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Population Genetic Structure of Escolar (Lepidocybium flavobrunneum) and a Molecular Phylogeny of the Trichiuroidea

Kirsten Brendtro

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POPULATION GENETIC STRUCTURE OF ESCOLAR
*(LEPIDOCYBIUM FLAVOBRUNNEUM)*
AND
A MOLECULAR PHYLOGENY OF THE TRICHIUROIDEA

A Thesis
Presented to
The Faculty of the School of Marine Science
The College of William and Mary

In Partial Fulfillment
Of the Requirements for the Degree of
Master of Science

By
Kirsten S. Brendtro
2006
This thesis is submitted in partial fulfillment
of the requirements for the degree of
Master of Science

Kirsten S. Brendtro

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Escolar (Lepidocybium flavobrunneum) is a large, mesopelagic fish species that inhabits tropical and temperate seas throughout the world. Escolar is a common bycatch in pelagic longline fisheries that target tuna and swordfish. Few studies have explored the biology and natural history of escolar, and little is known regarding the population structure of the species. To evaluate the genetic basis of population structure of escolar throughout their range, we surveyed genetic variation over an 806 base pair fragment of the mitochondrial control region. In total, 225 individuals from six geographically distant locations throughout the Atlantic (Gulf of Mexico, Brazil, South Africa) and Pacific (Ecuador, Hawaii, Australia) were analyzed. A neighbor-joining tree of haplotypes based on maximum likelihood distances revealed two highly divergent clades (δ = 4.85%) that were predominantly restricted to the Atlantic and Indo-Pacific ocean basins. All Atlantic clade individuals occurred in the Atlantic Ocean and all but four Pacific clade individuals were found in the Pacific Ocean. The four Atlantic escolar with Pacific clade haplotypes were all found in the South Africa collection. These four individuals as well as four representative individuals from each of the Atlantic and Pacific collections were subsequently analyzed for variation at the nuclear ITS-1 gene region. Again, divergent Atlantic and Pacific clades were resolved (δ = 1.95%) and the four South Africa escolar with Pacific mitochondrial control region haplotypes all had ITS-1 gene region sequences that clustered with the Pacific escolar, suggesting that these individuals were recent migrants from the Indo-Pacific. Due to the high divergence and geographic separation of the Atlantic and Pacific clades, as well as reported morphological differences between Atlantic and Indo-Pacific specimens, consideration of the Atlantic and Indo-Pacific populations as separate species or subspecies may be warranted, though further study is necessary. In the least, two genetically distinct populations should be recognized within the species.

Escolar belongs to the family Gempylidae. Fishes of the families Gempylidae and Trichiuridae, often grouped into the superfamily Trichiuroidea, are benthopelagic and mesopelagic members of the suborder Scombroidei. Like those of many scombroid fishes, the phylogenetic relationships of the families Gempylidae and Trichiuridae remain in question. Uncertainty exists regarding the grouping of the two families, the monophyly of the Gempylidae, and the placement of the gempylid Lepidocybium basal to all other trichiurids. To address these questions, phylogenetic analyses using mitochondrial COI and ND2 and nuclear TMO-4c4 gene regions were performed. Included in the analyses were 11 species of gempylids (representing 11 of 16 genera), 6 species of trichiurids (representing 5 of 10 genera), and several outgroup taxa. Analyses of concatenated data sets with maximum likelihood and Bayesian methods yielded fairly consistent hypotheses of the relationships of gempylid and trichiurid fishes. As has been found in previous studies, the Gempylidae and Trichiuridae formed a monophyletic group within the Scombroidei, supporting the grouping of these two families into the superfamily Trichiuroidea. Monophyly of the family Trichiuridae was also supported, but this family fell within the Gempylidae, resulting in a paraphyletic Gempylidae. Lepidocybium, which has been previously considered the most basal member of the trichiurids, was found in the basal cluster of the Gempylidae, as were Ruvettus and Neopinnula.
POPULATION GENETIC STRUCTURE OF ESCOLAR

(*LEPIDOCYBIUM FLAVOBRUNNEUM*)

AND

A MOLECULAR PHYLOGENY OF THE TRICHIUROIDEA
INTRODUCTION

Deep water fishes, which inhabit the mesopelagic (200 – 1000 m), bathypelagic (1000 – 4000 m), and abyssopelagic (>4000 m) environments, are less accessible, and therefore more difficult to study than epipelagic and coastal species. Consequently, very little is known about the biology of deep-sea fauna. To date, few studies have investigated the population structure of any mesopelagic or benthopelagic fish, and the evolutionary relationships of many deep-sea fishes are poorly understood. This study aimed to clarify these relationships for fishes in the superfamily Trichiuroidea. Using molecular genetic techniques, both intraspecific and interspecific relationships were investigated in efforts to elucidate stock structure for a single species, and to produce a phylogenetic hypothesis of relationships among two families of mesopelagic and benthopelagic fishes.

*Lepidocybium flavobrunneum* (Smith 1849), commonly called escolar, is a cosmopolitan marine fish most often placed in the family Gempylidae. Escolar is a large species that is thought to reach a maximum length of 200 cm (Nakamura and Parin 1993), but the largest specimen documented in the literature is 184 cm total length (Schwartz 1997). The body shape of escolar is semifusiform and slightly compressed, and can be distinguished by a prominent keel flanked by two supplementary keels on the caudal peduncle, and a single, sinuous lateral line (Nakamura and Parin 1993). The coloration of escolar is a uniform dark brown to black.
Escolar are found throughout tropical, subtropical and temperate seas, but likely do not occur in the northern Indian Ocean (Nakamura and Parin 1993). Escolar generally inhabit waters with surface temperatures of at least 23° C (Maskimov, 1970) over the continental slope at depths of 200 m and more (Nakamura and Parin 1993). Adult escolar have been found in epipelagic layers of the water column (Nakamura and Parin 1993), and comparisons of day and night catchability indicate escolar exhibit diel migrations towards the surface at night, presumably for feeding (Ward and Myers 2005a). Escolar feed primarily on squids, crustaceans, and fishes such as dolphin fish, snake mackerels, and small tunas (Maskimov 1970; Nakamura and Parin 1993). The growth rate of escolar is estimated to be relatively fast, with females experiencing a higher rate of growth compared to males (Maskimov 1970). Female fish may reach sexual maturity as small as 33 cm fork length (Maskimov 1970). Seasonal oscillations in catch of escolar suggest that this species may have migratory patterns for reproduction and feeding similar to other large pelagic species (Milessi and Defeo 2002). Escolar larvae appear to be more prevalent near islands, suggesting that spawning occurs in near-shore areas, adjacent to oceanic islands or continental landmasses (Nishikawa 1982). The larvae are planktonic and occur in the greatest densities at the surface layer of the water column (upper 1.5 m; Nishikawa 1987).

Except for a small Japanese fishery that targeted escolar from 1978 – 1980 (Nishikawa and Warashina 1988), escolar has primarily been captured incidentally in pelagic longline fisheries that target tuna and swordfish, and has either been retained for sale or discarded. The market for escolar varies throughout the world, primarily due to the purgative properties of its meat when ingested (Berman et al. 1981; Yohannes et al. 2002; Feldman et al. 2005), attributable to the high levels of non-digestible wax esters in the flesh (Berman et
al. 1981; Schwartz 1997; Nichols et al. 2001). As a result of the purgative aspects of escolar, the U.S. Food and Drug Administration advised against importation and marketing of this fish in the U.S. (USFDA 2001). Nonetheless, escolar continues to be imported into the U.S. (pers. obs.), commanding retail prices upwards of $10 per pound, and is often marketed as “white tuna” in sushi restaurants (D. Kerstetter, pers.comm.).

As previously noted, escolar are currently placed within the family Gempylidae. The Gempylidae and Trichiuridae are two families included in the suborder Scombroidei, and together comprise the superfamily Trichiuroidea. Distinguishing characters of the two families are: Gempylidae – body semifusiform or elongate and moderately compressed; large, forked caudal fin; two nostrils; two clearly separated dorsal fins, and the first dorsal fin is longer than the second (excluding finlets); and Trichiuridae – body very elongate and extremely compressed; small, forked caudal fin or caudal fin absent; a single nostril; two continuous dorsal fins separated by a notch, and the second dorsal fin is longer than the first (Nakamura and Parin 1993).

The family Gempylidae is comprised of 16 genera and approximately 24 species, including the snake mackerels, snoeks, gemfishes, sackfishes, escolars, and the oilfish (Nelson 2006). Most gempylids are large voracious predators that swim fast in pursuit of prey. However, Paradiplospinus and Diplospinus capture prey by waiting in ambush. Many species reach maximum lengths greater than 1 m, but Diplospinus, Paradiplospinus, Nealotus, Neoeppinula, Rexichthys, Thrysites, Tongaichthys, and some species of Rexea have maximum sizes less than 50 cm (Nakamura and Parin 1993). Gempylids inhabit mesopelagic and benthopelagic environments, usually between 200 and 500 m depth, but some exhibit diel migrations to the surface at night. Other than Thrysites atun and Rexea solandri, no
fisheries target gempylid species. However, many species, including *Ruvettus pretiosus* and *Lepidocybium flavobrunneum*, are caught as bycatch in pelagic longline fisheries.

The family Trichiuridae includes 10 genera and approximately 39 species of cutlassfishes, hairtails, scabbardfishes, and frostfishes (Nelson 2006). Trichiurids are voracious predators that catch fish, squid, and crustaceans by waiting in ambush. While most species grow to maximum lengths greater than 50 cm, some grow to upwards of 2 m. Trichiurids are benthopelagic and usually inhabit the continental shelf and slope or underwater oceanic ridges down to 2000 m in depth. Commercial fisheries target several trichiurids, including *Trichiurus*, *Eupleurogrammus*, *Lepturacathus*, *Lepidopus*, and *Aphanopus*.

**Objectives**

The purpose of this thesis project was to investigate the relationships of fishes in the superfamily Trichiuroidea. Using molecular genetic characters, I examined both intraspecific and interspecific relationships of these fishes. In chapter one, I assessed the population genetic structure of the gempylid *Lepidocybium flavobrunneum*, commonly known as escolar, using mitochondrial control region sequence data. Escolar samples were collected from six geographically distant locations throughout the Atlantic and Pacific oceans to test for genetic heterogeneity among collections, within and between ocean basins. As little is known regarding the biology and life history of escolar, this study adds to our understanding of this species and provides a genetic perspective of its stock structure. In the second chapter, the systematic relationships of species from the Gempylidae and Trichiuridae were analyzed. Sequence data of two mitochondrial genes and one nuclear gene were used to
develop hypotheses of the evolutionary relationships of the two families and among species within each family. Results of this study build upon previously presented molecular and morphological phylogenetic hypotheses to better understand the evolutionary history of this group of fishes.
CHAPTER I:
POPULATION GENETIC STRUCTURE OF ESCOLAR

(LEPIDOCYBIUM FLAVOBRUNNEUM)
Introduction

Understanding spatial population structure is essential for effective fishery management. Classically, the stock structure of fishes was inferred through analyses of morphological and life history characters, but since the advent of molecular genetic techniques, genotypic data have allowed for improved characterization of population structure (Allendorf et al. 1987; Dizon et al. 1992; Stepien and Kocher 1997). Furthermore, molecular genetics provides a useful means to evaluate population structure of marine fishes that are relatively rare and may be less accessible for study (Avise 1998).

An exemplar species is escolar (*Lepidocybium flavobrunneum*, Smith 1849), a large, mesopelagic fish with a cosmopolitan distribution in tropical and temperate seas (Nakamura and Parin 1993). While generally one species of escolar is recognized, morphological differences have been found between Atlantic and Pacific specimens. The first dorsal pterygiophore inserts into the second interneural space in escolar specimens from the Atlantic (like other gempylids), while the first pterygiophore inserts into the third interneural space in Pacific escolar (similar to scombrids) (Collette et al. 1984). It has also been suggested that vertebral count (pre-caudal + caudal = total) varies between specimens from the Atlantic (16 + 15 = 31) versus the Indo-Pacific (17 + 15 = 32) (Collette et al. 1984). In addition, other than the Smith’s (1849) type specimen of *Lepidocybium flavobrunneum*, which was collected near the Cape of Good Hope, South Africa, two additional descriptions of the species have been presented from the Pacific Ocean [*Xenogrammus carinatum* (Waite 1904) and *Lepidosarda retrigramma* (Kishinouye 1926)] and one from a specimen from the Atlantic Ocean [*Diplogonurus maderensis* (Noronha 1926)], though currently all are considered synonyms for *L. flavobrunneum* (Nakamura and Parin 1993).
Escolar are not generally targeted by any fishery, and catch of escolar was extremely rare prior to the expansion of pelagic longline fisheries in the 1960s. In the mid-1970s, when many longline vessels switched to deeper gear deployments, fishing to depths greater than 200 m (Ward and Myers 2005a), bycatch of escolar became more common. Despite the rising levels of catch, many aspects of escolar biology are poorly understood (Maskimov 1970; Nishikawa 1982; Schwartz 1997; Milessi and Defeo 2002). Escolar is thought to be a "potential" highly migratory species (FAO 1994) with seasonal oscillations in catch rates suggesting migratory patterns similar to those of other large pelagic species (Maskimov 1970; Milessi and Defeo 2002). These seasonal movements may be attributed to feeding and reproductive behaviors. As evident from catch data of escolar in both the eastern North Atlantic and western South Atlantic, this species appears to migrate to frontal zones high in productivity for feeding during a portion of the year, then travel to lower latitudes to spawn (Maskimov 1970; Milessi and Defeo 2002). Escolar larvae are predominantly found near islands, suggesting that spawning occurs in near-shore areas, adjacent to oceanic islands or continental landmasses (Nishikawa 1982). The larvae are planktonic and occur in greatest densities at the surface layer of the water column (upper 1.5 m; Nishikawa 1987).

Little is known regarding the stock status of escolar. The catch of escolar has increased over the past few decades. Maximum reported catch rates in Pacific Ocean pelagic longline fisheries reached 0.8-3.1 per 1000 hooks through the 1990s (Ward et al. 2004). Increases in catch have led one to question the susceptibility of escolar to overfishing. Milessi and Defeo (2002) found that the mean size of escolar caught in the southeastern Atlantic decreased 40% over 15 years (1982-1996), a time period of significant fishing effort. They also found that the proportion of escolar and other bycatch species to target species
increased during this time period, a trend seen in other fisheries for large pelagic species (Ward and Myers 2005b). Though the increased proportion of bycatch may be due to a relative decrease in the abundance of target species, these trends, in conjunction with the decrease in mean size of escolar, suggest that escolar in some areas may be facing overexploitation.

