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# FINDING NEEDLES IN THE HAYSTACK: SINGLE COPY MICROSATELLITE LOCI FOR *AEDES JAPONICUS* (DIPTERA: CULICIDAE)

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*Abstract.* First identified in three North American states in 1998, *Aedes japonicus japonicus*, the Asian bush mosquito, has since spread to 21 states, plus Ontario in Canada, northern France, and Belgium. Analyses of the introduction and expansion of this potentially deadly disease vector will be radically improved by including powerful genetic markers like microsatellites. Useful microsatellite loci have, however, been difficult to identify for mosquitoes in the genus *Aedes* because of the high amount of repetitive DNA in these species. We isolated single-copy DNA from *Ae. j. japonicus* and then used a standard enrichment method to identify regions containing microsatellites. Here we describe seven polymorphic microsatellite loci that were tested in American populations of *Ae. j. japonicus*. These loci were also found to be polymorphic in two other of the four *Ae. japonicus* subspecies and in *Aedes koreicus*.

## INTRODUCTION

In addition to the practical understanding required for successful control programs, population genetic studies of introduced disease vectors can provide excellent opportunities to examine evolutionary processes in complex systems. Genetic analyses enable us to locate putative origins of multiple introductions[1,2] and evaluate differing vectorial capacity across populations.[3,4] Changes in genetic makeup associated with introductions and expansions,[5,6] as well as new associations with local or introduced hosts and pathogens, create dynamic systems that are amenable to examination and even experimentation.[7] Change can be measured by comparison both to populations in the original range and, in some situations, to the earlier stages of the introduction. Though these systems are invaluable, thorough analyses are rare.[8]

The Asian bush mosquito, *Aedes (Finlaya) japonicus japonicus* Theobald (Diptera: Culicidae), was first collected outside its native range of northeast Asia[9] in 1998. Although it is unclear when it was first introduced to the United States, in 1998 three collections of *Ae. j. japonicus* were made independently in three different states (CT, NY, and NJ).[10,11] The fact that the extensive surveys associated with *Aedes albopictus*[12] failed to uncover *Ae. j. japonicus* prior to 1998 argues that it must have been introduced no earlier than 1992.[10] From three states in 1998, *Ae. j. japonicus* has since expanded in North America to a total of 19 U.S. eastern states (CT, DE, GA, IN, MA, ME, MD, NC, NH, NJ, NY, OH, PA, RI, SC, TN, VA, VT, and WV), Quebec, Canada, and the western state of Washington. *Ae. j. japonicus* is extremely common in many northeastern states,[10,13] although its presence in southern and more western states is still very localized.[14,15] Breeding populations were also found in France in 2000[16] and Belgium in 2002 (Schaffner F, personal communication). In July 2004, *Ae. j. japonicus* were collected on the Island of Hawaii (Burham Larish L, personal communication).

In its native range in northern Japan, Korea, and Eastern Russia, *Ae. j. japonicus* is mostly a forest and low-density mosquito that is not considered an important disease vector, although it is known to be a laboratory vector of Japanese encephalitis.[17] In contrast, American *Ae. j. japonicus* have been shown to be effective vectors of West Nile virus,[18,19] Eastern equine encephalitis,[20] La Crosse virus,[21] and St. Louis encephalitis.[22] The expansion of *Ae. j. japonicus* in the United States has rivaled that of *Aedes albopictus* in its speed and current abundance.[23] This expansion was unexpected considering the restricted and relatively unchanged ranges of two other introduced *Aedes* species: *Aedes togoi* was introduced in the 1960s to the state of Washington[24] and *Aedes bahamensis* to Florida in 1986,[25] but neither has spread beyond a few contiguous counties.[26,27] In contrast, *Ae. j. japonicus* spread from three U.S. states to 21 in only 6 years.

After introduction, the average genetic makeup of *Ae. j. japonicus* may change as a result of bottlenecks and possibly new selection pressures.[6,28] This might lead to behavioral and/or physiologic changes that could reveal the potentially critical role of this species as a disease vector. Indeed its current abundance in states like Pennsylvania, New Jersey, and New York, where it has become one of the most abundant species in both rural and urban environments[10,13] seems to indicate change. Investigating the genetic makeup associated with the introduction and range expansion of *Ae. j. japonicus* in the United States will allow us to test hypotheses of evolutionary change as well as examine the role of multiple introductions of phenotypically divergent populations. To do so we require highly polymorphic genetic markers such as microsatellites. Microsatellites are our marker of choice because of their relative ease of use, hypervariablity, and low cost compared with sequencing.[29] Although processing large numbers of samples is fairly inexpensive, the investment associated with microsatellites comes during the development process that is both time and equipment intensive.

