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# Development and characterization of thirteen novel microsatellite markers for the Huanglongbing vector, *Diaphorina citri*

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**ABSTRACT**

We isolated and characterized a total of 13 microsatellite loci from the Asian citrus psyllid, *Diaphorina citri.* Loci were screened in 24 individuals from the Rio Grande Valley in southern TX, USA. The number of alleles per locus ranged from 3 to 9, observed heterozygosity ranged from 0 to 0.542, and the probability of identity values ranged from 0.0054 to 0.07. These primers were then tested in multiplex reactions with previously developed primers to test compatibility. In conjunction with previously developed primers, these new loci may improve our ability for examining population structure, movement and history of an important agricultural disease vector.

*Additional index words*: : Asian citrus psyllid, Citrus Greening disease, multiplex, PCR primers, SSR, STR

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*Diaphorina citri* Kuwayama (Hemiptera: Psyllidae), the Asian citrus psyllid, may be the most significant pest of citrus plants around the world. While the insect damages the host during feeding and reaches large population sizes, the majority of damage originates from bacteria transmitted during feeding. Huanglongbing (HLB), or Citrus greening disease, is a devastating disease of Citrus believed to be caused by the fastidious, phloem-limited bacteria *Candidatus* Liberibacter asiaticus (CLas), *Ca.* L. africanus (CLaf), and *Ca.* L. americanus (CLam; Jagoueix et al., 1994; Garnier et al., 2000; Teixeira et al., 2005a, b; Pietersen et al., 2010). In the New World, these bacteria are vectored by the *D. citri* and transmitted to the plant during feeding (Capoor et al., 1967; Martinez and Wallace, 1967; Lallemand et al., 1986; Bové, 2006). The Asian citrus psyllid is highly invasive. The establishment of *D. citri* in the United States, first reported in Florida in 1998, was believed to be caused through the transport of infested plant material (Bové, 2006; Boykin et al., 2012). Symptoms of HLB are usually detected in trees several years after the Asian Citrus psyllid becomes established in an area (Bassanezi et al., 2011; Gottwald, 2010). Characterizing the population structure of newly introduced Asian Citrus psyllid populations may provide means to detect the pathway of this pest to other citrus growing regions. Twelve polymorphic microsatellite markers were developed by Boykin et al. (2007). We attempt to increase the number of available microsatellite markers and use them in a multiplex system to aid in characterizing populations of *Diaphorina citri*.

## METHODS and RESULTS

***Library construction.*** Total DNA was extracted from a pool of twenty *Diaphorina citri* individuals collected from Edinburg, Texas, using the DNeasy tissue kit protocol (Qiagen, Valencia, CA) for the construction of a microsatellite library. DNA was then serially enriched twice for microsatellites using three probe mixes following Glenn and Schable (2005) with the changes described in Lance et al. (2010). There were two primary changes to the Glenn and Schable (2005) protocol. First, a different linker was used (SimpleX-11 Forward 5'- AAACGAGCGAGCGAAGT -3' and SimpleX-11 Reverse 5'-pACTTCGCTCGCTCG -3'). Second, the enriched libraries were sequenced on a Roche 454 using titanium chemistry following standard Roche 454 library protocols (454 Life Sciences, a Roche company, Branford CT). All methods for sequencing, microsatellite identification, primer design, and primer screening are as described in Lance et al. (2010) with the exception that the sequence GTTT was added to primers without the universal CAG tag addition (Brownstein et al., 1996).

From the sequences containing three base or greater repeat motifs, we designed forty-eight primer pairs. The forty-eight primer pairs were tested for amplification success and polymorphism using DNA obtained from eight individuals from Edinburg, TX. PCR amplifications were performed in a 12.5 mL volume (10 mM Tris pH 8.4, 50 mM KCl, 25.0 mg/ml BSA, 0.4 mM unlabeled primer, 0.04mM CAG-tag labeled primer, 0.36mM fluorophore-labeled universal

primer, 3.0 mM $MgCl_2$, 0.8 mM dNTPs, 0.5 units AmpliTaq Gold® Polymerase (Applied Biosystems), and 20 ng DNA template) using an Applied Biosystems GeneAmp 9700 (Table 1).

