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Full Length Article

Genetic Diversity of *Cercospora arachidicola* Associated with Peanut Early Leaf Spot in Shandong Province of China

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Abstract

Early leaf spot caused by *Cercospora arachidicola* is the major destructive foliar disease of cultivated peanut in Shandong province of China. Understanding the genetic variability of this pathogen is crucial for evolutionary comprehension and breeding strategies. The genetic diversity of *C. arachidicola* isolates obtained from different peanut growing regions of Shandong province were assessed using inter-simple sequence repeats (ISSR) markers. Thirteen ISSR primers generating polymorphic, clearly discernible and reproducible patterns were screened out for further analysis. A total of 113 distinct bands were amplified, of which 85.8% were polymorphic, suggesting high values of polymorphism among the isolates. Cluster analysis using UPGMA indicated that all the isolates tested were separated into three distinct generic groups. The genetic relatedness of *C. arachidicola* isolates roughly coincided with geographical origin. Analysis of molecular variance (AMOVA) revealed that the observed genetic variation mainly existed within populations, and the variation among populations was weak. This study characterized the genetic diversity of *C. arachidicola* isolates for the first time, which will provide necessary genetic information for effective management practices. © 2021 Friends Science Publishers

Keywords: Gene flow; Genetic distance; Genetic variation; ISSR; Polymorphism

Introduction

Peanut (*Arachis hypogaea* L.), an important oilseed crop in tropical and subtropical regions of the world (Kumar and Kirti 2011), is widely cultivated for its high protein and oil content in seeds (Vasavirama and Kirti 2012; Kumar and Kirti 2015). Early leaf spot caused by *Cercospora arachidicola* is the major destructive foliar disease of cultivated peanut (Vasavirama and Kirti 2012) in Shandong production region of China. Symptoms of this disease progress from the formation of small necrotic lesions on the leaves, petioles, or stems to yellowing and finally premature defoliation (Melouk 1978). Yield losses up to 50% were common due to *C. arachidicola* infection without adequate control (Luo *et al*. 2005).

Understanding genetic diversity and population structure of plant pathogens is important because it will assist in providing fundamental information about its speciation, evolutionary comprehension, ecological studies and breeding strategies (Ghaffari *et al*. 2014). Moreover, the level of genetic variability within or among populations is crucial for species survival and adaptive capacity to changing eco-environments (Teixeira *et al*. 2014; Pirondi *et al*. 2015). Nevertheless, little is known about the genetic variation of *C. arachidicola*. Population genetic studies of pathogens are also essential for the development of effective disease control including resistant varieties, fungicide regimes and cultivation practices (Rampersad 2013). Therefore, the genetic diversity of *C. arachidicola* isolates could provide valuable references for developing improved disease management strategies in peanut fields.

Molecular markers have proven to be powerful tools for the characterization, relatedness and genetic variability of plant, animal and microbial species (Wolfe 2005; Yamada *et al*. 2016). Restriction fraction length polymorphism (RFLP) (Krishnamoorthy *et al*. 2015), amplified fragment length polymorphism (AFLP) (Onaga *et al*. 2015), random amplification of polymorphic DNA (RAPD) (Patricia *et al*. 2003), sequence-related amplified polymorphism (SRAP) (Mahmoud 2016) and ISSR have

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been widely applied to assess genetic diversity in plant pathogenic fungi. Among these molecular markers, ISSR is more sensitive, reliable and reproducible owing to higher annealing temperatures and longer primer sequences (Xu *et al*. 2013). This technique specifically amplifies regions of the genome flanked by two inverted microsatellite repeats (Xiao and Gong 2006). The ISSR makers have been successfully used in population genetic studies of a number of fungi (Mousavifard *et al*. 2014; Arif *et al*. 2015; Pasini *et al*. 2016). Therefore, ISSR is a helpful means for studies of genetic diversity in *C. arachidicola*.