Isolating microsatellites for mosquitoes in the genus *Aedes* (or *Ochlerotatus*[30]) has proven problematic in the past.[31] Although microsatellite regions are present in *Aedes* mosquitoes,[31] they are commonly duplicated throughout the genome so that a single set of primers will amplify several different loci. These "locus families" render the primers useless for standard population analysis that require single-locus markers with Mendelian inheritance.[32] To avoid this problem, we used a protocol designed to remove highly repetitive DNA and survey only single copy DNA for the presence of microsatellite regions.[33]

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## MATERIALS AND METHODS

We obtained 88 frozen larvae of *Ae. j. japonicus* from the colony at Rutgers University and extracted their DNA using a DNeasy Tissue Kit from Qiagen (Valencia, CA) including the optional RNAse step. The resulting 34.5 μg of DNA were sonicated to generate fragments ranging from 300 to 1,000 base pairs (bp). To eliminate interference from secondary structure, the sample was heated to 95°C for 10 minutes then promptly chilled on ice for 10 minutes before sonication.

Following Elsik and Williams,[33] approximately 25 μg of DNA (average size of approximately 700 bp) were cleaned by ethanol precipitation and resuspended in 25 μL (1 μg/μL) of 0.4 M phosphate buffer (PB), denatured at 100°C for 10 minutes and allowed to reanneal slowly at 60°C for 20 hours (derived from the $C_0T$ 1000 for *Ae. albopictus*).[34,35] During this period the repetitive regions reassociate and, by interrupting this process before the DNA has completely reannealed, we were able to recover single-stranded low copy DNA. This DNA is separated from the double stranded DNA with the use of a hydroxyapatite (HAP) column, which was prepared in 0.03 M PB with a pH of 7. The DNA was applied to the column and washed with 3–5 mL of 0.03 M PB. Single-stranded DNA was recovered with a 1 mL wash of 0.12 M PB. Double stranded DNA was collected with a 1 mL wash of 0.4 M PB. The recovered DNA was cleaned using a QIAquick PCR Purification column (Qiagen). This recovered low copy DNA is biotinylated and used to fish out the matching low copy sequences from a separate linker-ligated pool of DNA.

Approximately 1 μg of sonicated DNA isolated from 3 *Ae. j. japonicus* specimens was digested with mung-bean exonuclease (New England Biolabs [NEB], Beverly, MA) to remove single-stranded overhangs, dephosphorylated using calf intestinal phosphatase (NEB), and ligated to SNX linkers following the guidelines in Hamilton and others.[36,37] Both this pool of DNA and the biotinylated low copy DNA were denatured (95°C for 10 minutes) and hybridized (ratio of 1:2) overnight at 65°C in 6X SSC and 1% SDS.[38] Streptavidin coated beads (Dynabeads, Oslo, Norway) were used to isolate the SNX-linked low copy DNA.[33]

The SNX-linked low copy DNA was denatured and hybridized with biotin end-labeled oligonucleotides $(GT_{15})$, $(GA_{15})$, $(CAC_{10})$, $(GCT_{10})$, $(GGT_{10})$, $(GTC_{10})$ at 55°C and hybridized with $(AAC_{10})$ and $(CAT_{10})$ repeats at 45°C. As in Keyghobadi and others,[36] streptavidin-coated magnetic beads (Dynabeads) were used to isolate the DNA fragments that hybridized to the biotynelated repeats. Those fragments were digested with N*he*I (NEB) and ligated to *Xba*I-cut, dephosporalated pbluescript (SK+ plasmid; Stratagene, La Jolla, CA), transformed into *Escherichia coli* XL1-Blue MRF' cells (Stratagene), and plated onto selective agar medium. According to the guidelines in Keyghobadi and others,[36] positive colonies were identified and sequenced.

Initially we screened all microsatellite loci using a panel of 14 specimens from locations across the United States and Japan collected for a previous study.[39] Once a panel of informative loci was established, we examined their polymorphism in eleven sets of eight individuals obtained from the Vector Control offices in twelve eastern Pennsylvania counties (Susquehanna, Wayne, Wyoming, Pike, Monroe, Northampton, Lehigh, Berks, Bucks, Montgomery, Chester). We also examined loci OJ5 and OJ338 in 70 specimens from Japan (Sapporo, Obihiro, Tokyo, Hiroshima, Nagasaki, Saga).[39] To examine the broader usefulness of the loci, we went further and tested the panel of seven microsatellites on two other field collected subspecies of *Ae. japonicus* and related species obtained from collaborators in Japan, Korea, and the United States: *Aedes japonicus yayamensis*, *Aedes japonicus shintienensis*, *Aedes koreicus, Aedes albopictus,* and *Aedes aegypti*. Furthermore, to confirm the microsatellite loci were inherited following Mendelian assortment,[36,40] we examined the parents and progeny of two family groups with 20 and 17 offspring respectively, We created the families by setting aside male and female pupae and subsequently force mating the females to individual males.

