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TECHNICAL NOTE

Isolation and characterization of 8 novel microsatellites for the black abalone, *Haliotis cracherodii*, a marine gastropod decimated by the withering disease

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Abstract The black abalone, *Haliotis cracherodii*, is an intertidal marine gastropod that used to be extremely abundant before overharvesting and an outbreak of withering syndrome lowered its numbers to, in some sites, few individuals. A set of eight microsatellite markers was developed and tested for polymorphism using 41 individuals from two populations. Loci were highly polymorphic with expected heterozygosities ranging from 0.86 to 0.96, a likely signature of past large population size. These markers will be useful in tracking the fate of such a vulnerable species that is likely to experience a severe bottleneck in the next few generations.

Keywords *Haliotis cracherodii* · Black abalone · Withering disease · Bottleneck · Microsatellites

Black abalone (*Haliotis cracherodii*) are intertidal marine gastropods that occur from northern California, USA, to Baja California, Mexico (Neuman et al. 2010). Although they have been considered less desirable than most abalone species, they were heavily harvested, particularly in southern California and Mexico, with a peak in the 1970s, when 2 million pounds were harvested in California in 1973 alone (Karpov et al. 2000). In addition to unsustainable harvesting, a bacterial infection, known as withering syndrome, broke out in the early 1980s (Altstatt et al. 1996). Withering syndrome (WS) is caused by a bacterial infection, "withers" the snail's foot and eventually causes death. The spread of the disease now extends over almost the entire range of the species. If no action is taken, it is estimated that *H. cracherodii* will decline by at least 80 % over a period of three generations (from approximately 1975 to 2015), extending into the past and the future (Smith et al. 2003). Consequently, the species qualifies as Critically Endangered under criterion A4 of the IUCN red list (Smith et al. 2003) and in 2009 was listed as endangered under the USA Endangered Species Act.

High levels of interpopulation genetic divergence across California suggest that larval dispersal is restricted (Gruenthal and Burton 2008) despite the dispersal phase extending up to 15 days (Morse et al. 1979) and a range of ocean fronts that sweep recruitment pulses up to 200 km along the Californian coast (Woodson et al. 2012). Recruitment of new individuals is strongly and positively linked to local abundance of adults. Hence in areas with few adults due to losses from disease or harvest, recruitment is typically non-existent. Due to these life history traits, we are now in a situation where very few adult individuals born before the withering outbreak are present. Therefore, we expect individuals to show high levels of genetic diversity, as a reflection of their past history, yet the near future is likely to result in very severe bottlenecks, accompanied by a rapid decline in genetic diversity (Hamm and Burton 2000; Gruenthal and Burton 2008). With these unique predictions in mind, we decided to develop microsatellite markers for *H. cracherodii,* the black abalone and test the idea that there would be high levels of

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genetic diversity in two populations with different characteristics: one severely affected and one not affected by withering syndrome. The black abalone population in Point Arguello, California, was decimated by WS in the mid-1990s and has not recovered to date. There has been little to no recruitment in the area for the past 12 years. By contrast, the population at Andrew Molera (north of Point Arguello) has not yet been affected by withering disease and there has been no decline in the population over the same period of time. Moreover, recruitment continues to occur at Andrew Molera.

Genomic libraries enriched for microsatellite motifs were constructed by Genetic Identification Services (GIS, http://www.genetic-id-services.com; Chatsworth, CA, USA). Libraries were built using a sample containing 100 μg of genomic DNA extracted from muscle tissue from one individual *H. cracherodii* collected in Año Nuevo, California, following a standard phenol–chloroform procedure (Sambrook et al. 1989). Libraries were enriched for CA, CATC, TACA, and TAGA motifs. GIS sequenced 74 microsatellite-containing clones using universal M-13 primers. We tested 8 of these microsatellites, which were determined to have flanking sequences of length sufficient for primer design using Designer PCR version 1.03 (Research Genetics, Inc.). Amplification reactions were carried out in a total volume of 13 μL containing 11 μL of 1.1 PCR Mastermix (Thermo Scientific), 0.625 μL of both 20 μM primers and approximately 2 ng of DNA template. 35 PCR cycles were run at denaturation, annealing and amplification temperatures of 94, 52, and 72 °C, for 30, 40, and 30 s respectively. Each microsatellite amplification was diluted with nH2O (1:20), mixed with Applied Biosystems GeneScan 500 ROX and size standard, run on an ABI 3100 automated sequencer, and scored using the software GENEMAPPER version 3.7 (Applied Biosystems).

