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PRIMER NOTE

Microsatellite DNA markers for population-genetic studies of Atlantic bluefin tuna (*Thunnus thynnus thynnus*) and other species of genus *Thunnus*

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Abstract

Twenty-five microsatellites from Atlantic bluefin tuna (*Thunnus thynnus thynnus*) were characterized. All 25 microsatellites were polymorphic; the number of alleles among up to 56 individuals surveyed ranged from two to 23. Atlantic bluefin tuna are highly exploited and major questions remain as to stock structure and abundance in the eastern and western North Atlantic. The microsatellites will be useful in testing stock-structure hypotheses and in generating estimates of effective population size. The polymerase chain reaction primer sets developed also amplified identifiable alleles in three other species of genus *Thunnus*: *T. albacares* (yellowfin tuna), *T. alalunga* (albacore tuna) and *T. obesus* (bigeye tuna).

Keywords: Atlantic bluefin tuna, genus *Thunnus*, microsatellites

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Atlantic bluefin tuna (*Thunnus thynnus thynnus*) are among the world's largest and most economically valuable fish and are, therefore, highly exploited, with the current spawning stock biomass much less than that needed to generate sustainable yields (ICCAT 2000). A highly contentious issue is whether two discrete stocks, one in the eastern North Atlantic and one in the western North Atlantic, exist. Historical evidence for the stock structure of bluefin tuna in the North Atlantic was reviewed in Magnuson *et al*. (1994) and included genetic, life history and other (e.g. parasite distribution) characters. McDowell *et al*. (2002) briefly reviewed more recent studies (Broughton & Gold 1997; Takagi *et al*. 1999; Reece & Graves, unpublished data) that employed hypervariable, nuclear-encoded markers (microsatellites and single-copy-nuclear DNA). These latter studies, however, were inconclusive on the issue of discrete stocks. A second, no less contentious issue, concerns the abundance of Atlantic bluefin tuna and whether differences in abundance exist between the (putative) eastern and western stocks. Here, we report the characterization of 25 microsatellite markers from Atlantic bluefin tuna DNA. The markers should prove useful in testing stock-structure hypotheses (models) and in generating estimates of effective population size (e.g. Hauser *et al*. 2002; Turner *et al*. 2002) as a genetic means to estimate abundance.

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The methods used to generate microsatellites generally followed Broughton & Gold (1997). Approximately 400 ng of bluefin genomic DNA were digested with *Sau* 3AI. Fragments in the range 400–1000 bp were excised from a 1% agarose gel and ligated using T4 DNA ligase (BRL) into a multicloning site within the LacZ gene of a Pgem-3Zf(+) vector, transformed into DH10B-Beta electrocompetent cells (Life Technology) via electroporation and plated onto Luria-Bertani (LB)/ampicillin/IPTG/X-Gal plates. Colonies with inserts (white) were picked and transferred to LB-filled microplates for further screening of transformants; cells from individual wells of a microtiter plate were collected and used to inoculate a Hybond nylon membrane (Amersham) on top of a slab of LB agar plus ampicillin. Each colony was spotted twice to eliminate false positives. After overnight incubation at 37 °C, colonies were lysed and DNA affixed to a membrane. Appropriate 'prehybridized' membranes were probed with separate cocktails of γ^{32}P-labelled di-, tri- and tetraoligonucleotides. A total of 150 candidate microsatellites (out of 291 'positive' clones) were polymerase chain reaction (PCR) amplified using

Table 1 Summary data for microsatellites developed from Atlantic bluefin tuna (*Thynnus thunnus thunnus*)

