_{ST} among the four populations of *Phytophthora capsici* isolates in asexual and sexual progenies were calculated with POPGENE version 1.3.1, and the result are shown in Table 4. The G_{ST} values among different asexual and sexual progeny populations greatly differed, ranging from 0.0702 to 0.2582. In general, the G_{ST} between populations of self-oospore progenies and zoospore progenies of isolate HX2 was lowest (0.0702), while

Table 2. Genetic diversity of *Phytophthora capsici* isolates in asexual and sexual progenies

Population	Polymorphic bands	Polymorphism (%)	(*h*)[a]	(*Is*)[b]
$HX2ZG_1$	55	77.46	0.2264	0.3470
$HX2S_1$	57	80.28	0.2593	0.3882
$FY2S_1$	49	69.01	0.2104	0.3187
OG_1	68	95.77	0.3256	0.4867
Sexual population	71	100.00	0.3395	0.5043
Asexual population	55	77.46	0.2264	0.3470

[a]Nei's (1972) gene diversity; [b]Shannon's information index, as a measure of gene diversity

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Table 3. Nei's unbiased measures of genetic identity and genetic distance among populations of *Phytophthora capsici* isolates in asexual and sexual progenies

Population	$HX2ZG_1$	$HX2S_1$	$FY2S_1$	OG_1
$HX2ZG_1$	–	0.9103	0.8094	0.9014
$HX2S_1$	0.0562	–	0.7995	0.9453
$FY2S_1$	0.1919	0.2238	–	0.8254
OG_1	0.1038	0.0940	0.2115	–

Nei's (1972) genetic identity (above diagonal) and genetic distance (below diagonal)

Table 4 Genetic differentiation coefficient among populations of *Phytophthora capsici* isolates in asexual and sexual progenies

Population	$HX2ZG_1$	$HX2S_1$	$FY2S_1$	OG_1
$HX2ZG_1$	–			
$HX2S_1$	0.0702	–		
$FY2S_1$	0.1994	0.2504	–	
OG_1	0.1227	0.1288	0.2582	–

that between populations of self-oospore progenies of isolate FY2 and hybrid oospore progenies from isolates HX2 and FY2 was highest (0.2582).

Analysis of genetic variation between asexual and sexual progeny population. Genetic variation was analyzed in populations with two different reproductive modes, i.e., asexual reproduction and sexual reproduction. It was found that the total genetic variation (H_t) of the population was 0.3127, and the genetic variation (H_s) of the population was 0.2838, suggesting that 90.76% of the genetic diversity was caused by variation within the population. The G_{ST} of the population was 0.0925, indicating that there was low but still potentially important genetic differentiation level between asexual and sexual populations according to Wright's criterion (Wright 1978).

Cluster analysis among asexual and sexual offspring. Clustering analysis of different asexual and sexual offspring individuals using the UPGMA method was carried out by NTSYS-pc 2.1 biological software. The results showed that the genetic backgrounds of each strain were not the same, with a genetic similarity coefficient between strains of 0.54 to 0.73. There was a certain degree of crossover among different asexual and sexual offspring of each population in different branches of the system clustering diagram.

DISCUSSION

Asexual reproduction, hybridization, and self-crossing all play key roles in the genetic diversity of pathogens under natural conditions (Grunwald et al. 2003). To date, studies on the epidemiology and population structure of *P. capsici* have been conducted around the world, indicating that asexual or sexual reproduction drives overall diversity and population structure within individual countries. Research has shown that asexual reproduction plays an important role in the development of disease during a growing season (Lamour & Hausbeck 2002). Although many studies have suggested that oospores are the main propagules of *P. capsici* survival *in vitro*, the effect of sexual reproduction on the population structure of *P. capsici* has been rarely investigated. In South America (i.e., Peru and Argentina) where clonal lineages survive multiple years and are spread widely (Hurtado-González et al. 2008; Hulvey et al. 2011). The situation is different in the USA and South Africa where *P.capsici* populations have a high level of genotypic and genic diversity presumably as a result of sexual reproduction (Dunn et al. 2010; Yin et al. 2012). In this study, we investigated the effects of asexual and sexual reproduction on the population structure of *P. capsici* using the ISSR technique. The results revealed that sexual reproduction promoted the diversity of *P. capsici*, giving rise to difficulties in disease control.

The reproductive pattern determines the variation and adaptability of a pathogen population and, affects the genetic diversity among individuals within the population, leading to different degrees of genotypic diversity. Mutation and mitotic recombination is the main source of variation in clones (Goodwin

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et al. 1995; Dobrowolski et al. 2003). In most cases, mutations do not cause significant phenotypic changes, but it is unlikely that all the genetic variations observed by molecular markers are due to mutations. Sexual reproduction is another potential cause of genetic variation. Since genetic diversity in clones is distributed among individuals, it is more meaningful to measure genotypic diversity than genetic diversity. In sexually reproducing populations, gene diversity often must be measured in terms ofenetic diversity. In this study, ISSR analysis determined that the genetic diversity of sexual progeny was significantly higher than that of asexual progeny, that is, sexual reproduction can rapidly increase the genetic diversity of *P. capsici* and promote the complexity of the pathogen population structure, and sexual reproduction significantly influenced this structure. The data reported here support the conclusions of previous studies. Previous studies have shown that both A1 and A2 mating types of *P. capsici* were detected in a single-pepper field in Anhui, China (Qi et al. 2012; Li et al. 2017). The co-occurrence of both A1 and A2 mating types in pepper fields increases the possibility of sexual reproduction, oospore production and genetic variation of *P. capsici* (Li et al. 2012; Castro-Rocha et al. 2016). A low level of genetic variation was found in Northwest Spain, Bulgaria, southern Italy and southeastern Spain, where only only one (A1 or A2) mating type has been reported (Heiser & Smith 1953; Ilieva & Vintanov 1980; Pennisi & Agosteo 1998; Silvar et al. 2006).

The ability of asexual spores to survive multiple years has a great influence on the genetic structure of pathogen populations and led the genetic homogeneity (Hurtado-Gonzales et al. 2008; Gobena et al. 2012). However, the clones of *P. capsici* are limited by time and space and differ by year or field (at least 1 km apart) (Lamour & Hausbeck 2002). For example, the sexual fruiting bodies or spores of many pathogens are the surviving structures for overwintering or over summer. At the beginning of each growing season, the pathogen population may be composed of a series of new genotypes. Both A1 and A2 isolates of *P. capsici* from the same site in the northwest of Anhui does not prove that sexual reproduction is occurring, it does mean that sexual reproduction is possible (Fry et al. 1992; Li et al. 2017). The combination of high levels of genetic variation, thick-wall oospores and the development of polycyclic asexual diseases make *P. capsici* a formidable pathogen. Once *P. capsici* has established itself in one place, it can be very difficult to control. For these diseases, limiting the occurrence of sexual reproduction and reducing the spread of asexual propagators can have certain prevention effects. In cultivation practice, the use of resistant varieties to control *P. capsici* may be difficult because populations maintain a high diversity (Lamour & Hausbeck 2002; Dunn et al. 2010). Limit water including planting in well-drained locations, planting on high beds, the use of carefully controlled irrigation (such as drip irrigation) are often the most effective way to limit the dissemination of *P. capsici*. Moreover, it is necessary to remove the diseases and residues to reduce the source of initial infection.

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