Available online at www.sciencedirect.com

SCIENCE DIRECT®

Molecular Phylogenetics and Evolution 31 (2004) 833–851

MOLECULAR PHYLOGENETICS AND EVOLUTION

www.elsevier.com/locate/ympev

Molecular phylogenetics and biogeography of transisthmian and amphi-Atlantic needlefishes (Belonidae: *Strongylura* and *Tylosurus*): perspectives on New World marine speciation

H.M. Banford,[a,b,c,*] E. Bermingham,[b] and B.B. Collette[c]

[a] *Department of Biology, State University of West Georgia, Carrollton, GA 30118, USA*
[b] *Smithsonian Tropical Research Institute, Apartado 2072, Balboa, Panama*
[c] *National Marine Fisheries Service, National Systematics Laboratory, National Museum of Natural History, Washington, DC 20560, USA*

Received 3 September 2002; revised 15 October 2003

Abstract

Phylogenetic relationships among New World and eastern Atlantic species in the belonid genera *Strongylura* and *Tylosurus* were hypothesized using 3689 bp of nucleotide sequence; including the entire mitochondrial (mtDNA) ATP synthase 6 and 8 genes; partial cytochrome *b*; 12S and 16S ribosomal genes; and introns and exons, 2 and 3 of the nuclear-encoded creatine kinase B gene. Concordant mtDNA and nuclear genealogies permitted well-supported inference of species relationships within *Strongylura* and *Tylosurus*, and of the chronology of diversification in the two genera. Our phylogenetic hypothesis permitted an assessment of Rosen's [Syst. Zool. 24 (1975) 431] model of species diversification across the eastern Atlantic to eastern Pacific marine biogeographic track. The spatial predictions of the Rosen model were generally supported, but not the temporal predictions. Furthermore, long branches leading to terminal Belonidae indicated that many species have persisted for millions of years or that nucleotide substitution rates were elevated for some clades. Though heterogeneity of nucleotide substitution rate was indicated across some belonid lineages, molecular clock estimates were used to hypothesize biogeographic scenarios for *Strongylura* across the eastern Pacific and Atlantic region. Furthermore, use of a molecular clock indicated; that early diversification among contemporary *Strongylura* may have been initiated by changes in Atlantic Ocean circulation precipitated by closure of the Tethys Sea; and provided approximate dates for the isolation of the freshwater species on the American continents.
© 2003 Elsevier Inc. All rights reserved.

Keywords: Historical biogeography; Phylogenetic systematics; Mitochondrial DNA; Creatine kinase; Speciation

1. Introduction

Two vicariant events—the Cretaceous opening of the Atlantic Ocean and the Pliocene closure of the Central American isthmus—have been hypothesized as the principal and chronologically ordered causes of marine speciation in the tropical Atlantic and eastern Pacific Oceans (Rosen, 1975). Rosen's eastern Pacific/eastern Atlantic track (EP/EA) model suggests that, for many globally distributed taxa (families and genera), New World and eastern Atlantic tropical marine species form monophyletic assemblages, with eastern Atlantic species ancestral to derived New World species. Within these groups, the most recent relationships are hypothesized to be among tranisthmian sister taxa, or geminates, distributed in the eastern Pacific and western Atlantic (Jordan, 1908), whereas amphi-Atlantic relationships date to the opening of the Atlantic Ocean (Rosen, 1975). The numbers and distributions of species representing many putatively monophyletic assemblages indicate that the EP/EA track model, at best, represents an oversimplification of the earth history events underlying New World marine speciation. Our recent biogeographic analysis of the EP/EA *Scomberomorus regalis* species group (Banford, 1998; Banford et al., 1993) provided a phylogenetically based assessment of the Rosen model and reinforces a more complex view of EP/EA marine speciation. Here we continue our assessment of the EP/EA biogeographic track through a molecular systematic

* Corresponding author. Fax: +1-770-836-6633.
E-mail address: hbanford@westga.edu (H.M. Banford).

1055-7903/$ - see front matter © 2003 Elsevier Inc. All rights reserved.
doi:10.1016/j.ympev.2003.10.013

analysis of New World and eastern Atlantic Belonidae in the genera *Belone*, *Strongylura*, and *Tylosurus*.

