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. The loci have di- or trinucleotide repeat motifs and are highly variable with 10 to 22 alleles per locus. Observed heterozygosity ranged from 0.278 to 0.950 for the 78 individuals genotyped. One locus is sex-linked. No linkage between loci was found. All loci amplified consistently among phylogeographic as well as host-plant related samples and proved highly informative for population genetic studies.

INTRODUCTION

Epidemiological cycles of vector-transmitted diseases are greatly influenced by the dispersal ability and the host specificity of the vector (Weintraub & Beanland, 2006). The polyphagous planthopper *Hyalesthes obsoletus* is the main 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 vector and host-strains of the pathogen, and hence two epidemiological disease cycles. 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 *E. 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 polynuclotide kinase and gamma-P32-ATP (Hartmann Analytic, Braunschweig, Germany). Hybridization was performed at 40°C in 6 × SSC / 1% sodium-dodecylsulfate overnight, followed by washes at 50°C in 2 × SSC and 1 × SSC (1 × SSC contains 0.015 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 labelling, 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 55/59/60/64°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

TABLE 1. Characteristics and summary statistics for microsatellite loci for *Hyalesthes 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.

Locus GenBank Accession no.	Repeat motif	Primer sequences (5′–3′)	T_a^1 (°C)	No. of alleles D-U/D-C/ I/IL/total	Allele size range (bp)	D-U		D-C		I		IL	
						H_O^2	H_E	H_O	H_E	H_O	H_E	H_O	H_E
Hob B82 HM046815	$(CA)_{10}$	F: TGTAAGCACAAGAGACCTATCG R: CTCTTCCCTTCGTCAACG	59	7/11/10/14/17	131–171	0.650	0.754	0.650	0.826	0.778	0.843	0.800	0.905
Hob C147[3] HM046818	$(CA)_{21}$	F: GGTGTTTTTCCTCTACTGTCTGAG R: GGACATGGCTACGTTCAACA	60	5/11/8/13/18	141–205	0.750	0.569	0.900	0.901	0.556	0.744	0.278*	0.941
Hob E96 HM046820	$(GA)_{15}$ (interrupted by four T)	F: CGGCGTAAATTTGGAGAGAA R: ATCCCTCTTCCCCTTCTTCA	64	4/10/8/16/22	160–218	0.550	0.671	0.647	0.775	0.529*	0.818	0.526*	0.895
Hob F56 HM046823	$(CCA)_{12}$	F: AAGGCGCACGTTTCTACTGT R: TCGAAAATCGCGTTATCAGTC	60	8/9/5/13/14	164–215	0.700	0.681	0.650	0.812	0.500	0.692	0.950	0.873
Hob F84 HM046824	$(CA)_{13}$	F: CCACCTTTTCCCCTTATGAA R: GAGACTCCAGTTGCCACACA	60	5/8/3/10/10	237–260	0.700	0.628	0.950	0.792	0.556	0.584	0.600	0.794
Hob G85 HM046828	$(CA)_{10}$	F: AGCAAACACCTGCCTCTGAA R: CCAAAATTAGCGAACCGAAC	64	6/8/6/15/18	236–272	0.526*	0.771	0.400*	0.699	0.353*	0.533	0.650*	0.927
Hob H120 HM046830	$(CA)_3TA(CA)_7$	F: AACTCTCATGCGGACCAGAC R: AAGGGGATGGGTAGAACGAC	60	5/7/8/11/12	239–267	0.750	0.638	0.850	0.803	0.944	0.814	0.684	0.886

[1]T_a = annealing temperature. [2]GENEPOP v. 4.0.10 (Raymond & Rousset, 1995) was used to calculate H_O (observed heterozygosity), H_E (expected heterozygosity). [3]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 ($P < 0.05$).

DNA per reaction) was used. The mastermix contained a final concentration of 1 × QIAGEN Multiplex PCR Master Mix, which provides 3 mM $MgCl_2$, 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 populations (Bacharach) were syntopic and collected on *Urtica dioica* (N = 20) and *Convolvulus arvensis* (N = 20), respectively. Italian specimens (N = 18) were collected near Rome on *Urtica dioica*. Israel specimens (N = 20) were collected at several sites in the Central Golan Heights on *Vitex agnus-castus*. Genetic diversity indices, Hardy-Weinberg probabilities, and population differentiation were calculated with Genepop version 4.0.10 (Raymond & Rousset, 1995). Genetic differentiation among populations, F_{ST}, was calculated using the method of Weir & Cockerham (1984). Null alleles were tested using Micro-Checker version 2.2.3 (van Oosterhout et al., 2004) applying a Bonferroni correction for multiple comparisons (Rice, 1989).

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 in all populations. Locus C147 was sex-linked; males were always homozygous whereas females showed Hardy-Weinberg proportions. Locus E96 showed deviations from Hardy-Weinberg proportions in two populations and

TABLE 2. Pairwise F_{ST} values based on 78 individuals of *Hyalesthes obsoletus* 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. Overall F_{ST} = 0.12. All F_{ST} values were highly significant (p < 0.0001).

	D-U	D-C	I
D-C	0.12		
I	0.15	0.11	
IL	0.15	0.07	0.13

locus G85 showed significant but similar levels of homozygote excess in all populations.

The estimate of genetic differentiation among all populations was high for polymorphic microsatellite loci, F_{ST} = 0.12. Pairwise F_{ST} values are shown in Table 2. All F_{ST} 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).

DISCUSSION

The microsatellite loci reported here amplify consistently in *H. obsoletus* from diverse phylogeographic 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 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 and might therefore not represent a single population.

The loci were able to detect population differentiation within and between geographic regions, as is indicated by high F_{ST} values ranging from 0.07 to 0.15. The Israel population is phylogenetically divergent from German and Italian populations, which belong to two related but separate post-glacial expanding lineages (Johannesen et al., 2008). In Germany, populations from different host plants showed significant genetic differentiation. The evidence provided here for geographic as well as host-plant related genetic variance show that the seven loci are highly informative for gaining insights into both host-race evolution in *H. obsoletus* and epidemiological cycles of *H. obsoletus*-transmitted diseases.

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