The aim of this study was to assess the gene diversity of *C. arachidicola* isolates using ISSR markers, in order to determine the genetic variability within and among populations from different peanut growing areas in Shandong province of China. This study will provide valuable information for effective management practices.

Materials and Methods

Fungal isolates

Samples with characteristic *C. arachidicola* lesions were collected from different peanut growing areas of Shandong province in China. Single spores of the fungi were isolated from necrotic leaf spots and incubated on PDA medium at 25°C. In total, 42 *C. arachidicola* isolates were used for the present study. Reference isolates and their origins were listed in Table 1.

DNA extraction

We cultured the fungal mycelia in potato dextrose broth at 25°C for 3–5 d on an orbital shaker. Harvested mycelia were lyophilized by liquid nitrogen. Genomic DNA was extracted from lyophilized mycelia using the CTAB method (Doyle 1987). DNA concentrations were quantified by a spectrophotometer and DNA integrity was assessed by 1% agarose gel electrophoresis.

ISSR-PCR amplification

Ten representative isolates of *C. arachidicola* were selected to initially screen one hundred ISSR primers which have di- or tri- nucleotide repeats. And the primers that generated more clear and polymorphic amplified bands were used for subsequent work. PCR reactions were performed using TaKaRa PCR Amplification Kit (TaKaRa, China). The optimum annealing temperature was determined for individual primers (Table 2). DNA sequences were amplified in the Eppendorf Mastercycler using the following profile: denaturation step at 95°C for 10 min, following by 30 cycles of 30 s at 94°C, 30 s at a specific temperature for annealing, and 1 min extension at 72°C, and then 10 min at 72°C for final extension. The amplified bands were analyzed using Bio-Rad ChemiDoc MP imaging system. All PCR amplifications were repeated twice for each isolate to confirm their reproducibility.

Data analysis

The ISSR-amplified fragments were converted into a binary matrix (1 and 0) for statistical analysis. The observed number of alleles (*Na*), effective number of alleles (*Ne*), Nei's gene diversity (*He*), Shannon's information index (*I*), gene flow (N_m), Nei's population differentiation (G_{ST}), genetic identity and genetic distance were performed by POPGENE 1.32. We generated UPGMA dendrogram using NTSYS-PC 2.10e. AMOVA was assessed to analyze genetic diversity indices associated with different geographical locations, which was performed using AMOVA 15.5 for Windows.

Results

Genetic diversity

To evaluate the genetic diversity of the 42 samples, a total of 100 UBC ISSR primers were screened using ten representative strains of *C. arachidicola* and finally 13 ISSR primers (Table 2) that produced polymorphic, clearly discernible and reproducible patterns were selected for further study. The ISSR amplification using 13 selected primers produced a total of 113 distinct bands for 42 strains. An average of 8.7 bands per primer was shown, with the minimum of 6 (864) and the maximum of 11 (808 and 809) (Table 2). The polymorphism rate ranged from 66.7% (864) to 100% (890), with a mean of 85.8% polymorphism (Table 2). Clearly detectable PCR fragments ranged from 150 to 3000 bp in size.

At the population level, *Na* was estimated to be 1.2983 and *Ne* was 1.1970. Polymorphism existed among different populations and the genetic diversity parameters were shown in Table 3. *He* ranged from 0.0762 to 0.1367, with an average of 0.1145, and *I* were calculated from 0.1148 to 0.2032, with a mean of 0.1694 (Table 3). The highest level of genetic diversity was presented in the population of YT, whereas the lowest level of genetic diversity was shown in JN population. The results of AMOVA indicated that the genetic variation values were statistically significant ($P<0.001$), 30.18% among the populations and 69.82% within populations (Table 4).

