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TECHNICAL NOTE

Development of primers for 29 microsatellite loci in spotted seatrout (*Cynoscion nebulosus*)

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Abstract Twenty-nine microsatellites were isolated from genomic DNA of spotted seatrout (*Cynoscion nebulosus*). The microsatellites were screened across 10–25 individuals from a single population sampled in the Lower Laguna Madre, Texas. All microsatellites were polymorphic with the number of alleles per locus ranging from 3 to 25. Expected heterozygosity ranged from 0.34 to 0.96. No deviations from Hardy–Weinberg equilibrium or linkage disequilibrium were observed following correction for multiple simultaneous tests.

Keywords *Cynoscion nebulosus* · Sciaenidae · Microsatellites

The spotted seatrout (*Cynoscion nebulosus*) is a widely distributed estuarine species that supports important recreational fisheries in the United States. Spotted seatrout populations in some areas are perceived to be impacted by excessive fishing pressure, loss of habitat, and catastrophic environmental factors (Clark et al. 2003; Hutchings and Reynolds 2004). Management strategies for this species are based on detailed stock assessments which generally include attempts at identification and conservation of genetic variability (Policansky and Magnuson 1998).

Efforts by some states to augment spotted seatrout natural populations with stockings of hatchery-produced juveniles make detection of genetic subdivision critical to the design of broodfish acquisition and juvenile stocking strategies. Microsatellite markers may also be useful as genetic 'tags' to assess stocking success (Carson et al. 2009; Karlsson et al. 2008) and to evaluate the genetic diversity, habitat selection, and migration of stocking cohorts.

Microsatellites are considered a robust molecular tool for fine scale population studies, including identification of fish stocks (Riccioni et al. 2010). Genetic studies of spotted seatrout utilizing microsatellite markers (Gold et al. 2003; Ward et al. 2007; Anderson and Karel 2010) developed for red drum (*Sciaenops ocellatus*) have been conducted across most of the range of the distribution of the species. Although cross-species microsatellite markers are widely applied and considered cost effective, their use may lead to erroneous genotyping due to low detection of polymorphism, allele dropout, or nonspecific amplifications (Barbara et al. 2007). In this note we describe the development of novel microsatellites markers for spotted seatrout.

Genomic DNA from a single male sampled from the Lower Laguna Madre on the south Texas coast was extracted using the DNeasy Blood and Tissue Kit following the manufacturer's protocol. (QIAGEN, Inc.: Valencia, CA). A partially enriched microsatellite genomic library was constructed employing methods described by Reddy et al.

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Table 1 Primer sequences and summary polymorphism for 29 microsatellite loci isolated from *Cynoscion nebulosus*

