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TECHNICAL NOTE

PCR primers for nuclear-encoded microsatellites of the groupers *Cephalopholis fulva* (coney) and *Epinephelus guttatus* (red hind)

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Abstract Twenty-one nuclear-encoded microsatellites were isolated from an enriched genomic DNA library of coney, *Cephalopholis fulva*, and characterized in both *C. fulva* and red hind, *Epinephelus guttatus*. The microsatellites include 16 dinucleotide repeats, two trinucleotide repeats, and three tetranucleotide repeats. An additional 11 microsatellites, isolated originally from an enriched genomic DNA library of *E. guttatus*, were characterized in both *E. guttatus* and *C. fulva*. Both grouper species support important commercial and recreational fisheries in the western Atlantic along the coasts of North, Central, and South America.

Keywords PCR primers · Microsatellites · Coney · Red hind · Groupers

The coney, *Cephalopholis fulva*, and the red hind, *Epinephalus guttatus*, are groupers that belong to the family Serranidae and range in the western Atlantic Ocean from North Carolina (USA) to southern Brazil (Ferreira et al. 2008; Sadovy et al. 2008). Due to their wide distribution and relative abundance, both have been listed as species of least concern (Ferreira et al. 2008; Sadovy et al. 2008). However, the disappearance of many larger grouper species has generated increased fishing pressure on both coney and red hind, two of the smaller grouper species distributed worldwide (Nemeth 2005). The two species are particularly susceptible during annual spawning aggregations that form during the species' reproductive season (Nemeth 2005; Sadovy et al. 2008). Among other needs for informed management of the two species in the western Atlantic Ocean are data on population or stock structure of both species across their range.

Here, we report development of polymerase chain reaction (PCR) primers for 21 nuclear-encoded microsatellites from a genomic DNA library of *C. fulva*, characterized in both *C. fulva* and *E. guttatus*. Additionally, primers for 11 microsatellites, developed previously from a genomic DNA library of *E. guttatus* (Ramirez et al. 2006), were characterized in both *C. fulva* and *E. guttatus*. Nuclear-encoded microsatellites are well suited for population-genetic analysis and identification of stocks that cross geo-political boundaries (Ward 2000).

Whole genomic DNA was extracted from muscle tissue of a single individual of *C. fulva*, using a DNeasy Blood and Tissue Kit (Qiagen); two separate digestions were performed with *Hae*III and *Rsa*I (New England BioLabs). The digestions were deactivated, mixed together, and linkers attached with T4 DNA ligase (Promega). Linkers were prepared beforehand following the protocol of Glenn and Schable (2005), using the SuperSNX24 Forward and SuperSNX24 + 4P Reverse oligonucleotides. The double-stranded linker forms an *Xmn*I restriction site when it dimerizes (Hamilton et al. 1999). Therefore, the *Xmn*I restriction endonuclease was included in the DNA/linker ligation reaction to digest linker dimers. DNA/linker fragments ranging in size from 400 to 1,000 bp were excised from a 2% agarose gel and cleaned with a QIAquick gel extraction kit (Qiagen). These fragments were amplified with PCR using the SuperSNX24 Forward as the primer, and the PCR product was visualized on an agarose gel (with ethidium bromide staining) to verify success of linker ligations to genomic DNA fragments. Ten

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microliters of the size-selected genomic DNA/linker fragments (not the PCR product) were hybridized in a 100 µl reaction [6× SSC (0.9 M NaCl, 0.09 M sodium citrate) final concentration] with 50 pmoles of 3′-biotin-modified $(CA)_{13}$ oligonucleotides. The hybridization mixture was heated at 95°C for 10 min and then kept at 55°C for 75 min. During the hybridization period, 50 µl of streptavidin-coated magnetic beads (Dynabeads M-280, Invitrogen) were washed three times with 100 µl of 6× SSC. After the third wash, the beads were suspended in 100 µl of a bead block buffer, designed to lower the affinity of the beads for genomic DNA fragments and increase the targeted affinity for the biotin-modified oligonucleotides (St. John and Quinn 2008), and mixed gently on a shaking platform for 1 hour. The bead block buffer was made by combining 30 ml of 10× PBS (1.4 M NaCl, 0.03 M KCl, 0.1 M Na_2HPO_4, 0.02 M KH_2PO_4), 240 ml sterile water, and 0.6 g non-fat dry milk (SACO Foods, Inc., Middleton, WI); boiling the solution in a microwave for 10 s; adding 30 ml of 1% SDS and inverting the solution several times to mix; and finally cooling the solution to room temperature prior to use. After shaking in the bead block buffer, the beads were washed three more times with 100 µl of 6× SSC.

