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TECHNICAL NOTE

Isolation and characterization of microsatellite markers in southern flounder, *Paralichthys lethostigma*

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Abstract Thirty-six dinucleotide microsatellite markers were isolated from an enriched genomic library of southern flounder, *Paralichthys lethostigma*. Genotypes at all 36 microsatellites conformed to Hardy–Weinberg expectations, following Bonferroni correction for multiple tests executed simultaneously; analysis with MICRO-CHECKER indicated the possibility of null alleles at two of the microsatellites. The microsatellites characterized in this study will be useful for further evaluation of southern flounder stock structure and for assaying potential genetic impacts of stock enhancement programs.

Keywords Microsatellites · *Paralichthys lethostigma* · Southern flounder

Southern flounder, *Paralichthys lethostigma*, supports important recreational and commercial fisheries in the southeastern United States (Froeschke et al. 2011; Smith and Scharf 2010). Prior genetic studies employing allozymes (Blandon et al. 2001) and sequences of mitochondrial DNA (Anderson et al. 2012) have indicated separate stocks of southern flounder in the Gulf of Mexico and along the Atlantic coast. Because of declining stock sizes, restoration via stock enhancement of southern flounder is currently under consideration or in progress in several US states (Miller et al. 2010). Here, we report the development of primers for 36 microsatellites from an enriched southern flounder genomic DNA library. Genetic markers such as microsatellites provide fisheries managers with tools that can be used to assess stock structure, assay genetic variability within stocks, and to evaluate the genetic impacts of stock enhancement (Blankenship and Leber 1995; Ward 2000; Kohlmann et al. 2003; Saillant et al. 2009).

Protocols used to generate the microsatellite-motif enriched library followed procedures outlined in Renshaw et al. (2010). Genomic DNA was extracted from muscle tissue of a single individual, using a DNeasy Blood and Tissue Kit (Qiagen). The hybridization mixture of size-selected genomic DNA/linker fragments and 3′-biotin-modified $(CA)_{13}$ oligonucleotides was heated to 95 °C for 10 min and then kept at 58 °C for 75 min. Positive (white) clones were picked with sterile toothpicks, placed in 96-well culture plates with 200 µl LB broth (containing 50 µg/ml ampicillin and 8 % glycerol), and incubated overnight at 37 °C to increase the density of all cultures. Two culture plates were sent to the Interdisciplinary Center for Biotechnology Research at the University of Florida (http://www.biotech.ufl.edu) for sequencing with the M13 forward primer. Sequences were edited and vectors trimmed with SEQUENCHER 4.1 (Gene Codes); clones containing viable microsatellite motifs were identified with Simple Sequence Repeat Identification Tool (SSRIT, http://www.graene.org/db/markers/ssrtool); primer pairs were developed with PRIMER3 (http://frodo.wi.mit.edu).

A total of 67 unlabeled primer pairs were ordered from Integrated DNA Technologies (Coralville, Iowa); the forward primers included a 21 bp 5′-tail-sequence (5′-GCCTCGTTTATCAGATGTGGA-3′) that enabled the fluorescent labeling of fragments during PCR amplifications (Karlsson et al. 2008). The 5′-tail-sequence primer was labeled with one of three fluorescent dyes: 6-FAM, HEX,

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Table 1 Summary data for 36 microsatellites characterized in southern flounder, *Paralichthys lethostigma*

