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A Molecular Technique for Specific Identification of Western Atlantic Ocean Scombrids and an Analysis of a Larval Scombrid Assemblage off the Kona Coast of Hawaii

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A MOLECULAR TECHNIQUE FOR SPECIFIC IDENTIFICATION OF WESTERN
ATLANTIC OCEAN SCOMBRIDS AND AN ANALYSIS OF A LARVAL
SCOMBRID ASSEMBLAGE OFF THE KONA COAST OF HAWAII

A Thesis

Presented to

The Faculty of the School of Marine Science

The College of William and Mary in Virginia

In Partial Fulfillment

Of the Requirements for the Degree of

Master of Science

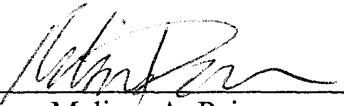
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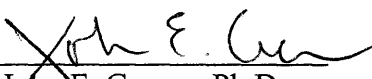
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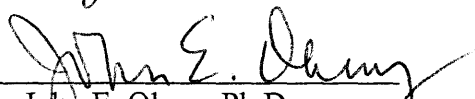
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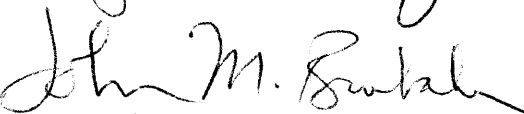

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ABSTRACT

The identification of scombrid fishes (e.g. tunas, mackerels, bonitos) is difficult at early life history stages. Molecular markers provide a means for positive identification when diagnostic morphological characters are not present or difficult to interpret. This project was undertaken to develop a molecular marker that could distinguish among scombrid species at any life history stage or physical condition.

The first chapter of this thesis describes the evaluation of the mitochondrial cytochrome *c* oxidase subunit I (COI) gene as a molecular marker for the specific identification of the 17 members of the family Scombridae common to the western Atlantic Ocean. A 950 base pair region in the COI gene was sequenced from up to 20 individuals of each species, and suites of nucleotide polymorphisms that unambiguously distinguish among these scombrid species were identified. A shorter 250 base pair fragment of COI proved to be sufficient for species identification and was better suited for analyzing degraded tissue samples. The utility of the COI marker was demonstrated with the specific identification of scombrid larvae collected in the Florida Straits and scombrid remains from the stomachs of large pelagic predators.

The second chapter of this thesis describes the application of the shorter COI fragment to identify the scombrid larval assemblage off the Kona Coast of Hawaii, 19 to 26 September 2004. Molecular and morphological techniques were used to determine species composition of scombrid larvae taken in 43 ichthyoplankton tows conducted in upper surface waters (10 and 14 m), primarily at night. All 872 scombrid larvae collected were identified to species, 29% unambiguously from morphological criteria and the remaining 71% were identified using the short COI fragment. Yellowfin tuna and skipjack tuna dominated the larval composition almost equally, with frequencies of 48% and 45%, respectively, and 5% of the larvae were identified as albacore. The frequency of albacore is higher than that reported in previous studies of scombrid larval assemblages around the Hawaiian Islands, and indicates increased spawning in this area.

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INTRODUCTION

Scombrid background

Scombrid fishes (e.g. tunas, mackerels, bonitos) are important worldwide for their economic and ecological value. The family Scombridae comprises 50 species of epipelagic fishes in 15 genera (Collette et al. 2001). The diagnostic characters for the family are an elongate and fusiform body, five to ten finlets behind the dorsal and anal fins, and at least 2 caudal keels (Collette 2003). This family is divided into four tribes, in order from more primitive to advanced: Scombrini, Scomberomorini, Sardini, Thunnini (Collette and Nauen 1983). An important morphological adaptation in the Thunnini is a countercurrent heat exchange system in the circulatory system that allows the fish to maintain body tissue temperatures warmer than the surrounding water (Collette et al. 2001). It has been suggested that this endothermic capacity has allowed niche expansion of the species in this tribe into cooler waters and facilitated a cosmopolitan distribution (Collette and Nauen 1983; Block et al. 1993). Reproduction in the Scombridae occurs via batch spawning in tropical and subtropical waters, with pelagic eggs that hatch into planktonic larvae (Collette and Nauen 1983).

Scombrid fisheries

Many scombrid species support lucrative fisheries in the Atlantic and Indo-Pacific oceans. Tunas (bluefin, albacore, yellowfin, bigeye, skipjack) are most commonly taken with longline, purse seine or pole-and-line gear (NMFS 1999a; NMFS 1999b). In the Atlantic Ocean, bluefin tuna, albacore, and bigeye tuna are currently assessed as overfished and yellowfin tuna are considered fully fished (NMFS 1999a). In the Pacific Ocean, bigeye tuna have been assessed as fully fished, while yellowfin tuna are regarded as fully fished in the eastern-tropical Pacific (NMFS 1999b). Because most scombrids are highly migratory species, international cooperation is necessary for effective management. The Inter-American Tropical Tuna Commission (IATTC) is an international organization responsible for management of tuna fisheries in the eastern Pacific Ocean (east of 150W) and management for the rest of the Pacific is under the purview of the Commission for the Conservation and Management of Highly Migratory Fish Stocks in the Western and Central Pacific Ocean (WCPFC). Within the Atlantic Ocean, the International Commission for the Conservation of Atlantic Tunas (ICCAT) provides recommendations for the management of tunas.

Effective management of scombrid stocks requires a thorough understanding of their biology. Recruitment can be quite variable in these species, so knowledge of the spatial and temporal distribution of spawning, as well as those factors that affect survival of early life history stages, is essential for proper management. Such studies require accurate identification of eggs, larvae and juveniles.

Scombrid identification

Because of their importance, scombrids have been well studied morphologically and specific identification at the adult level is unambiguous (Collette and Nauen 1983). The only difficulty in identification at the adult stage is with the two species of *Auxis* that are usually identified only to genus level (Richards 2006). However, early life history stages (eggs, larvae, juveniles) of this family are difficult to identify. Even though there are identification guides for nearly all scombrid larvae (Richards 2006; Nishikawa and Rimmer 1987), identification of this stage is difficult using morphological criteria, especially larvae in the genus *Thunnus*. Many studies of larval scombrids have been constrained by inability to identify all specimens due to damaged condition or limitations of morphological identification criteria (Boehlert and Mundy 1994; Beckley and Leis 2000). Furthermore, identification of juvenile or adult scombrids is not possible in situations where diagnostic morphological features are not recognizable, such as a fillet or degraded tissues in predator stomachs.

Molecular markers

Molecular markers can provide a means for positive identification when morphological characters are not present or difficult to interpret. Various molecular markers (allozymes, PCR-RFLP (polymerase chain reaction/ restriction fragment length polymorphism), multiplex assay, sequence analysis, microsatellites) have been employed in numerous fish identification studies (Morgan 1975; Daniel and Graves 1994; Rocha-Olivares 1998; Kirby and Reid 2001; McDowell and Graves 2002; Delghandi et al. 2003; Hyde et al. 2005; Perez et al. 2005). While each of these molecular techniques has

advantages and disadvantages, the advent of PCR-based methods has greatly expanded the ease, cost-effectiveness, and amount of information yielded from molecular analyses. PCR-RFLP analysis of an amplified gene region is practical to use, but it becomes more difficult with increasing number of species to find unique or unambiguous fragment patterns that will identify each species. Sequencing allows for one to distinguish among many species because individual nucleotides are surveyed, greatly increasing genetic resolution, and therefore the number of species-specific characters. The mitochondrial (mtDNA) genome has been favored for molecular analyses, including sequence analysis, because it is haploid and sequencing can be done directly. In this study, a portion of the mitochondrial genome was sequenced for the purpose of specific identification of the 17 scombrids occurring in the western Atlantic.

Objectives

The first objective was to develop a molecular marker for the specific identification of the 17 scombrids common to the western Atlantic Ocean. The ability of the mitochondrial gene cytochrome *c* oxidase subunit I (COI) to discriminate among these species was evaluated by sequencing this region in up to 20 reference samples of each of the 17 species. Degraded tissues may have fragmentary DNA, and so the shortest section necessary to be diagnostic was determined. This marker was then applied in situations where morphological identification of putative scombrids was problematic or impossible, such as with early life history stages and degraded tissues in stomach contents. The second objective was to apply this molecular marker in addition to morphological criteria to assess the species composition of the scombrid larvae collected

off the Kona coast of Hawaii, 19 to 26 September 2004. As many larvae as possible were identified morphologically, and the remainder were analyzed using the COI sequence analysis method. The species composition was compared to other studies of larval scombrids performed in Hawaiian waters that only used morphological criteria for larval identification.

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CHAPTER 1. Specific identification of western Atlantic Ocean scombrids using mitochondrial DNA cytochrome *c* oxidase subunit I (COI) gene region sequences

INTRODUCTION

Members of the family Scombridae (tunas, skipjack tuna, mackerels, bonitos, etc.) are important components of pelagic ecosystems, with several species supporting large commercial and recreational fisheries throughout the world's oceans. Proper identification of these species at all life stages and in various conditions, such as degraded stomach contents, is essential for effective management and to better understand early life history characteristics and ecological relationships in the pelagic ecosystem. In addition, specific identification of processed tissues or fillets is necessary for enforcement of fisheries management regulations.

While specific identification of adult scombrids is essentially unambiguous (Collette and Nauen 1983), identification is problematic in situations where morphological characters are difficult to interpret (early life history stages) or missing (fillets, digested stomach contents). Identification of early life history stages of scombrids has been especially challenging. Scombrid eggs are very similar in appearance and can only be separated by pigment characters that become lost after preservation (Richards 2006). Larvae of the genus *Thunnus* are particularly difficult to identify. Specific identification of these larval stages requires clearing and staining for vertebral precaudal/caudal count and position of the first closed hemal arch, and *T. albacares* and *T. obesus* can only be separated by pigment presence or absence (Richards

2006). Juvenile stages (15-60 mm SL) of *Thunnus* species cannot be identified unambiguously because the development of body pigmentation obscures diagnostic larval characteristics, and meristic counts are broadly overlapping (Nishikawa and Rimmer 1987).

Molecular markers can provide a means for positive identification when morphological identification is uncertain. Various molecular markers have been used to identify fish eggs and larvae including allozymes (Morgan 1975), polymerase chain reaction (PCR)/ restriction fragment length polymorphism (RFLP) analysis (Daniel and Graves 1994; McDowell and Graves 2002), multiplex PCR (Rocha-Olivares 1998; Hyde et al. 2004) and sequencing (Kirby and Reid 2001; Perez et al. 2005). Many of these techniques have been used to identify scombrids. Allozymes have been successfully used to discriminate between early juveniles of *T. obesus* and *T. albacares* (Graves et al. 1988) as well as between adult Pacific northern bluefin tuna (*Thunnus thynnus orientalis*) and southern bluefin tuna (*Thunnus maccoyii*) (Ward et al. 1995). Several studies have used PCR/RFLP analysis to identify species of the scombrid tribes Thunnini and Sardini (Chow et al. 2003) as well as eight species of the genus *Thunnus* (Chow and Inoue 1993). In addition, sequencing of a mitochondrial gene region has been used to identify *Thunnus* species (Bartlett and Davidson 1991; Ram et al. 1996; Quintero et al. 1998; Terol et al. 2002). While each of these techniques has advantages and disadvantages, sequencing provides the highest level of resolution as it shows genetic differences at the nucleotide level. While a few studies have used sequence analysis to identify scombrids, these investigations were limited as they only distinguished between a few species, used a region that revealed considerable intraspecific variation, had limited sample sizes, or

encountered problems with non-specific amplification (Bartlett and Davidson 1991; Ram et al. 1996; Quintero et al. 1998; Terol et al. 2002).

The mitochondrial genome has been preferred for analysis in many genetic studies as it has a high number of copies per cell which facilitates PCR amplification, and because the presence of a single allele makes it possible to sequence products directly (Awise 1994). Many mitochondrial gene regions (cytochrome b, ND4, 16S, COI) have been successfully used for fish identification studies (Bartlett and Davidson 1991; McDowell and Graves 2002; Hyde et al. 2005; Lopez and Pardo 2005). These gene regions display different levels of genetic variation as a result of different evolutionary rates. While variation is necessary to highlight interspecific differences, too much variation can be problematic for primer design. Because of this, the use of a conserved region is advantageous for effective amplification across many species.

