

GENETIC DIVERSITY IN BLUEFIN TUNA (*THUNNUS THYNNUS*) : A PROGRESS REPORT*Ahlquist, J.*¹**SUMMARY**

This paper summarizes the state of molecular genetics research relating to stock structure of bluefin tuna (Scombridae: *Thunnus thynnus*). It augments a presentation by the author at the Bluefin Tuna Otolith Micro-constituents Workshop held at the National Marine Fisheries Service Charleston Laboratory, Charleston, SC, April 30, 1997. It incorporates data presented at a Research Report Meeting, May 5, 1997, of the Cooperative Institute for Fisheries Molecular Biology (FISHTEC) at the University of South Carolina, Columbia, SC, and a Marine Forensics Workshop: Identification of Highly Migratory Species, also held at the Charleston Laboratory, September 15-16, 1997. Because of many of these data are preliminary in nature, they should not be cited without the permission of the individual investigators.

RÉSUMÉ

Ce document récapitule l'état de la recherche génétique moléculaire relative à la structure du stock de thon rouge (Scombridae : *Thunnus thynnus*). Il complète un exposé de l'auteur présenté au Bluefin Tuna Otolith Microconstituents Workshop tenu au National Marine Fisheries Service Charleston Laboratory, à Charleston, SC, le 30 avril 1997. Il inclut des données présentées au Research Report Meeting, du 5 mai 1997, du Cooperative Institute for Fisheries Molecular Biology (FISHTEC) à la University of South Carolina, Columbia, SC, et à un Marine Forensics Workshop : Identification of Highly Migratory Species, également tenu au Charleston Laboratory, les 15-16 septembre 1997. Du fait que nombre de ces données sont de nature préliminaire, ils ne doivent pas être cités sans l'accord des chercheurs.

RESUMEN

Este documento resume el estado de investigación de la genética molecular relacionada con la estructura de stock del atún rojo (Scombridae: *Thunnus thynnus*). Amplia una presentación del autor en Bluefin Tuna Otolith Microconstituents Workshop celebrado en National Marine Fisheries Service Charleston Laboratory, Charleston, SC, el 30 de abril, 1997. Incorpora datos presentados en Research Report Meeting, 5 de mayo, 1997, de Cooperative Institute for Fisheries Molecular Biology (FISHTEC) en la Universidad de South Carolina, Columbia, SC, y Marine Forensics Workshop: Identification of Highly Migratory Species, también celebrado en el Laboratorio de Charleston, 15 y 16 de septiembre de 1997. Dado que muchos de estos datos son por naturaleza preliminares, no deberán ser citados sin autorización de los investigadores.

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I have prepared this paper in a telegraphic style to convey information concisely and, hopefully, clearly. The reader who wishes a lucid account of how modern genetic tools are employed on a variety of problems involving real organisms should consult Avise (1994) and Avise and Hamrick (1996). More technical, but no less readable, information on the strengths and weaknesses of each technique are given in the volume edited by Hillis *et al.* (1996).

I. The species: Bluefin tuna (*Thunnus thynnus*)

- ▶ Largest member of the family Scombridae, found in both Atlantic and Pacific Oceans.
- ▶ Important commercial and recreational fishery, with giant individuals reaching values ex vessel of tens of thousands of dollars.
- ▶ High value has led to overexploitation, depleted stocks, and concern for the future of the species.
- ▶ Management of tunas is a major international problem, involving the governments and fisheries of over a half dozen principal countries.

II. The problems

- ▶ How can we identify the samples going into the main bluefin tuna study? Rapid, unambiguous means of species identification, especially larvae, is essential.
- ▶ How can we provide for proper management without first determining stock structure?
- ▶ Genetic history of populations. What has happened to the bluefin tunas which formerly spawned near Norway, off the Brazilian coast, and in the Black Sea?
- ▶ What is the phylogeographic structure of the bluefin tuna? How does its spatial distribution relate to historical biogeography?

Specifically, FISHTEC (The Cooperative Institute for Fisheries Molecular Biology) convened a workshop on "Genetics of highly migratory oceanic pelagic fishes: Bluefin tuna" on 20-22 July 1994 to assess our knowledge of the stock structure of the species and to propose specific goals to be addressed by molecular genetic methods. See Dean and Woodley (1994) for further details.

