Fisheries Science 65(4), 571-576 (1999)

PCR Primers for Microsatellite Loci in Tuna Species of the Genus *Thunnus* and its Application for Population Genetic Study

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(Received December 28, 1998)

Microsatellite loci were isolated from a size-selected genomic library of Pacific northern tuna *Thunnus thynnus orientalis*, and PCR primer sets to amplify four loci were designed. Investigation on genetic polymorphism at these loci in the Pacific northern bluefin tuna sample ($n=35$–40) revealed high degree of length polymorphisms in all loci, in which number of alleles per locus ranged from 8 to 23 and observed heterozygosity from 0.533 to 1. These primer sets were applied to Atlantic northern bluefin tuna *T. t. thynnus*, albacore *T. alalunga*, bigeye tuna *T. obesus* and yellowfin tuna *T. albacares*, detecting polymorphism in all loci comparable with those of Pacific northern bluefin tuna. Significant differences in the allele frequency were observed between Pacific and Atlantic northern bluefin tuna samples. These primer sets developed for Pacific northern bluefin tuna appeared to be useful for amplifying homologous microsatellite loci in the other *Thunnus* tuna species, and may have great potential as indicators for genetic variability within and between samples of tuna species of the genus *Thunnus*.

Key words: microsatellite, *Thunnus* tuna species, genetic variability, population genetics

All tuna species of the genus *Thunnus* are important for commercial fisheries. Clarifying their stock structure may be very important information for proper stock assessment and management strategy. Molecular genetic approaches have been available as tools for investigating fish stock structure. Allozyme analysis has been employed for investigating stock structure of Atlantic northern bluefin tuna *Thunnus thynnus thynnus*[1,2] and yellowfin tuna *T. albacares*.[3] However, these investigations have failed to find substantial genetic differentiation among local samples of these tuna species because of insufficient genetic polymorphism in allozyme gene markers. Mitochondrial DNA analyses revealed large genetic differentiation between Atlantic and Pacific northern bluefin tuna populations. However, mtDNA analysis alone has no power to cope with population mixture nor can they verify whether a local sample is from a single panmictic population. Recently, nucleic microsatellites consisting of tandem arrays of di-, tri-, or tetra- nucleotide sequences flanked by regions of unique DNA sequences[4] have been shown to be highly polymorphic particularly in fish.[5] Microsatellites are among the most likely to confirm with the assumption of neutrality and have proved its power for distinguishing geographically isolated subpopulations.[6] Few studies have been carried out on microsatellite variation analysis of *Thunnus* species (Atlantic northern bluefin tuna *T. thynnus thynnus*).[7]

In the present study, we report the isolation of microsatellite loci of the Pacific northern bluefin tuna *Thunnus thynnus orientalis* and PCR primers designed to amplify these microsatellite loci. These PCR primers were applied to the other tuna species of the genus *Thunnus*, and genetic differentiation within species was investigated.

Materials and Methods

Fish Samples and DNA Preparation

Collection information of fish samples used are presented in Table 1. For constructing genomic DNA library, high molecular weight DNA samples were extracted from blood of three fresh Pacific northern bluefin tuna caught off Japan. Crude DNA samples were extracted from frozen or ethanol-preserved muscle tissues of Atlantic northern bluefin tuna *T. thynnus thynnus*, albacore *T. alalunga*, bigeye tuna *T. obesus* and yellowfin tuna *T. albacares*. The standard SDS-phenol/chloroform procedures[8] were used for both tissues. Extracted DNA were disolved in TE buffer and stored at 4°C prior to PCR analysis.

Isolation of Microsatellites from Bluefin Tuna

A genomic library was constructed by using basically the same procedure as Takagi *et al.*[9] High molecular weight DNA samples extracted from 3 individuals of Pacific northern bluefin tuna were pooled, digested with *Hae*III, *Hinc*II and *Rsa*I and electrophoresed on agarose gel. Fragments ranged between 400 and 2000 bp were excised and subjected to phenol/chloroform extraction. The digested DNA samples were ligated into the *Sma*I site of PUC18

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plasmid vector which was used for transforming DH5α starin of *E. coli*. Positive transformants were screened with [^{32}P]-labeled $(GT)_{15}$ synthetic oligonucleotide. Sequencing of positive clones was performed by 373 DNA sequencer (Applied Biosystems Inc.) using a *Taq* dye-terminator cycle sequencing kit (Perkin-Elmer Cetus, Norfolk) with universal M13 reverse and forward primers.

