

Population genetic structure of escolar (*Lepidocybium flavobrunneum*)

Kirsten S. Brendtro · Jan R. McDowell · John E. Graves

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Abstract Escolar (*Lepidocybium flavobrunneum*) is a large, mesopelagic fish that inhabits tropical and temperate seas throughout the world, and 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 its population structure. 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 ($\delta = 4.85\%$) 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 found in the South Africa collection. The nuclear ITS-1 gene region of these four individuals was subsequently analyzed and compared to the ITS-1 gene region of four individuals from the South Africa collection with Atlantic clade haplotypes as well as four representative individuals each from the Atlantic and Pacific collections. 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 they 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.

Introduction

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 relatively homogenous global populations (skipjack tuna, Graves et al. 1984; yellowfin tuna, Scoles and Graves 1993; southern bluefin tuna, Grewe et al. 1997; and wahoo, Garber et al. 2005) to population differentiation between ocean basins (albacore tuna, Viñas et al. 2004; bigeye tuna, Alvarado Bremer et al. 1998; Martínez et al. 2005; Chiang et al. 2006; and blue marlin, Buonaccorsi et al. 2001) to structure within ocean basins (Atlantic bluefin tuna, Carlsson et al. 2007; sailfish, Graves and McDowell 2003; striped marlin, McDowell and Graves 2008, and swordfish, Alvarado Bremer et al. 1996; Reeb et al. 2000). It is difficult to predict where other pelagic species will fall along this continuum of population structure.

Escolar (*Lepidocybium flavobrunneum*, Smith 1849) is a large, mesopelagic fish with a cosmopolitan distribution in

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K. S. Brendtro · J. R. McDowell · J. E. Graves (&)
School of Marine Science,
Virginia Institute of Marine Science,
College of William and Mary,
Gloucester Point, VA 23062, USA
e-mail: graves@vims.edu

tropical and temperate seas (Nakamura and Parin 1993). While a single species of escolar is generally recognized, morphological differences have been found between Atlantic and Indo-Pacific specimens. The first dorsal pterygiophore inserts into the second interneural space in escolar specimens from the Atlantic (like other gempylids) but inserts into the third interneural space in Indo-Pacific escolar (similar to scombrids) (Collette et al. 1984). In addition, it has been noted 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).

Many aspects of escolar biology are poorly understood (Maskimov 1970; Nishikawa 1982; Schwartz 1997; Milessi and Defeo 2002). Escolar are 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 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 are thought to reach maturity by 30–35 cm, or earlier (Maskimov 1970) and 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).

Escolar are not generally targeted by any fishery, and catches were 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 and total catch has increased over the past few decades. Maximum reported catch rates in Pacific Ocean pelagic longline fisheries reached 0.8–3.1 per 1,000 hooks through the 1990s (Ward et al. 2004). Increases in catch have led to questions about the susceptibility of escolar to overfishing, though little is known regarding the stock status of the species. 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 they may be facing overexploitation. No management measures are currently in place, and catch of this species is not routinely reported.

In this study, we evaluated the population structure of escolar using mitochondrial DNA control region sequence data. Specifically, we used samples collected from throughout the Atlantic and Pacific oceans to test 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 (Fig. 1), and muscle tissue samples were taken from approximately 20–50 individuals at each location with the exception of the U.S. Mid-Atlantic where only 5 samples were available. 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 isolation and amplification

High molecular weight genomic DNA was extracted from each tissue sample using either a proteinase K digestion and phenol/chloroform/isoamyl alcohol extraction following the protocol of Sambrook and Russell (2001), a Proteinase K-Chelex extraction (Estoup et al. 1996), or a DNeasy[®] tissue kit (QIAGEN, Inc., Valencia, CA) following the manufacturers’ protocols. All isolation methods were equally successful.

The mitochondrial control region of each escolar specimen was amplified using the primers 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 AAC C 3′; this study), and ESCR (5′ CGG ATA CTT GCA TGT GTA AG 3′; this study). The two reverse primers were designed from sequences of escolar that were generated using primers Pro-F and 12SAR-3′ (Martin and Palumbi 1993). PCR amplifications were conducted using the Qiagen Taq PCR Core Kit (Qiagen, Valencia, CA) in 25 μ l volumes containing 0.25 μ l template following the manufacturer’s protocol with the addition of 0.5 μ l bovine serum albumin (BSA; 10 mg/ml). For amplification of DNA extracted by Chelex beads, 2.5 μ l 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:

