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Direct Evidence for Mendelian Inheritance of the Variations in the Ribosomal Protein Gene Introns in Yellowfin Tuna (*Thunnus albacares*)

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Abstract: Restriction fragment length polymorphism found in the S7 ribosomal protein gene introns of yellowfin tuna (*Thunnus albacares*) was compared between a single pair of parents and their offspring. The sizes of the first intron (*RP1*) and second intron (*RP2*) amplified by polymerase chain reaction were 810 bp and 1400 bp, respectively. The dam and sire had different restriction types from one another in *Hha*I and *Rsa*I digestions for *RP1* and in *Dde*I, *Hha*I, and *Scr*FI digestions for *RP2*. Putative genotypes in both introns of 64 larvae were found to be segregated in Mendelian proportions. Genotype distributions in a wild yellowfin tuna sample (n = 34) were in Hardy-Weinberg proportions, and observed heterozygosity ranged from 0.149 to 0.388. This study presents novel Mendelian markers, which are feasible for tuna population genetic study and pedigree analysis.

Key words: EPIC-PCR, ribosomal protein gene intron, RFLP, yellowfin tuna, Mendelian marker.

INTRODUCTION

Molecular genetic techniques incorporating polymerase chain reaction (PCR) have become conventional and potent tools for fish population genetic studies and pedigree analysis. Homospecific primers are designed for PCR-based assays on nuclear genomes such as microsatellites and anonymous single-copy sequences. Alternatively, an exon-primed intron-crossing PCR (EPIC-PCR) strategy has proven and wide use among genetically divergent taxa (Palumbi and Baker, 1994; Chow and Hazama, 1998; Quattro and Jones, 1999). This analysis uses universal primers designed from conserved exon sequences. One of the major problems with using conserved exon primers is the amplification of multiple PCR products, since the primer sequence may be conserved between duplicated genes, paralogous genes, and sometimes pseudogenes. Amplification of multiple fragments has been observed in several fish species (Chow and Hazama, 1998; Chow and Takeyama, 1998; Quattro and Jones, 1999). Even when a single fragment is amplified, it

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should be tested by subsequent variant analysis to determine whether the amplicon was derived from a single gene locus. Although crossing experiments are the best way to prove the Mendelian inheritance of the variants, it has been almost impossible to conduct them in highly mobile, large pelagic fish such as tunas and billfishes.

Capture and maintenance of a spawning population of yellowfin tuna (*Thunnus albacares*) has been achieved at the Inter-American Tropical Tuna Commission (IATTC) Achotines Laboratory in Los Santos Province, the Republic of Panama, where eggs and larvae were obtained from a single spawning pair. We have analyzed samples from the parents and their hatched larvae to test for Mendelian inheritance of nuclear DNA variants as detected by EPIC-PCR. This is the first direct evidence for Mendelian inheritance of nuclear DNA variations in a highly mobile, large pelagic fish species.

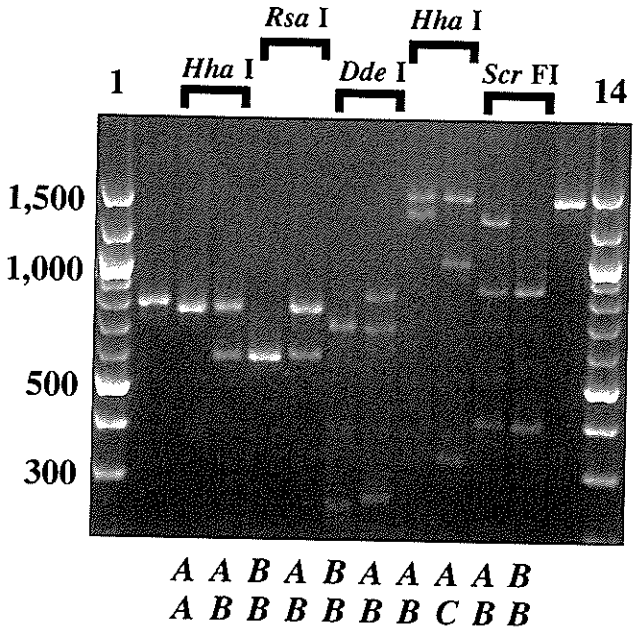

Figure 1. Restriction profiles of *RP1* and *RP2* fragments of the yellowfin tuna parents. Lanes were numbered in order from left to right. Fragment sizes of molecular marker (100-bp DNA ladder, New England BioLabs, Inc.) (lanes 1 and 14) are shown on the left. Lanes 2 to 6 are *RP1* and lanes 7 to 13 are *RP2*. Undigested PCR products were loaded in the 2nd and 13th lanes. Lanes 3 and 4, *Hha*I digestion; lanes 5 and 6, *Rsa*I; lanes 7 and 8, *Dde*I; lanes 9 and 10, *Hha*I; and lanes 11 and 12, *Scr*FI. In each digestion, samples from dam and sire were loaded on the left and right, respectively. Putative genotypes are represented at the bottom.

