Only one peak was detected for each locus, which is consistent with the haploid genome of *M. phaseolina*. Because they are haploids, observed heterozygosity for all loci is 0 and is not included in Table 1. Allele number per locus ranged from 6 to 22 except for locus MP19, which only had produced one allele for all samples. The average number of alleles per locus was 12.15. Allelic richness was calculated by dividing the number of alleles at a locus by the number of samples, and ranged from 0.018 to 0.400 when null alleles are included. We were able to uniquely identify all 55 haploid fungi samples using these 12 loci, presumably because of the large number of alleles at each locus. Average pairwise genetic distance within all soybean samples was 65.23% similar using Nei's Minimum (Nei 1973). Within each soybean population, the average pairwise genetic distance was 68.60% and 70.90% for soybean collection sites 1 and 2, respectively. We expect to use these loci for future population genetics studies as well as for identification of *M. phaseolina*. We are testing cross-amplification of these loci in other pathogens as well. Optimization of simple sequence repeat (SSR) amplification was relatively straightforward, and we expect the remaining 33 SSR loci to be useful in future studies.

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# Tri- and tetranucleotide microsatellites in dhufish *Glaucosoma hebracium* (Perciformes)

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### Abstract

**Thirteen polymorphic tri- and tetranucleotide microsatellite markers are reported for the exploited marine dhufish (*Glaucosoma hebracium*) from southwestern Australia. Variation was assessed among 48 individuals collected from a single locality. Most loci had 2–5 alleles, although one had more than 20 alleles, with corresponding observed heterozygosities of 0.02–0.81. These loci were also polymorphic in congeneric species.**

*Keywords*: genome, *Glaucosoma hebracium*, microsatellite, pufferfish, SSR, VNTR

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The dhufish (*Glaucosoma hebracium*) is distributed along the southwestern coast of Australia, and subjected to both commercial and recreational fisheries (Wise *et al*. 2007). Recent depletion of the fishery is suggested by a decline in the proportion of older fish in the population, and current exploitation exceeds international benchmarks (Wise *et al*. 2007). As adults appear somewhat sedentary based on preliminary tagging and otolith chemistry data (Wise *et al*. 2007), dispersal may be predominantly mediated via the larvae, and could represent an important source for recruitment to depleted populations. Here we report the isolation and characterization of tri- and tetranucleotide microsatellites for this species, to facilitate future studies of population structuring, to aid in the management of this resource.

Genomic DNA libraries were constructed and enriched for the following tri- and tetranucleotide repeat sequences: ATG, CAG, CAGA, CATC. These selections were made based on the relative frequencies of motifs in another perciform, the pufferfish *Takifugu rubripes* (Edwards *et al*. 1998; Takagi 2003), and probes that were available for enrichment by Genetic Identification Services (Chatsworth, California).

DNA library construction, enrichment and screening were as described previously (Jones *et al*. 2002). Genomic DNA was partially restricted with a cocktail of seven blunt-end cutting enzymes (*Rsa*I, *Hae*III, *Bsr*B1, *Pvu*II, *Stu*I, *Sca*I, *Eco*RV). Fragments in the size range of 300–750 bp were ligated to adaptor molecules (5′-AAGCTTCCGTCGTTTT-ACAACGTCGTGG-3′, 5′-ACGACGTTGTAAAACGACGG-AAGCTT-3′) and subjected to magnetic bead capture (CPG, Inc.), using biotinylated capture molecules (biotin-labelled probes for the motifs listed above) in a protocol provided by the manufacturer. Captured molecules were amplified by polymerase chain reaction (PCR) using one of the adaptor molecules as a primer, and restricted with *Hin*dIII to remove the adapters. The resulting fragments were ligated into the *Hin*dIII site of pUC19, and then electroporated into *Escherichia coli* DH5α cells. Recombinant clones were sequenced on an ABI 377 (Applied Biosystems Inc.), using ABI PRISM dye terminator cycle sequencing methodology.

