GENETIC VARIATION IN ATLANTIC YELLOWFIN TUNA (*Thunnus albacares*) TO ASSESS STOCK STRUCTURE AND REPRODUCTIVE VARIANCE

A Thesis

by

TIFFANY TALLEY FARNHAM

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

December 2003

Major Subject: Wildlife and Fisheries Sciences

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Approved as to style and content by:

Jaime Alvarado-Bremer (Chair of Committee)	Jay Rooker (Member)
Ayal Anis (Member)	John Gold (Member)
Robert Brown (Head of Department)	

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ABSTRACT

Genetic Variation in Atlantic Yellowfin Tuna (*Thunnus albacares*) to Assess Stock Structure and Reproductive Variance. (December 2003)

Tiffany Talley Farnham, B.S., Williams College

Chair of Advisory Committee: Dr. Jaime Alvarado-Bremer

The population genetic structure of Atlantic yellowfin tuna (*Thunnus albacares*) has received little attention despite the substantial fishing mortality of juveniles caused by purse seining around fish aggregating devices in the Gulf of Guinea targeting multi-species schools that also include similarly sized skipjack tuna (*Katsuwonus pelamis*) and bigeye tuna (*T. obesus*). We used sequence data from 355 bp of the mitochondrial control region I as well as six microsatellite loci to examine: (1) population structure, and (2) to look for evidence of reproductive variance. We analyzed two samples of adults from the Gulf of Mexico (GOM) and one sample of early juveniles (20-50 mm) from the Gulf of Guinea (GOG). We found no evidence of geographic or temporal differentiation among the samples. Accordingly, the null hypothesis of panmixia for yellowfin tuna in the Atlantic Ocean could not be rejected. A sudden expansion analysis based on mtDNA control region I sequence data of yellowfin tuna was highly significant. Time estimates for expansion were between 40,000 and 80,000 years before present. The associated high levels of homoplasy could be masking any existing population structure. Additional sampling from additional locations and across several years will be needed to test the hypothesis of panmixia. We also provide preliminary evidence of the Allendorf-Phelps effect, which may contribute to reproductive variance. This is the first evidence of this effect in any other tuna or pelagic species. Data indicates that early juveniles sharing the same mtDNA control region I haplotype were caught in the same tow and had a significant probability of half-

sibship status as calculated from their haplotype and genotype at one microsatellite locus through kinship analysis. Sampling throughout the spawning season and across several years, as well as analysis with additional microsatellite loci that have a more even distribution of alleles, will be needed to more fully identify the sibling status of larvae and early juveniles caught in the same tow as well as the extent of this reproductive variance.

This thesis is dedicated to my husband, Tim, who now appreciates being a thesis widow. It may have been a rough road getting here, but your love and support have made the end that much sweeter (let's go Av's!). Here's also to Big T and Double D, the best listeners and cuddle buddies there ever were.

ACKNOWLEDGMENTS

First and foremost, I thank my advisor Dr. Jaime Alvarado for convincing me to stick with it even when I felt that one more Friday trip from College Station, to Galveston, to Houston for the weekend, was going to be the end of my sanity at the very least. I'm almost convinced it was worth it. Thanks to our post-doc, Dr. Jordi Vinas, for helping with so much of the analysis and always being ready with an explanation as to why/how/what, and former lab member Holly Stephens for paving the way. I would also like to thank the members of my committee, Dr. John Gold, Dr. Jay Rooker, and Dr. Ayal Anis for their thoughtful comments and suggestions that have greatly improved this thesis. Thanks must also be extended to Bernard Stequert, various members of Jay Rooker's lab, Dr. Richard Shaw at Louisiana State University, and SEAMAP for providing me with all of my samples.

Most importantly, however, I thank the friends and family who have supported me along the way, whether your interest in the genetics of yellowfin tuna was feigned or real, you were all great listeners. Most notably, I thank my husband Tim who cooked, cleaned, and took such good care of me while I was writing this thesis that I considered stringing it out as long as possible; my parents, Dick and Sonnie Talley, who have been there from the beginning with nothing but love and support for whatever it is I have chosen to do; my LITTLE brother Rick for being such a wonderful, thoughtful, caring person; Sara Black for sharing my pain over the last couple of months, misery certainly does love company; Andrea Gilkinson, my co-president; Tory Nicholson; and to all the others who know who they are, how lucky can one girl be?

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CHAPTER I

INTRODUCTION

The genetic population structure of yellowfin tuna (*Thunnus albacares*) from the Atlantic Ocean has received little attention. The majority of studies on this species have examined samples from the Pacific Ocean, where approximately 70% of the yellowfin tuna harvest is obtained (FAO 1994). Garcia (1994) reviewed the principal commercially targeted tuna species and found that 14 of 20 global tuna stocks recognized by FAO Fisheries, and including Atlantic yellowfin tuna, were overfished or depleted, emphasizing the need for investigation in this basin as well. This need is particularly relevant in light of evidence indicating fishing technologies in the past 20 years have altered schooling behaviors, and therefore vulnerability, of juvenile yellowfin tuna. Fonteneau et al. (2000), in a 1981-2 survey in the Gulf of Guinea, reported that the majority of juvenile yellowfin tuna were found in open water in mixed-species schools with skipjack (*Katsuwonus pelamis*) and small bigeye (*T. obesus*) tuna. By 1999, however, all of the mixed species schools of small tuna were found under artificial fish aggregating devices (FADs) used to target skipjack and other bait species; the remaining unassociated schools were primarily monospecific. Use of FADs has become widespread in the Gulf of Guinea since 1991, accounting for up to 75% of the present day tuna catch in that area (Menard et al. 2000). Accordingly, yellowfin tuna in the Atlantic are experiencing not only increasing fishing pressure on adults but dramatic increases in pressure on the younger individuals as well. These actions threaten sustainability of the fishery as well as the genetic diversity of the population.

This thesis follows the style and format of Conservation Biology.

Yellowfin Tuna Life History and Management

Yellowfin tuna is a cosmopolitan species, occurring in most tropical and subtropical waters between 45° N and 40° S in the Atlantic (Collette and Nauen 1983), and spawning primarily in the Gulf of Guinea (ICCAT 2002). Spawning also occurs in the Gulf of Mexico and in the southeastern Caribbean off Venezuela (Richards et al. 1990, Lang et al. 1994, Arocha et al. 2000) although the relative contribution of each of these spawning areas to the population is unknown. The size and spawning frequency of females differs significantly between the Gulf of Mexico and the southeast Caribbean, suggesting two distinctive spawning groups. Females in the Gulf of Mexico spawn on average every 3.18 days from May to August, whereas the females off Venezuela are larger and spawn on average every 1.47 days from July to September (Arocha et al. 2001). Little information is available on the spawning frequency in the Gulf of Guinea, but it is hypothesized to be similar to that in the southeastern Caribbean (ICCAT 2002)

Spawning of yellowfin tuna is believed to occur year-round between 15° N and 15° S in offshore waters. In the U.S., spawning has been reported during the summer months from the 200 m isobath out to the Exclusive Economic Zone (EEZ) boundary (NMFS 1999). Larvae seem to be limited to temperatures above 24° C and salinities greater than 33 parts per thousand (ppt) in the Gulf of Guinea (Richards and Simmons 1971), though the highest growth rates in the Gulf of Mexico in the vicinity of the Mississippi River discharge plume were reported to be near 31 ppt and 29° C (Lang et al. 1994). Both juveniles/subadults (<110 cm fork length [FL]) and adults (>110 cm FL, 3 years of age) are found primarily in pelagic waters from the surface to 100 m deep and between 18° C and 31° C; juveniles are generally found closer to shore (NMFS 1999). As the fish grow, they move to deeper water and extend their range into higher latitudes.

Based almost exclusively on a limited number of trans-Atlantic tag recoveries, yellowfin tuna are currently managed as a single, panmictic stock in the Atlantic (NMFS 2001, ICCAT

2002). The U.S. Cooperative Tagging Center (CTC) tagged 9,220 yellowfin tuna between 1956 and 1998, with the majority of fish tagged in the Gulf of Mexico and along the east coast of the United States. There were 364 documented recoveries with about 50 recovered in the Gulf of Guinea (Oritz 2001). The majority of the recovered fish were tagged in the northeast Atlantic. Interestingly, tagging studies in the Pacific suggest that yellowfin tuna migrate on the scale of hundreds of kilometers (Bayliff 1979). In the Atlantic, yellowfin tuna are believed to leave the Gulf of Guinea when they reach around 65-75 cm FL and head toward the northwestern Atlantic, returning later to spawn. The large proportion of intermediate size yellowfin tuna caught in surface fisheries off Cape Verde, with a similar size distribution to the surface fishery in the western Atlantic, has been interpreted to suggest that the Cape Verde Islands serve as one of the stages in the east-west migration (Hallier and Vieira 1996).

In spite of such high migratory behavior, there is preliminary evidence of population subdivision within the Atlantic. In June 2000, a concentration of yellowfin tuna, with a large proportion of females greater than 70 kg, was harvested in the inner Gulf of Guinea (Bard and Devo 2001). These individuals could be part of a sedentary sub-population that spawns close to the northern coast of the Gulf of Guinea. Arocha et al. (2001) reported capture of spawning females near the equator in the western Atlantic in March of 2000. These individuals were not included in their analysis of the northwestern Atlantic spawning season because of the discrete geographical separation, but could be a separate spawning area that contributes to the fishery off Brazil's coast. In addition, the likely lack of exchange between the Gulf of Mexico spawning group and those spawning in the southeastern Caribbean, with those spawning in the Gulf of Mexico similar in size to the fish that leave the Gulf of Guinea, could indicate another sub-population off of Venezuela (Arocha et al. 2001). It should be noted, however, that while sub-populations may exist, migration and gene flow among these and the main population could

occur at levels sufficient (in the order of a few individuals per generation) to genetically homogenize the basin into a single population (Waples 1998). Genetic analyses may help to resolve this and other issues, including reproductive variance, as it has been done in other species (e.g. cod, *Gadus morhua*, Ruzzante et al. 1996; whiting, *Merlangius merlangus*, Rico et al. 1997; eulachon, *Thaleichthys pacificus*, McLean and Taylor 2001; and albacore tuna, *Thunnus alalunga*, Vinas et al. 2003).

The remainder of this thesis is organized into four additional chapters. Each of the Chapters, II through IV, is written as a stand-alone paper that, with some modification, will be submitted for publication to a peer-reviewed journal. Chapter II explores a variety of DNA extraction methods and three mitochondrial loci for the purpose of forensic identification of larvae of a variety of species. The larvae and early juveniles identified as yellowfin tuna, in addition to two adult samples, were used to test the hypothesis of a single Atlantic stock in Chapter III. Both a segment of mitochondrial DNA (mtDNA) and six microsatellite loci were used for this purpose. A combined analysis of the mtDNA and microsatellite data to verify whether the associations between maternal lineage and sampling (juveniles caught in the same tow and sharing the same haplotype) could be the result of reproductive variance is given in Chapter IV. Finally, Chapter V summarizes the conclusions of this thesis.

CHAPTER II

FORENSIC IDENTIFICATION OF SCOMBRID LARVAE

Introduction

Larval specimens of genus *Thunnus*, less than 7 mm standard length (SL), are notoriously difficult to identify based on morphological and meristic characters (Lang et al. 1994, Richards et al. 1990, Graves et al. 1988). Larvae of yellowfin (*Thunnus albacares*) and bigeye (*T. obesus*) are the most difficult to differentiate and there is no reliable method to distinguish between them. For example, Richards et al. (1990) purportedly identified bigeye tuna larvae in trawls from the Gulf of Mexico but later retracted this, in light of evidence that Atlantic bigeye tuna spawn only in the eastern equatorial regions (Richards pers. comm. *in* Alvarado Bremer et al. 1998). Analysis of pigment patterns and osteological characteristics can lead to reliable identification of larvae, but not in every case as many diagnostic characters for species identification are not always present in all specimens (Richards et al. 1990). In addition, as tuna larvae grow, juvenile pigment patterns can begin to obscure diagnostic larval patterns, making nearly impossible species identification of early juveniles between 15 and 60 mm SL (Graves et al. 1988).

Unambiguous identification of adult tuna specimens also can be difficult in certain instances. Tissue samples used for a variety of analyses are often obtained from carcasses or dressed fish and these can be easily misidentified in the absence of diagnostic morphological characters. This kind of tissue is often provided by on-board observers, port agents, and commercial and/or recreational fishermen. In contrast, an entire fish is not as likely to be misidentified by an expert, except for black market fishing (Bartlett and Davidson 1991). However, labels can be removed, writing can fade, records can be misplaced, or the wrong name

can simply be written on the specimen. Finally, mislabeling also can occur in the canning industry where a variety of fish species might be processed yet labeled as a single species.

Proper identification of specimens can help elucidate schooling patterns and behavior of young tunas, in addition to providing information on the reproductive biology and species distribution (Takeyama et al. 2001), through the collection of temporally and spatially discrete samples consisting of multiple species uniform in size. For instance, adult yellowfin tuna (>110cm) are found primarily in monospecific schools assorted by size, whereas juvenile yellowfin tuna (averaging 46 cm; Menard et al. 2000) school with bigeye tuna and skipjack tuna (*Katsuwonus pelamis*) of similar size (Fonteneau et al. 2000). Larval tuna are believed to associate with members of the same spawning event, given their exposure to similar oceanographic conditions and currents (NMFS 1999). However, there is no evidence of the actual ontogenetic timing when mixed species-schooling behavior begins.

Population genetic studies also require proper identification of samples since misidentification can significantly alter results by introducing new alleles or skewing allele frequencies (Peter Grewe, pers. comm.). Certain population characteristics, such as the levels of reproductive variance, may be inferred from the genetic analysis of larval data and such information can help improve the management of the commercially important tuna species. However, for these data to be useful, explicit species identification is necessary.

Recently, molecular genetic techniques have enabled scientists to unambiguously confirm the identity of fresh, frozen, and alcohol-fixed tuna specimens. Unambiguous specific identification of larval tunas less than about 5 mm, and those where larval and juvenile pigment patterns are confounded (those between about 15 mm and 60 mm), is only possible using genetic techniques. Many molecular techniques have been utilized for this purpose, including allozymes, restriction fragment length polymorphisms (RFLPs), and direct sequencing (Bartlett

and Davidson 1991, Alvarado Bremer et al. 1998, Quinteiro et al. 1998, Takeyama et al. 2001). However, not all of these genetic techniques can differentiate among species of *Thunnus*. In addition, canned tuna and specimens preserved in formalin have proven difficult to identify genetically because of the low quality and/or low-quantity of DNA extracted from these samples, using standard extraction methods. Further, heat treatment used in canning results in denaturation of muscle proteins as well as degradation and fragmentation of DNA (Quinteiro et al. 1998, Terol et al. 2002); whereas formalin appears to inhibit proteolysis, with DNA remaining associated with protein complexes and precipitating out during standard extraction procedures (Shedlock et al. 1997). The success of early methods of DNA extraction from formalin-fixed specimens was highly variable, with one study noting a "collection effect" that was most likely the result of variability in fixation conditions and the handling of tissue afterward (France and Kocher 1996). Shedlock et al. (1997) reported an isolation protocol that increased the quality and quantity of DNA extracted from formalin-fixed tissue and led to an 82% PCR success rate for two fragments over 450 bp (base pairs) long. Amplification of fragments of this size was not considered previously possible. While DNA extraction from formalin-fixed tissue has improved, many of these samples, as well as canned specimens and those preserved in contaminated or low-quality solutions, remain problematic.

Quinteiro et al. (1998) and Terol et al. (2002) utilized primers targeting short fragments, 126 bp and 171 bp, respectively, of the mitochondrial cytochrome *b* gene to identify tuna species from canned samples. However, initial results in our lab with a few specimens did not yield conclusive results about species identification when sequences of either fragment were submitted as BLAST (Basic Local Alignment Search Tool) searches to the National Center for Biotechnology Information (NCBI), by returning equal identity scores with several species of *Thunnus*. One aim of this study was to develop a DNA extraction protocol and genetic

technique for formalin-fixed and ethanol preserved larvae and early juveniles that would allow amplification of mtDNA fragments of at least ~450 bp and that also would allow use of microsatellite markers. Data from the mitochondrial control region I, in which high levels of nucleotide diversity have been reported for tunas (Alvarado Bremer et al. 1998), could then be utilized in studies of population genetics in general, and of reproductive variance in particular. Together with microsatellites, these data could serve as a fingerprint for identification of potential siblings.