Materials and Methods

Fish Sample

Young yellowfin tuna of 49 to 60 cm fork length were captured off Achotines, Los Santos Province, Panama, in September and October of 1996. They were transferred alive to Achotines Laboratory, where they were placed in a concrete reserve broodstock tank (8.5-m diameter by 3-m depth, 170,235-L capacity). In April of 1997 the population of 8 yellowfin tuna in the tank began spawning, and they spawned nearly daily.

Mortalities from infections or striking the tank walls reduced the tank population to 4 by mid-September of 1997. On October 10, 1997, two of these individuals were sacrificed in an attempt to leave a single breeding pair in the tank. This attempt was successful, and the single pair spawned on 6 occasions before being sacrificed on October 24, 1997. Eggs spawned by this single breeding pair were collected and transferred to a 300-L hatching tank. The resulting larvae were collected and fixed in a storage buffer (0.25 M EDTA, pH 8.0, 20% dimethylsulfoxide, saturated with NaCl) (Seutin et al., 1991) 3 to 4 hours after hatching. Muscle tissue of the parents, collected when they were sacrificed, was placed in the storage buffer. Tissue samples of wild yellowfin tuna for genetic analysis were taken from the laboratory collection of the National Research Institute of Far Seas Fisheries, Japan.

Genetic Analysis

The conventional phenol-chloroform method was used to extract crude DNA from all specimens. To avoid contamination, a body part of each larva posterior to the anus was dissected and subjected to DNA extraction. Universal PCR primers to amplify the first intron (*RP1*) and second intron (*RP2*) of the S7 ribosomal protein gene in fish were designed by Chow and Hazama (1998). The primer sequences to amplify the first intron (*RP1*) were 5′-TGGCCTCTTCCTTGGCCGTC-3′ (S7RPEX1F) and 5′-AACTCGTCTGGCTTTTCGCC-3′ (S7RPEX2R), and those for the second intron (*RP2*) were 5′-AGCGCCAAAATAGTGAAGCC-3′ (S7RPEX2F) and 5′-GCCTTCAGGTCAGAGTTCAT-3′ (S7RPEX3R). The PCR reaction mixture contained 0.25 U *Taq* DNA polymerase (Perkin-Elmer Cetus, Norfolk, Va.), 0.2 mM of each dNTP, 1.0 µl of manufacturer-supplied 10 × buffer, 2 mM $MgCl_2$, 50 ng of each primer, and 10 to 50 ng of template DNA, in a final volume of 10 µl. Amplification was carried out with an initial denaturation at 95°C for 1 minute, followed by 30

Table 1. Genotype of Yellowfin Tuna Parents and Genotype Frequencies in Their Offspring at *RP1* and *RP2* Intron Loci

		Genotypes of parents		Genotype frequencies of the offsprings*					
Loci	Enzymes	Dam	Sire	*AA*	*AB*	*BB*	*AC*	*BC*	χ^2
RP1	*Hha*I	*AA*	*AB*	37 (32)	27 (32)				1.563†
	*Rsa*I	*BB*	*AB*		27 (32)	37 (32)			1.563†
RP2	*Dde*I	*BB*	*AB*		37 (32)	27 (32)			1.563†
	*Hha*I	*AB*	*AC*	15 (16)	12 (16)		21 (16)	16 (16)	2.625†
	*Scr*FI	*AB*	*BB*		28 (32)	36 (32)			1.000†

*Expected number of genotype is in parentheses.
†Not significant.

cycles of amplification (denaturation at 95°C for 0.5 minute, annealing at 62°C for 1 minute, and extension at 72°C for 2 minutes, with a final extension at 72°C for 10 minutes). A battery of 9 restriction endonucleases was applied to both introns: *Bst*UI, *Dde*I, *Hae*III, *Hha*I, *Nla*III, *Nla*IV, *Rsa*I, *Scr*FI, and *Taq*I (New England Biolabs, Inc.). PCR products and those digested by endonuclease were subjected to electrophoresis in a 2.5% agarose gel (Biogel, BIO101 Inc.) in TBE buffer (50 mM Tris, 1 mM EDTA; and 48.5 mM boric acid.