Our analysis of the EP/EA marine biogeographic track combines spatial and temporal measures of phylogenetic relationships. Our spatial analysis focuses on the *Strongylura marina* species group because it is hypothesized to be monophyletic (Cressey and Collette, 1970) and has an EP/EA geographic distribution (Fig. 1). The group contains a single eastern Atlantic species (*S. senegalensis*), four western Atlantic species (*S. marina*, *S. timucu*, *S. notata*, and a species that has not been described from coastal Brazil) and two eastern Pacific species (*S. exilis* and *S. scapularis*). In addition, the *S. marina* species group includes two freshwater species, *S. hubbsi* from the Rio Usumacinta of Mexico and Guatemala, and *S. fluviatilis* of the Esmeraldas region of Ecuador's Pacific slope and the Rio Atrato on Colombia's Atlantic slope. We assessed monophyly of the *S. marina* species group using multiple Indo-west Pacific (IWP) species of *Strongylura* and the belonid genera, *Tylosurus*, *Ablennes*, and *Belone*, as outgroups. Our temporal analysis used molecular markers representing two linkage groups to provide independent estimates of genetic distances and time, based on the concept of a molecular clock (Zuckerkandl and Pauling, 1965).

We sequenced 3689 nucleotide pairs from six gene regions, mitochondrial and nuclear, for representatives of each belonid species considered herein. We reasoned that congruence across independent genealogies and consistent genetic distance ratios for unlinked genetic markers would increase confidence in our inference of branch order and chronology for species relationships among Atlantic and eastern Pacific Belonidae. In addition, our data permitted us to discuss the use of different gene regions, with different mutation rates, to provide phylogenetic resolution at various taxonomic levels, from close sister species to inter-generic relationships.

2. Materials and methods

2.1. Sampling

Our phylogenetic analysis included all species representing the *S. marina* species group and New World *Tylosurus*; when possible we collected individuals from

