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ISOLATION AND CHARACTERIZATION OF NINE MICROSATELLITE LOCI FROM THE SYCAMORE LACE BUG *CORYTHUCHA CILIATA* (HEMIPTERA: TINGIDAE)

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ABSTRACT

The sycamore lace bug, *Corythucha ciliata* (Say) (Hemiptera: Tingididae) of North America, is an invasive pest of plane and sycamore trees (*Platanus* spp.) (Proteales: Plantanaceae), and has invaded many countries. To explore the population genetic structure and the invasion route by which *C. ciliata* reached China, we developed 9 highly polymorphic microsatellites loci by the FIASCO method. Polymorphism of the 9 loci was assessed in 48 individuals from 2 populations (Guiyang and Nanjing) in China. The number of alleles per locus ranged from 2 to 13. The observed (H_O) and expected (H_E) heterozygosities varied from 0.146 to 0.958 and 0.290 to 0.849, respectively, in Guiyang population. Likewise H_O and H_E varied from 0.483 to 0.739 and 0.443 to 0.865, respectively, in Nanjing population. Two loci (CA15 and GA365) showed significant deviations from the Hardy-Weinberg equilibrium (HWE) in Nanjing population. Moreover, loci CA200&GT26, GT26&TG100, and TG100&GA365 showed significant linkage disequilibria (LD) in the Guiyang population ($P < 0.01$), and loci GT26 and GA5 ($P < 0.01$) showed significant linkage disequilibria (LD) in the Nanjing population. Finally, we found 2 types of mutational events that could generate the new alleles, but the main mutation mechanism for the newly developed microsatellites was slippage in the repeat motif and in the flanking region. In future work, the nine loci identified here will be used to study the population genetic structures of *C. ciliata* populations in China and in putative regions of their origin, and investigate the probable route by which the pest reached China.

Key Words: microsatellite, *Corythucha ciliata*, population genetics, invasive routes

RESUMEN

El chinche encaje del sicómoro, *Corythucha ciliata* (Say) (Hemiptera: Tingididae) de América del Norte, es una plaga invasora de plataneros y sicómoros (*Platanus* spp.) (Proteales: Plantanaceae), y ha invadido muchos países. Para explorar la estructura genética de la población y la ruta de invasión por el cual *C. ciliata* llegó a China, hemos desarrollado 9 loci de microsatélites altamente polimórficos por el método FIASCO. Se evaluó el polimorfismo de los 9 loci en 48 individuos de 2 poblaciones (Guiyang y Nanjing) en China. El número de alelos por locus varió de 2 a 13. El heterocigosis observado (H_O) y el esperado (H_E) varian de 0.146 a 0.958 y de 0.290 a 0.849, respectivamente, en la población de Guiyang. Del mismo modo el H_O y H_E variaron de 0.483 a 0.739 y 0.443-0.865, respectivamente, en la población de Nanjing. Dos loci (CA15 y GA365) mostraron desviaciones significativas del equilibrio de Hardy-Weinberg (EHW) en la población de Nanjing. Por otra parte, loci CA200 y GT26, GT26 y TG100 y TG100 y GA365 mostraron un desequilibrio de ligamiento (DL) significativo en la población de Guiyang ($P < 0.01$), y loci GT26 y GA5 ($P < 0.01$) mostraron un desequilibrio de ligamiento (DL) significativo en la población de Nanjing. Por último, encontramos 2 clases de eventos mutacionales que podrían generar los nuevos alelos, pero el principal mecanismo de la mutación de los microsatélites desarrollados recientemente fue el deslizamiento en la repetición de motivos y en la región de flanqueo. En trabajos futuros, los nueve loci identificados aquí se utilizarán para estudiar la estructura genética de la población de de *C. ciliata* en China y en regiones putativas de su origen, e investigar la ruta probable por el cual la plaga llegó a China.

Palabras Clave: microsatélites, *Corythucha ciliata*, genética de poblaciones, rutas invasoras

The sycamore lace bug, *Corythucha ciliata* (Say) (Hemiptera: Tingidae) is a new invasive insect species in China from North America (Halbert et al. 1998) that previously had invaded many countries including France, Germany, Chile, Korea, Japan (Wulf et al. 1987; Prado 1990; Chung et al. 1996; D' Aguilar et al. 1997; Tokihiro et al. 2003). In invaded locations, *C. ciliata* feeds primarily on leaves of plane and sycamore trees (*Platanus* spp.; Proteales: Plantanaceae), reducing photosynthesis and promoting diseases, which may be followed by death of the foliage.

