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Using fluorescently-labeled M13-tailed primers to isolate 45 novel microsatellite loci from the arboviral vector *Culex tarsalis*

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Abstract

Culex tarsalis Coquillett (Diptera: Culicidae) is a highly efficient arbovirus vector. Spatial and temporal heterogeneity have been observed in *Cx. tarsalis* for phenotypic traits including autogeny, virus susceptibility and host preference. Genetic differences between populations may in part explain these observations. Using the M13-tailed primer method, we identified 45 novel polymorphic microsatellite markers from microsatellite-enriched *Cx. tarsalis* genomic libraries. The M13-tailed primer method was more efficient in identifying useful loci than traditional screening by acrylamide gel electrophoresis. These markers will be useful for investigating genetic questions in this important vector mosquito.

Keywords

Culex tarsalis; microsatellites; disease vector; mosquito; West Nile Virus; genetic marker

Culex tarsalis Coquillett (Diptera: Culicidae) is an important North American arboviral vector of West Nile Virus (WNV), Western Equine Encephalitis Virus (WEEV) and St. Louis Encephalitis Virus (SLEV) (Reeves and Milby 1990, Goddard *et al.* 2002; 2003, Reisen 2003). Spatial variation in the ability of wild *Cx. tarsalis* populations to transmit WNV orally and vertically has been documented (Goddard *et al.* 2002; 2003). Several recent QTL studies have linked pathogen susceptibility in mosquito vectors to within-species genetic variation, such as in *Aedes aegypti* and dengue virus and *Anopheles gambiae* and *Plasmodium falciparum* (Menge *et al.* 2006, Bennett *et al.* 2005, Gomez-Machorro *et al.* 2004). The contribution of genetic differences among *Cx. tarsalis* populations to variations in arboviral susceptibility is currently unknown. We previously identified 12 *Cx. tarsalis* microsatellites as part of an ongoing effort to develop markers suitable for genetic studies in this mosquito (Rasgon *et al.* 2006). Here, we report the use of an optimized marker screening process to identify 45 new polymorphic microsatellite loci in the *Cx. tarsalis* genome. Together with previous work, this set of 57 markers provides a suite of tools that can be used to characterize genetic structure and investigate relationships between genetic and phenotypic variation in *Cx. tarsalis*.

Wild adult *Cx. tarsalis* were collected from Adams, County, Nebraska using CDC light traps, placed into 100% ethanol and transported to the Johns Hopkins Bloomberg School of

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Public Health. Genomic DNA was extracted by salt extraction/ethanol precipitation as previously described (Rasgon and Scott 2003). Construction of four *Cx. tarsalis* microsatellite-enriched genomic libraries (AC, AG, CAG and ATG) has been described previously (Rasgon *et al.* 2006). From each library, we randomly picked and sequenced 10-30 clones. Plasmid DNA was isolated using Qiaprep columns (Qiagen, Valencia, CA) and inserts sequenced using primer M13F on an ABI PRISM 3100 Avant Genetic Analyzer with BigDye chemistry (Applied Biosystems, Foster City, CA). PCR primers were designed using Primer3 (http://biotools.umassmed.edu/bioapps/primer3_www.cgi). Primers were redesigned up to 2 times for loci that did not amplify consistently.

Four microsatellite loci were PCR-amplified using previously described reaction mixtures and amplification conditions (Rasgon *et al.* 2006). Reliable amplification for each primer set was confirmed using 1% agarose gel electrophoresis on wild-caught individuals. Forward primers for these four loci were then 5' labeled with fluorescent HEX or 6-FAM for PCR amplification and assessment of allele sizes on a capillary sequencer (described below).