One of the most conserved protein-coding genes in mitochondrial (mt) DNA is cytochrome *c* oxidase subunit I (COI) (Brown 1985). COI is critical for cellular energy production and this functional importance constrains its evolution (Rawson and Burton 2002). The high level of conservation of COI allows for the design of a unique primer pair that successfully amplifies the same fragment across the diverse members of the Scombridae. Previous work has taken advantage of COI for broad taxonomic studies (eleven invertebrate phyla, Folmer et al. 1994; 11 animal phyla, Hebert et al. 2003), but COI has also been useful to distinguish closely related genera in species identification (3 copepod genera, Bucklin et al. 1999). Because COI is informative for distinguishing species across and within many different taxa, it is well suited for identification across a family as diverse as the Scombridae. In this study, a molecular key is developed based

on the mitochondrial COI region for the specific identification of the 17 scombrids present in the western Atlantic Ocean.

MATERIALS AND METHODS

Tissue samples were obtained from up to 20 specimens of each of the 17 scombrid species from the western Atlantic Ocean: *Acanthocybium solandri*, *Auxis rochei*, *A. thazard*, *Euthynnus alletteratus*, *Katsuwonus pelamis*, *Sarda sarda*, *Scomber colias*, *S. scombrus*, *Scomberomorus brasiliensis*, *S. cavalla*, *S. maculatus*, *S. regalis*, *Thunnus alalunga*, *T. albacares*, *T. atlanticus*, *T. obesus* and *T. thynnus*. All specimens were identified based on morphological characters. Tissue samples were either stored in DMSO buffer (Seutin et al. 1991) or frozen. Published COI sequences of *A. thazard* and *A. rochei* (Infante et al. 2004) were used to supplement the number of samples for these species. Collection information is provided in Table 1.

To evaluate the efficacy of COI as a marker to identify scombrids, specimens of larval scombrids stored in ethanol were obtained from David Richardson and Robert Cowen, Rosenstiel School of Marine and Atmospheric Science, University of Miami. In addition, stomach content samples containing putative scombrids were collected from blue marlin and white marlin caught in recreational fishing operations out of Cape May, NJ, USA and La Guaira, Venezuela. Putative scombrids were removed from the marlin stomachs dockside and rinsed with water. Either a muscle sample was removed and placed in DMSO buffer (Seutin et al. 1991) or the whole fish was frozen until analysis.

Total genomic DNA was extracted from adult tissues of known scombrid species using a standard phenol/chloroform isolation protocol (modified from Sambrook and Russell 2001). A series of extractions was performed on each sample using equilibrated phenol, followed by phenol: chloroform: isoamyl alcohol (25:24:1) and finally chloroform: isoamyl alcohol (24:1). Following extraction, DNA was precipitated with ethanol. For larval fishes, one eyeball (right eyeball when available) was removed and rinsed with distilled water. DNA was extracted from this tissue using proteinase-K and Chelex beads (Bio-Rad Laboratories, Hercules, CA) (Estoup et al. 1996). Each larva was photographed using a digital camera attached to a stereomicroscope via a photo tube, capturing as much detail as possible for future morphological or meristic analysis.

Primers that amplify the COI gene region across the scombrid family were designed using conserved regions of seven scombrid COI sequences (*Auxis rochei*, *A. thazard*, *Euthynnus alletteratus*, *Katsuwonus pelamis*, *Scomber scombrus*, *Thunnus alalunga*, and *T. thynnus*) available through GenBank (National Center for Biotechnology Information). Two sets of primers were developed that amplify a ~950 base pair (bp) fragment (long fragment) of the COI gene and a ~250 bp fragment (short fragment) located within the 950bp fragment:

950bp fragment:	LCOI 121	CTA AGC CAA CCA GGT GCC CTT CT
	HCOI 1199	AAT AGT GGG AAT CAG TGT ACG A
250bp fragment:	LCOI 646	AAT ACA ACC TTC TTC GAC C
	HCOI 947	GTT GGA ATT GCG ATA ATC

(The number in the primer name designates the position of the 5' end within the COI gene (1550bp)). All primers were ordered from Invitrogen Corporation (Carlsbad, California).

Polymerase chain reactions were performed on each known or putative scombrid sample. Each 25 μ L reaction consisted of 0.25 μ L template DNA, 2.5 μ L 10X PCR Buffer plus magnesium (QIAGEN, Inc., Valencia, CA), 0.5 μ L 10mM dNTP (QIAGEN), 0.25 μ L forward primer (100pm/ μ L), 0.25 μ L reverse primer (100pm/ μ L), 0.125 μ L *Taq* DNA polymerase (QIAGEN), 5.0 μ L BSA (bovine serum albumin) (1mg/mL) and 16.125 μ L sterile water (modified from McDowell and Graves 2002). Amplifications using Chelex extractions contained 2.5 μ L DNA template. Reactions were carried out in an MJ Research Corporation PTC-200 Peltier thermal cycler (Watertown, MA) under the following conditions: initial denaturation at 94°C for 4 minutes, followed by 35 cycles of 94°C for 1 min., 57°C for 1 min., 72°C for 2 min., a final extension at 72°C for 5 min., and final hold at 4°C. Amplifications done with the LCOI 646/ HCOI 1085 primers used an annealing temperature of 54°C, but were otherwise run using the same conditions.

Sequencing was performed on either gel-based or capillary-based automated sequencers. For gel-based sequencing, purified PCR (using ExoSAP; USB Corporation) products were cycle sequenced using a Thermo Sequenase Primer Cycle Sequencing Kit (Amersham Biosciences, Piscataway, NJ) and loaded onto a Li-Cor NEN IR² 4200 global sequencing system (Li-Cor, Lincoln, NE). The sequencing program eSeq version 2.0 was used to read sequences and to check base calls. For capillary-based sequencing, purified PCR products were cycle sequenced using a 1/8 dilution of the manufacturer's (Applied Biosystems BigDye) sequencing reaction protocol for a 5 μ L reaction: 0.25 μ L BigDye

reagent, 0.875 μ L 5X BigDye Buffer, 0.32 μ L primer, 1.0 μ L template (10-40ng for 1000bp product), 2.55 μ L water. The sequencing reaction products were loaded onto an ABI 3100 capillary sequencer (Applied Biosystems, Foster City, CA) and analyzed using the program Sequencing Analysis 5.1.1.

Both primer pairs successfully amplified samples taken from known scombrids. The longer fragment was used to generate information on the reference samples to identify those sites that discriminated between species. The internal primer pair was designed to amplify the shortest possible fragment that included informative sites. For the “unknown” samples (larvae, stomach contents), PCR was performed using the shorter primer pair. If the shorter primer pair generated an amplicon that differed in size from the targeted fragment, the sample was inferred to be a non-scombrid. In cases where the shorter primer pair did not generate a PCR product, universal COI primers (Folmer et al. 1994) were used as a positive control. If the universal primers generated an amplicon, I concluded the sample was a non-scombrid. If the universal primers did not result in a successful amplification, the sample was considered too degraded for analysis.

All sequences were edited using Sequencher version 4.2.2. Edited reference sequences (long fragment) of each species were aligned using the ClustalW program in MacVector version 7.2 to assess intraspecific variation. A consensus sequence of all haplotypes was generated for each species and these representative sequences were aligned to reveal informative interspecific differences using the program MEGA version 3.0 (Kumar et al. 2004). MacClade v. 4.07 (Maddison and Maddison 2005) was used to assess variability at each base position. For unknown samples, the species identity was

inferred by noting where the sample sequence clustered in a UPGMA tree using absolute number of differences between the consensus sequences.

Preliminary analyses suggested either misidentification or introgression in one sample identified as *S. regalis* based on morphology. To discriminate between misidentification and introgression between *S. maculatus* and *S. regalis*, the nuclear ITS-1 region was analyzed in four samples of each of these two species using the primers F-ITS-1 (5'GAG GAA GTA AAA GTC GTA ACA AGG3') and 5.8SR2 (5'GTG CGT TCG AAR KGT CGA TGA TCA AT3') (K. Johnson, Virginia Institute of Marine Science, unpublished). PCR products were cloned into the pCR4-TOPO vector (Invitrogen Corporation, Carlsbad, California) and three clones from each sample were sequenced. This fragment was amplified and sequenced as previously described for the long COI fragment, using the capillary sequencer. The only differences from the COI protocol were that 5 μ L of Q solution was used in the 25 μ L reaction and the annealing temperature used in the PCR was 45°.

RESULTS

Two amplicons were generated in this study, a long (945bp) and a short (264bp) fragment of the COI gene. Within the long fragment there were 279 (30%) variable sites and in the short fragment there were 64 (24%) variable sites. The vast majority (93.7%) of substitutions occurred at the third codon position, while only 5.7% occurred at the first position and 0.7% at the second position. There were no insertions or deletions within the COI regions analyzed.

The long fragment of COI exhibited a wide range of differences between the 17 species analyzed. The number of nucleotide differences between consensus sequences of each species ranged from 2 base changes (between *T. obesus* and *T. albacares* or *T. atlanticus*) to 152 (between *Scomber scombrus* and *Scomberomus cavalla*) (Table 2). The differences between species in the short fragment ranged from 1 base change (between *T. obesus* and *T. atlanticus* and between *S. maculatus* and *S. regalis*) to 48 (between *S. colias* and *A. thazard*). Within species, variation of the long fragment ranged from 0 in *Scomberomus brasiliensis* and *S. regalis* to 26 variable sites within *Sarda sarda*. Reference samples of *K. pelamis*, *T. albacares*, *T. obesus*, *A. rochei* and *A. thazard* included Atlantic and Pacific individuals, and thus the intraspecific variation observed in these species encompassed any inter-oceanic differences.

The positively identified samples of a species consistently grouped together in a UPGMA tree of all COI sequences in this study. A consensus sequence was generated for each species to serve as a representative of that species in a reference UPGMA tree (Fig. 1). A single *Scomberomorus regalis* sample had a COI sequence that was more similar to *Scomberomorus maculatus*. However, the ITS-1 sequence data indicated that the anomalous sample was indeed *S. regalis* suggesting introgression. The COI sequence of this sample was not included in the consensus sequence of *S. regalis*. The differences between these two species in the ITS-1 region are shown in Fig. 2.

From the consensus sequences, an unambiguous molecular key was developed that allowed identification of all 17 western Atlantic scombrids. Positions at which a species has a consistent, unique combination of nucleotide base pairs are indicated in Fig. 3. The shorter COI fragment also provided dependable species identification as it includes diagnostic sites. Clustering of an unidentified sample in the UPGMA tree was the quickest method of identification, but in cases where an individual did not clearly group with one species, discriminatory base positions were located in the unknown sequence and compared with the molecular key for identification.

Scombrid larvae from the Florida Straits in the western Atlantic Ocean were used to test the efficacy of this marker. These individuals were sufficiently large (4.5-12 mm) to be identified morphologically to genus; however, some were damaged, making specific identification based on morphological characters difficult if not impossible. Fifty-two scombrid larvae were identified based on DNA sequence and, when possible, using morphological characters following Richards (2006) and Nishikawa and Rimmer (1987). From these guides, the most useful morphological characters were: forebrain

pigment and ventral pigment spot in *K. pelamis*, lower jaw pigmentation in *E. alletteratus*, and lateral tail pigmentation in *A. thazard*. Morphological identification to species level was possible for 18 larvae, and in each case, morphological assignment supported genetic identification. The remaining 34 unknown larvae were identified to species solely by noting their placement with known samples in a UPGMA tree (Fig. 4).

To test the efficacy of the marker on degraded tissues, the short fragment was amplified from putative scombrids found in billfish stomach contents. The shorter fragment of COI was analyzed in the stomach contents as these tissues are generally deteriorated and therefore the DNA may also be degraded. When the sequences of these COI fragments were aligned to the known reference samples, nine samples clustered with *Auxis rochei* in the UPGMA tree (Fig. 5). Two samples from billfish stomach caught in Hawaii did not cluster with any of the scombrid species. The search engine Basic Local Alignment Search Tool (BLAST) (National Center for Biotechnology Information) was used to find the closest match between these samples and species in GenBank (National Center for Biotechnology Information). One sample shared 84% identity with the COI gene of *Myripristis berndti* (blotcheye soldierfish) and the other sample had 83% identity with the COI gene of *Hoplostethus japonicus* (flintperch).

DISCUSSION

Both the long and short COI fragments met the two requirements of a good molecular marker for scombrid identification: consistent interspecific differences and minimal intraspecific variation. Fixed differences between species in the long COI fragment range from 2 to 152 base pair differences. The short fragment is also sufficient for specific identification as it contains diagnostic differences.

