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Highly polymorphic microsatellites in the North American snakeweed grasshopper, Hesperotettix viridis

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Abstract

Microsatellite markers are preferred for fine-scale population genetic studies requiring high resolution. The grasshopper *Hesperotettix viridis* (Thomas) is an oligophagous species that feeds on composites and often exhibits locally restricted diets. Divergence in host plant use in some localities is seen where co-occurring subpopulations select alternate plant species, as expected with the evolution of host shifts and associated lineage divergence. To characterize the host-associated divergence patterns among populations of *H. viridis*, we developed markers from two microsatellite-enriched genomic libraries. Here we report the characterization and optimization of seven polymorphic di- and tri-nucleotide microsatellite loci for this species. One hundred and six individuals from 5 populations were tested for polymorphism. The number of alleles varied from 4 to 38 in all the populations. H_o ranged from 0.339 to 0.790. Homozygote excess was observed across loci, perhaps due to inbreeding. This is the first report of microsatellite markers for the subfamily Melanoplinae.

Keywords

Hesperotettix viridis, snakeweed grasshopper, microsatellite markers, local adaptation, host-associated divergence

Introduction

The grasshopper *Hesperotettix viridis* (Thomas) is oligophagous, feeding on composites (Asteraceae) in the tribe Astereae (Mulkern *et al.* 1969; Joern 1979; Parker 1984, 1985). When all feeding records for *H. viridis* are combined from across its broad geographic range, a moderate list of host species results (~ 35 spp.) (Pfadt 1994). However detailed feeding records of local populations show highly restricted diets, with populations of *H. viridis* often behaving almost as host specialists (Mulkern *et al.* 1969, Parker 1984, Traxler & Joern 1999). Moreover, it is not unusual to find nearby populations specializing on different host species (Traxler & Joern 1999). Microhabitat selection in *H. viridis* is also highly restrictive; individuals spend most of their time on host plants, and move only short distances to neighboring plants of the same species (Parker 1984).

Because broadly distributed species offer a spatial and temporal context for unraveling evolutionary forces that drive lineage diversification and speciation, *H. viridis* provides unique opportunities for investigating the evolutionary process of dietary divergence that may promote local adaptation and ultimately lineage divergence. Several factors could contribute to variation and species divergence and deciphering the importance of these factors is key to understanding the relative contributions of different evolutionary processes in genetic differentiation and speciation (Knowles & Richards 2005). To observe the fine-scale adaptive landscape, and to understand the underlying mechanisms of host shift, molecular markers that allow fine-scale phylogeographic resolution and that are able to detect recent demographic events are required. To our knowledge, this is the first report of microsatellite markers in the acridid (Orthoptera) subfamily Melanoplinae. However, several nuclear markers have been reported in Melanoplinae (Carstens & Knowles 2006).

Microsatellites are preferred for fine-scale population genetic studies due to their high degree of polymorphism and abundance in eukaryotic genomes (Grace *et al.* 2005). Several advantages in using microsatellites include: a) locus specificity, b) codominance, which allows separation of heterozygotes from homozygotes, c) high variability, which provides a wealth of information at finer scales than possible with other markers, and d) usefulness in detecting recent demographic events.

To develop microsatellite markers, high-quality genomic DNA was extracted from one individual of *H. viridis* using the DNA Easy Kit from QIAGEN. Genomic DNA was enriched for microsatellite repeats as described in Hamilton *et al.* (1999). The microsatellite enrichment protocol involved restriction of DNA into fragments of approximately 500 base pairs, by cutting with restriction enzymes *Rsa*1 and *Hae*111. This was followed by ligation of double-stranded *Snx* linkers to each end of the DNA fragments (SnxF 5′-CTAAGGCCTTGCTAGCAGAAGC-3, SnxR 5′-GCTTCTGC-TAGCAAGGCCTTAGAAAA- 3′). Enrichment was completed by hybridizing template DNA with biotinylated microsatellite probes containing di-, tri- and tetra-nucleotide repeats. DNA fragments with microsatellite repeats were then recovered from the hybridization buffer using Avidin D beads. Eluted DNA fragments were recovered by PCR amplification using *Snx* primers. Enriched PCR fragments were cloned into a plasmid vector (Invitrogen), followed by transformation into chemically competent *Escherichia coli* cells. Positive clones were picked using blue-white selection. The inserts were isolated and amplified by PCR, using T3 (5'-AATTAACCCTCAC-TAAAGGG-3) and T7 (5-TAATACGACTCACT ATAGGG-3) primers. Five hundred and thirty PCR fragments were thus amplified from the clones. PCR amplifications were carried out on an Eppendorf Master Cycler (Eppendorf Inc.) in a 30 µl-reaction volume. The reaction mixture contained 11.75 µl of water, 6 µl of 5x Taq buffer, 3 µl of 25 mM $MgCl_2$, 3 µl of 2 mM dNTPs, 1.8 µl of each primer (10 µg/µl), 0.15 µl of 5U Taq DNA Polymerase (Promega) and 2.5 µl of DNA. Cycling conditions included an initial denaturation step of 94°C for 3 min followed by 35 cycles of 95°C for 30 s, 50°C for 45 s and 72°C for 30 s and concluded with a final annealing