No management measures are currently in place for escolar, and catch of this species is not routinely reported. In fact, the charter for the International Commission for the Conservation of Atlantic Tunas (ICCAT) explicitly states that the organization will not study or monitor any gempylid species (ICCAT 1969). In recent years, several regional fishery management organizations have recorded landing statistics for escolar, yet often escolar are incorrectly grouped with or misidentified as a closely related gempylid, oilfish (*Ruvettus pretiosus*), or a distantly related centrolophid, rudderfish (*Centrolophus niger*) (Bromhead and Wise 2005). Even if proper records of escolar landings existed, it would be difficult to assess the status of the species as virtually nothing is known of their population structure.

In general, marine fishes have greater intraspecific gene flow and reduced population structure compared to freshwater fishes, likely due to fewer barriers to dispersal in the marine environment (Ward et al. 1994; Graves 1998). This is especially true for species with planktonic eggs and larvae and/or highly migratory adults (Graves 1998; Waples 1998). However, a range of population structure is exhibited by highly migratory marine fishes from homogenous global populations (*Katsuwonus pelamis*, Graves et al. 1984; *Thunnus albacares*, Scoles and Graves 1993; *Thunnus maccoyii*, Grewe et al. 1997; *Acanthocybium solandri*, Garber et al. 2005) to population differentiation between ocean basins (*Makaira nigricans*, Buonaccorsi et al. 2001; *Thunnus alalunga*, Viñas et al. 2004; *Thunnus obesus*,...
Alvarado Bremer et al. 1998; Martínez et al. 2005; Chiang et al. 2006) to structure within ocean basins (*Xiphia gladius*, Alvarado Bremer et al. 1996; Reeb et al. 2000; *Istiophorus platypterus*, Graves and McDowell 2003). It is difficult to predict where escolar will fall along this continuum of population structure.

Several molecular genetic techniques are available to elucidate fish stock structure, but over the past 15 years, DNA-level polymorphisms have been increasingly used (Park and Moran 1994). One molecular character that is commonly surveyed is the mitochondrial genome, and with its fast rate of evolution, lack of recombination, and maternal inheritance, it has proven to be an appropriate character for the analysis of population structure (Brown et al. 1979; Avise et al. 1987) and the designation of population units for conservation purposes (Moritz 1994). Portions of the non-coding mitochondrial control region, or displacement loop (D-loop), have been found to have considerable variation in teleost fishes (Lee et al. 1995). For species with low genetic diversity, such as most marine fishes (Ward et al. 1994), it is important to survey a locus with significant variation to detect a signal of population structure. Furthermore, control region sequence data have successfully highlighted population differentiation within other highly migratory fishes with considerable dispersal capabilities (e.g., Alvarado Bremer et al. 1996; Reeb et al. 2000; Viñas et al. 2004).

In this study, mitochondrial DNA control region sequence data were analyzed to evaluate the population structure of escolar. In particular, we tested for the presence of population structure within and between collections from the Atlantic and Pacific oceans with the null hypothesis that escolar comprise a single genetic stock.
Materials and Methods

Sample Collection

Escolar were collected at various locations throughout the Atlantic and Pacific oceans (Figure 1), and muscle tissue samples were taken from approximately 20-50 individuals at each location. Samples were collected from six geographical areas: the western North Atlantic [U.S. Mid-Atlantic (USM, \( n = 5 \)) and the Gulf of Mexico (GM, \( n = 25 \))], the western South Atlantic [Brazil (BR, \( n = 25 \))], the eastern South Atlantic [South Africa (SA, \( n = 22 \))], the western South Pacific [eastern Australia (AU, \( n = 50 \))], the central North Pacific [Hawaii (HI, \( n = 46 \))], and the eastern Pacific [Ecuador (EC, \( n = 52 \))]. All tissue samples were frozen at the time of collection or stored in either DMSO buffer (Seutin et al. 1991) or 95% ethanol. Frozen samples were eventually transferred to DMSO buffer for long-term storage.

DNA Extraction

High molecular weight genomic DNA was extracted from each tissue sample using a proteinase K digestion and phenol/chloroform/isoamyl alcohol extraction protocol (modified from Sambrook and Russell 2001). Subsequent extractions with buffered phenol, phenol:chloroform:isoamyl alcohol (25:24:1), and chloroform:isoamyl alcohol (24:1) were performed, and DNA was precipitated from the aqueous phase with an equal volume of isopropanol and 0.04x volume 5M sodium chloride. DNA was collected with high-speed centrifugation, washed with 70% ethanol to remove excess salts, dried with vacuum centrifugation, and resuspended in 0.1X TE buffer, pH 8.0. Alternatively, total genomic DNA was extracted using Chelex beads (Sigma-Aldrich, St. Louis, MO) or DNeasy® tissue kit (QIAGEN, Inc., Valencia, CA) following the manufacturers’ protocols.
PCR Amplification

The mitochondrial control region of each escolar specimen was amplified using the polymerase chain reaction (PCR). The primers used in this study were designed from the conserved regions flanking the control region: Pro-F (5’ CTA CCY CYA ACT CCC AAA GC 3’; K. Gray, unpublished), Phe-R (5’ GTA AAG TCA CGA CCA CCA AAC C 3’; this study), and ESCR (5’ CGG ATA CTT GCA TGT GTA AG 3’; this study). Pro-F is similar to Pro-5’ (Palumbi 1996), but was modified to include degeneracies. The two reverse primers were designed from sequences of escolar that were generated using primers Pro-F and 12SAR-3’ (Palumbi 1996). The reverse primers occur in the 12S RNA gene region and Pro-F occurs in the tRNA Proline. All primers were produced by Invitrogen Corporation (Carlsbad, CA).

Each 25 µl PCR amplification contained 0.25 µl template DNA, 2.5 µl 10X PCR Buffer plus magnesium (QIAGEN), 0.5 µl 10 mM dNTP (QIAGEN), 0.25 µl of each 100 µM forward and reverse primer, 0.125 µl Taq, DNA polymerase (QIAGEN), 0.5 µl BSA (bovine serum albumin; 10 mg/ml), and sterile filtered water. For amplification of DNA extracted by Chelex beads, 2.5 µl of template DNA was used, and sterile filtered water adjusted accordingly. The reactions were performed on a MJ Research Corporation PTC-200 Peltier thermal cycler (Watertown, MA) using the following conditions: 94°C initial denaturation for 2 min., followed by 40 cycles of 94°C denaturation for 1 min, 50°C annealing for 30 sec., 72°C extension for 2 min., and a final extension at 72°C for 5 min. followed by a 4°C hold.

For a limited number of individuals from Atlantic and Pacific collections, a nuclear gene region was amplified and analyzed to determine if results from a biparentally inherited molecular marker were consistent with those from analyses of the mtDNA control region. Sequences of the internal transcribed spacer 1 (ITS-1) gene region, which occurs between the
5.8S and 18S ribosomal RNA genes, were analyzed for a total of 12 escolar samples (four from the South African collection, two each from the Gulf of Mexico, Brazil, and Australia collections, and one each from the Hawaii and Ecuador collections). ITS-1 was amplified using the primers F-ITS1 (5’ GAG GAA GTA AAA GTC GTA ACA AGG 3’) and 5.8SR1 (5’ ATT CAC ATT AGT TCT CGC AGC TA 3’) (K. Johnson, unpublished). PCR was performed in 10 µl volumes containing, 0.2 µl template DNA, 1.0 µl 10X PCR Buffer plus magnesium (QIAGEN), 0.2 µl 10 mM dNTP (QIAGEN), 0.1 µl of each 100 µM forward and reverse primer, 0.05 µl Taq, DNA polymerase (QIAGEN), 0.2 µl BSA (bovine serum albumin; 10 mg/ml), 2 µl Q-solution (QIAGEN), and sterile filtered water. The reactions were performed on a MJ Research Corporation PTC-200 Peltier thermal cycler (Watertown, MA) using the following conditions: 94°C initial denaturation for 4 min., followed by 40 cycles of 94°C denaturation for 1 min, 54°C annealing for 1 min., 72°C extension for 2 min., and a final extension at 72°C for 5 min. followed by a 4°C hold.

All PCR products were run on an agarose gel, stained with ethidium bromide and visualized under UV-light, to verify that a single fragment of the correct size had been amplified. PCR products of the control region were purified using EXOSAP (USB Scientific, Cleveland, OH) or column filtration with QIAquick® PCR purification kit (QIAGEN), following manufacturers’ instructions. PCR products from the nuclear gene ITS-1 were cloned into a plasmid vector using the TOPO-TA plasmid cloning system (Invitrogen Corportation) prior to sequencing. Fresh PCR products were ligated into the TOPO 2.1 plasmid vector and transformed into competent TOP10 *Escherichia coli* bacterial cells. *E. coli* cells were applied to Luria-Bertani (LB) agar plates containing ampicillin and 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (x-gal) and grown up overnight at
37°C. Colonies containing the recombinant plasmid (white colonies) were screened against colonies not containing the insert (blue colonies) to confirm that the plasmid contained the insert of interest. In short, DNA was extracted from the *E. coli* using phenol:chloroform:isoamyl alcohol (25:24:1), and then electrophoresed on a 1% agarose gel matrix to determine if the plasmids contained the insert. Recombinant plasmids were subsequently grown overnight in 3 ml of LB liquid media containing ampicillin. Cloned fragments were isolated and purified with QIAprep Spin Miniprep kits (QIAGEN) following manufacturer's instructions. The concentration of all purified cloned fragments and PCR products was measured using a Biomate-3 UV spectrophotometer (Thermo Spectronic, Rochester, NY). Four clones were sequenced for each of the 12 escolar specimens.

**DNA Sequencing and Sequence Analyses**

Purified PCR products and cloned fragments were cycle sequenced in forward and reverse directions using ABI PRISM® BigDye™ Terminator v 3.0 Cycle Sequencing Kits (Applied Biosystems, Foster City, CA), with minor modifications of the manufacturer's recommendations. Each forward and reverse sequencing reaction contained the following: 0.5 µl Big Dye® Terminator (Applied Biosystems), 1.0 µl 5X Sequencing Buffer, 0.3 µl primer, approximately 20 ng of purified PCR product, and sterile filtered water to at total reaction volume of 5 µl. Sequencing reactions were performed on an MJ Research Corporation PTC-200 Peltier thermal cycler with the following conditions: 96°C for 1 min., followed by 25 cycles of 96°C for 10 sec., 50°C for 5 sec., and 60°C for 4 min., and finally a 4°C hold. Sequencing products were precipitated by the addition of 0.75 µl 3M sodium acetate (pH 4.6), 15.625 µl nonadenatured 95% ethanol, and 3.625 µl sterile filtered water to
each 5 μl sequencing reaction. Following precipitation, each sample was resuspended in 20 μl Hi-Di formamide (Applied Biosystems) and denatured for 2 min. at 95°C. 10 μl of the resuspended sequencing reaction was transferred to a 96-well plate and placed in the ABI PRISM® 3130 Genetic Analyzer using 50 cm capillaries and POP7 matrix.

All sequences were analyzed using Sequencing Analysis software v 5.2 (Applied Biosystems). Forward and reverse sequences were aligned and edited using Sequencher version 4.2.2 (Gene Codes Corp., Ann Arbor, MI). All edited sequences were aligned using the ClustalW algorithm for multiple alignments (Thompson et al. 1994) in MacVector version 7.2 (Oxford Molecular Ltd, Madison, WI). The number of variable sites, including indels, transitions and transversions were calculated in ARLEQUIN version 3.0 (Excoffier et al. 2005). Sequences were designated as a specific haplotype, and each haplotype was submitted to GenBank.

Population Genetic Analyses

The program ARLEQUIN (Excoffier et al. 2005) was used to calculate the number and frequency of mtDNA control region haplotypes in each collection, and to estimate haplotype diversity \( h \) and nucleotide sequence diversity \( \pi \). Haplotype diversity \( h \) measures the probability of drawing a unique haplotype in a sample from consecutive draws, and nucleotide sequence diversity \( \pi \) is the mean number of nucleotide differences between all pairs of haplotypes in a sample (Nei 1987). The number of polymorphic sites \( S \) and mean number of pairwise differences \( k \) were also calculated for each collection.

The most appropriate nucleotide substitution model for the mtDNA control region sequences was determined from a series of likelihood ratio tests executed in ModelTest
version 3.7 (Posada and Crandall 1998). The resulting model and its parameters were used in PAUP* 4.0 (Swofford 2000) to produce a neighbor joining tree (Saitou and Nei 1987) of maximum likelihood distances. Support of the internal branches was tested using bootstrap resampling (Felsenstein 1985) with 1000 replicates.

To assess the hierarchical subdivision of genetic diversity of the mtDNA control region, an analysis of molecular variance (AMOVA) (Excoffier et al. 1992) was conducted in ARLEQUIN. The AMOVA partitioned variation between ocean basins, among collections within ocean basins, and among individuals within collections using genetic distances between haplotypes calculated under the Tamura and Nei (1993) nucleotide substitution model. This model, designed for mitochondrial control region sequence data, allows for excess transitions, unequal nucleotide frequencies, and variation among rates of substitution at different sites. The significance of each variance component was tested with 10 000 permutations. In addition, estimates of population pairwise \( \Phi_{ST} \) values, analogues to F-statistics obtained by the ratio of the estimated variance due to differences among populations to estimated total variance (Michalakis and Excoffier 1996), were calculated in ARLEQUIN and used as a measure of genetic distance between populations. The squared distance matrix between haplotypes used to calculate \( \Phi_{ST} \) values was also used to create a minimum spanning network of haplotypes (Excoffier et al 1992). A median-joining network was drawn using the software program Network version 4.200 (Bandelt et al. 1999).

Tests for the assumption of selective neutrality of the mtDNA control region sequences were performed using Tajima’s D (Tajima 1989) and Fu’s \( F_S \) (Fu 1997) with the program ARLEQUIN. Fu’s \( F_S \) is especially useful as a measure of departure of population equilibrium due to expansion. Population expansion was also analyzed by a test of mismatch
distribution of the observed number of differences between haplotypes. Further tests of population expansion were implemented using Hapending’s raggedness index (Harpending et al. 1993).

Sequence divergence between mtDNA clades was used to estimate time of separation of the Atlantic and Pacific lineages. For the first method, estimates of corrected nucleotide sequence divergence per site (δ) and rate of mutation per site per year (μ) were used to back-calculate the time since divergence (T) with the formula: T = δ/(2μ). The second method to estimate time since population divergence (T) uses the average number of nucleotide differences within and between populations while taking into account differences in population size (Gaggiotti and Excoffier 2000). For this method, an estimate of τ calculated in ARLEQUIN with 1000 replicates was used in the formula: T = τ/2u, where u = mμ. Rogers and Harpending (1992) define u to be the mutation rate of the entire region being analyzed, while m is the total aligned sequence length and μ is an estimate of the mutation rate per nucleotide. For both methods, a mutation rate of 3.6 x 10^{-8} per site per year was employed, following the reported control region mutation rate estimated by Donaldson and Wilson (1999) using geminate pairs of snook on either side of the Isthmus of Panama.