Detection of Polymorphism by PCR and PAG Electrophoresis

Primers were designed to hybridize with unique regions flanking the core sequences of the microsatellites using Oligo™ (N. B. I. Inc.). The reverse primer was 5'end-labelled with [γ^{32}P]ATP. PCR was carried out using a MJ Thermal cycler (MJ research Inc.). The 6 μl of reaction contained 10ng of template DNA, 2.5 μM each unlabeled primer, 0.25 μM labeled reverse primer, 10 mM Tris-HCl (pH 8.3), 1 mM $MgCl_2$, 50 mM KCl, 0.01% gelatin, 1.75 μM each dNTPs and 0.25 unit of *Taq* Polymerase (Applied Biosystems Inc.). The PCR was programmed for 7 cycles of initial amplification (denaturation at 94°C for 1 min, annealing at 52°C for 30 s, and extension at 72°C for 30 s) followed by 33 cycles of secondary amplification (denaturation at 90°C for 30 s, annealing at 52°C for 30 s, and extension at 72°C for 30 s). Following amplification the sample was mixed with an equal volume of denaturing stop dye (Pharmacia Inc.), heated at 95°C for 15 min, and electrophoresed on 8% polyacrylamide gel (7 M urea, 8% acrylamide). Drying on gels and autoradiography were as described in Sambrook *et al.*[10] Alleles of each locus were sized relative to a M13 sequence ladder.[11] Designation of loci and alleles were given by following the abbreviated scientific name and fragment size.[12] Genotype frequencies in each sample at each locus were tested for conformity to Hardy-Weinberg equilibrium[13] using Arlequin version 1.1.[14] Allele frequencies were then used to calculate population pairwise FSTs by Arlequin version 1.1 and Nei's genetic distance[15] by Phylip-PC version 3.57c.[16]

Table 1. Catch locality, date and mean fork length of the tuna species used in the study

Area sample	(*n*)	Date	Mean fork length ± SD
Pacific northern bluefin tuna			
Off Japan (Tosa Bay)	(48)	26 Jul.–29 Aug. 1995	20.7±1.6
Atlantic northern bluefin tuna			
N. W. Atlantic (42N, 52-63W)	(48)	Nov.-Dec. 1992	193.2±44.9
Mediterranean (Messina Strait)	(39)	Mar.-May. 1994	Adults
Albacore			
E. Australia (18-20S, 154-155E)	(32)	4-7 Aug. 1993	9.5±0.5
Bigeye tuna			
Off Philippines (Celebes Sea)	(32)	26, 27 Jul. 1993	28.1±4.0
Yellowfin tuna			
Off Philippines (Celebes Sea)	(32)	21 Sep. 1993	28.3±5.6

Results

Detection of Microsatellite Loci and Genetic diversity in Northern Bluefin Tuna

Among 41 positive clones obtained, presence of microsatellite was directly confirmed in 10 clones by partial nucleotide sequencing. Among 10 PCR primer sets designed to flank these microsatellite regions, fragment amplification was observed in seven primer sets, and scorable and constant amplification of DNA fragments were obtained in 4 primer sets. The nucleotide sequences of 4 sets of PCR primers to amplify these four gene loci (designated *Ttho-1**, *-4**, *-6** and *-7**) are presented in Table 2. A highly polymorphic profile in a locus (*Ttho-6**) of Pacific northern bluefin tuna is shown in Fig. 1.