## RESULTS

An estimated 875 ng of single-stranded low copy DNA was recovered from the HAP column and used to develop a suite of microsatellites (Table 1). Our enrichment was successful, with an estimated 15,000 positive colonies from a total of approximately 70,000 (21%). A total of 233 positive clones

TABLE 1
Characterization of seven microsatellite loci in *Aedes japonicus japonicus*

| Locus | Primer sequences (5′–3′) | Repeat motif | No. of alleles | Allele size range (bp) | $H_O$ | $H_E$ |
|---|---|---|---|---|---|---|
| OJ5[A] | F: CACGAAGTCTGGAAGATCTGG<br>R: fam-ATTCGTGCAGCGAAATCTG | $(GTT)_6(GCT)_3(GTT)$ | 5 | 144–157 | 0.52 | 0.69 |
| OJ10[A] | F: GCTTGTCCTGGCTAAGTACTGC<br>R: ned-CGGTAATGTCCACCTGATTG | $(GTT)(GTG)(GTT)_8$ | 8 | 114–137 | 0.72 | 0.80 |
| OJ70[B] | F: CGTTGACAAAGCTCATCTGC<br>R: ned-TGATCTCCAACGGAAGTATGC | $(GCT)_4(GTT)_2(GCT)_2(GTT)$<br>$(GCT)_2(GTT)_2(GCT)_7$ | 6 | 186–212 | 0.51 | 0.64 |
| OJ85[A] | F: ned-CATAAAGCAGCAAGCACAGC<br>R: TGTCTTCCGGATTGATTTCC | $(CAG)_6$ | 4 | 161–173 | 0.28 | 0.32 |
| OJ100[B] | F: fam-CGCATTCCTCAAACCCTAAC<br>R: TCGGTCCGAGGGAAAAAC | $(GT)_5$ | 3 | 180–189 | 0.42 | 0.52 |
| OJ187[A] | F: hex-AAATCAGCTGCCAGTGCAAG<br>R: TGTGTACTTTGCGGTGAAGG | $(CGA)_{11}$ | 5 | 135–157 | 0.53 | 0.65 |
| OJ338[B] | F: ned-TCTCCTGATCCTGAAGAAGC<br>R: AGGGAGCAGAGCAACACTTG | $(CAA)_{10}$ | 9 | 134–179 | 0.78 | 0.80 |

F indicates forward primer and R indicates reverse primer. "fam," "hex," and "ned" indicates that primer is labeled with fluorescent tag (6-FAM, HEX, or NED, respectively; Applied Biosystems). The superscript letter ("A" or "B") after the locus refers to the multiplex it is a part of. Number of alleles and allele size range as well as observed and expected heterozygosity ($H_O$ and $H_E$, respectively) were measured in a panmictic subset of 88 individuals from eastern Pennsylvania. GenBank accession nos. AY994255–AY994261.

were sequenced but there was a substantial amount of repetition in the sequences recovered. The most common locus was found 42 times (~18% of clones sequenced), and only three microsatellite sequences were completely unique. Twenty eight clones did not have a well defined microsatellite region, 6 sequences had little or no flank to design primers in, and 13 had microsatellite repeats and were used to design primers using Primer3 software.[41] A total of 18 primer pairs were designed for those 13 loci; 7 loci proved to be polymorphic and useful in population analysis. Of the rejected loci, one locus was not single-copy (a GT repeat), four often did not amplify suggesting a large number of null alleles, and one locus was monomorphic.

The PCR conditions for all seven microsatellite loci are the same and were optimized as two different multiplexes (Table 1) that were run separately in an automated sequencer. Final concentrations of the PCR reagents in a 20 μL volume are as follows: 1x PCR buffer (GeneAmp, Applied Biosystems Inc., ABI), 2.5 mM MgCl2, 0.2 μM of each primer, 200 μM of each dNTP, 0.2 mg/mL BSA, and 1 unit of AmpliTaq DNA polymerase (ABI). The PCR amplification was performed in MJ Research PTC100 and 200 thermocyclers and was preceded by a 5-minute denaturation at 96°C, followed by 30 cycles of 30 seconds at 94°C, 30 seconds at 54°C, and 30 seconds at 72°C. The amplification ended with an elongation step of 5 minutes at 72°C. The PCR products were sized in an ABI3100 in GENESCAN mode, binned and sized with GeneMapper 3.5 (ABI).