Variation of the nuclear ITS-1 sequences produced for the 12 escolar specimens was assessed using ARLEQUIN and MEGA 3.1 (Kumar et al. 2004), and a neighbor-joining tree of uncorrected p distances was produced in PAUP*. 
Results

The mitochondrial control region and flanking tRNAs were sequenced for 225 escolar specimens. The length of the control region ranged from 881 to 883 bp aligning to 886 bp, except for one individual from the Ecuador collection which had a much larger control region resulting from four 55 bp repeats in the 5' end of the fragment. Aligned sequences were trimmed to 806 bp for analyses. This fragment contained 106 polymorphic sites consisting of 88 transversions, 23 transitions, and 8 indels (Table 1). From the 225 individuals, a total of 145 haplotypes was found (Table 2). Haplotype diversity ($h$) for each sampling locale ranged from 0.867 to 0.990 with an overall value of 0.982. Nucleotide diversity ($\pi$) values ranged from 0.003 to 0.018 (overall $\pi = 0.026$).

The hierarchical likelihood ratio tests in MODELTEST selected HKY + I + $\Gamma$ (Hasegawa et al. 1985) as the most appropriate nucleotide substitution model. Under this model, the transition/transversion ratio was 8.870, the proportion of invariable sites 0.796, and gamma shape parameter 0.585. The overall observed base frequencies were $A = 0.32$, $C = 0.21$, $G = 0.16$, $T = 0.30$. A neighbor-joining phylogeny using maximum likelihood distances grouped all escolar sequences into two highly divergent clades (Figure 2), which were almost exclusively distributed in different ocean basins. All escolar with Atlantic clade haplotypes were found only in the Atlantic Ocean, and most escolar with Pacific clade haplotypes (99.1%) were found in the Pacific Ocean (Figure 3). Four individuals with Pacific clade haplotypes were found in the Atlantic Ocean, all occurring in the South Africa collection. A median-joining network of haplotypes also depicted the two clades with two star-shaped clusters connected by a long branch (Figure 4).
The HKY + I +Γ evolutionary model was not available in ARLEQUIN, so the Tamura and Nei (1993) model, which was designed from control region data, was chosen to perform AMOVA tests and estimates of pairwise $\Phi_{ST}$. We considered grouping the escolar specimens caught in the U.S. mid-Atlantic (USM) with the Gulf of Mexico (GM) collection, but a pairwise comparison revealed a significant difference between the two collections (data not shown), likely resulting from the small sample size from USM ($n = 5$). These sequences were omitted from population-level analyses due to the small sample size but were included in the Atlantic Ocean grouping for ocean and clade analyses.

The overall AMOVA found most of the variation (78.48%) among collections with only 21.58% occurring among individuals within collections (Table 3). The amount of variation partitioned within collections was even lower (16.29%) when the South Africa samples with Pacific clade haplotypes were excluded from the analysis. The variation among collections was almost entirely attributed to variation between oceans with a $\Phi_{ST}$ of 0.866, $p = 0.0000$. The AMOVA revealed a small but significant variation among collections within oceans ($\Phi_{SC} = 0.052, p = 0.0004$), but when the individuals from South Africa with Pacific clade haplotypes were excluded from the analyses, the value was not significant ($\Phi_{SC} = 0.006, p = 0.0939$). Analyses of each ocean separately yielded non-significant values of $\Phi_{ST}$ among collections within the Pacific Ocean ($\Phi_{ST} = -0.004, p = 0.7992$), but significant values were found within the Atlantic Ocean both including and excluding the South Africa samples with Pacific clades haplotypes ($\Phi_{ST} = 0.113, p = 0.0000$ and $\Phi_{ST} = 0.041, p = 0.0284$, respectively). To visualize the variation among haplotypes from different collections in the Atlantic Ocean, a neighbor-joining tree of haplotypes was constructed (Figure 5).
Pairwise $\Phi_{ST}$ values were used to determine possible population structuring within and between ocean basins (Table 4). Highly significant pairwise differentiation was found between all Atlantic and Pacific collections. Population comparisons of Pacific collections revealed no significant differences between collections. Conversely, small but significant differences were found among Atlantic collections. The Gulf of Mexico and Brazil collections were not found to be significantly different, but significant differences were found between the South Africa collection and both the Gulf of Mexico and Brazil collections when the four individuals with Pacific clade haplotypes were included ($p = 0.004$ and $0.000$, respectively). However, when the Pacific clade individuals in the South Africa collection were excluded from the analyses, significant differences only remained between South Africa and Brazil ($p = 0.009$).

The mismatch distribution of all haplotypes was bimodal (Figure 6), due to the large pairwise differences between the two clades and small differences within each clade. Mismatch distributions of each clade were unimodal, which is indicative of a population undergoing expansion (Rogers and Harpending 1992). Also, values of the sum of squared deviations (SSD) and Harpending’s raggedness indices ($Hri$) were both low and not significantly different from the sudden population expansion model (Table 4). In addition, Fu’s $F_S$ statistics were negative and significantly different ($p < 0.01$) from expectations of neutrality for each clade and for all samples combined, which is indicative of a departure from population equilibrium due to expansion. Though often more conservative, Tajima’s $D$ statistic was also negative and significant for each clade, also suggestive of population expansion, but no significant deviation from neutrality was found for pooled samples.
Using a mutation rate of 3.6% per site per million years (Donalson and Wilson 1999) and an interclade net nucleotide sequence divergence of 4.85%, an estimate of time since divergence (T) using was 0.67 million years (my). The value of τ estimated between the two clades in ARLEQUIN was 35.211, and when applied to the formula for coalescence time, the time since divergence from a common ancestor was estimated to be 0.61 my.

Four nuclear ITS-1 sequences were produced for each of the four South Africa individuals with Pacific clade mtDNA control region haplotypes as well as four representative individuals from each the Atlantic and Pacific oceans. The ITS-1 gene region analyzed varied in size from 717 to 735 bp. The 48 ITS-1 sequences aligned to 740 bp and contained 58 polymorphic sites (37 transitions, 6 transversions, and 21 indels), 40 of which were parsimony informative. The neighbor joining tree of uncorrected p distances divided the sequences into two clades, one containing individuals collected in the Pacific and South Africa collections and the other containing the remaining Atlantic individuals (Figure 7). The net nucleotide sequence divergence (δ) between clades was 1.95%, and there were five fixed nucleotide differences. The nucleotide diversity within clades was 0.93% and 1.17% for the Atlantic and Pacific clades, respectively. The diversity among the four cloned sequences within an individual ranged from 0.42 – 1.33%, with an overall mean of 0.72%.
Discussion

The length of the escolar mtDNA control region is similar to that reported for other scombroids (sailfish: 839 – 855 bp, McDowell 2002; bluefin tuna: 868 bp, Carlsson et al. 2004; wahoo: 889 – 894, Garber et al. 2005). Though one specimen had a much longer control region due to tandem repeats, similar variations have been noted for other fish species (Lee et al. 1995).

High variability was found within the control region in escolar, with 13% of the 806 nucleotides examined exhibiting polymorphisms. A large number of control region haplotypes were found (145 from 225 individuals), 93% of which were only present in one or two individuals. This resulted in a high haplotype diversity \( h = 0.867 - 0.990 \), which is consistent with the high mitochondrial control region haplotype variability reported for several scombroid species \( h \approx 0.99 \), Alvarado Bremer et al. 1996; Reeb et al. 2000; Viñas et al. 2004; Garber et al. 2005; Martínez et al. 2005; Chiang et al. 2006). Overall nucleotide diversity of the control region \( \pi = 0.026 \pm 0.013 \) was fairly high, similar to other scombroids including swordfish \( (0.022, \) Reeb et al. 2000), bluefin tuna \( (0.015, \) Carlsson et al. 2004), albacore \( (0.054, \) Viñas et al. 2004), bigeye tuna \( (0.054, \) Martínez et al. 2005), and wahoo \( (0.053, \) Garber et al. 2005). However, estimates of intraclade nucleotide diversities \( \pi = 0.003 - 0.006 \) were an order of magnitude lower than most intraclade diversities reported for other scombroids \( (\text{bluefin tuna}, 0.011 - 0.020, \) Carlsson et al. 2004; swordfish, 0.009 – 0.026, Alvarado Bremer et al. 2005; bigeye tuna, 0.028 – 0.037, Martínez et al. 2005).
Phylogeography

Phylogenetic analyses based on mitochondrial control region sequence data revealed the presence to two highly divergent lineages in escolar. The two escolar clades differed by a net nucleotide sequence divergence (δ) of 4.85%. Evidence of two mitochondrial clades sharing similar levels of divergence has been reported for blue marlin (δ = 5.17%, Graves and McDowell 1995, 2003; Buonaccorsi et al. 2001), sailfish (2.6%, Graves and McDowell 1995, 2003; McDowell 2002), swordfish (4.1%, Alvarado Bremer et al. 2005), bigeye tuna (4.9 – 7.0%, Alvarado Bremer 2005; Martínez et al. 2005), and wahoo (13.6%, Garber et al. 2005). Within wahoo, two mitochondrial clades were found evenly distributed throughout the Atlantic and Pacific oceans. In contrast, blue marlin, sailfish, swordfish, and bigeye tuna each has one clade that occurs predominantly, if not exclusively, in the Atlantic Ocean, while the other clade is found ubiquitously distributed throughout the Atlantic and Indo-Pacific ocean basins. Like these latter species, escolar has one clade that occurs only in the Atlantic and another clade that is found in both ocean basins. However, the “ubiquitous” clade of escolar is almost exclusively found in the Pacific (99.1%). Only four individuals with Pacific clade haplotypes were found in the Atlantic, all of which occurred in the South Africa collection.

Based on analyses of the maternally inherited mitochondrial control region, the four South Africa escolar with Pacific control region haplotypes may represent recent migrants from the Indian Ocean, or the products of a historical migration that subsequently interbred with other Atlantic escolar. In the case of recent migrants, one would expect the nuclear genes, which undergo recombination, to resemble those of Pacific individuals. Alternatively, if migrants have been breeding with Atlantic conspecifics for several generations, one would
expect nuclear genes to be similar to Atlantic individuals. Results from analyses of the nuclear gene region ITS-1 demonstrated that all four South Africa escolar under question grouped with samples from the Pacific collections, consistent with recent migration. These results differ from those found with nuclear analyses of other large pelagic species with two mitochondrial clades in the Atlantic. Nuclear genotypes were randomly distributed among the two mitochondrial clades of bigeye tuna (Durand et al. 2005), blue marlin (Buonaccorsi et al. 2001), and sailfish (McDowell 2002), which is consistent with historical isolation of populations followed by secondary contact and interbreeding between lineages.

Atlantic escolar from South Africa with Pacific clade mtDNA control region and nuclear ITS-1 genotypes likely represent recent migration from the Indian Ocean. The genetic and geographic divisions of the two escolar mitochondrial lineages are similar to those found for white marlin (Tetrapturus albidus) and striped marlin (T. audax), which occur in the Atlantic and Indo-Pacific oceans, respectively. The two marlin species have a lower interspecific control region nucleotide sequence divergence than the divergence between escolar lineages (2.25%, Graves and McDowell 2003; 4.85%, this study), and a similar leakage of Indo-Pacific striped marlin into Atlantic waters off South Africa has been reported based on morphological characters (Talbot and Penrith 1962). In addition, analyses of nuclear microsatellite loci can unambiguously differentiate between white marlin and striped marlin (Graves and McDowell unpublished data). The taxonomic status of white marlin and striped marlin has been debated due to great morphological (Nakamura, 1985) and molecular (reviewed in Graves and McDowell 2003) similarities of the two species, but they continue to be recognized as separate species (Collette et al. in press). In addition to the genetic differences between the two escolar lineages, slight morphological differences have
been reported between escolar from the Atlantic and Pacific oceans (Collette et al. 1984). The first dorsal pterygiophore inserts into the second interneural space in escolar specimens from the Atlantic (like other gempylids), while the first pterygiophore inserts into the third interneural space in Pacific escolar (similar to scombrids) (Collette et al. 1984). Also, vertebral counts may vary for escolar in the Atlantic versus Indo-Pacific (16 + 15 = 31 and 17 + 15 = 32, respectively) (Collette et al. 1984). As both molecular and morphological differences exist between geographically isolated lineages of escolar, it is possible that the two populations represent different species or subspecies. The type-locality of *Lepidocybium flavobrunneum* (Smith 1849) is the Cape of Good Hope, South Africa, so it is possible that this type specimen may have belonged to either the Atlantic or Indo-Pacific lineage. Other names have been suggested for escolar in the Pacific population [*Xenogrammus carinatum* (Waite 1904) and *Lepidosarda retrigramma* (Kishinouye 1926)] and the Atlantic population [*Diplogonurus maderensis* (Noronha 1926)], yet they are currently considered synonyms to *L. flavobrunneum*. While these names are available for the Atlantic and Indo-Pacific escolar lineages, further genetic analyses in conjunction with morphological analyses are necessary to help clarify any need for taxonomic revision of the species. In particular, these studies should include more samples from the eastern South Atlantic and western Indian Oceans.

The presence of two deep evolutionary lineages in the escolar mitochondrial genome is similar to several other highly migratory, pelagic fishes. The occurrence of two mitochondrial clades has been attributed to vicariance during the Pleistocene that separated Atlantic and Indo-Pacific populations of tropical and temperate species (Graves and McDowell 1995, 2003; Alvarado Bremer et al. 1998, 2005; Chow et al. 2000; Garber et al. 2005; Martínez et al. 2005). This explanation is likely applicable for escolar as well.
Though major vicariant events during the Pleistocene such as the rise of the Isthmus of Panama (3.6 – 3.5 mya, Coates et al. 1992) and the inception of the cold-water Benguela Upwelling along the southeast coast of Africa (2.0 – 2.5 mya, Shannon 1985), contributed to the isolation of the Atlantic and Indo-Pacific conspecifics, the separation of Atlantic and Indo-Pacific populations of escolar and other highly migratory species likely resulted from more recent glaciation events.