To estimate levels of variation at each microsatellite locus, three samples of northern bluefin tuna (one Pacific and two Atlantic) were analyzed. All these 4 loci were found to be highly polymorphic, in which observed heterozygosity (*ho*) ranged from 0.538 at *Ttho-1** of N. W. Atlantic sample to 1 at *Ttho-7** of off Japan and Mediterranean samples (Table 3). Similar variations were observed among samples, in which *Ttho-6** had the largest number of alleles (15 to 23) followed by *Ttho-7** and *-4** (10 to 15), and *Ttho-1** having the least number of alleles (6 and 8). No significant discrepancy between observed and expected number of genotypes was observed in all loci, as well as between observed (*ho*) and expected (*he*) heterozygosities. Mean heterozygosity (*Ho*) was very high ranging from 0.701 to 0.865, and no large diffference in the mean heter-

Table 2. Nucleotide sequence and genetic variability of four *Thunnus thynnus* microsatellite PCR primers

Locus	Repeat motif*a	Primer sequence (5'-3')*b	Annealing temp.*c	No. of samples	No. of alleles	Size (bp)*d	Hetero-zygosity*e
*Ttho-1**	(GT)	F: AAACGCTCCAGGCAAATGAC	50	(39)	8	175–189	0.533
		R: CATAGCACACCCATAGACAC					
*Ttho-4**	(CA)	F: CCTTCATCTTCAGTCCCATC	52	(39)	12	134–164	0.811
		R: CTGTTCATCTGTTCGCCC					
*Ttho-6**	(CA)	F: TTCTGCTTCTTTCTTCTGG	52	(35)	23	121–219	0.924
		R: GAAAACACAGGGATTATGG					
*Ttho-7**	(CA)	F: ACTGGATGAAAGGCGATTAC	52	(40)	15	198–230	0.884
		R: ACAGAGGAGCATAACAGAAAC					

*a=Core repeat motif from cloned *Thunnus thynnus* orientalis sequence.
*b=F and R refer to forward and reverse primer sequence respectively.
*c=PCR annealing temperatures were optimized for *Thunnus thynnus orientalis*.
*d=Estimated size of the PCR fragment when compared with M13 sequence fragmentes of known length.
*e=Estimated heterozygosity.

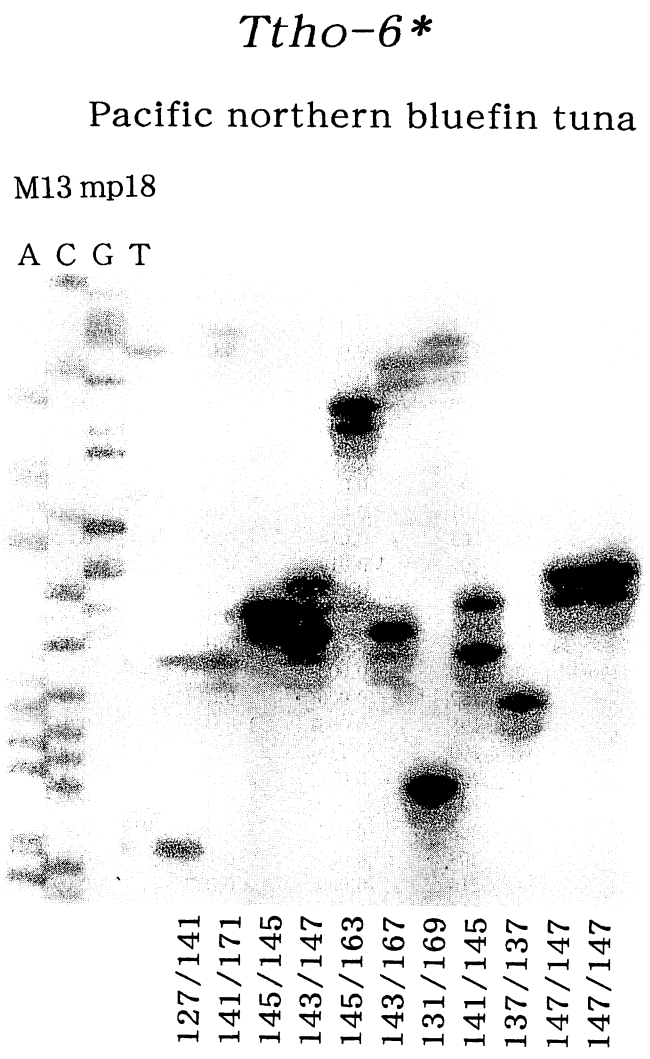

Fig. 1. Microsatellite variation in *Ttho-6** locus of *Thunnus thynnus orientalis*. The size standard is a sequence ladder of M13 mp18.