Microsatellite	Primer sequence (5′–3′)[a]	GenBank[b]	Repeat[c]	Clone size[d]	N/N_A^e	Size range[f]	H_E/H_O^g	P_{HW}^h
*Ple*1	CCCTGGCCCTCTGTAAGC^{HEX} GAATATTCAGCACATGGAAGC	JQ935980	$(CA)_{11}$	172	20/19	185–243	0.945/0.950	0.333
*Ple*2	TGTGAGGGACAGAGAGACTGG^{NED} CAGGCAGTCAACCTCCTACC	JQ935981	$(CA)_{24}$	195	18/23	195–259	0.975/1.000	1.000
*Ple*3	TCTTCGGGATCAGAACCAAC^{FAM} AGACAGTGCTGGGAAGAACG	JQ935982	$(CA)_{23}$	257	20/21	245–323	0.963/0.900	0.238
*Ple*5	AGTCTCCTGGGTCACAGTGC^{FAM} ATGGGCCATTTTTATGATGC	JQ935983	$(CA)_9$	269	20/7	283–295	0.665/0.450	0.135
*Ple*8	TCTGCCGTCGTTTTATCAGC^{FAM} TGTTTTCACAAAAACATTGACG	JQ935984	$(GT)_{26}$	260	19/10	255–289	0.896/0.895	0.798
*Ple*10	GACAGGGAGATGGGAAAGG^{NED} TTGAGCGACTCAACAACAGC	JQ935985	$(CA)_{17}$	224	18/19	235–295	0.960/1.000	0.594
*Ple*11	GTAGCGCTGTTGTTGAGTCG^{NED} ACCCACTAATGAAGCCAACG	JQ935986	$(GT)_9$	225	18/6	241–255	0.787/0.611	0.076
*Ple*12	TCCAGGTGGTCACAGAGAGG^{NED} GTGTGTTGCAGATGGAGACG	JQ935987	$(CA)_8$	208	19/2	227–229	0.478/0.526	1.000
*Ple*15	TCACAGATGAGCTGCATTCC^{NED} CTGTATGCTTCCAAAAGATATGG	JQ935988	$(TG)_{32}$	221	19/18	217–275	0.957/1.000	0.428
*Ple*18	TGTATGGCTCAACAAGACAGC^{FAM} TTTCACGCACAAACCTTACG	JQ935989	$(AC)_{22}$	298	19/19	288–398	0.946/1.000	1.000
*Ple*20	TGCTGCAAACATGTAAAAGG^{HEX} GAAGGAGTATGATGGCTCAAGG	JQ935990	$(AC)_8$	147	20/3	165–169	0.272/0.300	1.000
*Ple*22	TCCCTCGCCATTCTAAATCC^{HEX} AAGTGGAGAAATGAAGCTTGG	JQ935991	$(AC)_8$	195	18/7	211–223	0.806/0.833	0.728
*Ple*26	CCGTGTGTCCTTGTTAGAGC^{NED} CTCCCCTTTTTCCACTGTCC	JQ935992	$(TG)_7$	269	18/4	292–312	0.344/0.278	0.354
*Ple*27	GAAGTGCCGACTCAAGTGC^{FAM} TCCCGCTCTTACATCTCAGC	JQ935993	$(AC)_9$	254	19/12	270–320	0.778/0.895	0.655
*Ple*28	AAACCAAGCCCTCAAAAAGC^{NED} GTGGCTTCTGAAGTGCATCC	JQ935994	$(CA)_{32}$	231	18/18	215–271	0.951/0.944	0.672
*Ple*34	CATGGCTTATCCCTCTCTGC^{HEX} CTGACTGATCCTCCTGTCTGC	JQ935995	$(CT)_{20}$	117	20/17	132–166	0.927/0.950	0.572
*Ple*35	TGTATTTGCACACCCACTGC^{NED} CTGCTCCAATTTCAGACTGC	JQ935996	$(GT)_7$	245	19/5	259–269	0.538/0.474	0.538
*Ple*37	GGTAATTTCCAGCCTGTTGC^{NED} CCTGAAGCTCAGTGTTTTCG	JQ935997	$(GT)_{11}$	230	20/14	246–284	0.887/0.800	0.263
*Ple*38	TCCAGATTGTGACACACACG^{HEX} TCTGATCAGTCCCGCTTAGG	JQ935998	$(CA)_{11}$	166	18/7	184–202	0.748/0.722	0.316
*Ple*40	CGCCACATACAACATTGGAG^{HEX} TTCATTCCTGTCTGTAACCTGTG	JQ935999	$(AC)_9$	182	20/5	199–209	0.627/0.600	0.502
*Ple*41	TCTGGTGACTGGAACTACATGG^{HEX} GATGACAGCTGGGTGATGG	JQ936000	$(AC)_7$	89	20/17	109–175	0.890/0.850	0.473
*Ple*43	TCACGACACGACTCTACAAAGG^{HEX} CACAGAAAAACCTGAAACAACC	JQ936001	$(GT)_{22}$	113	20/14	116–172	0.746/0.800	0.410
*Ple*44	TGAATGTGCATGAACACAAGC^{NED} CGTATGTCTCTTTGTCTGTTTGC	JQ936002	$(AG)_{19}$	210	18/23	215–273	0.975/0.944	0.408
*Ple*46	TCGTGACATATGTTTTGAACAGC^{HEX} AATTCAGCCAGCCTCTATGC	JQ936003	$(GT)_9$	122	20/16	143–207	0.935/0.900	0.238