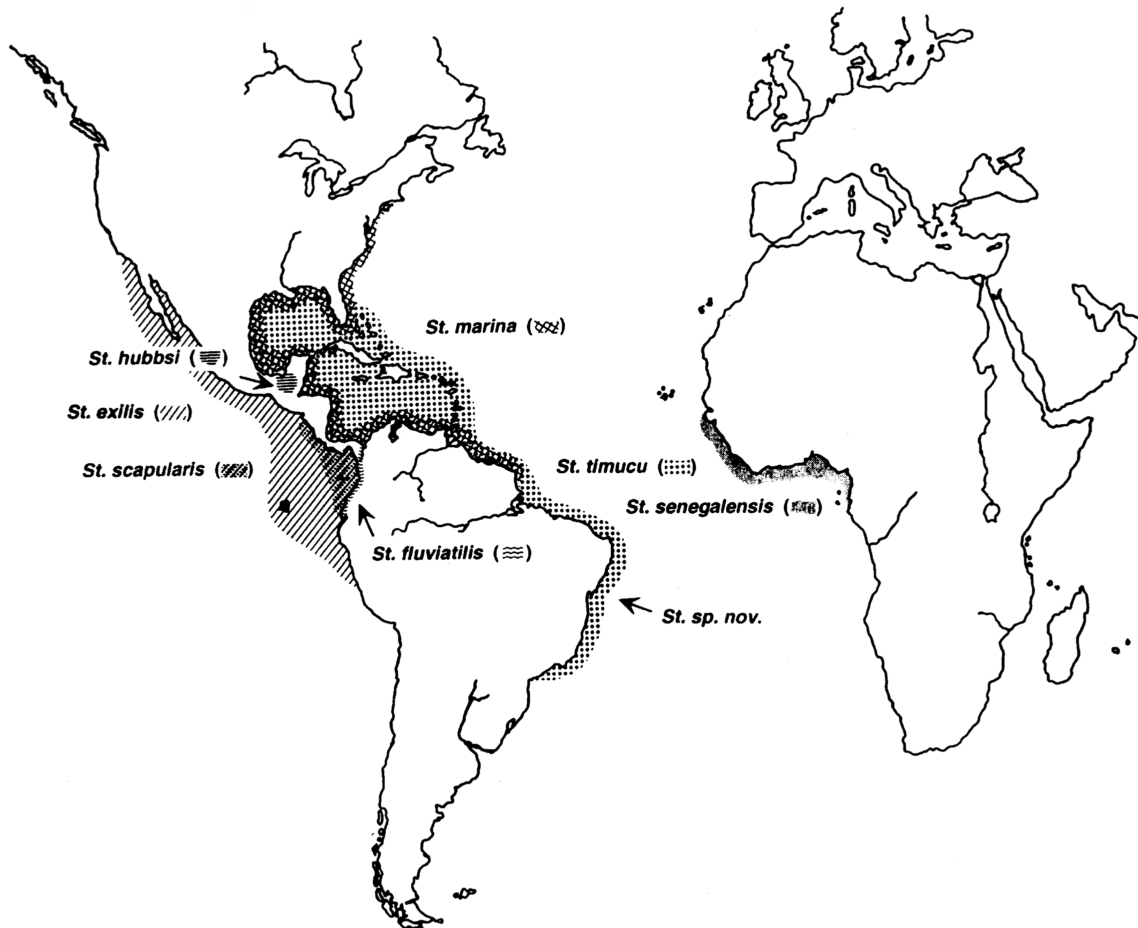

Fig. 1. Geographic distribution of *Strongylura marina* group species.

more than one location across a species range in order to provide some information regarding intraspecific DNA sequence polymorphism. Our choice of outgroup taxa was informed by a preliminary analysis which included additional Indo-West Pacific *Strongylura* species and a wider survey of belonid genera (not all data shown). No IWP *Strongylura* fell within the group of interest. This analysis identified the IWP *S. incisa*, the circumglobally distributed *Ablennes* and *Tylosurus*, and the putatively basal *Belone*, as informative outgroups for our in-depth molecular systematic analysis of the *S. marina* group. Due to unresolved higher order relationships within the Belonidae, we also included two non-belonid outgroup taxa, *Hyporhamphus unifasciatus* (Beloniformes: Hemiramphidae) and *Scomberomorus tritor* (Perciformes: Scombridae) to provide an unambiguous external rooting.

Gill tissue was removed from fresh specimens and stored in salt-saturated DMSO/EDTA buffer (Seutin et al., 1990) at 4 °C. After removal of tissue, fish were preserved in formalin as voucher specimens and subsequently stored in 70–75% ethanol. Specimens used in this study have been accessioned at the USNM, VIMS, and CU (Leviton et al., 1985) and at the Smithsonian Tropical Research Institute (STRI) (Bermingham et al., 1997a) (Table 1). All gene sequences presented in Table 1 have been accessioned in GenBank (Appendix A).

2.2. DNA sequencing

Genomic DNA was isolated by digesting approximately 0.2 g of gill tissue, rinsed with ddH_2O to remove salt, in 500 μl of 2× CTAB buffer (1 M Tris, 4 M NaCl, 0.5 M EDTA, CTAB, 2-mercaptoethanol, pH 8.0; Saghai-Maroof et al., 1984) with 10 μl of 100 mg/ml proteinase K and incubation at 56 °C for 2–4 h with rotation, followed by phenol–chloroform–isoamyl alcohol (PCI)(25:24:1 by volume) and chloroform–isoamyl alcohol (CI) (24:1) extraction. Samples were dialyzed at 4 °C in 1× TE buffer (10 mM Tris, 1 mM EDTA, pH 7.5) for 24–48 h. Genomic DNA samples were archived at −70 °C, working aliquots were maintained in the laboratory at −20 °C.

We used the mitochondrial ATP synthase 8 and 6 genes (ATPase8,6: 842 bp) to assay all individuals included in this report ($n = 56$) representing 18 belonid species and subspecies (Table 1). All species were genetically distinct and represented by monophyletic clusters of mtDNA haplotypes, however, this gene region did not provide a well-supported assessment of basal relationships in this group. To gain additional insight into the basal belonid relationships, we increased the number of nucleotides sampled by selecting addition gene regions with different molecular characteristics. We used prevailing taxonomy to choose a sub-sample of 15 individuals that were sequenced for the full complement of mitochondrial and the nuclear loci (3689 bp; Table 1). This included the 5′end of the mtDNA cytochrome *b* gene (800 bp) which was sequenced: (1) to increase the nucleotide sample of mtDNA protein coding genes, and (2) because cyt *b* is physically separated by roughly 8000 bp from the ATPase gene region within the mtDNA genome. Thus, comparison of the two gene regions can provide modest evidence against inadvertent amplification of a nuclear pseudogene (Bermingham et al., 1996; Zhang and Hewitt, 1996; but see also Lopez et al., 1994). We also sequenced 1005 bp of the mtDNA ribosomal genes representing portions of the 12S (429 bp) and 16S (576 bp) ribosomal subunits. These genes are reported to have lower substitution rates and different nucleotide saturation characteristics than mtDNA protein coding genes in fishes (Ortí and Meyer, 1997). Lastly, to provide an independent locus for phylogenetic analysis we sequenced 1042 bp of the nuclear-encoded creatine kinase B (CK) gene (Garber et al., 1990). This locus also represented a second gene region with a reduced nucleotide substitution rate relative to vertebrate mitochondrial protein-coding genes.