The Benguela Current, which presents a cold-water barrier between the Indian and Atlantic Oceans, has been found to be permeable by tropical and sub-tropical species due to the transfer of warm-water eddies from the Indian Ocean into the South Atlantic from retroflection of the Agulhas Current (Gordon 1985; Peeters et al. 2005). It has been suggested that this water transfer has allowed recent colonization of the eastern South Atlantic from the Indian Ocean (Rocha et al. 2005), and may have provided a corridor for gene flow among Atlantic and Indo-Pacific populations of escolar and other highly migratory species. However, the Agulhas Current has been found to diminish during glacial periods (Hutson 1980), which likely decreased the permeability of the Benguela cold-water barrier and contributed to the isolation of Atlantic and Indo-Pacific lineages of escolar. As a mesopelagic species that lives at depth, adult escolar may not be as affected by a cold-water barrier as epipelagic, tropical species. However, escolar appear to have a connection to the tropics, as fisheries data suggest that they migrate to lower latitudes to spawn (Maskimov 1970; Milessi and Defeo 2002). Thus, it is possible that the Benguela Current provided a sufficient barrier to escolar gene flow between the Atlantic and Indo-Pacific ocean basins during a glacial period. Significant glacial intervals have occurred approximately every 100 000 years for the past 700 000 years (Hewitt 1996), providing several instances where
isolation of the escolar lineages could occur. Though estimated times of divergence should be considered with caution, the estimates found for Atlantic and Indo-Pacific lineages of escolar (0.67 – 0.61 mya) are consistent with a glacial maxima that occurred approximately 0.68 – 0.62 mya (marine isotope stage (MIS) 16) (Gibbard and Kolfschoten 2004).

The occurrence of the Agulhas Retroflection is also a plausible reason for the presence of individuals with Pacific-like mtDNA control region and nuclear ITS-1 genotypes in the South Africa collection. As we are currently in an interglacial period, Agulhas eddies have been documented transferring Indian Ocean water around the Cape of Good Hope to the south Atlantic Ocean (Peeters et al. 2005). Agulhas eddies can penetrate through the depth range of escolar, pushing the 10°C isotherm as deep as 900 m (Gordon 1985). Escolar from the South Africa sample were captured near the Agulhas eddy corridor, so the individuals with Pacific clade haplotypes may have followed the warm-water isotherm of an eddy from the Indian Ocean into the Atlantic. Chow et al. (2000) suggest that bigeye tuna adults are capable of traversing between oceans via the Agulhas Retroflection, and they are likely to return to their ocean of origin to reproduce. In addition, a few Atlantic lineage bigeye tuna have been found in the Indian Ocean near Madagascar (n = 1) and Seychelles (n = 3) (Appleyard et al. 2002), so movement from Atlantic to Indian Ocean may likely occur as well. Additional sampling of escolar from different life stages along the east and west coasts of Africa would be necessary to determine if movement from the Atlantic to Indian Ocean is possible, and how far escolar from the Indo-Pacific lineage have penetrated into the Atlantic Ocean.
Population Structure and Demography

Hierarchical analysis of molecular variance indicated significant genetic differentiation among the six collections of escolar. The overall \( \Phi_{ST} \) calculated by pooling the six collections was high (\( \Phi_{ST} = 0.785, p < 0.00001 \)), with only 21.5% of the variance occurring within collections. This is unusual for marine fishes, which typically have much lower global fixation indices. For example, low but significant fixation indices are present in bigeye tuna (\( \Phi_{ST} = 0.22 \), Martínez et al. 2005), Atlantic mackerel (\( \Phi_{ST} = 0.02 \), Nesbø et al. 2000), and albacore (\( \Phi_{ST} = 0.041 \), Viñas et al. 2004). When escolar collections were grouped by ocean, almost all of the variation was partitioned among collections from different ocean basins (86.3%) rather than different collections from within oceans (0.7%). This can be attributed to the distribution of the two lineages in separate ocean basins. When the two lineages were divided, 90.5% of the variation occurred between lineages. Similarly high and significant variation (56.2 - 81.9%) has been found between lineages of cryptic fish species such as the goby, *Bathygobius soporator* (Lima et al. 2005).

Hierarchical partitioning of variance yielded a significant value of genetic differentiation among collections within ocean basins. However, an AMOVA of each ocean basin found no significant values of \( \Phi_{ST} \) in the Pacific collection (\( p = 0.801 \)), while significant \( \Phi_{ST} \) values were found for the Atlantic Ocean, both including and excluding the four South Africa samples with Pacific clade haplotypes (\( p = 0.0001 \) and 0.0288, respectively). Pairwise comparisons within oceans exhibited no significant \( \Phi_{ST} \) differences between Gulf of Mexico and Brazil collections, yet significant heterogeneity was found between each of these collections and the South Africa collection (\( p = 0.004 \) and 0.000,
respectively). When the four Pacific-like individuals were excluded from this analysis, only
differences between Brazil and South Africa remained (\( p = 0.009 \)). It is possible that gene
flow between Brazil and South Africa is limited, while connectivity exists between the Gulf
of Mexico and each of these locations. If escolar migrate to the tropics to spawn, as has been
suggested (Maskimov 1970), equatorial currents may allow transfer of larvae between distant
assemblages (i.e., South Africa and Gulf of Mexico) while restricting transfer between
others. It is also possible that this observed heterogeneity may be an artifact of small sample
size (BR, \( n = 25 \); SA, \( n = 18 \)). Further population-level analyses with microsatellite loci may
help clarify the intra-oceanic population structure of escolar. Non-significant pairwise \( \Phi_{ST} \)
comparisons between Pacific collections suggest homogenous gene flow of escolar within
the Pacific Ocean. A reverse pattern has been found for Atlantic white marlin (no geographic
population structure) and Indo-Pacific striped marlin (significant geographic heterogeneity)
with mtDNA control region sequences and microsatellite markers (McDowell and Graves in
press), and the authors attributed differences in population structure between the two species
to differing sizes of the two ocean basins.

Neutrality tests (Tajima’s D and Fu’s \( F_{S} \)) suggest that neither escolar lineage is in
population equilibrium. In addition, mismatch distributions for each clade were unimodal
and not significantly different from models of population expansion (by SSD and \( Hri \)).
These results support the hypothesis that each lineage has undergone significant population
expansion since separation.
Implications

This study uncovered two highly divergent mitochondrial lineages in escolar, which may represent more than population-level differentiation. Individuals analyzed at both the mitochondrial and nuclear loci consistently grouped into either the Atlantic or Pacific clade, and the distribution of the clades was nearly divided by ocean basin, suggesting that the two escolar lineages continue to be isolated with little, if any, contemporary gene flow. In addition to genetic differences, slight morphological differences between escolar from the Atlantic and Pacific have been reported (Collette et al. 1984). It is possible that the Atlantic and Indo-Pacific lineages of escolar may represent two separate species or subspecies, and further investigation into the taxonomic status of the escolar is warranted.

This is the first investigation into the population structure of escolar. As a common bycatch species that may experience overfishing (Milessi and Defeo 2002), it is important to understand the population structure of escolar to provide framework for meaningful stock assessments. Although no management practices or monitoring systems are currently in place for escolar, the significant inter-ocean genetic differences found for escolar suggest that populations from each ocean should be considered separate stocks.
Table 1. Summary table of population genetic statistics of escolar (*Lepidocybium flavobrunneum*) based on mitochondrial control region sequence data for each sampling location, each ocean, each clade, and overall samples.  Ts. = transitions, Tv. = transversions, and Indels = insertions or deletions. Values in parentheses include individuals from the South Africa collection with Pacific haplotypes.

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<td></td>
<td></td>
</tr>
<tr>
<td>Gulf of Mexico</td>
<td>25</td>
<td>12</td>
<td>19</td>
<td>0</td>
<td>0</td>
<td>19</td>
<td>0.867 ± 0.050</td>
<td>0.003 ± 0.002</td>
<td>2.757 ± 1.510</td>
</tr>
<tr>
<td>Brazil</td>
<td>25</td>
<td>17</td>
<td>21</td>
<td>1</td>
<td>1</td>
<td>22</td>
<td>0.943 ± 0.032</td>
<td>0.005 ± 0.003</td>
<td>3.525 ± 1.856</td>
</tr>
<tr>
<td>South Africa</td>
<td>18</td>
<td>13</td>
<td>18</td>
<td>0</td>
<td>1</td>
<td>19</td>
<td>0.928 ± 0.052</td>
<td>0.003 ± 0.002</td>
<td>2.510 ± 1.418</td>
</tr>
<tr>
<td></td>
<td>(22)</td>
<td>(17)</td>
<td>(46)</td>
<td>(9)</td>
<td>(7)</td>
<td>(61)</td>
<td>(0.952 ± 0.037)</td>
<td>(0.018 ± 0.010)</td>
<td>(14.850 ± 6.908)</td>
</tr>
<tr>
<td>Ecuador</td>
<td>52</td>
<td>44</td>
<td>40</td>
<td>5</td>
<td>1</td>
<td>45</td>
<td>0.983 ± 0.012</td>
<td>0.006 ± 0.003</td>
<td>4.924 ± 2.437</td>
</tr>
<tr>
<td>Hawaii</td>
<td>46</td>
<td>39</td>
<td>37</td>
<td>8</td>
<td>0</td>
<td>44</td>
<td>0.990 ± 0.008</td>
<td>0.006 ± 0.003</td>
<td>4.594 ± 2.298</td>
</tr>
<tr>
<td>Australia</td>
<td>50</td>
<td>36</td>
<td>32</td>
<td>7</td>
<td>0</td>
<td>37</td>
<td>0.966 ± 0.018</td>
<td>0.005 ± 0.003</td>
<td>3.730 ± 1.915</td>
</tr>
<tr>
<td><strong>By Ocean</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Atlantic</td>
<td>77</td>
<td>42</td>
<td>58</td>
<td>12</td>
<td>8</td>
<td>73</td>
<td>0.923 ± 0.022</td>
<td>0.009 ± 0.005</td>
<td>7.131 ± 3.382</td>
</tr>
<tr>
<td>Pacific</td>
<td>148</td>
<td>105</td>
<td>56</td>
<td>13</td>
<td>1</td>
<td>65</td>
<td>0.979 ± 0.007</td>
<td>0.006 ± 0.003</td>
<td>4.444 ± 2.204</td>
</tr>
<tr>
<td><strong>By Clade</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Atlantic Clade</td>
<td>73</td>
<td>38</td>
<td>38</td>
<td>3</td>
<td>2</td>
<td>41</td>
<td>0.914 ± 0.025</td>
<td>0.004 ± 0.002</td>
<td>3.246 ± 1.693</td>
</tr>
<tr>
<td>Pacific Clade</td>
<td>152</td>
<td>107</td>
<td>56</td>
<td>13</td>
<td>1</td>
<td>65</td>
<td>0.980 ± 0.007</td>
<td>0.006 ± 0.003</td>
<td>4.452 ± 2.207</td>
</tr>
<tr>
<td><strong>Overall</strong></td>
<td>225</td>
<td>145</td>
<td>88</td>
<td>23</td>
<td>8</td>
<td>106</td>
<td>0.982 ± 0.004</td>
<td>0.026 ± 0.013</td>
<td>21.334 ± 9.446</td>
</tr>
</tbody>
</table>
Table 2. Hierarchical variance partitioning and analysis of molecular variance (AMOVA) among escolar (*Lepidocybium flavobrunneum*) collections based on mitochondrial control region sequence data. Abbreviations for each collection: GM = Gulf of Mexico, BR = Brazil, SA = South Africa, EC = Ecuador, HI = Hawaii, and AU = Australia; *p*-values less than 0.05 were considered significant.

<table>
<thead>
<tr>
<th>Observed partition</th>
<th>Variance</th>
<th>% Total</th>
<th>(\Phi) Statistics</th>
<th>(p)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Overall (GM, BR, SA, EC, HI, AU) *</td>
<td>9.544</td>
<td>78.48</td>
<td>(\Phi_{ST} = 0.785)</td>
<td>0.0000</td>
</tr>
<tr>
<td>Among collections</td>
<td>2.617</td>
<td>21.52</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Within collections</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Overall (GM, BR, SA, EC, HI, AU) **</td>
<td>10.410</td>
<td>83.71</td>
<td>(\Phi_{ST} = 0.837)</td>
<td>0.0000</td>
</tr>
<tr>
<td>Among collections</td>
<td>2.026</td>
<td>16.29</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Within collections</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grouped by ocean (GM, BR, SA) (EC, HI, AU) *</td>
<td>17.354</td>
<td>86.27</td>
<td>(\Phi_{CT} = 0.863)</td>
<td>0.0000</td>
</tr>
<tr>
<td>Among oceans</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Among collections within oceans</td>
<td>0.144</td>
<td>0.71</td>
<td>(\Phi_{SC} = 0.052)</td>
<td>0.0004</td>
</tr>
<tr>
<td>Within collections</td>
<td>2.617</td>
<td>13.01</td>
<td>(\Phi_{ST} = 0.870)</td>
<td>0.0000</td>
</tr>
<tr>
<td>Grouped by ocean (GM, BR, SA) (EC, HI, AU) **</td>
<td>19.509</td>
<td>90.55</td>
<td>(\Phi_{CT} = 0.905)</td>
<td>0.0000</td>
</tr>
<tr>
<td>Among oceans</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Among collections within oceans</td>
<td>0.012</td>
<td>0.05</td>
<td>(\Phi_{SC} = 0.006)</td>
<td>0.0939</td>
</tr>
<tr>
<td>Within collections</td>
<td>2.026</td>
<td>9.40</td>
<td>(\Phi_{ST} = 0.906)</td>
<td>0.0000</td>
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<tr>
<td>Atlantic Ocean (GM, BR, SA)*</td>
<td>0.423</td>
<td>11.28</td>
<td>(\Phi_{ST} = 0.113)</td>
<td>0.0001</td>
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<tr>
<td>Among collections</td>
<td>3.329</td>
<td>88.72</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Within collections</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Atlantic Ocean (GM, BR, SA)**</td>
<td>0.064</td>
<td>4.09</td>
<td>(\Phi_{ST} = 0.041)</td>
<td>0.0288</td>
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<tr>
<td>Among collections</td>
<td>1.502</td>
<td>95.91</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Within collections</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pacific Ocean (EC, HI, AU)</td>
<td>-0.009</td>
<td>-0.39</td>
<td>(\Phi_{ST} = -0.004)</td>
<td>0.8013</td>
</tr>
<tr>
<td>Among collections</td>
<td>2.228</td>
<td>100.39</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Within collections</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>By Ocean (Atlantic, Pacific)*</td>
<td>17.578</td>
<td>86.61</td>
<td>(\Phi_{ST} = 0.866)</td>
<td>0.0000</td>
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<tr>
<td>Among Populations</td>
<td>2.718</td>
<td>13.39</td>
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<tr>
<td>Within Populations</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>By Clade (Atlantic, Pacific)</td>
<td>19.560</td>
<td>90.49</td>
<td>(\Phi_{ST} = 0.905)</td>
<td>0.0000</td>
</tr>
<tr>
<td>Among Populations</td>
<td>2.055</td>
<td>9.51</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Within Populations</td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

* Including Pacific-like samples from SA
** Omitting Pacific-like samples from SA
Table 3. Estimates of pairwise $\Phi_{ST}$ (below diagonal) and associated $p$-value (above diagonal) between collections of escolar (*Lepidocybium flavobrunneum*) based on mitochondrial control region sequence data (A) includes individuals from the South Africa collection with Pacific haplotypes; (B) excludes individuals from the South Africa collection with Pacific haplotypes. Bold $p$-values indicate significance ($p < 0.01$).

