academicJournals

Vol. 7(27), pp. 3556-3558, 5 July, 2013
DOI: 10.5897/AJMR2012.2336
ISSN 1996-0808 ©2013 Academic Journals
http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Short Communication

Development of polymorphic microsatellite markers from *Verticillium dahliae*, a pathogenic fungus of cotton

Li Zhi-Fang, Zhu He-Qin*, Feng Zi-Li, Zhao Li-Hong, Shi Yong-Qiang, and Yin Zhi-Xin

State Key Laboratory of Cotton Biology, Institute of Cotton Research of Chinese Academy of Agricultural Sciences, Anyang, Henan, 455000, China.

Accepted 10 May, 2013

Thirteen polymorphic microsatellite loci originating from the genome sequence were developed for cotton pathogenic fungus, *Verticillium dahliae*. The simple sequence repeats (SSR) markers were used to characterize the genotypic diversity of 30 isolates of *V. dahliae*. Thirteen loci displayed the number of alleles per locus (Na) ranged from 2 to 5 with an average of 3 and effective number of alleles ("NE") with a broader range (1.80 to 3.91). Unbiased expected heterozygosity ("HE") showed moderate to high levels of variation (0.43 to 0.74). Thirteen primer pairs rendered provide a new scope for population genetic analysis of *V. dahliae* on cotton.

Key words: Microsatellites, *Verticillium dahliae*, genotypic diversity, cotton Verticillium wilt.

INTRODUCTION

Verticillium dahliae Kleb. is the causal agent of vascular wilt that is a destructive disease of cotton crop, causing serious loses in most of the cotton-growing countries, including China (Xia et al., 1998). The resting structures of *V. dahliae*, called microsclerotia, could survive for several years in the absence of a host plant (Schnathorst, 1981). Isolates of *V. dahliae* on cotton exhibit different pathotypes by means of morphological and physiological traits as well as by virulence tests on different cotton cultivars (Bejarano-Alc´azar et al., 1996). It is obvious that *V. dahliae* on cotton exhibits great phenotypic as well as genetic diversity. Therefore it is necessary to find out effective tools for genetic plasticity analysis of this serious pathogen of cotton crop.

Although, random amplified polymorphic DNA (RAPD) (Perez-Artes et al., 2000) and amplified fragment length polymorphic (AFLP) (Collins et al., 2003) markers have been used to characterize molecular diversity in the populations of *V. dahlia*, dominance of RAPD and AFLP markers prevent the detection of heterozygotes in diploid species. Development of co-dominant, multi-allelic and sequence-specific microsatellite markers may enable the molecular genotypes at a number of loci to be determined, which can be more widely utilized for genetic diversity and genetic mapping of *V. dahliae*. Studies on the population structure of the *V. dahliae* on cotton have been limited partly because of a lack of useful and stable genetic markers. In this note, the development of thirteen simple sequence repeat markers for *V. dahliae* is reported.

MATERIALS AND METHODS

The genome sequence of *V. dahliae*, which can be downloaded at: http://www.broadinstitute.org/annotation/genome/verticillium_dahliae/MultiDownloads (Klosterman et al., 2011), was used for simple sequence repeat (SSR) exploring. A total of 5,418 SSR loci were identified in the genome of *V. dahlia*. The genome was scanned for search and investigation of SSRs loci with SciRoKo software (Li et al., 2012; Kofler et al., 2007). Abundance was removed after screening the whole genomic DNA. Primer pairs were designed from the flanking sequences to amplify the targeted SSR loci, using PRIMER3 software (Rozen and Skaletsky, 2000). The targeted amplicon size was set as 100-250bp, while the optimal annealing

*Corresponding author. E-mail: zhuheqin2012@163.com. Fax: +86-03722562280.

Abbreviations: **RAPD**, Random amplified polymorphic **DNA**; **AFLP,** amplified fragment length polymorphic; **SSR,** simple sequence repeat; **PCR,** polymerase chain reaction; **Na**, number of alleles; N**E**, effective number of alleles; **HO**, observed heterozygosities; **HE**, expected heterozygosities; **HWE,** Hardy–Weinberg equilibrium; **LD**, linkage disequilibrium; **NE**, effective number of alleles.

Table 1. Primer sequences and characteristics of 13 *Verticillium dahliae* microsatellites markers.