Amplifications using the polymerase chain reaction (PCR) (Saiki et al., 1985) were carried out in 50 μl reactions: 5 μl of 10× buffer (100 mM Tris, pH 8.3, 20 mM $MgCl_2$, 500 mM KCl, and 0.1% Gelatin), 4 μl $MgCl_2$, 5 μl of 2 mM dNTP, 0.25 μl *Taq* polymerase, 29.75 μl ddH_2O, 1–2 μl genomic DNA as template, and 2.5 μl of each 10 mM primer. For ATPase8,6 we used primers ATPase8.2, ATPase8.3, COIII.2, and COIII.3 (http://nmg.si.edu/bermlab.htm). For cyt *b* we used primers GLUDG-L and CB3-H (Palumbi et al., 1991). The mitochondrial ribosomal genes were amplified with primers 12SA-L and 12SB-H (Kocher et al., 1989) and 16Sar-L and 16Sbr-H (Palumbi et al., 1991). Creatine kinase was amplified with primers CK1-5′ and CK2-3′ (Palumbi et al., 1991). The ATPase and ribosomal genes were amplified as follows: 94 °C denaturation for 2 min followed by 30 cycles at 94 °C for 45 s, 54 °C annealing for 45 s, and 72 °C extension for 90 s, terminating with a 2 min extension at 72 °C. We used a touchdown protocol for cyt *b* and CK. Following denaturation, we employed 15 cycles of 94 °C for 45 s, 60 °C annealing, decreased by 0.4 °C each cycle, for 45 s, and a 72 °C extension for 1.5 min. The first 15 cycles were followed by 28 cycles of 94 °C for 25 s, 56 °C for 30 s, 72 °C for 1 min 15 s, terminating with a 5 min extension at 72 °C. The CK protocol varied in two important respects. First, due to presence of duplicated gene products in CK amplifications (Palumbi, 1996), we re-amplified the larger, 1 kb, fragment from stabs taken from each electrophoretically separated band. Second, CK PCRs were initiated with a 10 min, 95 °C denaturation rather than the 3 min, and 94 °C used for cyt *b*.

All PCRs were carried out on a MJ Research Inc. PTC-100 or 200 thermal controller. Amplification

Table 1
Belonidae material sequenced, geographic locale, and number of base pairs (bp) listed for each gene region