<table>
<thead>
<tr>
<th></th>
<th>Gulf of Mexico</th>
<th>Brazil</th>
<th>South Africa</th>
<th>Ecuador</th>
<th>Hawaii</th>
<th>Australia</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gulf of Mexico</td>
<td>*</td>
<td>0.122</td>
<td><strong>0.004</strong></td>
<td><strong>0.000</strong></td>
<td><strong>0.000</strong></td>
<td><strong>0.000</strong></td>
</tr>
<tr>
<td>Brazil</td>
<td>0.025</td>
<td>*</td>
<td><strong>0.000</strong></td>
<td><strong>0.000</strong></td>
<td><strong>0.000</strong></td>
<td><strong>0.000</strong></td>
</tr>
<tr>
<td>South Africa</td>
<td>0.122</td>
<td>0.141</td>
<td>*</td>
<td><strong>0.000</strong></td>
<td><strong>0.000</strong></td>
<td><strong>0.000</strong></td>
</tr>
<tr>
<td>Ecuador</td>
<td>0.902</td>
<td>0.897</td>
<td>0.765</td>
<td>*</td>
<td>0.913</td>
<td>0.557</td>
</tr>
<tr>
<td>Hawaii</td>
<td>0.905</td>
<td>0.900</td>
<td>0.760</td>
<td>-0.007</td>
<td>*</td>
<td>0.559</td>
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<tr>
<td>Australia</td>
<td>0.920</td>
<td>0.915</td>
<td>0.784</td>
<td>-0.002</td>
<td>-0.002</td>
<td>*</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Gulf of Mexico</th>
<th>Brazil</th>
<th>South Africa</th>
<th>Ecuador</th>
<th>Hawaii</th>
<th>Australia</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>B</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gulf of Mexico</td>
<td>*</td>
<td>0.119</td>
<td>0.169</td>
<td><strong>0.000</strong></td>
<td><strong>0.000</strong></td>
<td><strong>0.000</strong></td>
</tr>
<tr>
<td>Brazil</td>
<td>0.025</td>
<td>*</td>
<td><strong>0.009</strong></td>
<td><strong>0.000</strong></td>
<td><strong>0.000</strong></td>
<td><strong>0.000</strong></td>
</tr>
<tr>
<td>South Africa</td>
<td>0.016</td>
<td>0.083</td>
<td>*</td>
<td><strong>0.000</strong></td>
<td><strong>0.000</strong></td>
<td><strong>0.000</strong></td>
</tr>
<tr>
<td>Ecuador</td>
<td>0.902</td>
<td>0.897</td>
<td>0.897</td>
<td>*</td>
<td>0.913</td>
<td>0.557</td>
</tr>
<tr>
<td>Hawaii</td>
<td>0.905</td>
<td>0.900</td>
<td>0.900</td>
<td>-0.007</td>
<td>*</td>
<td>0.564</td>
</tr>
<tr>
<td>Australia</td>
<td>0.920</td>
<td>0.915</td>
<td>0.917</td>
<td>-0.002</td>
<td>-0.002</td>
<td>*</td>
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</table>
Table 4. Statistical tests of neutrality and estimates of population demography for escolar (Lepidocybium flavobrunneum) based on mitochondrial control region sequence data.

<table>
<thead>
<tr>
<th></th>
<th>Atlantic Clade</th>
<th>Pacific Clade</th>
<th>Overall</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>73</td>
<td>152</td>
<td>73</td>
</tr>
<tr>
<td>Mismatch Mean</td>
<td>3.26</td>
<td>4.42</td>
<td>22.86</td>
</tr>
<tr>
<td>Variance</td>
<td>4.32</td>
<td>4.49</td>
<td>446.73</td>
</tr>
<tr>
<td>SSD</td>
<td>0.000</td>
<td>0.002</td>
<td>0.035</td>
</tr>
<tr>
<td>p</td>
<td>0.761</td>
<td>0.780</td>
<td>0.167</td>
</tr>
<tr>
<td>Hri</td>
<td>0.012</td>
<td>0.016</td>
<td>0.007</td>
</tr>
<tr>
<td>p</td>
<td>0.937</td>
<td>0.581</td>
<td>0.665</td>
</tr>
<tr>
<td>Tajima's D</td>
<td>-1.941</td>
<td>-1.905</td>
<td>0.682</td>
</tr>
<tr>
<td>p</td>
<td>0.004</td>
<td>0.000</td>
<td>0.810</td>
</tr>
<tr>
<td>Fu's F_s</td>
<td>-26.305</td>
<td>-25.544</td>
<td>-23.645</td>
</tr>
<tr>
<td>p</td>
<td>0.000</td>
<td>0.000</td>
<td>0.005</td>
</tr>
</tbody>
</table>
Figure 1. Map of collection locations of escolar (*Lepidocybium flavobrunneum*) in the Atlantic and Pacific oceans. Samples were collected at seven geographically distant locations throughout the species’ range (GM = Gulf of Mexico; USM = United States Mid-Atlantic; BR = Brazil; SA = South Africa; EC = Ecuador; HI = Hawaii; AU = Australia).
HI (n = 46)
EC (n = 52)
USM (n = 5)
GM (n = 25)
BR (n = 25)
SA (n = 22)
AU (n = 50)
Figure 2. Neighbor-joining (NJ) tree of mitochondrial control region sequences using maximum likelihood distances calculated by the model HKY + I + Γ for escolar (*Lepidocybium flavobrunneum*) caught in the Atlantic and Pacific oceans. Collection location of each haplotype is indicated by color.
Collection Location
- Gulf of Mexico
- Brazil
- South Africa
- Ecuador

0.005
Figure 3. Map of the distribution of Atlantic (white) and Pacific (black) clade haplotypes of escolar (*Lepidocybium flavobrunneum*) for each sampling location in the Atlantic and Pacific oceans (GM = Gulf of Mexico; BR = Brazil; SA = South Africa; EC = Ecuador; HI = Hawaii; AU = Australia).
Figure 4. Median-joining network of haplotypes shows two distinct clades of escolar (*Lepidocybium flavobrunneum*) divided by 37 nucleotide changes.
Atlantic Clade

37 nucleotide differences

Pacific Clade
Figure 5. Neighbor-joining (NJ) tree using Tamura Nei (1993) distances of mitochondrial control region haplotypes from the Atlantic clade. A total of 38 haplotypes (A1 – A38) were found in the Atlantic clade. Collection location of each haplotype is indicated by color.
Figure 6. Mismatch distribution of pairwise differences between escolar (*Lepidocybium flavobrunneum*) mtDNA control region haplotypes (bars) and growth decline model (line) for (A) Atlantic clade \( (n = 73) \), (B) Pacific clade \( (n = 152) \), and (C) all samples \( (n = 225) \).
Figure 7. Neighbor-joining (NJ) tree of nuclear ITS-1 gene region sequences for four representative escolar (Lepidocybium flavobrunneum) samples from each the Atlantic and Pacific populations as well as four samples from the South Africa collection with Pacific mtDNA control region genotypes. Four cloned sequences (labeled A-D) were analyzed for each of the 12 samples. Samples are labeled according to collection location (GM = Gulf of Mexico; BR = Brazil; SA = South Africa; EC = Ecuador; HI = Hawaii; AU = Australia).
Atlantic Clade

Pacific Clade

0.001 substitutions/site
CHAPTER II:

MOLECULAR PHYLOGENY OF THE TRICHIUROIDEA
Introduction

The Gempylidae and Trichiuridae are two of 160 families that comprise the most speciose order of fishes, the Perciformes. Gempylids are benthopelagic, mesopelagic, and epipelagic fishes that include the snake mackerels, snoeks, gemfishes, sackfishes, escolars, and the oilfish. Trichiurids, which comprise the cutlassfishes, hairtails, scabbardfishes, and frostfishes, live predominantly in benthopelagic environments. These families along with Sphyraenidae (barracudas), Scombridae (mackerels and tunas), Xiphiidae (swordfish), and Istiophoridae (billfishes), comprise the suborder Scombroidei (Nelson 2006). The family Scombrolabracidae has been included in this suborder in the past, but due to its mixed scombroid and percoid characters, it is currently placed in its own suborder (Nelson 2006), though molecular evidence suggests that it may be a primitive member of the scombroids (Orrell et al. in press). Sphyraenidae has also been omitted from the suborder at times, and while Johnson (1986) found evidence that *Sphyraena* was the most primitive group of the Scombroidei, recent molecular sequence data places this family distant from other scombroids (Orrell et al. in press). In addition, placement of the billfishes (families Xiphiidae and Istiophoridae) within the Scombroidei has been contested (Nakamura 1985; Orrell et al. in press), with suggestions that these fishes originated elsewhere within the percoids.

In addition to the competing hypotheses of which families belong to the suborder Scombroidei, there is disagreement about lower taxonomic relationships within and among the Gempylidae and Trichiuridae. The Gempylidae and Trichiuridae are usually considered to be closely related (Regan 1919; Tucker 1956; Collette et al. 1984; Johnson 1986; Carpenter et al. 1995; Nakamura and Parin 1993; Gago 1997) and are sometimes grouped
into the superfamily Trichiuroidea; however, the relationship of the two families is unclear. Nakamura and Parin (1993) discussed the evolution of the two families based on general morphology and habitat, and two hypotheses of their evolution were considered: 1) mesopelagic ancestors gave rise to mesopelagic gempylids, which radiated into benthic trichiurids and epipelagic scombrids, and 2) trichiurids evolved from benthopelagic ancestors into benthic trichiurids and mesopelagic gempylids. The first hypothesis fits well with the fact that gempylid fishes have somewhat intermediate characteristics as compared to trichiurids and scombrids, including body form, coloration and markings, jaw teeth, tongue, and nostril shape (Nakamura and Parin 1993). Furthermore, the earliest fossils considered to be in the family Gempylidae, which date back to Campanian and Maastrichtian stages of the Late Cretaceous Epoch (83 – 65 mya), are otoliths that show affinities to the Recent gempylids *Lepidocybium flavobrunneum* and *Ruvettus pretiosus* (Nolf and Stringer 1996). It is possible that these ancestors radiated into the present-day gempylids, trichiurids and higher scombroids.

Detailed morphological studies have further investigated the phylogenetic relationships of the trichiurid fishes. In a study of several trichiurid fishes, Tucker (1956), placed members of the family Gempylidae in basal positions of the phylogenetic tree of the Trichiuridae (Figure 8). Based on an examination of 40 osteological and developmental characters, Collette et al. (1984) hypothesized that trichiurid fishes were sister to all other scombroids, and the gempylid fishes were more closely related to higher scombroids (Figure 9). Johnson (1986) contested these results with a cladistic study of morphological characters, claiming that fishes from these two families should be grouped into a single family, the Gempylidae, which includes *Lepidocybium* plus the tribes Gempylinae and Trichiurinae.
(Figure 10). Interestingly, the monotypic genus *Lepidocybium* was separated from the other gempylid species and found to be the most basal member of the group. An analysis of 62 morphological characters by Carpenter et al. (1995) agreed with Johnson’s rejection of a monophyletic Gempylidae, and they hypothesized a monophyletic Trichiuridae within the Gempylidae (Figure 11). In addition, the phylogeny of Carpenter et al. (1995) retained *Lepidocybium* as the most primitive member of the superfamily Trichiuroidea. A study of larval characters reaffirmed the monophyly of the Trichiuridae and placed gempylid fishes at the base of the phylogeny (Figure 12) (Gago 1997).

Molecular studies have failed to clarify the relationship between the families Gempylidae and Trichiuridae. Finnerty and Block (1993) included three species of gempylids and one trichiurid in a molecular phylogeny of the scombroids based on sequence analysis of the mitochondrial cytochrome *b* (*cyt b*) gene region. Though morphological studies usually agree on the grouping of the two families, this study found them to be paraphyletic with the trichiurid species *Trichiurus lepturus* falling out sister to gempylids + scombrids and the gempylid species placed within the scombrids (Figure 13). The two gempylid species included in a phylogeny based on cytochrome *c* oxidase II subunit (COX II) sequence data also were placed within the family Scombridae (Figure 14) (Dalziel et al. 2006), but no trichiurids were included in this study. Analyses of a nuclear gene region (TMO-4c4) grouped six gempylids and two trichiurids into one monophyletic group, more closely related to *Scombrolabrax* and *Pomatomus* than the scombrids (Figure 15) (Orrell et al. in press). In addition, the family Gempylidae was paraphyletic, as the trichiurids occurred within the group.
Few phylogenetic studies have investigated the relationships within the Gempylidae and Trichiuridae. Using morphological data, Tucker (1956) split the trichiurid fishes into three tribes: Aphanopodinae, which includes *Aphanopus, Benthodesmus*, and the gempylid *Diplospinus*; Lepidopodinae, including *Lepidopus, Assurger, Tentoriceps, Evoxymetopon*, and *Eupleurogrammus*; and Trichiurinae, which includes *Trichiurus* and *Lepturacanthus* (Figure 8). Carpenter et al. (1995) similarly found that *Diplospinous* was closely related to the trichiurids, though no interfamilial relationships were investigated (Figure 11). Gago’s (1997) phylogeny produced from an analysis of larval characters did not coincide with Tucker’s groupings within Trichiuridae (Figure 12). Gago (1997) did find a monophyletic group within the trichiurids containing *Benthodesmus, Lepidopus* and *Assurger*, but adult morphological characters did not support this grouping. *Aphanopus* was found to be the most primitive trichiurid, and *Trichiurus* and *Lepturacanthus* were found to be the most advanced fishes in the phylogeny. The newly described genus of the family Trichiuridae, *Demissolinea*, is considered to be closely related to the *Lepturacanthus* and *Trichiurus* (Burhanuddin and Iwatsuki 2003).

Studies of gempylid relationships are similarly confounded. A detailed morphological study by Russo (1983) found *Lepidocybium* to be the most primitive gempylid, followed by *Ruvettus*, while *Diplospinus* and *Paradiplospinus* were found to be the most advanced gempylids (Figure 16). The analysis of Carpenter et al. (1995) also hypothesized *Lepidocybium* to be the most primitive gempylid, but this was followed by *Tongaichthys* (Figure 11). Relationships between the other gempylids were not resolved. A larval character phylogeny by Gago (1997) again placed *Lepidocybium* as the basal member of the group, followed by *Nealotus* (Figure 12). The remainder of the species considered in
the study were divided into two groups, one including *Diplospinus, Eppinula, Paradiplospinus, Neoepippinula,* and *Rexea,* and the other group, which had a low support value, consisted of *Thyrsites, Thyrsitops, Gempylus, Nesiarchus, Promethichthys,* and *Ruvettus.* Other than the sister relationship of *Thyrsites* and *Thyrsitops,* no relationships within these groups were clarified. One molecular study examined six species of gempylids, and found that some of the species grouped together while others grouped with trichiurid fishes (Figure 15) (Orrell et al. in press).