This molecular key was developed to unambiguously identify all scombrids occurring in the western Atlantic Ocean, several of which have a circumtropical distribution. To evaluate the applicability of the marker outside the Atlantic, several Pacific conspecifics of some of the circumglobal species covered in this study were sequenced to identify any inter-ocean basin intraspecific differences. Previous studies have shown evidence of inter-oceanic differences in bigeye tuna and albacore based on other gene regions (Chow and Ushiyama 1995; Alvarado Bremer et al. 1998; Chow et al. 2000). The diagnostic sites still allowed for unambiguous identification of circumglobal species between ocean basins which is not unexpected given the high level of conservation in COI. The success of this marker in other ocean basins suggests that its utility may easily be extended to other scombrid species that occur elsewhere.

While the COI region has proven to be effective for species identification of scombrids, like other molecular markers it has limitations. Sequencing has drawbacks

including limitations imposed by cost and time, but this technology is being improved upon continually, making it quite an attractive high resolution technique for species identification. An alternative method using PCR/RFLP analysis of an amplified gene region (Chow and Inoue 1993; Daniel and Graves 1994; McDowell and Graves 2002; Chow et al. 2003) is practical, but it becomes more difficult with increasing number of species to find unique or unambiguous fragment patterns that will distinguish each species. Similarly, a multiplex assay increases speed and decreases cost of analysis, but requires the design of many species-specific primers which would be challenging given the number of species in this study.

Another concern with using only a mitochondrial marker is the possibility that introgression may lead to the misidentification of samples. Mitochondrial introgression has been previously reported in scombrids. The mitochondrial genome of the albacore, *Thunnus alalunga*, has introgressed onto the Pacific bluefin tuna *Thunnus orientalis* genetic background within the Pacific at a high frequency (98%) (Chow and Kishino 1995) and at a low frequency (5%; 6.8%) in the eastern Atlantic/ Mediterranean in Atlantic bluefin tuna *T. thynnus* (Vinas et al. 2003; Carlsson et al. 2004). Additionally, mitochondrial introgression has also been reported in the genus *Scomberomorus*. Banford et al. (1999) posited that the *S. regalis* mitochondrial genome has introgressed into *S. maculatus*. In the present study, one *S. regalis* sample clustered with *S. maculatus*, suggesting either misidentification or introgression. Results of this study indicate that introgression may be bidirectional, as the observed introgression is in the opposite direction as that seen in the study by Banford et al. (1999). Clearly there is a need for further analysis, including more *S. regalis* samples to adequately resolve this

issue. Until then, a nuclear marker should be employed in addition to the COI marker to verify species identity of any putative *S. regalis* or *S. maculatus* samples.

The demonstrated ability of this key to provide species identifications of scombrid larvae and scombrid remains in stomach contents indicates its potential for use in population studies, forensic analyses and early life history investigations. This marker has numerous applications, from verifying that samples are indeed the correct species in population studies employing analysis of nuclear microsatellite loci, to providing species level identification of fillets that are being sold illegally, which is critical for management enforcement (Lopez and Pardo 2005). In cases where morphological identification is not possible, a molecular key provides a reliable means of unambiguously identifying scombrid species.

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Table 1. Scombrid reference sample collection location and sample size.

Species name	Common name	Abbreviation	Catch location (no. of samples), date	Sample size
<i>Acanthocybium solandri</i>	Wahoo	ASOL	Cape May, New Jersey (12), 2004; Gulf of Mexico (5); Isla Mujeres, Mexico (1)	18
<i>Auxis rochei</i>	Bullet tuna	AUXR	Virginia (4), 2004; Philippines (1), 2003; Pacific (3) and Atlantic/ Mediterranean (8) from Infante et al. 2004.	16
<i>Auxis thazard</i>	Frigate tuna	AUXT	Panama, Pacific (5), 2004; Philippines (1), 2003; Seychelles (1), 2002; Spain (3) from Infante et al. 2004.	10
<i>Euthynnus alletteratus</i>	Little tunny	EUTH	Isla Mujeres, Mexico (10)	10
<i>Katsuwonus pelamis</i>	Skipjack tuna	SKJT	Virginia (16), 2004; Hawaii (3), 2004	19
<i>Sarda sarda</i>	Atlantic bonito	SARD	Mid-Atlantic Bight (1), 1994; Mediterranean (2), 2002	3
<i>Scomber colias</i>	Chub mackerel	CHMK	Gulf of Mexico (10); Virginia (4), 2004; Argentina (3); Ivory Coast (3), 1992	20
<i>Scomber scombrus</i>	Atlantic mackerel	ATLM	Massachusetts (10); Plymouth, England (10)	20

Species name	Common name	Abbreviation	Catch location (no. of samples), date	Sample size
<i>Scomberomorus brasiliensis</i>	Serra Spanish mackerel	SCBR	Brazil (2); Trinidad (1); Saba (1), 2006	4
<i>Scomberomorus cavalla</i>	King mackerel	KMCK	Panama City, Florida (2), 1994; South Carolina (2), 1996; Virginia (3), 2005; Maryland (1), 2005; Keys, Florida (1), 2006	9
<i>Scomberomorus maculatus</i>	Spanish mackerel	SPMK	South Carolina (13), 1997; Chesapeake Bay (5), 1994; Gulf of Mexico (2)	20
<i>Scomberomorus regalis</i>	Cero	CERO	Bahamas (2); Colombia (1)	3
<i>Thunnus alalunga</i>	Albacore	ALBC	Mid-Atlantic Bight (3), 2004; Atlantic (12); North Carolina (2), 2005	17
<i>Thunnus albacares</i>	Yellowfin tuna	YFT	Mid-Atlantic Bight (14), 2004; Hawaii (4), 2004	18
<i>Thunnus atlanticus</i>	Blackfin tuna	BLKF	Isla Mujeres (6), 2004; Florida Straits (5), 2004; North Carolina (7) 2005	18
<i>Thunnus obesus</i>	Bigeye tuna	BET	Florida Straits (14), 2004; Hawaii (4), 2004	18
<i>Thunnus thynnus</i>	Atlantic bluefin tuna	BLFT	Ocean City, Maryland (14), 2003; Spain (2), 1998; Mediterranean (2), 1998; New Jersey (2)	18

Table 2. Pairwise distances between western Atlantic scombrids based upon number of nucleotide differences for consensus haplotype *COI* sequences of each species. Differences in the longer fragment (945bp) are shown in the lower left portion of the matrix and differences in the shorter fragment (260bp) are shown in the upper right. The column labeled “Intra” contains the number of variable sites in the long fragment within each of the species in that row. (MEGA version 3.0 Kumar, Tamura, Nei 2004)

	ALBC	BLFT	BET	YFT	BLKF	AUXT	AUXR	EUTH	SKJT	SARD	ASOL	CHMK	ATLM	KMCK	SPMK	CERO	SCBR	Intra
ALBC																		
BLFT	7																	
BET	5	6																
YFT	2	4	3															
BLKF	1	2	1	2														
AUXT	2	2	3	2	2													
AUXR	2	4	2	4	2	2												
EUTH	25	23	25	23	23	23	23											
SKJT	17	17	17	17	17	17	17	17										
SARD	23	23	23	23	23	23	23	23	23									
ASOL	22	22	22	22	22	22	22	22	22	22								
CHMK	43	43	43	43	43	43	43	43	43	43	43							
ATLM	36	36	36	36	36	36	36	36	36	36	36	36						
KMCK	35	35	35	35	35	35	35	35	35	35	35	35	35					
SPMK	35	35	35	35	35	35	35	35	35	35	35	35	35	35				
CERO	35	35	35	35	35	35	35	35	35	35	35	35	35	35	35			
SCBR	36	36	36	36	36	36	36	36	36	36	36	36	36	36	36	36		

Figure 1. UPGMA tree based on absolute number of nucleotide differences between reference sequences. Each species group is a consensus sequence of all haplotypes of that given species. Species abbreviations are given in Table 1.

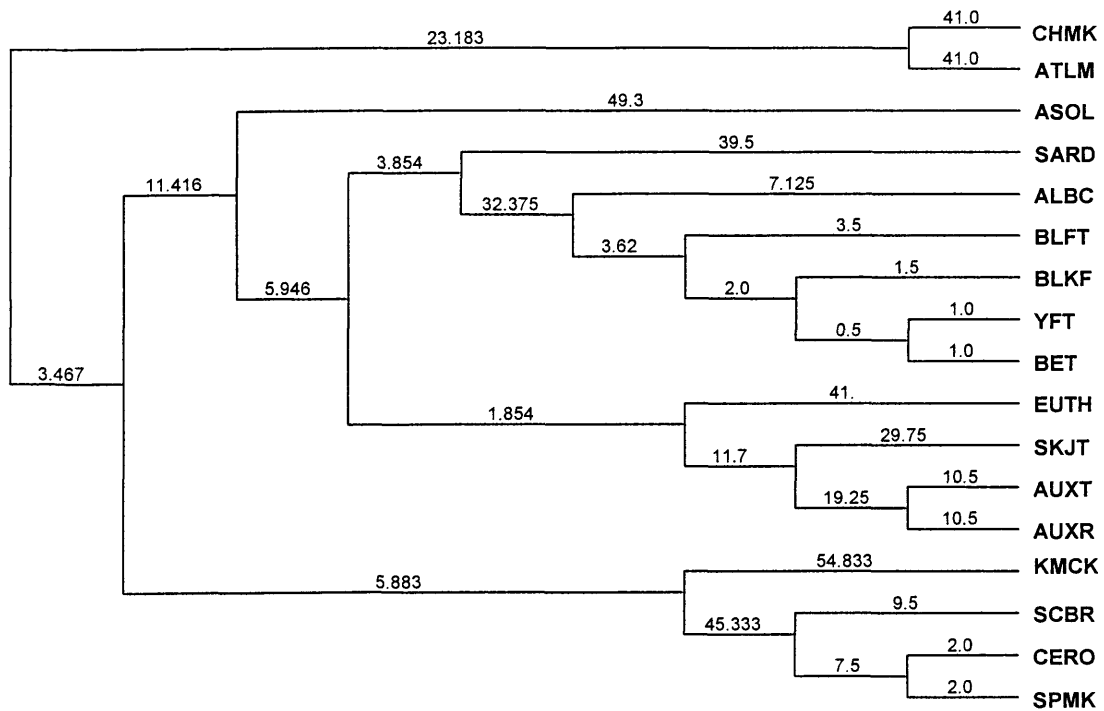


Figure 2. ITS-1 sequence alignment of *Scomberomorus maculatus* and *S. regalis* showing interspecific nucleotide differences. Insertions and deletions are shown between species by dash marks. Sequence names with the letters A, B, or C denote cloned PCR product and the other sequences are direct sequence of PCR product.