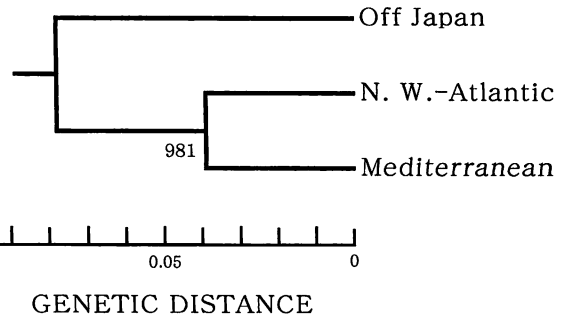

Fig. 2. UPGMA dendrogram and bootstrap value of Pacific northern bluefin tuna and 2 samples of Atlantic northern bluefin tuna.

ozygosity was observed among these northern bluefin tuna samples.

Allele frequencies at these four loci of three northern bluefin tuna samples are presented in Table 4. All samples shared the most common allele in all loci but *Ttho-6**. Significant difference in allele frequencies was observed between the off Japan and N. W. Atlantic samples ($F_{ST}=0.024$, $P<0.001$) and between the off Japan and Mediterranean samples ($F_{ST}=0.029$, $P<0.001$), while no significant difference was observed between N. W. Atlantic and Mediterranean samples ($F_{ST}=0.002$, $P>0.294$). Allele frequencies were used to calculate genetic distances between samples and a UPGMA dendrogram was constructed, showing close relationships between N. W. Atlantic and Mediterranean samples of Atlantic northern bluefin tuna and distinct status of Pacific northern bluefin tuna sample supported by considerably high bootstrap value (981 out of 1,000 randomization) (Fig. 2).

Application of PCR Primers to Different Thunnus *Tuna Species*

All four sets of PCR primers were found to successfully amplify scorable fragment patterns also in the other three

Table 3. Genetic variability for 4 microsatellite loci surveyed for northern bluefin tuna used in the study

		*Ttho-1**	*Ttho-4**	*Ttho-6**	*Ttho-7**	Mean
Pacific northern bluefin tuna						
Off Japan	No. of samples	(39)	(39)	(35)	(40)	
(Tosa Bay)	No. of allele	8	12	23	15	14.5
	Effective no. of allele*[1]	2.13	5.29	13.16	8.62	7.30
	Heterozygosity (*Ho*)	0.564	0.923	0.886	1.000	0.843
	(*He*)	0.531	0.811	0.924	0.884	0.788
	(*Ho*/*He*)	1.058	1.138	0.959	1.131	1.072
Atlantic northern bluefin tuna						
N. W. Atlantic	No. of samples	(39)	(38)	(39)	(37)	
(42N, 52-63W)	No. of allele	6	10	21	15	13.0
	Effective no. of allele	2.73	4.39	8.13	6.58	5.46
	Heterozygosity (*Ho*)	0.538	0.684	0.744	0.838	0.701
	(*He*)	0.633	0.772	0.877	0.848	0.783
	(*Ho*/*He*)	0.850	0.886	0.848	0.988	0.893
Mediterranean	No. of samples	(38)	(38)	(34)	(36)	
(Messina Strait)	No. of allele	6	12	15	12	11.3
	Effective no. of allele	2.73	6.58	7.35	8.77	6.36
	Heterozygosity (*Ho*)	0.684	0.921	0.853	1.000	0.865
	(*He*)	0.633	0.848	0.864	0.886	0.808
	(*Ho*/*He*)	1.081	1.086	0.973	1.129	1.067

*[1]: $=1/(1-He)$.