Nine clones from each motif-enriched library were sequenced, yielding seven, three, eight, and eight microsatellite-containing clones for the ATG, CAG, CAGA, and CATC motifs, respectively. Based on these results, a further 32 clones were sequenced from the ATG library, and 16 from each of the CAGA and CATC libraries, yielding 25, 15 and 15 microsatellite-containing sequences, respectively.

PCR primers were designed for 18 loci using PrimerSelect (DNASTAR). Genomic DNA was extracted from 48 individuals from the vicinity of Canal Rocks (–33.6692°S, 114.9972°E), using a simple 96-well silica-membrane approach (Epoch Biolabs). Forward PCR primers were 5′-end labelled with fluorescent dyes 6-FAM, Yakima Yellow, ATTO550, and ATTO565 (Microsynth) for detection on a 3730 DNA Analyser (Applied Biosystems Inc.). PCR was initially performed on subsets of four or five loci and 24 test individuals, to assess amplification and polymorphism, using the QIAGEN multiplex kit following manufacturer′s instructions for microsatellites. Amplifications were performed in 10 μL volumes comprising 5 μL of 2× QIAGEN Multiplex PCR Mastermix, 0.1 μM of each primer, and 2 μL DNA. Thermal cycling comprised 15 min at 95 °C, followed by 35 cycles of 94 °C for 30 s, 57 °C annealing for 90 s, and 72 °C extension for 60 s, and then a final 30-min extension at 60 °C subsequent to cycling. PCR products were diluted 1000-fold into a 20-μL volume containing 50% HiDi Formamide and 0.015 μL GeneScan 500 LIZ (ABI) size standard, prior to electrophoresis on a 3730 DNA Analyser.

Five loci were monomorphic in the source species. The remaining 13 loci were then amplified simultaneously for an additional set of 24 individuals using the same PCR method as above, but with varying primer concentrations (listed in Table 1). Because locus *GheC102* amplified weakly with the other loci, this locus was also amplified individually using Bioline MangoTaq (0.5 U, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 1× reaction buffer) with 35 cycles of 94 °C for 30 s, 58 °C for 30, and 72 °C for 60 s. An initial denaturation for 3 min at 94 °C, and a final extension for 30 min at 72 °C were also employed. Dilution and electrophoresis were performed as previously, with 1000-fold dilution of both the multiplex and single-locus PCRs. Allele scoring was performed with GeneMapper (ABI).

Concordances of microsatellite genotype frequencies with those expected under Hardy–Weinberg equilibrium, and the independence of genotypes among loci, were examined using exact tests implemented by GenePop4 (Rousset 2008), followed by sequential Bonferroni correction of $P$ values (Rice 1989). GenePop was also employed to calculate observed and expected heterozygosity levels, and to estimate $F_{IS}$.

The number of alleles observed per polymorphic locus ranged from 2 to 22, but nine of the loci exhibited less than

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**Table 1** Locus repeat motif, number of alleles surveyed ($n$), number of distinct alleles ($A$), size range, observed and expected heterozygosities ($H_O$, $H_E$), GenBank Accession, and PCR primer sequence and concentrations for each of 13 microsatellite loci developed for *Glaucosoma hebracium*. Locus names indicate the species (*Ghe*) and clone identifier (e.g. *A127*)