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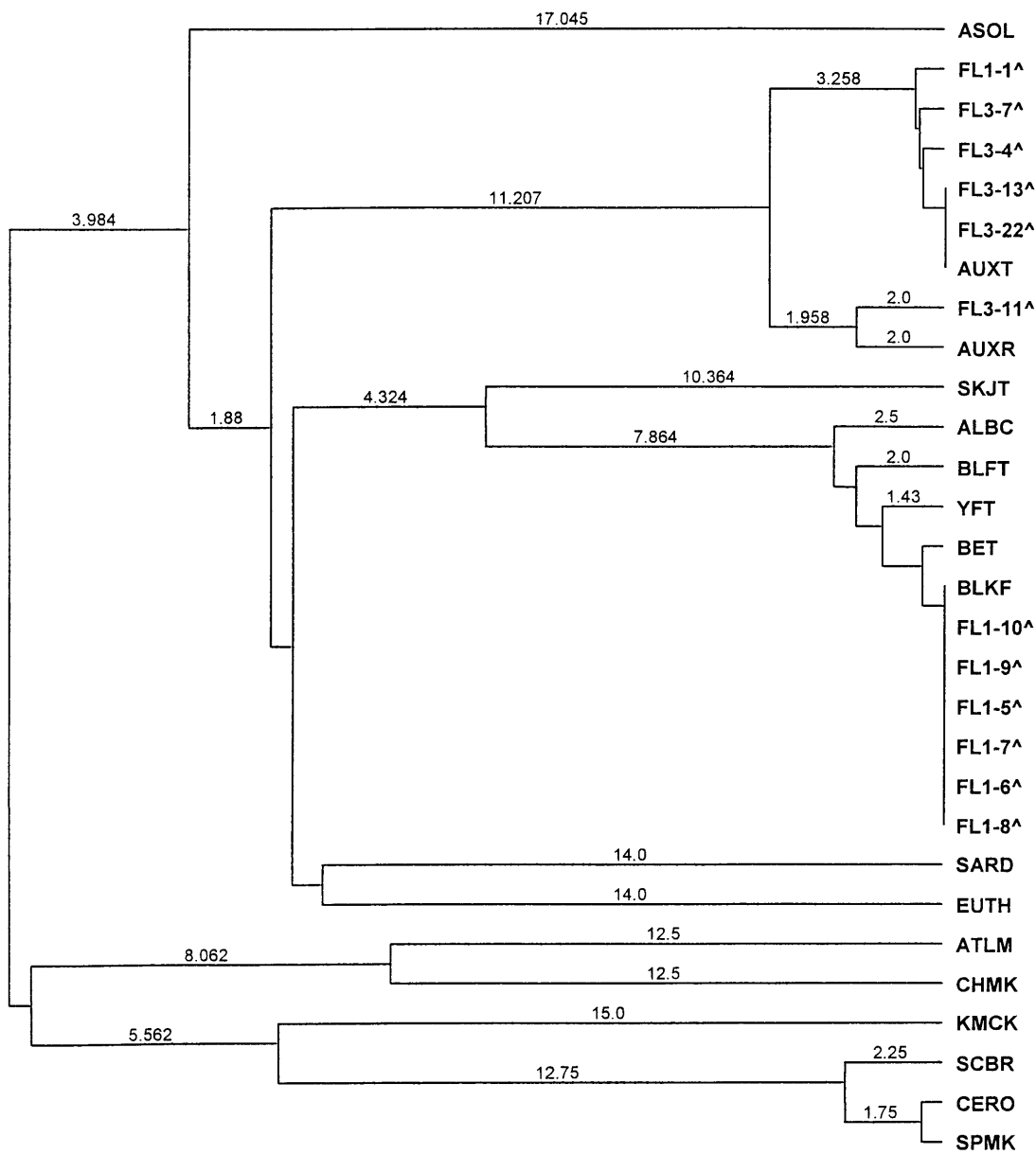
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SPMK1ITS  CCTGCGGAAGGATCATTACCGGTTTGCCCGCCCTGTGCGAGGGCGCGTGCAACAGTTGCCTACCCCCACGGCCGAGGGCCGGC
SPMK1AITS .....
SPMK2ITS .....
SPMK3ITS .....
SPMK3AITS .....
SPMK3BITS .....T.....
SPMK3CITS .....
SPMK1BITS .....
CER01ITS .....C.....
CER01AITS .....C.....
CER01BITS .....C.....
CER01CITS .....C.....
CER02AITS .....C.....
CER02BITS .....C.....
CER02CITS .....C.....
CER03ITS .....C.....
CER03AITS .....C.....
CER03BITS .....C.....
CER03CITS .....C.....
CER04ITS .....C.....
CER04AITS .....C.....
CER04BITS .....C.....
CER04CITS .....C.....

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888899999999990000000000111111111122222222223333333333334444444444555555555566666666667
678901234567890123456789012345678901234567890123456789012345678901234567890
SPMK1ITS  GCGGGTGTCCGCGGGTCCGGGTCCGGGTCCGGGTCCGGTATTCTGGCCCGGACGGGGCCGTTTACCTTTCGGCCCTT
SPMK1AITS .....C...A...C.....
SPMK2ITS .....C...C.....C.....
SPMK3ITS .....C...C.....C.....
SPMK3AITS .....A.....C...C.....C.....
SPMK3BITS .....C...C.....C.....
SPMK3CITS .....C...C.....C.....
SPMK1BITS .....C...C.....C.....
CER01ITS .....TC.....C...C.....C.....C.....
CER01AITS .....TC.....C...C.....C.....C.....
CER01BITS .....TC.....C...C.....C.....C.....
CER01CITS .....TC.....C...C...A.....C.....C.....
CER02AITS .....TC.....C...C.....C.....C.....
CER02BITS .....TC.....C...C.....C.....C.....
CER02CITS .....TC.....C...C.....C.....C.....
CER03ITS .....TC.....C...C.....C.....C.....
CER03AITS .....TC.....C...C.....C.....C.....
CER03BITS .....TC.....C...C.....C.....C.....
CER03CITS .....TC.....C...C.....C.....C.....
CER04ITS .....TC.....C...C.....C.....C.....
CER04AITS .....TC.....C...C.....C.....C.....
CER04BITS .....TC.....C...C.....C.....C.....
CER04CITS .....TC.....C...C.....C.....C.....

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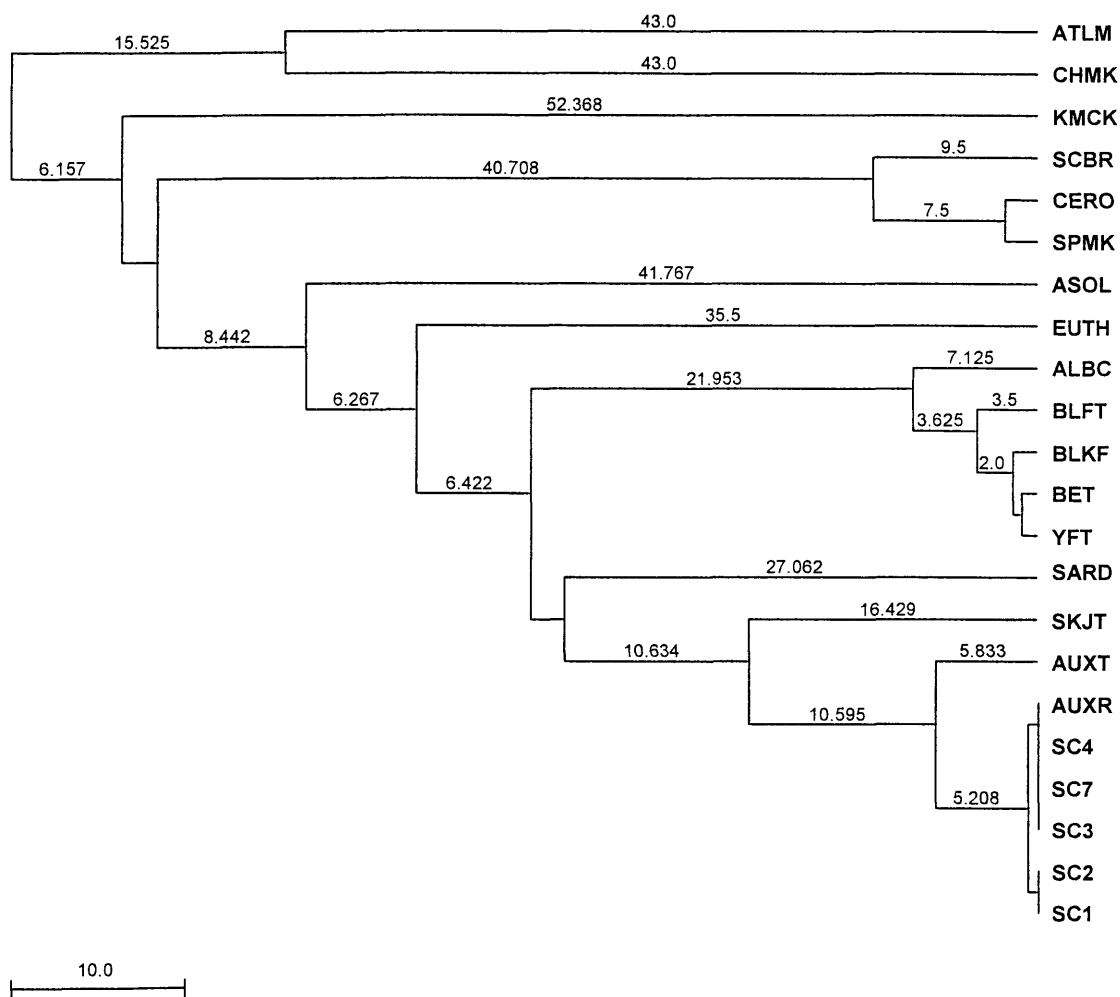

Figure 3. Molecular key of interspecific differences in the shorter COI fragment between consensus sequences. The sites that are useful in distinguishing very closely related species (i.e. *Thunnus albacares*/ *T. obesus*, *T. atlanticus*/ *T. obesus* and *Scomberomorus regalis*/ *Sc. maculatus*) have an asterisk and are bolded. Species abbreviations are given in Table 1.

Figure 4. UPGMA tree including consensus sequences and a select number of unknown larval scombrid samples from Florida clustering with their respective species. Unknown samples are designated by a FL prefix.

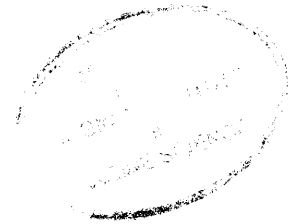


2.0

Figure 5. UPGMA tree of consensus reference sequences and unknown billfish stomach content samples. Unknown samples are denoted by a number with a SC prefix. Five of the nine stomach content samples analyzed are shown here. All nine samples cluster with *A. rochei*.



CHAPTER 2. Specific identification of scombrid larvae collected off the Kona coast of
Hawaii using COI sequence analysis



INTRODUCTION

Members of the family Scombridae (tunas, skipjack tuna, mackerels, etc.) are important components of pelagic ecosystems, with several species supporting large commercial and recreational fisheries throughout the world's oceans. Bigeye tuna (*Thunnus obesus*), yellowfin tuna (*T. albacares*), albacore (*T. alalunga*) and skipjack tuna (*Katsuwonus pelamis*) are important components of pelagic fisheries that operate in Hawaii's exclusive economic zone (Boggs and Ito 1993; Xi and Boggs 1996). Little is known about the distribution, abundance, ecology and behavior of early life history stages of these species around Hawaii, but it is this early period that is crucial to understanding survival and recruitment to fishable stocks (Sund et al. 1981).

The composition of scombrid larvae in the central Pacific, particularly around Hawaii, has been described as being dominated by *T. albacares*, *K. pelamis*, and *Auxis* spp (frigate and bullet tuna) (Strasburg 1960; Miller 1979; Boehlert and Mundy 1994). The other scombrid larvae that could be encountered around Hawaii are: albacore, bigeye tuna, *Acanthocybium solandri* (wahoo), *Euthynnus affinis* (kawakawa), *Sarda orientalis*, and *Scomber australasicus* (Collette and Nauen 1983). Boehlert and Mundy (1994) found and identified only a few albacore, bigeye tuna, wahoo, and kawakawa larvae out of hundreds collected in their surveys. While the incidence of mature adult albacore, wahoo and bigeye tuna would indicate that spawning could occur in this area, there have

been few confirmed collections of the larvae of these species. An examination of albacore gonads collected within 20 miles of the Hawaiian Islands suggests that some spawning has occurred during the summer in the vicinity of the islands (Otsu and Uchida 1959). An analysis of bigeye tuna adult gonads suggests that spawning occurs well offshore, to the southwest of the island of Hawaii (Nikaido et al. 1991), but the presence of larvae, especially smaller sizes, would be a more direct means to show that spawning has occurred in the region (Prince et al. 2005).

Larval tuna are found in abundance near land masses, especially tropical and subtropical islands (Boehlert and Mundy 1994). Gilmartin and Revelante (1974) hypothesize that nutrient-rich waters near the Hawaiian Islands contribute most to favorable conditions for spawning and larval survival. Additionally, physical oceanographic features such as eddies may act to retain larvae near the islands in waters that are favorable for growth and survival (Boehlert and Mundy 1993; Seki et al. 2002). Most studies of near shore abundance of scombrid larvae around Hawaii have taken place around Oahu (Higgins 1970; Miller 1979; Boehlert and Mundy 1994) and few studies of larval scombrids have been conducted specifically off the Kona coast of the big island of Hawaii. The studies off Oahu showed a high concentration of scombrid larvae close to land on the leeward side of the island. These observations and the finding that the Kona coast may be a “hot spot” for billfish spawning (Hyde et al. 2005) suggest that this area may also be an ideal spawning area for scombrids.

Proper identification of young stages is essential to better understand early life history characteristics of each species. While identification of scombrid adults is unambiguous (Collette and Nauen 1983), specific identification of early life history

stages is problematic as many morphological characters are difficult to interpret. Scombrid eggs are very similar in appearance and can only be separated by pigment characters that become lost after preservation (Richards 2006). Larvae of the genus *Thunnus* are especially challenging to identify. Specific identification requires clearing and staining to determine the position of the first closed hemal arch for vertebral precaudal/caudal count, but yellowfin tuna and bigeye tuna can only be separated by the presence or absence of certain pigment characters (Richards 2006). For example, it is not possible to separate larvae of yellowfin tuna from albacore prior to the appearance of black pigment cells at the tip of the lower jaw in yellowfin tuna at 4.5 mm SL (Matsumoto et al. 1971). Juvenile stages (15-60 mm SL) of *Thunnus* species cannot reasonably be identified because the development of body pigmentation obscures diagnostic larval characteristics, and meristic counts are broadly overlapping (Nishikawa and Rimmer 1987).

