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MICROSATELLITE MARKERS FOR *ALEUROCANTHUS SPINIFERUS* (HEMIPTERA: ALEYRODIDAE) AND THEIR POTENTIAL USE IN WHITEFLIES

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Abstract

The citrus (or orange) spiny whitefly, *Aleurocanthus spiniferus* (Quaintance) (Hemiptera: Aleyrodidae), is an important pest of various economic crops such as citrus and tea, causing severe economic losses. However, the population genetics of *A. spiniferus* is poorly understood, both in China and in other countries. To improve our knowledge of the population structure and genetic variation of this species, 10 polymorphic microsatellite loci for *A. spiniferus* were developed and characterized using FIASCO (Fast Isolation by AFLP of Sequences Containing Repeats). Our results showed that the polymorphic information contents (PIC) of all 10 loci were greater than 0.5, showing a high degree of polymorphism. The number of alleles ranged from 12 to 27 across 60 individuals from 6 populations. In addition, the expected heterozygosity (H_E) and the observed heterozygosity (H_O) ranged from 0.851 to 0.958 and from 0.621 to 0.850, respectively. Interestingly, all loci deviated from the Hardy-Weinberg equilibrium, although - according to our study - they were not affected by the species' specific reproductive strategy, and thus must be related to other unknown factors. Furthermore, linkage disequilibrium analysis revealed that C19-2 and D14, and also I20 and F12 showed linkage disequilibrium. Cross-species amplification was also tested in 5 closely related whitefly species (*Aleurodicus disperses* Russell, *Pealius mori* (Takahashi), *Aleuroclava aucubae* (Kuwana), *Bemisia tabaci* (Gennadius) MEAM1 and *B. tabaci* MED) in this study. Nine pairs of primers were successfully amplified at different levels except for F12. In particular, A21-2, D14, F13-2, and F42-2, coupled with I20 were successfully amplified across all the above species. Consequently, the 10 loci identified here can be used to study the population genetic structure of *A. spiniferus* and other closely related whitefly species.

Key Words: *Aleurocanthus spiniferus*, disequilibrium population genetic structure, genetic variation, microsatellite, cross-species amplification

Resumen

La mosca blanca espinosa de los cítricos (o naranja), *Aleurocanthus spiniferus* (Quaintance) (Hemiptera: Aleyrodidae), es una plaga importante de diversos cultivos económicos como los cítricos y el té, que causa graves pérdidas económicas. Sin embargo, la genética de las poblaciones de *A. spiniferus* es poco conocida, tanto en China como en otros países. Para mejorar nuestro conocimiento de la estructura de la población y la variación genética de esta especie, se desarrollaron y caracterizaron 10 loci microsatélites polimórficos para *A. spiniferus* utilizando FIASCO (Aislamiento Rápido por AFLP de Secuencias que Tienen Repeticiones, ARASTR). Nuestros resultados mostraron que el contenido de información polimórfica (CIP) de todos los 10 loci fueron mayor de 0.5, mostrando un alto grado de polimorfismo. El número de alelos varió de 12 a 27 a través de 60 individuos de 6 poblaciones. Además, la heterocigosidad esperada (H_E) y la heterocigosidad observada (H_O) oscilaron 0.851 a 0.958 y 0.621 a 0.850, respectivamente. Curiosamente, todos los loci se desviaron del equilibrio de Hardy-Weinberg, aunque de acuerdo a nuestro estudio ellos no fueron afectados por la estrategia reproductiva específica de la especie, y por lo tanto deben estar relacionados con otros factores desconocidos. Además, el análisis de desequilibrio de ligamiento reveló que C19-2 y D14, y también I20 y F12 mostraron desequilibrio de ligamiento. La amplificación de cruzar-especie también fue probada en 5 especies de mosca blanca estrechamente relacionadas (*Aleurodicus dispersus* Russell, *Pealius mori* (Takahashi), *Aleuroclava aucubae (*Kuwana), *Bemisia tabaci* (Gennadius) MEAM1 y *B. tabaci* MED) en este estudio. Se amplificaron con éxito nueve pares de cebadores a diferentes niveles con la excepción de F12. En particular, se amplificaron con éxito A21-2, D14, F13- 2, y F42-2 junto con I20 a través de todas las especies anteriores. En consecuencia, los 10 loci identificados aquí pueden ser utilizados para estudiar la estructura genética de la población de *A. spiniferus* y otras especies de mosca blanca estrechamente relacionadas.

