Highly polymorphic di- and trinucleotide microsatellite markers for the grapevine yellows disease vector *Hyalesthes obsoletus* (Auchenorrhyncha: Cixiidae)

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**Abstract.** Seven polymorphic microsatellite loci were developed for the planthopper *Hyalesthes obsoletus*, vector of stolbur 16SrXII-A phytoplasma, a pathogen of many Solanaceae crops and responsible for the economically important grapevine yellows disease Bois noir (BN) in Europe. The epidemiology of BN is primarily determined by the abundance of the vector on field bindweed (*Convolvulus arvensis*) and stinging nettle (*Urtica dioica*) (Maixner et al., 1995). Phenological differences in the life cycle of the vector on these two plants (Maixner et al., 2009) and the presence of plant-specific stolbur strains (Langer & Maixner, 2004) indicate host-races of the vector and determine its dispersal ability. However, the ability to test for genetic host races of the vector and determine its dispersal ability has been hampered by the extremely little genetic polymorphism in the markers so far analysed (mtDNA, RAPD-DNA, allozymes). This lack of polymorphism is most likely due to founder effects resulting from recent range expansion into large parts of the current European distribution (Johannesen et al., 2008). In the present paper, the development of microsatellite genetic markers that facilitate the study of the evolution of host races in *H. obsoletus* and the epidemiology of *H. obsoletus*-transmitted diseases are reported.

**MATERIAL AND METHODS**

Genomic DNA was purified (DNeasy Kit, Qiagen, Hilden, Germany) and sheared by nebulization. The size fraction between 1 and 2.5 kb was electro-eluted from a 1% agarose gel, end-repaired using Klenow enzyme and T4 DNA polymerase, blunt-end ligated into dephosphorylated, SmaI-digested pUC 18 plasmid vector and transformed into electro-competent *Escherichia coli* DH10B cells (Amid et al., 2001). After blue-white selection, about 1000 white colonies were analyzed for the presence of microsatellite-containing inserts by colony filter hybridization.

Synthetic oligonucleotides representing a mixture of the various di- and trinucleotide motifs (30 mers) were radio-labelled using T4 polynucleotide kinase and gamma-P32-ATP (Hartmann Analytic, Braunschweig, Germany). Hybridization was performed at 40°C in 6 × SSC / 1% sodium-dodecysulfate overnight, followed by washes at 50°C in 2 × SSC and 1 × SSC (1 × SSC contains 0.15 M trisodium citrate and 0.15 M sodium chloride). Autoradiography was performed using Kodak X-Omat films (Sigma-Aldrich, St. Louis, USA).

Plasmid DNA from positive clones was isolated in a 96-well format (Qiagen) and sequenced by the Sanger method using DyeTerminator chemistry (Applied Biosystems, Weiterstadt, Germany). Sequencing reactions were separated on an ABI3730 sequencer by a commercial service (StarSeq, Mainz, Germany). Bioinformatic identification of microsatellite stretches was performed with the program SciRoKo (Kofler et al., 2007).

Eighteen loci with repeat motifs were isolated (GenBank Accession no. HM046814–HM046831). For fluorescent labeling, the cost-efficient one-tube single-reaction nested PCR method described by Schuelke (2000) was used first. An 18-bp M13 primer was added to the 5′ end of each forward primer and a fluorescent-labelled M13 primer was added to the PCR. PCR amplification was performed using PuReTaq Ready-To-Go PCR beads (GE Healthcare, Munich, Germany) following PCR conditions described in Schuelke (2000). Cycling conditions were: 5 min at 94°C, 30 cycles of 30 s at 94°C, 45 s at 72°C, followed by 8 cycles of 30 s at 94°C, 45 s at 53°C, 45 s at 72°C, with a final extension of 15 min at 72°C. Samples were scored on an ABI3130 sequencer using 11.7 μl HiDi formamide, 0.3 μL ROX 500 standard (Applied Biosystems), and 1 μl of the PCR product. Loci were genotyped using GeneMapper 4.0 software (Applied Biosystems).