Methods

Larval and juvenile specimens from the Gulf of Guinea were sorted by Bernard Stequert, who excluded all non-scombrid specimens. All juvenile specimens were captured with a pelagic trawl between 10 and 50 meters depth and between latitudes 2°49' and 2°50' N and longitudes 7°15' and 10°36' W. Larval specimens were captured with a bongo net (300 μ and 1000 μ mesh sizes), an Omori net (1000 μ mesh size), or a hydrobois net (200 μ mesh size) between 10 and 50 meters depth and latitudes 0° and 4°00' N and longitudes 9°30' and 16°00' W. Formalin-fixed specimens were captured around various oil platforms in the Gulf of Mexico during several sampling trips in 1996, 1999, and 2000 with a surf net, bongo net, OL (off light trap, 20 m downstream of the platform), or SL (surface light, within the legs of the platform). These specimens were sorted in Richard Shaw's lab at Louisiana State University (LSU), with only specimens of *Thunnus* shipped to our lab. DNA was extracted from alcohol-preserved (either ethanol or isopropanol) larvae (less than 10 mm) and juveniles (averaging around 30 mm), and from formalin-fixed larvae, using methods described in Appendix A, including Chelex® (Bell and Grassle 1997), phenol-chloroform (Sambrook et al. 1989), a small tissue method (Simpson et al. 1999), and a method for formalin-fixed tissue (Shedlock et al. 1997). The various methods

used for different samples, as well as the success rate of each method based on the proportion of PCR products that could be sequenced are given in Table 2-1.

The majority of larval DNA extracted with the phenol-chloroform method (Sambrook et al. 1989) did not yield any PCR product and were therefore re-extracted with two different methods: one for small tissue amount (Simpson et al. 1999) and one for formalin-fixed tissue (Shedlock et al. 1997). Both methods were followed as described in the original papers (outlined briefly in Appendix A), except that the volumes listed in Shedlock et al. (1997) were reduced by half to adjust for the small tissue sizes. Samples also were resuspended in varying volumes of water, from 10 l to 50 l, depending on the amount of tissue used in extraction. The small tissue protocol of Simpson et al. (1999) was used because numerous larvae were less than 5 mm and the resulting concentration of DNA was expected to be extremely low. The Shedlock method was used because the extra washes and greater amounts of proteinase K, in addition to the use of DTT and RNase, might improve the success rate of PCR amplification, even in those specimens not fixed with formalin, by removing other *Taq* inhibitors.

Table 2-1 Different DNA extraction methods used on preserved larval and juvenile tissue, with their success rates (based on successful sequencing of PCR amplicons). Method 1 is the Chelex® (Bio-Rad) extraction (Bell and Grassle 1997); Method 2 is the phenol-chloroform extraction (Sambrook et al. 1989); Method 3, the small tissue amount extraction from Simpson et al. (1999); and Method 4 is the formalin-fixed tissue extraction from Shedlock et al. (1997). Preservation quality was based on visual examination of the specimens [see text].

Sample Type (preservation method)	Preservation Quality	Extraction Method	N	Amplified Fragment(s)	Success Rate
Larvae (alcohol)	Variable	2	177	CR I	11% (20/177)
			107*	cyt*b*	8% (9/107)
Larvae (alcohol)	Variable	3	70	CR I	16% (11/70)
			48*	cyt*b*	38% (18/48)
Larvae (formalin)	Good	4	45	CR I	42% (19/45)
	Good	4	10	Cyt*b*/CR I	70% (7/10)
Juvenile (alcohol)	Good	1	33	CR I	100% (33/33)
Juvenile (alcohol)	Variable	2	79	CR I	67% (53/79)

*These specimens are most of the 157 (method 2) and 59 (method 3) that failed to amplify the 450 bp mitochondrial control region I (CR I) fragment and were thus re-amplified with the 171 bp cytochrome *b* (cyt*b*) primers.

DNA from formalin-fixed *Thunnus* larvae was extracted using the method outlined in Shedlock et al. (1997) except for the modifications listed above. PCR amplification and sequencing followed methods described in Appendix B. Sequences were submitted directly as BLAST (Basic Local Alignment Search Tool) searches through the NCBI (National Center for Biotechnology Information) website (http://www.ncbi.nlm.nih.gov) and without prior alignment with other known tuna sequences for identification purposes.

Amplification of the mitochondrial control region I (CR I) was performed according to the method in Appendix B. PCR products (amplicons) were visualized by electrophoresis on a 1% agarose gel stained with 0.1 □g/ml ethidium bromide. For those samples where the control region I failed to amplify, another amplification was performed with primers targeting two smaller fragments of the mitochondrial cytochrome *b* (cyt*b*) gene; amplification of a 126 bp fragment was performed according to Quinteiro et al. (1998), and amplification of a 171 bp fragment followed Terol et al. (2002). All amplicons were cleaned and sequenced as outlined in Appendix B. The sequence alignment editor, BioEdit (Hall 1999), was used to edit sequences, which were then aligned using ClustalW (Thompson et al. 1994). Positively identified species were used as references to facilitate alignment. A phylogenetic tree that included sequences from the unknown specimens as well as from positively identified species, which served as references, was constructed in MEGA (Kumar et al. 2001) via neighbor-joining (N-J) and based on Tamura-Nei distances. Positive species identification was validated with a bootstrap test (1000 iterations). Sequences also were submitted as BLAST searches to confirm species identity.

Results

Amplification

The preservation quality of all specimens was very heterogeneous. Some specimens apparently were preserved in high quality, high percentage alcohol, whereas others were preserved in lower quality, low percentage alcohol or contaminated solutions, resulting in a flaccid consistency of the specimens. Other specimens were totally desiccated and discolored from having lost the alcohol during shipment or storage. The Chelex® extraction method, used for several juvenile samples, yielded a 100% amplification success rate (33/33). Of these, 24 were identified as yellowfin tuna, six as frigate mackerel (*Auxis thazard*), two as skipjack tuna (*Katsuwonus pelamis*), and one as little tunny (*Euthynnus alletteratus*), all in excellent preservation condition. The amplification success was lower (67%) when DNA was extracted with the phenol-chloroform method, but was utilized for ill-preserved specimens as this tissue could not be pulverized with the plastic pestle used with the Chelex® method. DNA extracted from muscle tissue worked consistently better than that from eyeball, either amplifying when DNA extracted from the eye failed or yielding a stronger PCR product. Out of five desiccated and brown juvenile specimens, all collected at the same time, stored in the same vial and extracted with the phenol-chloroform method, three failed to amplify either the mtDNA control region I fragment or the 171 bp cytochrome *b* fragment. The control region I fragment did amplify in two of the specimens but the quality and quantity of the amplicon was too poor to sequence. In addition, 17 of the remaining 21 specimens that could not be identified were apparently preserved in a low quality solution as the fish were flaccid, as opposed to stiff when preserved in a high percentage (>70%), high quality ethanol or isopropanol.

DNA extracted from alcohol-preserved larval and juvenile specimens and using the phenol-chloroform method yielded better PCR products than did DNA extracted from juveniles

with the Chelex method. However, the phenol-chloroform method was inconsistent among alcohol-preserved larvae, with only 28 of 183 (14%) specimens having been successfully sequenced at one of the two mtDNA loci, control region I or the 171 bp cyt*b*. Not all of the larval specimens that failed to amplify at the control region I were re-amplified at the cyt*b* region as several were labeled as non-tuna when they were sent. Among those that failed to amplify with either primer pair, it was noticed that 13 individuals in one set were tinted green and 10 individuals of another set were completely desiccated and brown. The DNA appeared degraded in at least 25 other specimens as evidenced by low molecular-weight smears on a gel loaded with total genomic DNA.

The formalin-preserved samples, extracted with the Shedlock et al. (1997) method, had a higher success rate of PCR amplification for both the control region I and the 171 bp cyt*b* fragments (the 126 bp fragment was not used). Close to 65% of the specimens were amplified successfully at the mitochondrial control region I although only 42% specimens produced amplicons of sufficient quality and quantity to determine their DNA sequence. In contrast, 70% of the specimens amplified at the 171 bp cyt*b* region yielded amplicons of sufficient quality to be sequenced. However, as all specimens were supposedly *Thunnus*, and this region is not able to distinguish amongst members of this genus (see below), this fragment was amplified in only 10 of the individuals.

All of the specimens, both larval and juvenile, that were successfully amplified and sequenced at the mitochondrial control region I could be placed, with confidence, within a given species by alignment with known sequences, and assigned statistical confidence with the bootstrap test (Figure 2-1), and/or by submission to NCBI as BLAST searches. The topology shown in Figure 2-1 allows identification of specimens based on their placement in relation to individuals of known species. There were some instances where the bootstrap value was less

than 70, most notably within the yellowfin tuna grouping, but the unknown specimens that were identified based on this phylogeny, including yellowfin tuna, clustered in groups that had well-supported branches. In addition, the unknown specimens that were identified as yellowfin tuna based on this phylogeny (unknowns 9, 10, 7, 6, 8, 5, 4, and 3), were interspersed within the cluster of reference yellowfin tuna sequences.

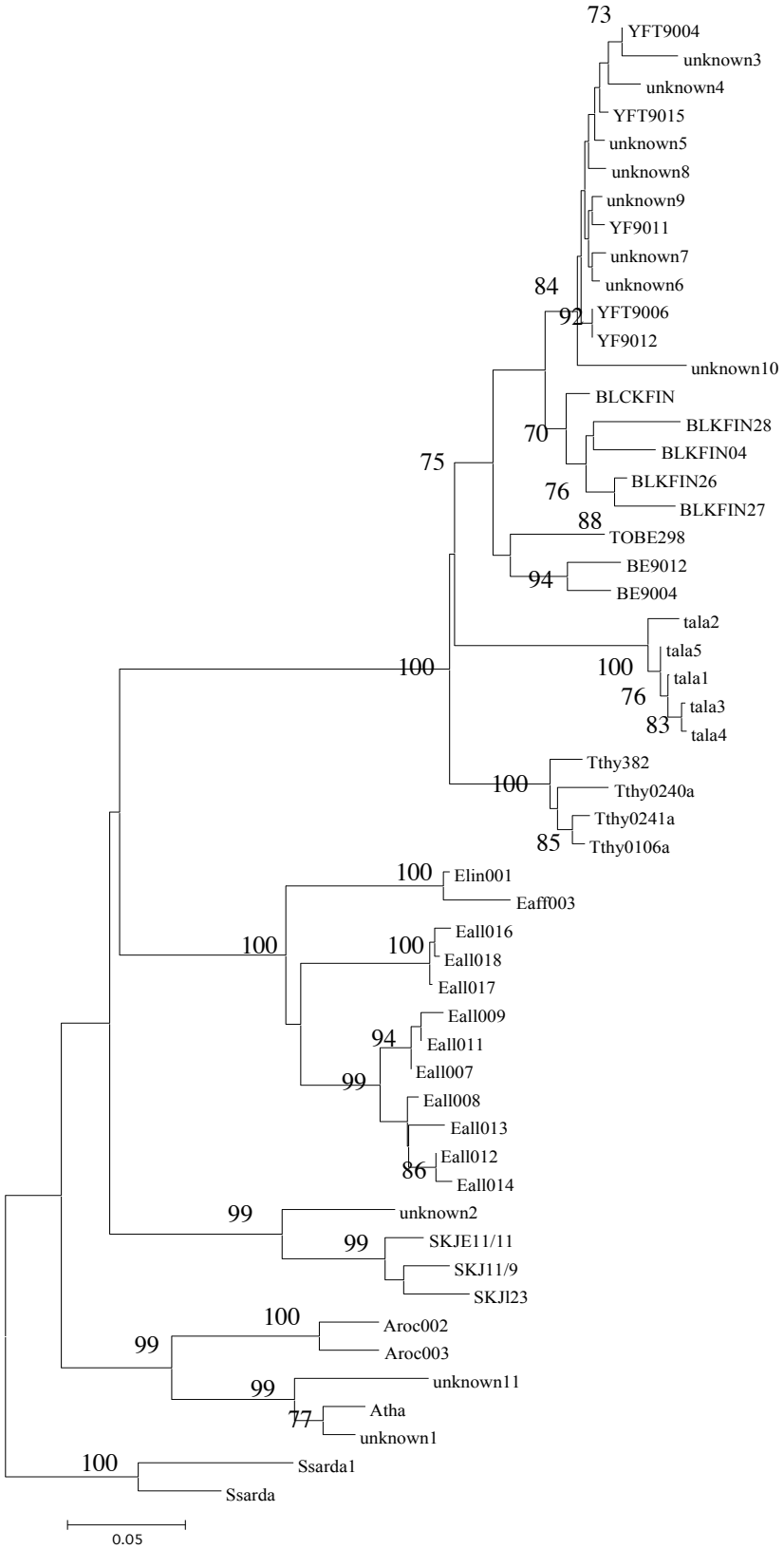
Since the 126 bp fragment of cytochrome *b* did not contain enough polymorphic sites to provide unambiguous identification of most species, this analysis was limited to only a few specimens. The 171 bp cytochrome *b* fragment amplified more readily in the larval samples than did the 450 bp control region I fragment. This cyt*b* fragment could be used to positively identify at least two species, skipjack and bullet tuna (*Auxis rochei*), via BLAST searches (phylogenetic trees were not constructed because of the lack of positively identified reference sequences available from numerous other species) but did not contain enough numbers of shared-derived characters to distinguish among members of genus *Thunnus* (Table 2-2). For instance, several submitted sequences produced a 99% identity match with three different species of *Thunnus*. Therefore, sequencing of this fragment was limited to ruling out non-*Thunnus* samples in order to concentrate efforts only on those likely to be yellowfin tuna larvae. After non-*Thunnus* individuals were excluded, several of the amplification parameters, including primer concentrations (from 5-10 pmol), number of cycles (from 28-40), and annealing temperatures (from 45-65°C), were altered in an attempt to increase the quality and quantity of the control region I PCR product for DNA sequencing with a certain degree (10%) of success.

Table 2-2 Comparison of species identification between phylogenetic analysis and BLAST searches. CR-I is the control region I fragment and cyt*b* is the 171 bp fragment of cytochrome *b*. Values in parentheses are the percent identity obtained from the BLAST search.

Number of specimens (type)	BLAST ID (cyt*b*)	Phylogenetic ID (CR-I)	BLAST ID (CR-I)
49 (juvenile)		*Thunnus albacares*	*Thunnus albacares*
11 (juvenile)		*Auxis thazard*	*Sarda sarda* (92%)
12 (juvenile)		*Auxis thazard*	*Katsuwonus pelamis* (93%)
7 (juvenile)		*Katsuwonus pelamis*	*Katsuwonus pelamis*
4 (larva)*	*Thunnus spp.*		
6 (larva)	*Euthynnus alletteratus*		
3 (larva)		*Euthynnus alletteratus*	*Euthynnus alletteratus*
8 (larva)	*Thunnus spp.*		*Thunnus albacares*
1 (larva)	*Thunnus spp.*		*Thunnus thynnus*
3 (larva)	*Auxis rochei*		

**- The control region I fragment failed to amplify in these specimens.*

Figure 2-1 Example of a neighbor-joining tree, using Tamura-Nei distance, constructed in MEGA for the purposes of species identification. *Sarda sarda* was used as an outgroup and no phylogenetic relationship among species is implied by the topology of the tree. Bootstrap values above 70 listed. YF/YFT= *Thunnus albacares*, BLCKFIN= *T. atlanticus*, tala= *T. alalunga*, TOBE/BE= *T. obesus*, Tthy= *T, thynnus*, Atha= *Auxis thazard*, Aroc= *A. rochei*, SKJ= *Katsuwonus pelamis*, Ssarda= *Sarda sarda*, Eall= *Euthynnus alletteratus*, and Elin= *E. lineatus*, Eaff= *E. affinis*.


