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# Microsatellite development and survey of variation in northern bluefin tuna (*Thunnus thynnus*)

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## Abstract

Decreasing population numbers of northern bluefin tuna (*Thunnus thynnus*) in the Atlantic Ocean have emphasized the need for information on intraspecific genetic variation. However, previous studies employing nuclear-encoded proteins and mitochondrial DNA have revealed little informative variation. We obtained 21 microsatellite loci in bluefin tuna. Survey of five of these loci in 59 individuals from five geographically widespread samples revealed extensive variation at the population level. Significant allele-frequency differences among samples were found under both of the frequently employed models of microsatellite evolution (i.e., infinite alleles and stepwise mutation). Primers developed for bluefin tuna microsatellites also allowed successful amplification in other scombrid species. These results indicate that microsatellites will be useful for future population studies of bluefin tuna and may also be useful for other tuna species.

## Introduction

Northern bluefin tuna (*Thunnus thynnus*) are among the largest marine teleost fishes, reaching over 3 m in length, weighing over 500 kg, and living upward of 30 years. These pelagic fish support important commercial and recreational fisheries, and their increasing market value has led to extensive exploitation (National Research Council, 1994). As a result, population numbers have substantially declined, and bluefin stocks have become the most depleted of any tuna species (Graves, 1996). These circumstances have led to increased international concern regarding stock conservation and management of fishing practices, particularly in the Atlantic Ocean.

Within the Atlantic, spawning of bluefin tuna is thought to be restricted largely to the Mediterranean Sea and Gulf of Mexico, although large numbers of individuals migrate across the northern Atlantic Ocean on seasonal cycles (Richards, 1976; National Research Council, 1994). Tag-recapture studies (Suzuki, 1991) suggest mixing of fish from Mediterranean and Gulf spawning grounds (i.e., fish tagged in the western Atlantic are recovered in the eastern Atlantic and vice-versa) occurs at a rate of about 3% per year (Suzuki, 1991). Although the extent of spawning-site fidelity among migratory adults is unknown (Graves, 1996), it could result in restricted gene flow and divergence of eastern and western forms. Effectively subdivided breeding populations are expected to maintain a greater amount of genetic variation and some variation could represent adaptation to local environments. As a result, effective conservation efforts for Atlantic bluefin tuna must incorporate knowledge of the amount of gene flow between spawning sites.

Results of prior genetic studies have failed to find differences between eastern and western Atlantic bluefin tuna. Two studies employing allozyme electrophoresis did not indicate allele-frequency differences or deviations from Hardy-Weinberg proportions among samples from the western and eastern Atlantic (Edmunds and Sammons, 1973; Thompson and Contin, 1979). Another study, employing partial sequences of the mitochondrial cyt *b* gene, found little informative variation in Atlantic bluefin (Bartlet and Davidson, 1991). Therefore, it remains unclear whether Atlantic bluefin tuna represent a large panmictic population or if there is restricted gene flow between spawning areas in the Mediterranean and Gulf.

In response to concerns about declining abundance of Atlantic bluefin tuna, we initiated an investigation of nuclear microsatellite markers to examine genetic variation and, ultimately, popula-

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tion structure in Atlantic bluefin tuna. Microsatellites are tandem arrays of short, repeated sequences with typically 2, 3, or 4 bp in each repeat unit (Tautz, 1989). Alleles are defined by differences in the number of repeat units that presumably are generated by slipped-strand mispairing (Levinson and Gutman, 1987) during DNA replication. Microsatellite loci are abundant in most eukaryotic genomes and tend to be highly polymorphic when the number of repeats is greater than about 10 (Weber, 1990). Allelic variation can be readily surveyed based on electrophoretic mobility of polymerase chain reaction (PCR) amplification products that include the repeat region (Weber and May, 1989). Because of their extensive variability and the large number of potential loci, microsatellites may be sensitive indicators of genetic variation and gene flow among populations and could be particularly informative in species that exhibit low levels of variation for other markers (Queller et al., 1993; Jarne and Lagoda, 1996). Microsatellites have been shown to be informative at the population level in a number of fish species including zebrafish (Goff et al., 1992), brown trout (Estoup et al., 1993), Atlantic salmon (Slettan et al., 1993), stickleback (Rico et al., 1993), Atlantic cod (Brooker et al., 1994), sea bass (Garcia de Leon et al., 1995), and northern pike (Miller and Kapuscinski, 1996).