Results

Strong amplification of a single fragment was observed in each set of primers used, and no apparent size difference in the amplified fragments was observed between female (dam) and male (sire) parents. The estimated sizes of these fragments from the two putative loci *RP1* and *RP2* were about 810 bp and 1400 bp, respectively. The dam and sire had different restriction profiles from one another in *Hha*I and *Rsa*I digestions for *RP1* and in *Dde*I, *Hha*I, and *Scr*FI digestions for *RP2* (Figure 1). Scorable restriction patterns were obtained in all 64 offspring used, and sums of the restriction fragments in each individual were not greater than the doubled size of undigested PCR products.

Genotypes in *RP1* of the dam and sire were respectively designated as follows: *AA* and *AB* in *Hha*I digestion; *BB* and *AB* in *Rsa*I digestion. Those in *RP2* were *BB* and *AB* in *Dde*I digestion; *AB* and *AC* in *Hha*I digestion; and *AB* and *BB* in *Scr*FI digestion. The genotypes of the parents and the frequencies in the offspring sample detected by these restriction enzymes are shown in Table 1. In all restriction digestions for both loci, no significant deviations were found between the observed number of genotypes and Mendelian expectations ($P > .1$). In *RP1*, the genotypes of dam were $HhaI^{AA}$ and $RsaI^{BB}$ (designated $HhaI^{AA}/RsaI^{BB}$), and those of sire were $HhaI^{AB}$ and $RsaI^{AB}$ (designated $HhaI^{AB}/RsaI^{AB}$). These combinations were perfectly maintained in the offspring. Linkage across *RP1* and *RP2* genotypes was observed. All $HhaI^{AA}/RsaI^{BB}$ offspring ($n = 37$) at *RP1* were $DdeI^{AB}$ at *RP2*, and all $HhaI^{AB}/RsaI^{AB}$ offspring ($n = 27$) at *RP1* were $DdeI^{BB}$ at *RP2*, indicating that these two loci were tandemly arrayed as expected.

The restriction endonucleases that detected variation in the parents were applied to a wild yellowfin tuna sample ($n = 34$) collected in 1993 from the Sulu Sea, Philippines. The wild sample was observed to share the same alleles with the larval sample in *RP1*. New allelic variants were observed in all digestions on *RP2*. The restriction profiles by *Hha*I and *Scr*FI digestions on *RP2* indicated another new allele (*D* for *Hha*I and *C* for *Scr*FI) (Figure 2). *Dde*I digestion revealed 13 restriction types among 34 individuals examined (Figure 3). The presence of a large number of alleles is obvious from the highly polymorphic restriction profile, which makes determination of homozygotes and heterozygotes virtually impossible. Genotype and allele frequencies in the wild sample for all endonuclease digestions, except for *Dde*I, are presented in Table 2. Observed heterozygosity ranged from 0.149 in *Scr*FI digestion for *RP2* to 0.388 in *Hha*I digestion for *RP1*, and observed and expected genotypes agreed well with Hardy-Weinberg proportions. Linkage between genotypes at *RP1* observed in the parents and offspring was also apparent in this wild sample. Nineteen individuals were $HhaI^{AA}/RsaI^{BB}$, 11 were $HhaI^{AB}/RsaI^{AB}$, and 2 were $HhaI^{BB}/RsaI^{AA}$. A new combination, $HhaI^{AB}/RsaI^{AA}$, was observed in 2 individuals. This suggests the presence of another allelic variant, in which the restriction sites of both enzymes are lost together.