73
YFT9004
unknown3
unknown4
YFT9015
unknown5
unknown8
unknown9
YF9011
unknown7
unknown6
84
92
YFT9006
YF9012
unknown10
BLCKFIN
70
BLKFIN28
BLKFIN04
75
BLKFIN26
76
BLKFIN27
88
TOBE298
BE9012
94
BE9004
tala2
tala5
100
100
tala1
76
tala3
83
tala4
Tthy382
100
Tthy0240a
Tthy0241a
85
Tthy0106a
100
Elin001
Eaff003
100
100
Eall016
Eall018
Eall017
Eall009
94
Eall011
Eall007
99
Eall008
Eall013
Eall012
86
Eall014
99
unknown2
99
SKJE11/11
SKJ11/9
SKJI23
100
Aroc002
99
Aroc003
99
unknown11
77
Atha
unknown1
100
Ssarda1
Ssarda
0.05

Biological Implications

The entire juvenile sample, with the exception of 6 specimens, and consisting of 110 specimens, was collected over a two-week period in the Gulf of Guinea in February of 1998. Table 2-3 shows the juvenile species that were collected together in the same tows. In all, it appears that at least *T. albacares*, *K. pelamis*, *A. thazard*, and *Euthynnus alletteratus* of similar size (around 30 mm in this case) can be found associated in the Gulf of Guinea.

Only two of 26 larval tows with multiple scombrids from the Gulf of Guinea yielded individuals that could not be unambiguously identified. One sample, consisting of six individuals, contained three *K. pelamis*, two *A. thazard*, and one individual that failed to amplify. The other sample consisted of nine specimens; two were *K. pelamis* but the rest failed to amplify. The alcohol-preserved and formalin-fixed larval specimens from the Gulf of Guinea and Gulf of Mexico that were successfully identified and collected in the same tow are listed in Table 2-4. Of the scombrids, at least *A. rochei* and *Thunnus spp.* (yellowfin and bluefin) are found together in the Gulf of Mexico.

Table 2-3 Species composition of individual tows for juveniles (20-50 mm) in the Gulf of Guinea from February 13-21 1998 in which multiple specimens were unambiguously identified. SL is standard length.

Sample name	N	Species identified	Mean SL and size range
CP1A	7	*T. albacares* (n=6), *Auxis thazard* (n=1)	16 mm, 12-19 mm
CP6	19	*T, albacares* (n=10), *A. thazard* (n=1)	20 mm, 16-26 mm
CP7	8	*T. albacares* (n=3), *A. rochei* (n=1), *K. pelamis* (n=1)	22.5 mm, 18-26 mm
CP12	10	*A. thazard* (n=9), *K. pelamis* (n-1)	28.5 mm, 27-33 mm
CP25	5	*T. albacares* (n=5)	41.2 mm, 35-46 mm
CP28-29	26	*T. albacares* (n=10), *A. thazard* (n=9), *K. pelamis* (n=2)	29.4 mm, 26-40 mm
CP42	13	*T. albacares* (n=12), *A. thazard* (n=1)	43 mm, 37-51 mm
CP43	6	*A. thazard* (n=3), *T. albacares* (n=1), *K. pelamis* (n=1), *Euthynnus alletteratus* (n=1)	59.2 mm, 49-66 mm

Table 2-4 Species composition of individual tows for larvae in the Gulf of Mexico (13-17, GC18, VK203, VK204, and MP259 samples) and Gulf of Guinea (MT8 and P1 samples) in which multiple specimens were unambiguously identified.

Sample name	N	Species identified	Mean SL and size range
13-17	5	*E. alletteratus* (n=3)	unknown
MT8	9	*K. pelamis* (n=2)	5.42 mm, 3.98-6.73 mm
P1	6	*K. pelamis* (n=3), *A. thazard* (n=2)	4.69 mm, 4.37-5.62 mm
GC18 N2S6	3	*A. rochei* (n=1), *T. albacares* (n=1), *T. thynnus* (n=1)	7.2 mm, 6-8.4 mm
GC18 N2S2	3	*E. alletteratus* (n=2)	4.0 mm, 3.5-4.5 mm
VK203 N1S1	2	*T. albacares* (n=1), *A. rochei* (n=1)	8.75 mm, 8.5-9.0 mm
VK203 N2S3	2	*T. albacares* (n=1), *A. rochei* (n=1)	10.35 mm, 10.0-10.7 mm
VK204 N2S2	2	*E. alletteratus* (n=1), *Cynoscion neubulosus* (n=1)	unknown
MP259 N1S1	2	*T. albacares* (n=2)	11.4 mm, 10.7-12.1 mm

Discussion

Amplification

The Chelex® method appears to be the most reliable and fastest DNA extraction method for good quality alcohol-preserved juvenile specimens regardless of species. However, phenol-chloroform is a standard DNA extraction method, used consistently throughout the literature, and can yield DNA from difficult specimens and tissue that cannot be ground according to the Chelex® protocol. This was exemplified by the amplification of two the five juvenile alcohol-preserved specimens that were totally dehydrated. However, all optimization attempts failed to improve upon these results, leading us to believe that DNA in these specimens was extremely degraded. In addition, the successful control region I amplification from specimens that appeared degraded, as evidenced by the low molecular weight smear results, concur with the results of France and Kocher (1996). These authors were unable to establish a correlation between apparent DNA quality and amplification, which led them to argue that electrophoresis was not a reliable method of determining whether extractions yielded sufficient DNA for PCR amplification and sequencing.

Surprisingly, formalin-fixed larvae had higher amplification and sequencing success rates than alcohol-preserved larval specimens. However, while the amplification of the

mitochondrial control region I from many of these formalin-fixed larvae was achieved, the PCR product was not of sufficient quantity and/or quality to generate a useable sequence. Similarly, several of the alcohol-preserved specimens amplified but could not be sequenced, though the overall amplification success rate for these specimens was lower than for the formalin-fixed individuals. Amplification of the 171 bp cytochrome *b* fragment was more successful in all larvae, regardless of fixation method, suggesting that DNA in most of these specimens was partially degraded or fragmented. One way to explain this DNA degradation is that specimens were kept under hot microscope lights while being sorted, prior to being properly fixed in ethanol. In addition, these results concur with France and Kocher (1996) regarding a "collection effect" due to variability in fixation conditions and handling of tissue afterward.

The Shedlock et al. (1997) method, although originally intended for formalin-preserved tissue, may increase the success rate in alcohol-preserved samples due to the numerous rinses in GTE, a solution that may bind, in addition to formalin, other substances that prevent PCR amplification. This method was initially performed on tissue from juvenile specimens and the quality of the PCR products was compared with those from the small tissue protocol. The amplicons obtained from templates extracted with either method were of similar quality and quantity, so the small tissue extraction method was used to re-extract DNA from larval samples because this protocol is fast and simple.

Several discrepancies emerged between our species identification, using a phylogenetic tree based on control region I sequences, and those obtained directly from BLAST searches of 171 bp cyt*b* sequences. The majority of tuna and tuna-like cyt*b* sequences in the NCBI database come from the Pacific Ocean where, for example, *Auxis thazard* is not found. The majority of the control region I reference sequences used in our tree, however, came from the Atlantic. In fact, the most common discrepancy was between specimens unambiguously identified as *A.*

thazard using the phylogenetic analysis of mitochondrial control region I sequences, yet identified as either *Sarda sarda* or *Katsuwonus pelamis* in the BLAST search results based on the same fragment (Table 2-2). The identification of *K. pelamis* as well as *A. thazard* and *A. rochei* via sequencing of the short cytochrome *b* fragment appear to be reliable (the entire sequence matched with 98-99% identity) but we limited this approach to determine *Thunnus* from non-*Thunnus* individuals and did not offer any statistical support (bootstrap) to this identity.

The formalin-preserved larvae were identified on the basis of morphological and meristic characters to belong to the genus *Thunnus* (Talat Farooqi, pers. comm.). However, of the 19 specimens that were sequenced and positively identified, 11 (almost 58%) did not belong to *Thunnus*. In addition, one of these non-*Thunnus* specimens was most likely not a scombrid, though this was likely a specimen vial mix-up as opposed to an identification error. This result emphasizes the need for unambiguous identification of specimens prior to further analysis.

Quinteiro et al. (1998) claim that unambiguous identification of *Thunnus* species can be obtained from the sequences of their 126 bp cyt*b* fragment. However, in the sequences given, the difference between yellowfin tuna and bigeye tuna consists of two transitions, and these fixed differences are unlikely to remain valid with increased sample sizes. While the 171 bp cyt*b* fragment (Terol et al. 2002) may be useful for distinguishing between *Thunnus* and non-*Thunnus* specimens, as shown here for this set of larvae, unambiguous identification cannot be obtained with either the 126 bp or 171 bp fragment has not been statistically validated for members of *Thunnus* and should not be relied on for identification of other species. Considering that Bartlett and Davidson (1991) could not statistically validate their identifications based on a larger fragment of the mitochondrial cyt*b* gene (307 bp), as the branches of their phylogenetic

tree collapsed with increased sample sizes and bootstrap analysis, it is unlikely that the shorter fragments contained within the larger fragment could perform any better.

Biological Implications

In the eastern Atlantic, older yellowfin tuna juveniles are caught together with similarly sized bigeye (*T. obesus*) and skipjack (*K. pelamis*; Fonteneau et al. 2000). Until now, there was no evidence of when associations of these species began. By using molecular genetic techniques to identify species in a number of separate tows, we found that *T. albacares*, *A. thazard*, *K. pelamis*, and *E. alletteratus*, of similar sizes within tows and ranging from 20 mm to 40 mm long between tows, are at least sampled together in the Gulf of Guinea and potentially schooling. However, an estimate of the ratio of species in each aggregation could not be determined because of the limited number of samples and specimens within each sample. Further sampling is needed to verify the nature of these associations in the Gulf of Guinea but could be the first evidence of the ontogenetic timing of the mixed-species schooling behavior.

We also found that larval *K. pelamis* and *A. thazard* are associated with each other in the eastern Atlantic, while larval *A. rochei*, *T. albacares*, and *T. thynnus* are sampled together in the Gulf of Mexico. Given that larvae are not strong swimmers and are highly susceptible to currents and other oceanographic conditions, these species could be spawning in the same spatial and temporal time frames with the larvae being kept together by these conditions. These early associations, then, may lead to later schooling between similarly sized and shaped individuals of various species as swimming ability improves. Other species than those discussed may also occur in these larval aggregations but could not be identified and/or were not sampled.

Several biases exist in the collection and handling of our samples that could lead to explanations other than schooling of why different species were sampled together. First, the type of gear used (bongo net, surf net, hydrobois net, etc) could bias collections, possibly favoring

slower or less reactive individuals or species. Second, scombrids collected in the Gulf of Guinea were sorted from non-scombrids by Bernard Stequert prior to shipping to our lab, removing other potential species associations. Third, sorting in the lab resulted in the exclusion of those specimens whose control region I sequences fell outside our set of known sequences and were thus not identified to the specifies level. All three biases could result in elimination of potential predators or prey not belonging to scombrid fish from these sample tows. Schooling, however, is a possible explanation for the association observed in those specimens, given the size uniformity of individuals within tows as well as the presence of the same species types in several tows with heterogeneous mean lengths, i.e. similar species associations in samples of differing lengths.

Other genetic techniques have been used to unambiguously distinguish among *Thunnus* species, including RFLP (restriction fragment length polymorphism) of the mitochondrial ATCO fragment (Chow and Inoue 1993, Takeyama et al. 2001). The gene diversity (0.996) levels of the control region I, however, make this locus useful not only for forensic identification of samples but also for population genetic studies, as shown in Alvarado et al. (1998). While the short, 171 bp, cytochrome *b* fragment can be used to identify certain species as well as differentiate *Thunnus* from non-*Thunnus* specimens, this locus does not have the power to distinguish among *Thunnus* species nor do identifications stand up to bootstrapping (Bartlett and Davidson 1991). Therefore, the mitochondrial control region I can serve the double purpose of allowing specific identification of specimens as well as to provide data for genetic population studies within and between *Thunnus* species.

CHAPTER III

POPULATION STRUCTURE OF ATLANTIC YELLOWFIN TUNA

Introduction

Yellowfin tuna (*Thunnus albacares*) in the Atlantic are considered for management purposes to belong to one panmictic population (=stock) by the International Commission for the Conservation of Atlantic Tunas (ICCAT 2002). This hypothesis is supported by tag-recapture data indicating transatlantic movement of fishes from the northwest Atlantic to the Gulf of Guinea, time-area size frequency distributions, locations of fishing grounds (caught continuously from the Gulf of Guinea west to off the coast of Brazil), and the general acceptance of the equatorial zone of the Gulf of Guinea as the main spawning ground (ICCAT 2002). However, spawning has also been documented in the Gulf of Mexico and southeastern Caribbean off Venezuela (Richards et al. 1990, Lang et al. 1994, Arocha et al. 2000), although the relative contribution of each of these areas to the entire population is unknown. Such separate spawning areas might imply separate stocks or substantial heterogeneity in the distribution of Atlantic yellowfin tuna (ICCAT 2002).

Proper stock assessment and management requires knowledge of a species' population structure (Ward 1995, Stocker 1999, Takagi et al. 1999, Chow et al. 2000). Current statistical models used to assess tuna stocks in the Atlantic utilize numerous biological factors, including age-at-length, yield-per-recruit, recruitment, fishing mortality, and spawning stock biomass, with the majority derived from reported fisheries data. In the last decade, the study of population structure, and more recently that of the levels of genetic variation within populations, have both become an integral part of the management strategies of species experiencing substantial fishing pressure (Bentzen et al. 1996, Ruzzante et al. 1996, Waples 2002a). Despite the fact that clarification of the genetic population structure of yellowfin tuna in the Atlantic could be very

valuable in stock assessment, it has received very limited attention even though landings of yellowfin tuna in the Atlantic appear close to the maximum sustainable yield (MSY) level and fishing effort and fishing mortality may be in excess of the levels associated with MSY.

In addition to considerable fishing effort on adults, vulnerability of juvenile yellowfin tuna to fishing effort has increased dramatically in the past 20 years (ICCAT 2002). Furthermore, fishing technologies may have altered the schooling behaviors of tunas in the Atlantic. Fonteneau et al. (2000) reported that the majority of juvenile yellowfin tuna in a 1981-2 survey in the Gulf of Guinea, were found in mixed-species schools in open water along with skipjack (*Katsuwonus pelamis*) and small bigeye tuna (*Thunnus obesus*). However, by 1999 all of the mixed species schools of small tuna were found under fish aggregating devices (FADs) used to target skipjack and other bait species, while the remaining unassociated schools were primarily monospecific (Fonteneau et al. 2000). Vulnerability of juveniles to sustained fishing effort could have important consequences on the genetic structure, particularly if reproductive variance (unequal production of viable offspring among females) in yellowfin tuna is substantial.

Several studies have examined population genetics of yellowfin tuna but none have focused primarily in the Atlantic. Scoles and Graves (1993), using mitochondrial (mt) DNA haplotype variation, detected notable variation among five Pacific and one Atlantic samples, but no significant intra- or inter-oceanic differentiation in haplotype frequencies. Elliott et al. (*in* Ward 1995) obtained similar results using restriction fragment length polymorphism (RFLP) of the entire mtDNA genome. Ward et al. (1997) analyzed allozyme and mtDNA data and suggested distinct Pacific, Indian, and Atlantic stocks, though only included one sample from the Atlantic. Ely et al. (1999) included two locations in the Atlantic, as well as Pacific samples, but their sample sizes were too small and geographically restricted (Gulf of Mexico and east coast of Florida, combined n=26) to derive any evidence of structure within this basin. That study

compared sequences of the mtDNA control region I in 80 yellowfin tuna from one Pacific and the two Atlantic locations but failed to detect any geographic association of the haplotypes. The only study that has detected heterogeneity in yellowfin tuna within an ocean basin is that of Ward et al. (1994). That study combined the restriction analysis of the entire mtDNA genome with two enzymes with the analysis of five allozyme loci. A significant difference between eastern and western Pacific samples was found at one allozyme locus. While this could indicate two separate stocks within the Pacific, the possibility of differential selection in the presence of gene flow could not be ruled out (Ward et al. 1994). Further examination of the genetic structure of this species, both within and between basins, will thus require more sensitive techniques.

