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**Short communication**

# Twenty-five new polymorphic microsatellites for the eastern mosquitofish, *Gambusia holbrooki* (Actinopterygii : Poeciliidae), an invasive species in Australia

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**Abstract.** We isolated 25 new polymorphic microsatellite markers from the eastern mosquitofish, *Gambusia holbrooki*. Initially, 454 shotgun sequencing was used to identify 1187 loci for which primers could be designed. Of these 1187, we trialled 48 in the target species, 40 of which amplified a product of expected size. Subsequently, those 40 loci were screened for variation in 48 individuals from a single population in Canberra, Australia. Twenty loci were in Hardy–Weinberg equilibrium and polymorphic, with observed heterozygosity ranging from 0.04 to 0.72 (mean: $0.45 \pm 0.18$) and the number of alleles per locus ranged from 2 to 5 (mean: $3.20 \pm 1.05$). These loci will be useful in understanding genetic variation, paternity analysis and in managing this species across both its native and invasive range.

**Additional keywords:** 454 GS-FLX, shotgun sequencing.

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The eastern mosquitofish, *Gambusia holbrooki* (Girard, 1859), is a small, live-bearing poeciliid fish native to south-eastern North America (Meffe 1991; Benejam *et al*. 2009). It is invasive in every continent except Antarctica and is the most widely distributed freshwater fish (Benejam *et al*. 2009). *Gambusia holbrooki* was intentionally released in Australia in 1925 for mosquito control (Peakall and Smouse 2006; Ayres *et al*. 2010). Its subsequent prolific invasion has had, and continues to have, significant impacts on Australian freshwater ecosystems (Komak and Crossland 2000; Pyke 2008; Reynolds 2009). These microsatellite loci, developed from an Australian *G. holbrooki* population, are therefore an important and powerful tool in the arsenal of researchers working for the conservation of freshwater ecosystems throughout Australia.

Genomic DNA (5 µg) was isolated from tissue from nine individuals of *G. holbrooki* with the Qiagen DNeasy Animal Tissue Mini Kit (Qiagen Inc., Valencia, CA) as per the manufacturer's protocol. The DNA was then sent to the Australian Genome Research Facility in Brisbane, Australia, for shotgun sequencing on a Titanium GS-FLX (454 Life Sciences/Roche FLX) following Gardner *et al*. (2011). The sample occupied 12.5% of a plate and produced 192 650 individual sequences (http://dx.doi.org/10.5061/dryad.jd183), with an average fragment size of 346, with a total microsatellite density (total number of bases of microsatellites in one Mb of sequences) of 294.28 (Meglécz *et al*. 2010). We used the program QDD ver. 1.0 (Meglécz *et al*. 2010) to screen the raw sequences with at least eight di-, tetra- or penta-base repeats, remove redundant sequences, and design primers (automated in QDD using Primer3: Rozen and Skaletsky 1999) for 1187 loci with PCR product lengths of 80–480 base pairs.

We followed the procedure outlined in Gardner *et al*. (2011) and 48 loci were chosen for further development. Initially, the loci were trialled for amplification in eight individuals in 10-µL reaction volumes containing 1× buffer, using 0.5 U Promega DNA polymerase, 2.5 mM $MgCl_2$, 0.2 mM of each dNTP, 200 nM each forward and reverse locus-specific primers and 10–50 ng gDNA. The following PCR conditions were used: one initial denaturation at 94°C for 3 min, then denaturation for 30 s at 94°C; annealing: one cycle each at 60°C, 58°C, 56°C, 54°C and 52°C, then 35 cycles at 50°C for 30 s; extension: 72°C for 45 s, and a final extension step of 72°C for 10 min. PCR products were visualised on a 2.0% agarose gel stained with SYBR® Safe (Invitrogen). Forty loci amplified an unambiguous product of the expected size. Each forward primer was 5′ labelled with a fluorescent tag: 6-FAM (GeneWorks), NED, PET or VIC (Applied Biosystems). The 40 loci were then screened for variation in 48 individuals from a single population from Canberra, Australia, at the Australian Genome Research Facility. The 40 loci were amplified in 6-µL reaction volumes containing

**Table 1. Characterisation of polymorphic loci**

Primer sequences, GenBank accession number, repeat motif, and diversity characteristics of 25 microsatellite loci from *Gambusia holbrooki*: Dye codes F, N, V, and P indicate that loci were 5′ labelled with the dyes 6-FAM, NED, VIC and PET respectively; *N* indicates the sample size; *N*a indicates number of alleles; *Ho* and *He* indicate observed and expected heterozygosity respectively; PIC indicates polymorphic information content; HWE *P* indicates the probability that loci are out of Hardy–Weinberg Equilibrium (values below 0.05 are shown in bold)

