

## Development and characterization of 17 polymorphic microsatellite loci in the faucet snail, *Bithynia tentaculata* (Gastropoda: Caenogastropoda: Bithyniidae)

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**Abstract** *Bithynia tentaculata* (Linnaeus, 1758), a snail native to Europe, was introduced into the US Great Lakes in the 1870's and has spread to rivers throughout the Northeastern US and Upper Mississippi River (UMR). Trematode parasites, for which *B. tentaculata* is a host, have also been introduced and are causing widespread waterfowl mortality in the UMR. Waterfowl mortality is caused by ingestion of trematode-infected *B. tentaculata* or insects infected with parasites released from the snails. We isolated and characterized 17 microsatellite loci from the invasive faucet snail, *B. tentaculata* (Gastropoda: Caenogastropoda: Bithyniidae). Loci were screened in 24 individuals of *B. tentaculata*. The number of alleles per locus ranged from 2 to 6, observed heterozygosity ranged from 0.050 to 0.783, and the probability of identity values ranged from 0.10 to 0.91. These new loci provide tools for

examining the origin and spread of invasive populations in the US and management activities to prevent waterfowl mortality.

**Keywords** Microsatellite · PCR primers · SSR · STR · *Bithynia* · Faucet snail

Since 2002, large scale (~70,000 birds) die-offs of 15 species of Mississippi river waterfowl have been caused by infection with trematode parasites carried by an invasive aquatic snail, *Bithynia tentaculata* (Wilkins and Otto 2006). This snail was introduced into Lake Michigan from its native range in Europe and Asia in the early 1870s (Mills et al. 1993). Since that time the snail has extended its range into the Great Lakes (except Lake Superior), rivers throughout the northeastern United States, the Wolf River system in Wisconsin and the Upper Mississippi River pools 6–13 (UMR). *B. tentaculata* and its parasites have also been introduced into a number of additional, apparently isolated habitats in the Upper United States including Lake Winnibigoshish and Twin Lakes (MN) and St. Georges Lake, Rattlesnake Reservoir and Smith Lake in Montana. This study was intended to develop microsatellite markers to determine origin of the North American *B. tentaculata* populations and determine colonization routes within U.S. lakes and rivers to facilitate actions to deter further spread of this invasive species.

Total genomic DNA was extracted from several milligrams of *B. tentaculata* foot tissue using CTAB lysis buffer (Saghai-Marooof et al. 1984) and 10 µL Proteinase K (10 mg/ml). After incubation overnight at 37°C, a phenol–chloroform extraction was used to remove cell membranes and mucus, then DNA was precipitated using isopropanol and resuspended in Tris–EDTA with RNase. Enrichment

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**Table 1** Details for 17 polymorphic microsatellite loci developed for *Bithynia tentaculata*