The collaborators at this workshop identified three areas of critical importance and posed them as sets of null and alternate hypotheses, which follow.

1. The null hypothesis (H_0):
There are no genetic differences between those fish spawned in the Gulf of Mexico and those spawned in the Mediterranean.
versus the alternate hypothesis (H_a):
There are genetic differences between the fish spawned in the Gulf of Mexico and those spawned in the Mediterranean.
2. The null hypothesis (H_0):
Genetic variation of a year class does not change over time,
versus the alternative hypothesis (H_a):
Genetic variation of a year class does change over time.
3. The null hypothesis (H_0):
There is no genetic variation among year classes,
versus the alternative hypothesis (H_a):
There is genetic variation among year classes.

III. The Molecules

- ▶ Mitochondrial DNA (mtDNA) - the small amount of genetic material associated with the mitochondrion, one of the cellular organelles. In vertebrates the mitochondrial genome is circular and contains about 16000 base pairs. Mitochondrial DNA is maternally inherited and does not undergo recombination. The mitochondrial DNAs of five teleosts have been completely sequenced: the carp (*Cyprinus carpio*), cod (*Gadus morhua*), rainbow trout

(*Oncorhynchus mykiss*), a lungfish (*Protopterus dolloi*) and *Crossostoma lacustre*, a species of river loach (Balitoridae [Homalopteridae *auctorum*]). Although mtDNA on the average evolves several times faster than the nuclear genome, thus making it ideal for population studies, there are conserved regions which permit the investigation of ancient divergences. For example, the mtDNA sequence of the coelacanth (*Latimeria*) has been compared to those of lungfish (Dipnoi) by Axel Meyer and Rafael Zardoya (see Roush, 1997) and various tetrapods. The results seem to suggest that the lungfish are closer to the lineage leading to tetrapods than is the coelacanth, *contra* conventional thinking.

- ▶ Nuclear DNA - the main body of genetic material, containing in fish over 2 billion nucleotide pairs. Several portions of the nuclear genome are important to investigators.
 - Nuclear coding genes. These are genes which produce a protein or enzyme.
 - Introns of well characterized nuclear genes (see diagram and discussion below).
 - Microsatellites (a new and powerful technique, currently receiving considerable attention (see diagram and discussion below).
 - Anonymous nuclear genes, for which one may not know a function (see discussion below).

IV. The Tools

- ▶ PCR (polymerase chain reaction - produces many copies of a given DNA segment)
- ▶ Cloning (does the same thing as PCR except that one has insert the copies of target DNAs ligated in to plasmids (or viral or phage vectors) and grow them in colonies of bacteria)
- ▶ Restriction enzymes - bacterial endonucleases which cleave the DNA molecule at specific locations (usually 4-6 bases) producing a pattern of different sized bands known as a restriction fragment length polymorphism (RFLP)
- ▶ Electrophoresis (the process by which proteins or nucleic acids are separated by charge and molecular size under the influence of an electric current).
- ▶ DNA sequencing (the determination of the actual sequence of nucleotides - adenine, guanine, cytosine, or thymine - of a gene or a portion thereof)

V. Status and progress of work

A. NOAA Fisheries, Charleston Lab:

Cheryl Woodley, Laura Webster, John Bemiss, Marty Ball, Jon Ahlquist

- ▶ Serves as a "clearinghouse" for the acquisition of material from collectors in the field, a repository for storage of samples, and distribution of samples to various investigators.
- ▶ The main research task is the identification of tuna species and other scombrids (as "outgroups") for management and law enforcement purposes.
- ▶ RFLP patterns (see example below) from cytochrome *b* region of mtDNA of five species are diagnostic, but polymorphism within species complicates analysis.
- ▶ Sequence data are needed to verify RFLP information. We will compare the RFLP fragment lengths from actual sequences to see how well they match with sequences determined by electrophoresis. Current data suggest that the lengths as determined from gels are longer than those determined by sequencing, but this does not compromise the use of existing patterns for the purposes of identification.
- ▶ Five of seven species of the genus *Thunnus* are currently represented. Material from all tuna species needs to be collected before database is unequivocal.