All eight primer pairs were polymorphic and successfully amplified most samples of *H. cracherodii* (see Table 1). Forty-one individuals taken from two California populations, Andrew Molera and Point Arguello, were sampled by cutting a minute amount of epipodium, a method shown previously not to harm the individuals. Individuals were then genotyped to estimate allelic diversity and calculate average observed and expected heterozygosities at these 8 loci. We scanned our data for null alleles using MICROCHECKER (van Oosterhout et al. 2006), for each locus, calculated expected and observed heterozygosities, deviations from Hardy–Weinberg (HW) equilibrium and presence or absence of linkage disequilibrium using ARLEQUIN version 3.11 (Excoffier et al. 2005). We identified 13–33 alleles per locus, and no significant linkage disequilibrium was observed for any pair of loci ($P > 0.05$ for all comparisons). Expected heterozygosity values were high in both populations, ranging from 0.86 to 0.96. For the majority of the loci, the highest expected heterozygocity found at Point Arguello where the withering syndrome has decimated the black abalone. Such

Table 1 Characterization of eight polymorphic microsatellite loci for black abalone, *Haliotis cracherodii* from a sample of 41 individuals

Locus	Primer seq	Repeat	Na	Pa	nw	Amp. size	Repeat range	(Molera)		(Pt Arguello)	
								Ho	He	Ho	He
HCH_A2	F: 5′-TAACGCTTGACATCCATCC-3′ R: 5′-AAGAAGATTTGCTGGTGAGTG-3′	$(CA)_{39}$	31	30	39	275	16–60	0.809	0.956	0.555	0.962
HCH_A7	F: 5′-TTCGGTGACTTCAACAGAAG-3′ R: 5′-GCACTATAAATCGGCATCAG-3′	$(CA)_{27}$	21	11	41	186	7–35	0.667	0.946	0.800	0.904
HCH_A11	F: 5′-TAACGAGACATTGTGCATTATG-3′ R: 5′-GGATGAAATGAAAGATGTGAGA-3′	$(CA)_{29}$	22	15	41	276	15–48	0.809	0.928	0.600	0.860
HCH_A12	F: 5′-ATCCTGTGAAGTGCATGTTC-3′ R: 5′-CCTGCTGCTCCCCATATA-3′	$(CA)_{67}$	33	27	40	295	20–98	0.500	0.943	0.400	0.982
HCH_B4	F: 5′-TTTACCCAACTTCCAGATTG-3′ R: 5′-GCCAGGTTTCAATACTGATG-3′	$(CATC)_{8}$	19	13	39	262	8–30	0.684	0.917	0.600	0.893
HCH_B11	F: 5′-GACGTGAAGAACAGCACAGAC-3′ R: 5′-ATGCAAGTTATGCAAGTTTGC-3′	$(CATC)_{12}$	13	3	41	275	6–19	1.000	0.923	0.800	0.880
HCH_C2	F: 5′-CCAAGTCCTTGCCTAAATGTC-3′ R: 5′-GTGCATGTGTGTATGTGCAATA-3′	$(TACA)_{29}$	24	12	40	220	14–38	0.700	0.955	0.700	0.952
HCH_C8	F: 5′-GTCGTTTCACACGTTCAATAAG-3′ R: 5′-GGTAGGAATTGGAGTCTCTCAA-3′	$(TACA)_{28}$	21	8	41	239	13–36	0.666	0.946	0.700	0.959

Columns correspond to: Microsatellite name, *F* forward and *R* reverse primer sequence, repeat motif, number of alleles per locus (Na), number of private alleles (Pa), number of individuals successfully amplified (nw), amplification size, repeat number range, and (Ho) observed and (He) expected heterozygosities for the two tested populations. GenBank accession numbers are EU781587–EU781594

high expected heterozygosity is consistent with a very large population size, which is known to have existed before the withering syndrome outbreak. The discrepancies between observed and expected heterozygosity at each locus in each of the populations are generally greater at Andrew Molera, which can be explained by the difference between historically large population sizes and the currently reduced populations. Yet, considering the low number of samples available in the field, it is likely that it is not possible to recover enough individuals to correctly sample the original diversity, pre-withering disease, and fully sample the genetic diversity that existed then. As predicted, there were no significant differences in genetic diversity between the two sampled populations. We expect these differences to increase in the future, as the widely infected populations show the effects of genetic bottlenecking on successive generations. This new set of microsatellite markers will help in assessing the black abalone's past, present, and future population dynamics.

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