| Locus | Repeat sequence | GenBank Accession | $n$ | $A$ | Size range (bp) | $H_O$ | $H_E$ | $F_{IS}$ | Primer sequences (5′–3′; forward, reverse) | (Primer) µM | Forward dye label |
|---|---|---|---|---|---|---|---|---|---|---|---|
| *GheA010* | $(ATC)_{11}$ | FJ409066 | 96 | 3 | 117–123 | 0.56 | 0.56 | –0.010 | AAGCCCAACGGATCAAAGAACAA<br>GAGCCGGTGGAGACAATAACAGTG | 0.100 | ATTO565 |
| *GheA108* | $(CTG)_6(ATG)_{10}$ | FJ409067 | 96 | 7 | 133–151 | 0.69 | 0.72 | 0.050 | AACGTCCACTTTCCAACC<br>ACTCAGCCTGTCTTTATCATC | 0.050 | ATTO550 |
| *GheB008* | $(CAG)_8$ | FJ409068 | 96 | 3 | 142–151 | 0.38 | 0.44 | 0.140 | CGGAGGGCTGGGCTGGAAATAA<br>GGCTGGCGCTGTTGACGAAATG | 0.100 | Yakima Yellow |
| *GheD002* | $(TGGA)_8$ | FJ409069 | 96 | 3 | 170–186 | 0.58 | 0.50 | –0.179 | ACACTTGTTATCTTGGCTGCTCTG<br>CAATCATCCTTCCGTCCATCTGTC | 0.075 | ATTO565 |
| *GheC105* | $(CTGY)_7CTAT(CTGY)_5$ | FJ409070 | 96 | 2 | 188–192 | 0.02 | 0.02 | 0.000 | AGGCACACGGGATAACAATAAATA<br>TGTGAGTGTGAAAGCCTGATAGAG | 0.100 | ATTO550 |
| *GheA110* | $(ATC)_{11}$ | FJ409071 | 96 | 5 | 199–223 | 0.31 | 0.37 | 0.162 | CCCGCGCCACCTCCCACTC<br>GTCCTGTACGCCTCAGTCAACCCAGAG | 0.075 | Yakima Yellow |
| *GheA104* | $(ATC)_8$ | FJ409072 | 96 | 3 | 233–239 | 0.56 | 0.55 | –0.021 | GAGAATCTGTCCATCGGGTGTTA<br>CAGTCAGTTTGGTGTTTGCTTTGTTC | 0.100 | ATTO565 |
| *GheD101* | $(CATC)_{13}(CGTC)_2$ | FJ409073 | 90 | 9 | 278–310 | 0.73 | 0.68 | –0.067 | GCGCGGACCGGGACTGAA<br>AAGGGGAGATGGGTGGATGAAGG | 0.100 | ATTO565 |
| *GheD102* | $(GATG)_8$ | FJ409074 | 92 | 4 | 301–317 | 0.69 | 0.63 | –0.092 | CTGCCATGTTCTACTTCTCCACTA<br>CCATTTTCGGTTTCTGTTTGA | 0.100 | ATTO550 |
| *GheA127* | $(ATG)_{13}$ | FJ409075 | 90 | 4 | 303–318 | 0.25 | 0.44 | 0.429 | CAATCGTCCTTATGGCTTCAC<br>TATCTTCACTGGCTTAATGGTCTC | 0.150 | FAM |
| *GheC102* | $(GTCT)_{16}$ | FJ409076 | 80 | 22 | 335–427 | 0.81 | 0.77 | –0.059 | ATCAAGACAAGCAGACGCCACCAG<br>GCAGCAGCAGCATAAAGTCCAGAG | 0.350 | Yakima Yellow |
| *GheA114* | $(ATC)_{10}$ | FJ409077 | 80 | 3 | 377–386 | 0.17 | 0.16 | –0.076 | AGGGGCTGGAGATGACAATG<br>**GTTT**CTGACCAGGAATCCCACTATGA* | 0.100 | ATTO565 |
| *GheA129* | $(RTC)_{11}$ | FJ409078 | 80 | 3 | 382–394 | 0.50 | 0.48 | –0.046 | ATGAAGGGAGATGAAGTGTTTGAG<br>**GTTT**GCGCCAGGCTGAGATGAC* | 0.100 | ATTO550 |
| *GheA003* | $(CAT)_{11}$ | FJ409079 | 48 | 1 | 241 | 0.00 | 0.00 | | GAGAACTGACCCCCAAAACATCC<br>GTAAAATCAACAAAAAGAATCCCAGAAA | 0.100 | ATTO550 |
| *GheA005* | $(TCA)_8$ | FJ409080 | 48 | 1 | 387 | 0.00 | 0.00 | | GTTCTGCCATAAAGCCACAT<br>**GTTT**CCCAGGGGTTCAGTCAA* | 0.100 | FAM |