Palabras Clave: *Aleurocanthus spiniferus*, desequilibrio estructura genética de la población, variación genética, microsatélites, amplificación entre especies

The citrus spiny whitefly *Aleurocanthus spiniferus* (Quaintance) (Hemiptera: Aleyrodidae), is a pest of citrus (*Citrus* spp.; Rutaceae) and tea (*Camelia sinensis* (L.) Kuntze; Ericales: Theaceae), and several serious outbreaks of a tea-infesting whitefly in China and Japan have been attributed to this species over the last 20 yr (Kanmiya et al. 2011). The citrus spiny whitefly affects host plants by removal of plant sap, but whiteflies also cause indirect damage by producing honeydew, which promotes sooty mold. In addition to citrus and tea, this whitefly infests many other host plants, as noted in Evans (2008). Furthermore, *A. spiniferus* is an EPPO (European and Mediterranean Plant Protection Organization) quarantine species that has recently been moved from the A1 to the A2 list (Anonymous 2011). It is worth mentioning that commonly *A. spiniferus* reproduces sexually and infrequently by arrhenotoky (Huang et al. 1999), which may influence the Hardy-Weinberg equilibrium (see Discussion).

Because *A. spiniferus* is a well-known insect pest, some aspects of its biology, behavior, ecology, and management have been thoroughly investigated (Van den Berg et al. 2000; Han and Cui. 2002; Guo et al. 2006; Muniappan et al. 2006; Peng et al. 2010; Niu et al. 2014). In contrast, little is known about the genetic diversity and population structure of *A. spiniferus*, and no microsatellite markers have been developed for this species. Fu & Han (2007) preliminarily analyzed the genetic diversity of *A. spiniferus* from 7 populations in eastern China using random amplified polymorphic DNA (RAPD). Kanmiya et al (2011) proposed new specific status for tea- infesting populations of the nominal citrus spiny whitefly *A. spiniferus* using molecular marker (mtCOI gene), morphological and acoustic analysis to distinguish it from *A. spiniferus* that constitutes the citrus-infesting population. However, genetic diversity for *A. spiniferus* has not been comprehensively investigated, and it is poorly understood. Such gaps in our knowledge highlight the need for the development of highly polymorphic and informative molecular markers in order to accurately assess and monitor genetic variation in and population structure of *A. spiniferus* populations.

Microsatellite DNA, also known as simple sequence repeats (SSRs), is a technique that has been widely used in many areas of research (Carleton et al. 2001; Peterlunger et al. 2003). In the field of entomology, it has been used for gene mapping, population genetic structure analysis, identification of genetic relationships, and genetic map constructions (Gorman 1997; Zenger et al. 2005). In this study, we developed 10 high polymorphic microsatellite markers for *A. spiniferus*, and cross-species amplifications were also conducted of the 5 closely related whitefly species (*Aleurodicus disperses* Russell, *Pealius mori* Takahashi, *Aleuroclava aucubae* Kuwana, *Bemisia tabaci* (Gennadius) MEAM1 and *B. tabaci* MED). These markers are expected to be used widely in studies on the genetic diversity and population genetic structure of whitefly species in their native and invasive ranges and in tracing their global invasion history.

Materials and Methods

Aleurocanthus spiniferus samples were collected from tea plants in 6 provinces: Shandong (N 34.73° E 117.29°), Jiangsu (N 32.41° E 119.42°), Zhejiang (N 30.19 ° E 120.09°), Hunan (N 28.20° E 113.09°), Guizhou (N 27.66° E 107.60°), and Yunnan (N 25.13° E 102.75°) in China. At first, we did not identify the gender of the 60 *A. spiniferus* individuals, until we noticed that all 10 study loci deviated from Hardy-Weinberg equilibrium. To ensure the accuracy of our results, another 48 female individuals from Jiangsu (N 32.41° E 119.42°) and Sichuan (N 30.57° E 104.07°) provinces were tested later. Gender was determined as per Dubey & Ko (2012). All specimens (including those from the 5 closely related whitefly species) were preserved in 100% ethanol and stored at -20 °C until DNA extractions were performed.