Locus name and GenBank accession no.	Repeat motif	Primer sequence (5′-3′)	Size range (bp)	*N(n)/k*	H_O	H_E	*P* value
Cne_06A HM223219	$(TG)_{20}$	F: ACCATGTAGAGGGCAGAGA	164–154	16(16)/4	0.3636	0.6364	0.1074
		R: GGTATGGGAAAGGGAGGAAA					
Cne_08A HM223219	$(TGA)_{10}$	F: CTGATTGGCAGATTGGACCT	268–238	25(25)/6	0.5600	0.4555	0.8978
		R: TCTTTGTCTTTCTCTGCTGCTG					
Cne_10A HM223246	$(TG)_{20}$	F: TGTGCTCCAACTGCTGCTAT	200–184	16(16)/3	0.6250	0.6875	0.0128
		R: TATGCAGAATTCGCCCTCTC					
Cne_15A HM223220	$(TG)_{21}$	F: TGCTCTCTCTTGCTTGTGCT	272–248	25(25)/5	0.5417	0.6746	0.5417
		R: CAAACAAACAATCAGGCTTACA					
Cne_23A HM223221	$(CA)_{22}$	F: GGAAGCTGCAAACACACAAC	234–184	16(16)/5	0.3750	0.3407	1.00
		R: CATGTGCCATGTCCAAGAAG					
Cne_42A HM223223	(CA)36	F: TGTTCGGCTCTGTGTCTCAC	238–228	16(16)/6	0.5556	0.7320	0.2288
		R: GTGTGTGTGTGTCCGCTCTC					
Cne_49A HM223224	$(TG)_{12}$	F: AACGGACGTGGACACAAAG	358–222	25(25)/23	0.7600	0.9543	0.0064
		R: AGGAAGTCAACCATGGCAAG					
Cne_62A HM223225	$(CA)_{22}$	F: TAGCCCTTGACGTCCTGTTT	184–122	25(25)/12	0.8400	0.8237	0.3562
		R: AGCCGTGTAAGCTGGTGATT					
Cne_64A HM223226	$(CA)_{27}$	F: TGAACACACCGCCACAGTTA	183–149	25(25)/12	0.8000	0.8526	0.5872
		R: GACCTTCTCCCTCTCCTTGC					
Cne_65A HM223244	$(TG)_{12}$	F: CTCCCTCACTCATCGATTCC	190–164	25(25)/9	0.6800	0.8033	0.4378
		R: GGGGCACACACAATCATACA					
Cne_69A HM223227	$(GA)_{27}$	F: CATACAGCTGCTGCCGAGT	174–156	25(25)/9	0.8000	0.7820	0.8874
		R: CTCCCTACAGCTCACAGCTC					
Cne_93A HM223243	$(CA)_{16}$	F: AACCACAGCTCAACAAACACA	176–136	25(25)/10	0.4800	0.5551	0.0482
		R: GTGTGGCATTTAGCCAGGAT					
Cne_16B HM223230	$(CA)_{17}$	F: GCAATGAAGTGAAGCTGTGG	176–162	25(25)/9	0.7391	0.8464	0.2486
		R: TGGCTCTAAAATCTATGGCACA					
Cne_42C HM223248	$(GGA)_{22}$	F: CAGCACAAACGTTCCAGAAG	260–233	16(15)/4	0.6154	0.5662	0.7199
		R: GAGTAGGCTATTCGGCGATG					
Cne_46C HM223249	$(TAGA)_{35}$	F: CCACCACGCATAGTCAAATG	276–188	16(16)/5	0.3750	0.3407	1.000
		R: CTGACCTGGCTTGCGTCTAT					
Cne_52C HM223250	$(TATC)_{53}$	F: CAACCACAGTCGATAGAGATAGAGA	220–532	25(25)/25	0.9200	0.9706	0.2886
		R: CCATGCAACACTGTCTGGTAA					
Cne_01D HM223232	$(CA)_{25}$	F: ACATTCCACCCCATCACTGT	216–232	25(25)/8	0.6000	0.6473	0.2055
		R: TGTTAATGTTCAACCCAATGC					
Cne_02D HM223233	$(TCTA)_{31}$	F: CCTGCGAAATGTTTGTGTGT	210–334	25(25)/18	0.9200	0.9420	0.1750
		R: CGGGTGGAGTACGTTTGAAT					
Cne_07D HM223234	(TC)16 $(CA)_8$	F: TGCAGCCATGCTTGTTATTC	210–180	25(25)/9	0.8400	0.8637	0.7930
		R: GCAGTGTCGCAGGAGTAGTG					
Cne_15D HM223235	$(GGA)_{15}$	F: TGTGTGTCTGGGAAAACGAG	310–230	16(16)/13	0.9375	0.0.9456	0.0310
		R: TCTTCCTCCTCCTCCTCCTC					
Cne_16D HM223236	$(TC)_{18}$	F: TCGTTCTCCTTCACCCACTC	270–260	10(10)/4	0.4632	0.4125	1.0000
		R: TCTCTCCCGGAAAAGTGTTG					
Cne_18D HM223237	$(AGG)_{27}$	F: GAAGAGGCTGTGAGGAGGTG	178–155	25(25)/6	0.7200	0.7608	0.9165
		R: GACAGCAGGAGGTGAAATCAG					
Cne_20D HM223247	$(TG)_{24}$	F: CGTCTCGTGCCTTAAAGGAG	164–134	25(25)/7	0.5000	0.6959	0.0049
		R: GTGGTGGGCTGTAAAACCTG					
Cne_22D HM223238	$(CA)_{11}$	F: AGGCAGCTGGAAGAAGACAA	418–206	25(25)/23	0.9130	0.9623	0.5387
		R: GTTAGGGCAGCAAGGAACTG					

Table 1 continued

Locus name and GenBank accession no.	Repeat motif	Primer sequence (5′-3′)	Size range (bp)	*N(n)/k*	H_O	H_E	*P* value
Cne_29D HM223239	$(CA)_{42}$	F: GCATGTGGCTGAAAGCAGT	226–120	25(25)/20	0.8750	0.9238	0.3548
		R: AAAGCACTTCGGCTTGACAT					
Cne_49D HM223241	$(CA)_{42}$	F: TTTGGATGTGTGAGGGAAAA	260–246	16(13)/6	0.5000	0.7899	0.0712
		R: ACCCGTGAAACGAGACAAAG					
Cne_50D HM223242	$(TCC)_{21}$	F: GATGGGTAGCAGAGCAGGAC	218–202	25(25)/5	0.5682	0.5600	0.1051
		R: GCAGCACTCAGACAGACAGC					
Cne_60D HM223245	$(TG)_{18}$	F: CAGCCCAAACTGTGCTATGA	370–314	16(13)/6	0.4615	0.4123	1.0000
		R: ATATCTTGCATGGGGAATGC					
Cne_93D HM223243	$(TCC)_{18}$	F: TCTCTCAAGTCTGCGCTTCA	188–154	16(16)/4	0.3333	0.4771	0.1486
		R: AGAGTGGGCCTCTGTTTTCA					