| Locus  | Primer sequence 5'→3'                                  | Repeat motif         | Size (bp) | <i>N</i> | <i>k</i> | <i>H<sub>o</sub></i> | <i>H<sub>e</sub></i> | PI   |
|--------|--|----------------------|-----------|----------|----------|----------------------|----------------------|------|
| Bite03 | F: AGACCTCCCAATGCTTCAGG<br>R: *GCAACGCTCAAGGCAGTTA     | (AGT) <sub>14</sub>  | 188–209   | 22       | 5        | 0.773                | 0.697                | 0.14 |
| Bite05 | F: ATGGCGCAGCTAGAGTTGTA<br>R: *CGTTTGCAAGGCACAGAGTT    | (ACTC) <sub>11</sub> | 328–370   | 21       | 5        | 0.476                | 0.703                | 0.14 |
| Bite09 | F: *CCTGGAGGAATCTGCTGGAA<br>R: TCAAGAGCATTGTATGTACCGC  | (GTT) <sub>8</sub>   | 206–219   | 23       | 6        | 0.783                | 0.749                | 0.10 |
| Bite14 | F: *GAATCTCCACCGCTTTGACG<br>R: CGACAAGGCACTTTACCACC    | (GTTT) <sub>6</sub>  | 400–404   | 20       | 2        | 0.050                | 0.050                | 0.91 |
| Bite15 | F: *GCACGTTGACCAATCTACG<br>R: TTGTCGGTCGCAAACATAGT     | (ACAT) <sub>6</sub>  | 316–320   | 23       | 2        | 0.217                | 0.485                | 0.38 |
| Bite16 | F: *GCATCAGAGCAGCCTTTA<br>R: CCATCCATGTTAGTGAGGCC      | (GTTT) <sub>6</sub>  | 296–318   | 20       | 4        | 0.600                | 0.711                | 0.14 |
| Bite18 | F: *GCTTACTGTCCTTCGGTG<br>R: CCACTAAACAACGTTACCCA      | (ACT) <sub>10</sub>  | 286–308   | 23       | 2        | 0.217                | 0.485                | 0.62 |
| Bite19 | F: *GCTTCAGCAATGATCAGAAGGG<br>R: CGGCTGTGGAGTACGTTATCA | (AAC) <sub>19</sub>  | 210–243   | 23       | 3        | 0.565                | 0.610                | 0.22 |
| Bite20 | F: *GGCTCTTCTTCATCTGGGC<br>R: GGTGGCTGTGTAGGCACTAT     | (ATC) <sub>7</sub>   | 185–203   | 24       | 3        | 0.250                | 0.223                | 0.62 |
| Bite21 | F: *GGGACTCAGCCTCGTCAATA<br>R: CCCATAACTCTGGGATGGT     | (GTTT) <sub>6</sub>  | 307–311   | 23       | 2        | 0.522                | 0.499                | 0.38 |
| Bite22 | F: *GGGAGTAGACCAAAGAGTCCA<br>R: ACATGAGATAGGCCTTGCGG   | (AGT) <sub>8</sub>   | 136–205   | 24       | 3        | 0.208†               | 0.612                | 0.23 |
| Bite29 | F: *TGCATCGGTGGGTCTGATTA<br>R: GCTAGCCTCGTATTTCCAGC    | (GTTT) <sub>8</sub>  | 228–240   | 23       | 5        | 0.565                | 0.639                | 0.18 |
| Bite38 | F: CTTTCAGCACTAACACAGGT<br>R: *CCCTCTTTCATTTCCGAGCG    | (AAGT) <sub>6</sub>  | 268–316   | 20       | 2        | 0.050                | 0.219                | 0.63 |
| Bite40 | F: GGCAGCAGCGTTATGTTAGAA<br>R: *GAAGTTGGCTCTGTAAGACCG  | (ATC) <sub>7</sub>   | 262–281   | 23       | 4        | 0.522                | 0.566                | 0.26 |
| Bite41 | F: GTCATACTGAAGTTTCGCTGCT<br>R: *AAGCCAACCACTGCTAGAT   | (AGT) <sub>10</sub>  | 156–159   | 24       | 2        | 0.125                | 0.187                | 0.68 |
| Bite45 | F: TCGGCGACGTATGTGAGATT<br>R: *GGACTCAACGGCCTACATC     | (AGT) <sub>7</sub>   | 186–192   | 22       | 3        | 0.636                | 0.662                | 0.19 |
| Bite48 | F: TTTCTAGCTGCAATAGCGCC<br>R: *AACCGTAAGGCGAGCAAAC     | (AAAC) <sub>8</sub>  | 222–228   | 23       | 2        | 0.261                | 0.287                | 0.55 |

The number of individuals genotyped is *N*; size indicates the range of observed alleles in base pairs and includes the length of the CAG tag; *k* is number of alleles observed; *H<sub>o</sub>* and *H<sub>e</sub>* are observed and expected heterozygosity, respectively; PI is the probability of identity for each locus

\* CAG tag (5'-CAGTCGGGCGTCATCA-3') label

† Significant deviations from Hardy–Weinberg expectations after Bonferroni corrections

procedures of Glenn and Schable (2005) were followed, with some exceptions. DNA was digested with restriction enzyme *RsaI* (New England Biolabs), ligated to double-stranded linkers, denatured and hybridized to biotinylated microsatellite oligonucleotide mixes (mix 2 = (AG)<sub>12</sub> (TG)<sub>12</sub> (AAC)<sub>6</sub> (AAG)<sub>8</sub> (AAT)<sub>12</sub> (ACT)<sub>12</sub> (ATC)<sub>8</sub>; mix 3 = (AAAC)<sub>6</sub> (AAAG)<sub>6</sub> (AATC)<sub>6</sub> (AATG)<sub>6</sub> (ACAG)<sub>6</sub> (ACCT)<sub>6</sub> (ACTC)<sub>6</sub> (ACTG)<sub>6</sub>; mix 4 = (AAAT)<sub>8</sub> (AACT)<sub>8</sub> (AAGT)<sub>8</sub> (ACAT)<sub>8</sub> (AGAT)<sub>8</sub>) from IDTDNA, then

