arthropods result from sex differences in development time? *The American Naturalist*, **169**, 245–257.
Eberhard WG (2001) Species-specific genitalic copulatory courtship in sepsid flies (diptera, sepsidae, microsepsis) and theories of genitalic evolution. *Evolution*, **55**, 93–102.
Hamilton MB, Pincus EL, Di Fiore A, Fleischer RC (1999) Universal linker and ligation procedures for construction of genomic DNA libraries enriched for microsatellites. *BioTechniques*, **27**, 500–507.
Ingram KK, Laamanen T, Puniamoorthy N, Meier R (2008) Lack of morphological coevolution between male forelegs and female wings in Themira (Sepsidae: Diptera: Insecta). *Biological Journal of the Linnean Society*, **93**, 227–238.
Kraushaar U, Blanckenhorn WU (2002) Population variation in sexual selection and its effect on size allometry in two dung fly species with contrasting sexual size dimorphism. *Evolution*, **56**, 307–321.
Kraushaar U, Goudet J, Blanckenhorn WU (2002) Geographical and altitudinal population genetic structure of two dung fly species with contrasting mobility and temperature preference. *Heredity*, **89**, 99–106.
Martin OY, Hosken DJ (2003) The evolution of reproductive isolation through sexual conflict. *Nature*, **423**, 979–982.
Nater A, Krützen M, Lindholm AK (2008) Development of polymorphic microsatellite markers for the livebearing fish *Poecilia parae*. *Molecular Ecology Resources*, **8**, 857–860.
Park SDE (2001) *Trypanotolerance in West African Cattle and the Population Genetic Effects of Selection*. University of Dublin, Dublin.
Rice WR (1989) Analyzing tables of statistical tests. *Evolution*, **43**, 223–225.
Rousset F (2008) Genepop'007: a complete re-implementation of the genepop software for Windows and Linux. *Molecular Ecology Resources*, **8**, 103–106.
Schuelke M (2000) An economic method for the fluorescent labeling of PCR fragments. *Nature Biotechology*, **18**, 233–234.
Van Oosterhout C, Hutchinson WF, Wills DPM, Shipley P (2004) Micro-Checker: software for identifying and correcting genotyping errors in microsatellite data. *Molecular Ecology Notes*, **4**, 535–538.
Weir BS, Cockerham CC (1984) Estimating F-statistics for the analysis of population structure. *Evolution*, **38**, 1358–1370. Data. Molecular Ecology Notes, 535–538.

doi: 10.1111/j.1755-0998.2009.02736.x

Isolation and characterization of 10 polymorphic microsatellite markers from striped marlin, *Tetrapturus audax*

C. M. PURCELL,* J. S. HARRISON† and S. EDMANDS*
**Department of Biological Sciences, University of Southern California, Los Angeles, CA 90089-0371, USA, †Department of Biology, Georgia Southern University, Statesboro, GA 30460, USA*

Abstract

We present the isolation and characterization of 10 microsatellite loci for striped marlin, *Tetrapturus audax*. Thirty individuals from each of four locations revealed that all loci were polymorphic with two to 31 alleles per locus. Observed levels of heterozygosity ranged from 0.3000 to 0.9667. Significant deviations from Hardy–Weinberg equilibrium were detected in two loci, TA105 in Hawaii and New Zealand and TA155 in Hawaii, and null alleles may be present in loci TA105 and TA155 in those locations, and in locus TA193 in Mexico. No significant linkage disequilibrium was detected in any pairwise-locus comparison.

Keywords: marlin, microsatellites, striped marlin, *Tetrapturus audax*

Received 31 December 2008; revision accepted 24 March 2009

Striped marlin, *Tetrapturus audax*, an important recreational and commercial species, occupies tropical and temperate waters in the Pacific and Indian Oceans (Nakamura 1985). While five microsatellite markers were developed for a different marlin species (Buonaccorsi & Graves 2000), none has been developed specifically for striped marlin. Because striped marlin is known to exhibit subtle genetic differentiation within the Pacific (Graves & McDowell 1994; McDowell & Graves 2008),

Correspondence: Catherine M. Purcell, Fax: (213) 740 8123; E-mail: purcellc@usc.edu

Table 1 Characteristics of 10 polymorphic microsatellite loci in striped marlin (*Tetrapturus audax*)