Agreement of previous morphological and molecular phylogenetic studies of the Gempylidae and Trichiuridae has been poor, and the relationship of trichiurid fishes remains unclear. This study attempts to resolve the relationships within and between the families Gempylidae and Trichiuridae with a multi-locus molecular phylogeny. For our analyses, nucleotide sequence from two maternally inherited mitochondrial gene region sequences, cytochrome *c* oxidase subunit I (COI) and NADH dehydrogenase subunit 2 (ND2), as well as a nuclear region, TMO-4c4 were used.

Both COI and ND2 were found to be “good” phylogenetic performers among the mitochondrial genes when tested against expected phylogenies of tetrapods and mammals (Zardoya and Meyer 1996). Of the 13 mitochondrial protein-coding genes, COI was found to be the most conserved in fishes (Meyer 1993). The subunits of cytochrome oxidase play a critical role in aerobic energy production and ATP synthesis, and consequently have been conserved due to natural selection (Schmidt et al. 2005). Thus, the low nucleotide sequence variability in COI, mostly found at third positions of codons in fishes (Beckenbach 1991; Meyer 1993), is appropriate for phylogenetic studies at the genus, family, and higher taxonomic levels. The ND2 gene is also a mitochondrial protein-coding gene, yet it is highly
variable compared to COI (Meyer 1993), and thus useful for determining relationships of more closely related species. ND2 has been used in several phylogenetic studies of teleosts (Banford et al. 1999; Thacker and Hardman 2005; Schelly et al. 2006).

For more complete understanding of the relationships within the Trichiuroidea, a nuclear gene region was also analyzed. We chose to clone and sequence the TMO-4c4 gene region, which was developed from the genomic library of the cichlid *Tropheus moorii* (Streelman and Karl 1997). This nuclear region has been shown to amplify across many teleost taxa and has been used for phylogenetic studies of fishes from the families Labroidei (Streelman and Karl 1997; Streelman et al. 2002; Westneat and Alfaro 2005) and Scombroidei (Orrell et al. in press). In addition, several of the TMO-4c4 sequences produced by Orrell et al. (in press) were available for this study and used in our analyses.

In the present study we focused on the following questions: 1) do gempylid and trichiurid fishes group together to form a monophyletic super-family Trichiuroidea, 2) are each of the gempylid and trichiurid families monophyletic, and 3) is the genus *Lepidocybium* the most primitive member of the trichiuroid group?
Materials and Methods

Sample Collection

Tissue samples of eleven species representing eleven of the sixteen genera of Gempylidae were collected as well as six species from five of the ten genera of Trichiuridae (Figure 17). Included in these samples were the gempylid genera: Diplospinus, Gempylus, Lepidocybium, Nealotus, Neopinnula, Nesiarchus, Paradiplospinus, Promethichthys, Rexea, Ruvettus, and Thyrsites, and the trichiurid genera: Aphanopus, Assurger, Benthodesmus, Lepidopus, and Trichiurus. In addition, five species from the family Scombridae, and one species each from Sphyraenidae, Scombrolabracidae, and Pomatomidae were chosen to serve as outgroups. When possible, samples of each species were obtained from both the Atlantic and Indo-Pacific oceans to account for intraspecific variation. All tissue samples were frozen at the time of collection or stored in either DMSO buffer (Seutin et al. 1991) or 95% ethanol. Frozen samples were eventually transferred to DMSO buffer for long-term storage. Several of the TMO-4c4 sequences used in the analyses were obtained from a study by Orrell et al. (in press) deposited in GenBank with accession numbers: DQ388068, DQ388070, DQ388078 – DQ388085, DQ388091, DQ388092, DQ388097, DQ388099, and DQ388103.

DNA Extraction

High molecular weight genomic DNA was extracted from each tissue sample using a proteinase K digestion and phenol/chloroform/isoamyl alcohol extraction protocol (modified from Sambrook and Russell 2001). Subsequent extractions with buffered phenol, phenol:chloroform:isoamyl alcohol (25:24:1), and chloroform:isoamyl alcohol (24:1) were performed, and DNA was precipitated from the aqueous phase with equal volume of
isopropanol and 0.04x volume 5M sodium chloride. DNA was collected with high-speed centrifugation, washed with 70% ethanol to remove excess salts, dried with vacuum centrifugation, and resuspended in 0.1X TE buffer, pH 8.0. Alternatively, total genomic DNA was extracted using DNeasy® tissue kit (QIAGEN, Inc., Valencia, CA) following the manufacturer’s instructions.

**PCR Amplification**

The polymerase chain reaction (PCR) was employed to amplify a portion of COI, ND2, and TMO-4c4 gene regions for each sample. The primers used for COI amplification were a combination of universal primers developed by Folmer et al. (1994), primers designed from known COI sequences of several scombrid fishes deposited in GenBank (Paine et al. in press), and primers designed for this study using the online software Primaclade (Gadberry et al. 2005) and COI sequences of a diversity of marine fishes available in GenBank (Table 5). The primers used to amplify a portion of the ND2 gene region were either designed by eye (K. Gray, unpublished) or with the use of Primaclade using sequences of scombroids and other fishes found in GenBank. The TMO-4c4 primers were developed by Streelman and Karl (1997). All primers were produced by Invitrogen Corporation (Carlsbad, CA).

PCR was performed in 25 µl volumes, each containing 0.25 µl template DNA, 2.5 µl 10X PCR buffer plus magnesium (QIAGEN, Inc., Valencia, CA), 0.5 µl 10 mM dNTP (QIAGEN), 0.25 µl of each 100 µM forward and reverse primer, 0.125 µl Taq DNA polymerase (QIAGEN), 0.5 µl BSA (Bovine Serum Albumin; 10 mg/ml), and 21.125 µl sterile filtered water. PCR was performed on a MJ Research Corporation PTC-200 Peltier thermal cycler (Watertown, MA) with the conditions: 94°C initial denaturation for 4 min,
followed by 40 cycles of 94°C denaturation for 1 min., 50-58°C annealing for 1 min., 72°C extension for 2 min., and a final extension at 72°C for 5 min. followed by a 4°C hold.

All PCR products were visualized on a 1.5 % agarose gel to determine if a single fragment of the correct size had been amplified. PCR products of COI and ND2 amplification were purified using EXOSAP (USB Scientific, Cleveland, OH) or column filtration with QIAquick® PCR purification kit (QIAGEN), following manufacturers’ instructions.

PCR products from the nuclear gene TMO-4c4 were cloned into a plasmid vector using the TOPO-TA plasmid cloning system (Invitrogen Corporation) prior to sequencing. Fresh PCR products were ligated into the TOPO 2.1 plasmid vector and transformed into competent TOP10 *Escherichia coli* bacterial cells. *E. coli* cells were applied to Luria-Bertani (LB) agar plates containing ampicillin and 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (x-gal) and grown up overnight at 37°C. Colonies containing the recombinant plasmid (white colonies) were screened against colonies not containing the insert (blue colonies) to confirm that the plasmid contained the insert of interest. In short, DNA was extracted from the *E. coli* using phenol:chloroform:isoamyl alcohol (25:24:1), and then electrophoresed on a 1% agarose gel matrix to determine if the plasmids contained the insert. Recombinant plasmids were subsequently grown overnight in 3 ml of LB liquid media containing ampicillin. Cloned fragments were isolated and purified with QIAprep Spin Miniprep kits (QIAGEN) following manufacturer’s instructions. The concentration of all purified cloned fragments and PCR products was measured using a Biomate-3 UV spectrophotometer (Thermo Spectronic, Rochester, NY). For each species, between one and five clones were sequenced.
Sequencing and Sequence Analyses

Purified PCR products and cloned fragments were cycle sequenced using ABI PRISM® BigDye™ Terminator v 3.1 Cycle Sequencing Kits following manufacturer's instructions (Applied Biosystems, Foster City, CA). Each forward and reverse sequencing reaction contained the following: 0.5 μl Big Dye® Terminator (Applied Biosystems), 1 μl 5X Sequencing Buffer, 0.3 μl primer, 20 – 40 ng purified PCR product and sterile filtered water to a total reaction volume of 5 μl. Sequencing reactions were carried out on a thermal cycler with the following conditions: 96°C for 1 min., followed by 25 cycles of 96°C for 10 sec., 50°C for 5 sec., and 60°C for 4 min., and a final hold at 4°C. Sequencing products were precipitated by the addition of 0.75 μl 3M sodium acetate (pH 4.6), 15.625 μl non-denatured 95% ethanol, and 3.625 sterile filtered water to each 5 μl sequencing reaction. Following precipitation, each sample was resuspended in 20 μl Hi-Di formamide (Applied Biosystems) and denatured for 2 min. at 95°C. 10 μl of the resuspended sequencing reaction was transferred to a 96-well plate and placed in the ABI PRISM® 3130 Genetic Analyzer using 50 cm capillaries and POP7 matrix.

All sequences were analyzed using Sequencing Analysis software v 5.2 (Applied Biosystems). Forward and reverse sequences were compared and edited using Sequencher version 4.2.2 (Gene Codes Corp., Ann Arbor, MI). For species with multiple samples or TMO-4c4 clones, sequences were aligned, and the consensus was used for analyses. All edited sequences of each species were aligned using the ClustalW algorithm in MacVector version 7.2 (Oxford Molecular Ltd, Madison, WI). Correct assignment of 1st, 2nd, and 3rd positions were determined for COI, ND2, and TMO-4c4 protein-coding genes by translating
the sequences in MacVector. Translations that produced a chain of amino acids without stop codons were determined to be the correct orientation.

Estimates of nucleotide composition, number of variable sites, and relative frequencies of transitions and transversions were performed with PAUP* version 4.0b10 (Swofford 2000). To examine sequence saturation within each data set, we plotted the number of transitions and transversions against uncorrected percent nucleotide sequence divergence for each of the 1st, 2nd, and 3rd positions and the entire gene region. These values were estimated using the program JaDis (Goncalves et al. 1999) and the values were plotted in Excel. A gene region or position within the sequence was deemed saturated (multiple substitutions at a single nucleotide position) when the relationship between the number of nucleotide changes and genetic distance reached an asymptote. Saturated positions, which can skew phylogenetic inferences, were eliminated from the data set for all parsimony analyses.

Before combining sequence data from different gene regions, partitioned homogeneity tests (Farris et al. 1994) implemented in PAUP* were used to test for compatibility between data sets. Incongruence length differences (ILDs) were examined by a heuristic search of 1 000 replicates and 100 random sequence additions, to determine if there were conflicts between the two mitochondrial gene regions (COI and ND2) and among the mitochondrial and nuclear gene regions (COI, ND2, and TMO-4c4). To reject the null hypothesis of congruence, trees produced from combined data sets must be significantly longer than trees randomly made from partitions of equal length.
Phylogenetic Analyses

Phylogenetic relationships were inferred by maximum parsimony, maximum likelihood, and Bayesian analyses for each gene region separately, combined mitochondrial gene regions, and over all mitochondrial and nuclear gene regions. Parsimony analyses were conducted in PAUP* using a heuristic search algorithm with tree-bisection-reconnection (TBR) branch swapping with 100 random sequence additions. All saturated positions were eliminated from the analyses, and the remaining nucleotides were unordered and equally weighted. Gaps were treated as missing data. Support for each node was evaluated with nonparametric bootstrap analysis (Felsenstein 1985) using 10 000 pseudoreplicates and 100 random sequence additions per replicate with TBR.

ModelTest version 3.7 (Posada and Crandall 1998) was used to determine the most appropriate evolutionary model for each gene region, combined mitochondrial genes, and overall gene regions. The models selected by the Akaike Information Criterion (AIC) were used in subsequent maximum likelihood and Bayesian analyses. Maximum likelihood analyses were performed using the program PAUP* using a heuristic search with TBR branch swapping with 10 random sequence additions. Bayesian analyses were conducted with the program MrBayes version 3.1.2 (Ronquist and Huelsenbeck 2003) using a computer cluster available with the Computational Biology Service Unit from Cornell University. For each gene region and the combined mitochondrial and overall data sets two metropolis coupled markov chain monte carlo (MCMCMC) analyses were run from random starting trees with three heated chains and one cold chain over five million generations, with sampling every 1000 generations. As a default in the program, the burn-in period was removed by eliminating the first 25% of the generations for analyses. The remaining trees
for both runs were combined to produce a 50% majority-rule consensus tree, and the posterior probability of each bipartition was used as a measure of node support.
Results

Sequences of the COI, ND2, and TMO-4c4 gene region obtained for the phylogenetic analyses were 944 bp, 952 bp, and 511 bp respectively, resulting in a concatenated mitochondrial data set of 1896 bp and an overall data set of 2407 bp. The base composition was biased against guanine for the mitochondrial genes (%G = 18.6 and 13.8 for COI and ND2, respectively) and against cytosine for TMO-4c4 (%C = 18.9) (Table 6). A chi-square test of homogeneity of base composition between species was not significant for the COI and TMO-4c4 gene regions, but it was significant for ND2 ($p = 0.013$). The combined mitochondrial genes also had significant base frequency heterogeneity ($p = 0.011$), but the test of base homogeneity for the overall data set was not significant.

The number of base changes was plotted against the percent pairwise differences for each gene region to infer the degree of saturation at each position. In both mitochondrial data sets, saturation was found for third position transitions (Figures 18 and 19). Consequently, all third positions of the COI and ND2 data sets were removed for the parsimony analyses, resulting in data sets of 629 bp and 635 bp fragments for the COI and ND2 gene regions, respectively. The TMO-4c4 gene region was not found to be saturated at any position (Figure 20), so all nucleotide positions were included in subsequent analyses.

Due to poor amplification of the ND2 sequence fragment for the outgroup taxon *Scombrolabrax*, sequencing yielded approximately 600 bp of the ND2 gene region. Trimming all of the other sequences to this length would have resulted in a loss of over 100 parsimony informative sites with third positions removed, so *Scombrolabrax* was not included in the analyses of the ND2 gene region or the concatenated mitochondrial and overall data sets.
Partition homogeneity tests of the parsimony data sets, which did not include 3rd positions for mitochondrial genes, found congruence between mitochondrial ($p = 0.967$) and among mitochondrial and nuclear ($p = 0.428$) gene region analyses. Similarly, compatibility of gene regions was found using the entire sequence lengths (3rd positions included; $p = 0.675$ and 0.383 for mitochondrial and overall data sets, respectively). Therefore, gene region sequences were combined for parsimony, maximum likelihood, and Bayesian analyses.