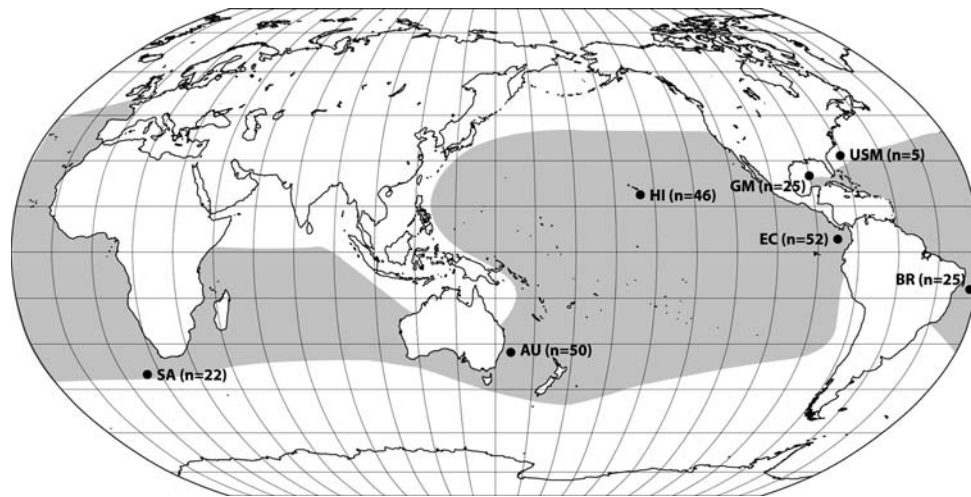


Fig. 1 *Leptodocybium flavobrunneum*. Map of collection locations of escolar in the Atlantic and Pacific oceans. Samples were collected from six geographical areas: the western North Atlantic (United States Mid-Atlantic (USM, $n = 5$)) and the Gulf of Mexico (GM, $n = 25$)), the west-

ern 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, $n = 52$))

94°C initial denaturation for 2 min., followed by 40 cycles of 94°C denaturation for 1 min, 50°C annealing for 30 s, 72°C extension for 2 min, and a final extension at 72°C for 5 min followed by a 4°C hold.

The internal transcribed spacer 1 (ITS-1) nuclear gene region was analyzed for a total of 16 escolar samples (eight 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 using the Qiagen Taq PCR Core Kit (Qiagen, Valencia, CA) in 10 μ l volumes according to the manufacturer's protocol using 0.2 μ l template DNA and with the addition of 0.2 μ l BSA (bovine serum albumin; 10 mg/ml) and 2 μ l Q-solution (QIAGEN). The reactions, all of which resulted in a single band, 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 and resulted in amplification of a single band.

All PCR products were verified on an agarose gel and subsequently purified using EXOSAP (USB Scientific, Cleveland, OH) or column filtration with QIAquick® PCR purification kit (QIAGEN), following manufacturers' instructions. Since ITS-1 occurs in multiple copies as tandem repeats throughout the genome, PCR products from the nuclear ITS-1 gene region were cloned into a plasmid

vector using the TOPO-TA plasmid cloning system (Invitrogen Corporation), and cloned fragments were isolated and purified with QIAprep Spin Miniprep kits (QIAGEN), both following manufacturers' 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 16 escolar specimens to observe heterozygotes and variation among multiple copies within a genome.

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), following manufacturer's recommendations and run on an ABI PRISM® 3130 Genetic Analyzer using 50 cm capillaries and POP7 matrix. All sequences were analyzed using Sequencing Analysis software version 5.2 (Applied Biosystems). Forward and reverse sequences were aligned and edited using Sequencher version 4.2.2 (Gene Codes Corp., Ann Arbor, MI). 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 insertions and deletions (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 (accession numbers: EU046723–EU046995).

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), nucleotide sequence diversity (π), the number of polymorphic sites (S), and mean number of pairwise differences (k). 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 1,000 replicates.

Estimates of population pairwise Φ_{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. Probability values were not corrected for multiple testing. The squared distance matrix between haplotypes used to calculate Φ_{ST} values was also used to create a minimum spanning network of haplotypes (Excoffier et al. 1992). In addition, 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. The significance of all analyses were tested with 10,000 randomizations of the data.