| Locus | Dye | Primer sequence (5′–3′) | GenBank accession number | Repeat motif | *N* | Allele size range | *N*a | *Ho* | *He* | PIC | Null allele frequency | HWE *P* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Locus 01 | F | F: TTGCTGACAGAGACACAGCC<br>R: GCTGCACCCACACTGACTAA | JX845687 | $(AGAT)_{12}$ | 48 | 129–195 | 4 | 0.208 | 0.209 | 0.197 | n.a. | 0.55 |
| Locus 03 | P | F: CCAACGTAACAAACTGCCTTC<br>R: TGATTTTGTTTGCTCGTTGC | JX845688 | $(ATTT)_{10}$ | 48 | 149–171 | 2 | 0.500 | 0.499 | 0.375 | n.a. | 0.990 |
| Locus 04 | V | F: GTGGCATCGTGTCTTTCTGA<br>R: CACTCCACATATGCACAAAGG | JX845689 | $(CTGT)_{10}$ | 46 | 137–159 | 3 | 0.652 | 0.612 | 0.54 | n.a. | 0.066 |
| Locus 06 | N | F: AGTTAGCTGCTGGCAAGGAC<br>R: AGCTGCTGTGGTTCAGACTTT | JX845690 | $(GTCT)_{8}$ | 48 | 106–114 | 3 | 0.208 | 0.225 | 0.206 | n.a. | **0.000** |
| Locus 10 | P | F: CGCTCTTCACAGTTCTTCCC<br>R: AGGAGGACGTCACCACAAAG | JX845691 | $(CTT)_{12}$ | 48 | 112–118 | 2 | 0.042 | 0.209 | 0.04 | n.a. | 0.883 |
| Locus 11 | N | F: ACACTAAGGGTCCATGCTGC<br>R: GGCAGGAAACCAACCAGTTA | JX845692 | $(TAT)_{8}$ | 48 | 109–112 | 2 | 0.125 | 0.041 | 0.11 | n.a. | 0.644 |
| Locus 13 | V | F: ATCGTGTTGGGATGTGACAA<br>R: TGCGCACCTTACTCATCTTG | JX845693 | $(CCA)_{10}$ | 47 | 185–188 | 2 | 0.447 | 0.409 | 0.326 | n.a. | 0.532 |
| Locus 14 | V | F: GCTCCTCTTTGCTGCTTCAT<br>R: AGGAGGAAACCATAACGGCT | JX845694 | $(ATG)_{11}$ | 47 | 118–136 | 4 | 0.511 | 0.550 | 0.494 | n.a. | 0.364 |
| Locus 16 | P | F: AGCCCAAAGACGAAGTTGAG<br>R: GGCGTCAATCCGTCTACAGT | JX845695 | $(TCA)_{14}$ | 48 | 205–223 | 3 | 0.583 | 0.575 | 0.495 | n.a. | 0.828 |
| Locus 17 | N | F: TGAAACGGAATGAGAGTAGAGACA<br>R: TTTCCATCAATGCAAGACCA | JX845696 | $(ATCC)_{13}$ | 48 | 107–147 | 2 | 0.417 | 0.413 | 0.328 | n.a. | 0.954 |
| Locus 18 | F | F: TTTTCATCTCCTGTTATCTCCTGTT<br>R: TGGTAATAATGAAGTGGAAGCAGA | JX845697 | $(TCCA)_{11}$ | 42 | 97–101 | 2 | 0.190 | 0.172 | 0.157 | n.a. | 0.495 |
| Locus 19 | P | F: TCAGAGTCTATCTCATCTTTCTGAGTC<br>R: AAAGCGAAGAGCTGCTGAAC | JX845698 | $(AAT)_{12}$ | 47 | 245–311 | 4 | 0.511 | 0.563 | 0.47 | n.a. | 0.874 |
| Locus 22 | F | F: GTTGGACAAGAAGGCCTGG<br>R: TGTGACTGCACCACAGCATA | JX845699 | $(TAT)_{25}$ | 48 | 155–194 | 4 | 0.563 | 0.558 | 0.517 | n.a. | 0.707 |
| Locus 23 | N | F: CAGGAGATGCTCAACCACG<br>R: CTGCTCAGCCCAGGTAAGAC | JX845700 | $(AC)_{15}$ | 47 | 233–239 | 3 | 0.447 | 0.507 | 0.434 | n.a. | **0.018** |
| Locus 25 | V | F: CTGGTCCATCCTTCTCCAAA<br>R: CAGGAGAACATTCCTCGGTC | JX845701 | $(TG)_{12}$ | 48 | 159–165 | 2 | 0.479 | 0.498 | 0.374 | n.a. | 0.793 |
| Locus 27 | V | F: CTGGAATTGTTTGCCAAGGT<br>R: ACAGCCCACGTAACCTCATC | JX845702 | $(AC)_{22}$ | 48 | 140–150 | 5 | 0.708 | 0.707 | 0.657 | n.a. | 0.777 |
| Locus 29 | N | F: TTGTCACATTCGTCTCCAGC<br>R: GCTGCGTGTTCCATATTTGTT | JX845703 | $(AC)_{12}$ | 47 | 120–130 | 4 | 0.723 | 0.720 | 0.669 | n.a. | 1.000 |
| Locus 32 | P | F: GTCCTGAGTCCCTGGAGGA<br>R: TTGGAGCAGAGCAGGTTACA | JX845704 | $(AC)_{16}$ | 46 | 114–160 | 4 | 0.652 | 0.526 | 0.438 | n.a. | **0.001** |
| Locus 34 | N | F: TCAAAGAAATTGAGATTTAACCACC<br>R: ACACTGAGGCCGCTGTAACT | JX845705 | $(CAG)_{10}$ | 47 | 86–104 | 3 | 0.489 | 0.421 | 0.378 | n.a. | 0.325 |
| Locus 35 | N | F: TGGAATGGTCCAAATTCCTC<br>R: AAATGCCTACCTTAGCAACAGC | JX845706 | $(TGT)_{18}$ | 48 | 81–129 | 5 | 0.333 | 0.453 | 0.399 | 0.110 | **0.000** |
| Locus 36 | V | F: AACTCACTGAGAGCGCCTGT<br>R: TTTCTTAGACCGCAACCCAC | JX845707 | $(ATA)_{12}$ | 39 | 82–106 | 3 | 0.469 | 0.485 | 0.434 | n.a. | 0.267 |
| Locus 37 | P | F: TCCATGTGAACAGAGTCGGA<br>R: CATCAACGGCCAAATTCATA | JX845708 | $(GAT)_{18}$ | 47 | 89–149 | 5 | 0.489 | 0.569 | 0.496 | n.a. | **0.000** |
| Locus 38 | V | F: CAGTGGTACCGGGTCAGAAC<br>R: GTCCAGCAGAGGTTCAATCG | JX845709 | $(CTG)_{16}$ | 48 | 97–106 | 3 | 0.604 | 0.574 | 0.506 | n.a. | 0.141 |
| Locus 40 | F | F: CATTAGGAGATGAAATTATCAAGGG<br>R: ACAGACATTTCTGATCATAACCCA | JX845710 | $(ATA)_{11}$ | 47 | 84–87 | 2 | 0.362 | 0.370 | 0.301 | n.a. | 0.883 |
| Locus 41 | N | F: AACAGATTAAGAGCATGTGGCT<br>R: GCTCAGTGCACCACAAAGAA | JX845711 | $(ATT)_{13}$ | 47 | 90–120 | 4 | 0.426 | 0.561 | 0.475 | 0.112 | 0.281 |