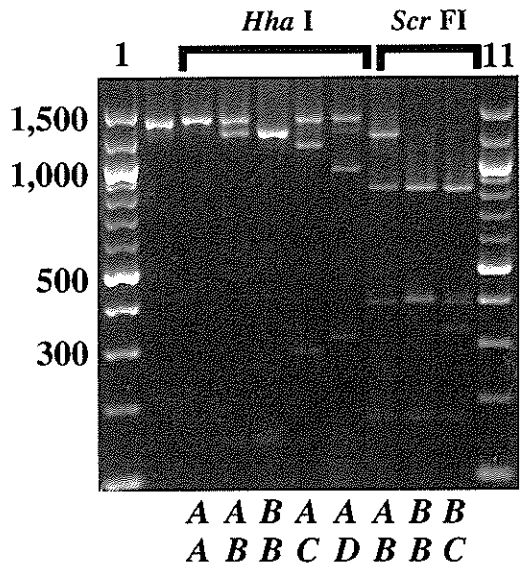

Figure 2. *Hha*I (lanes 3 to 7) and *Scr*FI (8 to 10) restriction profiles of *RP2* observed in wild yellowfin tuna sample. Fragment sizes of molecular marker (100-bp DNA ladder) (lanes 1 and 11) are shown on the left. Undigested fragment was loaded in the second lane. Putative genotypes are represented at the bottom.

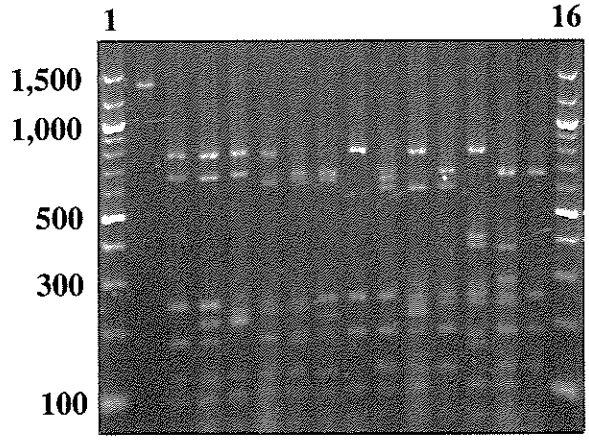

Figure 3. *Dde*I restriction profiles of *RP2* observed in the wild yellowfin tuna sample. Fragment sizes of molecular marker (100-bp DNA ladder) (lanes 1 and 16) are shown on the left. Undigested PCR product was loaded in the second lane. Thirteen types were observed among 34 individuals examined.

Discussion

This study provides the first direct evidence of Mendelian inheritance of nuclear DNA variants in highly mobile, large pelagic fish. Annilo et al. (1998) reported the existence of 10 to 12 members of the S7 ribosomal protein gene family in mice and assumed that most of them were processed pseudogenes. Unexpectedly, primer sets used in this study amplified predominantly single PCR products for *RP1* and *RP2* among distant fish species (Chow and Hazama, 1998). These results suggest that the copy number of the S7 ribosomal protein gene may be much lower in fish than in mammals. Alternatively, priming site sequences in pseudogenes or other copies, if any occur in fish, may have been differentiated enough to avoid multilocus priming.

The RFLP assay on EPIC-PCR may have inherent problems, however. Interpretation of allelic variation becomes very difficult, as shown in the *Dde*I digestion of the wild yellowfin tuna DNA, although genotype variation alone may be informative. It would also be very difficult to detect cryptic alleles that may be sensed by multiple restriction digestions. Thus the RFLP assay on EPIC-PCR may be limited to detecting allelic variants with a single enzyme digestion, providing a relatively low level of polymorphism comparable with allozyme analysis. However, enzyme expression may change ontogenetically as observed in bonefish (Pfeiler et al., 1990), or vary from tissue to tissue, making DNA assay the preferred choice, especially when larvae are used or limited types of tissue are available. The simplicity and cost-effective of the procedure for detecting nDNA

Table 2. Genotype and Allele Frequencies, χ^2 Values for Hardy-Weinberg Proportions and Observed and Expected Heterozygosities at *RP1* and *RP2* Loci in a Wild Yellowfin Tuna Sample

	RP1		*RP2*	
Genotypes	*Hha*I	*Rsa*I	*Hha*I	*Scr*FI
AA	19	4	21	0
AB	13	11	6	3
BB	2	19	1	29
AC	—	—	4	0
AD	—	—	2	—
BC	—	—	0	2
A	0.750	0.279	0.794	0.044
B	0.250	0.721	0.118	0.927
C	—	—	0.059	0.029
D	—	—	0.029	—
χ^2	0.008*	1.381*	1.632*	1.460*
ho	0.388	0.328	0.358	0.149
he	0.381	0.408	0.357	0.140

*Not significant.

variation demonstrated in this study may be suitable for large-scale genetic stock studies and pedigree analysis in the hatchery, especially when analysis of large sets of genotype data is necessary.

Acknowledgments

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