Number of individuals assayed (*N*), number of individuals successfully amplified (*n*), number of alleles identified (*k*), observed heterozygosity (H_O), expected heterozygosity (H_E) under Hardy–Weinberg equilibrium, *P* values for deviation from Hardy–Weinberg expectation (Bonferroni adjusted critical value = 0.002)

(2001). Biotinylated oligonucleotides synthesized by Sigma-Genosys (Woodland, TX) were used for enrichment which included $(AG)_9$, $(TG)_8$, $(AAG)_5$, $(AGC)_4$, $(ATC)_4$, $(AAT)_4$, $(AGG)_9$, $(ACGC)_5$, $(ACTC)_5$, $(ACTG)_5$, and $(AGAT)_5$. A total of 500 transformed bacterial colonies putatively positive for microsatellite inserts were individually picked from LB agar plates and amplified via polymerase chain reaction (PCR) on an Eppendorf Mastercycler (Eppendorf North America, Inc.: Westbury, NY) containing 10.0 µl of Go Taq Green Master Mix (Promega: Madison, WI), and 1.0 µl each of 50 µM modified M13 (WM13-R: 5′-AGCGGATAACAATTTCACACAGG-3′) and TOPO (TOP-R1: 5′-GCCAGTGAATTGTAATACGAC-3′) primers in a final 20 µl reaction volume. The amplification protocol employed an initial denaturation step of 1 min at 94°C, followed by 40 cycles of 1 min at 94°C, 1 min at 58°C annealing temperature, 1 min at 72°C, and a final extension step of 2 min at 72°C. The PCR products were purified with Excella Pure 96-Well UF PCR Purification Plates (Edge Biosystems: Gaithersburg, MD) using a Vac-Man 96 Vacuum Manifold (Promega: Madison, WI).

Sequencing reactions were performed with the WM13 and TOP-R1 primers using the Genome Lab DTCS Quick Start Kit for Dye Terminator Cycle Sequencing (Beckman Coulter, Inc.: Fullerton, CA) adjusting the final volume to 12 µl. Products were separated via capillary electrophoresis using the CEQ8800 Genetic Analysis System, chemistry, and protocols (Beckman Coulter, Inc.: Fullerton, CA). Forward and reverse sequences were aligned and corrected by hand using SEQUENCHER 4.8 (GeneCodes: Ann Arbor, MI). High quality microsatellite repeats were found in 250 of 500 clones sequenced for screening. Primers annealing to flanking regions of the microsatellite regions were designed for 100 clones using the program PRIMER3 (Rozen and Skaletsky 2000). Each forward primer had an M13 sequence tail added to its 5′-end to allow for fluorescent labeling with a WellRED D4 M13 primer following the protocol described by Schuelke (2000).

The PCR mixture for individuals screened to assess marker usefulness included 6.5 µl Illustra Hot Start Master Mix (GE Healthcare: Piscataway, NJ), 0.5 µl each of 10 µM forward primer with an M13 tail at the 5′ end, 10 µM reverse primer, 10 µM WellRED D4 labeled M13 primer, and 1 µl of DNA template ($\sim$100 ng/µl) for a final 12 µl reaction volume (modified from Schuelke 2000). PCR amplification was performed using the following profile: 95°C for 5 min, then 40 cycles at 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min, and 72°C for 5 min. Fluorescent visualization on the CEQ8800 Genetic Analysis System sample setup included 1 µl of PCR product in 20 µl of sample loading solution and 0.5 µl of CEQ DNA Size Standard 400 or Size Standard 600, as appropriate. The resulting microsatellite fragment data were analyzed and scored using the CEQ 8800 Fragment Analysis Module software. A total of 100 primer pairs were screened using 10–25 samples. Of these, 29 primer pairs produced scorable polymorphic patterns which were deposited in GenBank. Composite genotypes were prepared for each individual. Collected data were analyzed with GenePop ver. 1.2 (Raymond and Rousset 1995; Rousset 2008). The number of alleles per locus ranged from 3 to 25. Observed heterezygosity ranged from 0.33 to 0.94. No deviations from Hardy–Weinberg equilibrium and linkage disequilibrium were observed (Table 1) following corrections for multiple simultaneous comparisons (Rice 1989).

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