Sequence divergence between mtDNA clades was used to estimate time of separation of the Atlantic and Pacific lineages using two different methods. 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: $T = \delta/(2\mu)$. The second method to estimate time since population divergence (T) used the average number of nucleotide differences within and between populations while allowing for differences in ancestral population size (Gaggiotti and Excoffier 2000). For this method, an estimate of τ calculated in ARLEQUIN with 1,000 replicates was used in the formula: $T = \tau/2u$, where $u = m\mu$. 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 3.6×10^{-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 16 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 (the total length of this sequence was not determined due to problems with sequencing caused by the repeats). 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). Indels were treated as informative in all analyses. From the 225 individuals, a total of 145 haplotypes was found (Table 1). Haplotype diversity (h) within each collection ranged from 0.867 in the Gulf of Mexico sample to 0.990 in the Hawaii sample with a global value of 0.982. Nucleotide diversity (π) values ranged from 0.003 in the Gulf of Mexico sample to 0.018 in the South Africa sample (global $\pi = 0.026$). Overall, (h) was slightly higher in the Pacific than in the Atlantic (0.979 vs. 0.923), while (π) was higher in the Atlantic (0.009 vs. 0.006).

The hierarchical likelihood ratio tests in MODELTEST selected HKY + I + Γ (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 (Fig. 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 (97.4%) were found in the Pacific Ocean. Four individuals with Pacific clade haplotypes were found in the Atlantic Ocean, all occurring in the South Africa collection.

The overall AMOVA based on a Tamura and Nei (1993) model and excluding the U.S. mid-Atlantic (USM) sample due to its small sample size, found most of the variation (78.48%) among collections with only 21.52% occurring among individuals within collections (Table 2). The

Table 1 *Lepidocybium flavobrunneum*: summary table of population genetic statistics of escolar based on mitochondrial control region sequence data for each sampling location, each ocean, each clade, and overall samples

	<i>n</i>	No. of haplotypes	No. of Ts.	No. of Tv.	Indels	Polymorphic sites (<i>S</i>)	Haplotype diversity (<i>h</i>) ± SE	Nucleotide diversity (π) ± SE	Mean No. of pairwise differences (<i>k</i>)
By location									
Gulf of Mexico	25	12	19	0	0	19	0.867 ± 0.050	0.003 ± 0.002	2.757 ± 1.510
Brazil	25	17	21	1	1	22	0.943 ± 0.032	0.005 ± 0.003	3.525 ± 1.856
South Africa	18	13	18	0	1	19	0.928 ± 0.052	0.003 ± 0.002	2.510 ± 1.418
	(22)	(17)	(46)	(9)	(7)	(61)	(0.952 ± 0.037)	(0.018 ± 0.010)	(14.850 ± 6.908)
Ecuador	52	44	40	5	1	45	0.983 ± 0.012	0.006 ± 0.003	4.924 ± 2.437
Hawaii	46	39	37	8	0	44	0.990 ± 0.008	0.006 ± 0.003	4.594 ± 2.298
Australia	50	36	32	7	0	37	0.966 ± 0.018	0.005 ± 0.003	3.730 ± 1.915
By ocean									
Atlantic	77	42	58	12	8	73	0.923 ± 0.022	0.009 ± 0.005	7.131 ± 3.382
Pacific	148	105	56	13	1	65	0.979 ± 0.007	0.006 ± 0.003	4.444 ± 2.204
By clade									
Atlantic Clade	73	38	38	3	2	41	0.914 ± 0.025	0.004 ± 0.002	3.246 ± 1.693
Pacific Clade	152	107	56	13	1	65	0.980 ± 0.007	0.006 ± 0.003	4.452 ± 2.207
Overall	225	145	88	23	8	106	0.982 ± 0.004	0.026 ± 0.013	21.334 ± 9.446

Values in parentheses include individuals from the South Africa collection with Pacific haplotypes

Ts. transitions, Tv. transversions, Indels insertions or deletions

amount of variation partitioned within collections was even lower (16.29%) when the South Africa individuals with Pacific clade haplotypes were excluded from the analysis. The variation among collections was almost entirely attributed to variation between oceans with a Φ_{ST} of 0.863, $P < 0.0001$. The AMOVA revealed a small but significant variance due to 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 Φ_{ST} among collections within the Pacific Ocean ($\Phi_{ST} = -0.004$, $P = 0.8013$), but significant values were found within the Atlantic Ocean both including and excluding the South Africa samples with Pacific clades haplotypes ($\Phi_{ST} = 0.113$, $P = 0.0001$ and $\Phi_{ST} = 0.041$, $P = 0.0288$, respectively).

Pairwise Φ_{ST} values were used to determine possible population structuring within and between ocean basins (Table 3). 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 $P < 0.001$, 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 ($P = 0.009$).