Microsatellite loci are highly polymorphic nuclear DNA markers that have been shown to exhibit high levels of allelic variation in marine fish in general, and in members of the genus *Thunnus* in particular (Brooker et al. 1994, Takagi et al. 1999, DeWoody and Avise 2000). These genetic markers are abundant within vertebrate genomes, are inherited in a Mendelian fashion, and are more likely to conform to the assumption of neutrality than allozymes or other nuclear or mitochondrial DNA markers (Wright and Bentzen 1994, Jarne and Lagoda 1996). Microsatellites also have been used to detect intraspecific population structure in marine populations where other markers have been unsuccessful (Ruzzante et al. 1996, Rico et al. 1997, McLean and Taylor 2001). In the first study utilizing microsatellite markers in yellowfin tuna, Appleyard et al. (2001) detected significant heterogeneity at one of five microsatellite loci between samples from the central/western Pacific Ocean and samples from the eastern Pacific Ocean. Taken all together, microsatellites markers are an excellent choice to study the fine-scale population structure of yellowfin tuna in the Atlantic.

In this chapter, a segment of the mitochondrial control region and six microsatellite loci were examined in yellowfin tuna larvae and juveniles collected from the Gulf of Guinea and

larvae and adults collected from the Gulf of Mexico. The genetic examination of larval cohorts can provide information concerning recruitment (Graves et al. 1988) and reproductive variance (Ruzzante et al. 1996), important determinants of the genetic structure of a population, more readily than the study of adults (Chapman et al. 1999). Direct sequencing of the mitochondrial control region I was used for three reasons: (1) to identify larval and juvenile specimens (see Chapter II); (2) to combine with microsatellite data to examine the population structure (this Chapter); and (3) to serve as a potential maternal identification for use in the analysis of reproductive variance (see Chapter IV).

Methods

Juveniles from the Gulf of Guinea were collected over a two-week period in February of 1998. Larvae from the same area were obtained in January and August 1997, late January to early February 1998, and late November to early December 1998 (described in Chapter II). All samples were preserved in alcohol; scombrids were sorted from non-scombrids prior to arrival in our lab. Adults from the Gulf of Mexico were collected during the summers of 2002 and 2003. Tissues initially were frozen and then transferred to 95% ethanol. Collection information for formalin-fixed larvae is given in Chapter II.

DNA from all specimens was extracted according to methods outlined in Appendix A. The mtDNA control region I was amplified and sequenced according to the protocol listed in Appendix B; amplification and sequencing of microsatellite loci were performed as described in Appendix C.

Sequences were edited, aligned, and identified as described in Chapter II. A second N-J tree was constructed, using only positively identified yellowfin tuna sequences, to view whether any phylogeographic pattern with the distribution of haplotypes was present. Recently expanded

populations are characterized by a star-like phylogeny where haplotypes are separated by only one to two mutational steps. Accordingly, an unrooted phenogram was constructed to look for evidence of population expansion. Arelquin 2.0 (Schneider et al. 2000) was used to calculate the average number of nucleotide differences and nucleotide diversity. In addition, this program was used to estimate F_{ST} values, and conduct an analysis of molecular variance (AMOVA), using Tamura-Nei distance matrix based on a Gamma α parameter of 0.58. This α value was estimated empirically from our mitochondrial control region I data using ModelTest (Posada and Crandall 1998).

Microsatellite loci were analyzed with the GeneScan 3.7 program (Applied Biosystems, Foster City, California), which reads the size of each fragment directly. GENEPOP 3.3 (Raymond and Rousset 1995) was used to calculate allele frequencies, number of alleles per locus, genic (allelic) differentiation between pairs of populations, and the χ^2 (chi-squared) value between pairs of populations across all loci. Arlequin was used to conduct both global and locus-by-locus AMOVA, with corresponding F_{ST} values. However, F_{ST} assumes a low mutation rate and that the result of a mutation event is independent of the prior allelic state; both of these assumptions are not satisfied at microsatellite loci. Slatkin's (1995) R_{ST} parameter is a more appropriate statistic for microsatellite data as it accounts for higher mutation rates assumes a stepwise mutation process. This parameter makes other assumptions that are not satisfied by our data, such as populations of equal sample size and equivalent variances at all loci. Therefore, the genetic differentiation parameter, R_{HO}, was calculated using R_{ST} Calc (Goodman 1997). This statistic is an unbiased estimator of Slatkin's R_{ST} that accounts for differences in variance between loci and differences in sample size between populations, and is the most appropriate and reliable statistic for our microsatellite data.

The population parameters θ and τ also were estimated in Arlequin for the control region I sequences. A statistical test of mutation neutrality was carried out using Fu's (1997) F_S test and Tajima's D test. Fu's F_S test was used to infer the population history of yellowfin tuna in the Atlantic. The F_S value tends to be negative when there is an excess of recent mutations, and therefore a large negative value of F_S will be taken as evidence against the neutrality of mutations, an indication of deviation caused by population expansion and/or selection (Su et al. 2001). The estimated τ value can be transformed to estimate time since expansion using the formula $\tau = 2\mu t$, where μ is the mutation rate for sequence and generation and t is the time since expansion (Slatkin and Hudson 1991).

Results

MtDNA control region variability

A total of 355 bp corresponding to the first domain of the mtDNA control region (control region I) were characterized for adults (n=138; n=69 for each of the two adult samples), juveniles (n=49), and larvae (n=9). Only one larva from the Gulf of Guinea samples was identified as a yellowfin tuna; it was pooled with juveniles in ensuing analyses. In addition, formalin-fixed larvae collected from the Gulf of Mexico were not included in data analysis due to small sample size and substantial amounts of missing data. Results including these specimens, however, are listed in Appendix D.

Considerable genetic variation was found in all three samples, adults sampled from the Gulf of Mexico (GOM) in 2002, adults sampled from the GOM in 2003, and juveniles sampled from the Gulf of Guinea (GOG) in 1998 (Table 3-1). Among adults, 20.4% of the control region I sites were polymorphic in the 2002 sample, 19.3% in the 2003 sample, and 14.2% among the GOG juvenile specimens. Values of haplotypic (gene) diversity were extremely high in all

samples and in the combined data (Table 3-1). In addition, nucleotide diversities, ratios of transitions to transversions, and mean number of pairwise differences within each sample and for the combined data are listed in Table 3-1. The mean number of pairwise differences among the three samples, when corrected by the mean number of pairwise differences within each sample, yielded very low and statistically insignificant values of differentiation among samples (Table 3-2).

Table 3-1 Genetic variation at the mtDNA control region I for each of the three samples. Adult02 refers to the adult specimens collected in the Gulf of Mexico in 2002 (n=69), Adult03 to the adult specimens collected in the Gulf of Mexico in 2003 (n=69), and Juv98 to juvenile specimens collected in the Gulf of Guinea in 1998 (n=49). PiX is the mean number of pairwise differences within each population. All values were calculated in Arlequin.

	Adult02	**Adult03**	**Juv98**	**Combined**
Gene diversity	0.997	0.997	0.994	0.996
Nucleotide diversity	0.029 +/-0.014	0.030 +/-0.015	0.025 +/-0.013	0.024 +/- 0.012
Transition/transversion	5.42	6.30	7.83	3.82
PiX	10.224 +/- 4.725	10.659 +/- 4.913	8.839 +/-4.146	8.974

Table 3-2 Population comparison parameters as calculated in Arelquin from control region I sequences. The first comparison is between the two adult samples and the other two between each adult sample and the juvenile sample. F_{ST} is the amount of variation attributed to differences between the populations and PiXY is the corrected mean number of pairwise differences between populations.

	GOM:GOM	**GOM:GOG**	**GOM:GOG**
Pairwise F_{ST}	0.0032	0.0027	0.0080
F_{ST} P values	0.2072	0.3153	0.0541
PiXY	0.0293	0.0266	0.0755
PiXY P values	0.2091	0.9182	0.9364

The 2003 GOM adult sample and the GOG juvenile sample each had six haplotypes present more than once, while the 2002 GOM adult sample had five. Of these, three were unique to each of the 2002 and 2003 GOM adult samples and four were unique to the GOG juvenile sample, all with a frequency of just two or three individuals. While the 2002 and 2003 GOM adult samples had eight haplotypes in common, the 2002 GOM adult sample had seven haplotypes in common with the GOG juvenile sample, and the 2003 GOM adult sample had six.

All haplotypes were interspersed throughout the phylogenetic tree. Thus, there was no obvious phylogeographic association of individual haplotypes or sets of lineages with a particular region. However, when the frequency distribution was examined in detail, it was found that those GOG juveniles sharing the same haplotype were collected in the same tow, with one exception.

An AMOVA of control region I sequences (Table 3-3) revealed that an overwhelming proportion of the variance (99.55%) is contained within the samples, with a small (0.45%) and statistically insignificant (P= 0.111) difference between samples. The global F_{ST} was similarly low at 0.0045 as were each of the population pairwise F_{ST} values (Table 3-3 and 3-2, respectively).

Table 3-3 Analysis of molecular variance (AMOVA) results comparing juvenile and adult nucleotide sequence data of the mitochondrial control region I, as calculated in Arlequin.

Source of variation	d.f.	Sum of squares	Variance components	% of variation
Among populations	2	11.328	0.0199 Va	0.45
Within population	184	816.672	4.4384 Vb	99.55
Total	186	828.000	4.4583	
Fixation Index F_{ST}=	0.00446			
Va and F_{ST}: p=	0.1105 +/- 0.011			

Microsatellite genetic variation

All six microsatellite loci were polymorphic within each of the two GOM adult samples and the GOG juvenile sample. The number of alleles per locus was similar in each sample and ranged between 2 and 18, with a mean of 10.67. AMOVA over all populations and all loci revealed that 99.83% of the variation was found within the samples, while only a small and insignificant (0.17%, P=0.189) proportion of the variation could be attributed to differences between samples. The global (across all samples and all loci) F_{ST} value was correspondingly low (0.0017). The F_{ST} value for the comparison between the 2002 GOM adults and the GOG juveniles over all loci appeared to indicate a difference (F_{ST}= 0.0101, P= 0.018), though χ^2

analysis of homogeneity in allele frequency between this pair of samples across all loci was not significant (Table 3-4).

None of the comparisons in allele frequency distribution between pairs of samples at each locus were significantly different (Table 3-4; Figure 3-1). Borderline differentiation was observed at locus 125 between each of the GOM adult samples and the GOG juvenile sample. In addition, a locus-by-locus AMOVA suggested a difference among all three samples for locus 125 (F_{ST}= 0.0165, P= 0.0244). However, none of these comparisons were significant before and/or after Bonferroni correction. In contrast, R_{ST} estimates between pairs of samples across all loci and a across all samples and all loci revealed no differentiation (Table 3-5). In addition, the comparisons across all samples for a single locus yielded small R_{ST} values ranging from –0.00708 to 0.01642 indicating homogeneity at all of the loci.

Table 3-4 Genic (allele frequency) differentiation between pairs of yellowfin tuna samples at each microsatellite locus as calculated in GenePop. Sample designations are the same as stated in Table 3-1 and values are probabilities with standard errors in parentheses except for across all loci where the values are χ^2, (degrees of freedom), and p-value. Bonferroni-corrected p-value (0.05/6)= 0.0083

Locus	Adult02:Adult03	Adult02:Juv98	Adult03:Juv98
113	0.1200 (0.0098)	0.0522 (0.0050)	0.1805 (0.0111)
208	0.6753 (0.0068)	0.6249 (0.0076)	0.3476 (0.0111)
125	0.2952 (0.0109)	0.0590 (0.0049)	0.0640 (0.0072)
144	0.6985 (0.0031)	1.0000 (0.0000)	0.6586 (0.0033)
Ttho-1	0.4147 (0.0128)	0.3596 (0.0144)	0.3762 (0.0135)
Ttho-4	0.2001 (0.0100)	0.1450 (0.0090)	0.1981 (0098)
Across all loci	13.161 (12) 0.3574	18.414 (12) 0.1037	17.065 (12) 0.1472

Table 3-5 Summary of the genetic differentiation R_{HO} calculations from R_{ST} Calc. Comparisons are across all loci with 100 permutations. Sample designations are the same as stated in Table 3-1. VC- value averaged over the variance component, loci- value averaged over loci.

	Adult02:Adult03	Adult02:Juv98	Adult03:Juv98	Across all loci and samples
R_{HO} (VC)	-0.00532	0.0051	0.00023	-0.00157
P	0.96	0.19	0.40	0.68
R_{HO} (loci)	-0.00535	0.00353	-0.00035	-0.00178
P	0.97	0.21	0.44	0.69

Genetic differences between Pacific and Atlantic specimens

Initial inspection of the microsatellite data appeared to indicate substantial differences in allele frequency when compared to data reported by Appleyard et al. (2001). However, a closer examination of both sets of data suggests that the differences may be due to mobility differences related to the use of different genetic analyzer equipment (ABI 377 used by Appleyard et al. [2001] compared to ABI 310 used in this study). After adjusting for a shift in mobility by aligning the most frequent allele(s), a general agreement in allele frequencies for loci 125 and 144 were observed. The frequencies of the most common alleles in loci 113 and 208, however, appear to differ (Figure 3-2 A□D). Specifically, the frequency of allele 5 at locus 113 (as designated in Figures 3-2A and B) from this study is less than that in the Philippine 94/95 and California 91/92 samples, and greater than that in the Mexico sample from Appleyard et al. (2001). The frequency range over allele 8 from this study is also greater than the range over the five Pacific samples. In addition, the frequency of allele 3 at locus 208 is greater in our study than in Appleyard et al. (2001) and the frequency of allele 5 is less.

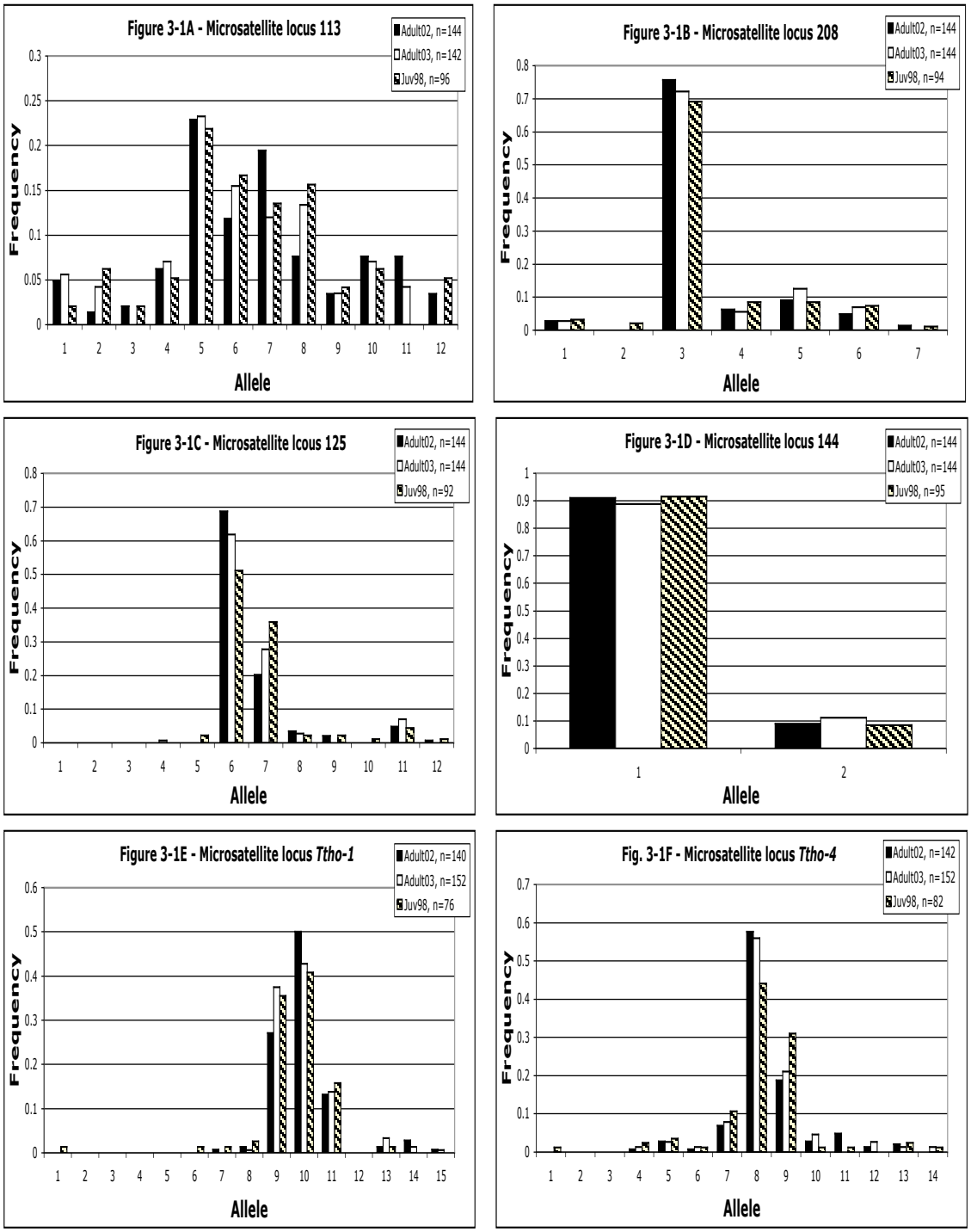


Figure 3-1 (A– F)– Allele frequencies for the six dinucleotide microsatellite loci in all three samples. Sample designations are the same as those used in Table 3-1 and "n" is the number of alleles. The size of each allele is listed in Appendix C.