Species	Catalog No.	Spec.ID No.	Location	ATPase8,6	Cytb	12S	16S	CK
Ablennes hians	USNM347819	STRI-4732	Philippines, IWP	842	800	429	576	1042
Belone belone	No voucher	AB-02-97	Italy, EATL	842	800	429	576	
Strongylura exilis	STRI00192	STRI-3096	Panama, EPAC	842				
S. exilis	STRI00192	STRI-3097	Panama, EPAC	842	800	429	576	1042
S. fluviatilis	Col. Bogata	STRI-1485	Colombia, Atrato	842	800	429	576	
S. fluviatilis	STRI00193	STRI-1486	Colombia, Atrato	842		429		
S. fluviatilis	Quito, uncat.	STRI-6133	Ecuador, Esmeraldas	842	800	429	576	1042
S. fluviatilis	Quito, uncat.	STRI-6134	Ecuador, Esmeraldas	842		429		
S. fluviatilis	Quito, uncat.	STRI-6138	Ecuador, Esmeraldas			429		
S. hubbsi	CU	GU-5-96	Guatemala, Usumacinta	842		429		
S. hubbsi	CU	GU-6-96	Guatemala, Usumacinta	842	800	429	576	1042
S. incisa	USNM348248	STRI-4702	Philippines, IWP	842	800	429	576	1042
S. incisa	USNM348249	STRI-4711	Philippines, IWP		800	429	576	1042
S. marina	USNM-pending	HB18	Florida, Gulf coast, WATL	842		429		
S. marina	USNM-pending	HB19	Florida, Gulf coast, WATL	842				
S. marina	USNM-pending	HB22	Florida, Gulf coast, WATL	842				
S. marina	USNM-pending	HB268	Chesapeake Bay, WATL	842	800	429	576	1042
S. marina	USNM-pending	HB270	Chesapeake Bay, WATL	842		429		
S. marina	USNM-pending	HB404	Brazil, WATL	842	800	429	576	1042
S. marina	USNM-pending	HB408	Brazil, WATL	842	800	429		
S. marina	USNM-pending	HB53	Florida, east coast, WATL	842		429		
S. notata	USNM344599	HB159	Cuba, WATL	842	800	429	576	
S. notata	USNM-pending	HB72	Florida, east coast, WATL	842				
S. notata	USNM-pending	HB82	Florida, east coast, WATL	842	800	429	576	1042
S. notata	USNM-pending	HBO1	Florida, Gulf coast, WATL	842				
S. notata	USNM-pending	HBO2	Florida, Gulf coast, WATL	842		429		
S. scapularis	STRI00194	STRI-3051	Panama, EPAC	842	800	429	576	1042
S. senegalensis	USNM-pending	STRI-6605	Ghana, EATL	842	800	429	576	1042
S. senegalensis	USNM-pending	STRI-6606	Ghana, EATL	842		429		
S. timucu	STRI00197	STRI-3817	Panama, WATL	842		429		
S. timucu	STRI00179	STRI-3846	Panama, WATL	842				
S. timucu	STRI00196	STRI-4308	Honduras, WATL	842	800	429	576	1042
S. timucu	STRI00196	STRI-4310	Honduras, WATL	842				
S. timucu	USNM342925	HB180	Cuba, WATL			429		
S. timucu	USNM342925	HB181	Cuba, WATL	842				
S. timucu	USNM342925	HB190	Cuba, WATL	842		429		1042
S. timucu	USNM342925	HB191	Cuba, WATL	842	800	429		
S. timucu	USNM-pending	HB401	Brazil, WATL	842		429		
S. timucu	USNM-pending	HB405	Brazil, WATL	842		429		
Tylosurus acus acus	USNM337725	BBC11BERM	Bermuda, WATL	842	800	429	576	
T. acus acus	USNM337725	BBC13BERM	Bermuda, WATL	842				
T. acus imperialis	No voucher	GO-1-95	Israel, EATL	842	800	429	576	
T. acus imperialis	No voucher	GO-2-95	Israel, EATL	842				
T. acus melanotus	USNM348245	STRI-4700	Philippines, IWP	842	800	429	576	
T. acus melanotus	USNM348246	STRI-4704	Philippines, IWP	842				
T. acus melanotus	STRI00181	STRI-5109	Panama, EPAC	842	800	429	576	1042
T. acus melanotus	STRI00181	STRI-5110	Panama, EPAC	842				
T. pacificus	STRI00182	HB955	Panama, EPAC	842	800	429	576	
T. pacificus	STRI00182	HB954	Panama, EPAC	842				
T. acus rafale	USNM-pending	STRI-6516	Senegal, EATL	842	800	429	576	1042
T. acus rafale	USNM-pending	STRI-6536	Cote d'Ivoire, EATL	842				
T. crocodilus crocodilus	USNM339006	HB166	Tonga, IWP	842	800	429	576	
T. crocodilus crocodilus	STRI00185	STRI-3837	Panama, WATL	842	800	429	576	1042
T. crocodilus crocodilus	STRI00187	STRI-2579	Panama, WATL	842				
T. crocodilus crocodilus	USNM348292	BBC4PHIL	Philippines, IWP	842	800	429	576	

Table 1 (*continued*)

Species	Catalog No.	Spec.ID No.	Location	ATPase8,6	Cytb	12S	16S	CK
T. crocodilus fodiator	STRI00184	STRI-5111	Panama, EPAC	842				
T. crocodilus fodiator	STRI00183	HB959	Panama, EPAC	842	800	429	576	1042
Hyporhamphus unifasciatus	STRI00198	STRI-3819	Panama, WATL	842	800	429	576	1042
Scomberomorus tritor	No voucher	STRI-4017	Angola, EATL	842	800	429	576	