Restriction Fragment Length Polymorphisms (RFLP)

Bacterial endonucleases cut the DNA strand every time they recognize their specific sequence. In the example below the bases in a strand of DNA are represented by (^), and the endonuclease specific sequence by ACGT.

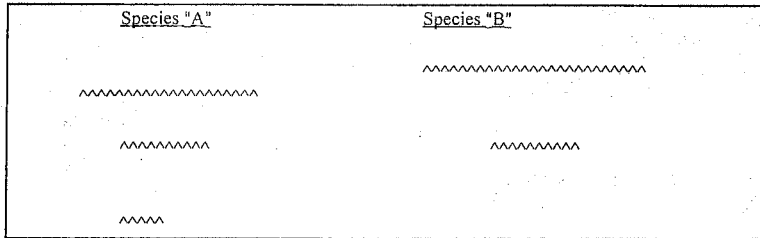
In Species "A" there are four restriction sites which produce three fragments of different length. These are resolved by electrophoresis in the diagram below. In Species "B", however, a

mutation has occurred at one of the sites, as indicated by the arrow. The restriction enzyme no longer recognizes this site, so it makes only two fragments.

Species "A" -ACGT~~~~~ACGT~~~~~ACGT~~~~~ACGT-
 Species "B" -ACGT~~~~~ACGT~~~~~AGGT~~~~~ACGT-



Separate by electrophoresis; smaller fragments move farther in the gel.



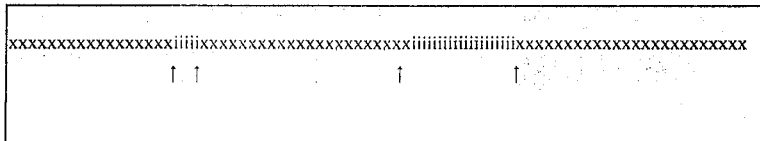
B. Virginia Institute of Marine Science, Gloucester Point, VA:
 John E. Graves and Kimberly S. Reece

1. Variation in the introns of the actin gene.

Introns occur in the genomes of all eukaryotic organisms.

- ▶ They can be thought of as an intervening (or spacer) sequence of a functional gene.
- ▶ They occur between exons, which are transcribed to form the functional part of a gene
- ▶ Since the introns are spliced out (removed) before a protein is manufactured by a cell, they appear to be non-functional and hence have high mutation rates (changes occur randomly in accordance with genetic drift and thus accumulate monotonically with respect to time).

Think of a typical gene as containing exons (x) and introns (I), also indicated by arrows.



Reece and Graves (unpublished) have found two introns within the actin gene which show variation in restriction sites of the nature that could lead to usable data statistically. One of these is the actin gene 1 (long intron) which has a length of about 1200 base pairs (bp); the other is called the actin gene 2 short intron, and is about 600 bp in length.

2. Anonymous loci

Graves and Reece have also isolated several single copy anonymous loci from the nuclear gene. Anonymous loci are just that - we don't know what they do. The researcher uses a pair of randomly constructed primers to amplify a piece of the genome. Using primers for marlin, Graves and co-workers have amplified two of five anonymous loci in tunas and are examining these for variation.

C. Texas A&M University, College Station, TX:
 John R. Gold and Richard E. Broughton

Broughton and Gold (unpublished) thus far have identified and characterized six microsatellite loci, five of which are variable in bluefin tuna. Microsatellites (or "micros") are short nucleotide

sequences that are tandemly repeated in the genome. They may be as simple as two nucleotides, as the following example shows:

Stock "A" -~~~~~GAGAGAGAGA~~~~~

Stock "B" -~~~~~GAGAGAGAGAGAGAGAGA~~~~~

In Stock "A" the dinucleotide (GA) is repeated 5 times, in Stock "B" it is repeated 10 times. These products may be separated electrophoretically on gels according to size, giving the profiles shown diagrammatically below.

Stock "A" (mother)	Stock "B" (father)	Offspring
	GAGAGAGAGAGAGAGAGA	gagagagagagagagaga
GAGAGAGAGA		gagagagaga

Why do microsatellites work well?