Loci that produced consistent results were amplified in two QIAGEN Multiplex PCR reactions with four and three fluorescent labelled primers, respectively (mix 1: B82, F56, F84, and H120, annealing temperature 60°C; mix 2: E96, G85, and C147, annealing temperature 62.5°C). For the multiplex PCR, a PCR volume of 10 μl (8.5 μl mastermix and 1.5 μl DNA of c. 50 ng
DNA per reaction) was used. The mastermix contained a final concentration of 1 × QIAGEN Multiplex PCR Master Mix, which provides 3 mM MgCl₂, and 0.2 µM of each primer. Cycling conditions were: 30 s at 95°C, 30 cycles of 30 s at 94°C, 90 s at 60/62.5°C, 90 s at 72°C, followed by a final extension of 10 min at 72°C.

Genetic variability and amplification consistency was tested in two German, one Italian, and one Israel population. German specimens (N = 20) were collected near Berlin, Germany (D-U [IL], N = 20, and D-C [C. arvensis], N = 20), Italy (I), N = 18, and Israel (IL), N = 20. Overall FST values were highly significant (P < 0.0001). Bayesian clustering analysis performed with Structure 2.1 (Pritchard et al., 2000) for individuals sampled on different host plants at the syntopic German site clustered all individuals, except one, according to the host plant on which they were collected (proportion of membership to bindweed = 0.915, nettle = 0.936).

**RESULTS**

Seven polymorphic microsatellite loci produced consistent results. These loci amplified consistently in all 78 specimens. 10–22 alleles per locus (mean 15.86) were recorded. The expected heterozygosity per locus ranged from 0.533 to 0.941 (Table 1). The loci B82, F56, F84, and H120 obeyed Hardy-Weinberg proportions. Locus B82 showed deviation from Hardy-Weinberg proportions in all populations. Locus C147 was sex-linked.

**LOCUS CHARACTERISTICS**

Table 1. Characteristics and summary statistics for microsatellite loci for Hyalales obsoletus based on 78 individuals from four populations sampled in Germany (D-U [U. dioica], N = 20, and D-C [C. arvensis], N = 20), Italy (I), N = 18, and Israel (IL), N = 20.

