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# Microsatellite loci for the white-dotted mosquito (*Culex restuans*), a principal vector of West Nile virus in North America

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## Abstract

We characterized the first microsatellite loci in the white-dotted mosquito, *Culex restuans*, a critical early spring West Nile virus vector. An enrichment protocol yielded 960 positive clones of which we sequenced 300. We designed primers to amplify 29 unique di-, tri- and tetranucleotide microsatellites and chose 17 that amplified consistently across populations and were polymorphic. We developed three multiplex primer combinations for all 17 loci. A survey of 44 individuals revealed two to 20 alleles across loci, and expected heterozygosity ranging from 0.17 to 0.89. These markers will allow examination of the life history of this mysterious early season encephalitis vector.

### Keywords

*Culex restuans*; microsatellite; multiplex; West Nile virus

While over 30 species of mosquitoes have been found positive for the West Nile virus (WNV), Kilpatrick and colleagues (2005) suggested that *Culex pipiens* L. and *Culex restuans* Theobald may be responsible for up to 80% of human WNV infections in the northeastern USA. *Culex restuans* is a spring species, unlike *C. pipiens*, and it appears to be the critical vector of WNV early in the transmission season (Andreadis *et al*. 2001). *Culex restuans* occurs from California to North Carolina, from southern Canada to Honduras (Strickman & Darsie 1988), and locally adapted populations may differ in epidemiologically significant traits. Highly polymorphic molecular markers will allow the examination of life history and ecological traits of this species and more informed exchange of information across epidemiological studies.

We isolated microsatellite loci from *C. restuans* using a modified version of the enrichment protocols described by Hamilton *et al*. (1999) and further refined by Glenn & Schable (2005). Approximately, 3 μg of DNA from four mosquitoes from the state of New York were digested with *Hae*III, and blunt-end ligated to double-stranded SNX linkers. The ligation was divided into two and hybridized to different groups of biotin-end labelled oligonucleotides using the conditions in the polymerase chain reaction (PCR) program OligoHyb. [Group 1: $(AAC)_{10}$, $(CAT)_{10}$, $(GT)_{15}$, $(GA)_{15}$; Group 2: $(GTC)_{10}$, $(CAC)_{10}$,



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$(GCT)_{15}$, $(GGT)_{15}$]. The hybridization reactions were mixed with iron beads coated with streptavidin (Dynabeads) and incubated for 30 min at 43 °C after which a series of baths of increasing stringency removed most of the unattached DNA that lacks repeats. After denaturation, the single-stranded DNA recovered from the beads was amplified using primers to the linkers using the PCR conditions in Keyghobadi *et al*. (2004). The amplified DNA was cloned into bacteria using a TOPO TA cloning kit (Stratagene) with a transformation efficiency of 90%. Out of 3750, 960 white colonies were transferred into 96-well plates containing Luria-Bertani liquid broth with 15 μg/mL of ampicillin, and grown for 48 h at 37 °C with moderate shaking. From these we chose 300 colonies that were amplified and sequenced using M13 primers.

We found 220 unique inserts that contained microsatellites, of which 98 had more than four repeats and enough flank to design primers. We refrained from using loci with more than 20 repeats, especially in the case of dinucleotide motifs, because in our experience those loci are hard to score consistently. Primers were designed for 29 loci focusing on an even mix of di- and trinucleotide motifs, using 'Primer 3′ software (http://frodo.wi.mit.edu/primer3/input.htm); often we designed multiple primers from the same sequence to create multiplexes. We optimized 17 microsatellite loci into only three PCRs (Table 1). In a multiplex (MP) amplification, up to six primer pairs labelled with up to three different colours (Applied Biosystems) are added simultaneously to a single PCR and processed together.

The variability of each locus was assessed in 44 individuals of *Cx. pipiens* from Albany, New York (Table 1). PCRs were in 20-μL final volume and contained 1× buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl), 2.0 mM $MgCl_2$ (MP1 and MP2, 2.5 mM for MP3), 150 μg/mL BSA, 200 μM each dNTP, 0.2 μM each primer (except for loci Crest2T4_30 and Crest1T2.83a that need 0.3 μM), 0.5 U *Taq* polymerase (Applied Biosystems), and 1 μL DNA template (~5 ng). Thermal cycling was performed on an MJ Research Peltier machine: 5 min at 96 °C; 30 cycles of 30 s at 96 °C, 30 s at annealing temperature (MP1 and MP2 = 52 °C; MP3 = 55 °C), 30 s at 72 °C; 5 min at 72 °C. PCR products were electro-phoresed and detected on a 3100 Model capillary sequencer (Applied Biosystems).

Although we routinely examine the inheritance of the loci by testing them in known family groups (Keyghobadi *et al*. 2004), *Cx. restuans* refuses to mate in captivity. Instead, we tested 44 specimens all collected in a restricted area of Albany county, New York, and examined departures from Hardy–Weinberg equilibrium. We observed two to 20 alleles per locus, and expected heterozygosities ranged from 0.17 to 0.89 (Table 1). Statistical tests for Hardy–Weinberg and linkage equilibrium were conducted in GenePop (Raymond & Rousset 1995). A significant heterozygote deficiency was observed for two loci, Crest1T2.83a and Crest1T4.76, suggesting null alleles. All pairwise tests of linkage disequilibrium between loci were nonsignificant after sequential Bonferroni correction. These microsatellite loci are highly polymorphic and suitable for population studies in *Cx. restuans.*