Genetic relationship

To ascertain the relationships among *C. arachidicola* isolates selected for the present study, the UPGMA tree was performed based on Nei's genetic distance matrices. The results of cluster analysis demonstrated that all the strains tested were divided into three distinct groups (Fig. 1). Most of the isolates from the same region clustered together in UPGMA tree. In this analysis, populations from JN, TA and

Table 1: *Cercospora arachidicola* isolates used in the present study

Isolate No.	Geographic origin
C703, C724, C732, C735, C765, C766	Ji'nan (JN)
C520, C544, C551, C555, C567, C568	Tai'an (TA)
C130, C133, C151, C159, C193, C235	Weihai (WH)
C7, C21, C56, C78, C89, C90	Yantai (YT)
C293, C295, C296, C341, C346, C367	Linyi (LY)
C411, C423, C473, C489, C491, C502	Rizhao (RZ)
C821, C822, C836, C864, C881, C890	Dongying (DY)

Table 2: Features of ISSR primers

Primer	Primer motif	Annealing temperature (℃)	No. of amplified loci	No. of polymorphic loci	Percentage polymorphism (%)
807	(AG)nT	52.2	9	8	88.9
808	(AG)nC	54.6	11	9	81.8
809	(AG)nG	54.6	11	10	90.9
810	(GA)nT	52.2	8	7	87.5
811	(GA)nC	54.6	9	7	77.8
816	(CA)nT	52.2	9	8	88.9
823	(TC)nC	54.6	5	4	80.0
836	(AG)nYA	53.9	10	8	80.0
864	(ATG)n	42.6	6	4	66.7
888	BDB(CA)n	53.8	9	8	88.9
889	DBD(AC)n	53.0	10	9	90.0
890	VHV(GT)n	53.0	7	7	100.0
891	HVH(TG) n	52.6	9	8	88.9

B = C, G, or T; D = A, G, or T; H = A, C, or T; V = A, C, or G.

Table 3: Genetic diversity of *Cercospora arachidicola* populations

Population	*Na*	*Ne*	*He*	*I*
JN	1.2124	1.1257	0.0762	0.1148
TA	1.2212	1.1345	0.0806	0.1209
WH	1.3363	1.2207	0.1283	0.1901
YT	1.3628	1.2328	0.1367	0.2032
LY	1.3274	1.2246	0.1288	0.1894
RZ	1.3363	1.2225	0.1288	0.1906
DY	1.2920	1.2184	0.1219	0.1767

Na, number of different alleles; *Ne*, effective number of alleles; *He*, Nei's gene diversity; *I*, Shannon's information index

Table 4: Analysis of molecular variance (*AMOVA) within and among Cercospora arachidicola* populations

Source	d.f.	Sum of squares	Variance components	Percentage variation	P value
Among populations	6	167.381	3.356	30.18%	<0.001
Within populations	35	271.667	7.762	69.82%	<0.001
Total	41	439.048	11.118	100%	

DY clustered together, population from LY clustered closely with population from RZ, while populations from WH and YT formed a cluster. The dendrogram obtained from UPGMA roughly coincided with the geographical distribution of these populations. Matrices of genetic identities and genetic distances were calculated from pairwise comparisons based on ISSR analysis. The results indicated that the genetic identity values ranged from 0.8919 (RZ/DY) to 0.9948 (WH/YT) (Table 5). In contrast, the genetic distance values varied from 0.0052 (WH/YT) to 0.1144 (RZ/DY) (Table 5). This phenomenon was congruent with the results of ISSR cluster analysis.

Gene flow and genetic differentiation

The gene differentiation coefficient (G_{ST}) calculated to assess the genetic differentiation among different populations was 0.3812, indicating 38.12% of the total variation. This result showed moderate genetic heterogeneity according to Wright's qualitative guideline. The gene flow (N_m) values among different populations were estimated based on G_{ST} (Table 6). *N*m ranged from 1.0246 (JN/WH) to 8.0448 (WH/YT).

