

Characterization of 12 polymorphic microsatellite loci in *Ifremeria nautiliei*, a chemoautotrophic gastropod from deep-sea hydrothermal vents

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Abstract *Ifremeria nautiliei* is deep-sea provannid gastropod endemic to hydrothermal vents at southwest Pacific back-arc spreading centers. Twelve, selectively neutral and unlinked polymorphic microsatellite loci were developed for this species. Three loci deviated significantly from Hardy–Weinberg expectations. Average observed heterozygosity ranged from 0.719 to 0.906 (mean $H_O = 0.547$, $SD = 0.206$). Three of the 12 loci cross-amplified in two species of Alviniconcha (Provannidae) that co-occur with *I. nautiliei* at Pacific vent habitats. Microsatellites developed for *I. nautiliei* are being deployed to study connectivity among populations of this species colonizing geographically discrete back-arc basin vent systems.

Keywords Back-arc basin · Chemoautotrophic · Hydrothermal vent · *Ifremeria nautiliei* · Microsatellite · Provannid gastropod

Deep-sea hydrothermal vents are discrete habitats that occur throughout the ocean at plate margins and other volcanically active settings. Biomass-dominant taxa endemic to vent systems are key targets for understanding population structure and connectivity at vents (e.g., Hurtado et al. 2004; Shank and Halanych 2007; Fusaro

et al. 2008). Commercial grade polymetallic sulfide deposits are often associated with hydrothermal vents, making some vents candidates for mining operations (Rona 2003). Defining natural conservation units for key vent-endemic organisms can inform best practices for minimizing the environmental impact of mining activities (Halfar and Fujita 2002).

Ifremeria nautiliei is a deep-sea vent-endemic provannid gastropod from the western Pacific (Bouchet and Warén 1991), where it is a dominant primary consumer at geographically isolated back-arc basins (Desbruyères et al. 2006). The species is dependent on chemoautotrophic endosymbionts for nutrition (Suzuki et al. 2006), and it creates habitat for a diverse assemblage of other vent organisms (Urakawa et al. 2005).

Ifremeria nautiliei DNA was enriched for microsatellite-containing motifs following Glenn and Schable (2005). Genomic DNA was isolated from ethanol-preserved foot tissue by Chelex-Proteinase K extraction. Tissue (10–30 mg) was digested with 120 µg Proteinase K (Bioline: Taunton, MA) in 600 µl 10% Chelex-100 resin (Bio-Rad: Hercules, CA) overnight at 60°C, heated to 100°C for 15 min, and centrifuged at 10,000 rpm for 5 min. Supernatant was digested with *RsaI*, ligated to SNX linkers, hybridized with Biotinylated oligonucleotide probes (Mix 2 and Mix 3, Glenn and Schable 2005), bound to Streptavidin magnetic beads, and amplified with SNX-F primer. Enriched DNA was transformed via Topo TA vector (Invitrogen: Carlsbad, CA) into α -Select electrocompetent cells (Bioline: Taunton, MA). Clones were sequenced on an ABI 3730xl DNA Analyzer (Applied Biosystems: Foster City, CA). Repetitive elements were located using Msatfinder (Thurston and Field 2005) and flanking primers were designed with Primer3 software (Rozen and Skaletsky 2000). Forward primers were

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designed with a T3-tail (5'-ATTAACCCTCACTAAAGG GA-3') to allow the attachment of FAM-labeled (6-carboxy-fluorescein) T3- primer (Schuelke 2000).

Genomic DNA template was diluted 1:10 for 20 µl polymerase chain reactions (PCRs) prepared as follows: 2 µl template, 2 µl 10× PCR Buffer (200 mM Tris, pH 8.8; 500 mM KCl; 0.1% Triton X-100, 0.2 mg/ml BSA), 2 µl MgCl₂ (final concentrations indicated in Table 1), 1 µl 2.5 mM dNTP's, 0.2 µl 10 µM forward primer, 0.8 µl 10 µM reverse primer, 0.8 µl 10 µM 5'-FAM-labeled T3 primer (Eurofins: Huntsville, AL), and 0.2 µl Taq polymerase (1 unit, Bioline: Taunton, MA). Reactions were run under the following conditions: 95°C 4 min; 25 cycles of 94°C 45 s, T_A °C 15 s (indicated in Table 1), 72°C 45 s; 8 cycles of 94°C 45 s, 53°C 30 s, and 72°C 45 s; final extension 72°C for 10 min.