Using a mutation rate of 3.6% per site per million years (Donaldson and Wilson 1999) and a net nucleotide sequence divergence of 4.85% between clades, an estimate of time since divergence (*T*) 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.

The nuclear ITS-1 region was cloned for each of the four South Africa individuals with Pacific clade mtDNA control region haplotypes as well as for four South Africa individuals with Atlantic clade haplotypes. Four representative individuals from other locations in each the Atlantic and Pacific oceans were also cloned for a total of 16 samples. Since ITS-1 occurs as multiple copies throughout the genome, four sequences were generated from the clones from each individual (encompassing both variation among multiple copies within a genome, and variation due to heterozygosity). The ITS-1 gene region analyzed varied in size from 717 to 735 bp. Sequences aligned to 740 bp and contained 58 polymorphic sites (37 transitions, 6 transversions, and 21 indels), 40 of which were parsimony informative. A neighbor-joining tree of uncorrected *p* distances separated the sequences into two clades, one containing clones of

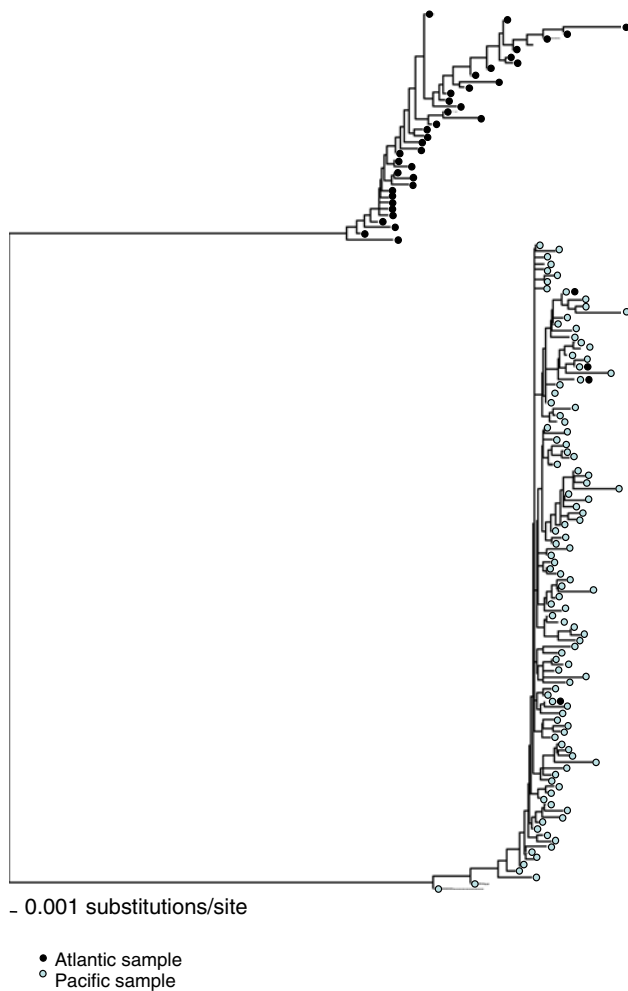


Fig. 2 *Lepidocybium flavobrunneum*. Neighbor-joining (NJ) tree of mitochondrial control region sequences using maximum likelihood distances calculated by the model HKY + I + Γ for escolar depicting Atlantic and Pacific clades. Samples collected in the Atlantic are represented by *filled circles* and samples collected in the Pacific are represented by *open circles*

individuals collected in the Pacific plus the four South Africa individuals with Pacific mtDNA clade haplotypes, and the other containing the clones of the remaining Atlantic individuals, including the South Africa samples with the Atlantic clade mtDNA haplotypes (Fig. 3). 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 to 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 ($h = 0.867\text{--}0.990$), 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).

Population structure

Hierarchical analysis of molecular variance indicated significant genetic differentiation among the six collections of escolar. The overall Φ_{ST} calculated by pooling the six collections was high ($\Phi_{ST} = 0.785$, $P < 0.0001$), 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 grouped separately, 90.5% of the variation occurred between lineages ($\Phi_{ST} = 0.905$, $P < 0.0001$). Lima et al. (2005) reported similar levels of variation (56.2–81.9%) between reproductively isolated lineages of cryptic fish species such as the goby, *Bathygobius soporator*.