Discussion

Shandong is the main peanut-growing province in China, and the peanut production amounted to 2,500 kilotons, covering acreage up to approximately 800,000 hectares. However, at the late stage of peanut growth and development, peanut plants are infected by leaf spots which evidently reduce the yields. Genetic diversity of plant pathogen populations present in a particular locality is especially valuable to enhance epidemiological studies and

Table 5: *Pairwise Nei's genetic identity and genetic distance among Cercospora arachidicola* populations

Population	JN	TA	WH	YT	LY	RZ	DY
JN	****	0.9823	0.8986	0.9045	0.9133	0.9027	0.9485
TA	0.0179	****	0.9121	0.9181	0.9168	0.9079	0.9344
WH	0.1069	0.0920	****	0.9948	0.8938	0.8984	0.8945
YT	0.1004	0.0855	0.0052	****	0.8922	0.8963	0.8935
LY	0.0907	0.0869	0.1123	0.1140	****	0.9804	0.9016
RZ	0.1023	0.0966	0.1072	0.1095	0.0197	****	0.8919
DY	0.0529	0.0679	0.1115	0.1126	0.1036	0.1144	****

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

Table 6: Gene flow (N_m) and Nei's population differentiation (G_{ST}) among populations of *Cercospora arachidicola*

Population	JN	TA	WH	YT	LY	RZ	DY
JN	****	3.3579	1.0246	1.1247	1.1813	1.0665	1.7911
TA	0.1296	****	1.1905	1.3155	1.2529	1.1452	1.4928
WH	0.3279	0.2958	****	8.0448	1.2482	1.2978	1.2206
YT	0.3078	0.2754	0.0585	****	1.2736	1.3171	1.2524
LY	0.2974	0.2852	0.2860	0.2819	****	4.5172	1.3010
RZ	0.3192	0.3039	0.2781	0.2752	0.0997	****	1.1972
DY	0.2182	0.2509	0.2906	0.2853	0.2776	0.2946	****

Above diagonal was gene flow (N_m), below diagonal was genetic differentiation (G_{ST}).

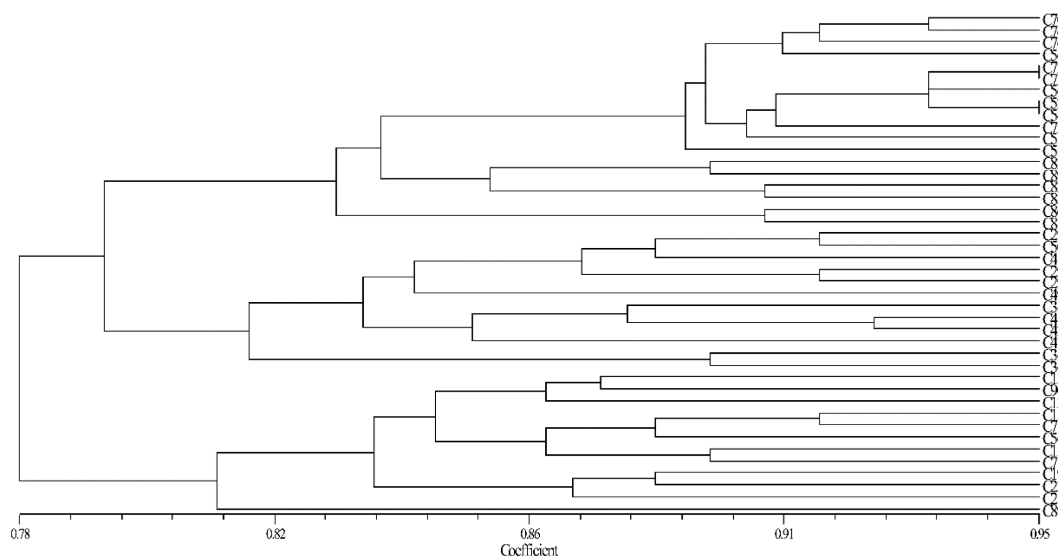

Fig. 1: UPGMA clustering of 42 *Cercospora arachidicola* isolates based on Nei's genetic distance

implement effective control strategies (Lurá *et al*. 2011). Although more has been learned about the genetic diversity in many plant pathogenic fungi, so far, no exhaustive knowledge about that has been available within *C. arachidicola* isolates. The genetic diversity of *C. arachidicola* isolates obtained from different areas of Shandong was characterized first based on ISSR molecular marker.