*The 5′ end of the reverse primer has been modified to read 'GTTT', to improve a-tailing of PCR products by *Taq* polymerase (Brownstein *et al*. 1996).

six alleles (Table 1). Expected heterozygosities ranged from 0.16 to 0.81, except for locus *GheC105* in which only two alleles and one heterozygous individual was observed ($H_E = 0.02$). Significant deviation from Hardy–Weinberg expectations was only observed for *GheA127* ($P = 0.0023$), and genotypes appeared independent among loci (smallest $P = 0.006$, but none was significant following sequential correction for 78 simultaneous tests). The deviation from Hardy–Weinberg at locus *GheA127* is in the direction of heterozygote deficiency. The presence of null alleles at this locus was suggested by analyses using Micro-Checker 2.2.3 (Van Oosterhout *et al*. 2004), which indicated a general excess of homozygotes for each allele size class, and an estimated null allele frequency of 0.13–0.28, depending on the method employed.

The microsatellites were tested on other *Glaucosoma* spp. without additional optimization of PCR conditions, and in the majority they successfully amplified products and exhibited polymorphism in *G. buergeri*, *G. magnificum*, and *G. scapulare* (Table 2).

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**Table 2** Number of alleles and allele size range (in bp) of microsatellite loci developed for *Glaucosoma hebracium* in congeneric species. Numbers in parentheses after species name represent the number of individuals surveyed. '—' indicates no discernable amplification

| | Species | | |
|---|---|---|---|
| Locus | *G. buergeri* (5) | *G. scapulare* (4) | *G. magnificum* (4) |
| *GheA010* | 3, 117–123 bp | 3, 111–123 bp | — |
| *GheA108* | 1, 131 bp | 3, 132–148 bp | 1, 134 bp |
| *GheB008* | 2, 148–151 bp | 1, 148 bp | 1, 126 bp |
| *GheD002* | 6, 162–198 bp | 5, 182–206 bp | 2, 162–166 bp |
| *GheC105* | — | — | — |
| *GheA110* | 3, 200–221 bp | 7, 214–235 bp | 2, 213–216 bp |
| *GheA104* | 2, 250–253 bp | 5, 252–261 bp | — |
| *GheD101* | 3, 273–281 bp | 5, 273–289 bp | — |
| *GheD102* | 2, 313–317 bp | 2, 305–317 bp | — |
| *GheA127* | 4, 303–312 bp | 2, 317–320 bp | 4, 276–285 bp |
| *GheC102* | 5, 338–410 bp | 2, 316–320 bp | 3, 320–340 bp |
| *GheA114* | 1, 379 bp | 4, 370–385 bp | 1, 376 bp |
| *GheA129* | 2, 404–407 bp | — | — |
| *GheA003* | 2, 242–245 bp | 3, 238–253 bp | 3, 209–215 bp |
| *GheA005* | 4, 396–399 bp | 2, 387–390 bp | 5, 390–402 bp |

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# Development of 30 microsatellite markers for dab (*Limanda limanda* L.): a key UK marine biomonitoring species

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## Abstract

**Dab (*Limanda limanda*) are the principal target fish species in offshore biomonitoring programmes in the UK; however, detailed knowledge of genetic structure and connectivity among sampling locations is unavailable. Here, the isolation and characterization of 30 polymorphic microsatellite loci for dab is described. The number of alleles per locus ranged from 2 to 42, with observed heterozygosities ranging from 0.089 to 1. These loci will enable high resolution of genetic population structure and dynamics of dab around the British Isles.**

*Keywords*: biomonitoring, dab, ecotoxicology, flatfish, microsatellites, population genetics

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