PCR products were diluted 1:10 and 1 µl added to 0.05 µl LIZ500 size standard and 8.95 µl water (MCLab, San Francisco CA) and denatured at 95°C for 10 min. Size-

fragment analysis was conducted on an ABI 3730xl DNA analyzer. Chromatograms were scored using Genemarker v1.8 (SoftGenetics LLC: State College, PA).

Deviation from Hardy–Weinberg Equilibrium (HWE), heterozygote excess and deficiency, and linkage disequilibrium were tested with Genepop version 4.0.10 (Rousset 2009) and corrected for multiple tests using sequential Bonferroni (Rice 1989). Presence of null alleles, stutter, and large allele dropout were assessed using MicroChecker (1,000 randomizations; van Oosterhout et al. 2004). The software LOSITAN (50,000 simulations; Antao et al. 2008) was used to detect loci potentially under selection.

Out of approximately 2,500 clones screened, 105 primer pairs were developed. Twelve loci were polymorphic and reproducible. All 12 loci were assessed on 71 *Ifremeria nautiliei* individuals from Manus Basin. Four loci (Ifr20A, Ifr25, Ifr73, and Ifr94) deviated significantly from HWE (Table 1) but only Ifr20A, Ifr25, and Ifr73 remained significant after sequential Bonferroni correction ($k = 12$).

Table 1 Polymorphic loci (GenBank accessions GU361800–GU361811) from 71 *Ifremeria nautiliei* individuals

| Locus | Repeat motif | Primer sequence (5'-3') | [MgCl] (mM) | T_A (°C) | Allele size range | N_A | H_O | H_E | P | <i>Alviniconcha</i> species cross amplification (# positive/# total) |
|--------|--------------|---|-------------|------------|-------------------|-------|-------|-------|--------|--|
| Ifr20A | (TC)7 | F: GCGAGCGCTCATATACATGC R: GCCCAGTCACCCACTATGGG | 1.0 | 62.0 | 143–181 | 14 | 0.41 | 0.86 | 0.000* | <i>Alviniconcha</i> type 1 (0/3) <i>Alviniconcha</i> type 2 (0/3) |
| Ifr25 | (ATGTT)20 | F: TCGAACGACAACCTTAATTCC R: CTTACACTGTCCCGTCATCG | 2.0 | 60.5 | 147–290 | 28 | 0.70 | 0.92 | 0.000* | <i>Alviniconcha</i> type 1 (0/3) <i>Alviniconcha</i> type 2 (0/3) |
| Ifr40 | (CAAA)6 | F: GTTCTGTCATGTGACAGATGC R: AGGCCAACCGTTTAACCAC | 1.0 | 56.0 | 202–218 | 3 | 0.51 | 0.52 | 0.611 | <i>Alviniconcha</i> type 1 (0/3) <i>Alviniconcha</i> type 2 (0/3) |
| Ifr43 | (AG)7 | F: CTGCAACTGAAACCCACAAG R: TCAAGTGTGCTCCAAGCATC | 2.0 | 62.0 | 174–200 | 6 | 0.62 | 0.66 | 0.088 | <i>Alviniconcha</i> type 1 (0/3) <i>Alviniconcha</i> type 2 (0/3) |
| Ifr52 | (TTG)7 | F: AGTGATAGGCCTGGTGTTC R: GACTAGGAACTCCCCACAAGC | 2.0 | 64.0 | 217–226 | 3 | 0.63 | 0.57 | 0.700 | <i>Alviniconcha</i> type 1 (0/3) <i>Alviniconcha</i> type 2 (0/3) |
| Ifr68 | (TG)8 | F: CGAATGACCCTGCAACTGTA R: GCTGGTCTTGTTCCTCAAGC | 2.0 | 62.3 | 221–231 | 4 | 0.63 | 0.64 | 0.792 | <i>Alviniconcha</i> type 1 (0/3) <i>Alviniconcha</i> type 2 (0/3) |
| Ifr73 | (ACTA)4 | F: CATGTCAGCCCTGATGTATCC R: TGCTCTTTTGAATGCCTTCG | 2.0 | 59.5 | 249–253 | 2 | 0.06 | 0.18 | 0.000* | <i>Alviniconcha</i> type 1 (0/3) <i>Alviniconcha</i> type 2 (0/3) |
| Ifr78 | (GT)6 | F: CGTAAGCTTAGCCGGACTGG R: CAAACAAACGTCCATGATGC | 2.0 | 60.5 | 246–250 | 5 | 0.41 | 0.48 | 0.321 | <i>Alviniconcha</i> type 1 (3/3) <i>Alviniconcha</i> type 2 (2/3) |
| Ifr86 | (AG)9 | F: CCCCTCTATGTCACCAGTCG R: GAAGAAGGCGTCAGAAATGG | 2.0 | 56.0 | 227–231 | 3 | 0.30 | 0.32 | 0.459 | <i>Alviniconcha</i> type 1 (0/3) <i>Alviniconcha</i> type 2 (0/3) |
| Ifr93 | (TG)5 (CT)10 | F: ATCTGGCTGGTCTGTCTTGG R: GGCTACATAACGCACTGAGG | 2.0 | 62.3 | 252–283 | 20 | 0.80 | 0.91 | 0.130 | <i>Alviniconcha</i> type 1 (0/3) <i>Alviniconcha</i> type 2 (0/3) |
| Ifr94 | (CAAA)3 | F: TTGAGTCGACAGCCAGACC R: CTAAGTCCGGTCTTTGATGG | 2.0 | 63.5 | 258–279 | 5 | 0.30 | 0.35 | 0.050 | <i>Alviniconcha</i> type 1 (2/3) <i>Alviniconcha</i> type 2 (2/3) |
| Ifr103 | (TTAG)3 | F: TTGACAGAACTTTGGTGTGG R: CACTATTGCAGGCTGACTGG | 2.0 | 60.5 | 222–364 | 4 | 0.51 | 0.53 | 0.488 | <i>Alviniconcha</i> type 1 (3/3) <i>Alviniconcha</i> type 2 (3/3) |