Genomic DNA was extracted using the salting-out method as described by Teng et al. (2009). The enriched library was constructed by FIASCO (Fast Isolation by AFLP of Sequences Containing Repeats) protocol according to Zane et al. (2002) with slight modifications in terms of PCR amplification conditions. The genomic DNA was first digested with the restriction enzyme *Mse*I (BioLabs, Beijing, China) and approximately 250 ng of DNA was ligated to 1μg *Mse*I adaptor (5'-TACTCAGGACTCAT-3' /5'-GACGATGAGTCCTGAG - 3'). The digestion-ligation products was diluted (1:10) and amplified with adaptor-specific primers (5'-GATGAGTCCTGAGTAAN-3', *Mse*I-N) in 20 µL reactions containing 1 × PCR buffer (10 mM Tris-HCl, pH 9.0, 25 °C; 50 mM KCl), $MgCl_2$ 1.5 mM, dNTPs 250 µM, *Mse*I-N 0.5 µM, 1 U of *Taq* DNA polymerase (TaKaRa, Dalian, China) and 5 µL diluted digestion-ligation DNA. The PCR conditions were 5 min at 94 °C followed by 20 cycles of 30 s at 94 °C, 1 min at 53 °C, 1 min at 72 °C with a final extension of 10 min at 72 °C. The PCR products were hybridized with biotinylated $(AG)_{12}$ and $(GT)_{12}$ for 1 h at 68 °C, respectively, after denaturation for 5 min at 95 °C. The DNA fragments that hybridized to biotinylated probes were selectively captured by streptavidin-coated magnetic beads (Streptavidin Magnesphere Paramagnetic Particles, Promega, Shanghai, China). Microsatellite-rich fragments were amplified by *Mse*I-N primers for 35 cycles and cloned into an *Escherichia coli* strain (DH5α) using pGEM-T Easy vectors (Promega). Insert-positive bacterial clones identified by blue-white selection were amplified using M13 primers and visualized by agarose gel electrophoresis. A total of 132 positive clones were chosen for sequencing.

Because the flanking regions of some sequences were too short, and only 41 pairs of primers were designed and synthesized in the end. In addition, we attached FAM, HEX and TAMRA fluorophores at the 5' ends of each forward primer. PCR was conducted in 25 µL volumes containing PCR buffer, 1.5 mM MgCl2, 200 µM of each dNTP, 50 ng genomic DNA, 0.75 U *Taq* polymerase, and 4 pmol of primer. Amplification included an initial denaturation step at 94 °C for 4 min, followed by 42 cycles of 50 s at 94 °C, 50 s at 54–63 °C depending on the primer pair (Table 1), 1 min at 72 °C, and a final extension for 10 min at 72 °C. The PCR products of 3 fluorophores (FAM, HEX and TAMRA) were mixed in a ratio that was based on the brightness of bands visualized by agarose gel electrophoresis. Parameters relevant to multiplex analysis of 10 loci are included in Table 1. PCR products were analyzed using an ABI 3730XL DNA sequencer. Electropherograms were derived using Gene Scan 4.0 and used to deduce DNA fragment sizes using Gene Mapper 4.0 (Sangon Biotech, Shanghai).

Cross-species amplification was tested in 5 closely related whitefly species (*Aleurodicus dispersus*, *Pealius mori*, *Aleuroclava aucubae*, *Bemisia tabaci* MEAM1 and *B. tabaci* MED). DNA samples from 10 individuals of each species were tested.

The number of alleles at each polymorphic locus, size range, and heterozygosities (both observed and expected) were calculated using Cervus 2.0 software (Marshall et al. 1998). Deviation from Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium at each locus were calculated using GenePop 3.4 software (Rousset 2008). The null allele frequency was calculated using Micro-Checker (Van Oosterhout et al. 2004).

Results

The 41 pairs of primers described above were used for screening microsatellite polymorphism. Twenty-eight loci successfully amplified the target regions, while only 10 loci revealed high microsatellite polymorphisms. All of the polymorphic information contents (PIC) of these 10 primers were greater than 0.5. The number of alleles ranged from 12 to 27 across the 60 individuals tested. The expected heterozygosity (H_E) and the observed heterozygosity (H_O) ranged from 0.851 to 0.958 and from 0.621 to 0.850, respectively (Table 1). Null allele frequencies at those loci ranged from 0.0352 to 0.1619 and all loci exhibited no significant evidence of null alleles, but deviated from Hardy-Weinberg equilibrium based on testing of 60 unsexed individuals. Table 2 provided characteristics of the loci for *A. spiniferus* from 48 female individuals in which all loci also deviated from the Hardy-Weinberg equilibrium, and no loci exhibited significant evidence of null alleles. In addition, loci C19-2 and D14, and also I20 and F12 manifested linkage disequilibrium after sequential Bonferroni correction.

The results of cross-species amplification showed that all loci could be used for amplification at different levels except F12 (Table 3). In particular, loci A21-2, D14, F13-2, F42-2, and I20 were readily amplified in all 5 additional whitefly species.