Parsimony analysis of each gene region yielded four equally parsimonious trees (EPTs) for COI (length = 2250, retention index (RI) = 0.296), seven for ND2 (length = 2584, RI = 0.291), and six for TMO-4c4 (length = 286, RI = 0.635). Both ND2 and COI sequence fragments had a large number of parsimony informative sites (302 and 351, respectively), while TMO-4c4 had 70 parsimony informative sites (Table 7). For the combined mtDNA data, 650 of 1264 bp were parsimony informative and parsimony analysis yielded a single most parsimonious tree of length 4776, RI = 0.289. Adding the nuclear TMO-4c4 data to this analysis rendered a single most parsimonious tree of length 5109, RI = 0.298.

For the concatenated data sets, there was low bootstrap support (< 50%) for most nodes on the trees (Figure 21A, B). Support was high for joining the scombrids *Auxis rochei* and *Auxis thazard* (100) and moderate for joining *Sarda* to these two species (mitochondrial $= 73$, overall $= 63$). The joining of *Diplospinus* and *Paradiplospinus* was also strong (mitochondrial $= 96$, overall $= 100$), as was the sister relationship of *Gempylus* and *Thyrsites* (mitochondrial $= 71$, overall $= 93$). For the mitochondrial data set, the sister relationship of *Aphanopus* and *Benthodesmus* had moderate support (65), and for the overall data set, *Promethichthys* and *Nealotus* joined with low support (55).
The individual mitochondrial gene regions also had little bootstrap support of the nodes (Figure 21C, D). Parsimony analysis of TMO-4c4 yielded six EPTs and a greater number of bootstrap-supported nodes (Figure 21E). The grouping of the gempylid and trichiurid taxa with Pomatomus, and Scombrolabrax taxa had low support (53), but the grouping of trichiurids into a monophyletic clade had higher support (80).

The entire length of sequence for each gene region and concatenated data sets was used for maximum likelihood and Bayesian analyses. ModelTest found the GTR + I + Γ model to be the most appropriate model for each gene region and the combined mitochondrial genes, and the most appropriate model for the overall data set was the TVM + I + Γ (Table 7). Each of these models was employed in the maximum likelihood analyses with PAUP*, and the GTR + I + Γ model was used for each gene region and each data partition of the concatenated data sets in the Bayesian analyses with the program MrBayes.

The maximum likelihood analyses of the concatenated mitochondrial and overall data sets each produced similar trees (Figures 22A, B, respectively). Both trees were rooted by Sphyraena and Pomatomus, and all scombrid species branched into a monophyletic group. The Gempylidae + Trichiuridae formed a monophyletic group, and while the trichiurid species formed a monophyletic group, the gempylid species were paraphyletic. Lepidocybium, Ruvettus, and Neoeppinula formed the most basal group of the gempylid + trichiurid clade. All other gempylids were found sister to the trichiurids, and they were split into two clades, one including Gempylus, Nesiarchus, and Thyrsites, and the other including Rexea, Paradiplospinus, Diplospinus, Promethichthys, and Nealotus. Maximum likelihood analyses of individual gene regions resulted in slightly different trees, though several of the sister group relationships were the same (Figure 22C-E).
Results of the Bayesian analyses of concatenated data sets were similar to the maximum likelihood results. Many nodes of the Bayesian 50% majority-rule trees had high or moderate posterior probabilities. For the combined mtDNA, *Sphyraena* was the root of the tree, and *Pomatomus* grouped with the scombrids with low support (60; Figure 23 A), but for the combined mitochondrial and nuclear data set, *Pomatomus* did not group with the scombrids (Figure 23 B). The family Scombridae formed a highly supported monophyletic group with both the mitochondrial and overall data sets (97 and 100, respectively). Though there was low support for the trichiurid + gempylid grouping for the mitochondrial data set (54), this group had high support for the overall data set (97). Neither of the combined data sets found the family Gempylidae to be monophyletic, yet both provided high support for a monophyletic Trichiuridae (100). Though many of the relationships among gempylid taxa were unresolved, some interspecific relationships were clarified with high support.

*Paradiplospinus* was found to have a sister relationship to *Diplospinus* for both data sets (100), and these two taxa in addition to *Promethichthys* and *Nealotus* form a monophyletic group with high support (100) that is sister to *Rexea* (mitochondrial = 94, overall = 96). Some relationships were confounded between the two concatenated data sets, as the mitochondrial data set found high support for the sister relationship of *Nesiarchus* and *Thyrsites* (100), the overall data set found *Gempylus* to be sister to *Thyrsites* (99). Bayesian analyses of individual gene regions revealed some of the same relationships, such as a monophyletic Trichiuridae (COI (100) and TMO-4c4 (100)), monophyletic Scombridae (COI (100) and ND2 (58)), and monophyletic grouping of the Trichiuridae + Gempylidae (TMO-4c4 (98)) (Figure 23C-E). However, many of the phylogenetic relationships varied by gene region.
Discussion

Phylogenetic relationships within and between the families Gempylidae and Trichiuridae were examined using concatenated mitochondrial and mitochondrial + nuclear gene regions. The combined data sets yielded fairly consistent phylogenetic hypotheses when inferred by maximum likelihood and Bayesian analyses. Parsimony analyses were less consistent, and many relationships were unresolved. Few nodes of the parsimony 50% majority-rule consensus trees were supported by bootstrapping, and the EPTs had low retention and rescaled consistency index values. Thus, for the most part, only maximum likelihood and Bayesian analyses were informative. In addition, the results from concatenated data sets were more thoroughly considered because analyses of individual gene regions yielded varying phylogenetic hypotheses, and combining multiple gene regions has been shown to produce more reliable phylogenies (Mattern 2004).

The Superfamily Trichiuroidea

Many morphological (Johnson 1984; Carpenter et al. 1995; Gago 1997) and molecular (Orrell et al. in press) phylogenetic studies of the Trichiuridae and Gempylidae have found that these two families form a monophyletic group. As early as Regan (1919), the Gempylidae and Trichiuridae have been grouped into the superfamily Trichiuroidea. While gempylid and trichiurid species were divided in a molecular phylogeny based on cyt b sequences (Finnerty and Block 1993), this study found fairly strong support for monophyly for the trichuroid fishes. Monophyletic clusters of the families Gempylidae and Trichiuridae were found for both concatenated mitochondrial and overall data sets with both maximum likelihood and Bayesian analyses. Support values were not calculated for maximum
likelihood analyses, and while low posterior probability support was found for the grouping of the trichiuroid fishes for the mitochondrial data sets (54), high support was found for the overall data set (97). Thus, the grouping of the Gempylidae and Trichiuridae into a monophyletic superfamily appears to be a strongly supported taxonomic hypothesis.

The superfamily Trichiuroidea was found to be more closely related to members of the family Scombridae than other outgroup taxa. Other than *Pomatomus* grouping with the scombrids with low support (60) in the Bayesian analysis of the combined mitochondrial data set, *Pomatomus* and *Sphyraena* had unresolved relationships at the base of the phylogeny. However, maximum likelihood and Bayesian analyses of the individual COI and TMO-4c4 gene regions found *Pomatomus* and *Scombrolabrax* to be more related to the trichiurid taxa than the scombrids. Similar results were found by Orrell et al. (in press), also by using TMO-4c4 sequence data.

*The Family Trichiuridae*

The monophyly of the Trichiuridae has been hypothesized by several studies (Tucker 1956; Collette et al. 1984; Carpenter et al. 1995; Gago 1997), and no one has contested these relationships. Results of this study support a monophyletic Trichiuridae. Both concatenated mitochondrial and overall data sets clustered the trichiurid fishes into a monophyletic group with maximum likelihood and Bayesian analyses, and support of this clade was high with a posterior probability of 100 for both data sets.

Interspecific relationships among the trichiurids varied slightly between the concatenated data sets, but both maximum likelihood and Bayesian analyses arrived at similar tree topologies. Two clades were found for the combined mitochondrial data sets,
one including *Aphanopus* and *Benthodesmus* with a posterior probability of 99, and the other including *Trichiurus, Lepidopus caudatus, L. altifrons*, and *Assurger* with a posterior probability of 100. Tucker (1956) joined *Aphanopus* and *Benthodesmus* into the tribe Aphanopodinae that also included the gempylid *Diplospinus*. However, the overall concatenated data set of this study dissolved the *Aphanopus* and *Benthodesmus* clade, and *Benthodesmus* fell out sister to all other trichiurids, while *Aphanopus* was sister to all other trichiurids minus *Benthodesmus*. The other clade (including *Trichiurus, Lepidopus caudatus, L. altifrons*, and *Assurger*) was hypothesized for both concatenated data sets with posterior probabilities of 100. Tucker (1956) hypothesized that *Assurger* and *Lepidopus* were in the same tribe Lepidopodinae with *Tentoriceps, Evoxymetopon*, and *Eupleurogrammus*, but he included *Trichiurus* in the tribe Trichiurinae with *Lepturacanthus*. *Tentoriceps, Evoxymetopon, Eupleurogrammus*, and *Lepturacanthus* were unavailable for this study, so direct comparison with Tucker’s findings was not possible. None of the interspecific relationships of trichiurids hypothesized by Gago (1997) using larval characters coincided with the results of this study. He found *Assurger, Lepidopus*, and *Benthodesmus* to be closely related, but this hypothesis was not supported in this study.

Interestingly, none of the concatenated data sets revealed a sister relationship between the two *Lepidopus* species. *L. altifrons* was found sister to *Assurger* with both concatenated data sets, and *L. caudatus* was either found sister to this grouping or this grouping plus *Trichiurus*. Addition of the other four *Lepidopus* species may change this topology, yet further study of species from this genus and closely related taxa (e.g., *Assurger* and *Trichiurus*) is warranted to investigate possible revision of this genus.
The Family Gempylidae

Though the proposed phylogeny of Russo (1983) suggests the family Gempylidae is monophyletic, the monophyly of the family has been questioned (Johnson 1986), and several studies have found the Gempylidae to be paraphyletic (Johnson 1986; Carpenter et al. 1995; Gago 1997; Orrell et al. in press). Results of this study also indicate that the family Gempylidae is paraphyletic with the trichiurid fishes falling within the clade of gempylids. In both mitochondrial and concatenated data sets analyzed with maximum likelihood, the trichiurid cluster appears as an advanced clade within the Gempylidae. This is similar to the findings of Johnson (1986) and Gago (1997) where trichiurid fishes are placed in advanced position on a tree with gempylid species at the base. Carpenter et al. (1995) also proposed that the trichiurid cluster branched from within the gempylids, and more specifically placed *Diplospinus* at the base of this branch. The paraphyly of the Gempylidae was not as clearly resolved with Bayesian analyses of the two concatenated data sets, but monophyly of the family was not evident.

Paraphyly of the Gempylidae was demonstrated for both concatenated data sets analyzed with maximum likelihood. One clade of gempylids occurred sister to the trichiurids and one clade occurred sister to all other gempylids + trichiurids. This configuration is similar to that proposed by Johnson (1986) with *Lepidocybium* as the basal member sister to other gempylids + trichiurids. However, for this study the basal clade was comprised of *Lepidocybium, Ruvettus* and *Neoepinnula*. Though few trichiurid species were analyzed, Orrell et al. (in press) also found *Lepidocybium + Ruvettus* to be sister to other gempylids + trichiurids. Results of this study indicate that it is possible for *Lepidocybium* to be the most basal member of the family Gempylidae as hypothesized by Russo (1983), Johnson (1984),
Carpenter et al. (1995), and Gago (1997), though other species (e.g., *Ruvettus* and *Neoepinnula*) are also potential candidates for this position on the tree. Russo (1983) hypothesized *Ruvettus* and *Neoepinnula* to be positioned closer to the base of the gempylid phylogeny with *Ruvettus* sister to all gempylids minus *Lepidocybium* and *Neoepinnula* in the next-most basal unresolved group. It should be noted that these relationships were not resolved by Bayesian analyses. *Ruvettus + Neoepinnula* had poor support (52) and the relationship with *Lepidocybium* was unresolved for the combined mitochondrial data set. For the overall data set, Bayesian analysis did not resolve the relationships of *Lepidocybium* and *Ruvettus* to the other gempylid species, and *Neoepinnula* grouped with *Gempylus + Thyrsites* with low support (53).

The other species of gempylids that did not group with the basal clade were divided into two sub-clades with maximum likelihood analyses of both concatenated data sets. One sub-clade included *Gempylus, Nesiarchus,* and *Thyrsites,* while the other group was comprised of *Rexea, Paradiplospinus, Diplospinus, Promethichthys,* and *Nealotus.* For both mitochondrial and overall combined data sets, this latter grouping was recognized with high support by posterior probabilities from Bayesian analyses (94 and 96), and *Rexea* was found to be sister to the other four species. The grouping of *Paradiplospinus, Diplospinus,* *Promethichthys,* and *Nealotus* also had high posterior probability support for both data sets (100), and revealed a highly supported sister relationship between *Paradiplospinus* and *Diplospinus* (100). While most of the gempylid groupings inferred from this study differ from previous hypotheses, the pairing of *Diplospinus* and *Paradiplospinus* was found by Russo (1983), and both of these taxa were present in an unresolved clade in Gago’s (1997) study of larval characters.
Conclusions

With a survey of 17 of the approximately 63 species, representing 16 of the 26 genera of the trichiuroid fishes, this study is the most comprehensive molecular phylogeny of this group of fishes to date. Results of this study support the joining of the gempylid and trichiurid fishes into a monophyletic superfamily, the Trichiuroidea. This superfamily was found to be sister to members of the family Scombridae, yet some individual gene analyses placed Pomatomus and Scombrolabrax more closely related to the trichiuroids. As has been found in previous studies, the trichiurid species formed a monophyletic clade, which was positioned within the Gempylidae. Consequently, the monophyly of the Gempylidae was not supported, as one clade of gempylids formed sister relationships with the Trichiuridae, while other members were positioned at the base of this grouping. The basal gempylids were comprised of Lepidocybium, which was previously hypothesized as the most primitive gempylid, as well as Ruvettus and Neoeppinula. While many major taxon groupings and sister relationships of the Trichiuroidea were resolved with this study, further work surveying a greater number of species over additional gene regions is necessary to more fully understand the evolutionary relationships of this group of fishes.

Overall, the phylogenetic relationships resolved for trichiuroid fishes in this study appear congruent with those hypothesized by Johnson (1986). Consistent with his suggestions, we recommend that the two families Gempylidae and Trichiuridae be joined into one family, the Gempylidae consisting of three tribes: Gempylinae, Trichiurinae, and Lepidocybinae. However, contrary to Johnson’s results, Lepidocybinae is likely comprised of multiple species, including Lepidocybium, Ruvettus and Neoeppinula. Further analyses including more species are recommended to clarify this grouping.
Table 5. Primers and primer sequences used in PCR amplification and cycle sequencing of COI, ND2, and TMO-4c4 gene regions of the Trichiuroidea.