Table 4. Allele frequency for 4 microsatellite loci surveyed for Northern bluefin tuna used in the study

Locus	Allele (bp)	Pacific	Atlantic	
		Off Japan	N. W. Atlantic	Mediterranean
*Ttho-1**	175	0.013	0.000	0.000
	177	0.051	0.026	0.013
	179	0.115	0.192	0.211
	181	0.667	0.526	0.526
	183	0.077	0.231	0.211
	185	0.026	0.000	0.026
	187	0.013	0.000	0.000
	189	0.038	0.013	0.013
	193	0.000	0.013	0.000
*Ttho-4**	134	0.013	0.013	0.000
	142	0.051	0.040	0.053
	144	0.333	0.329	0.263
	146	0.205	0.303	0.171
	148	0.077	0.066	0.105
	150	0.141	0.066	0.105
	152	0.038	0.132	0.158
	154	0.013	0.013	0.026
	156	0.064	0.000	0.026
	158	0.013	0.000	0.013
	160	0.000	0.000	0.013
	162	0.038	0.000	0.039
	164	0.013	0.013	0.000
	172	0.000	0.026	0.026
*Ttho-6**	121	0.014	0.000	0.000
	127	0.029	0.013	0.000
	131	0.086	0.128	0.132
	133	0.000	0.013	0.000
	135	0.000	0.051	0.044
	137	0.071	0.051	0.074
	139	0.043	0.013	0.000
	141	0.114	0.026	0.132
	143	0.129	0.128	0.118
	145	0.071	0.218	0.265
	147	0.129	0.179	0.015
	149	0.057	0.000	0.000
	151	0.000	0.000	0.015
	153	0.014	0.013	0.000
	157	0.000	0.013	0.000
	161	0.000	0.013	0.074
	163	0.029	0.026	0.029
	165	0.014	0.013	0.015
	167	0.029	0.000	0.044
	169	0.043	0.000	0.000
	171	0.029	0.013	0.000
	175	0.000	0.013	0.000
	177	0.014	0.000	0.000
	179	0.000	0.013	0.000
	185	0.000	0.000	0.015
	187	0.000	0.038	0.015
	193	0.000	0.000	0.015
	201	0.014	0.013	0.000
	203	0.014	0.000	0.000
	205	0.014	0.013	0.000
	215	0.014	0.000	0.000
	217	0.014	0.000	0.000
	219	0.014	0.000	0.000
*Ttho-7**	180	0.000	0.000	0.014
	182	0.000	0.014	0.000
	198	0.088	0.041	0.042
	200	0.138	0.027	0.000
	202	0.025	0.054	0.000
	204	0.050	0.027	0.083
	206	0.025	0.054	0.153
	208	0.025	0.176	0.111
	210	0.000	0.027	0.097
	212	0.000	0.027	0.000
	214	0.025	0.027	0.028
	216	0.013	0.014	0.111
	218	0.013	0.135	0.097
	220	0.188	0.297	0.181
	222	0.150	0.041	0.069
	224	0.113	0.041	0.014
	226	0.113	0.000	0.000
	228	0.025	0.000	0.000
	230	0.013	0.000	0.000