step at 72°C for 10 minutes. The PCR products were run on a 1% agarose gel and fragments containing inserts of ~>300 bp with a single clean band were selected, and sequenced. Three hundred and sixteen samples were selected for sequencing. PCR products from the selected clones were purified and sequenced on an ABI 3730xl automated sequencer at the Advanced Genetic Technologies Center (AGTC) at the University of Kentucky, USA. Sequencing analysis revealed 295 clones with microsatellite inserts.

Fragments with simple sequence repeats and enough flanking sequence from which to design primers were retained. One hundred and ninety-five clones without sufficient flanking regions from which to design primers were discarded. Oligonucleotide primers flanking both sides of the microsatellite repeat were designed for 99 loci using Primer 3 software (http://frodo.wi.mit.edu//) and further checked using Oligo Analyzer ver. 3.0, primer analysis software (Integrated DNA Technologies Inc: http://www.idtdna.com/analyzer/applications/oligoanalyzer) and then synthesized at the Integrated DNA Technologies, Coralville, IA.

These PCR fragments were then fluorescently labeled in a 3-primer PCR reaction (Schuelke 2000) and visualized by an ABI 3730 DNA analyzer. This low-cost method of genotyping involves three primers: the forward primer of each locus which contains the M13 (-21) universal sequence (18bp) at the 5′ end (5′- TGTAAA ACGACGGCCAGT 3′), a locus-specific reverse primer and a third fluorescently labeled HEX-M13 (-21) universal primer: HEX-TGTAAA ACGACGGCCAGT-3′ (5-HEX= hexachloro-6-carboxy-fluorescine). PCR amplifications were carried out on an Eppendorf Master Cycler (Eppendorf Inc.) in 20-µl reaction volume as above, except for adding the third primer. To optimize annealing temperatures, we used gradient PCR for each primer set, which included an initial denaturation step of 94°C for 3 min, followed by 35 cycles of 95°C for 30 s, 48-60°C (12-step gradient design) for 30 s and 72°C for 30 s followed by 8 cycles of 95°C for 30 s, 53°C for 30 s and 72°C for 30 s for the fluorescently labeled m13 primer, and concluded with a final annealing step of 72°C for 15 min. Optimum temperatures were determined for each primer set by visualizing the bands of PCR products on a 1% agarose gel. For the fragments that amplified, the temperature that gave the best amplification (clear and bright band without multiple bands) was selected, and this temperature was used in further analysis. Of the 99 primer sets designed for microsatellites, 52 primer sets amplified a single fragment. Out of these, 6 were later discarded due to amplification inconsistencies.

DNA from 12 individuals was prepared for 46 primer sets in 20 µl reaction volumes and amplified using the optimal amplification temperature for each primer set, determined as described above. These samples were then prepared for ABI genotyping by diluting the PCR product 1:4 times in DNA-grade water. Four µl of the diluted PCR product was mixed with 8 µl of the size standard/formamide mixture (mixture of 1.25% Genescan 400 (Rox) size standard and 98.75% Hi-Di Formamide) and denatured for 4 min at 95°C. The denatured product was run on an ABI Genotyper at the Gene Expression Facility at Kansas State University, Manhattan, USA. The alleles were scored in Genemarker ver. 1.70 (SoftGenetics Inc).

Monomorphic loci and loci that produced inconsistent peaks were discarded. Optimization, which included varying the concentrations of various PCR components and varying annealing temperatures, was conducted for each polymorphic locus to minimize nonspecific amplification. Thirty-nine loci that produced aberrant results were discarded. Seven loci that gave consistent results were retained for further analysis. Optimized primers for the seven polymorphic loci were then tested on 106 individuals from five populations of *H. viridis* in Kansas [Ellis, *Gutierrezia* (n=26); Ellis, *Solidago* (n=23); Cimmaron, *Gutierrezia* (n=8), Seward; *Gutierrezia* (n=19); Marysville, *Solidago* (n=30)]. Each homozygous genotype and 10% of all heterozygotes were twice amplified independently to assess allelic drop-out.

All seven loci were polymorphic (0.95 criterion, Hartl & Clark 1997). The number of alleles varied from four in Hvir 97 to 38 in Hvir 32. Expected heterozygosity values ranged from 0.486 to 0.964, while observed heterozygosities ranged from 0.339 to 0.790 (Table 1).