**Table 1.** Details for 13 polymorphic microsatellite loci developed for *Diaphorina citri.* The size indicates the range of observed alleles in base pairs and includes the length of the CAG tag; number of individuals genotyped is *N*; *k* is number of alleles observed; $H_o$ and $H_e$ are observed and expected heterozygosity, respectively; PI is the probability of identity for each locus, and TD refers to the touchdown protocol used for PCR (see text).

| Locus | Primer Sequence 5' --> 3' | Repeat motif | Size (bp) | N | *k* | $H_o$ | $H_e$ | PI | TD |
|---|---|---|---|---|---|---|---|---|---|
| Dici04 | F: *ACTCATCACATGCCTTCCG<br>R: GTTTCCAATGTTGTGAGCCGTTTC | AGG(11) | 251-278 | 22 | 3 | 0.273 | 0.584 | 0.024 | 65 |
| Dici07† | F: *CATCATCTCCACCCAGCC<br>R: GTTTCGTCGGAATGCAGCTTATGG | ACC(7) | 105-174 | 21 | 9 | 0.286 | 0.744 | 0.0089 | 60 |
| Dici08 | F: *CTAAGCACCGCACGATTC<br>R: GTTTACCACGAGATCAGTGACGAG | AAG(8) | 120-156 | 21 | 5 | 0.333 | 0.615 | 0.02 | 65 |
| Dici10† | F: *CTGCTGGTAACCTTGTCC<br>R: GTTTCAGACGCTCTACCCTCCAAG | AGAT(10) | 304-332 | 22 | 3 | 0.000 | 0.169 | 0.07 | 65 |
| Dici14† | F: *GGATAATTCTCTCAGACGCG<br>R: GTTTCAAGAAGTGCACATCCCTCC | AGG(8) | 149-179 | 22 | 7 | 0.455 | 0.825 | 0.0054 | 65 |
| Dici15† | F: *GGTCATGCACGTTCACTTG<br>R: GTTTAGCTCGTCATTTACAGGTCAAC | AAGT(15) | 171-335 | 24 | 6 | 0.250 | 0.665 | 0.016 | 60 |
| Dici20† | F: *TGATCAGGTCTGGCTTTCTTC<br>R: GTTTAGTGCTGGATATAGGGTGCC | ACTC(10) | 292-364 | 23 | 6 | 0.435 | 0.708 | 0.012 | 65 |
| Dici23† | F: *TTTCAGTTCCAACAGCTCCC<br>R: GTTTGAACTGCATCACCTGGGAAC | AAGT(10) | 151-251 | 24 | 9 | 0.542 | 0.772 | 0.0081 | 55 |
| Dici25† | F: GTTTACAGATGAGAGGACGCTGAG<br>R: *GCAAGAACACAACACCGAGC | AGG(9) | 138-180 | 23 | 5 | 0.304 | 0.721 | 0.012 | 65 |
| Dici27 | F: GTTTACGCGTTATCTCTGACCTGG<br>R: *GCAGTAGTTCCCACAAGTAGC | AAC(8) | 300-306 | 24 | 2 | 0.500 | 0.497 | 0.038 | 65 |
| Dici29† | F: GTTTACTGATGGATGAGGACGCG<br>R: *TTGTGTTTCGTGCTTCAGGG | AAC(8) | 153-216 | 21 | 9 | 0.238 | 0.711 | 0.011 | 55 |
| Dici40 | F: GTTTGAAAGCCGGGTGATGTTCTC<br>R: *CTCACCTCAGCAACTTGG | AAG(9) | 133-160 | 24 | 6 | 0.542 | 0.717 | 0.013 | 55 |
| Dici45 | F: GTTTGCTTGGACAATGGACATAGGC<br>R: *TACTCATAGCATGCCCGTCC | AAC(12) | 155-230 | 24 | 4 | 0.417 | 0.633 | 0.02 | 65 |

* indicates CAG tag (5'- CAGTCGGGCGTCATCA-3') label; † indicates significant deviations from Hardy-Weinberg expectations after Bonferroni corrections.

Touchdown thermal cycling programs (Don et al., 1991) encompassing a 10°C span of annealing temperatures ranging between 65-55°C (TD65), 60-50°C (TD60) or 55-45°C (TD55) were used for all loci. Touchdown cycling parameters consisted of an initial denaturation step of 5 min at 95°C followed by 20 cycles of 95°C for 30 s, highest annealing temperature (decreased 0.5°C per cycle) for 30 s, and 72 °C for 30 s; and 20 cycles of 95 °C for 30 s, lowest annealing temperature for 30 s, and 72 °C for 30 s. PCR products were run on an ABI-3130xl sequencer and sized with Naurox size standard prepared as described in DeWoody et al. (2004), except that unlabeled primers started with GTTT (Lance et al., 2010; Brownstein et al., 1996). Results were analyzed using GeneMapper version 3.7 (Applied Biosystems).