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 Φ_{ST} in the Pacific collection ($P = 0.8013$), while significant Φ_{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

Table 2 *Lepidocybium flavobrunneum*: hierarchical variance partitioning and analysis of molecular variance (AMOVA) among escolar collections based on mitochondrial control region sequence data

	Observed partition		Φ statistics	<i>P</i>
	Variance	Total (%)		
Overall (GM, BR, SA, EC, HI, AU) ^a				
Among collections	9.544	78.48	$\Phi_{ST} = 0.785$	0.0000
Within collections	2.617	21.52		
Overall (GM, BR, SA, EC, HI, AU) ^b				
Among collections	10.410	83.71	$\Phi_{ST} = 0.837$	0.0000
Within collections	2.026	16.29		
Grouped by ocean (GM, BR, SA) (EC, HI, AU) ^b				
Among oceans	19.509	90.55	$\Phi_{CT} = 0.905$	0.0000
Among collections within oceans	0.012	0.05	$\Phi_{SC} = 0.006$	0.0939
Within collections	2.026	9.40	$\Phi_{ST} = 0.906$	0.0000
Atlantic Ocean (GM, BR, SA) ^b				
Among collections	0.064	4.09	$\Phi_{ST} = 0.041$	0.0288
Within collections	1.502	95.91		
Pacific Ocean (EC, HI, AU)				
Among collections	−0.009	−0.39	$\Phi_{ST} = −0.004$	0.8013
Within collections	2.228	100.39		
By Ocean (Atlantic, Pacific) ^a				
Among Populations	17.578	86.61	$\Phi_{ST} = 0.866$	0.0000
Within Populations	2.718	13.39		
By Clade (Atlantic, Pacific)				
Among Populations	19.560	90.49	$\Phi_{ST} = 0.905$	0.0000
Within Populations	2.055	9.51		

GM Gulf of Mexico, BR Brazil, SA South Africa, EC Ecuador, HI Hawaii, AU Australia

P-values less than 0.05 were considered significant

^a Including Pacific-like samples from SA

^b Omitting Pacific-like samples from SA

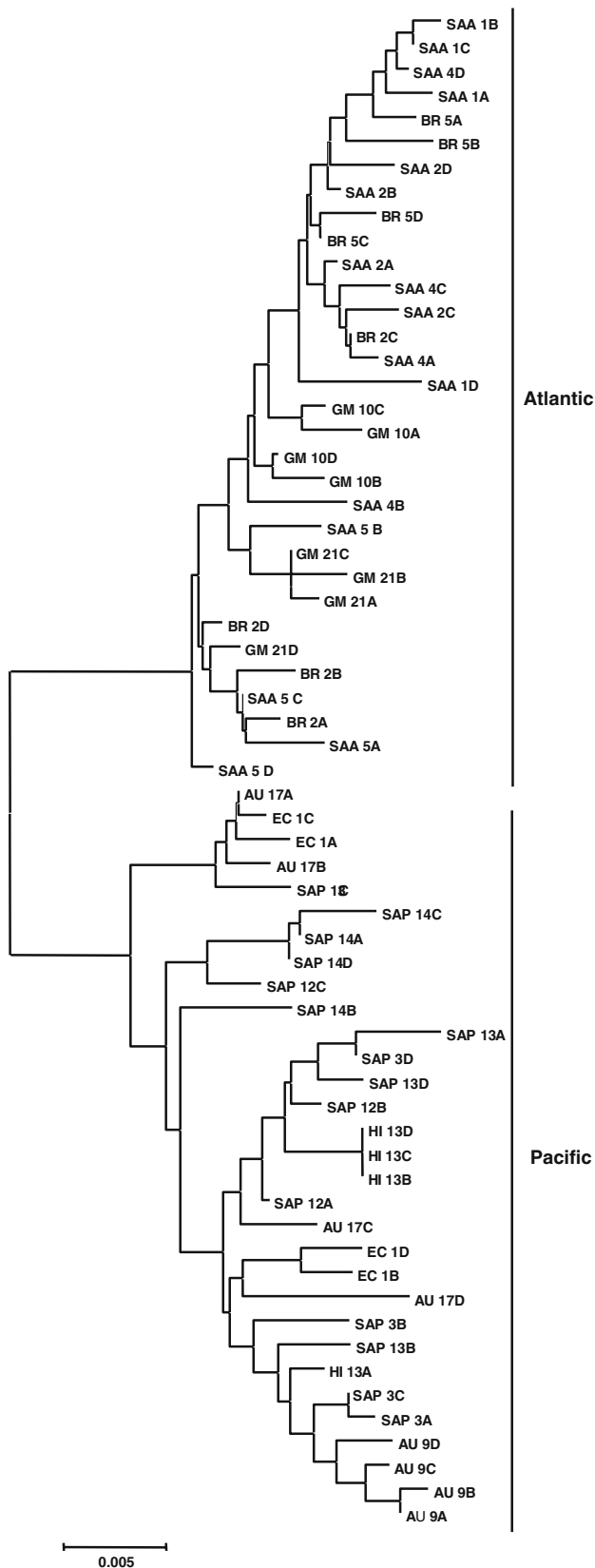
Table 3 *Lepidocybium flavobrunneum*: estimates of pairwise Φ_{ST} (below diagonal) and associated *P*-value (above diagonal) between collections of escolar based on mitochondrial control region sequence data (A) includes individuals from the South Africa collection with Pacific haplotypes; (B) excludes individuals from the South Africa collection with Pacific haplotypes