<table>
<thead>
<tr>
<th>Name</th>
<th>Primer Sequence</th>
<th>Reference</th>
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<tr>
<td><strong>COI</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LCO1490</td>
<td>GGT CAA CAA ATC ATA AAG ATA</td>
<td>Folmer et al. 1994</td>
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<tr>
<td>LCOI 121</td>
<td>CTA AGC CAA CCA GGT GCC CTT CT</td>
<td>M. Paine, in press</td>
</tr>
<tr>
<td>HCOI 1199</td>
<td>AAT AGT GGG AAT CAG TGT AGC A</td>
<td>M. Paine, in press</td>
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<tr>
<td>COI 34D</td>
<td>CAY AAA GAY ATC GGC ACC CT</td>
<td>This study</td>
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<tr>
<td>COI 1273D</td>
<td>ARG AAF TGY TGB GGG AAG AA</td>
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<tr>
<td>COI 104</td>
<td>TGC TYA TCC GAG CYG AAC</td>
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<td>COI 1336</td>
<td>TGT GTT TCA AAG RGT RTA GGC</td>
<td>This study</td>
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<tr>
<td><strong>ND2</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ND2B-L</td>
<td>TAA GCT TTY GGG CCC ATA C</td>
<td>K. Gray</td>
</tr>
<tr>
<td>ND2E-H</td>
<td>CRR TTA GGR CTT TGA AGG C</td>
<td>K. Gray</td>
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<tr>
<td>ND2F</td>
<td>CTH TTG GGC CCA TAC CCC</td>
<td>This study</td>
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<tr>
<td><strong>TMO-4c4</strong></td>
<td></td>
<td></td>
</tr>
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<td>TMO-4c4F</td>
<td>CCT CCG GCC TTC CTA AAA CCT CTC</td>
<td>Streelman et al. 1997</td>
</tr>
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<td>TMO-4c4R</td>
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<td>Tmo-1-6f</td>
<td>GAA AAG AGT GTT TGA AAA TGA</td>
<td>Westneat and Alfaro</td>
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<td>2005</td>
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Table 6. Size and nucleotide composition of each of the three gene regions surveyed in this study, as well as concatenated mitochondrial (ND2 and COI) and overall (ND2, COI, and TMO-4c4) data sets. In addition, the table includes a chi-squared test of homogeneity of base frequencies.

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<th>C</th>
<th>G</th>
<th>T</th>
<th>X²</th>
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<td>TMO-4c4</td>
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<td>0.316</td>
<td>0.162</td>
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<td>0.262</td>
<td>0.289</td>
<td>0.182</td>
<td>0.267</td>
<td>79.402</td>
<td>69</td>
<td>0.184</td>
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Table 7. Summary statistics of parsimony and maximum likelihood analyses of COI, ND2, and TMO-4c4 DNA sequences in the Trichiuroidea. Abbreviations: parsimony informative (PI), equally parsimonious tree (EPT), retention index (RI), rescaled consistency index (RC), proportion of invariable sites (I), alpha shape parameter (Γ).

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<th>Maximum likelihood</th>
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<td></td>
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<tr>
<td>COI</td>
<td>944 (629)</td>
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<td>302</td>
<td>4</td>
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<tr>
<td>Overall</td>
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<td>720</td>
<td>1</td>
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<td>TVM + I + G</td>
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Figure 8. Proposed phylogeny of the trichiuroid fishes based on morphological characters studied by Tucker (1956; Fig. 23).
Figure 9. Phylogenetic tree of scombroid fishes based on 40 morphological and developmental characters hypothesized by Collette et al. (1984; Fig. 312).
Figure 10. Cladogram of hypothesized relationships of the Scombroidei and immediate outgroups as proposed by Johnson (1986; Fig. 1).
Figure 11. Cladistic hypothesis of the evolutionary relationships of the Scombroidei and immediate outgroups as proposed by Carpenter et al. (1995; Fig. 5) resulting from a strict consensus of 1,682 equally parsimonious trees from the analysis of 62 morphological and developmental characters.
Figure 12. Phylogenetic hypothesis of the Trichiuroidea proposed by Gago (1997; Fig. 2) from a fifty percent majority-rule consensus tree of 87 equally parsimonious trees obtained by branch-and-bound analysis of larval characters. Numbers indicate the percentage of trees that support those nodes.
Figure 13. Phylogenetic hypothesis of the Scombroidei and outgroup taxa from the single most parsimonious tree based on 600 bp of the mtDNA cytochrome b gene region (Finnerty and Block 1993; Fig. 2).
Figure 14. Phylogenetic hypothesis of the Scombroidei and outgroup taxa from a consensus of 3 equally likely maximum likelihood trees based on sequence data of COX II gene region (Dalziel et al. 2006; Fig. 1).
Figure 15. Phylogenetic hypothesis of the Scombroidei and outgroup taxa from a maximum likelihood analysis of TMO-4c4 sequence data (Orrell et al. in press; Fig. 2).
scnDNA TMO-4c4 Maximum Likelihood Analysis

ML model=GTR+I+G
-ln L=3955.95541
Figure 16. Phylogenetic hypothesis of the Gempylidae based on non-homoplastic morphological characters (Russo 1983; Fig. 52).
Figure 17. Species of Gempylidae, Trichiuridae and outgroup taxa included in this molecular phylogenetic study.
<table>
<thead>
<tr>
<th>Gempylidae</th>
<th>Trichiuridae</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diplospinus multistriatus</td>
<td>Aphaneus carbo</td>
</tr>
<tr>
<td>Gempylus serpens</td>
<td>Assurger anzac</td>
</tr>
<tr>
<td>Lepidocybium flavobramaenum</td>
<td>Berthodesmus simonyi</td>
</tr>
<tr>
<td>Nealeactus tripes</td>
<td>Lepidopus altifrons</td>
</tr>
<tr>
<td>Nestarchus nasutus</td>
<td>Lepidopus caudatus</td>
</tr>
<tr>
<td>Neosomnuda americana</td>
<td>Trichiurus lepturus</td>
</tr>
<tr>
<td>Paradiplospinus</td>
<td></td>
</tr>
<tr>
<td>Promethichthys prometheus</td>
<td>Scombridae</td>
</tr>
<tr>
<td>Rexea solandri</td>
<td>Acanthocybium solandri</td>
</tr>
<tr>
<td>Ruvettus pretiosus</td>
<td>Sarda sarda</td>
</tr>
<tr>
<td>Thyrsites atun</td>
<td>Auxis rochei</td>
</tr>
<tr>
<td>Sphyraenidae</td>
<td>Auxis thazard</td>
</tr>
<tr>
<td>Sphyraena barracuda</td>
<td>Scomber scombrus</td>
</tr>
<tr>
<td>Pomatomidae</td>
<td>Scombrolabracidae</td>
</tr>
<tr>
<td>Pomatomus saltatrix</td>
<td>Scombrolabrax heterocephis</td>
</tr>
</tbody>
</table>
Figure 18. Nucleotide substitution patterns observed in pairwise comparisons of the mtDNA COI gene region in the Trichiuroidea and outgroup taxa. The number of transitions (squares) and transversions (diamonds) observed in (A) first, (B) second, and (C) third positions were plotted against pairwise uncorrected sequence divergence to investigate sequence saturation for each nucleotide position.
Figure 19. Nucleotide substitution patterns observed in pairwise comparisons of the mtDNA ND2 gene region in the Trichiuroidea and outgroup taxa. The number of transitions (squares) and transversions (diamonds) observed in (A) first, (B) second, and (C) third positions were plotted against pairwise uncorrected sequence divergence to investigate sequence saturation for each nucleotide position.
Figure 20. Nucleotide substitution patterns observed in pairwise comparisons of the nuclear TMO-4c4 gene region in the Trichiuroidea and outgroup taxa. The number of transitions (squares) and transversions (diamonds) observed in (A) first, (B) second, and (C) third positions were plotted against pairwise uncorrected sequence divergence to investigate sequence saturation for each nucleotide position.
Figure 21. Hypotheses of phylogenetic relationships of the Trichiuroidea and outgroup taxa based on parsimony analyses of (A) concatenated mitochondrial COI and ND2, (B) concatenated mitochondrial COI and ND2 and nuclear TMO-4c4 sequence data, as well as individual (C) COI, (D) ND2, and (E) TMO-4c4 gene regions. Trees represent the 50% majority-rule consensus of the equally parsimonious trees, and numbers above each bipartition indicate percent of trees supporting the node. Nodal support was also assessed with bootstrapping (1000 pseudoreplicates, 100 random sequence additions), and numbers below each data bipartition indicate bootstrap support (if >50%).
Figure 21 continued.
Figure 22. Hypotheses of phylogenetic relationships of the Trichiuroidea and outgroup taxa based on maximum likelihood analyses of (A) concatenated mitochondrial COI and ND2, (B) concatenated mitochondrial COI and ND2 and nuclear TMO-4c4 sequence data, as well as individual (C) COI, (D) ND2, and (E) TMO-4c4 gene regions. All likelihood analyses were performed with 100 random sequence additions.
Figure 22 continued.
Figure 22 continued.
Figure 23. Hypotheses of phylogenetic relationships of the Trichiuroidea and outgroup taxa based on Bayesian analyses of (A) concatenated mitochondrial COI and ND2, (B) concatenated mitochondrial COI and ND2 and nuclear TMO-4c4 sequence data, as well as individual (C) COI, (D) ND2, and (E) TMO-4c4 gene regions. Trees represent the 50% majority-rule consensus of the equally parsimonious trees, and numbers above each bipartition indicate the posterior probability from 5 million generations sampled every 1000 generations.
Figure 23 continued.
Figure 23 continued.
CONCLUSIONS

Population structure of escolar (*Lepidocybium flavobrunneum*) was evaluated with nucleotide sequence data of the mitochondrial control region. The geographic distribution of molecular variation was not consistent with a global, panmictic stock of escolar, and the null hypothesis of a single genetic stock was rejected. Nearly all of the nucleotide sequence variation was partitioned between the Atlantic and Pacific collections. Variation among collections within oceans was negligible, and pairwise comparisons revealed slight population differentiation among the Atlantic collections and no heterogeneity among the Pacific collections. However, the population structure found among the Atlantic collections may be an artifact of small sample size.

Analyses revealed two highly distinct clades of haplotypes, which were almost exclusively divided between the Atlantic and Pacific oceans. The geographical separation of the two lineages is indicative of vicariant isolation, and the amount of divergence between clades suggests that separation of lineages likely occurred during a period of glaciation in the Pleistocene. All specimens with Atlantic clade haplotypes were found in the Atlantic Ocean, and all but four specimens with Pacific clade haplotypes were found in the Pacific Ocean. The four escolar individuals with Pacific clade haplotypes were all caught in the South Africa sample. These individuals may represent migrants from the Indian Ocean around the Cape of Good Hope, but further analyses using a biparentally inherited nuclear gene must be performed to verify this.
The high nucleotide sequence divergence and near geographical isolation of the Atlantic and Pacific escolar lineages, as well as previously reported morphological differences between Atlantic and Pacific specimens suggest that each lineage may represent a separate species or subspecies. Further study regarding the taxonomic status of escolar is warranted. Analyses of various life history stages of escolar caught along the east and west coasts of Africa using biparentally inherited, nuclear loci would be necessary to test for contemporary gene flow between lineages.

Phylogenetic analyses of two mitochondrial (COI and ND2) and one nuclear (TMO-4c4) gene regions were conducted for the superfamily Trichiuroidea. Included in this study were eleven species of Gempylidae and six species of Trichiuridae. The specific phylogenetic hypotheses that I addressed were: 1) the monophyly of the superfamily Trichiuroidea within the suborder Scombroidei, 2) the monophyly of each the Gempylidae and Trichiuridae families, and 3) the placement of *Lepidocybium* basal to all trichiurid fishes. Consistent with several morphological and molecular studies, my analyses supported the monophyly of the superfamily Trichiuroidea. In addition, the family Trichiuridae formed a monophyletic clade within the Gempylidae. The family Gempylidae was found to be paraphyletic with analyses supporting the sister grouping of one clade of gempylids with the trichiurids, while the other clade was positioned sister to the other gempylids + trichiurids. *Lepidocybium* was positioned within the basal clade of the Gempylidae, as were *Ruvettus* and *Neoepinnula*. Many of the sister relationships between species within the Gempylidae and Trichiuridae were consistent among the different types of analyses within this study, but other relationships were either poorly supported or unresolved. To more clearly resolve the relationships within the Gempylidae and Trichiuridae, additional taxon sampling would be
necessary. Future work should include samples from the genera unavailable for this study as well as more species within genera. Analyses of this study did not group two congeneric *Lepidopus* species as sister taxa, so consideration of multiple species from each genus may contribute to a different hypothesis of trichiurid relationships. Future work should also consider using more or different mitochondrial and nuclear gene regions. Though even the most conserved mitochondrial region (COI) was found to be saturated at 3rd position transitions for this study, use of a more variable region like the mitochondrial control region may help elucidate relationships among closely related taxa. A different nuclear gene region with greater variation may also be useful, as TMO-4c4 had only 70 parsimony informative sites of 511 bp.
APPENDICES
Appendix 1. Mitochondrial control region haplotype frequencies for escolar 
(*Lepidocybium flavobrunneum*) from seven collection locations (BR = Brazil; GM = Gulf 
of Mexico; USM = U.S. Mid-Atlantic; SA = South Africa; EC = Ecuador; HI = Hawaii; 
AU = Australia). Haplotypes designated as A or P to indicate either belonging to the 
Atlantic or Pacific clade, respectively.
<table>
<thead>
<tr>
<th>Haplotype</th>
<th>BR</th>
<th>GM</th>
<th>USM</th>
<th>SA</th>
<th>EC</th>
<th>HI</th>
<th>AU</th>
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Kirsten Senger Brendtro was born in Petoskey, Michigan on April 1, 1982, the daughter of Paul Brendtro and Mary Senger. She graduated as co-valedictorian from Charlevoix High School, Charlevoix, MI in 2000. She went on to earn a B.S. in Zoology, concentration Marine Biology at Michigan State University, East Lansing, MI in the spring of 2004 where she also worked in a molecular genetics laboratory in conjunction with the Howard Hughes Undergraduate Research Scholar Program. Also prior to graduation, she worked in a marine biotoxicology laboratory in Charleston, SC in the summer 2003 with a Research Experience for Undergraduates Program with the College of Charleston Grice Marine Lab. In the fall of 2004, she entered the Masters of Science program at the College of William and Mary, School of Marine Science.