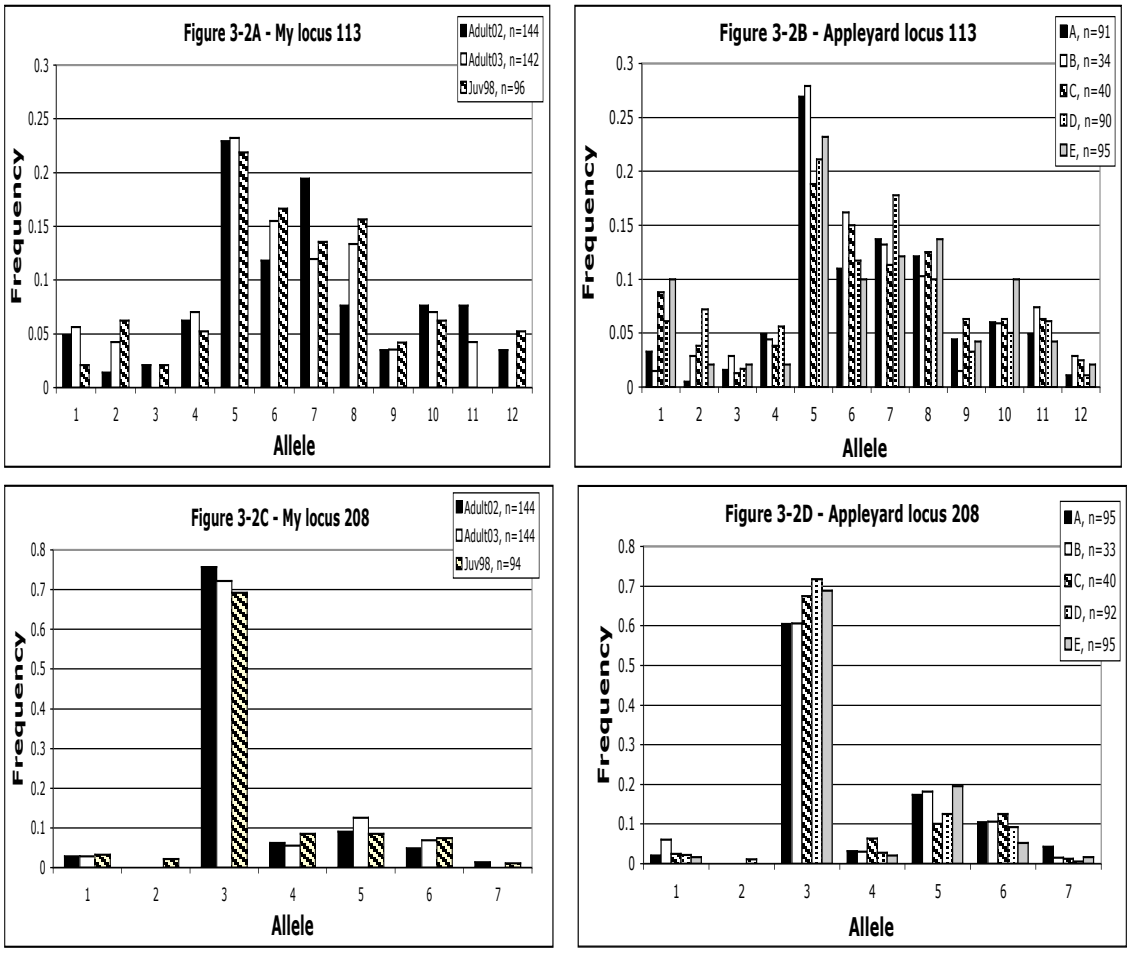


Figure 3-2 (A–D) Comparison of the allele frequencies of loci 113 and 208 between my three samples and five of the samples from Appleyard et al. (2001). A= Philippines 94/95; B= California 91/92; C= Mexico 91/92; D= Fiji 95/96; E= Coral Sea 91/92. Sample designations for our data are the same as stated in Table 3-1. N in the Appleyard data is the number of fish whereas for our samples, n is the number of alleles.

Mismatch distribution and population expansion

The pairwise sequence difference among the yellowfin tuna control region I sequences was calculated using Arlequin and the mismatch distribution is shown in Figure 3-3. The resulting star-like topology of the N-J tree constructed from the aligned mitochondrial control region I haplotypes can be seen in Figure 3-4. Estimated parameters from the sudden expansion model in Arlequin included $\tau = 8.475$, $\theta_0 = 0.030$ (diversity before an expansion), and $\theta_1 =$

682.813 (diversity after an expansion). The time since expansion was estimated using first a lower bound mutation rate of 5% per million years as well as an upper bound mutation rate of 10% per million years for mitochondrial control region I sequences, based on the rates discussed in Volckaert et al. (2002) and the references therein. These mutation rates, with a generation time of three years, estimate that the yellowfin tuna population began to expand in the Atlantic ~40,000-80,000 years ago.

Mutation neutrality tests were conducted in two ways. First, all of the individual sequences were considered as one population. Second, the specimens were separated into three subpopulations based on the time of their collection, the 2002 GOM adults, the 2003 GOM adults, and the GOG juveniles. Fu's test of selective neutrality was statistically significant for the combined data set (F_S= -24.438 P=0.000) and for all three subpopulations (Table 3-6). Tajima's and Fu's statistical tests were also conducted on both the single Atlantic population and on each of the subpopulations. The results of these tests are shown in Table 3-6.

Table 3-6 Summary of estimations and selective neutrality tests as estimated in Arelquin with yellowfin tuna control region I sequences. Values in parentheses for the estimates are standard deviations and those in the other tests are p-values. Sample designations are the same as previously noted.

	Adult02	**Adult03**	**Juv98**	**Pooled**
Tajima's estimate	14.155 (3.745)	13.738 (3.906)	11.214 (3.647)	16.004 (4.144)
Tajima's D	-1.383 (0.079)	-1.268 (0.101)	-1.189 (0.120)	-1.499 (0.056)
Fu's estimate	8.346 (4.340)	8.561 (4.451)	7.363 (3.972)	8.340 (3.903)
Fu's F_S Test	-24.791 (0.00)	-24.756 (0.00)	-24.994 (0.00)	-24.438 (0.000)

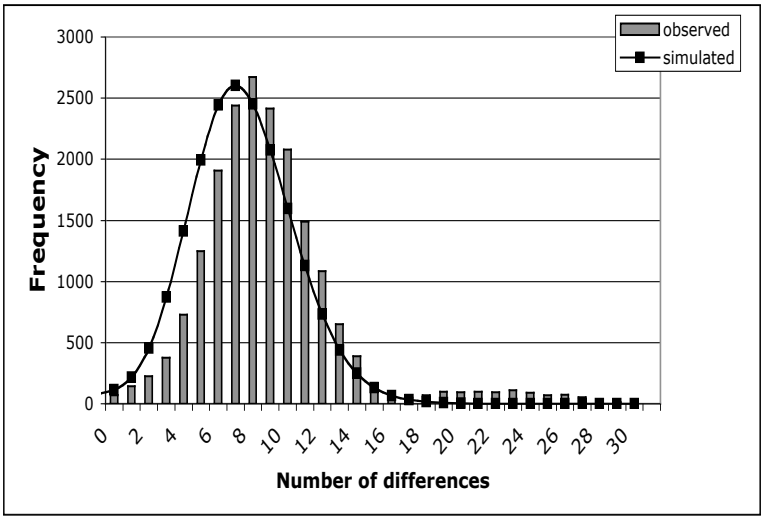


Figure 3-3 Mismatch distribution as calculated in Arlequin with 152 mitochondrial control region I haplotypes from 188 adult and juvenile Atlantic yellowfin tuna specimens.

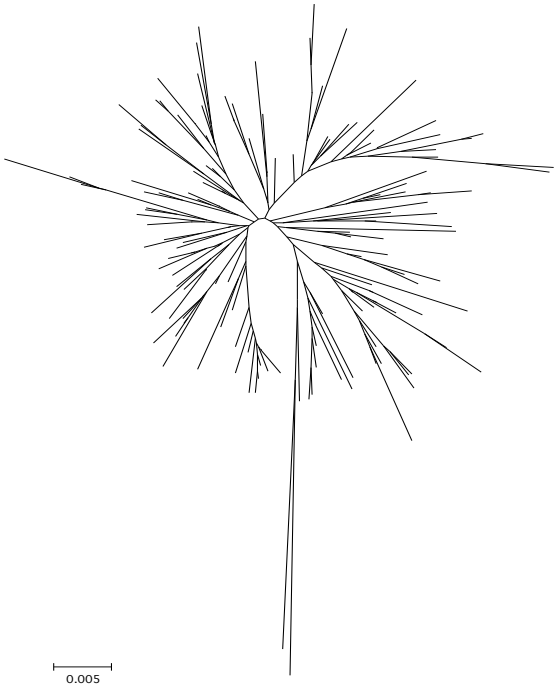


Figure 3-4 Star-like phylogeny of the 152 Atlantic yellowfin tuna control region I haplotypes constructed in MEGA.

Discussion

Six microsatellite loci and the mtDNA control region I were used to test the hypothesis of a single stock in the Atlantic by comparing juveniles collected in the Gulf of Guinea to adults collected over two years in the Gulf of Mexico. Control region I sequences were also used to examine the likelihood of a population expansion in the Atlantic yellowfin tuna's history. Significant amounts of genetic variation were found in the control region I; most haplotypes were unique, although the nucleotide diversity was less than half that observed in other tuna such as albacore (*T. alalunga*; Vinas et al. 1999) and bigeye (*T. obesus*; Alvarado Bremer et al. 1997). Considerable amounts of genetic variation were also found in the microsatellite loci surveyed in all three samples. These values are similar to those reported by Appleyard et al. (2001).

Structure within the Atlantic

Phylogenetic analysis of control region I sequences revealed no obvious phylogeographic pattern separating the three samples of yellowfin; haplotypes from all samples were interspersed throughout the tree. AMOVA of the mtDNA data revealed that only a small fraction (0.45%) of the total variation was a result of differences among samples. A correspondingly low F_{ST} value was estimated and none of the pairwise population comparisons differed significantly. Thus, the analysis of mtDNA control region I data failed to reject the hypothesis of panmixia for yellowfin tuna in the Atlantic.

Although a previous study (Ely et al. 1999) using the control region I had failed to find difference both within the Atlantic and between the Atlantic and the Pacific Oceans, we felt that the examination of this fragment was justified for three reasons. First, I examined a longer segment of the same region. Second, the sampling of the Atlantic by Ely et al. (1999) was geographically restricted to the western part of this basin. Third, my sample sizes were larger

than those in Ely et al. (1999). In an analogous study in albacore (*T. alalunga*), Vinas et al. (2003) were able to detect significant differences between the Atlantic and the Mediterranean, that were insignificant using smaller sample sizes (Vinas et al. 1999). For Atlantic yellowfin tuna, however, neither increasing the length of the control region I fragment, nor the number of individuals, helped to resolve structure within this basin with mitochondrial data.

Six microsatellite loci were employed to examine the population genetics of Atlantic yellowfin tuna. Results of a global AMOVA revealed no differences among samples. Chi-squared analyses between pairs of populations across all loci similarly detected no heterogeneity. However, the comparison of adults from the GOM and early juveniles from the GOG is a problematic as the adults could have migrated from the GOG, and therefore not representative of the total diversity present in the Atlantic basin. On the other hand, the F_{ST} value (0.01) calculated between the 2002 GOM adults and the GOG early juveniles indicated a difference between these two samples when compared across all loci. In addition, allelic differentiation between pairs of samples and locus-by-locus AMOVA across all samples appear to suggest some level of differentiation at locus 125. However, this locus was difficult to score in several of the juvenile specimens. Specifically, when the size of an allele could not be determined unambiguously, it was scored as missing data in the analysis, potentially biasing the results. In addition, this finding is not supported when compared to the results of the R_{ST} calculation as none of the R_{HO} values for either sample or locus comparisons were significant. Therefore, the null hypothesis of panmixia of yellowfin tuna in the Atlantic basin cannot be rejected with the microsatellite data surveyed here.

The comparison of the microsatellite data from Appleyard et al. (2001) with that presented here suggests that there may be statistically significant differences between the Pacific and the Atlantic Oceans in the frequency of alleles at two of the loci. However, due to potential

shifts in mobility direct comparisons are not possible between results generated in different genetic analyzers. In order to validate this result, the shift in mobility needs to be confirmed by assaying Pacific yellowfin tuna samples in our genetic analyzer. If this difference were real it would be the first statistically significant evidence for differences between yellowfin tuna in the Pacific Ocean and those in the Atlantic Ocean.

Both Scoles and Graves (1993) and Elliott et al. (*in* Ward 1995) detected substantial amounts of variation between samples from the Pacific and from the Atlantic, but fail to detect statistical differences. Ward et al. (1997) did find a difference between two groups of yellowfin tuna samples at one of the five allozyme loci examined: the eastern Pacific and Indian Ocean fish were statistically different from the Atlantic Ocean and west-central Pacific Ocean fish at the *GPI-A** locus. These authors argue that, because there are no migration routes between the eastern Pacific and the Indian Ocean that avoid the west-central Pacific, and between the Atlantic and west-central Pacific that avoid the Indian Ocean, there is reason to believe that there are four stocks of yellowfin tuna (Ward et al. 1997). While this may be true, and indeed separate stocks within each ocean basin is highly probably because of restricted migration, local selection pressure differences could not be ruled out as the cause of this observed difference.

The differences detected by Ward et al. (1997) were between the western Pacific Ocean and the Indian Ocean at both *GPI-A** and mtDNA. Although no differences were detected between the Gulf of Mexico and Pacific Ocean samples, no potential corridor to explain this homogeneity can be identified given that the Indian Ocean is different from both basins. Yellowfin tuna moving from the Indian Ocean into the Atlantic could change the allele frequencies in the south Atlantic Ocean to be genetically intermediate between the western Atlantic Ocean and the Indian Ocean. Chow et al. (2000) found admixture of bigeye tuna (*Thunnus obesus*) in the genotypes of PCR-RFLP analysis of two mitochondrial segments

around South Africa. The oceanographic influence of the Agulhas current favor the movement of subadult and adult bigeye tuna into the Atlantic and may prevent the movement of fish in the other direction. In fact, Chow et al. (2000) provided evidence suggesting that migration or immigration by the Indo-Pacific bigeye tuna into the Atlantic Ocean is ongoing or had occurred recently. As no previous study has incorporated samples from the eastern Atlantic Ocean or around South Africa, the potential movement of yellowfin tuna around South Africa has not been examined.