Here we report development and characterization of microsatellite loci for northern bluefin tuna. Several loci were used to investigate the distribution of genetic variation among samples from the North Atlantic, Mediterranean, and Gulf (*T. t. thynnus*), and from the Pacific (*T. t. orientalis*). We also used PCR primer sets developed for bluefin tuna to investigate the degree of conservation of microsatellites among other scombrid species.

## Results

### *Characterization of microsatellites*

Thirty of 864 genomic clones hybridized with at least one of five simple-sequence repeat probes, and nucleotide sequences of 24 of the inserts were determined. No microsatellite-like repeats were found in three of the clones, although each was quite large and was not sequenced completely. Primer sets for amplification were designed for 10 loci, six of which produced scorable products (Table 1). Primer sets were designated as scorable if they repeatably produced either one or two strong bands on autoradiographs of polyacrylamide gels, however, in many cases fainter amplification artifacts were observed. Among six scorable loci, the number of alleles ranged from 1 to 22, with a positive association between number of repeats and number of alleles at a locus. One locus (7) was monomorphic in all individuals examined.

### *Distribution of microsatellite variation*

Samples of bluefin tuna were obtained from five localities in three regions: two were from the Mediterranean, one from the northwest Atlantic, one from the Gulf of Mexico, and one from the Pacific. Fifty-nine individuals were scored for five variable (and one invariant) loci. Allele frequencies by locality are shown in Figure 1. The less variable loci (15, 16, and 38) were characterized by a common allele with a few others at low frequency, whereas the more variable loci (20 and 26) rarely had alleles with a frequency greater than 0.5. Mean sample size per locus and mean observed heterozygosity for each locality were as follows: MedV, 12.0 and 0.517; MedT, 7.0 and 0.380; NwAtl, 21.8 and 0.443; GulfM, 7.4 and 0.389; PacC, 8.6 and 0.439. Observed frequencies of heterozygotes across all loci were consistently less than Hardy-Weinberg expectations, but were always within one standard error of the estimated expected value (not shown). Fixation indices and associated $\chi^2$ tests indicated three cases of significant departure from Hardy-Weinberg equilibrium proportions (Table 2); all three involved locus 26, the locus with the greatest number of alleles. In each case, there was a deficiency of heterozyotes.

Measures of population subdivision (Table 3) were determined by assuming either an infinite alleles model (IAM) where all alleles differ by an equal amount, or a stepwise mutation model (SMM) where differences among alleles are based on the number of mutational steps between them (assuming a change of one repeat unit per mutation). Wright's (1978) $F_{ST}$ (employing IAM) indicated significant allele-frequency differences at loci 16, 20, and 26, and for all loci combined (Table 3). $\phi_{ST}$ is an analog of $F_{ST}$ that employed the SMM (Excoffier et al., 1992). $\phi_{ST}$ indicated allele-frequency differences at loci 16, 26, and 38, and all loci combined (Table 3). Although both mutation models yield significant frequency differences when all loci are considered, differences in the two models were observed at loci 20 and 38.

Allele-frequency homogeneity among samples was tested using the Monte Carlo randomization procedure of Roff and Bentzen (1989). This method is similar to the $\chi^2$ contingency test of $F_{ST} = 0$ (assuming IAM), except that the test distribution is