For the remaining 41 microsatellite loci, we used the M13-tailed primer method (Boutin-Ganache *et al.* 2001) to label amplicons for visualization on the capillary sequencer. Forward primers were 5'-tailed with the 23 base pair M13 (uni-43) sequence (AGGGTTTTCCTCAGTCACGACGTT), such that the entire forward primer would look like 5'-AGGGTTTTCCTCAGTCACGACGTTXXXXXXXXXXXXXXXXXXXX-3', where the X's denote the microsatellite-specific primer sequence (Table 1). PCR was conducted in 10 µl reactions containing 0.8 units Taq polymerase, 1.0 µl 10X ThermoPol buffer (New England Biolabs, Ipswich, MA), 0.2 mM each dNTP, 1 µM each microsatellite-specific primer, 0.5 µM 5'-fluorescently labeled M13 (uni-43) primer and 0.5 µl template DNA. The M13 (uni-43) primer was 5'-fluorescently tagged with HEX, 6-FAM or NED to facilitate multiplexing. Amplicons were amplified using a PTC-200 Peltier thermocycler (Biorad, Hercules, CA) under the following reaction conditions: 95 °C for 5 min, 10 cycles of 94 °C for 30 s, a primer-specific annealing temperature (see Table 1) for 1 min, and 72 °C for 30 s, 27 cycles of 94 °C for 30 s, 55 °C for 1 min and 72 °C for 30 s, followed by a 10 min extension at 72 °C. Most primers amplified at an annealing temperature of 57 °C. Gradient PCR was used to determine optimal annealing temperatures for primers that failed to amplify at 57 °C.

Mosquitoes from Adams County, NE (N = 15-20) were assayed for allelic variation on an ABI-3100 Avant capillary sequencer (Applied Biosystems, Foster City, CA). Allele sizes were automatically determined using GeneScan v. 3.7 with an internal ROX-500 size standard (Applied Biosystems). Deviations from Hardy-Weinberg expectation (HWE) were calculated with exact tests using Arlequin software (Schneider *et al.* 2000). Linkage disequilibrium (LD) between all pairs of loci was calculated using GenePop (Raymond and Rousset 1995) with a Bonferroni correction for multiple tests.

We designed primers for 83 identified unique microsatellite-containing sequences. Twenty-two never amplified consistently despite primer redesign. Of the remaining 61 potential loci, 12 appeared to be multi-copy and 4 were not polymorphic, and were not investigated further. The remaining 45 loci were polymorphic. Allele number ranged between 3 and 20. Sixteen loci were in HWE. The remaining loci showed evidence of heterozygote deficiency, likely due to the presence of null alleles (Table 1). Significant linkage disequilibrium was detected between loci CUTB101 and CUTB219.

We found the M13-tailed primer method to be a highly efficient protocol to isolate microsatellite markers from *Cx. tarsalis*. Due to the expense of purchasing individually-labeled primers for all putative loci, in our previous study we used acrylamide gel

electrophoresis to identify promising markers prior to their resolution on the capillary sequencer (Rasgon *et al.* 2006). In this study, the use of fluorescently-labeled M13-tailed primers allowed us to eliminate the acrylamide step and economically screen all primer sets directly on the capillary sequencer. At a fraction of the cost, we were able to generate almost 4 times as many usable markers in approximately one third the time compared to our previous study. The tailed primer method facilitates sequencer multiplexing for economical marker resolution, as the fluorescent label can be changed at will. While we did not do so in this report, it is also possible to use different primer tail sequences to conduct multiplexed PCR of multiple loci simultaneously (Missiaggia and Grattapaglia 2006).

The ecology and population dynamics of *Cx. tarsalis* have been well studied (Reisen *et al.* 1992; Reisen and Lothrop 1995; Reisen *et al.* 1995; Reisen *et al.* 1996), but genetic studies have been lacking due to the absence of genetic markers and tools for this mosquito. In concert with our previous efforts, we have developed a suite of 57 microsatellite loci that can be used to investigate the genetics of *Culex tarsalis*. We anticipate that these markers will be highly useful for the medical entomology community for conducting population genetic and mapping studies (Black *et al.* 2002) in *Cx. tarsalis* and possibly other *Culex* species.

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Table one

List of identified polymorphic *Cx. tarsalis* microsatellite loci. Locus names are followed by GenBank accession numbers. *N*, number of mosquitoes assayed. M13, AGGTTTCCAGTCACGACGTT tail attached to the 5' end of the forward primer.