After removing the final 6× SSC wash from the beads, the hybridization mix was added to the beads and incubated at room temperature for 30 min. The beads were then rinsed with the following series of washes to remove non-target DNA fragments: twice with 200 µl of 1× W/B Buffer (1 M NaCl, 0.01 M Tris–HCl pH 7.5, 0.001 M EDTA); twice with 200 µl of 2× SSC (0.3 M NaCl, 0.03 M sodium citrate); twice with 200 µl of 1× SSC (0.15 M NaCl, 0.015 M sodium citrate); and twice with 200 µl of 1× SSC pre-heated to 50°C. Microsatellite-containing DNA fragments were eluted at 95°C with 100 µl of PCR-grade water. The quantity of enriched DNA was increased with PCR, using the SuperSNX24 Forward oligonucleotide as the primer, and PCR products were cleaned with QIAquick PCR Purification Kits (Qiagen).

Cleaned PCR products were ligated into the pCR®2.1-TOPO® vector (Invitrogen), and transformed into *Escherichia coli* (One Shot® TOP10 Chemically Competent Cells, Invitrogen). Transformed cells were plated onto X-Gal/IPTG Luria-Bertani (LB) agar with 100 µg/ml of ampicillin and grown overnight at 37°C. Positive (white) colonies were picked with sterile toothpicks, placed in 96-well tissue culture plates with 200 µl LB broth (containing 100 µg/ml of ampicillin), and incubated at 37°C overnight to increase density of the cultures. Inserted DNA fragments were amplified using the 555 and 837 (vector-based) primers from Makova and Patton (1998), band cut and cleaned with QIAquick gel extraction kits (Qiagen), and sequenced in both directions using the same primers and ABI PRISM BigBye Terminator version 3.1. Products were electrophoresed on an ABI 3100 automated DNA sequencer (Applied Biosystems); sequences were edited and vectors trimmed with SEQUENCHER 3.0 (Gene Codes). Primer pairs were developed using PRIMER3 (http://frodo.wi.mit.edu/). Primer pairs for microsatellite sequences previously isolated from an *E. guttatus* enriched genomic library and available on GenBank (Ramirez et al. 2006) were also developed using PRIMER3.

Unlabelled primers for the newly developed primer sequences as well as previously described primer pairs for *E. guttatus* (Ramirez et al. 2006) were tested for amplification by screening four individuals of each species. A total of 38 primer pairs were chosen for further evaluation and the forward primer from each pair was labeled with one fluorescent label of Dye Set D (Applied Biosystems): 6-FAM, HEX, or NED. Each primer pair was further evaluated by genotyping 25 individuals of *C. fulva*, sampled from waters off the west coast of the island of St. Croix, and 26 individuals of *E. guttatus*, sampled from waters off the west coast of Puerto Rico. PCR products were run on an ABI 377 automated sequencer. Alleles were sized using the GENESCAN®-400 HD ROX Size Standard (Applied Biosystems); allele sizing and scoring were performed using GENESCAN® 3.1.2 and GENOTYPER® version 2.5 software. Genetic variability of the microsatellite markers was measured by the number of alleles, expected heterozygosity, and observed heterozygosity. Fisher's exact test, as implemented in GENETIC DATA ANALYSIS (Lewis and Zaykin 2001), was used to test for significant departure from the expectations of Hardy–Weinberg equilibrium at each microsatellite locus and for departure from genotypic equilibrium at each pair of loci. The presence of null alleles was explored using MICROCHECKER (Van Oosterhout et al. 2004).