Locus	Repeat motif	Size (bp)	Primer sequence (5′–3′)	T_A	Primer type	Primer (μM)	$MgCl_2$ (mM)	N_A	Size range (bp)	Accession number
TA024	$(TGAG)_{13}$	221	F: CTCTGTCTCTGTCCCTCCTTTTTCA-***D3 label***	65	Specific	0.3	1.5	16	200–272	FJ644628
			R: ACCCTTTTCACCCCCATACGC			1				
TA105	$(CTAT)_{19}$	174	F: AAGCTAAAATATCCAGCAGTCACAC-***D3 label***	60	ZipPrim	0.7	1.5	21	178–290*	FJ644620
			R: TCTGAGTCCCTAAAACAAGGTATCTC			1				
TA149	$(GT)_{18}$	173	F: ATTCTCCCCCTCTCCCTGTAGCC-***D4 label***	55	ZipPrim	0.7	1.5	4	199–205*	FJ644619
			R: ATCCACAACCCTCCTCCCAATGT			1				
TA155	$(GA)_{26}$	201	F: GGTTTCCTATCACATCACCAAATGA-***D2 label***	60	ZipPrim	0.7	3.5	6	205–233*	FJ644622
			R: AGAAGCACACAGCCAGAACG			1				
TA157	$(GT)_{17}$	130	F: ACCTGCGGACTTGAGGAGGAA-***D4 label***	60	Specific	0.3	1.5	5	128–136	FJ644626
			R: CTTCACCCGTCTAACACATCCAAC			1				
TA162	$(GT)_{15}$	216	F: TGACAAGGAAAGTGTTGACTGATGG-***D3 label***	65	Specific	0.3	1.5	17	180–228	FJ644624
			R: GGACGAGTGCGATTTGAGTTTATC			1				
TA164	$(CA)_4(CGCA)_7$	178	F: GTCAGAAGAGGTGATGTTGACCAG-***D2 label***	60	Specific	0.3	1.5	10	163–213	FJ644623
			R: GCAGCGTTGTTTTATACCTAGTTTTC			1				
TA193	$(CTAT)_{22}(AT)_3(AG)_{13}$	220	F: TGATATGAACTGCTTTAGCCAGCT-***D3/D4 label***	60	ZipPrim	0.7	2.5	31	228–304*	FJ644621
			R: GAAGGAAAATTACAAAAACTGCGTTA			1				
TA218	$(GATA)_9$	109	F: TGGGATCTACCTGACCAGAATC-***D2 label***	60	ZipPrim	0.7	2.5	2	130–134*	FJ644627
			R: TGAAGAGGGTAAAAAGGTTAAAGTG			1				
TA235	$(CTAT)_4(CATC)_5GTT(CTAT)_{11}$	180	F: AAGCCCTTTATTTTTCCTAAATT-***D3 label***	55	Specific	0.3	2.5	17	176–226	FJ644625
			R: GATGGTATTAGCACTGTGGAAAG			1				

Size is the number of base pairs between the primers in the cloned allele. T_A is the PCR annealing temperature. The Beckman WellRED fluorescent label used for each locus is listed after the primer sequence (Note: only forward primers or forward-zip primers were labelled). Primer type describes if the locus was scored with specifically labelled fluorescent primers (Specific) or with modified unlabelled forward primer containing a 25-bp zip-code tag (ZipPrim). Primer concentrations are listed for both primer types (Note: ZipPrim loci also used 0.25 μM fluorescently labelled zip-code tags per reaction), along with $MgCl_2$ concentrations for each locus. The number of alleles per locus (N_A), and the size ranges of those alleles are shown (*Inclusion of the 25-bp zip-code tag in the size range). GenBank Accession numbers are also listed for each locus.

we developed 10 microsatellite markers from a *T. audax* library to further resolve population genetic structure in this species.

A microsatellite-enriched library was generated following the modified protocol of Hamilton *et al.* (1999). Total genomic DNA was isolated from frozen striped marlin heart tissue (DeWoody 2002). Twenty-five micrograms of genomic DNA was digested with *Hae*III, *Rsa*I and *Nhe*I, followed by incubation with Klenow polymerase to fill in overhangs left by *Nhe*I. The digest was dephosphorylated with shrimp alkaline phosphatase, and then double-stranded SNX linkers (Hamilton *et al.* 1999) were blunt-end ligated to the digest. The product was amplified with SNX primers using polymerase chain reaction (PCR) and cleaned with a PCR purification column (Qiagen).