	Gulf of Mexico	Brazil	South Africa	Ecuador	Hawaii	Australia
A						
Gulf of Mexico	–	0.122	0.004	0.000	0.000	0.000
Brazil	0.025	–	0.000	0.000	0.000	0.000
South Africa	0.122	0.141	–	0.000	0.000	0.000
Ecuador	0.902	0.897	0.765	–	0.913	0.557
Hawaii	0.905	0.900	0.760	−0.007	–	0.559
Australia	0.920	0.915	0.784	−0.002	−0.002	–
B						
Gulf of Mexico	–	0.119	0.169	0.000	0.000	0.000
Brazil	0.025	–	0.009	0.000	0.000	0.000
South Africa	0.016	0.083	–	0.000	0.000	0.000
Ecuador	0.902	0.897	0.897	–	0.913	0.557
Hawaii	0.905	0.900	0.900	−0.007	–	0.564
Australia	0.920	0.915	0.917	−0.002	−0.002	–

Bold *P*-values indicate significance (*P* < 0.01)

within the Atlantic Ocean exhibited no significant Φ_{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-



◀ **Fig. 3** *Lepidocybium flavobrunneum*. Neighbor-joining (NJ) tree of nuclear ITS-1 gene region sequences for four representative escolar 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 16 samples. Samples are labeled according to collection location (*GM* Gulf of Mexico; *BR* Brazil; *SAA* South Africa Atlantic clade; *SAP* South Africa Pacific clade; *EC* Ecuador; *HI* Hawaii; *AU* Australia)

level analyses with microsatellite loci may help clarify the intra-oceanic population structure of escolar. Non-significant pairwise Φ_{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 (Graves and McDowell 2006), and the authors attributed differences in population structure between the two species to differing sizes of the two ocean basins.

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 ($\delta = 5.17\%$, 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 et al. 2005; Martínez et al. 2005), and wahoo (13.6%, Garber et al. 2005). However, estimates of intra-clade nucleotide diversities ($\pi = 0.003$ – 0.006) were an order of magnitude lower than most intraclade diversities reported for other scombroids (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). 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 (97.4%). Only four individuals with Pacific clade haplotypes were found in the Atlantic, all of which were restricted to 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 from the Pacific that have subsequently interbred with Atlantic escolar. In the case of recent migrants, one would expect the alleles of 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 the alleles of nuclear genes to be similar to Atlantic individuals. Results from analyses of the nuclear gene region ITS-1 demonstrated that sequences from all four South Africa escolar with Pacific clade mtDNA haplotypes grouped with sequences from samples from the Pacific collections, consistent with recent migration.

These results differ from those found with nuclear analyses of some 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. However, the genetic and geographic divisions of the two escolar mitochondrial lineages are similar to those found for another set of highly migratory Atlantic and Pacific congeners; white marlin (*Kajikia albida*) and striped marlin (*K. 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 (McDowell and Graves, unpublished data). The taxonomic status of white marlin and striped marlin has been debated due to great morphological (Nakamura, 1985) and molecular similarities of the two species (reviewed in Graves and McDowell 2003), but they are currently recognized as separate species based on morphological and genetic differences (Collette et al. 2006).

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). As both molecular and

morphological differences exist between geographically isolated lineages of escolar, it is probable 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. In addition, a combination of molecular and morphological work on the same individuals, which include the use of co-dominant nuclear markers such as microsatellites, should be conducted to confirm their status as separate species and to look for evidence of interbreeding.

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, 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 of many species, 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 retroreflection 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 glacial periods. 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 maximum that occurred approximately 0.68–0.62 mya (marine isotope stage (MIS) 16) (Gibbard and Van Kolfshoten 2004).

The occurrence of the Agulhas Retroflexion is 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 Retroflexion, 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.

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