Discussion

Traditionally, microsatellite markers are developed by extensive screening for microsatellite containing clones through repetitive hybridization of a repeat motif probe to a large number of random clones (Rassmann et al. 1991). Such an isolation strategy resulted in low rate of the number of positive clones (containing microsatellites) detection (Zane et al. 2002). Using modified protocols of Hamilton et al (1999) and Glenn et al (2000) to construct and clone genomic libraries increased proportions of inserts that contained tandem repeat arrays. A great number of microsatellite repeat regions have been detected, sequenced and used to design specific flanking primers for microdatellite amplification.

Our studied loci indicated a high degree of polymorphism (PIC > 0.5), and can be successfully used to study the population genetic structures of *A. spiniferus* and other closely related whitefly species. The 10 loci still deviated from Hardy-Weinberg equilibrium when another 48 female whiteflies were tested, although we avoided the influence factor of reproduction (Table 2), which was consistent with the findings of Kobmoo et al. (2009), who found that 4 out of 10 loci in *Ceratosolen fusciceps* Mayr (Hymenoptera: Agaonidae) deviated from the Hardy-Weinberg equilibrium (HWE), and the global test of deviation from the HWE equilibrium was significant ($P < 0.01$) even when only females were tested. This deviation occurred because the involved individuals were indeed inbreeding. It is important to note that the HWE test is a low power statistical test to identify genotyping errors (Leal 2005). Thus, the reasons for deviating from the HWE by *A. spiniferus* may be due to other factors such as mutation, natural selection, small population size (genetic drift and population bottle necks) or presence of population substructures including Wahlund's effect (http://www.dorak.info/genetics/popgen.html). Specifically, Martins et al. (2012) reported that 11 of the 15 microsatellite loci in *Planococcus citri* (Hemiptera: Pseudococcidae) significantly deviated from the HWE, probably due to data structuring, since individuals from 2 significantly distant geographic areas were used. Probably this is a potential factor affecting the HWE for *A. spiniferus*, since we used 48 females from Jiangsu (N 32.41° E 119.42°) and Sichuan (N 30.57° E 104.07°) provinces for testing. Moreover, small population size results in random sampling errors as well as unpredictable

TABLE 1. CHARACTERISTICS OF POLYMORPHIC MICROSATELLITE LOCI OF *ALEUROCANTHUS SPINIFERUS* FROM 60 INDIVIDUALS COLLECTED FROM TEA PLANTS IN 6 PROVINCES (SHANDONG, JIANGSU, ZHEJIANG, HUNAN, GUIZHOU, AND YUNNAN) IN CHINA.

Locus	Genbank Accession no.	Repeat motif	Primer sequences (5'-3')	Size range (bp)	Ta (°C)	NA	HO	HE	PIC	Null freq
A21-2	JF422773	$(GA)_6GC(GA)_{36}$	F: CTGGTGATGGACAAGATA R: GACATTGAGACTGTGATAAC	254-284	61	14	0.830	0.919	0.903	0.0469
B48-2	JF422774	$(GT)_7AT(GT)_3$	F: CAGTTTAGCCCTTTTAC R: AATAGCAGTTCAGTCCC	164-206	54	21	0.800	0.922	0.908	0.0656
C19-2	JF422775	$(CT)_{25}$	F: CTCGCATTCCCTTCACCC R: ACATCGCCCAACTGCCTT	179-207	65	15	0.850	0.922	0.907	0.0352
D14	JF422776	$(GT)_{24}$	F: GCTGCTATCCCCACTCT R: AACGATTTGCTCGCCTC	203-257	63	20	0.846	0.926	0.911	0.0404
F12	JF422777	$(GT)_{25}GA(GT)_{11}$	F: GGTTAGGCTGGGTAAAAT R: ACACCTCGGTAGGATAGT	225-281	63	27	0.717	0.958	0.948	0.1394
F13-2	JF422778	$(CA)_{31}$	F: ACGATTTGCTCGCCTCA R: GCTGCTATCCCCACTCT	188-244	63	21	0.828	0.928	0.915	0.0536
F42-2	JF422779	$(CA)_{23}$	F: AACGATTTGCTCGCCTCA R: CTGCTATCCCCACTCTGT	187-243	63	18	0.732	0.930	0.917	0.1173
I20	JF422780	$(TG)_{17}$	F: CTAAAGGGACTCTGGTTC R: GGTAGTTGCGTAAGGTGT	133-161	56	12	0.621	0.851	0.825	0.1494
I29	JF422781	$(CA)_{22}$	F: AACGATTTGCTCGCCTCAG R: TGCTGCTATCCCCACTCTG	190-242	63	17	0.706	0.918	0.902	0.1267
I47-2	JF422782	$(GA)_6GC(GA)_{36}$	F: CGTGTAACCCAACATAACCC R: TCCTCGGAAGTGACCAGAT	245-277	59	14	0.635	0.877	0.856	0.1619