Although the physiology and ecology of *C. ciliata* and its control with pesticides have been investigated (Yoon et al. 2008; Ju et al. 2010, 2011a, 2011b), its population genetics is unknown. To better control this pest, an increased understanding of its population genetics and invasion routes is needed. Microsatellites markers can help identify the origins of newly established populations of invasive species, as well as their genetic makeup and their routes of migration (Behura 2006; Ascunce et al. 2011). However, the microsatellite loci of *C. ciliata* have not been reported and few expressed sequence tags (ESTs) of *C. ciliata* are available in the public domain, hindering the study of the population genetics and routes of migration of this species. Consequently, we used the FIASCO method (fast isolation by AFLP of sequences containing repeats) with slight modifications to develop an enriched library of microsatellite loci (Zane et al. 2002) to screen polymorphic loci. Here, we present sequences of 9 microsatellite loci for *C. ciliata*.

Materials and Methods

Samples and DNA extraction

Adult *C. ciliata* samples were collected in 2010 from Guiyang, Guizhou Province and Nanjing, Jiangsu Province, China. Samples were identified and preserved in 100% ethanol, and then stored at -20 °C. Genomic DNA was extracted from individual samples using AxyPrep Multisource Genomic DNA Miniprep Kit and stored at -20 °C until needed for PCR.

Isolation of Microsatellite Markers

We used the FIASCO (fast isolation by AFLP of sequences containing repeats) method with slight modifications to develop an enriched library of microsatellite loci (Zane et al. 2002). The genomic DNA was first digested with the restriction enzyme MseI (BioLabs, Beijing, China) and ligated to MseI AFLP adaptor (5'-TACTCAGGACTCAT-3'/5'-GACGATGAGTCCTGAG-3') in a total volume of 25 µL containing 250 ng of genomic DNA, 1 × OnePhorAll buffer, 5.0 mM DTT, 50 µg/mL BSA, 1.0 µM adaptor, 200 µM ATP, 2.5 U of MseI (NEB), and 1.0 U of T4 DNA ligase (Promega). The reaction was then incubated at 37 °C for 3 h. The digestion-ligation products was diluted (1:10) and amplified with adaptor-specific primers (5'-GATGAGTCCTGAGTAAN-3', MseI-N) in 20 µL reactions containing 1 × PCR buffer (10 mM Tris-HCl, pH 9.0, 50 mM KCl), $MgCl_2$ 1.5 mM, dNTPs 250 uM, MseI-N 0.5 uM, 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 26 cycles of 30 s at 94 °C, 1 min at 53 °C, 1 min at 72 °C with a final extension time of 10 min at 72 °C. After denaturation at 95 °C for 5 min, the PCR products were hybridized for 1 h at 68 °C with biotinylated $(GA)_{12}$, $(GT)_{12}$, $(CA)_{25}$ and $(TG)_{18}$, respectively. The DNA fragments hybridized to biotinylated probes were selectively captured by streptavidin coated magnetic beads (Streptavidin Magnesphere Paramagnetic Particles, Promega, Shanghai, China). The microsatellite-enriched DNA fragments were purified using PCR Cleanup Kit (Axygen, www.axygen.com)and then amplified by MseI-N primers for the following PCRconditions: 3 min at 95 °C followed by 26 cycles of 30 s at 95 °C, 30 s at 53 °C, 45 s at 72 °C with a final extension time of 30 min at 72 °C. The purified PCR products were ligated into pGEMT Easy vectors (Promega, www. promega.com) and then transformed into *Escherichia coli strain* (DH5α). The positive clones were identified by PCR using M13 primers and visualized by agarose gel electrophoresis. All positive clones were sequenced in both directions using the BigDye Terminator Sequencing Kit (Applied BioSystems) and the ABI 3730XL Genetic Analyzer (PE Applied Biosystems, San Francisco, California, USA) with 2 vector-specific primers and internal primers for primer walking. Microsatellite sequences were identified by software SSRHunter 1.3 (Li & Wan 2005). Primer pairs for each microsatellite locus were designed by Primer Premier 5.0 software (http://www.premierbiosoft.com/primerdesign/).