**Table 1.** Characteristics of six bluefin tuna microsatellite loci.

| Locus | Repeat sequence | Repeat length* | Repeat type† | Primer sequences 5′-3′ |
|---|---|---|---|---|
| Tth7 | CA/GT | 15 | Perfect | ATCCGTAAAGACGAAAACAT |
| | | | | AGGGATGTCAGTTCCTTTAG |
| Tth15 | CA/GT | 15 | Perfect | ACCGCAATCCTCCTGTAGAC |
| | | | | CACATCGTCCATCCAGTTAC |
| Tth16 | CA/GT | 24 | Imperfect | CCCGTGGACTTGACTACACA |
| | | | | AGTGCCTCCCTCTCTTCTCC |
| Tth20 | CA/GT-CT/CA | 36 | Compound | TCACAGTTAGGGGATTAGAC |
| | | | | CTTTGACCACCCACTTTGTC |
| Tth26 | CA/GT | 28 | Perfect | GCAGAGCCAGAGTTTGATAC |
| | | | | GCATAAAGACTGGAGACAGC |
| Tth38 | CA/GT | 17 | Imperfect | ATAAACAAACCCCCTTGAAT |
| | | | | AGTGTGGAAAGAGCCCTGCC |

* Number of repeats in cloned microsatellite.

† After Weber (1990).

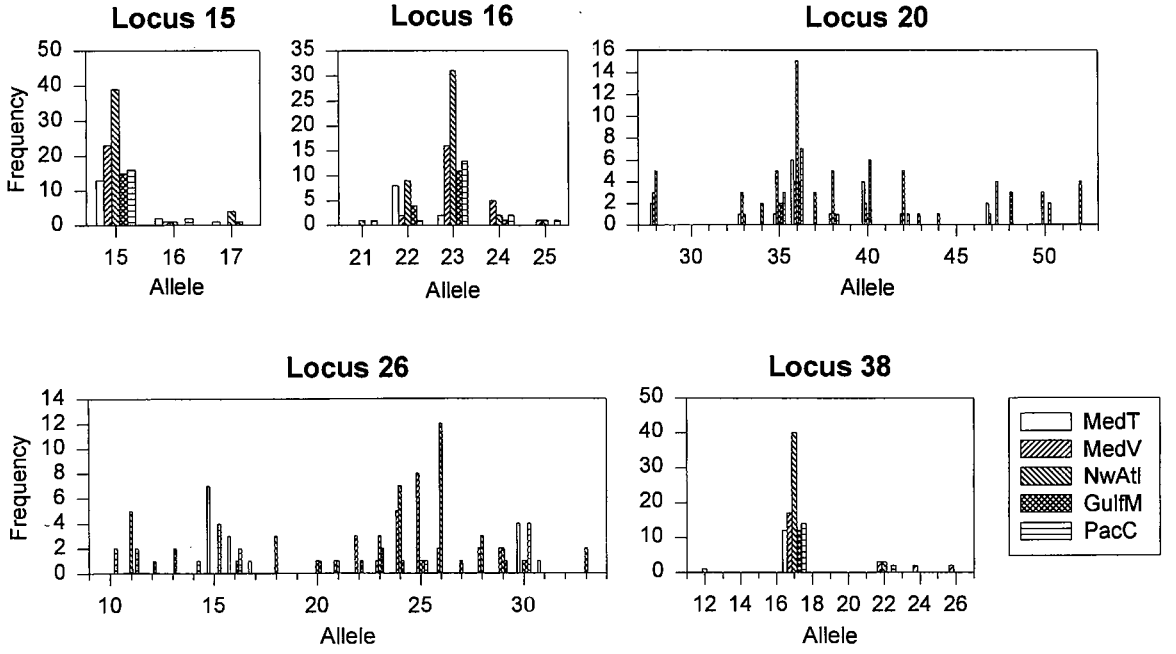


**Figure 1.** Allele absolute frequencies for five microsatellite loci in five geographic samples of bluefin tuna. MedT = central Mediterranean Sea, MedV = western Mediterranean Sea, NwAtl = northwestern Atlantic Ocean, GulfM = Gulf of Mexico, PacC = western Pacific Ocean.

generated by randomizing the original matrix in order to reduce the problem of small sample sizes for rare alleles. Significant heterogeneity was observed in combined analysis of all loci and all samples, as well as for the three loci exhibiting significant $F_{ST}$ values across all samples (Table 4). Significant heterogeneity was found between the two samples from the Mediterranean (Table 4) as well as for most other pairwise comparisons, but did not appear to be corellated with geographic distance among samples.