Statistical tests were conducted to test for Hardy-Weinberg equilibrium and linkage disequilibrium using ARLEQUIN ver. 3.1 (Excoffier *et al.* 2005) and *FIS* values were estimated using F_{STAT} ver. 2.9.3.2 (Goudet 2001). Tests for deviation from Hardy-Weinberg equilibrium showed significant differences in observed and expected heterozygosity at all seven loci.

Testing for null alleles using MICROCHEKER ver. 2.2.3 (Van Oosterhout *et. al.* 2004) indicated that null alleles may be a problem at some of the loci. But results of mating studies conducted in our lab (Grace *et. al.* unpub. data) reveal high levels of assortative mating based on host-plant type. Results of the mating studies, coupled with the fact that *H. viridis* populations in Kansas are highly fragmented in nature, suggest that inbreeding is occurring in these populations. High *FIS* values observed in populations support this hypothesis. (*FIS* values: Ellis, *Gutierrezia* = 0.301; Ellis, *Solidago* =0.313; Cimmaron, *Gutierrezia* =0.275 ; Seward, *Gutierrezia* =0.282 ; Marysville, *Solidago* =0.332).

The observed deviations in Hardy-Weinberg equilibrium are likely due to the highly fragmented nature of the *H. viridis* populations, to inbreeding as a result of assortative mating, or to both. Significant multilocus linkage disequilibrium was also detected in the populations (Table 2). Several factors could result in significant multilocus disequilibrium among microsatellite loci as observed in other studies, due to factors such as high inbreeding (Ferreira *et al.* 2007, Machado *et al.* 2004) or local adaptation (Ferreira *et al.* 2007). High levels of inbreeding were observed in all the populations screened. Deficit of heterozygotes and linkage disequilibrium between loci represents bimodality in the study area where parental forms (two host forms) predominate and are maintained by strong reproductive isolation. The observed deficit could also be a result of grouping two genetically differentiated forms together that mate assortatively. Even though we cannot rule out linkage, based on mating preference studies conducted in our lab and inbreeding coefficient values observed, we interpret that inbreeding, rather than physical linkage between specific markers, is the most probable reason for the observed linkage disequilibrium.

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Table 1. Characteristics of seven polymorphic microsatellite loci in *Hesperotettix viridis*. H_O, observed heterozygosity and H_E, expected heterozygosity; * observed and expected heterozygosities are significantly different from each other. (Genbank accession numbers given below locus description).

Loci	Repeat Motif	Primer sequence (5′-3′)	T_M	No. of Alleles	Size Range of Alleles	H_O	H_E
Hvir54 FJ948482	GT-23	F: CATGTTTGACCACGTTCCTG R:TGGTTCTCTTTTGCCGATTC	59C-25CY 55C-10CY 53C-8CY	30	178-248	0.433*	0.946
Hvir58 FJ948483	GT-11	F: TCCTTGCTCCACACCTATCC R: GGCAGATAGGCACACGAAAA	59C-25CY 55C-10CY 53C-8CY	32	194-272	0.790*	0.945
Hvir73 FJ948484	CT-10	F: CGGCAGGTCGTAAAGGAG R:CCAGTAGCAGTGGGAGGAAG	59C-25CY 55C-10CY 53C-8CY	31	189-271	0.679*	0.934
Hvir22 FJ948479	CA-23	F: CAGTCTACCATGCACATTAGC R:TTGCCAAATCCCAAAAGAC	48C-35CY 53C-8CY	34	205-277	0.788*	0.956
Hvir97 FJ948485	GTT-6	F: TCCGTATGCAGTAGCTCTCG R:CTTTGCATATCAATATGTTACC	48C-35CY 53C-8CY	4	175-223	0.352*	0.486
Hvir32 FJ948480	GA-23	F: ATACGCCGTGAATGTTTCAA R:AGCTGTGACCTTTCGGACAA	56C-35CY 53C-8CY	38	238-350	0.717*	0.964
Hvir50 **FJ948481**	GT-25	F: AACACACACGGCGAGATGTA R: GAGAAGAGGCGAGGACAGG	56C-35CY 53C-8CY	24	187-239	0.339*	0.936

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Table 2. Linkage disequilibrium between pairs of microsatellite loci in five populations of *Hesperotettix viridis*. Shaded areas indicate significant linkage disequilibrium with exact *p* values. (Adjusted *p* value for 5% nominal level is 0.0023).

Marker	Hvir54	Hvir58	Hvir73	Hvir22	Hvir97	Hvir32	Hvir50
Hvir54	*	0.000	0.000	0.000	0.400	0.000	0.000
Hvir58		*	0.063	0.045	0.00	0.009	0.063
Hvir73			*	0.000	0.009	0.081	0.000
Hvir22				*	0.227	0.000	0.000
Hvir97					*	0.145	0.000
Hvir32						*	0.000
Hvir50							*