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Short Paper

Isolation and characterization of single copy nuclear DNA markers in Atlantic bluefin tuna *Thunnus thynnus*

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The Atlantic bluefin tuna *Thunnus thynnus* is the most valuable bony fish, mainly inhabiting the northern hemisphere of the Atlantic Ocean including the Mediterranean Sea and Gulf of Mexico.[1] The population status has been a prime concern, the and issue of whether two distinct eastern and western populations of bluefin tuna exist in the North Atlantic has been challenged using many approaches.[2] Molecular genetic approaches using highly variable microsatellite and mitochondrial control region sequences have been inconclusive.[3,4] Highly polymorphic genetic markers are potentially capable of detecting subtle signals of population subdivision on a small scale and over short periods of time. However, these techniques usually require large numbers of individuals for statistical robustness and may not be practical for estimating the stock mixing ratio because of the inherent large variance. Single nucleotide polymorphism in a nuclear gene intron and restriction assay on a coding region of mitochondrial DNA revealed diagnostic genetic difference between ocean samples of the swordfish *Xiphias gladius* and bigeye tuna *Thunnus obesus*,[5,6] which were not well resolved by analyses using highly polymorphic DNA markers.[7,8] These results indicate that further development and application of other DNA markers including fewer polymorphic markers are necessary. We have attempted to isolate single copy nuclear DNA (scnDNA) loci from the Atlantic bluefin tuna (ABT) and investigated the genetic variability.

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Primer information to amplify eight scnDNA loci of ABT is presented in Table 1. Four are introns of protein coding genes, and the others are anonymous DNA regions. We adopted primers designed in exon regions for amplifying introns of fish calmodulin (*CAM*), glucose-6-phosphate dehydrogenase (*G6PD*) and S7-ribosomal protein (*S7RP*) genes.[9–11] A primer set (TropEx3F: 5′-AACTTGAGCTGGCTGAGAAG-3′ and TropEx4R: 5′-GCCCTGTTCTCAATGACCTT-3′) were designed from a database search for tropomyosin (*TROP*). DNA fragments amplified using these intron targeted primer sets were cloned, and the nucleotide sequences were determined to design internal specific primers. Four anonymous nuclear DNA loci (*F1*, *F2*, *F3* and *Q2*) were obtained through following protocol. Polymerase chain reaction (PCR) was performed at a low annealing temperature (37°C) in the first two cycles using two arbitrarily chosen independent primers (~20 mer), and the PCR products were directly cloned into plasmids. Plasmids inserted with 300–700-bp fragments were screened and the nucleotide sequences were determined to design internal primers. Representative sequences of these introns and anonymous regions are available from DDBJ (AB291547–AB291552, AB300375, AB300376). These eight primer sets were applied to a total of 65 individuals of ABT (subadults and adults) caught in the north-west Atlantic (41–42°N, 55–64°W) in November 1992 to January 1993. The PCR reaction mixture (10 μL) contained 0.2 U *Taq* DNA polymerase (Perkin Elmer Cetus, Norfolk, VA, USA), 0.2 mM of each dNTP, 1 μL of the 10 × buffer supplied by the manufacturer, 2 mM $MgCl_2$, 10 pmol of each primer and

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Table 1 Primer sequences for eight single copy nuclear DNA loci isolated in the Atlantic bluefin tuna *Thunnus thynnus*

Locus[†]	Primers[‡]	Primer sequence	Ta (°C)[§]	Size of amplified fragments (bp)	Accession no.
CAM	CAMint4F5RLF	CGGGTATTTGACAAGGTAAA	60	387	AB291547
	CAMint4F5RLR	TGGAAACATGCCGTATGGGA			
G6PD	G6PDint4F	CCTGAAGGTACAACTTGACC	55	377, 484	AB291548
	G6PDint4R	TGGGCCAGAGAGTGAAAACA			
S7RP	RP2intF	CGGATGAAAGGAGAGATCAT	55	334, 350	AB291549
	RP2intR2	TAGGTGATGTCTGGACAGAA			
TROP	Trop238F	GAGAGGTGGGTTCTTTAGAG	60	231, 238	AB300376
	Trop238R	AGAAGTAAGGAGCTGACCTG			
F1	F1F	AGCATGCAGTAGACCCAGGA	50	302, 330	AB291550
	F1R	ACTTTGTCTCCGCCTTTCCG			
F2	F2F	AGCTGTAACTGCAATATGGT	60	305, 340	AB291551
	F2R	GTATTCGTAATCTGATCCGG			
F3	F3F	TCGCAGACCTGGTGAAAATG	55	320–360	AB291552
	F3R	TTCGCCGCAAGTCTGGATAA			
Q2	Q2F	ACTTTTACCACCAGCCAGGT	55	386	AB300375
	Q2R	GCTGCTACTAGCATAACACA			

[†]See text for loci.
[‡]F and R, forward and reverse primers, respectively.
[§]Annealing temperature.