*Interspecific amplification of microsatellites*

Interspecific conservation of microsatellites was examined by testing for amplification of the five variable loci in six related scombrid species: yellowfin

**Table 2.** Fixation indices (F) and results of $\chi^2$ tests for deviation from Hardy-Weinberg equilibrium proportions for five samples of bluefin tuna.*

| | Sample | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | MedV | | MedT | | NwAtl | | GulfM | | PacC | |
| Locus | F | *P* | F | *P* | F | *P* | F | *P* | F | *P* |
| 15 | −0.043 | 1.000 | 0.220 | 0.072 | 0.337 | 0.103 | −0.067 | 1.000 | −0.125 | 0.796 |
| 16 | −0.159 | 0.058 | −0.250 | 0.705 | 0.207 | 0.678 | −0.365 | 0.736 | 0.027 | 0.608 |
| 20 | 0.040 | 0.142 | 0.340 | 0.038† | 0.065 | 0.616 | 0.158 | 0.013† | −0.033 | 0.138 |
| 26 | 0.379 | **0.001** | −0.067 | 0.622 | 0.108 | **0.005** | 0.349 | 0.020† | 0.390 | **0.001** |
| 38 | −0.244 | 0.945 | 1.000 | 1.000 | −0.080 | 0.984 | 1.000 | 1.000 | −0.143 | 0.782 |

* Significant results are indicated by boldface.

† Not significant when corrected for multiple tests (Rice, 1989).

**Table 3.** Allele frequency heterogeneity among samples.

| Locus | $F_{ST}$ | $P$*† | $\Phi_{ST}$ | $P$†‡ |
|---|---|---|---|---|
| 15 | .033 | .393 | −.018 | .700 |
| 16 | .213 | .007 | .118 | <.001 |
| 20 | .070 | <.001 | .026 | .154 |
| 26 | .121 | <.001 | .128 | .008 |
| 38 | .090 | .225 | .116 | <.001 |
| Mean | .106 | <.001 | .074 | <.001 |

* $P$ values for contingency $\chi^2$ test of hypothesis that $F_{ST} = 0$.

† All values <.05 were significant when corrected for multiple tests (Rice, 1989).

‡ Probability of having a more extreme value of $\Phi_{ST}$ by chance alone.

**Table 4.** Homogeneity tests of allele distributions.

| | Comparison | |
|---|---|---|
| Loci | All localities | MedV-MedT |
| All | <.001 | <.001 |
| 16 | .008 | <.001 |
| 20 | <.001 | .356 |
| 26 | <.001 | <.001 |

tuna (*Thunnus albacares*), blackfin tuna (*Thunnus atlanticus*), bigeye tuna (*Thunnus obesus*), albacore tuna (*Thunnus alalunga*), skipjack tuna (*Katsuwonus pelamis*), and king mackerel (*Scomberomorus cavalla*). Amplification with bluefin primers produced either scorable bands that were similar in length to those of bluefin tuna, or many bands of varying length and intensity (Table 5). Scorable results for 24 of 30 tests indicates a reasonable level of priming site conservation among the species.

**Table 5.** Cross-specific amplification with bluefin tuna primers (+ = successful amplification).

| | Primer set | | | | |
|---|---|---|---|---|---|
| | 15 | 16 | 20 | 26 | 38 |
| Yellowfin tuna | + | + | + | + | + |
| Blackfin tuna | + | + | + | − | + |
| Bigeye tuna | + | + | + | + | + |
| Albacore tuna | + | + | + | + | + |
| Skipjack tuna | + | + | − | − | + |
| King mackerel | + | + | − | − | − |

## Discussion

### *Characterization of microsatellites*

The number of microsatellite-containing clones in the bluefin genomic library was 3.5% of total clones examined, a frequency comparable to that found in Atlantic cod (Brooker et al., 1994), and somewhat higher than that found in sea bass (Garcia de Leon et al., 1995), brown trout (Estoup et al., 1993), and northern pike (Miller and Kapuscinski, 1996). Although five different repeat probes were used to identify microsatellite loci, we found a strong bias toward CA/GT repeats. Loci with larger numbers of repeats tended to be more variable, yet locus 26 has a greater number of alleles than might be expected based on mean allele length (Table 1). On the whole, the frequency and variability of microsatellites in bluefin tuna seem fairly typical of vertebrates, including fishes (Brooker et al., 1994; Jarne and Lagoda, 1996).