***Test for locus heterozygosity and suitability for multiplexing*.** From the 48 primers sets developed, 13 primer sets successfully amplified polymorphic amplicons. Subsequently, we assessed the variability of these 13 polymorphic loci on 24 specimens collected from a across the Rio Grande Valley of Texas. Conditions and characteristics of the loci are provided in Table 1. We estimated the number of alleles per locus (*k*), observed and expected heterozygosity ($H_o$ and $H_e$), and probability of identity (PI) using GenAlEx v6.4 (Peakall & Smouse, 2006). Tests for deviations from Hardy-Weinberg equilibrium (HWE) and for linkage disequilibrium were conducted using GENEPOP v4.0 (Rousset, 2008). These 13 primer pairs consistently amplified high quality PCR product that exhibited polymorphism (Table 1). After Bonferroni correction for multiple comparisons of the 13 primer sets, eight loci showed significant deviations from expectations under Hardy-Weinberg equilibrium (HWE) and no linkage disequilibrium was detected for any of 78 paired loci comparisons. The deviations from HWE are not unexpected as this species is an invasive pest with rapidly fluctuating population sizes resulting from flushing plant material and pesticide treatments.

**Table 2.** Multiplex PCR primer sets (i.e., panels) used in this study along with fluorescent dye choice and PCR conditions used for each reaction.

| Panel | Locus | Dye | PCR Conditions |
|---|---|---|---|
| 1 | Dici08 | Vic | 95° 3 min [95° 30 sec, 65° 30 sec, 72° 30 sec] X 20, [95° 30 sec, 55° 30 sec, 72° 30 sec] X 30, 72° 5 min |
| | Dici04 | 6-Fam | |
| | Dici20 | Vic | |
| 2 | Dici25 | Vic | 95° 3 min [95° 30 sec, 65° 30 sec, 72° 30 sec] X 20, [95° 30 sec, 55° 30 sec, 72° 30 sec] X 30, 72° 5 min |
| | Dici45 | 6-Fam | |
| | Dici27 | Vic | |
| 3 | B_Dci09[a] | 6-Fam | 95° 3 min [95° 30 sec, 60° 30 sec, 72° 30 sec] X 20, [95° 30 sec, 50° 30 sec, 72° 30 sec] X 30, 72° 5 min |
| | B_Dci12[a] | Vic | |
| | B_Dci07[a] | 6-Fam | |
| 4 | Dici23 | Vic | 95° 3 min [95° 30 sec, 60° 30 sec, 72° 30 sec] X 15, [95° 30 sec, 50° 30 sec, 72° 30 sec] X 45, 72° 5 min |
| | B_Dci04[a] | 6-Fam | |
| 5 | B_Dci06[a] | Vic | 95° 3 min [95° 30 sec, 45° 30 sec, 72° 60 sec] X 35, 72° 5 min |
| | B_Dci5[a] | 6-Fam | |
| 6 | Dici14 | Vic | 95° 3 min [95° 30 sec, 60° 30 sec, 72° 60 sec] X 35, 72° 5 min |
| | B_Dci02[a] | 6-Fam | |
| | Dici10 | Vic | |
| 7 | Dici40 | 6-Fam | 95° 3 min [95° 30 sec, 55° 30 sec, 72° 60 sec] X 35, 72° 5 min |
| | Dici15 | Vic | |

Overlap in size for a particular panel and optimal annealing temperatures prevented using Dici07 and Dici29 for testing in multiplex reactions (See multiplex panel development). However, these two alleles still performed well as single reactions and could possibly be of use for population genetics. The remaining eleven loci were identified as potential markers to be used in multiplex reactions.

The 24 specimens were also tested using the twelve microsatellite loci developed by Boykin et al. (2007) in singleplex reactions. *B_Dci01*, *B_Dci03*, *B_Dci08*, *B_Dci10*, and *B_Dci11* did not amplify consistently, with failure rates greater than 50%. Even after troubleshooting, amplification success was poor. Therefore, only seven among 12 microsatellite loci developed by Boykin et al. (2007) were selected for the evaluation multiplexing capability with 11 loci identified in the current study.