Six of the putative microsatellite markers were removed from further consideration due to inconsistent amplifications, unreliable scoring, or lack of allelic variability. Summary data for the 32 experimentally tractable microsatellite loci (19 polymorphic for the samples of *E. guttatus* and 24 polymorphic for the samples of *C. fulva*) are presented in Table 1. For *E. guttatus*, the number of alleles detected per polymorphic microsatellite ranged from two (*Cfu*25) to 24 (RH_GATA_002); expected heterozygosity ranged from 0.040 (*Cfu*25) to 0.960 (RH_GATA_002), while observed heterozygosity ranged from 0.040 (*Cfu*25) to 1.000 (RH_GATA_023); genotypes at all 19 polymorphic microsatellites conformed to the expectations of Hardy–Weinberg equilibrium following Bonferroni correction (Rice 1989). MICROCHECKER identified possible null alleles at three microsatellites: RH_GATA_015, RH_GATA_018, and *Cfu*57. For *C. fulva*, the number of alleles detected per polymorphic microsatellite ranged from 2 (*Cfu*9, *Cfu*14,

Table 1 Summary data for 32 microsatellites characterized for red hind, *Epinephelus guttatus*, and coney, *Cephalopholis fulva*

Microsatellite	Primer sequence (5′–3′)[a]	GenBank[b]	Repeat[c]	Clone size[d]	T_A^e	N/N_A^f	Size range[g]	H_E/H_O^h	P_{HW}^i
RH_CA_002	**CTCGTTACCACATCGGGACT**[F]	DQ223785	$(CA)_{25}$	153	57	26/14	129–157	0.900/0.923	0.549
	AACACTGGCTGGTTTGCACT				57	24/24	133–187	0.951/0.917	0.150
RH_CA_004	**GAGAACGACATTCCAGCACA**[H]	DQ223784	$(CA)_{14}$	180	54	26/15	186–232	0.895/0.923	0.770
	TGTGTGACCAGAAACCAGGA				54	25/6	180–190	0.614/0.680	0.990
RH_CA_008	**AGTTGCCCAGGTTACACGAG**[H]	DQ223787	$(CA)_{21}$	216	54	26/18	207–253	0.925/0.885	0.656
	TTGGGTCCTGGCATTTAGAG				54	25/6	206–216	0.703/0.560	0.197
RH_GATA_002	**CTCGACAGTGGACAAGGTCA**[H]	DQ223791	$(GATA)_{34}$	238	57	26/24	142–274	0.960/0.923	0.169
	AAGGGCATGATGGGAAATG				NA	NA	NA	NA	NA
RH_GATA_003	**GGGCAATTTGGTTCTTCACA**[F]	DQ223790	$(GATA)_7$	205	54	26/6	196–216	0.765/0.731	0.118
	TGTCAATGCCACAGGATACA				NA	NA	NA	NA	NA
RH_GATA_015	**AATTCCACTCATGTGGCTCTG**[F]	DQ223821	$(GATA)_{13}$	167	51	25/10	147–183	0.898/0.720	0.026
	CCGTGCTTGATCCAACTCTT				NA	NA	NA	NA	NA
RH_GATA_018	**TACAGATTGCCGCCCACTAC**[F]	DQ223822	$(GATA)_{19}$	194	54	26/23	145–217	0.949/0.769	0.009
	CACTTCACCCACCTCCTGTC				NA	NA	NA	NA	NA
RH_GATA_023	**GGTGATGGACAAATGTTTGGTA**[F]	DQ223823	$(GATA)_{17}$	224	60	26/13	212–260	0.923/1.000	0.682
	TACCATCAGCTCTGCCTGTC				NA	NA	NA	NA	NA
RH_GATA_034	**GGACCAGACGCACTGGTAGT**[H]	DQ223825	$(GATA)_{30}$	197	48	25/12	171–219	0.904/0.800	0.471
	ATACAGCGCTGGAGGATTTG				48	25/11	171–211	0.869/0.760	0.044
RH_GATA_057	**CATGCCAGACTGTTGCTGTT**[H]	DQ223830	$(GATA)_{11}$	243	57	26/15	219–279	0.922/0.962	0.950
	TGCAACACAGAGATGGGATT				NA	NA	NA	NA	NA
RH_GATA_065	**ACTGTGAACGAGGCGTGAG**[H]	DQ223834	$(GATA)_{14}$	173	57	26/10	153–189	0.879/0.808	0.255
	TTTCCCCAGTTTGCTAATGC				NA	NA	NA	NA	NA
*Cfu*9	**CAAGTCTGATGCCAAAATTGA**[H]				60	26/1	198	0.000/0.000	1.000
	CCGTGGTGGGTTTCTTACC	FJ798803	$(CA)_7$	203	60	25/2	203–205	0.040/0.040	1.000
*Cfu*10	**CGCCTCCCGTACATACAGAT**[N]				60	26/4	197–205	0.149/0.154	1.000
	CCTCACCCGGTTTGAATATG	FJ798804	$(CA)_5$	188	60	25/1	187	0.000/0.000	1.000
*Cfu*14	**CGGGGAAGTGTTTTATTCCA**[N]				54	26/3	161–165	0.358/0.423	1.000
	TGTGTGCATTTGTGCTGTGT	FJ798805	$(CA)_7$	158	54	25/2	157–159	0.040/0.040	1.000