### *Distribution of microsatellite variation*

Samples of bluefin tuna from different localities were treated as "populations" largely as a matter

of convenience. Given the long-lived and highly migratory nature of bluefin tuna, current sample groups are probably not Mendelian populations in the traditional sense. However, genotypic frequencies within samples were generally within expectations of Hardy-Weinberg equilibrium. The lone exception was locus 26, where a deficiency of heterozygotes was observed in three samples. Apparent heterozygote deficiency can result from an undetected "null" allele; however, no null homozygotes were observed at locus 26 and without specific crosses, presence of a null allele cannot be confirmed. As significant deviations from Hardy-Weinberg expectations were detected at only one locus, we infer that mating within these samples of bluefin tuna does not deviate substantially from random.

Tests of allele-frequency homogeneity indicated genetic differentiation among combined *T. thynnus* samples at several loci. There also were significant genetic differences between most pairwise comparisons among samples, including the two from the Mediterranean for loci 16 and 26. Curiously, where differences were not significant (i.e., between MedT and PacC for loci 16 and 26), those samples were not in close geographic proximity. These results might be expected if there has been recent long-distance dispersal between some localities.

The IAM and SMM models yielded different results for two loci (Table 3). If data fit the IAM, alleles identical in state will be identical by descent (i.e., there is no homoplasy), and $F_{ST}$ is an appropriate measure of subdivision. However, when data fit the SMM, $F_{ST}$ will tend to underestimate the degree of subdivision (Slatkin, 1995) and indices that account for allele lengths appear to provide better estimates (Slatkin, 1995; Rousset, 1996). Differences between $F_{ST}$ and $\phi_{ST}$ for loci 20 and 38 (Table 3) are likely due to the distribution of allelic-size differences within and among samples. For locus 38, where three of five size variants are unique to single samples, $\phi_{ST}$ emphasizes these differences to a greater degree than $F_{ST}$. However, for locus 20, where samples share a wide range of similar (but not identical) sized alleles, $F_{ST}$ suggests greater differentiation than $\phi_{ST}$.

Due to the limited availability of bluefin tuna, sample sizes for the present study were small. This precludes any firm conclusions regarding population structure. However, small sample sizes often result in insufficient power to reject the null hypothesis of no population subdivision. Therefore, our results are provocative to the extent that they suggest the possibility of allele frequency differences among geographic samples. Microsatellites in bluefin tuna appear to be abundant and exhibit a wide range of variability among loci, suggesting they will be of great utility in future population studies.

*Interspecific amplification of microsatellites*

Heterospecific amplification using bluefin primers indicated a fairly high level of sequence similarity in microsatellite regions among scombrid fishes. With one exception, all loci produced scorable results within the genus *Thunnus*, while fewer scorable results were obtained in *Katsuwonus* and *Scomberomorus*. These results are generally consistent with molecular phylogenetic relationships proposed for scombrid fishes (Block et al., 1993; Chow and Kishino, 1995). Although locus homology has not been conclusively demonstrated (e.g., via sequencing), production of similar sized amplification products suggests conservation of microsatellites among tunas.

## Experimental Procedures

Genomic DNA samples for 59 individual bluefin tuna were provided by the United States National Marine Fisheries Service laboratory in Charleston, South Carolina, U.S.A. Individuals were obtained from five localities: two from the Mediterranean (northeast of Valencia, Spain, $n = 12$; and the Gulf of Taranto, Italy, $n = 8$), one from the northwest Atlantic (off the northeastern coasts of the United States and Canada, $n = 22$), one from the Gulf of Mexico (near the Florida Keys, $n = 8$), and one from the eastern Pacific (Cortez Bank, off San Diego, Calif., U.S.A., $n = 9$).