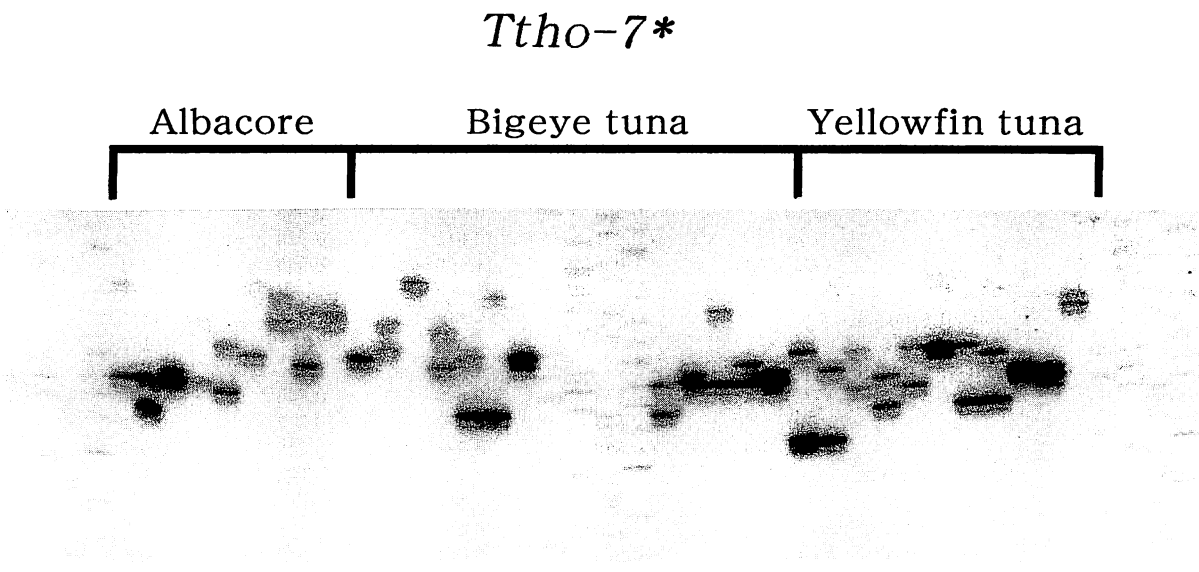

Fig. 3. Microsatellite electrophoretic pattern of *Ttho-7** locus from albacore, bigeye tuna and yellowfin tuna. The size standard is a sequence ladder of M13 mp18.

Table 5. Genetic variability for 4 microsatellite loci surveyed for tuna species used in the study

			*Ttho-1**	*Ttho-4**	*Ttho-6**	*Ttho-7**	Mean
Albacore							
E. Australia	No. of samples		(31)	(32)	(31)	(32)	
(18°-20°S, 154°-155°E)	No. of allele		7	15	7	14	10.8
	Effective no. of allele*[1]		3.62	7.14	2.34	8.93	5.51
	Heterozygosity	(*Ho*)	0.871	0.688	0.548	0.906	0.753
		(*He*)	0.724	0.860	0.573	0.888	0.761
		(*Ho*/*He*)	1.203	0.800	0.956	1.020	0.995
Bigeye tuna							
Off Philippines	No. of samples		(32)	(31)	(16)	(26)	
(Celebes Sea)	No. of allele		12	10	22	18	15.5
	Effective no. of allele		2.24	5.18	17.54	11.76	9.18
	Heterozygosity	(*Ho*)	0.563	0.806	1.000	0.923	0.823
		(*He*)	0.554	0.807	0.943	0.915	0.805
		(*Ho*/*He*)	1.016	0.999	1.060	1.009	1.021
Yellowfin tuna							
Off Philippines	No. of samples		(31)	(25)	(23)	(24)	
(Celebes Sea)	No. of allele		7	9	5	16	9.3
	Effective no. of allele		3.36	4.48	1.38	11.64	5.22
	Heterozygosity	(*Ho*)	0.581	0.880	0.217	0.792	0.618
		(*He*)	0.702	0.777	0.274	0.914	0.667
		(*Ho*/*He*)	0.828	1.133	0.792	0.867	0.905

*[1]: $=1/(1-He)$.

Thunnus tuna species without modifying PCR condition. Highly polymorphic profiles in a locus (*Ttho-7**) of these three speceis are shown in Fig. 3, and genetic variability indices at four loci are presented in Table 5. Notably low variation was observed in *Ttho-6** of yellowfin tuna sample, in which the number of alleles was 5 and observed heterozygosity (*ho*) was 0.217. Whereas, relatively to highly polymorphic patterns were observed in the other loci of yellowfin tuna and in all loci in the other species, in which observed heterozygosity (*ho*) ranged from 0.548 at *Ttho-6** of albacore sample to 1 at *Ttho-6** of bigeye tuna sample. Mean heterozygosity (*Ho*) was highest in bigeye tuna sample (0.823) followed by albacore (0.753), and that of yellowfin tuna was lowest (0.618).