Micro-satellite	Primer sequence (5′–3′)	Repeat sequence	Length of the cloned allele	Annealing temp. (°C)	N/N_A	Size range (bp)	H_E/H_O	P_{HW}
Tth 4	F: GAAACGCAGCCGGAGAGGAAAGAG R: AATGTGAGGGGGATGGGAGCTTGT	$(GT)_{16}$	210	60	50/22	198–244	0.934/0.960	0.648
Tth 14	F: AAGATGGGGGTACAAACAAG R: TTTTTCATACCGGAGGACTC	$(AC)_{11}$	134	60	49/8	124–138	0.661/0.735	0.534
Tth 17	F: TCTGTGAGCATCACGTTACTG R: TCTGAAGCGACTGCATTCT	$(CA)_7GA(CA)_5$	99	60	48/2	99–101	0.417/0.375	0.498
Tth 112	F: TAGCAACAAGCAGTTAGAGA R: GAAAGTCCTATCAATCAAG	$(TC)_5TA(TC)_5TT(TC)_4$	131	57	54/14	115–143	0.850/0.759	0.082
Tth 114	F: TCCAGAAGATATTCCCTCATTTGTA R: AAGCCGTCAGCGAAGCCTAAC	$(CTTT)_2(CT)_6$	185	57	49/4	185–199	0.650/0.653	0.781
Tth 152	F: ATGCCGCTCTGATGAGGTTA R: CTTGTTCTTCCCCGACACTG	$(CT)_{10}$	175	60	49/3	171–179	0.237/0.245	0.302
Tth 157	F: CAAGAGGCTTAAAGCAAACTC R: CATGAATGGGTTCCTTCATC	$(CA)_{13}$	129	60	55/6	123–133	0.638/0.582	0.050
Tth 178	F: AGACATCTGCAGGAAGTG R: AAAGAACTGCAACATGACA	$(CCT)_7CTT(CCT)_4(CT)_2$	150	57	50/5	140–152	0.407/0.380	0.102
Tth 185	F: AGCCTTCGATGCACCCGTCTTAC R: ATCATGCTTCCACTGCCCACTCTC	$(TG)_{11}$	142	60	50/6	142–178	0.572/0.480	0.007
Tth 204	F: CCTGTGGAGCCATGACAG R: TTTAATTTCCCTAGTGTCCTGAT	$(TG)_{14}$	157	60	55/9	143–161	0.697/0.691	0.016
Tth 207	F: GTGTGTCGGATGCTGATT R: ATAGATGGATTGTTATGTCTTGT	$(GA)_8GC(GA)$	270	57	52/6	268–278	0.741/0.750	0.331
Tth 208	F: GAGAGGGAAAGCAAAGAAG R: GTTGAGCTGCTGACACAGA	$(GA)_{18}$	158	60	56/22	148–204	0.914/0.821	0.056
Tth 217	F: ACTTTCCCTACCTGGTGATAT R: GTTGTAACCACTAATGGTAAAC	$(CA)_{18}$	225	57	51/17	219–259	0.855/0.745	0.011
Tth 226	F: ATTGTGCATACACCCACAC R: ACACTGTGCTGAACTCAACTTA	$(CA)_{20}$	155	57	48/12	149–175	0.880/0.792	0.157
Tth 254	F: TGGGAGACAGTGACATACGAG R: CACACCAAACAAAGGATTACT	$(AG)_{21}$	107	60	50/23	79–135	0.923/0.880	0.350
Tth 260	F: TTTATCCTCAGATTTGATATG R: GTGTCTGCTTGTATTTGTGT	$(AC)_2A(AC)_{26}$	128	57	32/14	114–144	0.879/0.719	0.002
Tth 1-31	F: ATGCACAAGTCATTTATCACCT R: AGATGCATGGATTACATTCTACC	$(AC)_{11}$	102	60	50/13	96–136	0.874/0.900	0.064
Tth 7-16	F: TTCCTTCAGGACCAATAAAGTATC R: TCAGAGCTGCTAGCATGTATGTAG	$(TATC)_{17}$	126	60	50/13	94–162	0.859/0.860	0.640
Tth 10-43	F: ATTTTTACCTGGCTACATCTATCT R: CACACCGGCGATTTTGAG	$(TCTA)_9$	125	57	48/8	109–137	0.768/0.312	0.000*
Tth 12-29	F: CATACATACACATCTACATTGAACG R: CACCAACAAGTACTGTAGATATGC	$(GT)_{10}AC(GT)_7$	111	60	49/8	93–113	0.836/0.796	0.169
Tth 23-6	F: TTAAATTTGCAAGGGTATGATG R: CCTTCTTCTTGATTGTCTTTGTAA	$(AT)_6$	92	57	46/3	86–94	0.320/0.065	0.000*
Tth 16-2	F: TGAGTTCCCAATTACACT R: CTGTAGCATCGTCACAGT	$(GT)_{10}(TACA)_2$	103	60	47/13	89–119	0.862/0.957	0.295
Tth 62	F: GGTATATGTGTTTGTAGGCGTGTG R: TTTTCCCAATGCGACTGATGA	$(GT)_{16}$	103	60	34/6	95–107	0.632/0.647	0.798
Tth 211	F: ATAAACACACCCTTACTCACT R: TATTCTTTTCCTAACCATTCT	$(CA)_{13}$	192	57	45/12	173–201	0.883/0.756	0.002
Tth 265	F: TCGGTGGGAGGGAGACGC R: CAGGTGGTGCAATTAATGGAAAA	$(CA)_{17}$	197	60	46/12	187–211	0.773/0.783	0.546

*Actual probability values were 0.0006.

The polymerase chain reaction primer sequences are forward and reverse; the forward primer was labelled for genotyping. Repeat sequence indicates the repeat motif of the cloned allele.

N, number of individuals assayed; N_A, number of alleles detected; size range, alleles thus far uncovered; H_E and H_O, expected and observed heterozygosity, respectively; P_{HW}, probability that genotype proportions conform to expectations of Hardy–Weinberg equilibrium.