Concentration of MgCl ([MgCl]), annealing temperature (T_A), size range of alleles (base pairs), number of alleles (N_A), observed heterozygosity (H_O), expected heterozygosity (H_E), and probability of deviation from Hardy–Weinberg Equilibrium (P) are reported. Significant deviation from Hardy–Weinberg Equilibrium after sequential Bonferroni correction is indicated with an asterisk. Cross-amplification with *Alviniconcha* phylotypes are reported from 3 individuals tested for each species

Four loci (Ifr20A, Ifr25, Ifr73, and Ifr93) showed significant homozygote excess ($P < 0.05$) and remained significant after Bonferroni correction ($k = 12$). Micro-checker indicated the presence of null alleles at four loci (Ifr20A, Ifr25, Ifr73, and Ifr93), and two loci (Ifr20A and Ifr73) showed evidence of scoring errors due to stutter. There was no evidence for linkage disequilibrium among loci nor did LOSITAN indicate selection at any locus.

All markers were tested for cross amplification in two phylotypes of *Alviniconcha* (*Alviniconcha* type 1 and *Alviniconcha* type 2; Kojima et al. 2001) using reaction conditions optimized for *Ifremeria nautiliei*. Three loci (Ifr78, Ifr94, and Ifr103) showed positive cross-amplification (Table 1).

To our knowledge, this is the first report of microsatellite markers developed for a deep-sea vent gastropod. Microsatellite markers have been developed for three other deep-sea hydrothermal-vent-endemic organisms (*Riftia pachyptila*: Fusaro et al. 2008; *Branchiopolynoe seepensis*: Daguin and Jollivet 2005; *Bathymodiolus childressi*: Carney et al. 2006). *I. nautiliei* microsatellite markers are being deployed on *I. nautiliei* populations in the western Pacific to explore connectivity within and among Manus, Fiji, and Lau Basins. The data generated will establish a baseline of genetic diversity to assess changes caused by volcanic eruptions, mining, or other disruptive events.

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