PCR Amplification and Genotyping

Each primer pair was screened against 48 individuals of *C. ciliata.* Three kinds of fluorophores (FAM, HEX and TAMRA) were tagged with forward primers at the 5'end of each pair. PCR amplifications were conducted in 25 µL volumes including 1 × PCR buffer (10 mM TrisHCl, pH 9.0, 50 mM KCl), 1.5 mM MgCl2, 200 FM of each dNTPs, 50 ng genomic DNA, 0.75 U of Taq DNA polymerase (TaKaRa), 4 pmol of each primer. Conditions for PCR amplification were

TABLE 1. CHARACTERISTICS OF NINE MICROSATELLITE LOCI OF *CORYTHUCHA CILIATA*: GENBANK ACCESSION NUMBER, REPEAT MOTIF, ALLELE SIZE RANGE, ANNEALING TEMPERATURE (TA), NUMBER OF ALLELES (N_a), OBERSERVED HETEROZYGOSITY (H_o), EXPECTED HETEROZYGOSITY (H_E) AND VALUE FROM THE TEST FROM THE EXACT TEST FOR THE HARDY-WEINBERG EQUILIBRIUM (P-HW).

Locus	Accession no	Repeat motif	Primer sequences (5'-3')	Fluorophore	Size range (bp)	Ta	NA		HO		HE		P-HW	
							Guiyang	Nanjing	Guiyang	Nanjing	Guiyang	Nanjing	Guiyang	Nanjing
CA200	KF803195	(CA)11	F:CACTATTGGTCTCGTCGT R:CCATTTTACTCCACTTTC	FAM	176-233	52	10	13	0.958	0.636	0.849	0.735	0.0474	0.0990
CA146	KF803194	(CA)11	F:GAGGATGGGGAAAGGGTT R:GCTGGTTTGAGGGGGGAC	HEX	344-357	58	5	4	0.625	0.542	0.642	0.543	0.8114	0.0239
GT26	KF803198	(GT)10	F: CCATTTTACTCCACTTTC R: ATTGGTCTCGTCGTGATG	TAMRA	1173-195	52	9	7	0.905	0.556	0.828	0.762	0.3666	0.0202
GA70	KF803202	(CT)9	F: ACCACTCCACCTCCACCG R:AGTAAGGGCGAAGCAACA	TAMRA	179-195	55	3	3	0.542	0.583	0.630	0.443	0.4136	0.1910
TG100	KF803201	(GT)11	F: TTTCTCTGAAGACTGTGA R: GGGTGGGTAGCATCATTT	FAM	192-202	56	2	5	0.250	0.625	0.422	0.696	0.0603	0.0131
CA15	KF803200	(AC)7	F: AGGAATCTTCATCACATC R: TGACCGTTTCTTGACCAG	FAM	233-269	55	3	3	0.146	0.483	0.290	0.582	0.0901	0.0000*
CA7	KF803197	(CA)12	F: AGCCGTGCGGTAGCGAAT R: TTTGCGTTTTGAAGGAGC	TAMRA	347-362	54	3	11	0.542	0.739	0.583	0.865	0.9044	0.0388
GA365	KF803199	(AG)26	F: CCGACTCAGTACCACCGA R: GACTTCATAATGTCTCCC	TAMRA	168-219	54	5	9	0.750	0.500	0.733	0.793	0.8758	0.0039*
GA5	KF803196	(TC)13	F: CCCAGCCCTAGAGTCATT R: GCACCTCCCTCCTTTTCA	HEX	310-369	57	4	9	0.391	0.727	0.397	0.849	1.0000	0.0383

*denotes a significant deviation from the Hardy–Weinberg equilibrium ($P < 0.01$), after sequential Bonferroni's correction of the significance threshold.