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**Table 1**

Characterization of 17 microsatellite loci in Culex restuans

| Locus | Primer sequences (5′-3′) | *M* | *A* | Allele range (bp) | $H_O$ | $H_E$ | *P* | Motif | GenBank Accession no. |
|---|---|---|---|---|---|---|---|---|---|
| Crest1T1.2d | F: ATTAGCTTCCCAGGCTCTCC<br>R: CGGAAAGCGGAATCGATAG(H) | 3 | 13 | 142–166 | 0.83 | 0.89 | 0.34 | $CA_{10}$ | FJ373274 |
| Crest1T1.17 | F: ACCACACCGATCATTATCCTC<br>R: GAAACATGGAAACGGCAAAC(N) | 3 | 8 | 131–153 | 0.72 | 0.73 | 0.58 | $GTT_6$ | FJ373273 |
| Crest1T1.30 | F: TCTTCGCGCAGAAACGTC(F)<br>R: ACTGCTGCTGGACAGTACCC | 3 | 7 | 118–130 | 0.68 | 0.77 | 0.38 | $CA_8$ | FJ373275 |
| Crest1T2.21 L | F: GCTGATCTTGGCTTGTGGTC(F)<br>R: AACTAAACAATGTCACGAGATACAAGG | 1 | 2 | 181–189* | 0.14 | 0.17 | 0.29 | $GAGT_5$ | FJ373276 |
| Crest1T2.23 | F: CAAGCCTTGGTCATGTGAG(H)<br>R: CGCGCCATTTTGTCTTTAC | 2 | 9 | 101–117 | 0.69 | 0.79 | 0.72 | $CA_7$ | FJ373277 |
| Crest1T2.33 | F: AGGGCACATGTTAAGCCTTG<br>R: TCGGAAATCGAATGATGTTG(F) | 2 | 6 | 88–100 | 0.81 | 0.75 | 0.34 | GA7 | FJ373278 |
| Crest1T2.54 | F: TCTCCTGTACGCTTGTGTGC(H)<br>R: CCTGGCTGATCTTTGGACTG | 1 | 4 | 103–112 | 0.4 | 0.43 | 0.48 | $CAA_6$ | FJ373279 |
| Crest1T2.83a | F: CCCATTCAAAGTTAAAAGATTGC(F)<br>R: GCCAAGACTGATTGGAAACG | 1 | 9 | 118–164 | 0.6 | 0.79 | < 0.01 | $CAA_5$ | FJ373280 |
| Crest1T3.7 | F: CAATCATCGAGGAGCATCAG(H)<br>R: TTGCGATGATCCTGTCGTAG | 1 | 6 | 132–152 | 0.42 | 0.43 | 0.62 | $CAA_4$ | FJ373281 |
| Crest1T4.76 | F: CGATATGGGAACGCGTTAGG(F)<br>R: GCAGTTTGAGAGGCTCAACG | 3 | 3 | 174–178 | 0.16 | 0.33 | < 0.01 | $TG_6$ | FJ373282 |
| Crest1T4.82 | F: GAAGTGTGAGCTAGGGCAAG(N)<br>R: CAATTCAATGCCAAATACCG | 1 | 10 | 156–168 | 0.67 | 0.63 | 0.49 | $GT_7$ | FJ373283 |
| Crest1T5.52 | F: GCGAGATTTCGTTGACAAAC<br>R: ACGAAGAGCCGAGGTACAAC(N) | 2 | 7 | 99–128 | 0.46 | 0.44 | 0.74 | $CAA_6$ | FJ373284 |
| Crest1T5.87 | F: TGTTTATCCTGGAGGAAATGAG<br>R: CCAAAGCCTTTCCTGTCATC(F) | 2 | 20 | 159–204 | 0.83 | 0.87 | 0.28 | $GT_9$ | FJ373285 |
| Crest2T2.25 | F: GACACGGAAGCGAAATTAAAC(N)<br>R: ACTCCAACCCGACAAGAAAC | 1 | 7 | 104–117 | 0.58 | 0.61 | 0.66 | GTC5 | FJ373286 |
| Crest2T2.56 | F: AAATTGCGAAAGGACGATTC<br>R: TCGATGCTTGAAGTTTATCACG(N) | 2 | 13 | 182–218 | 0.71 | 0.86 | 0.4 | TGC9 | FJ373287 |
| Crest2T4.30 | F: CCGGAAGTGAACCGCAAC(H)<br>R: TGTTGAAACCCACTGTTCTAAACC | 2 | 14 | 164–185 | 0.7 | 0.74 | 0.45 | $TC_9$ | FJ373288 |
| Crest2T4.71 | F: TCTCGTTTCGTCCATTCTCC<br>R: AACCACGCACGATGAGATTC(H) | 3 | 11 | 71–97 | 0.75 | 0.82 | 0.75 | $GTC_5$ | FJ373289 |

F and R indicate forward and reverse primer. The letter after the primer sequence (F, H, or N) indicates that primer is labelled with blue, green, or yellow fluorescence, respectively, and *M* indicates which multiplex it is part of. Number of alleles (*A*), their size range, observed ($H_O$) and expected ($H_E$) heterozygosities, and the *P* value of the comparison (*P*) were estimated from a sample of 44 individuals. We list the microsatellite motif we focused on, but other microsatellite types were often present in the sequences in GenBank.

* This locus amplifies in *Culex pipiens*. We have found a single allele (186) not found in *Culex restuans*.