***Multiplex panel development.*** Eighteen primer sets were sorted into seven multiplex PCR panels including either two or three loci each (Table 2). Determination of which primer sets to combine in a panel was based on similarity of PCR reaction conditions and dissimilarity in range of PCR product sizes to minimize fragment size overlap. To minimize miscalled alleles, distinct fluorescent dyes were used to differentiate potential markers in cases where overlap in sizes of the alleles may occur (Table 2). To test performance of the panels, seven *D. citri* individuals collected from Mission, Texas were tested to compare results of singleplex and multiplex reactions using PCR conditions described in Table 2. PCR products were analyzed on an ABI 3730XL DNA Analyzer, using the ABI Data Collection Program (v 2.0). The resulting data were visualized with Applied Biosystems PeakScanner v1.0 to determine fragment size, and sorted using the Excel Microsatellite Toolkit (v3.3.1, UC-Davis 2008; Tables 3 and 4). Data were then binned using PGDSpider (v2.0.3, Lischer & Excoffier 2012) and Tandem (v1.08, Matchiner & Salzburger 2009). A total of 122 out of an expected 133 singleplex chromatographs, and all 49 expected multiplex chromatographs, were produced for comparison (Tables 3 and 4).

Our testing of seven multiplex panels determined that four panels consisting of eleven loci generated results that matched singleplex reaction results and performed as appropriately as multiplex reactions with the chosen primer pairs. As shown in Tables 3 and 4, the panel 2 multiplex match identically with singleplex results. Multiplex panels 1, 3, and 4 differed from singleplex results by one mismatch. Four of the markers produced unexpected results (Tables 3 and 4). *Dici15* consistently failed both in singleplex and multiplex reactions. *Dici10* yielded fragments outside expected size ranges in singleplex reactions and failed to amplify in multiplex reactions. *Dici14* failed to amplify in several singleplex reactions (Table 3), but did amplify in the multiplex reactions. Further optimization may be required in order for *Dici14* to provide consistent signals. Finally, *B_Dci05* produced 330 bp fragment that appears to be extraneous based on the shape of the chromotograph signal. Additional testing

**Table 3.** Fragment size (bp) observed for each primer pair from singleplex reactions on each individual psyllid.

| Locus | *D. citri* #1 | *D. citri* #2 | *D. citri* #3 | *D. citri* #4 | *D. citri* #5 | *D. citri* #6 | *D. citri* #7 | (-) ctrl |
|---|---|---|---|---|---|---|---|---|
| Dici08[a] | 129 | 129 | 129 | 141 | 141 | 129/141 | 141 | 0 |
| Dici04[a] | 252 | 258/267 | 252 | 267/276 | 252 | 252 | 252 | 0 |
| Dici20[a] | 299/315 | 315 | 315 | 287 | 355 | 315 | 315/355 | 0 |
| Dici25[a] | 138 | 138 | 138 | 138 | 138 | 144/171 | 138 | 0 |
| Dici45[a] | 219 | 219 | 219 | 153/219 | 153 | 153 | 153/219 | 0 |
| Dici27[a] | 297 | 303 | 297/303 | 297 | 297/303 | 297 | 297/303 | 0 |
| B_Dci09[a] | 90 | 186 | 90 | 90 | 186 | 90 | 90/186 | 0 |
| B_Dci12[a] | 212 | 214 | 224 | 212/224 | 212/214 | 212 | 224 | 0 |
| B_Dci07[a] | 271 | 271 | 267/271 | 267 | 271/275 | 271 | 267/271 | 0 |
| Dici23[a] | 195 | 179/195 | 179 | 187 | 179/187 | 179/187 | 187/195 | 0 |
| B_Dci04[a] | 267/270 | 291/297 | 294/297 | 297 | 297 | 297 | 297 | 0 |
| B_Dci06[a] | 282/297 | 282 | 282/297 | 282/297 | 282/297 | 282 | 282/297 | 0 |
| B_Dci05[a] | 330/333 | 330/333 | 330/333/348 | 330/333 | 330/333/363 | 330/333 | 330/333/363 | 0 |
| Dici14 | 168 | --- | 153 | --- | --- | --- | 180 | 0 |
| B_Dci02[a] | 194 | 189/194 | 189 | 189/194 | 194 | 194 | 189/194 | 0 |
| Dici10 | 139/223 | 331 | 139/331 | 139/331 | 139 | 139/331 | 139 | 0 |
| Dici40[a] | 141/147 | 132/150 | 138/141 | 159 | 132/141 | 141 | 141 | 0 |
| Dici15 | --- | --- | --- | --- | --- | --- | --- | 0 |
| Dici07[a] | 136 | 136 | 136/142 | 136 | 136/142 | 142 | 142 | 0 |
| Dici29[a] | 153/192 | 153/192 | 213 | 153/210 | 153/213 | 153 | 147 | 0 |

[a] Suitable for use in population studies

is needed to determine if these primer sets should be combined in alternate panels or analyzed under different conditions.