Table 1 continued

Microsatellite	Primer sequence (5′–3′)[a]	GenBank[b]	Repeat[c]	Clone size[d]	N/N_A[e]	Size range[f]	H_E/H_O[g]	P_{HW}[h]
*Ple*50	TTTCCACTCCTCTCCTGTGC^{HEX} CATGTGACCAAGTAAAGAGATGG	JQ936004	$(GT)_{27}$	109	20/21	102–160	0.964/0.950	0.576
*Ple*52	GGGAGGGTGAGTGGTGAGAG^{HEX} TTGCCATGAAATGTAGATGC	JQ936005	$(GA)_{26}$	102	20/25	97–159	0.971/0.950	0.485
*Ple*53	TTTCATGGCAATTACAAACAGC^{HEX} TCCCTTTGTTGCAGTCTTCC	JQ936006	$(GT)_9$	107	20/11	122–148	0.882/0.900	0.436
*Ple*55	CGCAGAAACTCACACAAACC^{NED} AAGTCAGTCTGAGGCGATGG	JQ936007	$(GT)_8$	174	19/6	188–202	0.555/0.526	0.588
*Ple*56	GGAGAGGCTTTGTGAAGAAGG^{FAM} GGAGTTTCACATGAGCAAGG	JQ936008	$(CA)_{24}$	230	19/13	198–262	0.908/0.947	0.892
*Ple*57	GAATTACACACAAAATGCTGTCC^{NED} CTGGCTCAGAGTCAATGAGG	JQ936009	$(AC)_{12}$	204	20/14	218–246	0.903/1.000	0.159
*Ple*58	ACATCACGTGGTGAAGATGC^{FAM} TAAGGCTAAATGGGCTGAGG	JQ936010	$(AC)_{31}$	245	20/16	217–271	0.885/0.950	0.882
*Ple*60	GGCTGGACAGAGTAACACTCG^{NED} GCTACAATGCAAAGCAAAAGG	JQ936011	$(GT)_8$	207	19/8	225–243	0.674/0.474	0.018
*Ple*61	TCCATGAAACACACATATCTTGC^{NED} CTTGAGCATGTGCAAAATGG	JQ936012	$(AC)_{15}$	186	18/16	190–240	0.930/0.889	0.610
*Ple*62	TCCCATTTCAAAGGGTCTTC^{FAM} CCAGCAGAGCTTTTTGTGTG	JQ936013	$(CA)_{34}$	217	20/21	200–264	0.945/0.900	0.061
*Ple*63	GTGTGAAGAGGGCTCAGTGG^{FAM} AGGAGACGCATCATCAGACC	JQ936014	$(GT)_{12}$	219	17/14	239–319	0.904/0.824	0.535
*Ple*64	CATGCACTGGAGGTTGCTAA^{NED} ACAGCTGGCTTCACCCATAA	JQ936015	$(CA)_{22}$	188	20/17	203–249	0.946/1.000	0.918

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

[b] GenBank Accession number

[c] Repeat motif

[d] Size (in base pairs) of the allele in the sequenced clone

[e] N is the number of individuals assayed, N_A is the number of alleles identified

[f] Size range for alleles thus far detected (includes the 21 bp 5′-tail-sequence)

[g] H_E is the expected heterozygosity, H_O is the observed heterozygosity

[h] P_{HW} is the probability of deviation from Hardy–Weinberg expectations. The fluorescent 5′-tail-sequence label attached to the forward (top) primer is noted as 6-FAM^{FAM}, NED^{NED}, or HEX^{HEX}

or NED (Set D, Applied Biosystems). Fin clips were collected from 20 individuals sampled from Sabine Lake, Texas. DNA was extracted using a modified Chelex protocol (Estoup et al. 1996). PCR protocols followed procedures outlined in Karlsson et al. (2008) with one exception. Concentrations for both the reverse and 5′-tail-sequence primer (0.5 μM) were the same as in Karlsson et al. (2008); the concentration of the tailed, forward primer here was 0.05 μM. PCR amplifications were electrophoresed on an ABI 377 DNA Sequencer. Alleles were sized using the GENESCAN® 3.1.2 and GENOTYPER® version 2.5 software. Genetic variability of the microsatellite loci was measured by the number of alleles, expected heterozygosity (gene diversity), and observed heterozygosity. Fisher's exact tests, as implemented in GDA (Lewis and Zaykin 2001), were used to test for significance of departure from Hardy–Weinberg expectations at each microsatellite and for departure from genotypic equilibrium at pairs of microsatellites. MICRO-CHECKER (Van Oosterhout et al. 2004) was used to evaluate possible scoring errors at each marker due to stuttering, large allele dropout, and null alleles.

Of the initial 67 putative microsatellites identified, 36 primer pairs produced experimentally tractable amplifications (Table 1). The number of alleles ranged from two (*Ple*12) to 25 (*Ple*52); expected heterozygosity ranged from 0.272 (*Ple*20) to 0.975 (*Ple*2, *Ple*44), while observed heterozygosity ranged from 0.278 (*Ple*26) to 1.000 (*Ple*2, *Ple*10, *Ple*15, *Ple*18, *Ple*57, and *Ple*64). All individual microsatellites and microsatellite pairs conformed to Hardy–Weinberg expectations and genotypic equilibrium,

respectively, following Bonferroni correction for multiple tests (Rice 1989). Analysis with MICRO-CHECKER indicated two microsatellites (*Ple*5 and *Ple*60) with possible null alleles, but no evidence of possible scoring errors due to stuttering or large allele dropout. The 36 microsatellite loci characterized in this study can be used to further evaluate stock structure in southern flounder as well as assay for potential genetic impacts of stock enhancement programs.

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