PCR buffer, Bioline Immolase DNA polymerase and dNTPs based on the recommendations provided by Bioline (available from: www.bioline.com), 1.5 mM $MgCl_2$, 0.33 µM each forward and reverse primer and 15 ng gDNA. The following PCR conditions were used: 95°C for 5 min followed by 15 cycles at 94°C for 30 s, 60°C for 45 s (dropping 0.5°C per cycle), and 72°C for 45 s; followed by 17 cycles at 94°C for 30 s, 53°C for 45 s, and 72°C for 45 s; followed by 10 cycles at 94°C for 30 s, 50°C for 45 s, and 72°C for 45 s; and a final elongation step at 72°C for 10 min. The panel design was: Panel 1: Gh06, Gh15, Gh38, Gh44 and Gh45; Panel 2: Gh13, Gh17, Gh22, Gh32, Gh40 and Gh47; Panel 3: Gh04, Gh11, Gh12, Gh16 and Gh18; Panel 4: Gh07, Gh10, Gh21, Gh27 and Gh48; Panel 5: Gh28, Gh30, Gh35 and Gh46; Panel 6: Gh03, Gh14, Gh23, Gh31 and Gh41; Panel 7: Gh01, Gh25, Gh34 and Gh37; Panel 8: Gh19, Gh29, Gh42 and Gh43; Panel 9: Gh24 and Gh36. Of the 40 loci, 25 (62.50%) loci were polymorphic, 13 (32.50%) were monomorphic and alleles in 2 (5.00%) loci were ambiguous. For each of the polymorphic loci (25) we tested for Hardy–Weinberg Equilibrium (HWE) using GenAlEx 6.5 (Peakall and Smouse 2006) and found that five loci deviated from HWE (Table 1). For the 25 loci we also calculated number and range of alleles, observed and expected heterozygosity, polymorphic information content and estimated null allele frequencies using Cervus 3.0 (Kalinowski *et al*. 2007) (Table 1). We used Micro-Checker 2.2.3 (Van Oosterhout *et al*. 2004) to check each locus for further evidence of null alleles, scoring error due to stuttering, and large allele drop out. Two loci (Loci 23 and 41) showed significant null allele frequencies at the target site (Table 1). None of the loci showed evidence for large allele drop out, or evidence of scoring error due to stuttering. We checked all pairs of loci for linkage disequilibrium in Genepop; two locus pair comparisons (Locus 17 with both Locus 5 and 9) were significant after sequential Bonferroni adjustment. To maintain data independence in further analyses, Locus 17 should therefore not be used in conjunction with these possibly linked loci. These markers will be used to document the genetic diversity in *G. holbrooki* and to investigate patterns of paternity in selected laboratory lines, but more broadly will prove an important tool in the conservation of Australian freshwater ecosystems.

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