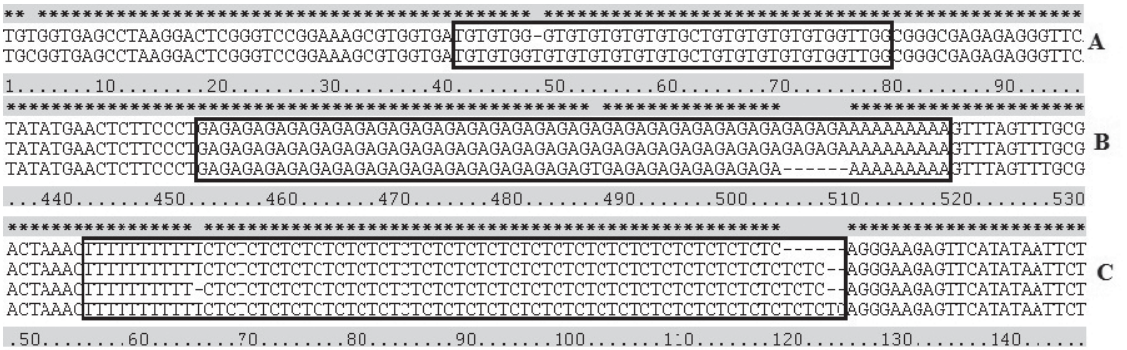

Fig. 1. Mutational events of *Corythucha ciliata* clone microsatellite sequences. A and B are the first type of mutational pattern; C is the second type of mutational pattern.

as follows: an initial denaturing at 94 °C for 4 min, followed by 42 cycles of 50 s at 94 °C, 50 s at 51–58 °C depending on the primer pair (Table 1), and 1min at 72 °C, followed by a final extension for 10 min at 72 °C. The PCR products of 3 fluorophores (FAM, HEX and TAMRA) were mixed in a ratio dependent on the brightness of bands visualized by agarose gel electrophoresis, respectively. The PCR products were run on an ABI 3730XL DNA sequencer and the electropherograms drawn through Gene Scan 4.0 were used to extract DNA fragment sizing details using Gene Mapper 4.0 software by Sangon Biotech (Shanghai) Co., Ltd.

Statistical Analysis

The number of alleles (N_A), observed heterozygosity (H_O), expected heterozygosity (H_E), and polymorphism information content (PIC) were calculated for each locus using Cervus version 3.0 (Marshall et al. 1998). The Hardy-Weinberg equilibrium (HWE) and genotypic linkage disequilibrium between pairs of microsatellites were tested by Genepop 3.4 (Raymond et al. 1995). Null allele frequencies were measured by MICRO-CHECKER 2.2.3 (van Oosterhout et al. 2004). All P-values were adjusted for multiple tests using the sequential Bonferroni method (Rice 1989).

Results and Discussion

Characteristics of Microsatellite Loci

Thirty five primers were designed based on 163 clones that contained a microsatellite sequence, but 9 microsatellite loci showed high polymorphisms (PIC > 0.5) (Table 1). The number of alleles per locus ranged from 2 to 13, with an average of 6 alleles per locus. The observed (H_O) and expected (H_E) heterozygosities varied from 0.146 to 0.958 and 0.290 to 0.849, respectively, in Guiyang population. The observed (H_O) and expected (H_E) heterozygosities varied from 0.483 to 0.739 and 0.443 to 0.865, respectively, in Nanjing population. The null allele frequency was lower than 0.1 in at least 1 population. After sequential Bonferroni corrections, 2 loci (CA15 and GA365) showed significant deviations from the Hardy-Weinberg equilibrium (HWE) in Nanjing population. This phenomenon may be caused by the Wahlund effect, the effect of evolutionary pressure during the process of invasion or the existence of a null allele. Loci CA200&GT26, GT26&TG100, TG100&GA365 showed significant linkage disequilibria (LD) in the Guiyang population ($P < 0.01$), and loci GT26 and GA5 ($P < 0.01$), showed significant linkage disequilibrium in Nanjing population. This phenomenon may be caused by genetic drift of the *C. ciliata* population after invasion. Consequently, the 9 loci can be useful for population genetics studies and explore invasive routes of *C. ciliata*.

Mutations of Microsatellite Sequences

In total, we obtained 1,385 bacterial colonies from the enriched library of microsatellite loci that used the biotinylated probes: $(GA)_{12}$, $(GT)_{12}$, $(CA)_{25}$ and $(TG)_{18}$. Two hundred and four recombinant clones were sequenced, but only 163 were successful. We analyzed these microsatellite sequences to determine the mutational model. We found 2 types of mutational events that could have generated the new alleles. First, the differences in numbers of repeat motifs caused size variation of alleles (Figs. 1A and 1B). Secondly, 2 different repeat motifs contributed to the allele-size variation (Fig. 1C). However, slippages in the repeat motif and flanking region were the main mutation mechanism for the newly developed microsatellites.

The findings of this study will allow us to elucidate genetic structure of the *C. ciliata*

populations in China and in putative regions of their origin, and thereby investigate the probable route by which the pest reached China, as well as its subsequent spread in China.

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