Previous work on scombrid larval distribution has been limited by dependence upon morphological identification. These types of analyses typically have many larvae that cannot be identified to species level because they are too small to have developed distinguishing characteristics, or are too disfigured (Strasburg 1960; Leis et al. 1991; Beckley and Leis 2000). Only half of the 227 *Thunnus* larvae that Boehlert and Mundy (1994) collected in their September surveys were large enough to be identified to species and the remainder had to be classified as *Thunnus* spp. Also, bullet and frigate tuna larvae generally have not been distinguished, and are listed as *Auxis* spp. (Higgins 1970; Boehlert and Mundy 1994). To fully utilize information from all scombrid larvae, a more

reliable method of identification is necessary to accurately describe the early life history characteristics of these species.

Molecular markers can provide a means for positive identification when morphological identification is uncertain or impossible (Morgan 1975; Bartlett and Davidson 1991; Ram et al. 1996; McDowell and Graves 2002; Perez et al. 2005). Previously, I developed a method for the identification of all scombrids occurring in the western Atlantic Ocean that utilizes sequence information from the COI gene region (Ch. 1). That study demonstrated that the marker may be applied to those species with circumtropical distribution as well. When morphological identification is limited because of a damaged sample or morphological characters are ambiguous, this molecular marker provides a means for unambiguous identification. In this study, the COI molecular marker was used in concert with morphological identification to describe species composition of scombrid larvae taken off the leeward coast of Hawaii in September 2004.

MATERIALS & METHODS

SAMPLE COLLECTION

Ichthyoplankton sampling was conducted off the Kona coast of Hawaii (Fig. 1) aboard NOAA R/V *Oscar Elton Sette*, 19 to 26 September 2004, using a 1.8 m Isaacs-Kidd Trawl with 0.5 mm mesh. A total of 43 tows was examined, each taken at 2.5 knots for 1 hour. Of these, 31 tows were performed at night, 27 of which were taken at 10 m and the other 4 tows were taken at 14 m depth. The nine preliminary daytime collections were stepped oblique tows at various two step intervals of 14 m/ 10 m, 8 m/ 14 m, 30 m/ 20 m, and 20 m/ 10 m. One tow was taken at 20 m and another was just below the surface (<1 m) in the early morning. Total collections spanned a distance of 80 km along the leeward coast over 2 days and subsequently 5 nights. Tows were carried out in all directions in an area from 1-16 km offshore. Bottom depth averaged 2000 m. Temperature of the top 50 m layer was at least 27.5°C and the wind speed during sampling was very light, never exceeding 10 knots.

Plankton samples were preserved in 95% ethanol and putative scombrid larvae were removed. Each sorted larva was given a unique identifying number and was stored in 95% ethanol and analyzed individually. Each larva was photographed using a digital camera attached to a stereoscope via a photo tube capturing as much detail as possible for

future morphological or meristic analysis. Total length was taken using a ruler and approximated to the nearest 0.5 mm.

APPLICATION OF MARKER TO IDENTIFY SPECIES

The morphological criteria of Nishikawa and Rimmer (1987) and Richards (2006) were used to identify as many scombrids as possible. The morphological characters used from these guides were: forebrain pigment and ventral pigment spot in *K. pelamis*, lower jaw pigmentation in *Euthynnus*, lateral tail pigmentation in *A. thazard*, and *A. solandri* are unique, especially the snout length, and are not confused with any other larvae. Well preserved larvae of *K. pelamis* (skipjack tuna), *E. affinis* (kawakawa), *A. solandri* (wahoo), and at some sizes, *Auxis spp.* (frigate and bullet tuna), were distinguished following the aforementioned identification criteria. Larval *Thunnus* are generally problematic and were distinguished following the COI (cytochrome *c* oxidase I) sequence analysis method developed previously (Ch. 1). This molecular identification method was also used for larvae that could not be identified because of damage or questionable morphological characters.

DNA was isolated, amplified, and sequenced following the short fragment COI sequence analysis method (Ch. 1). In cases where there was no amplicon, the reaction was repeated with both the COI primers and with universal COI primers. If there was no amplification, then it was inferred that this sample was too degraded or not a scombrid. All sequences were edited using Sequencher version 4.2.2. The species identity was inferred by noting where the sample sequence clustered in a UPGMA tree constructed of reference sequences using absolute number of differences (Ch. 1). In cases where an

unknown sample clustered between two species, the potentially informative base positions were located in the unknown sequence and compared to a molecular key. The positions at which a species has a consistent, unique combination of nucleotide base pairs are indicated in the molecular key shown in Fig. 2.

Preliminary genetic identification of a few larvae showed them grouping between the *T. albacares* (yellowfin tuna) and *T. obesus* (bigeye tuna) reference sequences. Only two base position differences separate these two species in the COI fragment, and upon referencing the molecular key, it appeared that only one of the bases was distinguishing, and it was unclear which one. To refine the key and ascertain species identity, part of the mitochondrial cytochrome *b* (cyt *b*) gene was sequenced in four each of known yellowfin tuna and bigeye tuna samples to provide another reference in addition to the unknown ambiguous larvae, from which cyt *b* was also sequenced. The primers used were cytbL686 (5'TCC TTG GTT TCG TGA TCC3') and cytbH982 (5'GGG TTC AGA ATA GGA ATT GG3'). All PCR and sequencing of cyt *b* was carried out in the same manner as for COI, with a 53°C annealing temperature.

Because introgression of albacore mtDNA into Pacific bluefin tuna (*T. orientalis*) has been observed previously (Chow and Kishino 1995), the possibility exists that bluefin tuna may be misidentified based on mtDNA characters alone. Preliminary analyses identified 43 larvae as albacore and to address the issue of misidentification, the nuclear ITS-1 region of these larvae was sequenced using the primers F-ITS-1 (5'GAG GAA GTA AAA GTC GTA ACA AGG3') and 5.8SR2 (5'GTG CGT TCG AAR KGT CGA TGA TCA AT3') (K. Johnson, Virginia Institute of Marine Science, unpublished). This

fragment was amplified and sequenced as previously described for the COI fragment, with a 45° annealing temperature and 5 μ L of Q solution was used in the 25 μ L reaction.

RESULTS

Forty-three ichthyoplankton tows yielded a total of 872 scombrid larvae. Scombrids were found in all collections except two daytime tows. The daytime tows averaged 2.3 ± 2.2 scombrids per tow and those taken at night averaged 24.3 ± 21.2 . Morphological characters were used to identify 29% of the samples and the remaining 71% were identified using the COI marker. All scombrid larvae were amplified successfully on the first attempt, with the exception of two of the specimens which amplified on the second effort.

Four of the scombrid larvae grouped between bigeye and yellowfin tuna and two of these (OES18-36, OES22-2) are shown in Fig. 3. Sequence information obtained from the cyt b mitochondrial gene helped to clarify four of these as yellowfin tuna and one as a bigeye tuna. In the Atlantic, site 882 discriminated yellowfin and bigeye tuna, but was not informative in the Pacific, so only site 870 was used to discriminate between these two species.

Preliminary analyses identified 43 albacore larvae using the COI marker. To rule out the possibility of misidentification, a portion of the nuclear ITS-1 region was sequenced from these larvae and from reference samples of bluefin tuna and albacore. The albacore larvae identified using the COI marker had ITS sequences more similar to

the known albacore samples and consequently were confirmed as albacore. The differences in the ITS-1 region for these two species are shown in Fig. 4.

Yellowfin and skipjack tuna larvae dominated the collections at frequencies of 48% and 45%, respectively. Yellowfin tuna were found in half the daytime tows and were represented in all but five of the nighttime tows. Skipjack tuna were not found in any daytime tows, but occurred in all but four nighttime tows. Albacore were found in 20 tows and comprised 5% of the scombrid larvae, while frigate tuna were found in five tows and made up 1% of the collections. Two wahoo larvae were encountered, both were taken in two daytime tows. Only one larva each of kawakawa and bigeye tuna were collected and both were taken at night. The species composition of all tows is represented in Fig. 5 and information on collections is given in Table 1.

The range of lengths and mean length (\pm SD) of the larvae collected of each species were: skipjack tuna 2.5-9 mm (4.1 mm \pm 1.3) with one individual juvenile skipjack tuna that measured 21 mm caught in the 50 m midwater trawl; yellowfin tuna 2.5-10 mm (5.1 mm \pm 1.5); albacore 3-10.5 mm (5.5 mm \pm 1.7); wahoo 4.5 and 9.5 mm (7 mm \pm 3.5); frigate tuna 2.5-6 mm (4.3 mm \pm 1.1); bigeye tuna 4 mm; kawakawa 2.5 mm. Between the commonly encountered larvae, the lengths of skipjack tuna were smaller on average than albacore or yellowfin tuna ($p < 0.05$). The length frequency distribution of the most common species collected (yellowfin tuna, skipjack tuna, and albacore) at all stations is presented in Fig. 6.

DISCUSSION

The COI molecular marker allowed for unambiguous species identification of all scombrid larvae collected off the Kona coast of Hawaii, many of which would have remained unidentified if only morphological criteria were used. Every specimen was discriminated to the species level, regardless of size or physical condition, a major limitation of previous studies that relied solely upon morphological identification. For example, previous studies were not able to distinguish past the generic level in *Auxis*, but the distinction between *A. thazard* (frigate tuna) and *A. rochei* (bullet tuna) was possible using sequence information from COI. Also, previous work has identified some larvae only as *Thunnus* spp., with many tentative specific identifications based on unreliable characteristics, such as one or two very small and hard to see ventral pigment spots which separate intact bigeye tuna larvae from yellowfin tuna. Conversely, in this study, one bigeye tuna larva was confidently identified using this molecular marker. Additionally, previous studies could not discriminate albacore from yellowfin tuna below 4.5 mm SL (Fig. 7), and in this study, 46% of the *Thunnus* larvae collected fell into this size range, but were successfully distinguished using this marker.

The use of the COI marker allowed for a complete description of species diversity of the larval assemblage collected. The diversity found in this study was greater than that observed by Boehlert and Mundy (1994) in their surveys taken around Oahu. Almost

half of the *Thunnus* larvae they collected in September could not be identified to species. Some of these *Thunnus* spp. might have been bigeye tuna, of which they reported 0 in September, or albacore, of which they positively identified only 9 in that month. Additionally, they could not distinguish to species for larvae of the genus *Auxis*, while the COI marker used in this study allowed for specific identification of several *A. thazard* larvae.

The findings in this study suggest that the Kona coast may be an important area for albacore, skipjack tuna and yellowfin tuna early life history stages. The Kona study was dominated by yellowfin and skipjack tuna almost equally, with 421 and 395 larvae, respectively, while in comparison, the September surveys of Boehlert and Mundy (1994) were dominated by *Thunnus* larvae (227 out of 365 scombrid larvae). Boehlert and Mundy encountered almost 75 *Auxis* spp. larvae in September, which outnumbered the 50 skipjack tuna they caught. This study only found nine frigate tuna, while encountering almost 400 skipjack tuna. Boehlert and Mundy found *Thunnus* spp. and skipjack tuna only when water temperatures were warmest, during September and June. During sampling off Kona, the water temperature was above 27°C, and may account for increased abundance of larval yellowfin and skipjack tuna.

An interesting finding in the larval assemblage collected was the number of albacore larvae encountered. Forty-three albacore larvae (5%) were found, which is considerably more than the nine (2.5%) found by Boehlert and Mundy in September off Oahu. The number of albacore discovered in this study suggests that the Kona coast may be an important area for early life history stages of this species. Not much is known about exact spawning locations of albacore; however, they are known to spawn in the

general vicinity of the Hawaiian Islands, with limited spawning to the east of the islands and more frequent spawning to the west (Ueyanagi 1969; Sund et al. 1981; Nishikawa et al. 1985). These studies report that spawning is centered around 20°N in the Pacific with Hawaii located on the northeastern border of this range. Identifying larval habitat at the perimeter of the spawning range is important in describing appropriate conditions for spawning (Boehlert and Mundy 1994).

Smaller larvae are a better indicator of spawning location, as postflexion larvae of scombroid billfish larvae actively move from spawning areas (Hyde et al. 2005). Length frequency distributions of skipjack tuna larvae were generally smaller than those of the albacore or yellowfin tuna larvae. I conclude that skipjack tuna larvae were hatched nearshore and this area is an important spawning ground for that species.