Table 2 Genotype frequencies at eight loci of bluefin tuna sample collected in North-West Atlantic

	CAM								*Q2*
Genotype	*Nla*III	*Nla*IV	*G6PD*	*S7RP*	*TROP*	*F1*	*F2*	*F3*	*Rsa*I
AA	4	7	19	0	49	0	7	2	57
AB		38	30	6	16	12	6	1	1
BB		18	14	54		51	49	1	
AC	30								2
AD	3							16	
AE	3							1	
BD								3	
BE								3	
CC	20								
CD	1								
CE								2	
CF	1								
DD								31	
DE								3	
n	62	63	63	60	65	63	62	63	60
H_O	0.61	0.60	0.48	0.10	0.25	0.19	0.10*	0.46*	0.05
H_E	0.54	0.49	0.50	0.10	0.22	0.17	0.27	0.52	0.05

*Significant heterozygote deficits ($P < 0.002$).
H_E, expected heterozygosity; H_O, observed heterozygosity.

10–100 ng of template DNA. Amplification was carried out with an initial denaturation at 96°C for 3 min followed by 30 cycles of amplification (denaturation at 96°C for 0.5 min, annealing for 0.5 min at temperatures shown in Table 1 and extension at 72°C for 0.5 min) with a final extension at 72°C for 10 min. Genotype scoring and allele determination were readily performed by conventional agarose gel (2–2.5%) electrophoresis (Fig. 1). Restriction fragment length polymorphism at *CAM* and *Q2* loci was detected by *Nla*III and *Nla*IV digestions for the former and *Rsa*I digestion for the latter, in which one to five fragments were observed in an individual but total size of all fragments never exceeded double the size of the amplified fragment. Fragments smaller than 100 bp are not shown. Length variations of amplified fragments were observed at another six

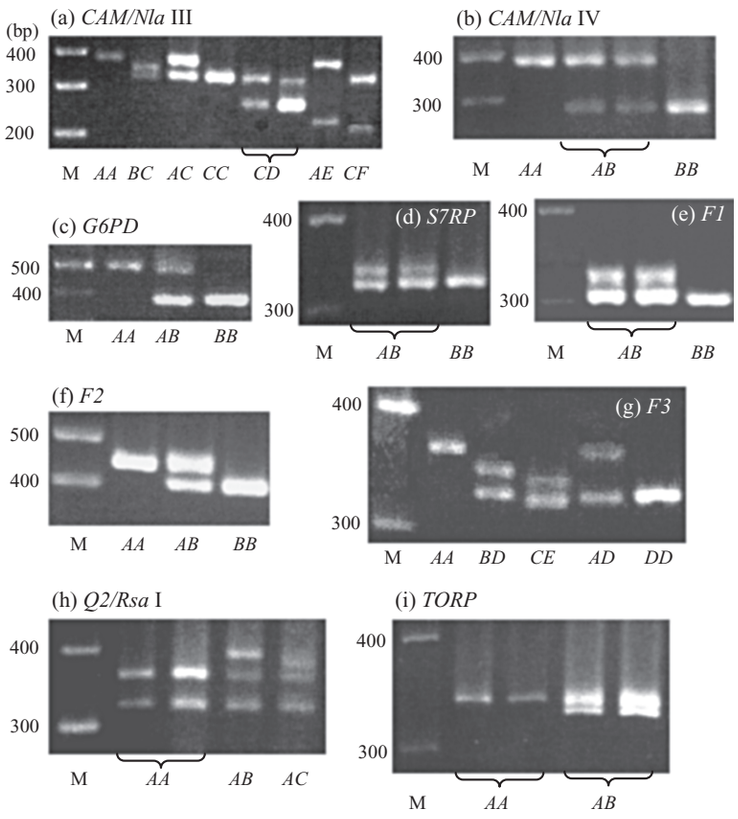

Fig. 1 Agarose gel electrophoresis showing variations at eight loci of the Atlantic bluefin tuna *Thunnus thynnus*, (a) *CAM/Nla*III, (b) *CAM/Nla*IV, (c) *G6PD*, (d) *S7RP*, (e) *F1*, (f) *F2*, (g) *F3*, (h) *Q2/Rsa*I and (i) *TROP*. Genotypes determined are shown at bottom of each lane. M, molecular marker. Size (bp) is shown along the left margin.

loci, where one or two banded patterns were observed, indicating the variation is caused by indels.

Genotype frequencies at these eight loci are shown in Table 2. Expected heterozygosity and number of alleles ranged 0.05–0.54 and 2–5, respectively. Significant heterozygote deficits were observed at two loci (*F2* and *F3*), which may be due to null alleles, large allele dropout or population mixture.

Successful PCR amplification and similar variation for these loci were observed in the closely related southern bluefin tuna *T. maccoyii* using the same amplification conditions.

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