It should be noted that, while we do not provide substantial evidence of differentiation between the Gulf of Guinea and the Gulf of Mexico, no samples from any other basin nor samples from the southeastern Caribbean or South America were analyzed. The present migration model for yellowfin tuna in the Atlantic states that once fish reach about 65-75 cm fork length in the Gulf of Guinea they migrate towards the northwestern Atlantic and at some point migrate back to spawn. In addition, all trans-Atlantic tag recoveries were from fish tagged off the east coast of the United States and recovered in the Gulf of Guinea (Oritz 2001). However, spawning has been documented in the southeastern Caribbean and the reproductive population there has been shown to differ significantly in size and spawning frequency from that in the Gulf of Mexico. In addition, there is the possibility of a subpopulation off the coast of Brazil (Arocha et al. 2001). Therefore, potential stocks may not simply be separated on and east-west basis in the Atlantic but also north-south in the western regions. To fully understand the stock structure in the Atlantic, all known spawning areas and any potential subpopulations need to be analyzed with mitochondrial and nuclear markers, as well as other techniques such as pop-up tags and otolith micro-chemistry. In addition, estimates of effective population size (N_e) for each sample may indicate separate sub-populations (=stocks) where analysis of allele frequencies indicates homogeneity.

Population Expansion

Finally, we provide evidence of significant population expansion within the last 40,000-80,000 years. The mismatch distribution is a better fit to the bell-like curve of a population undergoing exponential growth than a typical multi-modal curve of a population at equilibrium (Slatkin and Hudson 1991). The star phylogeny shown in Figure 3-4 is also indicative of a recent expansion in population size (Slatkin and Hudson 1991). While a star phylogeny could also result from a selective sweep of alleles, the mitochondrial control region is a neutral locus exhibiting high levels of haplotypic diversity. High levels of diversity from mtDNA RFLP analysis in Pacific yellowfin tuna have also been shown (Scoles and Graves 1993). Therefore, this alternative explanation for the shape of the unrooted phylogeny is unlikely. In addition, the calculated F_S values for each sample and for the combined data were similar to each other and all were significant, providing statistical evidence for a population expansion of yellowfin tuna in the Atlantic. Tajima's test of neutrality was also applied was insignificant for any of the individual samples and for the combined data. However, this was likely due to a lack of power in this test for population expansion (Fu 1997).

Assuming a constant mutation rate, the ratio of population size change is positively correlated with the Fu and Tajima θ estimates (Su et al. 2001). Comparing the two values of θ can help define how the population size has changed over time, given that Fu's estimator puts heavy emphasis on recent mutations while Tajima's puts more weight on ancient mutations (Fu 1997). Table 3-6 shows that Tajima's estimate of θ is about twice that of Fu's for the combined data set, and about 1.5 times larger for each of the individual samples, indicating that the population approximately doubled sometime farther in the past. This is supported by the calculated time of expansion of 40,000-80,000 years ago. While population expansion is not the only explanation for a significant F_S (Fu 1997), we have no evidence of population subdivision

and have seen no evidence of selection on the mitochondrial control region I in fish, thereby making population expansion the most likely reason for the significant F_S value. Similar recent expansion has also been suggested for Pacific yellowfin tuna using mtDNA data (Ward et al. 1994).

CHAPTER IV

REPRODUCTIVE VARIANCE

Introduction

Reproductive variance may have a large impact on population (stock) structure and/or the vulnerability of species to certain types of fisheries. It is possible that certain females produce a majority of the viable offspring in each spawning cycle in tuna populations, reducing the effective population size (N_e) several orders of magnitude as compared to the estimated adult census size (N; Hedgecock 1994). In fact, N_e /N ratios average only 0.11 among population samples of both terrestrial and aquatic vertebrates (Frankham 1995) and could be even lower in such highly fecund marine species (Type III survivorship) as tuna. For instance, Turner et al. (2002) found that the genetic effective size of red drum in the northern Gulf of Mexico is three orders of magnitude smaller than the adult census size. An even more extreme disparity was reported by Hauser et al. (2002) from a time series study of the overexploited New Zealand snapper, where the estimated the effective population size was five orders of magnitude smaller than the census size.

The reduced effective population sizes as compared to census size reported by Turner et al. (2002) and Hauser et al. (2002), among others, are thought to be small enough to cause the loss of alleles at neutral and weakly selected genes that may be crucial for the long-term adaptability of the species. Specifically, Ryman et al. (1995) showed that, at equilibrium and with a mutation rate to neutral alleles of 10^{-7}, a marine population with $N_e = 10^8$ will have an average of over 300 alleles per locus. A population with $N_e = 10^6$, on the other hand, will have an average of only 4 alleles per locus. A combination of overfishing, fluctuating population size and reproductive variance may decrease the effective population size faster than any of these factors would alone (Turner et al. 1999; Hedgecock 1994) creating the potential for loss of

genetic diversity even in what were once extremely large populations that have been reduced to around a million individuals. Thus, the examination of reproductive variance in yellowfin tuna is relevant to evaluate the potential impact of current fishing effort conducted on this species, particularly those incidentally targeting yellowfin juveniles. If reproductive variance is substantial in yellowfin tuna and the population is over harvested, as suggested by Garcia (1994), genetic variance within the population could be lost at an accelerated pace if the N_e becomes too small (Wright 1938).

Variance in reproductive success can arise from the existence of only a few largely successful females at each spawning event, or by chance, "sweepstakes" survival of broods (Hedgecock 1992, 1994). The former phenomenon, correlated reproductive success of individuals over seasons, may increase the variance in reproductive success in the population and further decrease N_e/N (Driscoll 1999). In species with substantial fecundity the opportunity for reduction in N_e/N due to family correlated survival can be considerable and, if the effective population size is decreased, the number of alleles at each locus could drop drastically with the potentially significant long-term evolutionary effects (Waples 2002b) mentioned previously. However, on the basis of the large amounts of genetic diversity observed at both mitochondrial and nuclear loci (see Chapter III), what is known about their reproductive biology and behavior (individuals spawn every couple of days, spawning seasons last for several months, and spawning aggregations are loosely structured; NMFS 1999), as well as the fact that the population appears to have remained stable even in the face of intense fishing pressure (ICCAT 2002), the potential for family correlated survival is not a likely scenario for Atlantic yellowfin tuna.

Alternatively, reproductive variance in yellowfin tuna could be caused by sweepstakes survival. Evidence of sweepstakes survival has been found in cod (Ruzzante et al. 1996) through

the direct comparison of microsatellite allele distribution in adults and larvae. This type of study, however, requires sampling of larvae/juveniles as well as adults from the spawning grounds, which is difficult to achieve in most species. For this reason, Chapman et al. (1999) outlined three null hypotheses to test the sweepstakes hypothesis in surveys of early life history (ELH) stages off cod: "1) genetic homogeneity should be expected within and among locations, 2) multiple loci should be in linkage equilibria, and 3) gene frequencies should be homogenous within and among year-classes". In this study we were able to test hypotheses 1 and 2, but not 3 since all of our ELH specimens were collected over a two-week period in a single year. This type of reproductive variance has not been demonstrated in tunas but if ELH subsets do exhibit haplotype frequency differences for mtDNA data and heterozygous deficiencies and departures from HWE expectations within and across these subsets for nuclear data, then variation in reproductive success can be hypothesized as the cause if the other assumptions of Hardy-Weinberg equilibrium (HWE) are met (random mating, infinite population size, no genetic drift, no selection, and no migration). Alternatively, reproductive variance could be inferred from the degree of kinship within ELH samples.

Methods

Juvenile specimens were collected in the Gulf of Guinea over a two-week period in February 1998 by Bernard Stequert (sampling details given in Chapter II). All specimens were stored in alcohol and scombrids were sorted from non-scombrids prior to arrival in our lab. DNA was extracted according to the method described in Appendix A, and the mitochondrial control region I and microsatellite loci were amplified and sequenced according to the methods outlined in Appendices B and C, respectively. The editing and alignment of mitochondrial

sequences, as well as the identification of specimens to the species level is described in Chapter II. Only unambiguously identified yellowfin tuna specimens were used in further analysis.

Microsatellite loci were analyzed with the GeneScan 3.7 program (Applied Biosystems, Foster City, California), which reads the size of each fragment directly. GENEPOP 3.3 (Raymond and Rousset 1995) was used to calculate observed heterozygosity (H_o), expected heterozygosity (H_e), allele frequencies, and the number of alleles per locus. The deviation from HWE within populations (F_{IS}) was then determined for each locus using a modified version of the Markov-chain random walk algorithm described by Guo and Thompson (1992). Linkage disequilibrium between pairs of loci, and departures from HWE were also estimated with Arlequin 2.0 (Schneider et al. 2000). The linkage disequilibrium analysis was used to determine whether genotypes at pairs of loci occur randomly. Results from these analyses were compared to those from adults sampled in the Gulf of Mexico in 2002 and 2003.

A Neighbor-joining (N-J) tree, based on the number of differences and with the pairwise deletion option, was constructed in MEGA with the control region sequences of identified yellowfin tuna sequences to look for shared haplotypes. The sampling date, sampling time, and microsatellite genotypes were compared for those specimens with the same haplotype. The hypothesized relationship of half-sibship between all pairs of individuals sharing the same haplotype was tested in the computer program Kinship 1.3.1 (Queller and Goodnight 1989) based on both the haplotype and microsatellite allele frequencies calculated from the adult data. This program determines significance empirically, by generating a series of pairs at random (using the allele frequencies and *r* settings) and determining what values of the likelihood ratio result.

Results

Probability tests of HWE revealed that all six loci were in equilibrium in both of the GOM adult samples. However, deviation from HWE for locus 125 in the GOG juvenile sample was highly significant in both GenePop (P< 0.0001) and Arlequin (P< 0.0001). Consequently, the HWE test over all six loci (Fisher method) for the combined samples was significant (χ^2= infinity, df= 12, P< 0.0001). The multilocus HWE test for heterozygote deficit was significant for the GOG juvenile sample (P< 0.0001) but not for either the 2002 GOM adult sample (P=0.2685, S.E.=0.0453) or the 2003 GOM adult sample (P=0.2358, S.E.=0.0453). None of the exact tests of HWE for each locus, where H1= heterozygote deficit, were significant in the GOM adults after Bonferroni correction for multiple testing, though a heterozygote deficit was detected in the GOG juvenile sample at loci 125 and 208 (Table 4-1). We decided to compare GOG juvenile samples by day and location of capture to identify the source of such deviation from HWE for these two loci. No deviations from HWE were detected when GOG juveniles were assigned to sub-samples after Bonferroni correction (Table 4-2). However, the 2/18/98 sub-sample did suggest a deviation at locus 125, as did the overall χ^2 value.

Results from the linkage disequilibrium tests for each pair of loci in all three samples revealed that the test between loci 125 and *Ttho-4* was significant in the 2003 GOM adult sample after Bonferroni correction for multiple testing (P= 0.0018, S.E.= 0.0133) and linkage was suggested between loci 113 and 208 in the GOG juvenile sample (P= 0.0408 +/- 0.00039). However, when all of the samples were pooled, none of the linkage disequilibrium tests remained significant.

Table 4-1 P-value (standard error) of Hardy-Weinberg test when H1= heterozygote deficit at each locus in each yellowfin tuna sample. Sample designations are the same as in Table 3-1 and all values were calculated in GenePop. Values in bold are significant after Bonferroni correction for multiple tests.

Sample	113	208	125	144	*Ttho-1*	*Ttho-4*
Adult02	0.0194 (0.007)	0.6781 (0.015)	0.2420 (0.014)	1 (/)	0.3984 (0.029)	0.9933 (0.005)
Adult03	0.4835 (0.030)	0.2269 (0.009)	0.2083 (0.012)	0.6079 (/)	0.2408 (0.019)	0.4130 (0.028)
Juv98	0.1177 (0.017)	**0.0052 (0.002)**	**0.0000 (0.000)**	1 (/)	0.1928 (0.025)	0.4825 (0.034)

Table 4-2 P-value (standard error) of the probability test for HWE in juvenile sub-samples with at least five specimens separated out by individual tow or date of capture. Sub-populations are labeled with the date they were collected. The χ^2 value calculated over all loci and all populations was 46.2 (P=0.0294)

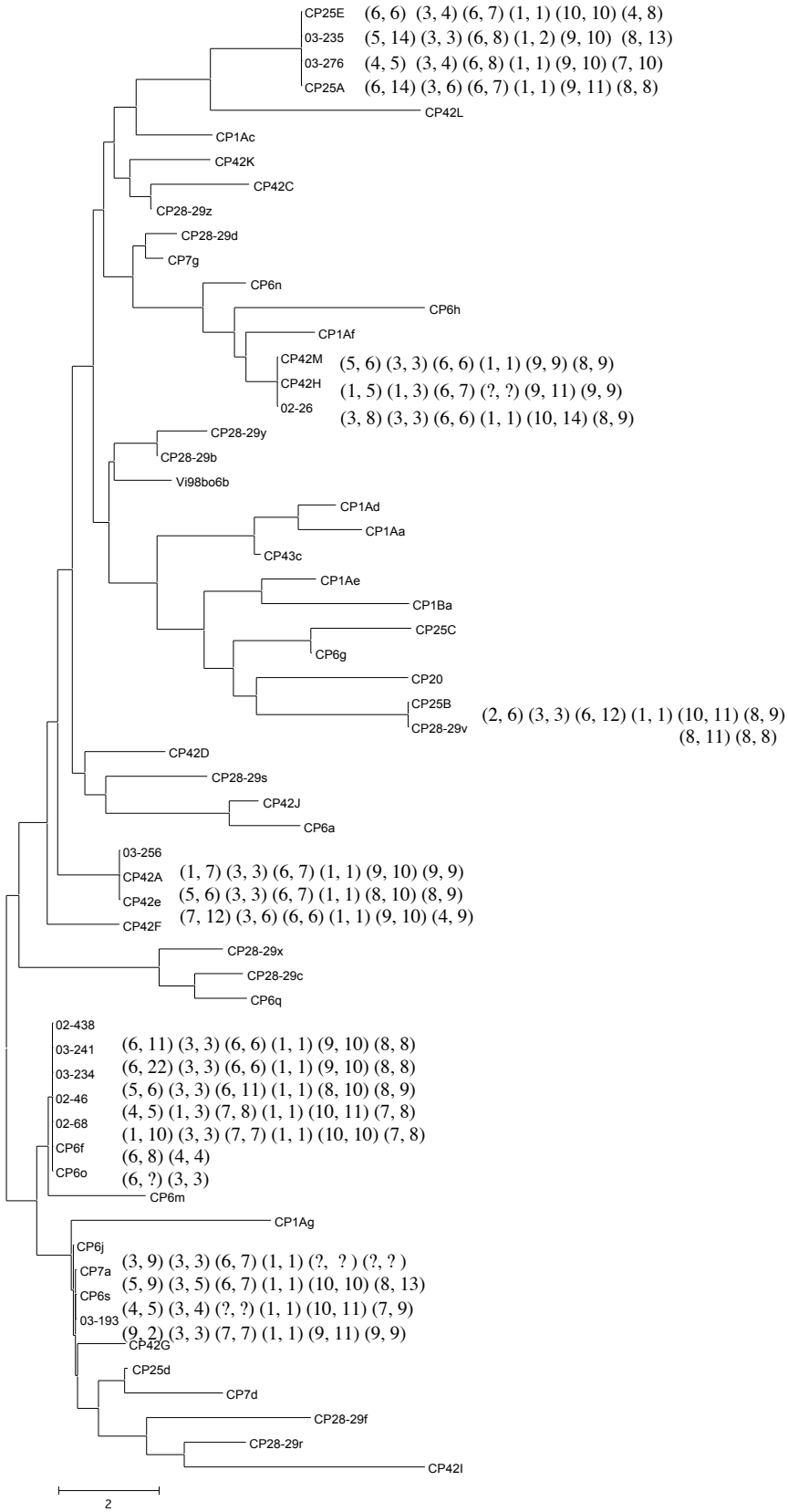
Sub-sample (n)	113	208	125	144	*Ttho-1*	*Ttho-4*
2/13/98 (7)	1 (0.000)	1 (/)	0.441 (/)	-	0.131 (0.007)	1 (/)
2/14/98 (10)	0.253 (0.025)	0.280 (0.012)	0.108 (/)	-	-	0.111 (/)
2/17/98 (5)	0.167 (0.013)	1 (/)	1 (/)	-	0.543 (/)	1 (/)
2/18/98 (9)	0.147 (0.015)	1 (/)	0.046 (/)	1 (/)	0.088 (/)	0.467 (0.011)
2/21/98 (13)	0.755 (0.022)	1 (/)	1 (/)	1 (/)	0.392 (0.015)	0.249 (0.014)

A close inspection of the N-J tree (Figure 4-1) revealed that juveniles sharing the same haplotype corresponded with one exception to individuals collected in the same tow (Table 4-3). Thus, we decided to conduct a combined analysis of mtDNA data with microsatellite data to verify whether such association between maternal lineage and sampling could be evidence of reproductive variance. In order to test whether those fish sharing the same mtDNA haplotype were indeed siblings from the same spawning event, their genotypes were compared across all six microsatellite loci, where available. There were six instances where two juveniles, and in one case three, shared the same haplotype. In all but one of the cases, at least one adult from the Gulf of Mexico also shared the same haplotype (Figure 4-1). Figure 4-1 lists the microsatellite alleles at each of the six loci for those specimens sharing the same haplotype.