<table>
<thead>
<tr>
<th>Locus</th>
<th>GenBank Accession no.</th>
<th>Repeat motif</th>
<th>Primer sequences (5'-3')</th>
<th>T&lt;sub&gt;a&lt;/sub&gt; (°C)</th>
<th>No. of alleles</th>
<th>D-U/D-C/I/IL</th>
<th>Allele size range (bp)</th>
<th>D-U</th>
<th>D-C</th>
<th>I</th>
<th>IL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hob B82</td>
<td>HM046815</td>
<td>(CA)&lt;sub&gt;10&lt;/sub&gt;</td>
<td>F: TGTAGACCAAGACACCTCTG G: RTTCTCCCTTGGTTCACG</td>
<td>59</td>
<td>7/11/10/14/17</td>
<td>131–171</td>
<td>0.650</td>
<td>0.754</td>
<td>0.650</td>
<td>0.826</td>
<td>0.778</td>
</tr>
<tr>
<td>Hoh C147</td>
<td>HM046818</td>
<td>(CA)&lt;sub&gt;10&lt;/sub&gt;</td>
<td>F: GGTGTTTTTTTCTACTGTCTGAG G: RGACATGCTCTGTCACAA</td>
<td>60</td>
<td>5/11/8/13/18</td>
<td>141–205</td>
<td>0.750</td>
<td>0.569</td>
<td>0.900</td>
<td>0.901</td>
<td>0.556</td>
</tr>
<tr>
<td>Hob E96</td>
<td>HM046820 (interpolated by four T)</td>
<td>(GA)&lt;sub&gt;10&lt;/sub&gt;</td>
<td>F: CGCGGTTAATTGAGGAGA G: ATCCCTGCTCCTTTCTTC</td>
<td>64</td>
<td>4/10/8/16/22</td>
<td>160–218</td>
<td>0.550</td>
<td>0.671</td>
<td>0.647</td>
<td>0.775</td>
<td>0.529*</td>
</tr>
<tr>
<td>Hob F56</td>
<td>HM046823 (CCA)&lt;sub&gt;10&lt;/sub&gt;</td>
<td>F: AAGGGCCAGCTTCTATCTT</td>
<td>R: TCGAAATTACGGTTACGTC</td>
<td>60</td>
<td>8/9/5/13/14</td>
<td>164–215</td>
<td>0.700</td>
<td>0.681</td>
<td>0.650</td>
<td>0.812</td>
<td>0.500</td>
</tr>
<tr>
<td>Hob F84</td>
<td>HM046824 (CA)&lt;sub&gt;10&lt;/sub&gt;</td>
<td>F: CCACCTTTTTTCTATGGA</td>
<td>R: GAGACTCCAGTTGGCCACACA</td>
<td>60</td>
<td>5/8/3/10/10</td>
<td>237–260</td>
<td>0.700</td>
<td>0.628</td>
<td>0.950</td>
<td>0.792</td>
<td>0.556</td>
</tr>
<tr>
<td>Hob G85</td>
<td>HM046828</td>
<td>(CA)&lt;sub&gt;10&lt;/sub&gt;</td>
<td>F: AGCAAAACCTGCTCTGGA</td>
<td>R: CCAAAATTAGCGAACAGGAA</td>
<td>64</td>
<td>6/8/6/15/18</td>
<td>236–272</td>
<td>0.526*</td>
<td>0.771</td>
<td>0.400*</td>
<td>0.699</td>
</tr>
<tr>
<td>Hob H120</td>
<td>HM046830</td>
<td>(CA),TA(CA)&lt;sub&gt;10&lt;/sub&gt;</td>
<td>F: AACCTCTCATGCGGACCA</td>
<td>G: AAGGGGATGGTGTAACAGC</td>
<td>60</td>
<td>5/7/8/11/12</td>
<td>239–267</td>
<td>0.750</td>
<td>0.638</td>
<td>0.850</td>
<td>0.803</td>
</tr>
</tbody>
</table>

The estimate of genetic differentiation among all populations was high for polymorphic microsatellite loci, F<sub>ST</sub> = 0.12. Pairwise FST values are shown in Table 2. All FST values were highly significant (P < 0.0001).

DISCUSSION

The microsatellite loci reported here amplify consistently in *H. obsoletus* from diverse phyllogeographic areas and host-plants. An overall departure from Hardy-Weinberg equilibrium was found for one locus, G85. Since there were no amplification failures at G85, and because the heterozygote deficit was constant across the four divergent populations, this indicates that a null-allele cannot alone explain the deficit (putative null allele frequencies = 0.11–0.16). No linkage was found between any locus pair, therefore all loci can be considered as independent. The Israel population was the only one with an overall deviation from Hardy-Weinberg proportions. This might be caused by the Wahlund effect as no amplification failures were observed. The Wahlund effect as no amplification failures were observed. The Wahlund effect is a reduction in expected heterozygosity of a population due to structuring into several subpopulations. Israel specimens where collected at several sites in the Central Golan Heights on *Vitis am尼斯-castus*. Genetic diversity indices, Hardy-Weinberg probabilities, and population differentiation were calculated with Genepop version 4.0.10 (Raymond & Rousset, 1995) was used to calculate Ho (observed heterozygosity), He (expected heterozygosity). *Sex-linked locus: The heterozygosity estimates for the German population were based only on females, whereas the sex of Italian and Israel populations were unknown. * denotes significant deviation from Hardy-Weinberg proportion (P < 0.05).
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REFERENCES


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