In the larval surveys performed by Boehlert and Mundy (1994) taken around Oahu in September, December, April and June, they had little representation by bigeye tuna, wahoo and kawakawa. In this study performed off Kona in September, I also encountered few larvae of these species. Many surveys have found lower abundance of bigeye tuna compared to other tuna species; this may be due to interspecific behavioral differences that cause a difference in sampling or catchability (Nishikawa et al. 1985). Also, despite the high fecundity of the cosmopolitan wahoo (Collette and Nauen 1983), their larvae are rarely encountered (B. Mundy, pers. comm.) and only two wahoo larvae were collected off Kona in September and 11 were taken in the Oahu study in September and June. Additionally, only one kawakawa larva was found off Kona, which is also rare, and agreed with Boehlert and Mundy, who only encountered five kawakawa larvae total in December, September, and June.

While the COI molecular marker offers many advantages for species identification, one limitation in using only a mitochondrial marker is the possibility that introgression may result in the misidentification of samples. Mitochondrial introgression has been previously reported in the genus *Thunnus*. Historically some hybridization events occurred between male bluefin tuna and female albacore, and the maternally inherited albacore mitochondria were subsequently retained in backcrossing between the hybrids and bluefin tuna (Chow and Kishino 1995). The mitochondrial genome of the albacore has introgressed into the bluefin tuna genetic background within the Pacific at a high frequency (98%) (Chow and Kishino 1995). Bluefin tuna are not commonly encountered around Hawaii (Boggs and Ito 1993; NMFS 1999) and are not known to spawn there (Nishikawa et al. 1985), so this small possibility of misidentification may not be a great concern when using this marker in the Pacific. This possibility of introgression was ruled out by sequencing the nuclear region ITS-1 of the 43 larvae that had been identified as albacore using COI, and confirming their identity as albacore larvae.

The Kona coast has not been the focus of many scombrid larval studies, and the information from the present study would support further investigations in this area. The successful identification of all scombrid larvae of any size and in any damaged condition indicates the potential of this molecular marker as a means for describing putative spawning grounds off the Kona coast of Hawaii and elsewhere. I recommend this approach for use in future ichthyoplankton surveys targeting scombrids, as it is especially useful to distinguish *Thunnus* and *Auxis* early life history stages to species.

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Table 1. Specifics of all tows sampled for scombrids with both the ship station number and my own numbering system of the tows (OES Tow). The time of each tow is given along with the depth at the end of the tow. Tow depth with two numbers denotes the depth of the first step of the tow followed by the second step, each step with equal time. "Molec ID" column gives the number identified using the COI marker in that tow. "Morph ID" column gives the number identified in that tow using the morphological criteria of Nishikawa and Rimmer (1987) and Richards (2006).

date	time	Tow Depth (m)	station #	OES Tow #	depth (m)	Yellowfin	Skipjack	Albacore	Bigeye	Frigate tuna	Kawakawa	Wahoo	Total #/ tow	Molec ID	Morph ID
19-Sep-04	0830-0930	8/14	1	1	2925			3				1	4	3	1
19-Sep-04	1000-1430	20	2	2	2608			1					1	1	
19-Sep-04	1530-1600	30/20	3	3	2041								0		
19-Sep-04	1700-0800	20/10	4	4	1703								0		
20-Sep-04	0900-0930	14/10	6	5	2084			2				1	7	6	1
20-Sep-04	1030-1100	14/10	7	6	1200								1	1	
20-Sep-04	1200-1200	14/10	8	7	168								3	3	
20-Sep-04	1300-1400	14/10	9	8	1217			1					4	4	
20-Sep-04	1600	50	10	10	2893								1	1	

Table 1 cont.

date	time	Tow Depth (m)	station #	OES Tow #	depth (m)	Yellowfin	Skipjack	Albacore	Bigeye	Frigate tuna	Kawakawa	Wahoo	Total #/ tow	Molec ID	Morph ID
21-Sep-04	2200-	10	20	9	1507	2	3	2					7	7	
21-Sep-04	2300-	14	21	31	599		6						6	4	2
22-Sep-04	0100-	10	22	32	1050	1							1	1	
22-Sep-04	0200-	10	23	33	2192		5						5		5
22-Sep-04	0300-	10	24	34	1733		22	1					23	1	22
22-Sep-04	0430-	10	25	35	954		31						31		31
22-Sep-04	2200-	10	40	11	2290	18	14						32	32	
22-Sep-04	2300-	14	41	12	2928	8	12						20	13	7
23-Sep-04	0030-	10	42	13	2555	2	6	3					11	8	3
23-Sep-04	0200-	14	43	14	2305	3	5						8	8	
23-Sep-04	0300-	14	44	15	2114	11	3						14	13	1
23-Sep-04	2230-	10	58	16	2445	8	7						15	15	
24-Sep-04	0000-	10	59	36	2955	28	15	6		4			53	35	18
24-Sep-04	0130-	10	60	17	3009	19	11						30	24	6

Table 1 cont.

date	time	Tow Depth (m)	station #	OES Tow #	depth (m)	Yellowfin	Skipjack	Albacore	Bigeye	Frigate tuna	Kawakawa	Wahoo	Total #/ tow	Molec ID	Morph ID
24-Sep-04	0230-0330	10	61	37	2746	32	22	1					55	33	22
24-Sep-04	0400-0500	10	62	18	2358	47	18	6					71	63	8
24-Sep-04	1800-1900	sfc	72	19	2872			1					1	1	
24-Sep-04	2100-2200	10	74	38	2977	2	1						3	2	1
24-Sep-04	2230-2330	10	75	39	3923	13	5	3					21	15	6
24-Sep-04	2330-0030	10	76	20	2463	12	1	1					14	14	
25-Sep-04	0100-0200	10	77	40	2017	4	3						7	5	2
25-Sep-04	0200-0300	10	78	41	2159	5	13		1				19	8	11
25-Sep-04	0400-0500	10	79	42	2040	5		1					6	6	
25-Sep-04	0500-1700	10	80	43	2076	20	10	5					35	27	8
25-Sep-04	1800-1930	sfc	90	21	1607	7	1	1					9	9	
25-Sep-04	2030-2100	10	92	22	1710	19	6						25	23	2
25-Sep-04	2200-2300	10	93	44	1793	9	16						25	9	16
25-Sep-04	2300-0000	10	94	23	2225	23	57				1		81	60	21
25-Sep-04	0000	10	95	24	2074	6	5	1					12	11	1

Table 1 Cont.

date	time	Tow Depth (m)	station #	OES Tow #	depth (m)	Yellowfin	Skipjack	Albacore	Bigeye	Frigate tuna	Kawakawa	Wahoo	Total #/ tow	Molec ID	Morph ID
26-Sep-04	0030-0130	10	96	25	2048	21							21	21	
26-Sep-04	0130-0230	10	97	26	2111	29	1						30	30	
26-Sep-04	0300-0400	10	98	27	2149	8	5			1			14	13	1
26-Sep-04	0400-0500	10	99	28	2047	13	70	1		1			85	40	45
26-Sep-04	0500-0600	10	100	29	1694	9	17	1		2			29	18	11
26-Sep-04	0630-0730	sub	101	30	2491	25	4	2		1			32	32	
	Total/ spp					421	395	43	1	9	1	2	872	620	252
	%					48.2798	45.2982	4.93119	0.115	1.032	0.115	0.229		71.100	28.8991
						Yellowfin	Skipjack	Albacore	Bigeye	<i>A. thazard</i>	<i>E. affinis</i>	Wahoo			

Figure 1. Map of Hawaiian Islands with scombrid larval collection area shown by striped region.

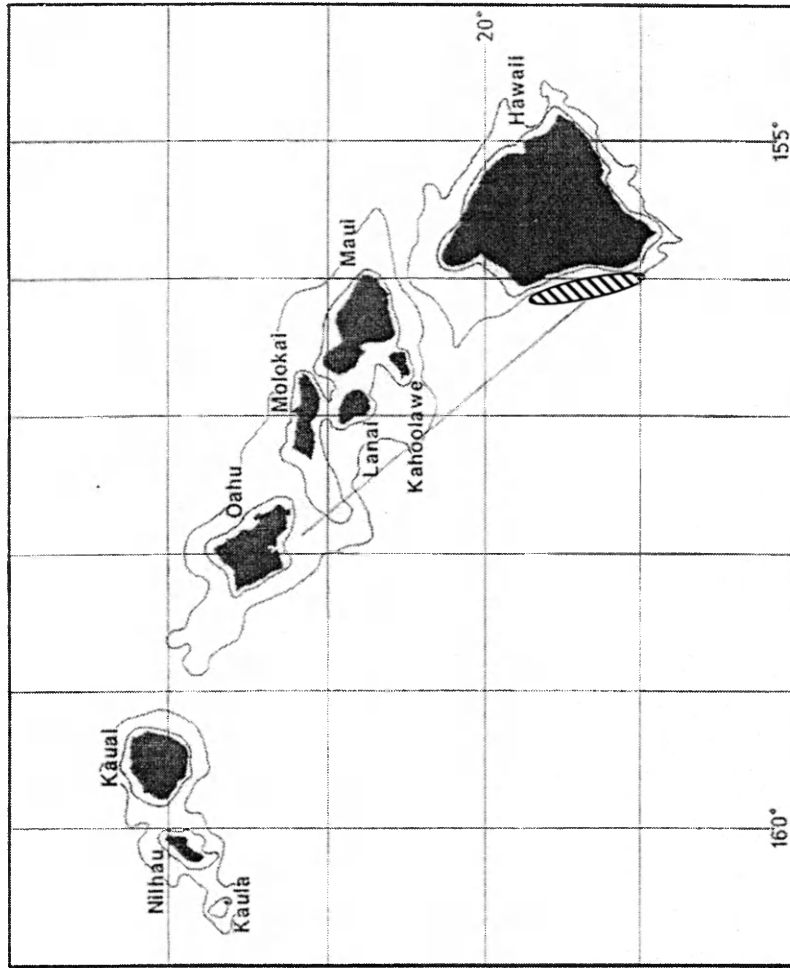
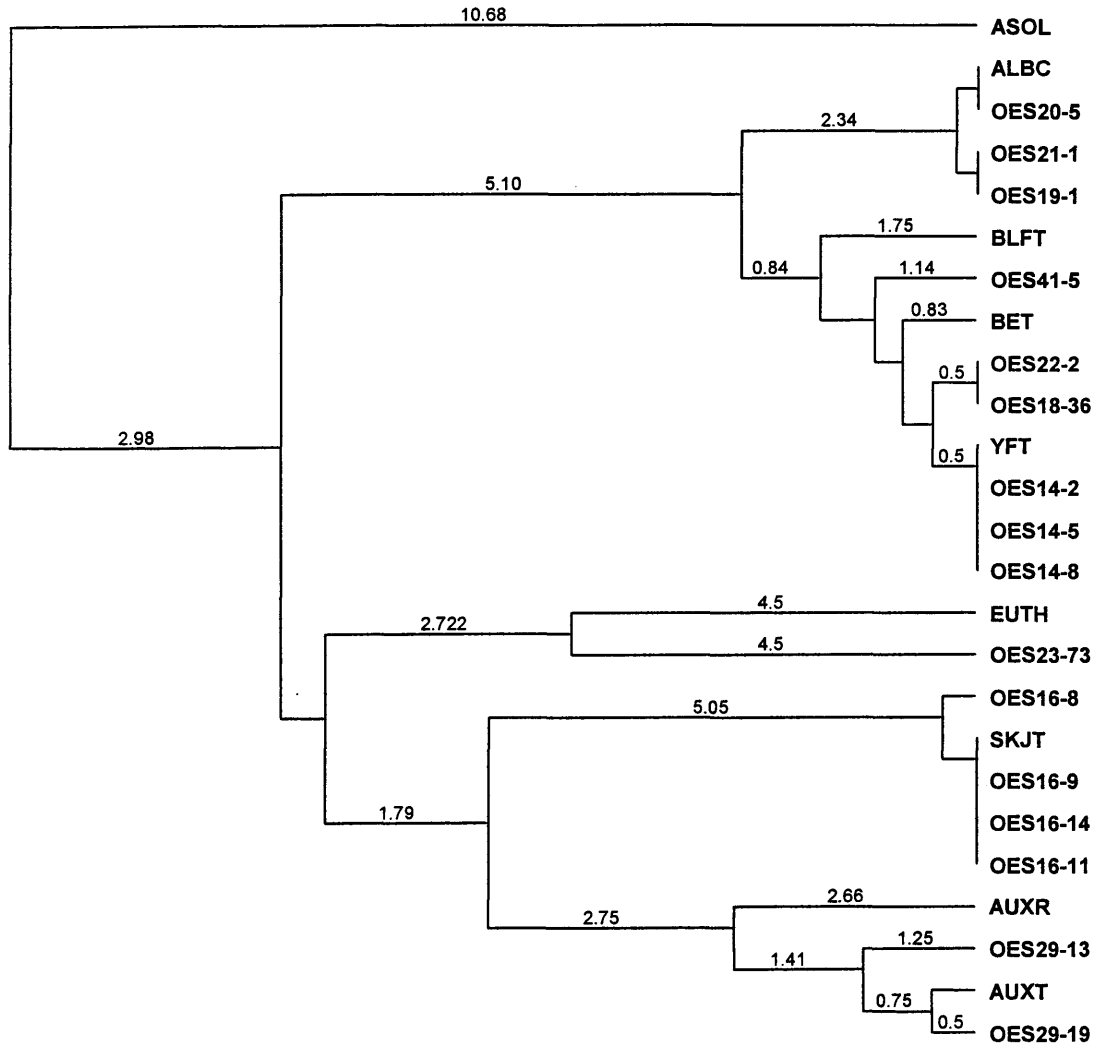


Figure 1. ---Track of the NOAA ship Oscar Elton Sette cruise OES-12 (OES-21), September 17-27, 2004.