Genbank accession no.	Locus	Primer sequence (5'-3')	Repeat motif	Size (bp)	Na	N_E	H_O	HE
JX122843	VdS17	F:AGGTCATGTTGAGGCGTGGT R:GGAGTCTCTATCGTCGTCGCATC	$(AG)_6$	176	3	2.87	0.64	0.65
JX122844	VdS21	F:GGACTTGGACATGGTGATGGAT R:CTTCCCTCCCTCACTTCTCTCAC	$(TG)_{12}$	158	4	3.91	0.49	0.74
JX122845	VdS39	F:GTGTCCCCTTGCTTTTGATCTCT R:GGGGACCAAGAACACTTTCACATA	$(TC)_{28}$	138	3	1.95	0.63	0.49
JX122846	VdS65	F:CCTGACAAGTACATCAGCAACGAT R:CGAGGTCATTACCAACATTACCTG	$(AG)_{12}$	141	2	1.80	0.48	0.44
JX122847	VdS68	F:ATCTGCACTTGCGTTTTATACGGT R:ACATCGCCTTCCTCCTTCATAC	$(AG)_{22}$	128	5	3.75	0.49	0.73
JX122848	VdS111	F:CTCGAAGCTGTCTGGCTTGAGAT R: GGCATCCATCTCTTCGATTTCGTA	(TTC)24	136	2	1.92	0.45	0.48
JX122849	VdS117	F:TTATCATCCCAGGACCATCCTAGA R:GTGAGCATGGCTGAGCTGGT	$(ATG)_{22}$	139	5	2.33	0.76	0.57
JX122850	VdS120	F:GGCGTCGGTCTACAGTATTACCAT R:ATTTTCACGCACTGTTCATGATTG	$(AG)_{14}$	216	2	1.87	0.49	0.46
JX122851	VdS127	F:GAGCTCAATGACGATTGCTTTTCT R:GTACATAGTACCTCGCAGTGGCCT	$(TG)_{18}$	152	2	1.90	0.48	0.43
JX122852	VdS137	F:GAAGTGCAAGACGAAACAGGTTG R:CTACACCAAATCGTCAGCCTTTG	$(AAGC)_{20}$	159	4	3.40	0.50	0.71
JX122853	VdS214	F:CTATGACTGCTGTCAGCCACATCT R:GTCTATGCGGTGAGTTGCACCC	$(ACG)_{20}$	156	2	1.91	0.48	0.48
JX122854	VdS231	F:AGATGGATCGAGGAGAGATGTGAT R:ATTTACCGACACCATCTCAGTGCT	$(TCC)_{19}$	142	3	1.84	0.64	0.46
JX122855	VdS244	F:AATCGGTGCAGACCCACAAG R:GTCGTCACTGATTCTCCGGCT	(CG)26	158	4	2.12	0.72	0.53

F, Forward primer; R, reverse primer; Na, number of alleles; NE, effective number of alleles; HO, observed heterozygosities; HE, expected heterozygosities.

temperature as 64°C. According to the SSR distribution and present frequency in the genome, three hundred primer pairs randomly targeted selected were used to test the phenotypic diversity of *V. dahliae* on cotton. The related PCR products contained mono-, di-, tri- and tetra-SSRs. The total of thirty isolates representing three different populations from Yangtze River Basin (10), Yellow River Basin (10) and Xinjiang Region (10) were screened for polymorphisms.

Polymerase chain reaction (PCR) reactions were carried out in a 10 µl volumes containing 1× PCR buffer (10 mM Tris-HCl pH 8.4, 50 mM KCl, and 2 mM MgCl2), 100 µM each dNTP, 0.02 µM Forward and Reverse primers, and 1 U of Taq DNA polymerase. Approximately, 10 ng of genomic DNA was used in each reaction. The reagents for PCR amplification were obtained from TAKARA Biotechnology (DaLian) CO. LTD. Primers were tested using TOUCHDOWN thermal cycling programs encompassing a 10°C span of annealing temperatures ranging between 65-55°C. Cycling parameters were: an initial denaturing step of 3 min at 95°C, followed by ten cycles of 30 s at 94°C, 30 s at the highest annealing temperature (annealing temperature was reduced by 1°C per cycle), 45 s at 72°C, followed by 30 cycles of 30 s at 94°C, 30 s at 50°C (for 60-50°C touchdown range), 45 s at 72°C, and a final extension time of 10 min at 72°C. PCR product was checked on 6% denatured acrylamide gel with a LI-COR 4200 DNA sequencer. Data were scored using SAGA software (LI-COR) and allele sizes were determined using IRDye 700 size standards (50–350 bp, LI-COR). The genetix software (Belkhir et al., 1996) was used to determine the number of alleles (Na) per locus and effective number of alleles (NE). Observed (HO) and expected (HE) heterozygosities were calculated with arlequin 3.0 (Excoffier et al., 2005).

Observed heterozygosity was calculated as the fraction of individuals that were heterozygous at a given locus and expected heterozygosity was calculated as $1-\sum n\ i=1pi2$; where n is the number of alleles at the locus and pi is the frequency of the ith allele. Tests of significant deviation from Hardy–Weinberg equilibrium (HWE) and linkage disequilibrium (LD) were performed using genepop version 3.4 (Raymond and Rousset, 1995).