Hybridization with biotin-labelled oligonucleotides, $(CATA)_8$, $(GATA)_8$, $(TCAG)_8$, was conducted using both the original linker ligation and PCR product. A total of 300 micrograms of streptavidin-coated magnetic beads (Promega) were added to the hybridization and mixed for 30 min prior to stringency washing and cleaning with a PCR purification column, followed by a second round of PCR with the SNX oligonucleotide primers.

Enriched DNA was digested with *Nhe*I and ligated into pBS II SK(+) Bluescript plasmids cut with *Xba*I. These plasmids were used to transform XL2-Blue MRF′ competent *Escherichia coli* cells, and the transformed cells were grown on LB ampicillin plates. Colonies containing inserts were amplified with universal M13 primers and sequenced on a Beckman-Coulter CEQ 8000. Primers (Operon Technologies) were developed for clones containing repetitive regions using the program Primer 3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi), and the repetitive regions were then screened for polymorphism.

Amplification conditions varied between microsatellite primer sets. Five microsatellites were amplified using specific fluorescent-labelled forward primers, while the remaining microsatellites were amplified with a modified unlabelled forward primer containing a 25-bp zip-code tag (Chen *et al.* 2000). A second stage of amplification using fluorescent primers complimentary to the zip-code tags resulted in labelled fragments 25 bp larger than the expected amplicon size. Both sets of fluorescent primers were Beckman WellRED D2, D3 or D4 dyes. PCR was conducted on both a MJ Research PTC-200 DNA Engine and an Applied Biosystems GeneAmp PCR System 9700 with the following conditions: 15 ng template DNA, 0.3–1 μM primers, 1.5–3.5 mM $MgCl_2$ (Table 1), 0.25 mM dNTPs, 10 mM Tris–HCl, 50 mM KCl and 0.3 U of *Taq* DNA polymerase in 12 μL total volume. Two cycling conditions were used; specifically labelled forward primers used the following conditions: initial denaturation at 94 °C for 4 min, 35 cycles of denaturation at 94 °C for 30 s, primer annealing between 55 and 65 °C (Table 1) for 35 s, followed by extension at 72 °C for 30 s. After 35 cycles, a 5-min final extension at 72 °C was used, followed by a hold at 8 °C. Primers with zip-code tags followed these cycling conditions: initial denaturation at 94 °C for 4 min, 22 cycles at 92 °C for 30 s, 55–65 °C (Table 1) for 35 s and 72 °C for 30 s. A second stage of cycling with 15 cycles at 92 °C for 30 s, 57 °C for 30 s and 72 °C for 30 s was included to amplify the zip-code tags, followed by a 5-min extension at 72 °C, and a hold at 8 °C. The PCR product was run on a Beckman Coulter CEQ 8000 sequencer following the manufacturer's protocol for fragment analysis, and fragments were visually scored.

The 10 microsatellite loci were amplified in a total of 120 samples from four populations (Table 2). Microsatellite polymorphism ranged widely, containing from two

Table 2 For each locus, the number of individuals (n) genotyped for each of the four locations, expected level of heterozygosity (H_E), observed level of heterozygosity (H_O) and F_{IS}-value

	Hawaii (n = 30)				Mexico (n = 30)				New Zealand (n = 30)				Southern California (n = 30)			
Locus	n	H_E	H_O	F_{IS}	n	H_E	H_O	F_{IS}	n	H_E	H_O	F_{IS}	n	H_E	H_O	F_{IS}
TA024	30	0.8622	0.8667	0.012	30	0.8628	0.9667	−0.104	29	0.8888	0.8276	0.086	30	0.8878	0.8000	0.116
TA105	30	0.8967	0.7000	**0.235***	30	0.9172	0.8667	0.072	26	0.9112	0.7308	**0.217***	25	0.8960	0.9200	−0.006
TA149	30	0.4750	0.4333	0.105	30	0.3750	0.5000	−0.318	26	0.5399	0.5769	−0.049	28	0.4011	0.3214	0.216
TA155	30	0.5272	0.3000	**0.445***	30	0.5867	0.6667	−0.120	27	0.4767	0.4815	0.009	26	0.3765	0.3077	0.202
TA157	30	0.5706	0.7667	−0.329	30	0.5194	0.4000	0.246	27	0.5384	0.6667	−0.220	27	0.5343	0.4074	0.255
TA162	30	0.8189	0.7000	0.162	30	0.7883	0.8000	0.002	29	0.8317	0.7931	0.064	29	0.7806	0.7586	0.046
TA164	30	0.5183	0.4667	0.116	30	0.4506	0.4667	−0.019	28	0.7213	0.7143	0.028	30	0.4367	0.4333	0.025
TA193	30	0.9472	0.9333	0.032	30	0.9078	0.8000	0.135	28	0.9082	0.8214	0.113	25	0.9128	0.8800	0.056
TA218	30	0.4994	0.5000	0.016	30	0.4994	0.4333	0.149	30	0.4861	0.7000	−0.426	30	0.5000	0.6000	−0.184
TA235	30	0.8261	0.8333	0.008	30	0.7967	0.8333	−0.029	29	0.8740	0.8621	0.031	30	0.8506	0.9333	−0.081