Sequences of the clones are listed in GenBank under Accession nos AY396520–AY396544.

universal M13 primers and the PCR products obtained were cleaned with p-20 DNA miniprep columns (Quiagen). Sequencing reactions were performed using the same M13 primers and run on an ABI 377 DNA sequencer (Applied Biosystem).

Microsatellite-like motifs were identified in about 75 of the clones. The PCR primers and approximate associated annealing temperatures were designed for 67 of these using PRIMER SELECT (GCG Wisconsin package, Oxford Molecular, Molecular Simulation, Inc.). Amplification by the primers was then tested under a variety of experimental conditions. Amplifications were performed in an Omn-E thermal cycler in 96-well plates and involved end-labelling (using T4 polynucleotide kinase) one primer of each pair with [γ^{32}P]-dATP. Reactions contained approximately 5 ng of sample DNA, 0.1 U of *Taq* DNA polymerase, 0.5 μM of each primer, 200 μM of each dNTP, 1 mM $MgCl_2$ and 1× *Taq* buffer, pH 9.0 (500 mM KCl, 100 mM Tris, 10% Triton-X 100), in a total volume of 10 μL. Amplifications consisted of 30 cycles of denaturation at 95 °C for 45 s, annealing at 57–60 °C for 30 s, extension at 72 °C for 1 min and final extension at 72 °C for 10–60 min. The PCR products were electrophoresed in 6% polyacrylamide gels and visualized by autoradiography.

A subset of 36 PCR primer pairs were tested for experimental tractability [reproducibility, consistency, range of allele size, frequency of 'stutter' bands (if present) and microsatellite polymorphism] by screening a panel of DNAs isolated from eight to 10 fish. A total of 25 primer pairs were then used to screen DNAs from up to 56 fish sampled from the northern Atlantic. Genotypes at polymorphic microsatellites were tested for conformity to Hardy–Weinberg (HW) expectations and for (pairwise) genotypic disequilibrium.

Summary data for the 25 microsatellites are given in Table 1. Estimates of expected and observed heterozygosity and F_{IS} were obtained using GENETIX version 4.05 (Belkhir *et al*. 1996–2002). The probability of departure from HW equilibrium (P_{HW}) and of genotypic disequilibrium between pairs of microsatellites were assessed using a Markov-chain method (Guo & Thompson 1992), as implemented in GENEPOP version 3.3 (Raymond & Rousset 1995) and using 5000 dememorizations, 500 batches and 5000 iterations per batch. Sequential Bonferroni correction (Rice 1989) was applied for all multiple tests performed simultaneously. The 25 microsatellites included a number of 'perfect' dinucleotide (15) and tetranucleotide (two) repeat motifs, five 'imperfect' repeat motifs (containing mixtures of dinucleotide repeats) and three compound repeats. Lengths of the cloned sequences ranged from 92 to 270 bp (GenBank Accession nos AY396520–AY396544). Annealing temperatures ranged from 57 to 60 °C and all 25 microsatellites were polymorphic, with the number of identified alleles ranging from 2 to 23 (avg. 10.3). Expected and observed heterozygosity over all microsatellites ranged from 0.237 to 0.934 and 0.245–0.960, respectively. Genotypes at two microsatellites (*Tth* 10-43 and *Tth* 23-6) differed significantly from HW expectations following sequential Bonferroni correction (Rice 1989). Estimated F_{IS} values for the two microsatellites were positive (0.596 for *Tth* 10-43 and 0.798 for *Tth* 23-6), possibly suggesting the presence of null alleles. Although the P_{HW} estimate of 0.006 for microsatellite *Tth* 211 was not significant following Bonferroni correction, the F_{IS} estimate (0.145) was positive and may indicate null allele(s) at this microsatellite as well. Two pairwise tests of genotypic disequilibrium (*Tth* 178 vs. *Tth* 185 and *Tth* 178 vs. *Tth* 207) were significant ($0.25 < P < 0.05$, adjusted) following Bonferroni correction and may indicate that the microsatellites are linked. The pairwise test between *Tth* 185 and *Tth* 207, however, was not significant ($P > 0.50$, adjusted).

The 25 primer pairs also were used to screen DNAs from up to 10 individuals each of yellowfin tuna (*T. albacares*), albacore tuna (*T. alalunga*) and bigeye tuna (*T. obesus*). All 25 primer pairs, except those for *Tth* 114 in *T. alalunga*, amplified scorable PCR products in all three species under the same conditions as those designed for *T. thynnus*. Identified alleles ranged from three to 16 (avg. 8.0) in *T. albacares*, two to 13 (avg. 7.2) in *T. alalunga* and two to 15 (avg. 7.7) in *T. obesus*.

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