*Development of microsatellite loci*

Approximately 400 ng of bluefin tuna genomic DNA was digested with restriction endonuclease *Sau*3AI. Fragments in the range of 400 to 1000 bp were excised from a 1% agarose gel. Approximately 200 ng of pGEM cloning vector (Promega Inc.) was digested with *Bam*HI. The digested vector was treated with shrimp alkaline phosphatase (NEB), plasmid and tuna DNAs were combined in a 2:1 ratio (tuna:vector), T4 ligase (BRL) was added, and the mixture was incubated overnight at 16°C. Recombinant molecules were used to transform *E. coli* cells by electroporation. Insert-containing (i.e., white) colonies were transferred to 96-well microtiter plates containing LB plus ampicillin, and overnight cultures from nine plates (864 clones) were transferred to an

8 × 12 cm nylon membrane using a robotic work station (Biomek 2000, Beckman, Inc.). Colonies were grown on the membrane and then lysed, and DNA was bound to the membrane by UV crosslinking. The oligonucleotides $(CA)_{15}$, $(GA)_{15}$, $(CCT)_7$, $(ATT)_7$, and $(GACA)_8$ were end-labeled with $\gamma^{32}P$ by T4 polynucleotide kinase (NEB) and then hybridized with the membrane-bound DNAs overnight at 60°C. After washing, the membrane was wrapped in cellophane and exposed to x-ray film. Plasmids from clones that hybridized with probes were digested with *Eco*RI and *Xba*I to excise inserts. Digests were separated on agarose gels, transferred to nylon membranes, and reprobed with the same oligonucleotides as above. This served to confirm the previous hybridization and allowed assessment of insert sizes.

Sequences were determined for clones that hybridized in both probing procedures (sequences are available from the senior author on request). Plasmids were purified with Qiagen tip-20s (Qiagen, Inc.), and DNA concentration was determined by fluorimetry. Cycle-sequencing reactions were performed using M13 forward or reverse primers and dye-labeled dideoxynucleotides for automated sequencing. Sequences were read on an ABI 377 automated sequencing system (Applied Biosystems, Inc.). Resulting sequences that confirmed presence and identity of simple-sequence repeats also provided flanking sequences from which to design oligonucleotide primers for amplification. Pairs of 20-mer primers were designed for each microsatellite locus using the computer program Oligo.

*Amplification and survey of variation*

Microsatellite genotypes were determined by PCR amplification and gel electrophoresis. Prior to amplification, one of the primers was labeled with $\gamma^{32}P$ by T4 polynucleotide kinase (30 min, 37°). PCR reactions contained approximately 5 ng of genomic DNA, 0.1 units *Taq* DNA polymerase, 0.5 μM each primer, 800 μM dNTPs, 1–2 mM magnesium ion, 1X *Taq* buffer pH 9.0 (Promega, Inc.), and sterile deionized water in a total volume of 10 μl. Thermal cycling was conducted in 96-well plates with the following parameters: denaturation 94°, 30 seconds; annealing (temperature variable), 1 minute; polymerization 72°, 30 seconds. Some loci were successfully amplified by employing temperature regimes whereby annealing temperature is gradually lowered through a series of PCR cycles. Three-microliter aliquots of each PCR reaction were electrophoresed in denaturing polyacrylamide "sequencing" gels. Gels were dried and exposed to x-ray film. Sizes of amplification products were determined by comparison with a known sequence ladder run on the same gel. Repeat numbers were inferred by subtracting the length of the flanking sequence.

*Analysis*

Cloned microsatellite sequences were characterized for type and number of repeats, and frequencies of alleles were determined for all individuals at selected loci. Several analyses were performed to quantify the distribution of allelic variation among samples. We employed an IAM of mutation to test for Hardy-Weinberg proportions, generate hierarchical *F* statistics, and test for allele-frequency homogeneity. Tests for Hardy-Weinberg equilibrium and generation of *F* statistics employed BIOSYS-1 (Swofford and Selander, 1981). Homogeneity of allele frequencies was tested with the Monte Carlo procedure of Roff and Bentzen (1989). A hierarchical analysis of molecular variance was performed with the program WINAMOVA (Excoffier et al., 1992). Microsatellite alleles were input as "haplotypes" and distances were in the form of squared differences in repeat number among alleles (i.e., the SMM was employed). Each locus was analyzed separately and results were subsequently combined as weighted means.

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