Significant deviations from Hardy–Weinberg equilibrium are denoted by (*) and bold numbering.

to 31 alleles (Table 1). Expected and observed levels of heterozygosity were calculated in GENETIX (Belkhir *et al*. 1996-2004). Observed levels of heterozygosity ranged from 0.3000 to 0.9667 (Table 2). Exact tests for deviations from Hardy–Weinberg equilibrium, F_{IS} and linkage disequilibrium were calculated in GENEPOP (1.2) (Raymond & Rousset 1995). Significant deviations from Hardy–Weinberg equilibrium were detected in two loci, locus TA105 in individuals from Hawaii (F_{IS} = 0.235) and New Zealand (F_{IS} = 0.217), and locus TA155 in Hawaiian samples (F_{IS} = 0.445), which could indicate the presence of null alleles in these loci. MICRO-CHECKER (van Oosterhout *et al*. 2004) revealed possible null alleles in locus TA105 in Hawaii and New Zealand, locus TA155 in Hawaii and locus TA193 in Mexico. None of the 45 pairwise-locus comparisons showed significant linkage disequilibrium after sequential Bonferroni correction.

The 10 microsatellite loci contain a sufficient amount of variation which is useful in evaluating population structure and gene flow in striped marlin, *Tetrapturus audax*.

Acknowledgements

This research was supported in part by the US Environmental Protection Agency STAR fellowship (FP-91655901), and by the Paxson Offield Family Foundation. Samples were provided by NMFS, the IATTC, Sofia Ortega-Garcia, Richard Keller Kopf and Valerie Allain. The authors thank Jason Curole and Marie Voigt for zip-code tag protocol assistance.

References

Belkhir K, Borsa P, Chikhi L, Raufaste N, Bonhomme F (1996-2004) GENETIX *4.05, Logiciel Sous Windows TM Pour la Génétique des Populations*. Laboratoire Génome, Populations, Interactions, CNRS UMR 5171, Université de Montpellier II, Montpellier, France.

Buonaccorsi VP, Graves JE (2000) Isolation and characterization of novel polymorphic tetra-nucleotide microsatellite markers from the blue marlin, *Makaira nigricans*. *Molecular Ecology*, **9**, 820–821.

Chen J, Iannone MA, Li MS *et al*. (2000) A microsphere-based assay for multiplexed single nucleotide polymorphism analysis using single base chain extension. *Genome Research*, **10**, 549–557.

DeWoody (2002) *DeWoody's Microsatellite Cloning Protocol* (Spring 2002). Available from http://www.agriculture.purdue.edu/fnr/html/faculty/DeWoody/pdfs/msatclngprtcl.pdf.

Graves JE, McDowell JR (1994) Genetic analysis of striped marlin *Tetrapturus audax* population structure in the Pacific Ocean. *Canadian Journal of Fisheries and Aquatic Sciences*, **51**, 1762–1768.

Hamilton MB, Pincus EL, Di Fiore A, Fleischer RC (1999) Universal linker and ligation procedures for construction of genomic DNA libraries enriched for microsatellites. *BioTechniques*, **27**, 500–507.

McDowell JR, Graves JE (2008) Population structure of striped marlin (Kajikia audax) in the Pacific Ocean based on analysis of microsatellite and mitochondrial DNA. *Canadian Journal of Fisheries and Aquatic Sciences*, **65**, 1307–1320.

Nakamura I (1985) *Billfishes of the World*. FAO Species Catalogue, vol. 5. FAO Fish. Synopsis, 5, 65 pp.

van Oosterhout C, Hutchinson WF, Wills DPM, Shipley P (2004) MICRO-CHECKER: software for identifying and correcting genotyping errors in microsatellite data. *Molecular Ecology Notes*, **4**, 535–538.

Raymond M, Rousset F (1995) GENEPOP (version 1.2): population genetics software for exact tests and ecumenicism. *Journal of Heredity*, **86**, 248–249.

doi: 10.1111/j.1755-0998.2009.02709.x