Acronyms: Ta, annealing temperature; N_A, observed number of alleles; H_O, observed heterozygosity; $H_{E,}$ expected heterozygosity; PIC, Polymorphic information con tent; Null freq, null allele frequency.

TABLE 2. CHARACTERISTICS OF POLYMORPHIC MICRESATELLITE LOCI FOR *ALEUROCANTHUS SPINIFERUS* FROM 48 FEMALE INDIVIDUALS FROM TEA PLANT S IN JIANGSU AND SICHUAN PROVINCES.

Locus	N_A	H_O	H_E	PIC	Null freq	P-HW
A21-2	13	0.632	0.784	0.749	0.0655	0.0000*
B48-2	19	0.814	0.908	0.890	0.0434	0.0000*
C19-2	17	0.310	0.853	0.830	0.4788	0.0000*
D14	21	0.977	0.874	0.853	-0.0724	0.0000*
F12	21	0.809	0.874	0.854	0.0058	0.0000*
F13-2	31	0.938	0.877	0.858	-0.0491	0.0068*
F42-2	26	0.872	0.785	0.752	-0.0715	0.0000*
I20	27	0.938	0.922	0.907	-0.0163	0.0000*
I29	16	0.636	0.790	0.768	0.0849	0.0032*
I47-2	32	0.800	0.876	0.859	0.0174	0.0000*

Acronyms: N_A, observed number of alleles; H_O, observed heterozygosity; H_E expected heterozygosity; PIC, Polymorphic information content; Null freq, null allele frequence; P-HW, value from the exact test for Hardy–Weinberg equilibrium; *denotes a significant deviation from Hardy–Weinberg equilibrium($P < 0.01$),after sequential Bonferroni's correction of the significance threshold.

TABLE 3. RESULTS OF CROSS-SPECIES AMPLIFICATION TESTS INVOLVING DNA SAMPLES FROM 10 INDIVIDUALS OF EACH OF 5 CLOSELY RELATED WHITEFLY SPECIES.

Locus	*Aleurodicus disperses* (*n* = 10)	*Pealius mori* (*n* = 10)	*Aleuroclava aucubae* (*n* = 10)	*Bemisia tabaci* MEAM1 (*n* = 10)	*Bemisia tabaci* MED (*n* = 10)
A21-2	√	√	√	√	√
B48-2	×	×	×	√	√
C19-2	×	×	×	√	√
D14	√	√	√	√	√
F12	×	×	×	×	×
F13-2	√	√	√	√	√
F42-2	√	√	√	√	√
I20	√	√	√	√	√
I29	√	×	×	×	×
I47-2	√	√	√	×	√

√, Successful amplification; ×, unsuccessful amplification.

genotype frequencies; and in small populations, departure from expected genotype frequencies occurs more easily. Probably this is another factor because we used 60 samples from 6 provinces (10 from each location) in the beginning. Furthermore, as mentioned above, a very high mutation rate in the population (typical mutation rates are < 10-4 per generation) and selection of one or a combination of genotypes (Ineichen & Batschelet 1975) also could violate the HWE. Clearly, further population genetic structure studies are urgently needed to assess these hypotheses.

Our results may also provide new insights about the reproductive mechanism of *A. spiniferus*. Since few individuals engaged in arrhenotokous reproduction, the reproductive strategies of this insect may not significantly affect Hardy-Weinberg equilibrium of the loci studied.

Microsatellites are particularly reliable for studying recent biological invasions (Zygouridis et al. 2009) and are a powerful means to detect the origin and invasion route of exotic insect species (Fonseca et al. 2010). Our cross-species amplification results indicated that the 10 microsatellite polymorphic loci may possess high versatility, and can be used to study gene flow, genetic diversity and population genetic structure of related whitefly species. Meanwhile, this information will facilitate the selection of molecular markers for *B. tabaci*, a controversial invasive species worldwide. Overall, these microsatellite markers show suitable resolution to meet our needs in subsequent studies and to give us insights into the genetic variability, gene flow and the mating system of whitefly species.

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