- ▶ They are present throughout the genome of vertebrates such as teleost fishes.
- ▶ There may be literally thousands of microsatellite loci available for study.
- ▶ They are highly variable, hence work well for discrimination among populations.
- ▶ They are inherited in a Mendelian fashion.
- ▶ With some work and a little luck, one can find loci which exhibit variation at a level that will enable one to answer the question at hand.

Gold and Broughton also have identified 15 additional microsatellite loci from bluefin tuna which they are in the process of characterizing for variation.

D. Baruch Institute, University of South Carolina:
 Joseph M. Quattro and colleagues

Dr. Quattro (unpublished data) has shown that a non-coding portion of the gene which codes for the enzyme lactate dehydrogenase (LDH VI - LDH6) harbors sufficient variation to pursue population level questions. This intron has been digested with the restriction enzymes *Ase I* and *Mwo I* which differentiate the three LDH6 alleles.

Two additional nuclear intron loci have been developed. They are the fifth intron of the creatine kinase locus (CK5) and the third intron of the aldolase-B locus (ALDB3). Although these loci have shown variation among a variety of teleost fishes, they have yet to be tested in bluefin tuna.

E. Department of Biological Sciences, University of South Carolina:
 Bert Ely, Jaime Alvarado Bremer, and colleagues

Dr. Ely's group (Alvarado Bremer et al., 1997) has sequenced approximately 400 base pairs from the mtDNA control region from all eight species of the genus *Thunnus*. They have also sequenced 371 bases of the D-loop region of the mitochondrial genome for the bluefin project (Ely, unpublished). Of 43 individuals, which either amplified or had reliable patterns, all were unique. Thus at face value, the D-loop contains too much variation to be useful. It was found, however, that the sequence information provided details for the use of restriction enzymes. One lineage was characterized by a unique *Afl III* site and another by an *Apo I* site.

Ely (personal communication, Marine Forensics Workshop: Identification of Highly Migratory Species, NOAA Fisheries Charleston Laboratory, September 15-16, 1997) found that by sequencing only a single nucleotide (for example thymine, T), unique patterns for the identification of tunas could be obtained, thus achieving a system as cost effective as one

requiring the use of two or three RFLP markers.

Ely's group also discovered other useful nuclear DNA markers. Two are anonymous genes (BFT 3-20 and BFT 3-42), each of which contain two alleles; a third is a portion of a translation elongation factor gene (ef-1), which had a variable *Hin* fl site.

VI. Summary of accomplishments to date

- ▶ A minimum of 12 loci from nuclear and mitochondrial genes have the *potential* of yielding data suitable for addressing the bluefin tuna problem.
- ▶ An additional 21 loci *may* provide such information.
- ▶ Other genetic markers may be discovered serendipitously.
- ▶ No single locus has so far proved to be the "magic bullet" which will unambiguously identify a bluefin tuna spawned in the Gulf of Mexico from one spawned in the Mediterranean, but the aggregate of data *should* provide a reliable answer.

VII. What needs to be done?

- ▶ Continue to characterize the loci which have been identified as variable.
- ▶ Compare samples from juvenile tuna known to have been spawned in the Gulf of Mexico with those from juvenile tuna known to have been spawned in the Mediterranean Sea. Part of this work will be done from preserved specimens of larval bluefin tuna from the Gulf of Mexico because it is unlikely that we will procure enough fresh specimens in the wild.
- ▶ Carry out the work on age classes as indicated in hypotheses (2) and (3) at the outset. At the outset, approximately 125 fish from each year class (1996, 1995, and 1994) will be targeted from Delmarva (Delaware, Maryland, and Virginia) and 65 fish from each year class from New Jersey to Massachusetts. This does not as yet solve the problem of procuring specimens of these age classes from the Mediterranean, nor does it fully negate the possibility that some of these individuals may have migrated westward across the Atlantic from the Mediterranean. We anticipate that this sampling scheme will not compromise the data, either for the DNA or otolith work. First, it is unlikely that these young fish will have crossed the Atlantic. Second, tagging studies seem to indicate that the predominant movement, when it occurs, is west to east.
- ▶ If all the above are successful, the problem remains of how to deal with a situation of potentially mixed stocks of large adults occurring in the mid-Atlantic.
- ▶ The difficulties of allocating mixed stocks among various national interests will continue to perplex fisheries managers and policy makers.

VIII. References

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