captured on magnetic streptavidin beads (Dyna). Unhybridized DNA was washed away and remaining DNA was eluted from the beads, amplified in polymerase chain reactions (PCR) using the forward SimpleX-2 as a primer. There were two primary changes to the Glenn and Schable (2005) protocol. First, a different linker was used (SimpleX-2 Forward 5'-AAAAGCTGCTGGCGAATC and SimpleX-2 Reverse 5'-pGATTTCGCCAGCAGC). Second, the enriched libraries were sequenced on a 454 Genome

Sequencer FLX System using titanium chemistry following standard Roche 454 library protocols (454 Life Sciences, a Roche company, Branford CT). Sequences were subjected to a 3' quality trim where only one base in the last 25 bases of the sequence contains a quality score less than 20 or alternatively contains one ambiguous base. CAP3 sequence assembly program (Huang and Madan 1999) was then used to assemble sequences at 98% sequence identity using a minimal overlap of 75 bp. Along with singlets, contigs of two or three sequences were searched for the presence of microsatellite DNA loci using the program MSATCOMMANDER version 0.8.1 (Faircloth 2008) and primers designed with Primer3 (Rozen and Skaletsky 2000). One primer from each pair was modified on the 5' end with an engineered sequence (CAG tag 5'-CAGTCGGGCGTCA TCA-3') to enable use of a third primer in the PCR (identical to the CAG tag) that was fluorescently labeled for detection.

Forty-eight primer pairs were tested for amplification and polymorphism using DNA obtained from eight individuals of *B. tentaculata*. PCR amplifications were performed in a 12.5  $\mu$ L volume (10 mM Tris pH 8.4, 50 mM KCl, 25.0  $\mu$ g/ml BSA, 0.4  $\mu$ M unlabeled primer, 0.04  $\mu$ M tag labeled primer, 0.36  $\mu$ M universal dye-labeled primer, 3.0 mM MgCl<sub>2</sub>, 0.8 mM dNTPs, 0.5 units JumpStart Taq DNA Polymerase (Sigma), and 20 ng DNA template) using an Applied Biosystems GeneAmp 9700. Touchdown thermal cycling programs (Don et al. 1991) encompassing a 10°C span of annealing temperatures ranging between 60 and 50°C were used for all loci (Table 1). Touchdown cycling parameters consisted of 20 cycles of 96°C for 30 s, highest annealing temperature of 60°C (decreased 0.5°C per cycle) for 30 s, and 72°C for 30 s; and 20 cycles of 96°C for 30 s, 50°C for 30 s, and 72°C for 30 s. PCR products were run on an ABI-3130xl (Applied Biosystems) sequencer and sized with Naurox size standard prepared as described in DeWoody et al. (2004), except that unlabeled primers started with GTTT. Results were analyzed using GeneMapper version 3.7 (Applied Biosystems). Seventeen of the tested primer pairs amplified high quality PCR product that exhibited polymorphism.

We assessed the variability of 17 polymorphic loci in 24 specimens that came from a site in Pool 7 of the Upper Mississippi River (Lake Onalaska). Conditions and characteristics of the 17 loci are given in Table 1. We estimated number of alleles per locus ( $k$ ), observed and expected heterozygosity ( $H_o$  and  $H_e$ ), and probability of identity (PI) using GenAlEx v6.0 (Peakall and Smouse 2006). Tests for deviations from Hardy–Weinberg equilibrium (HWE) and for linkage disequilibrium were conducted using GENEPOP v4.0 (Rousset 2008). After Bonferroni correction for multiple comparisons 1 locus showed significant deviation from expectations under HWE and no linkage

disequilibrium was detected for any pairs of loci out of 136 paired loci comparisons. These microsatellite loci will allow researchers to study the *B. tentaculata* invasion and assist management efforts. Specifically, we plan to use these markers to determine the origins of invasive *B. tentaculata* populations in the United States and examine levels of genetic diversity in invasive populations. This work has the potential to allow us to infer colonization routes and prevent further invasive spread of this species.

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