ISSR analysis which has been proved to be effective for population genetics could detect a higher polymorphism in comparison with other molecular markers (Gramaje *et al*. 2014). In previous studies, high polymorphisms were obtained based on ISSR markers (Rampersad 2013). In the present study, a total of 42 *C. arachidicola* isolates collected from Shandong province were subjected to the analysis of genetic diversity. Thirteen primers were screened out for ISSR analysis. And the results of ISSR for seven populations indicated high values of genetic diversity, with 85.8% polymorphisms. The selection of environmental habitat determines the population structure of pathogenic fungi (Wang *et al*. 2005). According to climatic conditions, terrain characteristics, soil types and cropping systems, Shandong peanut planting regions were divided into the eastern hilly area, the middle-southern mountain area, the western plain area and the northern plain area. The various ecological environments may contribute to the presence of high genetic variation. Weihai and Yantai located in the eastern hilly area which is the major peanut-producing regions of Shandong. Thus, the higher levels of genetic diversity in WH and YT populations may due to the wide cultivating areas in this region.

In this study, the cluster analysis obtained from ISSR markers indicated that all the isolates tested were separated into three distinct generic groups. In general, the genetic relatedness of *C. arachidicola* isolates was associated with geographical origin. Most of the isolates from the same region clustered together in UPGMA tree. Our findings coincide with those of the previous study which suggested that there exists a positive correlation between the genetic relationships and the geographical distance, and that geographical isolation represent a barrier to genetic exchanges among populations (Lamour and Hausbeck 2001). The results of AMOVA revealed that the observed genetic variation mainly existed within populations, and the variation among populations was weak. High levels of gene flow and low levels of genetic differentiation were found among *C. arachidicola* populations. Garant *et al*. (2007) indicated that N_m plays a vital role in genetic differentiation and diversity of species. A high level of N_m between Weihai and Yantai was shown in the present study, which indicated frequently genetic exchange among the two populations.

The reproductive mode has a major impact on the transmissibility and persistence of a pathogen, and thus influences population genetic structure (Rampersad 2013). Sexual recombination plays a large part in generating highly variable genomes (Lu *et al*. 2004). No sexual reproduction has been found for *C. arachidicola* yet, thus the origin of wide genetic variability which was assessed by ISSR markers is unclear. However, the effects of mutation or migration would be required to account for such genotype diversity (Mahuku *et al*. 2002). Groenewald *et al*. (2006) suggested that sexual reproduction may be active in some *Cercospora* species. For *C. arachidicola*, the possibility of infrequent sexual cycle cannot be rule out, even though the teleomorph heretofore has not been sought. Therefore, hybridization may be another potential explanation for genetic variation.

The high level of genetic diversity among isolates as identified in the present work indicates that *C. arachidicola* has a large evolutionary potential which was supposed to help overcome management strategies over time (Li *et al*. 2012). Therefore, it becomes quite necessary to understand the genetic variability of the pathogen in peanut fields for developing improved disease management practices including sustaining the durable nature of resistant cultivars as well as effective bio-agents.

Conclusion

Early leaf spot is the major destructive foliar disease of cultivated peanut in Shandong province which is one of the main production areas of peanut in China. In the present study, high values of genetic diversity were presented among *C. arachidicola* populations. The genetic relatedness of *C. arachidicola* isolates was associated with geographical origin. Moreover, the observed genetic variation mainly existed within populations, and the variation among populations was weak.

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Author Contributions

YG, LGM and XGL planned the experiments, FG, SY and JLZ interpreted the results, YG, SBW and JGW made the write up, JJM and ZHT statistically analyzed the data and made illustrations.

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