Discussion

The four sets of primers designed to amplify microsatellite loci of Pacific northern bluefin tuna were clearly demonstrated to be applicable for other congeneric tuna species. These phenomena are observed also in the former analysis of genus *Thunnus* and other scomber fishes.[7] Successful cross-amplification of microsatellites among these closely related species suggests that these microsatellites have been conserved in the homologous DNA regions no matter how the levels of variation have differentiated. Ward *et al.*[17] investigated genetic variation of Pacific northern bluefin tuna population ($\bar{n}=29$) using 35 allozyme gene loci. Of 8 loci found to be variable, relatively high polymorphism ($P_{.90}$) was observed only in 3 loci, in which expected heterozygosity ranged from 0.384 to 0.523. Maximum number of alleles per locus was 3 in their allozyme analysis, while that in the present study was 23. Elliott and Ward[18] also investigated allozyme variation in albacore, bigeye and yellwofin tunas. In a few loci showing relatively high variation, they found expected heterozygosity ranging from 0.295 to 0.619 and number of alleles per locus ranging from 2 to 5. Thus, it appeared that microsatellite loci may harbor a much larger amount of genetic variation than allozyme gene loci. Extremely low genetic variability of microsatellites shown by number of allele and heterozygosity has been reported in endangered species[19] and inbreeding population.[20] The levels of length variations observed in *Thunnus* tuna species are much higher than these endangered or inbreeding populations, and comparable with those found in other wild fish populations such as Atlantic cod ($He=0.757$)[5], red sea bream ($He=0.849$)[9], Japanese flounder ($He=0.875$)[21] and ayu ($He=0.784$).[19] Therefore, these tuna species may not be categorized as endangered or inbred species at the genetic variability point of view.

Broughton and Gold[7] found that differences between Pacific and Mediterranean northern bluefin tuna were not significant for two loci Tth 16 and 26. In our study, we observed the larger genetic divergence between Pacific and Atlantic samples in all of the loci exanined. The phylogenetic topology of separation between Pacific and Atlantic northern bluefin tunas obtained with UPGMA is supported by high bootstrap value and is congruent with previous mtDNA analysis.[22,23] Another concern is to assess genetic heterogeneity within Atlantic northern bluefin tuna population, by which one might evaluate genetic relationships between putative western and eastern stocks. Microsatellites found in this study were considerably polymorphic but detected little differentiation between N. W. Atlantic and Mediterranean samples. Much larger sample size might be necessary for comparing local samples when using highly polymorphic gene markers. Otherwise, standard sample such as larvae or juveniles from spawning ground in Mediterranean and Gulf of Mexico must be analyzed using these microsatellite genetic markers.