Table 4-3 Collection information for those GOG juveniles sharing the same haplotype. The number in parentheses indicates those specimens that share the same haplotype

	Collection date	**Collection time**	**Latitude**	**Longitude**
CP42a (1)	2/21/98	9:30	2°49 N	10°36 W
CP42e (1)	2/21/98	9:30	2°49 N	10°36 W
CP6f (2)	2/14/98	10:00	2°50 N	10°21 W
CP6o (2)	2/14/98	10:00	2°50 N	10°21 W
CP6s (3)	2/14/98	10:00	2°50 N	10°21 W
CP7a (3)	2/15/98	15:00	2°55 N	9°52 W
CP6j (3)	2/14/98	10:00	2°50 N	10°21 W
CP25a (4)	2/17/98	23:00	2°55 N	9°59 W
CP25e (4)	2/17/98	23:00	2°55 N	9°59 W
CP42m (5)	2/21/98	9:30	2°49 N	10°36 W
CP42h (5)	2/21/98	9:30	2°49 N	10°36 W
CP25b (6)	2/17/98	23:00	2°55 N	9°59 W
CP28-29v (6)	2/18/98	20:30	2°51 N	10°32 W

Figure 4-1 N-J phylogenetic tree of control region I sequences constructed in MEGA with number of differences and pairwise deletion. “CP” specimens are GOG juveniles and “02” and “03” specimens are GOM adults. The fragment size for the alleles of each microsatellite locus are listed in Appendix C and are ordered, in parentheses, as follows: 113, 208, 125, 144, *Ttho-1*, and *Ttho-4*. “?” indicates missing data. The complete N-J tree is in Appendix E.


CP25E (6, 6) (3, 4) (6, 7) (1, 1) (10, 10) (4, 8)
03-235 (5, 14) (3, 3) (6, 8) (1, 2) (9, 10) (8, 13)
03-276 (4, 5) (3, 4) (6, 8) (1, 1) (9, 10) (7, 10)
CP25A (6, 14) (3, 6) (6, 7) (1, 1) (9, 11) (8, 8)
CP42L
CP1Ac
CP42K
CP42C
CP28-29z
CP28-29d
CP7g
CP6n
CP6h
CP1Af
CP42M (5, 6) (3, 3) (6, 6) (1, 1) (9, 9) (8, 9)
CP42H (1, 5) (1, 3) (6, 7) (?, ?) (9, 11) (9, 9)
02-26 (3, 8) (3, 3) (6, 6) (1, 1) (10, 14) (8, 9)
CP28-29y
CP28-29b
Vi98bo6b
CP1Ad
CP1Aa
CP43c
CP1Ae
CP1Ba
CP25C
CP6g
CP20
CP25B (2, 6) (3, 3) (6, 12) (1, 1) (10, 11) (8, 9)
CP28-29v (8, 11) (8, 8)
CP42D
CP28-29s
CP42J
CP6a
03-256
CP42A (1, 7) (3, 3) (6, 7) (1, 1) (9, 10) (9, 9)
CP42e (5, 6) (3, 3) (6, 7) (1, 1) (8, 10) (8, 9)
CP42F (7, 12) (3, 6) (6, 6) (1, 1) (9, 10) (4, 9)
CP28-29x
CP28-29c
CP6q
02-438
03-241 (6, 11) (3, 3) (6, 6) (1, 1) (9, 10) (8, 8)
03-234 (6, 22) (3, 3) (6, 6) (1, 1) (9, 10) (8, 8)
02-46 (5, 6) (3, 3) (6, 11) (1, 1) (8, 10) (8, 9)
02-68 (4, 5) (1, 3) (7, 8) (1, 1) (10, 11) (7, 8)
CP6f (1, 10) (3, 3) (7, 7) (1, 1) (10, 10) (7, 8)
CP6o (6, 8) (4, 4)
(6, ?) (3, 3)
CP6m
CP1Ag
CP6j
CP7a (3, 9) (3, 3) (6, 7) (1, 1) (?, ?) (?, ?)
CP6s (5, 9) (3, 5) (6, 7) (1, 1) (10, 10) (8, 13)
03-193 (4, 5) (3, 4) (?, ?) (1, 1) (10, 11) (7, 9)
(9, 2) (3, 3) (7, 7) (1, 1) (9, 11) (9, 9)
CP42G
CP25d
CP7d
CP28-29f
CP28-29r
CP42I
2

Results from the half-sibling likelihood analysis in the Kinship program using the mtDNA haplotype frequencies as well as those for all six microsatellite loci were significant among the members of each group that shared the same haplotype, including the GOM adult specimens. Curiously, there was also significant evidence of kinship between several individuals that did not share the same haplotype. Although polymorphic, the leptokurtotic distribution (Figure 3-1) of the microsatellite allele frequencies, with the exception of locus 113, results in the majority of the fish sharing at least one allele per locus. Accordingly, the estimates of kinship using all seven loci are not informative about the true sibling status between individuals. However, when only the mtDNA and microsatellite locus 113 frequencies are used, significant probability of half-sibship was found only between those individuals sharing the same haplotype.

Discussion

Six microsatellite loci and a segment of the mitochondrial control region were used to examine whether variance in reproductive success could be detected in samples of juveniles collected in the Gulf of Guinea. The data are relevant to issues of stock structure and management of yellowfin tuna but are the result of sampling over a single nine-day period and, therefore, have no temporal implications.

Significant deviations from HWE expectations were only detected in the sample of juveniles. These deviations can be attributed to a deficit of heterozygotes. Often such deficit in microsatellite data is attributed to certain alleles failing to amplify, thus resulting in what could be interpreted as null alleles. Appleyard et al. (2001) reported a very small deviation of HWE for locus 208 and did not discard the possibility of null alleles. However, our adult sample conformed well to HWE for all loci. In contrast, the juvenile sample showed deviations from HWE at locus 125 and heterozygote deficits at loci 125 and 208. One possibility that could

explain such deviations would be the analysis of samples from an effectively small number of breeders at different spawning sites, known as the Allendorf-Phelps effect (Waples 1998), because such sampling does not conform to the assumption implicit to the null hypothesis that individuals sampled were drawn randomly from the global population. In this case, we reasoned that if reproductive variance exists in yellowfin tuna, individual tows would contain juveniles differing significantly in allele frequency from other tows since only a few females would be contributing disproportionately to that school. Accordingly, since juveniles from a particular tow would be more likely to be siblings, combining such heterogeneous samples most likely would not conform to HWE. No significant deviations from HWE were detected within the juvenile sub-samples when separated out by individual tow or date of capture, though the χ^2 value across all sub-populations and all loci was significant before Bonferroni correction. This lack of deviation, however, could be a result of the small sample sizes within each sub-sample.

There were six instances where two juveniles, and in one case three, shared the same haplotype, thus putatively the same mother. In addition, the individuals in three of the pairs shared at least one allele at five or six of the microsatellite loci. The adult specimens that shared the same haplotype with the pairs of juveniles often had at least one allele in common at most of the loci. While the results of Kinship using all seven loci were significant for several pairs of individuals, including ones that did not share the same haplotype, this program does not take into consideration sampling differences, for instance that certain individuals were collected in the same tow. In addition, five of the six microsatellite loci that we employed were not informative for this purpose as the frequencies of two or three alleles accounted for greater than 80% of the total number of alleles scored. Indeed, a significant probability of half-sibship was detected between individuals sharing the same haplotype when only the mtDNA and locus 113 data were

used. Therefore, microsatellite loci with a more even distribution of alleles will be required to adequately validate the half-sibling status of these juveniles.

In terms of Chapman et al.'s (1999) three null hypotheses, we can only reject that of homogeneity between locations for the juvenile sample. The deviation from HWE was highly significant for locus 125 and the heterozygous deficit for loci 125 and 208 was also significant. While the overall χ^2 value, and locus 125 within the 2/18/98 sub-sample, both suggested heterogeneity, neither was significant and, therefore, we cannot reject the null hypothesis of homogeneity within locations. However, the sample size for each sub-sample was extremely small, being no greater than 13. We also could not reject the null hypothesis of linkage equilibria. Disequilibrium was suggested between loci 113 and 208 but, again, this was not significant. Finally, we cannot reject the null hypothesis of homogeneity within and among year classes as these juveniles were collected only over a nine-day period. Accordingly, to properly test the hypothesis of homogeneity, extended sampling over the entire spawning season and across several years would be required, as patterns of genetic relatedness or differentiation that persist across time are unlikely to be caused by sampling artifacts (Garant et al. 2000).

How important or biologically relevant these findings are, depends on the sampling timing, with results from sampling during one of the tails of the spawning curve not being representative of the norm. Spawning occurs in the Gulf of Guinea from January through April, with February 14 used by ICCAT as the approximate mid-point of the peak spawning season (ICCAT 1999), and the juvenile samples were collected in mid-February, right around this mid-point. Levels of reproductive output have not been closely examined for yellowfin tuna in the Gulf of Guinea but given that the sample was obtained nearer the middle of the spawning period than the edge makes it more likely to have been during a peak in spawning than in one of the tails of the curve. In spite of these limitations, the results presented here are likely to be the first

preliminary evidence of a mechanism (Allendorf-Phelps effect) that may lead to reproductive variance in a tuna species or in any other highly migratory pelagic species. Regardless of the extent of reproductive variance, the Allendorf-Phelps signal has implications towards management in light of the intense fishing pressure on immature yellowfin tuna around FADs, with high juvenile mortality and consequent loss of genetic variation due to the removal of highly related units, and deserves further attention.

CHAPTER V

CONCLUSIONS

In this study, we have examined: 1) means of forensically identifying larvae and small juveniles of the genus *Thunnus*, 2) the population structure of yellowfin tuna in the Atlantic Ocean, and 3) potential reproductive variance in this species with the following conclusions:

- The Chelex® protocol is the fastest DNA extraction method and yields high quality and high quantity DNA from well-preserved tissue, other than formalin-fixed.
- Shedlock et al.'s (1997) method for DNA extraction from formalin-fixed tissue yields enough quality and quantity of DNA to sequence with a high success rate. This method also works well for tissue that has not been fixed in formalin by rinsing and binding other potential PCR contaminants.
- The 171 bp cytochrome *b* fragment described in Terol et al. (2002) can distinguish between *Thunnus* and non-*Thunnus* specimens, while the shorter fragment described in Quinteiro et al. (1998) is not as reliable for forensic identification purposes. By contrast, the mitochondrial control region I fragment unambiguously identifies members of *Thunnus*. The control region I can also be used for population genetic studies within and between species of *Thunnus* because of the high gene diversity. Specifically, it can be used for studies examining reproductive variance by providing a potential maternal fingerprint.
- *Thunnus albacares*, *Auxis thazard*, *Katsuwonus pelamis*, and *Euthynnus alletteratus* juveniles are sampled together in the Gulf of Guinea, potentially schooling, and larval *K. pelamis* and *A. thazard* are at least sampled together. In the Gulf of

Mexico, *A. rochei*, *T albacares*, and *T. thynnus* larvae are found together in the same tows.

- No difference was detected between the Gulf of Mexico adults and the juveniles collected in the Gulf of Guinea with either mitochondrial DNA or microsatellites. However, the other known spawning sites and potential sub-populations need to be sampled, and all over several years, to more fully understand the genetic structuring of yellowfin tuna in the Atlantic.
- There is potential significant difference in allele frequencies between Pacific and Atlantic yellowfin tuna at four loci developed by Appleyard et al. (2001). However, differences may be due to mobility shifts between different genetic analyzers employed, precluding definite conclusion about inter-oceanic differences.
- Based on mtDNA data, we provide evidence of a rapid population expansion between 40,000 and 80,000 years ago for yellowfin tuna in the Atlantic. One potential result of this expansion is that it could be masking existing population structure.
- Finally, we present possible evidence of reproductive variance from yellowfin tuna juveniles collected in the Gulf of Guinea. However, increased sample sizes and sample locations, spanning the whole spawning season and extending across several years, need to be analyzed to determine the magnitude of reproductive variance and its potential effects on the genetic diversity of yellowfin tuna.

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APPENDIX A

DNA Extraction Methods

Larvae

Method 2, Phenol-chloroform (Sambrook et al. 1989) One or both of the eyeballs, the posterior half, or the entire larvae was used for DNA extraction from the alcohol-preserved specimens, depending on the size of the individual. The extraction method was the same as above except 50 l of all solutions were used. Once dried, the DNA was resuspended in varying amounts of water from 15-30 l, depending on the amount of tissue used. A very low percentage of the samples extracted by this method yielded PCR products that could be sequenced so other methods were attempted.

Method 3, Small Tissue Extraction (Simpson et al. 1999) DNA was extracted from alcohol-preserved specimens according to Simpson et al. (1999) with a few exceptions. Briefly, the posterior half or the entire larvae, depending on the size, was digested in 30 l extraction buffer (50 mM KCl, 10 mM Tris-HCl pH 8.3, 2.5mM $MgCl_2$, 0.01% gelatin, and 0.9% Tween®20) and 10 l of 30mg/ml pK for 60 minutes at 65°C and denatured for 10 minutes at 94°C.

Method 4, Formalin-Fixed Tissue Extraction (Shedlock et al. 1997) This method (described in Shedlock et al. [1997]) initially entailed three 24-hour washes in excess volumes of 1X GTE (100 mM glycine, 10mM Tris-HCl pH8.0, and 1mM EDTA). Tissue was completely dried and digested in 250 l extraction buffer (1% SDS, 25 mM Tris-HCl pH 7.5, 100 mM EDTA) at 65°C for 24 hours in the presence of a high concentration of pK as well as DTT (dithiothreitol) and RNase. Samples were then extracted three times with buffered phenol and once with 25:24:1 P-C. Cold 100% ethanol was added and the samples were placed at -20°C for 24 hours. The tubes were spun at 14,000xG for 30 minutes, allowed to dry and resuspended in

varying amounts of water, from 15-40µl, depending on the amount of tissue used. This protocol was also used to extract DNA from those samples obtained from Richard Shaw at LSU, as they were initially preserved in formalin.