We examined the polymorphism of the seven informative markers across *Ae. j. japonicus* populations in eastern Pennsylvania (Table 1). We observed 3 to 9 alleles per locus and expected heterozygosities ranged from 0.32 to 0.80 (Table 1). Statistical tests for Hardy-Weinberg equilibrium and linkage were conducted in GENEPOP.[42] There was no significant deviation from Hardy-Weinberg equilibrium, and all pairwise tests of linkage disequilibrium between loci were nonsignificant especially after sequential Bonferroni correction. A significant linkage between OJ5 and OJ338 in the specimens from eastern Pennsylvania was not significant in tests using the specimens from Japan. Tests of inheritance of the seven loci in two family groups showed no significant departure from expected Mendelian patterns ($\chi^2$ test).

We found that several of the microsatellite loci were also polymorphic in *Ae. j. shintienensis* and *Ae. j. yayamensis* (Table 2) and, interestingly, were overall as variable or more variable in *Ae. koreicus*. When applied to *Ae. albopictus* and *Ae. aegypti,* the primers did not amplify.

TABLE 2

Variability of the panel of microsatellite loci developed for *Aedes japonicus japonicus* across related species (the allelic size range (in bp) is listed, followed by the number of alleles in parenthesis)

| Locus | Clone size (bp) | *Aedes japonicus yayamensis* ($N$ =8) | *Aedes japonicus shintienensis* ($N$ = 8) | *Aedes koreicus* ($N$ = 6) |
|---|---|---|---|---|
| OJ5 | 154 | 132 (1) | 132–135 (2) | 148 (1) |
| OJ10 | 134 | 155–168 (2) | 168–171 (2) | 172–183 (4) |
| OJ70 | 135 | 175–183 (2) | 183–186 (2) | 182–186 (4) |
| OJ85 | 176 | 111 (1) | 111–112 (2) | 111 (1) |
| OJ100 | 150 | 187 (1) | 187 (1) | 188 (1) |
| OJ187 | 150 | 108–128 (2) | 129–132 (2) | 135–151 (2) |
| OJ338 | 159 | 145–160 (2) | 132–151 (3) | 147–153 (3) |

## DISCUSSION

We successfully isolated seven microsatellite markers useful for fine scale genetic analysis. We found these markers to have a Mendelian inheritance and a high degree of polymorphism in Pennsylvania, as well as being informative in related species and subspecies. It seems that adding a few extra steps for the isolation of single-copy DNA paid off.

Previous attempts to isolate usable microsatellite loci from *Aedes* species have been generally frustrating, often unsuccessful, or marginally so[31,43] (D. Fonseca, unpublished). Huber and others[43] failed to develop microsatellites for *Ae. aegypti* without enrichment. Using an enrichment protocol, Fagerberg and others[31] were unable to isolate any useful microsatellites, but instead found only multi locus families.[31] With an enrichment of GT repeats, Fonseca (unpublished) similarly found only locus familes in *Ae. j. japonicus*. Although Huber and others[43] claimed they found three useful microsatellite loci for *Ae. aegypti* after using an enrichment protocol, most of the loci currently in use[44–47] were developed from sequences obtained from the *Ae. aegypti* Genome Project.[48] The drawback of this approach is that microsatellites are often in coding regions that are under selection and often have low polymorphism.[45] On the other hand, Behbahani and others[49] developed microsatellites for *Aedes polynesiensis* using an enrichment protocol apparently without any overt problem.

The success of our isolation of low copy microsatellite loci from *Ae. j. japonicus* was not without tribulation. We had a large amount of repetition in the clones, which may be partially explained by the proposition of Fagerberg and others[31] that some DNA fragments are more likely to be cloned than others (clone specific redundancy). Also, the use of PCR to create complements for the single-stranded DNA recovered from the streptavidin beads certainly generates redundant copies. Our efforts to isolate single-locus DNA reduced the risk of finding loci in locus families, but did not safeguard us from the repetition seen in the clones. To eliminate this problem we propose probing the colonies with the most repetitious flanking regions to avoid wasting resources resequencing the same locus.

The seven markers we describe have also proven to be informative for other subspecies in the *Ae. j. japonicus* complex as well as *Ae. koreicus*. Unlike mitochondrial DNA for which quasiuniversal primers are available,[50] microsatellites tend to be very species specific. Mutations accumulate in the flanking regions resulting in the amplification of a suite of primers in another species being inversely related to the evolutionary distance between the two species.[51] The fact that there was more polymorphism in *Ae. koreicus,* compared with the tested subspecies, agrees with the findings using sequence data (ND4, COII, and D2), that show *Ae. j. japonicus* is more closely related to *Ae. koreicus* than to *Ae. j. shintienensis* and *Ae. j. yayamensis* (D. Fonseca and others, unpublished data).

The microsatellite markers we report were developed to illuminate genetic differences in recently introduced populations. The newness of *Ae. j. japonicus* in the United States allows a unique opportunity to uncover some of the dynamics of introductions that are masked with time or obscured by less sensitive indicators. These primers will enable us to take an in depth look at patterns of expansion of this species in the

United States and possibly make inferences about putative source populations.

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