Figure 2. Molecular key of interspecific differences in the COI fragment between consensus sequences of each scombrid species. The sites that are useful in distinguishing very closely related species (i.e. *T. albacares*/*T. obesus*, *T. thynnus* from other *Thunnus*) have an asterisk. Species abbreviations with number of reference samples represented in each consensus sequence are: *Acanthocybium solandri* (ASOL) (21); *Thunnus alalunga* (ALBC) (17); *T. thynnus* (BLFT) (18); *T. obesus* (BET) (18); *T. albacares* (YFT) (18); *Euthynnus alletteratus* (EUTH) (10); *Katsuwonus pelamis* (SKJT) (19); *Auxis rochei* (AUXR) (16); *A. thazard* (AUXT) (10). Note that *Euthynnus alletteratus* occurs in the western Atlantic and is the congener of *E. affinis* that occurs in Hawaii. Since *E. affinis* is the only *Euthynnus* that occurs around Hawaii, the EUTH reference consensus sequence can be used to identify *E. affinis*.

Figure 3. UPGMA tree constructed based upon absolute number of nucleotide differences between consensus sequences and unknown larval specimens from Hawaii. Unknown larvae are designated by an OES prefix. Each species group is a consensus sequence of all haplotypes of COI of that given species. Species abbreviations are given in Figure 2. Samples OES41-5 (bigeye), OES22-2 (yellowfin), OES18-36 (yellowfin) were compared against the molecular key to verify species assignment. OES23-73 clusters nearest to EUTH (*E. alletteratus*) because it is the congener that occurs off Hawaii, *E. affinis*.



2.0

Figure 4. ITS-1 sequence alignment of *Thunnus alalunga* and *T. thynnus* samples from the Pacific showing interspecific nucleotide differences. Insertions and deletions are shown between species by dash marks.

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111111111122222222223333333333444444444455555555556666666666777777777788888888
1234567890123456789012345678901234567890123456789012345678901234567890123456789012345
ALBC11ITS ACCTGCGGA-GGATCATTACCGTTTGCCCGCCTGTGCGAGGGGCGCGCAACAGACGCCTCCGGACAGCTGAGGGCCAGAGG
ALBC5ITS .....A.....
ALBC9ITS .....A.....
BLFT20ITS .....-.....
BLFT18ITS .....-.....
BLFT19ITS .....-.....
BLFT17ITS .....-.....

111111111111111111111111111111111111111111111111111111111111111111111111111111111
888899999999000000001111111111222222222233333333334444444444555555555566666666667
6789012345678901234567890123456789012345678901234567890123456789012345678901234567890
ALBC11ITS GTAGGTCCTTCCCGCCGGT-CGGTCGCGGTGTCCTCCCGAGGACTTCCCGCGGCGGCGGCGGCGGTTGGTTCGTTCCCT
ALBC5ITS .....G..G.....
ALBC9ITS .....-.....
BLFT20ITS .....-.....C.GT.....
BLFT18ITS .....C.....-.....
BLFT19ITS .....C.....T.....
BLFT17ITS .....C.....T.....

111111111111111111111111111111111111111111111111111111111111111111111111111111111
777777778888888899999999990000000111111111122222222223333333333444444444455555
1234567890123456789012345678901234567890123456789012345678901234567890123456789012345
ALBC11ITS CCCCGACGGCCGAGGCGCG-CGCGGGCGTCTCCTCCCGAGACGCCCGCGCACACAACCTCCTCCTGGTCCGACCTCGTCCC
ALBC5ITS .....GG.....C.....
ALBC9ITS .....G.....C.....
BLFT20ITS .....-.....A.AC.....
BLFT18ITS ...GA.G.C.G-.....C.....A.AC.....
BLFT19ITS ...GA.G.C.G-.....C.....A.AC.....
BLFT17ITS ...GA.G.C.G-.....C.....A.AC.....

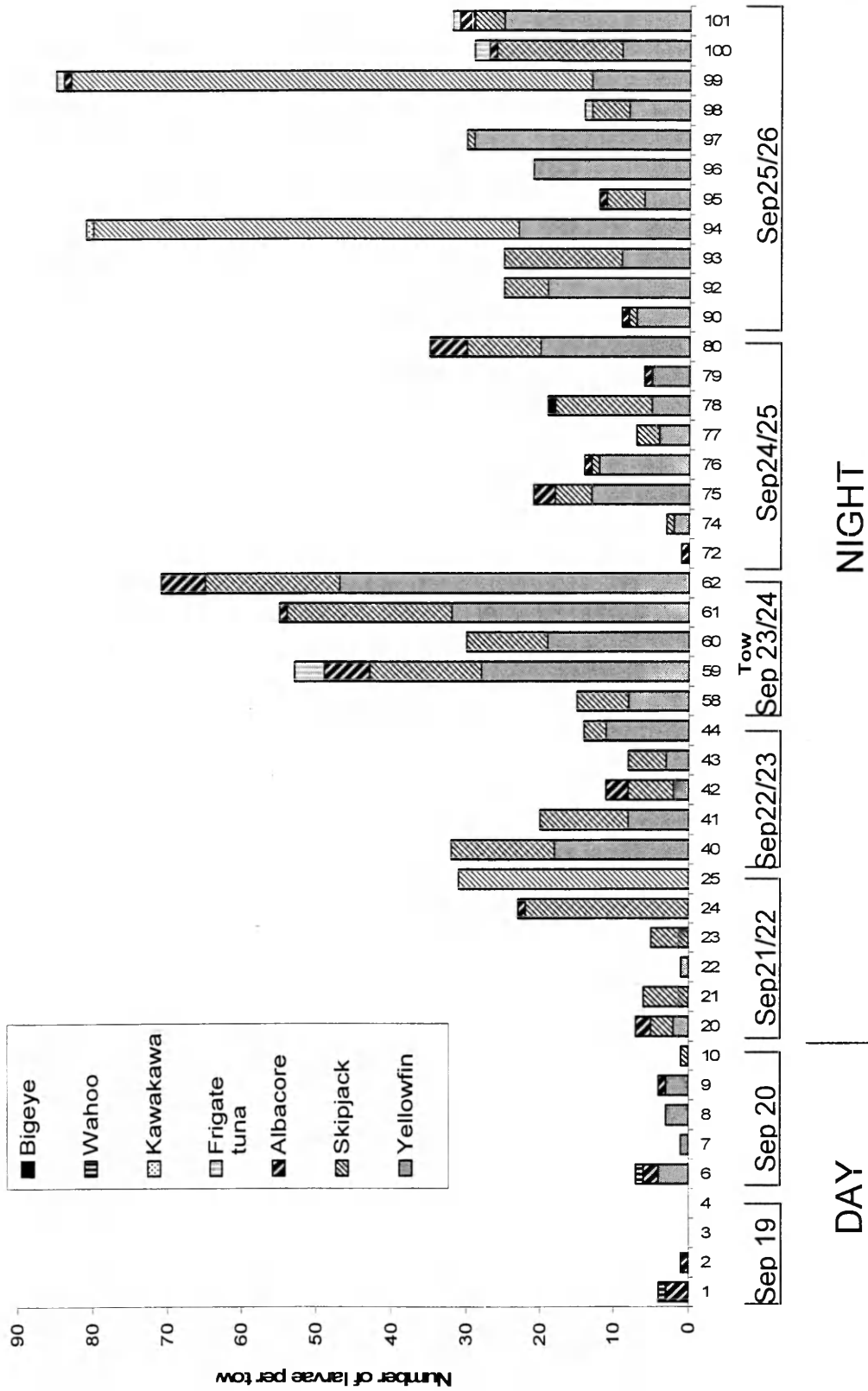
222222222222222222222222222222222222222222222222222222222222222222222222222222222
555566666666667777777778888888889999999999000000011111111122222222223333333333
6789012345678901234567890123456789012345678901234567890123456789012345678901234567890
ALBC11ITS GACCGGGCGGCTCCCATCTCTCCCGG-TGGGACACGCCTCGCGATCCCGCCGGCCAAAGCTCCGCGGGCTGACGGA
ALBC5ITS .....-.....
ALBC9ITS .....-.....
BLFT20ITS .....CT.....C.....C.....A..T.....
BLFT18ITS .....CT.....C.....C.....A..T.....
BLFT19ITS .....CT.....C.....C.....A..T.....
BLFT17ITS .....CT.....C.....C.....A..T.....

333333333333333333333333333333333333333333333333333333333333333333333333333333333
444444445555555555666666666677777777888888889999999999000000011111111222222
1234567890123456789012345678901234567890123456789012345678901234567890123456789012345
ALBC11ITS GCGGCGGTCGGACGTCGCGGGGCGCCCTCGCGGGGTCGGGGACCCCGTCCGGTGCACCGGGCTGGGCGGACACTCGGAACT
ALBC5ITS .....-.....
ALBC9ITS .....-.....
BLFT20ITS .....-.....GTC..GA.....
BLFT18ITS .....-.....GTC..GA.....
BLFT19ITS .....-.....GTC..GA.....
BLFT17ITS .....-.....GTC..GA.....

444444444444444444444444444444444444444444444444444444444444444444444444444444444
22223333333344444444445555555556666666666777777778888888899999999990000000011111111222222
6789012345678901234567890123456789012345678901234567890123456789012345678901234567890
ALBC11ITS AAACCTAAGCGCGGAAGCGGAGGCTTCGCCCCCGCCGACGCGCGCCCCGGTACCCAACTCTCCCCCTCCTTCGGAG
ALBC5ITS .....-.....
ALBC9ITS .....-.....
BLFT20ITS T...A.....C.....
BLFT18ITS T...A.....C.....
BLFT19ITS T...A.....C.....
BLFT17ITS T...A.....C.....

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Figure 5. Species composition of all tows numbered according to station number. Each bracket denotes all tows taken in that day or throughout a night consecutively. See Table 1 for information on each tow.



NIGHT

DAY

Figure 6. Length frequency distribution of the most common scombrid species collected: *Thunnus albacares* (yft), *Katsuwonus pelamis* (skj), *T. alalunga* (albc) from all scombrid targeted tows. Skipjack tuna were on average smaller than albacore and yellowfin tuna ($p < 0.05$).