References

1) P. H. Edmunds and J. I. Sammons: Genetic polymorphism of tetrazolium oxidase in bluefin tuna, (*Thunnus thynnus*) from the western North Atlantic. *J. Fish. Res. Bd. Can.*, **28**, 1053–1055 (1971).
2) P. H. Edmunds and J. I. Sammons: Similarity of genetic polymorphism of tetrazolium oxidase in bluefin tuna (*Thunnus thynnus*) from the Atlantic coast of France and the western North Atlantic. *J. Fish. Res. Bd. Can.*, **30**, 1031–1032 (1973).
3) R. D. Ward, N. G. Elliott, and P. M. Grewe: Allozyme and mitochondrial DNA variation in yellowfin tuna (*Thunnus albacares*) from the Pacific Ocean. *Mar. Biol.*, **118**, 531–539 (1995).
4) J. L. Weber and P. E. May: Abundant class of human DNA polymorphisms which can be typed using then polymorphic DNA markers. *Am. J. Hum. Genet.*, **44**, 388–396 (1989).
5) A. L. Brooker, D. Cook, P. Bentzen, J. M. Wright, and R. W. Doyle: Organization of microsatellites differs between mammals and cold-water teleost fishes. *Can. J. Fish. Aquat. Sci.*, **51**, 1959–1966 (1994).
6) P. Jarne and P. J. L. Lagoda: Microsatellites, from molecules to populations and back. *Trends in Ecology and Evolution*, **11**, 424–429 (1996).
7) R. E. Broughton and J. R. Gold: Microsatellite development and survey of variation in northern bluefin tuna (*Thunnus thynnus*). *Mol. Mar. Biol. Biotechnol.*, **6**, 308–314 (1997).
8) A. S. Harris, S. Bieger, R. W. Doyle, and J. M. Wright: DNA fingerprinting of tilapia, *Oreochromis niloticus*, and its application to aquaculture genetics. *Aquaculture*, **92**, 157–163 (1991).
9) M. Takagi, N. Taniguchi, D. Cook, and R. W. Doyle: Isolation and characterization of microsatellite loci from red sea bream *Pagrus major* and detection in closely related species. *Fisheries Sci.*, **63**, 199–204 (1997).
10) J. Sambrook, E. F. Fritsch, and T. Maniatis: Molecular Cloning, Cold Springer Harbor Laboratory Press, New York, 1989, pp. 6.45.
11) C. Yanisch-Perron, J. Vieira, and J. Messing: Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13 mp18 and pUC 19 vectors. *Gene*, **33**, 103–119 (1985).
12) J. B. Shaklee, F. W. Allendorf, D. C. Morizot, and G. S. Whitt: Gene nomenclature for protain-coding loci in fish. *Trans. Am. Fish. Soc.*, **119**, 2–15 (1990).
13) S. W. Guo and E. A. Thompson: Performing the exact test of Hardy-Weinberg proportion for multiple alleles. *Biometrics*, **48**, 361–367 (1992).
14) S. Schneider, J.-M. Kueffer, D. Roessli, and L. Excoffier: Arlequin version 1.1, University of Geneva, Switzerland,1997.
15) M. Nei: Estimation of average heterogeneity and genetic distance from a small number of individuals. *Genetics*, **89**, 583–590 (1978).
16) J. Felsenstein: Phylip version 3.5c, University of Washington, Seattle, 1989.
17) R. W. Ward, N. J. Elilott, and P. M. Grewe: Allozyme and mitochondrial DNA separation of Pacific northern bluefin tuna (*Thunnus thynnus orientalis*) from southern bluefin tuna (*Thunnus maccoyii*). *Mar. Freshwater Res.*, **46**, 921–930 (1995).
18) N. G. Elliott and R. D. Ward: Genetic relationships of eight species of Pacific tunas (Teleostei: *Scombridae*) inferred from allozyme analysis. *Mar. Freshwater Res.*, **46**, 1021–1032 (1995).
19) M. Takagi, E. Shoji, and N. Taniguchi: Microsatellite DNA polymorphism to reveal geographical divergence in ayu, *Plecoglossus altivelis*. *Fisheries Sci.*, **65**, 507–512 (1999).
20) N. Taniguchi, M. Takagi, and S. Matsumoto: Genetic evaluation of quantitative and qualititative traits of hatchery stocks for aquaculture in red sea bream. *Bull. Natl. Res. Inst. Aquacult.*, **3**, Suppl., 35–41 (1997).
21) M. Takagi, K. Yoshida, and N. Taniguchi: Isolation of microsatellite loci from Japanese flounder *Paralichthys olivaceus* and detection of PCR fragments with simple Non-RI methods. *Fisheries Sci.*, **65**, 486–487 (1999).
22) S. Chow and S. Inoue: Intra- and interspecific restriction fragment length polymorphism in mitochondrial genes of *Thunnus* tuna species. *Bull. Nat. Res. Ins. Far Seas Fish.*, **30**, 207–225 (1993).
23) S. Chow and H. Kishino: Phylogenetic relationships between tuna species of the genus *Thunnus* (Scombridae: Teleostei): Inconsistent implications from morphology, nuclear and mitochondrial genomes. *J. Mol. Evol.*, **41**, 741–748 (1995).