Table 1 continued

Microsatellite	Primer sequence (5′–3′)[a]	GenBank[b]	Repeat[c]	Clone size[d]	T_A^e	N/N_A^f	Size range[g]	H_E/H_O^h	P_{HW}^i
*Cfu*17	**CACCAGTCCAGCTCCAAACT^N**				NA	NA	NA	NA	NA
	TGGTCTTCATGGTCCTGTTG	FJ798806	$(AC)_7$	156	57	24/2	155–157	0.082/0.083	1.000
*Cfu*18	**TTCAGCGTATAGCCGGAGTC^F**				NA	NA	NA	NA	NA
	CCAGCGTCACTTACAAACCA	FJ798807	$(AC)_8$	201	60	25/3	202–214	0.280/0.240	0.478
*Cfu*20	**TGGCAGTTGATAATGCCAGA^N**				57	25/13	225–251	0.893/0.960	0.470
	GCGCCACATAACCTCTGTTT	FJ798808	$(CA)_8$	201	57	25/3	198–202	0.187/0.200	1.000
*Cfu*21	**AGGCTGACTAGGAGGCTGTG^F**				NA	NA	NA	NA	NA
	GTCACACAAAGGGGGTTGAC	FJ798809	$(CA)_{33}$	153	60	24/13	128–154	0.906/0.917	0.083
*Cfu*23	**AATGAGCTGCAGGACAAGGT^F**				NA	NA	NA	NA	NA
	TGTTCCTGCACAAAAACAGTG	FJ798810	$(CA)_{24}$	198	57	25/10	181–211	0.661/0.760	0.921
*Cfu*25	**AAGGAGACCAGTCCCCTGTT^N**				60	25/2	170–184	0.040/0.040	1.000
	TGCATGAGCATCAGTGTCTG	FJ798811	$(AC)_{10}$	201	60	24/5	195–203	0.708/0.750	0.053
*Cfu*26	**CACACCCACACCCACATAGA^H**				NA	NA	NA	NA	NA
	GCGAGCAACATGTCATCCTA	FJ798812	$(AC)_{11}$	184	60	25/6	181–191	0.681/0.440	0.005
*Cfu*43	**GATGCGAATGTGAAGTGTGC^F**				60	26/3	154–158	0.452/0.423	0.397
	GGGCTCATAAAACGAGCTGA	FJ798813	$(GT)_7$	172	60	23/4	175–183	0.437/0.391	0.759
*Cfu*46	**TGAACCAAATACACTGCATCC^N**				NA	NA	NA	NA	NA
	CCGGTGTATTTCTGCTTCCT	FJ798814	$(CA)_6$	185	57	25/2	184–186	0.184/0.040	0.005
*Cfu*52	**CGCTCAGATCAGCTCACAAA^N**				NA	NA	NA	NA	NA
	ATGTTGCATGAAGGCAGTGA	FJ798815	$(CA)_9$	235	60	25/10	234–282	0.790/0.760	0.287
*Cfu*57	**AGGTTTTCCAGACCCTTTCC^F**				60	25/17	255–293	0.856/0.680	0.033
	AACGCTCAAACTGAATGCAA	FJ798816	$(CA)_{14}$	226	60	25/9	216–232	0.816/0.840	0.312
*Cfu*69	**GTGATGAGTGCAGAGCAGGA^H**				NA	NA	NA	NA	NA
	TGGTGTGGTCTGCTGTGTTT	FJ798817	$(AGG)_9$	186	54	25/7	169–193	0.561/0.480	0.412
*Cfu*70	**AGACCACACCAACCGTGTTT^N**				57	26/5	186–201	0.372/0.269	0.073
	CGAGGCAGAAACCACAGAGT	FJ798818	$(AGG)_{12}$	194	57	25/6	177–198	0.735/0.640	0.536
*Cfu*72	**GCTTTTGCATAGTACCTTGTATAATTT^N**				NA	NA	NA	NA	NA
	AAGTTCAGCCTCCCTTGTTCT	FJ798819	$(ATAG)_{16}$	140	51	24/12	96–144	0.895/0.875	0.344
*Cfu*75	**CCCAGATTTGCCAAAGAAAC^F**				NA	NA	NA	NA	NA
	CCATTCAGATTTGGTTGGGTA	FJ798820	$(AGAT)_9$	201	57	25/16	181–229	0.903/0.880	0.836