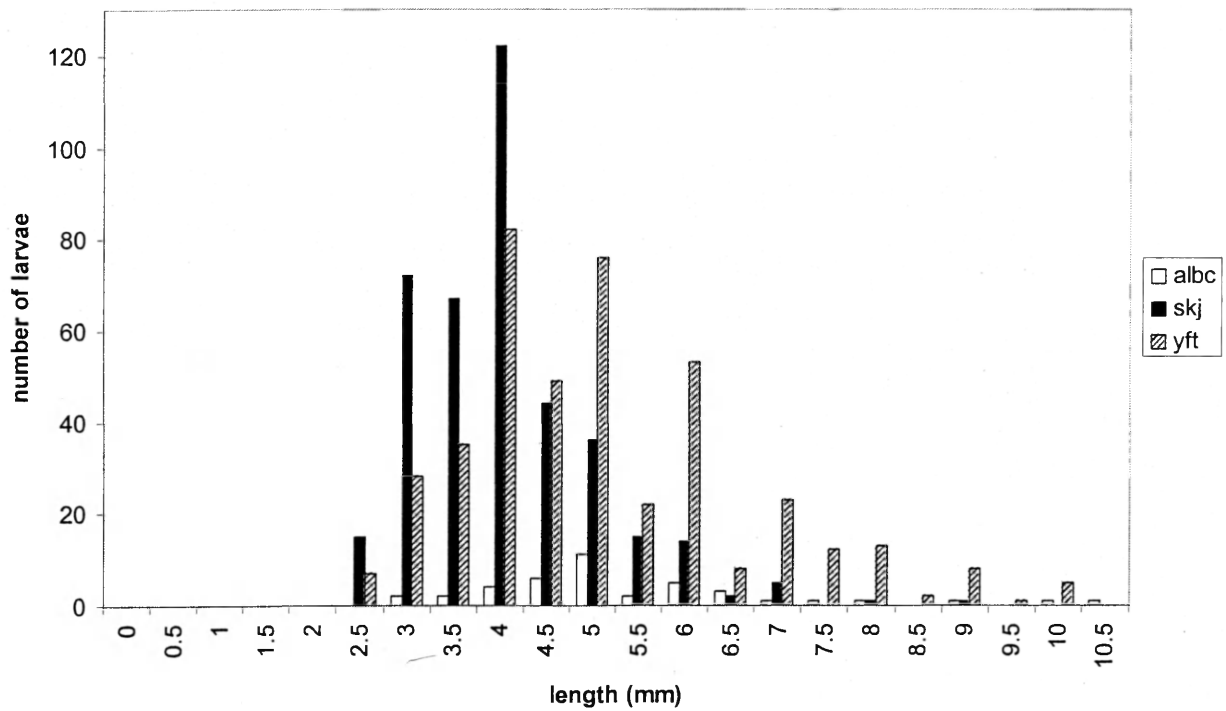
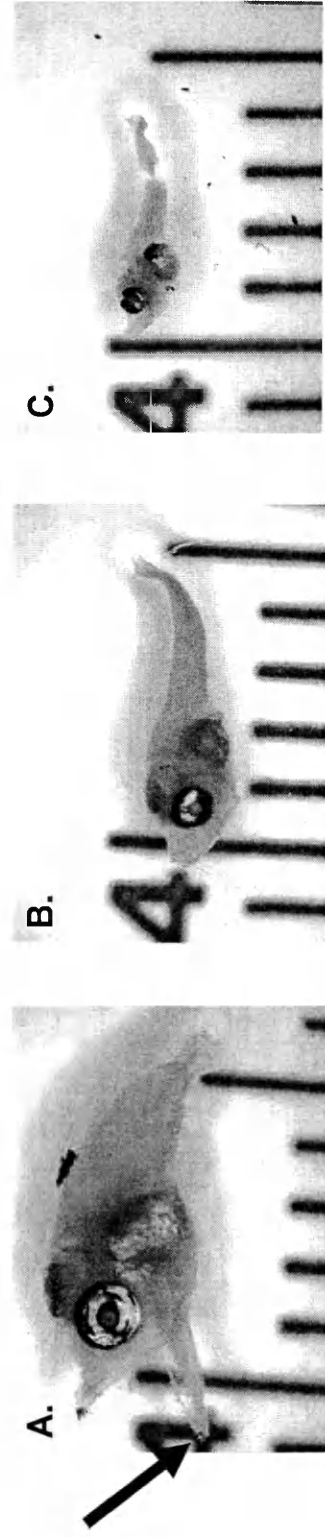


Figure 7. Photographs of *Thunnus* larvae identified using the COI molecular marker: A. yellowfin 7 mm; B. albacore 5.5 mm; C. albacore 4 mm. At sizes smaller than 4.5 mm SL, yellowfin tuna cannot be distinguished from albacore, and the larva pictured in (C) was identified using the molecular marker. Yellowfin tuna larvae larger than 5.5mm should have pigmentation at the tip of the lower jaw, as seen in (A) while albacore do not acquire this pigmentation (B) until they are larger than 7 mm.



CONCLUSION

The primary objective of this thesis was to develop a molecular marker to distinguish among the 17 scombrid species occurring in the western Atlantic Ocean. The COI sequence information obtained from this work successfully discriminated 15 of the 17 scombrid species occurring in the western Atlantic. This COI marker was found to be reliable even though in some instances there were only a few consistent nucleotide differences between different species. This marker worked on scombrid larvae from the Atlantic and on putative scombrid remains in large predator stomachs. Any early life history stage or tissue sample that belongs to the family Scombridae occurring in this area, with the exception of Spanish mackerel (*Scomberomorus maculatus*) and cero (*S. regalis*), can be identified using this marker.

A limitation of relying on a mtDNA marker is that misidentification could occur when using this technique if there is mitochondrial introgression between species. Introgression has taken place between albacore (*Thunnus alalunga*) and Pacific and Atlantic bluefin tuna (*T. thynnus* and *T. orientalis*), where mtDNA of the former has introgressed into the latter (Chow and Kishino 1995). The frequency of “albacore-like” mtDNA bluefin tuna is low (<5%) in the Atlantic, and the “albacore-like” mtDNA bluefin tuna is distinct from albacore, allowing specific identification of bluefin tuna with “albacore-like” mtDNA (Carlsson et al. 2004). However, this study also demonstrated

introgression of mtDNA between Spanish mackerel and cero, in a direction opposite to that observed by Banford et al. (1999). Consequently, unambiguous identification of these two species requires a nuclear marker, such as ITS-1 that was used in this study.

The COI molecular marker was successfully used to identify scombrid larvae collected off the Kona coast of Hawaii. The larval assemblage was identified completely using the COI marker in concert with morphological criteria. Unambiguous morphological identification was possible for 29% of the larvae, and most of these identified morphologically were skipjack tuna larvae. Morphological identification within the genus *Thunnus* was tentative, so all putative *Thunnus* larvae were identified using the molecular marker.

The description of species diversity of the larval assemblage demonstrated that the early life history stages of yellowfin tuna (*T. albacares*), skipjack tuna (*K. pelamis*) and albacore occur off the Kona coast. An unexpected result was that many more albacore were identified than has been found before in other areas around the Hawaiian Islands (Boehlert and Mundy 1994). Because albacore cannot be distinguished from yellowfin tuna below 4.5 mm, it is possible that previous studies which found larvae smaller than this size may have underestimated the contribution of albacore larvae to scombrid assemblages around the Hawaiian Islands. The results of this study suggest a greater contribution of albacore larvae near the Kona coast.

The selection of the COI gene suited the overall objectives for this study, but presented some limitations. The risk in relying on relatively few nucleotide differences in COI to discriminate among species was realized when the molecular key was used to identify Pacific larvae. A few inter-oceanic (intraspecific) nucleotide differences were

observed within *Thunnus* species that necessitated a slight revision of the key. In this instance, a gene with more variation may have revealed a greater number of interspecific differences. However, high levels of variation pose problems with primer design, efficiency of amplification, and intraspecific variation, and so this study opted for consistent, reliable results.

While the COI molecular marker was sufficient for the objectives of this study, a different approach may be necessary for a large scale, high-throughput study. For an ecological scale study, an identification technique will be needed that maximizes the number of samples that can be processed in the shortest amount of time and with minimal cost. A microarray would be very effective to process many samples and reduce processing time (Bell and Grassle 1998); however, this is quite expensive and has greater chance of producing false negative results. Multiplex assays have been used for billfish early life history stage identifications and this technique has been reported to be an effective means of identifying “hot spots” of billfish spawning in real-time (Hyde et al. 2005). Multiplex assay is one approach that could be used to identify scombrids more quickly, perhaps focusing only on *Thunnus* species so that designing multiple species-specific primers is more tenable. A different gene with more variation than COI could be targeted for primer development, in conjunction with the COI marker developed in this study, to find enough species-specific primers to support a multiplex assay.

The development of a rapid molecular technique for identification of early life history stages would provide a valuable tool for broad ecological studies of scombrid early life history. A faster technique that could identify many samples, together with a more comprehensive spatial and temporal sampling design, could provide much more

conclusive information on scombrid spawning and recruitment success off the Kona coast. And specifically, the occurrence of larvae onshore versus offshore and at various depths could be related to moon phase, physical oceanographic features, season and water temperature. In light of the results of this study, further investigation off the Kona coast is clearly warranted, and the molecular tool developed in this project can be useful for future studies as it can describe the complete species diversity present in assemblages of larvae.

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APPENDIX

DESCRIPTION OF SPAWNING AREAS OF SCOMBRIDS IN THIS STUDY IN THE
ATLANTIC AND PACIFIC OCEANS*Thunnus orientalis*

Pacific: Western extremity of the North Pacific Ocean, with their most eastern occurrence near 150°E longitude (just east of Japan).

Thunnus thynnus

Atlantic: Gulf of Mexico and in the Mediterranean Sea; also known to spawn in the Florida Strait (Richards 2006) and could potentially spawn in the mid-Atlantic (Lutcavage et al. 1999).

Thunnus alalunga

Pacific: Anti-equatorial distribution. In North, larvae occur to from Taiwan to vicinity of Hawaiian Islands, but not known how far the larvae are present in the central and eastern Pacific to the east of the Hawaiian Islands.

Atlantic: Larvae found off the east coast of Brazil and few off the coast of West Africa. In January-March, occur north of the equator.

Thunnus obesus

Pacific: Distributed widely in the equatorial area of the western, central and eastern Pacific. Despite spawning stock size, number of larvae taken per unit volume of water is less than other tuna species.

Atlantic: Few confirmed spawning events in western central Atlantic; occur in the equatorial area from Caribbean Sea to the Gulf of Guinea in October-December and in January-March, also occur in the east coast of Brazil in January-March. Richards and Potthoff (1974) say larvae of *Thunnus atlanticus* closely resemble bigeye tuna, so these reports may include blackfin tuna.

Thunnus albacares

Spawning occurs in core areas of distribution; larval distribution in equatorial waters is transoceanic year round with seasonal changes in larval density in subtropical waters (Collette and Nauen 1983).

Pacific: Distributed throughout the entire width of the equatorial Pacific.

Atlantic: Many larvae present over the equatorial area from the Caribbean Sea to the Gulf of Guinea.

Thunnus atlanticus

Atlantic: Spawning well offshore off Florida and in Gulf of Mexico (Collette and Nauen 1983).

Katsuwonus pelamis

Spawn equatorially year round and from spring to early fall in subtropical waters (Collette and Nauen 1983).

Auxis

Spawns throughout its range (Collette and Nauen 1983).

Pacific: Distribution land-related

Atlantic: Caribbean Sea and Gulf of Guinea of West Africa

Mediterranean: spawn in the western region

Euthynnus alletteratus

Atlantic: North coast of South America including the Caribbean Sea and in the Gulf of Guinea. Spawns in eastern and western Atlantic (Collette and Nauen 1983).

Euthynnus affinis

Pacific: Adults occur throughout Indo-Pacific, including oceanic islands with known spawning seasons in Philippine waters, around the Seychelles, off East Africa, and off Indonesia (Collette and Nauen 1983).

Sarda sarda

Atlantic: Adults occur throughout Atlantic, absent from much of the Caribbean and rare throughout western central Atlantic; larvae rarely encountered in coastal areas (Richards 2006); Spawns in the Mediterranean

Scomber colias

Atlantic: Western central Atlantic (Richards 2006).

Scomber scombrus

Atlantic: Western population spawns from Chesapeake Bay to Newfoundland; eastern population spawns in the Mediterranean, off southern England, northern France and in the North Sea (Collette and Nauen 1983).

Scomberomorus cavalla

Atlantic: Spawn in western Gulf of Mexico, northeastern Caribbean and northeastern Brazil (Collette and Nauen 1983).

Scomberomorus maculatus

Atlantic: Spawn in batches in Gulf of Mexico and along east coast of the USA (Collette and Nauen (1983).

Scomberomorus regalis

Atlantic: Life history not described extensively, but adults occur in the western central Atlantic and larvae are described (Richards 2006); spawning takes place around Puerto Rico (Collette and Nauen 1983).

Scomberomorus brasiliensis

Atlantic: Larvae have not been described, but adults occur along the continental shelf of the western central Atlantic, and spawning takes place off the northern coast of South America

Acanthocybium solandri

Cosmopolitan; fecundity believed to be high (Collette and Nauen 1983).

Pacific: Larvae rarely encountered (Mundy, pers. comm.)

Atlantic: Spawn throughout western central Atlantic, but larvae rare (Richards 2006).

VITA

Melissa Ann Paine was born in Orange, California on February 24, 1978. She grew up in Santa Barbara, California, where she graduated from Laguna Blanca School in 1996. The author went on to attain a Bachelor of Science degree in biology with a minor in environmental science from Revelle College at the University of California at San Diego in 2000. In San Diego, she worked at an environmental consulting firm after college and then as a molecular biologist at The Scripps Research Institute for a couple of years before starting graduate school. Melissa entered the graduate program at the Virginia Institute of Marine Science in the fall of 2003 and completed her Master's degree in Marine Science in April 2006.