## DISCUSSION

We developed and tested 13 novel microsatellites for *D. citri*, doubling the number available for use with the disease vector. In an effort to streamline characterization of individual psyllids, we screened 12 previously published markers (Boykin et al., 2007) for baseline data to use in designing multiplex reactions. By using a multiplex platform, costs and time required to analyze large sample sets can be reduced. Seven of the 25 primer sets were excluded outright from consideration for use in multiplex reactions. However *Dici07* and *Dici29* could still be used in single reactions. The 18 remaining loci were then tested to see if they are suitable for multiplex analysis of the pest. In all, we find 16 of the 25 markers are well suited for immediate use in population genetic studies on *D. citri* (Table 3). From these markers, four multiplex panels produced reliable, distinguishable amplicons (Table 4). However, primer concentration still requires optimization to normalize signal strength between each allele in a panel. Using a standardized primer concentration most likely affected the substrate competition during PCR resulting in loss of signal strength for the larger amplicons. This may be a cause of the null alleles observed in Panels 1 and 3. *Dici15* failed both in singleplex (Table 3) and multiplex (e.g., panel 7; Table 4) assay, and thus may be unreliable using PCR conditions described in Table 2. Further testing may be required using the touchdown PCR conditions used in the generation of this primer set *Dici14* should not be used since amplifications in singleplex and multiplex reactions were not consistent. Further optimization may help and testing for interference between primer sets may lead to possible usage in the future. *Dici40* should be restricted to singleplex reactions. Care is required when interpreting results from *B_Dci05* as artifact fragment may be produced with a unique peak shape occurring within the expected range. Without careful examination of the peak shape where the artifact fragment occurs, there is the potential for confusion which may lead to making a false call. With these cautions in mind, these markers may be useful for examination of population structure, movement, and history of *D. citri*.

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**Table 4.** Fragment size (bp) observed for each primer pair from multiplex reactions on each individual psyllid.

| Panel | Locus | *D. citri* #1 | *D. citri* #2 | *D. citri* #3 | *D. citri* #4 | *D. citri* #5 | *D. citri* #6 | *D. citri* #7 | (-) ctrl |
|---|---|---|---|---|---|---|---|---|---|
| 1[a] | Dici08 | 129 | 129 | 129 | 141 | 141 | 129/141 | 141 | 0 |
| | Dici04 | 252 | 258/267 | 252 | 267/276 | 252 | 252 | 252 | 0 |
| | Dici20 | 299/315 | 315 | 315 | 287 | 355 | 315 | 315 | 0 |
| 2[a] | Dici25 | 138 | 138 | 138 | 138 | 138 | 144/171 | 138 | 0 |
| | Dici45 | 219 | 219 | 219 | 153/219 | 153 | 153 | 153/219 | 0 |
| | Dici27 | 297 | 303 | 297/303 | 297 | 297/303 | 297 | 297/303 | 0 |
| 3[a] | B_Dci09 | 90 | 186 | 90 | 90 | 186 | 90 | 90/186 | 0 |
| | B_Dci12 | 212 | 214 | 224 | 212/224 | 212/214 | 212 | 224 | 0 |
| | B_Dci07 | 271 | 271 | 267/271 | 267 | 271 | 271 | 267/271 | 0 |
| 4[a] | Dici23 | 195 | 179/195 | 179 | 187 | 179/187 | 179/187 | 187/195 | 0 |
| | B_Dci04 | 291/294 | 291/297 | 294/297 | 297 | 297 | 297 | 297 | 0 |
| 5 | B_Dci06 | 282/297 | 282 | 282 | 282/297 | 282/297 | 282 | 282/297 | 0 |
| | B_Dci05 | 330/333 | --- | 330/333/348 | 330/333 | 330/333/363 | 330/333 | 330/333/363 | 0 |
| 6 | Dici14 | 168 | 165/180 | 153/168 | 180 | 165 | --- | 180 | 0 |
| | B_Dci02 | 194 | 189/194 | 189 | 189/194 | 194 | 194 | 189/194 | 0 |
| | Dici10 | --- | 331 | 331 | --- | --- | 331 | --- | 0 |
| 7 | Dici40 | 141/147 | 132/150 | 138/141 | 159 | 132/141 | 141 | 141 | 0 |
| | Dici15 | --- | --- | --- | --- | --- | --- | --- | 0 |

[a] Panel suitable for use in population studies

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