RESULTS AND DISCUSSION

Out of 300 candidate primer pairs, 13 polymorphic microsatellites primer pairs were selected based on successful amplifications and evidence of polymorphism. One pair of primers represented one simple sequence repeats locus. To test the potential use of the 13 microsatellite loci for genotypic diversity, we screened 30 *V. dahliae* isolates collected from *Verticillium* wilt affected cotton of Yangtze River Basin, Yellow River Basin and Xinjiang Region in China. Results showed that dinucleotide repeats loci were in the majority which contained eight ones, while three loci contained trinucleotide repeats and one was tetranucleotide repeats motif (Table 1). The number of alleles (Na) per locus ranged from two to five with an average of three. Polymorphic information content (PIC), which is also called as Simpson diversity index (1949),

represented moderate to high levels of variation from 0.43 to 0.74. Thirteen locus displayed effective number of alleles (NE) with a broad range from 1.80 to 3.91. The HE represented moderate to high levels of variation from 0.43 to 0.74. Observed heterozygosities also presented moderate to high levels of variation from 0.45 to 0.76. The loci were not in linkage disequilibrium, and only one marker (VdS244) showed evidence of deviation from Hardy–Weinberg expectation ($P < 0.05$).

Among these thirteen loci, VdS117 exhibited highest polymorphic diversity which had 5 alleles and effective number was 2.33, HE was 0.57 and HO genotypic diversity index was 0.76. Locus VdS21 also showed high polymorphic diversity, whose Ne, HE, Ne and HO were 3.91, 0.74 and 0.49, respectively (Table 1). Absence of an amplicon was scored as a null allele when the same results were obtained in at least two separate PCR, and the sample DNA and simple sequence repeat primer pair both gave positive results in control experiments using different primer/DNA combinations.

Based on our results, the different degree of polymorphism markers will be useful in polymorphic research including analysis of individual relatedness and parentage analysis.

The development of 13 primer pairs, mentioned above, is proved to be valuable extra tools for studying the population genetic diversity and evolution of the cotton pathogen *V. dahliae*. They also have the potential to strengthen the theory of *Verticillium* disease epidemiology caused by *V. dahliae*, such as gene flow, diversity structure of specific period or region, and distribution patterns worldwide.

ACKNOWLEDGEMENTS

This work was supported by “National Science and Technology Supporting Planed Project (2011BAD35B09)”. We are very grateful to Dr Muhammad Ibrahim Khaskheli for his comments and critical reading of the manuscript.

REFERENCES

Belkhir K, Borsa P, Goudet J, Bonhomme F (1996). GENETIX, Logiciel sous Windows pour la Génétique de Population. Laboratoire Génome et Populations. CNRS UPR 9060, Université de Montpellier II, Montpellier.

Bejarano-Alc´azar J, Blanco-L´opez MA, Melero-Vara JM, Jim´enez-D´iaz RM (1996). Etiology, importance, and distribution of Verticillium wilt of cotton in southern Spain. Plant Dis. 80:1233-1238.

Collins A, Okoli CAN, Morton A, Parry D, Edwards SG, Barbara DJ (2003). Isolates of Verticillium dahliae pathogenic to crucifers are of at least three distinct molecular types. Phytopathology 93:364-376.

Excoffier L, Laval G, Schneider S (2005). Arlequin ver. 3.0: an integrated software package for population genetics data analysis. Evol. Bioinformatics 1:47-50.

Klosterman SJ, Subbarao KV, Kang S, Veronese P, Gold SE, Thomma BPHJ, Zehua Chen ZH, Henrissat B, Lee YH, Park JS, Garcia-Pedrajas MD, Barbara DJ, Anchieta1 A, Jonge RD, Santhanam P, Maruthachalam K, Atallah Z, Amyotte SG, Paz Z, Inderbitzin P, Hayes RJ, Heiman DI, Young S, Zeng QD, Engels R, Galagan J, Cuomo CA, Dobinson KF, Ma LJ (2011). Comparative genomics yields insights into niche adaptation of plant vascular wilt pathogens. PLoS Pathog. 7:e1002137.

Kofler R, Schlö tterer C, Lelley T (2007). Sci RoKo: a new tool for whole genome microsatellite search and investigation. Bioinformatics 23:1683-1685.

Li ZF, Zhu HQ, Zhao LH, Feng ZL, Shi YQ, Yang JR (2012). Frequency and distribution of genome-based microsatellites in Verticillium dahliae. Afr. J. Microbiol. Res. 6(49): 7504-7511.

Perez-Artes E, Garcia-Pedrajas MD, Bejarano-Alcazar J, Jimenez-Diaz RM (2000). Differentiation of cotton-defoliating and nondefoliating pathotypes of Verticillium dahliae by RAPD and specific PCR analyses. Eur. J. Plant Pathol. 106: 507-517.

Raymond M, Rousset F (1995). Genepop: population genetics software for exact tests and ecumenicism. J. Heredity 86:248-249.

Rozen S, Skaletsky HJ (2000). Primer 3 on the WWW for general users and biologist programmers. In: Bioinformatic Methods and Protocols (eds Krawetz S, Misener S), 365-386. Humana Press, Totowa, New Jersey.

Schnathorst WC (1981). Verticillium wilt. In: Compendium of cotton disease. Watkins GM, eds. St. Paul, MN: Am. Phytopathol. Soc. pp. 41-44.

Xia Z, Achar PN, Gu B (1998). Vegetative compatibility groupings of Verticillium dahliae from cotton in mainland China. Eur. J. Plant Pathol. 104:871-876.