Juveniles

Method 1, Chelex® (modified from Bell and Grassle 1997)® Tissue from behind the gills was used for DNA extraction from juvenile samples. The end of a pipet tip was melted just enough to make a pestle, which was used to grind the tissue in the presence of 30µl of a 5% Chelex® solution in sterile water. The samples were denatured at 100°C for 10 minutes, treated with RNase at a final concentration of 50µg/ml and incubated for 30 minutes more at 37°C, and finally spun for 30 seconds at 14,000xG.

Method 2, Phenol-chloroform (Sambrook et al. 1989)® Tissue from behind the gills, or one eyeball, was used. The tissue was digested in 100µl of extraction buffer (TENS, 0.2 mg/ml proteinase K [pK]) overnight at 55°C, and treated with RNase as stated above. The digests were extracted once in 100µl buffer-saturated phenol and once in 100µl 25:24:1 phenol:chloroform:isoamyl (P-C) and precipitated with about 1.6M final concentration of ammonium acetate in cold (-20°C) 95-100% ethanol. Samples were stored at -20°C for a minimum of 1 hour, and up to overnight, then spun at 14,000xG for 12 minutes. The supernatant was gently poured off and tubes placed upside down to dry. The DNA pellet was resuspended in 50µl sterile water.

Adults

Small pieces of muscle were sub-sampled from the middle of larger muscle samples to avoid contamination from other individuals sampled at the same time, using knives that were not

cleaned between fish. This tissue was digested and extracted with the phenol-chloroform method as described above for the juvenile tissue with the exception of volume. The extraction procedure followed that of the juveniles except 200 l of each solution was used. DNA pellets were resuspended in 100 l sterile water.

APPENDIX B

MtDNA Amplification and Sequencing

One microliter (μl) of the DNA extractions were used as template in PCR reactions. The universal primers, L15998 and CSBD-H, were used to target a portion of the mtDNA control region (referred to hereafter and in the text at D-loop). PCR was performed in 13 μl reactions with the following final concentrations: 20mM Tris-HCl (pH 8.4), 50mM KCl, 1.5mM $MgCl_2$, 100μM of each dNTP, 15pM of each primer, and 1 unit of Platinum® *Taq* DNA polymerase (Invitrogen). The PCR conditions were optimized in our lab and are as follows: 94°C for 2 minutes; 35 cycles of 94°C for 30 seconds, 55°C for 45 seconds, 72°C for 1 minute; and a final extension at 72°C for 3 minutes.

Amplification of the shorter 126bp and 171bp cyt*b* fragments was conducted according to the methods in Quinteiro et al. (1998) and Terol et al. (2002), respectively, except for the reaction volumes, which were reduced to 12μl of cocktail and 1μl of DNA, with concentrations of all reagents remaining the same.

Successful amplification was determined by running 5μl of PCR product on a 1% agarose gel stained with 0.1 μg/ml ethidium bromide and visualized on a UV light table. The unincorporated primers and dNTPs were removed from the successfully amplified PCR products (amplicons) using ExoSAP-IT® (USB corporation, Cleveland, Ohio). The BigDye® Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer Corporation, Foster City, California) was used for the cycle sequencing according to package instructions. The cycle sequencing products were cleaned using the RapidXtract® Dye Terminator Removal Kit (Prolinx Corporation, Bothell, Washington) and sequenced in an ABI 310 genetic analyzer (Perkin-Elmer Corporation, Foster City, California).

Production of the RapidXtract™ Dye Terminator Removal Kit was halted near the end of this study and the cycle sequencing products from a few of the adult samples, as well as the Gulf of Mexico samples received from Dr. Richard Shaw and SEAMAP, were precipitated with the following method: to the 10µl cycle sequencing reaction product, 1µl of 3M ammonium acetate and 25µl of cold 95% ethanol was added. The tubes were inverted a few times and centrifuged for 25 minutes in a table-top microcentrifuge capable of holding two 8-tube strips. The supernatant was removed and 150µl of 70% ethanol was added. The tubes were again inverted, spun for 10 minutes, and the supernatant removed. The pellets were allowed to air-dry and then resuspended in 25µl of TSR (template suppression reagent). After sitting for a minimum of 5-10 minutes at room temperature, and up to overnight in a refrigerator, the tubes were vortexed and spun briefly, heated for 2 minutes at 95°C, and vortexed and spun briefly again after being chilled on ice. These products were placed directly into the ABI 310 genetic analyzer.

APPENDIX C

Microsatellite amplification and analysis

Four dinucleotide microsatellite loci, primers for which were developed specifically for yellowfin by Appleyard et al. (2001), were analyzed and included loci cmrTa-113, cmrTa-125, cmrTa-144, and cmrTa-208 (referred to in the text as 113,125,144, and 208). A fifth microsatellite loci, cmrTa-161, was excluded because initial screening revealed unacceptable levels of sub-bands or "stuttering" which made it impossible to score the alleles unambiguously. The forward primer of each primer pair was end-labeled with one of three fluorescent tags, FAM, HEX, or TET. Multiplex PCR was set up in 12 µl reactions with the following final concentrations: 10mM Tris-HCl (pH 8.3), 50mM KCl, 2.5mM $MgCl_2$, 100µM dNTP's, and 0.8 µM for each forward and reverse primer (as per Appleyard et al. 2001). For each reaction 1 µl of the DNA template and 0.5 unit of Platinum® *Taq* was used. The PCR conditions also followed those described in Appleyard (2001) as: 93°C for 10 minutes; 55°C for 15 seconds; 72°C for 2 minutes; 35 cycles of 93°C for 15 seconds, 54°C for 15 seconds, and 72°C for 2 minutes; and 72°C for 10 minutes.

Two other dinucleotide microsatellite loci, isolated from Pacific northern bluefin tuna, *Thunnus thynnus orientalis*, were also analyzed as the targeted fragments had previously been shown to amplify in yellowfin (Takagi et al. 1999). These included the *Ttho-1* and *Ttho-4* loci. The *Ttho-6* and *Ttho-7* loci were excluded because neither could be scored unambiguously. The forward primer of each of these primer pairs was also end-labeled with either the FAM (*Ttho-1*) or TET (*Ttho-4*) fluorescent tag. Multiplex PCR was set up in 12 µl reactions with the following final concentrations: 10mM Tris-HCl (pH 8.3), 1mM $MgCl_2$, 50mM KCl, 0.01% gelatin, 0.8µM of each *Ttho-1* primer, and 0.4µM of each *Ttho-4* primer. One-half unit of Platinum® *Taq* and 1 µl of the DNA template were used for each reaction. The PCR conditions followed that

described in Takagi et al. (1999) as: 7 cycles of 94°C for 1 minute, 52°C for 30 seconds, and 72°C for 30 seconds, followed by 33 cycles of 90°C for 30 seconds, 52°C for 30 seconds, and 72°C for 30 seconds.

Following PCR, the amplicons from the adult specimens were diluted 1:10 while the larval and juvenile products were left undiluted. For each reaction, 1 µl of the GeneScan® -500 TAMRA® Size Standard (ABI Prism, Applied Biosystems, Warrington, UK) was mixed with 22 µl of formamide. Twenty-two microliters of this cocktail was mixed with 1 µl of each amplified sample and denatured for 2 minutes at 95°C then placed directly on ice. The products were run in the ABI 310 genetic analyzer and analyzed with the GeneScan 3.7 program (Applied Biosystems, Foster City, CA).

Table A-1. Allele designation for the different-sized fragments at each of the six microsatellite loci.

Allele designation	**113**	**208**	**125**	**144**	***Ttho-1***	***Ttho-4***
1	103	133	148	172	165	124
2	105	135	150	174	167	126
3	107	137	152	176	169	128
4	109	139	154		171	130
5	111	141	156		173	132
6	113	143	158		175	134
7	115	145	160		177	136
8	117		162		179	138
9	119		164		181	140
10	121		166		183	142
11	123		168		185	144
12	125		170		187	146
13	127				189	148
14	129				191	150
15	131				193	152
16	133					154
17	135					156
18	137					158
19	139					160
20	141					162
21	143					
22	145					

APPENDIX D

Results for yellowfin tuna larvae collected in the Gulf of Mexico

A considerable amount of genetic variation was found in the D-loop region of this sample of yellowfin tuna larvae collected in the Gulf of Mexico: 14.1% of the sites were polymorphic, similar to that found in the juvenile sample collected in the Gulf of Guinea. In addition, the gene diversity was 1 (+/- 0.0524) as every haplotype was unique, the nucleotide diversity was 0.0735 +/- 0.0406, the transition-to-transversion ratio was 4.25, and the mean number of pairwise differences within the population was 23.459 +/- 11.416. While each of the haplotypes within this sample was unique, one specimen did share a haplotype with a juvenile collected in the Gulf of Guinea. There was no obvious phylogeographic association of the individual haplotypes from this sample, though three of the D-loop sequences were mapped outside of the close fit of the other haplotypes (differing from the next closest haplotype by ~14 differences), with one of the adult specimens from 2003.

Table A-2 Population comparison parameters as calculated in Arelquin from control region sequences. The population designations are the same as in Table 3-1 except for Larvae, which refers to the yellowfin tuna larvae collected in the Gulf of Mexico. F_{ST} is the amount of variation attributed to differences between the populations and PiXY is the corrected mean number of pairwise differences between populations.

	Adult02:Larvae	Adult03:Larvae	Juv98:Larvae
Pairwise F_{ST}	0.215	0.204	0.236
F_{ST} P values	0.000	0.000	0.000
PiXY	2.516	2.479	2.733
PiXY P values	0.000	0.000	0.000

When the larval data was added as a fourth population to the AMOVA, a significant difference was detected (Table A-3). The F_{ST} value calculated with the larval sample (0.0487) was greater by about a factor of 10 than without it (0.0042). The comparisons between this sample and each of the other three (Table A-2) also differed greatly. These pairwise

comparisons indicate that the larval sample differed from each of the three by about 20%. While only 0.42% of the variation was attributed to differences among the three samples without the larvae, when these specimens were added in this value jumped to 4.09%.

Table A-3 Analysis of molecular variance (AMOVA) results comparing nucleotide sequence data of the mitochondrial D-loop region from all four samples, as calculated in Arlequin.

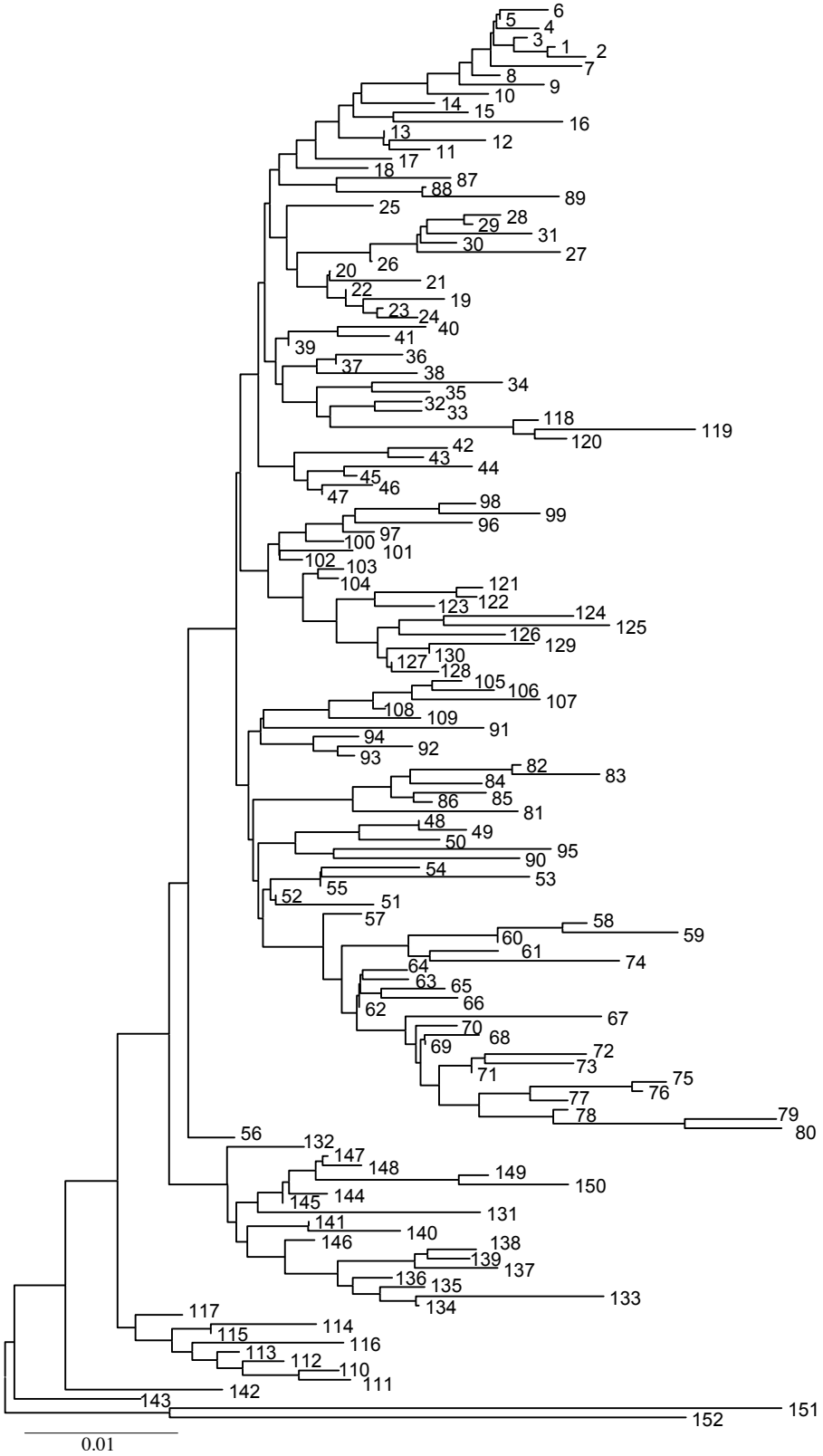
Source of variation	d.f.	Sum of squares	Variance components	% of variation
Among populations	3	47.206	0.23009 Va	4.09
Within population	192	1036.819	5.40010 Vb	95.91
Total	195	1084.025	5.63019	
Fixation Index F_{ST}=	0.0487			
Va and F_{ST}: p=	0.000 +/- 0.000			

The microsatellite data from the larval sample also differed from that of the other three. All six loci were polymorphic within this sample. However, while the allele frequencies among the juvenile and two adult samples were relatively close, several new alleles were introduced in the larval sample and the frequencies of the shared alleles were different (data not shown). GenePop could not estimate genic differences between samples for each loci, or χ^2 across all loci, because of the missing data at several loci in several specimens. Estimates of the population expansion parameters were possible and are shown in Table A-4. While Fu's F_S test was negative it was not significant as it was for each of the other three samples and for the pooled data.

Table A-4 Summary of θ estimations and selective neutrality tests as estimated in Arelquin with D-loop sequences from the larvae collected in the Gulf of Mexico. Values in parentheses for the θ estimates are standard deviations and p-value for Fu's test.

Estimates/Tests	Larvae
Fu's θ estimate	15.934 (9.175)
Tajima's θ estimate	14.717 (7.469)
Fu's F_S Test	-1.821 (0.117)

APPENDIX E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Publications:

Jessica A. Gorski, Tiffany Talley, Mengsheng Qiu, Luis Puelles, John L. R. Rubenstein, and Kevin R. Jones. 2002. Cortical excitatory neurons and glia, but not GABAergic neurons, are produced in the Emx1-expressing lineage. Journal of Neuroscience 22: 6309-6314.

Presentations:

Tiffany Talley Farnham, Bernard Stequert, and Jaime R. Alvarado Bremer. Preliminary analysis of the comparison in levels of variation between juvenile and adult yellowfin tuna samples from the Atlantic Ocean using both mtDNA and microsatellite data. International Commission for the Conservation of Atlantic Tunas (ICCAT) Yellowfin Tuna Species Group Meeting, July 21-26, 2003 Merida, Mexico.

Alvarado, J. R and T. Farnham. Genetic characterization of fish populations: What DNA can (and can't) tell you about the biogeography, reproductive biology and phylogenetics of tunas and billfishes. Texas Chapter of the American Fisheries Society, Annual Meeting 2003, January 28, 2003. Galveston, Texas.