Locus	5' Label	Core Repeat	T _a (°C)	Primers	Population	N	No. of alleles	Size Range	H _o	H _e	P
CUTA6	HEX	(AC) _n	60	F: 5'-ACTCACACCCGATTGTAGAG- ³ R: 5'-AGCCAGTCAGTCAGTCAGTG- ³	Adams Cty, NE	16	13	259-307	0.438	0.956	<0.001
DQ682664											
CUTA101	6-FAM	(AC) _n	56	F: 5'-GAGATTTTGCTGCAATTC- ³ R: 5'-CGATTTTCGCGTGTGAGTT- ³	Adams Cty, NE	16	5	91-111	0.688	0.790	0.101
DQ682665											
CUTA105#7	M13	(AC) _n	57	F: 5'-TCGCTTACTTCCACAT- ³ R: 5'-AGGACCCAACAACAGCAC- ³	Adams Cty, NE	18	9	244-282	0.111	0.859	<0.001
DQ682666											
CUTA109#7	M13	(AC) _n	57	F: 5'-CCATCACATTGAACATCACTT- ³ R: 5'-CGAGTTGCCGATAGAAGAT- ³	Adams Cty, NE	15	13	236-288	0.600	0.947	<0.001
DQ682667											
CUTA119	M13	(AC) _n	57	F: 5'-GCAGAGAAATCGAGTGCATA- ³ R: 5'-TCCCAAAITTAAGTTTACCCATG- ³	Adams Cty, NE	18	6	200-246	0.056	0.744	<0.001
DQ682668											
CUTA122	M13	(AC) _n	57	F: 5'-CCTACAAACCCGGTGAGAC- ³ R: 5'-GGTAAAGGGGATTTCATC- ³	Adams Cty, NE	15	5	200-216	0.333	0.363	1.00
DQ682669											
CUTA212	M13	(AC) _n AT(AC) _n	57	F: 5'-TTGTTAGACCCAGTCAGAG- ³ R: 5'-CCCCTGATTCCCAAAITTAAG- ³	Adams Cty, NE	15	7	235-269	0.000	0.871	<0.001
DQ682670											
CUTA214	M13	(AC) _n	57	F: 5'-TGTTCTGCAACACCTCTTC- ³ R: 5'-TGACATCTCTCCCGTCAG- ³	Adams Cty, NE	18	8	229-249	0.611	0.806	0.010
DQ682671											
CUTA216	M13	(AC) _n	57	F: 5'-GTGAGACCTGACGCTGAC- ³ R: 5'-GCGCAGTGGCAAAATTAAG- ³	Adams Cty, NE	20	5	227-237	0.200	0.319	0.015
DQ682672											
CUTA218	M13	(AC) _n	53	F: 5'-CGCTAAACACACTCGCCTAAG- ³ R: 5'-TCCACCAGACCGTAAAGTGC- ³	Adams Cty, NE	16	7	185-263	0.313	0.815	<0.001
DQ682673											
CUTA220	M13	(AC) _n	57	F: 5'-TGAGCAGGGGTGAGTTACAC- ³ R: 5'-CCAAATCGACGGGAAATTACA- ³	Adams Cty, NE	18	9	148-168	0.667	0.852	0.124
DQ682674											
CUTA221	M13	(AC) _n	57	F: 5'-CAACCCACAGATGAGAAAGAG- ³ R: 5'-AGCGTCAGCAAAAGTTGTC- ³	Adams Cty, NE	16	6	163-183	0.813	0.798	0.075
DQ682675											
CUTA226	M13	(AC) _n	57	F: 5'-CCAGTGTGAGTTATTCAC- ³ R: 5'-CTTCTCCGCTGTCAATGTC- ³	Adams Cty, NE	15	19	234-286	0.733	0.975	0.002
DQ682676											
CUTA229	M13	(AC) _n	57	F: 5'-CGAAAGTAGCAACGGAAATCTG- ³ R: 5'-CAITTCCTTTGTTTCAGTGTGC- ³	Adams Cty, NE	18	8	225-251	0.556	0.798	0.003
DQ682677											