Table 1 continued

Microsatellite	Primer sequence (5′–3′)[a]	GenBank[b]	Repeat[c]	Clone size[d]	T_A[e]	N/N_A[f]	Size range[g]	H_E/H_O[h]	P_{HW}[i]
*Cfu*80	**AGTGGGGCAAGTTTTGTTTG^H**				60	26/11	205–233	0.750/0.731	0.543
	ACAAGCGGGTAAGTTGCTGT	FJ798821	$(CA)_{14}$	172	60	25/5	167–177	0.622/0.560	0.071
*Cfu*82	**AGAGGGATGACAGACGGATG^F**				54	26/1	250	0.000/0.000	1.000
	ACTCCACCCACAGAGGACAC	FJ798822	$(GT)_7$	248	54	25/3	248–254	0.308/0.280	0.571
*Cfu*92	**TTGTGGCATAAGTGGATGGA^N**				NA	NA	NA	NA	NA
	GACTGGAGTGAAGCACCACA	FJ798823	$(GGAT)_9$	217	54	25/10	223–267	0.831/0.760	0.303

For each microsatellite, species-specific information for *E. guttatus* is given in the upper row; information for *C. fulva* is given in the lower row. The fluorescently labeled primer is in bold: 6-FAM^F, HEX^H, or NED^N. Novel microsatellites isolated and characterized in the present study are identified with *Cfu*; RH_CA_002, RH_CA_004, RH_CA_008, RH_GATA_002, and RH_GATA_003 were previously characterized in *E. guttatus* while clone sequences for RH_GATA_015, RH_GATA_018, RH_GATA_023, RH_GATA_034, RH_GATA_057, and RH_GATA_065 were given but the microsatellites were not characterized (Ramirez et al. 2006)

NA unavailable data due to inconsistent amplification or unreliable scoring

[a] Primer sequences are forward (top) and reverse (bottom)

[b] GenBank accession numbers

[c] Repeat indicates repeat motif

[d] Clone size is the size of the sequenced clone (in base pairs)

[e] T_A is annealing temperature in °C

[f] N is the number of individuals assayed and N_A is the number of alleles detected

[g] Size range refers to alleles thus far uncovered

[h] H_E and H_O are expected and observed heterozygosity, respectively

[i] P_{HW} represents the probability of deviation from Hardy–Weinberg expectations

*Cfu*17, *Cfu*46) to 24 (RH_CA_002); expected heterozygosity ranged from 0.040 (*Cfu*9, *Cfu*14) to 0.951 (RH_CA_002), while observed heterozygosity ranged from 0.040 (*Cfu*9, *Cfu*14, *Cfu*46) to 0.917 (RH_CA_002, *Cfu*21); genotypes at all 24 polymorphic microsatellites conformed to Hardy–Weinberg expectations following Bonferroni correction (Rice 1989). MICROCHECKER identified possible null alleles at two microsatellites: *Cfu*26 and *Cfu*46. All pairwise comparisons of microsatellites in both species did not deviate significantly from genotypic equilibrium following Bonferroni corrections (Rice 1989). The 32 microsatellites characterized in this study will prove useful for future population-genetic research in both *E. guttatus* and *C. fulva*.

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