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Locus	5' Label	Core Repeat	T _a (°C)	Primers	Population	N	No. of alleles	Size Range	H _o	H _e	P
DQ682693				R: 5'-GCGCATAAAAATACAGAGCAA-3'							
CUTB218	M13	(AG) _n	57	F: 5'-TGCTGAGGCGCTTTTACC-3'	Adams Cty, NE	16	15	150-214	0.188	0.976	<0.001
DQ682694				R: 5'-CCCTGGAAAAAGCATCAAACT-3'							
CUTB219	M13	(AC) _n	57	F: 5'-AACGAGCATTTGTCCATTGTG-3'	Adams Cty, NE	16	9	162-214	0.313	0.804	<0.001
DQ682695				R: 5'-CACATCCAGCAACTGTCC-3'							
CUTB223	M13	(AG) _n	57	F: 5'-CGATATTTTGCTCCACTTTTG-3'	Adams Cty, NE	16	7	153-167	0.813	0.782	0.998
DQ682696				R: 5'-AACTCTTCGGGGCTACACTG-3'							
CUTB228	M13	(AG) _n	57	F: 5'-CATCACCATCAATCGTTTCC-3'	Adams Cty, NE	16	11	143-181	0.750	0.819	0.288
DQ682697				R: 5'-GAAAACTTCGGGCACACAC-3'							
CUTC102	M13	(ATG) _n CTG(ATG) _n	57	F: 5'-GGAACCAAAATCATCAATAACC-3'	Adams Cty, NE	18	8	256-292	0.611	0.827	0.098
DQ682698				R: 5'-GCAACAAAAAGAAATCTTAGAAAC-3'							
CUTC201	M13	(ATG) _n	57	F: 5'-GCGAACTTTGTGCTTCGAC-3'	Adams Cty, NE	16	4	246-255	0.563	0.700	0.734
DQ682699				R: 5'-ATCATCATCTTCCACCAG-3'							
CUTC203	M13	(ATG) _n	57	F: 5'-AGGCCATGCAACATCCTTAC-3'	Adams Cty, NE	16	9	200-224	0.813	0.869	0.718
DQ682700				R: 5'-CGACTTTATCTAGGCGCTCTC-3'							
CUTD5#7	M13	(CAG) _n	57	F: 5'-GTAGGTA TTGGACGACTGTGTC-3'	Adams Cty, NE	18	3	169-178	0.111	0.162	1.000
DQ682701				R: 5'-GCAC TACAAACGGCAACAAT-3'							
CUTD10	M13	(CAG) _n	57	F: 5'-GGTCTCTTTTCAGACAACCAC-3'	Adams Cty, NE	15	5	214-232	0.267	0.710	<0.001
DQ682702				R: 5'-CGTTCATGTGTGATGTTGAAGTT-3'							
CUTD102	M13	(CAG) _n CAACAGCAA(CAG) _n	60	F: 5'-CAGTTCCAGCAGCAGTCA-3'	Adams Cty, NE	16	4	117-142	0.250	0.290	1.000
DQ682703				R: 5'-CAGGTGATGGGGGTGTAG-3'							
CUTD104	HEX	(CAG) _n CAA(CAG) _n (CAA) _n	60	F: 5'-CGCCAGTAACAGATTATGC-3'	Adams Cty, NE	16	9	124-155	0.938	0.833	0.546
DQ682704				R: 5'-GTTGTTGTGATTGTTGCTGTGT-3'							
CUTD203	M13	(CAG) _n CAC(CAG) _n CAA(CAG) _n	57	F: 5'-TATCCGGCAGCAGAACTTG-3'	Adams Cty, NE	16	4	224-236	0.375	0.726	0.017
DQ682708				R: 5'-ACAAGCACCAACAGCAAACTG-3'							
CUTD206	M13	(CAG) _n	57	F: 5'-CCCACTCATCTCAACC-3'	Adams Cty, NE	16	3	227-233	0.375	0.331	1.000
DQ682705				R: 5'-TGCTGCTGAAGGATTTCG-3'							
CUTD211	M13	(CAG) _n	57	F: 5'-TTCTGTTGTTGGGATTGCTG-3'	Adams Cty, NE	17	6	261-276	0.118	0.873	<0.001
DQ682706				R: 5'-GTCCGCACCCCTGAATTGTA-3'							
CUTD213	M13	(CAG) _n CAA(CAG) _n	57	F: 5'-CAACGGTAAACAGCACGA-3'	Adams Cty, NE	16	3	225-232	0.125	0.181	1.000
DQ682707				R: 5'-GTTTTCATTACAGCCCCACA-3'							