Outgroup taxa listed from other families; Hemiramphidae and Scombridae. Oceanic regions, eastern Pacific (EPAC), eastern Atlantic (EATL), western Atlantic (WATL), and Indo-West Pacific (IWP), are given to clarify exact locations, for example where countries are adjacent to more than one oceanic region.

products (PCR) were electrophoresed in a 2% TAE (0.04 M Tris–acetate, 0.001 M EDTA, pH 8.0) (Maniatis et al., 1982) *NuSieve GTG* LMP agarose gel and stained with EtBr to remove unincorporated dNTPs and undesirable PCR products. The visualized band (typically one) was cut out and melted at 70 °C for 10 min. The temperature was then decreased to 45 °C and after at least 10 min, 1 μl of *GELase* (Epicentre Technologies) was added and the solution was incubated for at least 3 h. Sequencing reactions were carried out with the *Taq DyeDeoxy Terminator Cycle Sequencing Kit* (Applied Biosystems, ABI). Cycle sequencing was carried out on a PTC-100 thermal controller as recommended by ABI and according to their protocol. Sequencing gel electrophoresis, data capture and gel analysis were accomplished on an ABI Prism 377 DNA Sequencer.

2.3. Phylogenetic analysis

Sequence data were aligned and edited using *MacClade* (version 3.07, Maddison and Maddison, 1997), *Macvector sequence analysis software* (version 4.5.2, Eastman Kodak Co., 1994), and *SeqEd* (version 1.0.3, Applied Biosystems, ABI, 1992). The mtDNA ribosomal gene regions were aligned in ClustalW (Thompson et al., 1994) with gap penalties varying from 5 to 15. Alignments were further modified by eye using Se-Al (version 1.0 alpha 1, Rambaut, 1995) to insure, for example, that indels were pushed out of inferred stem regions. The ribosomal component of the combined mtDNA data included only nucleotide sites that could be unambiguously aligned. The CK data were aligned using Se-Al in a straightforward manner owing to a small number of observed indels and the ease with which apparent homologs could be identified. The CK data were subsequently treated in two ways in the phylogenetic analyses: indels were coded either as missing data or as a single evolutionary event regardless of the length of the indel.

Nucleotide composition and bias were examined using *Phylogenetic Analysis Using Parsimony* (*PAUP** version 4.0.0d64, prerelease; Swofford, 1997 and version 4.0.b2, beta, 1999) and *Sequencer* (version 5.1.1, Kessing, 2000). Homogeneity of nucleotide frequencies across taxa was tested independently for each gene region (χ^2, $p > 0$, α 0.05) in *PAUP**.

We aligned our ribosomal subunit sequences to those of the red piranha, *Pygocentrus nattereri* (Teleostei: Characidae) (GenBank Accession Nos. U33558 and U33590). A secondary structure has been inferred for these sequences (Ortí and Meyer, 1997; Ortí et al., 1996), thus permitting us to estimate stem and loop sub-regions for the belonid ribosomal sequences. In our preliminary phylogenetic analyses we explored the data as individual gene partitions and in a variety of combinations. The individual gene partitions corresponded to, the six gene or gene regions, each ribosomal gene separated into loop and stem sub-regions, and CK split into exons and introns. Data combinations included the three mtDNA protein-coding genes, the two ribosomal genes, all mitochondrial genes, and all genes. The partition-homogeneity test (Farris et al., 1995) implemented in *PAUP** (Swofford, 1999) was used to test the homogeneity of the gene partitions before combining data for phylogenetic analysis. We ran 100 replicates of a heuristic search each time the partition-homogeneity test was employed.

Cladistic analyses for each gene and combined data set were carried out using *PAUP** and *MacClade*. Generally, we used the heuristic search in *PAUP** with TBR branch swapping and 1000 bootstrap replicates to generate parsimony cladograms. When possible branch-and-bound searches were conducted on taxonomically reduced data sets to compare to the heuristic results. The shortest observed cladograms were compared, using the KH test (Kishino and Hasegawa, 1989) and most parsimonious tree (MP) indices, to trees based on earlier, morphological-based hypotheses of belonid phylogenetic relationship.

We utilized the likelihood ratio test implemented in MODELTEST (Posada and Crandall, 1998) to select the model of molecular evolution used for the phylogenetic analyses of the different data sets. Using distance/likelihood options both the transition/transversion (Ts/Tv) ratio and gamma shape parameter, α, were iteratively generated with *PAUP**. Due to the large number of OTUs and characters, and the apparently high number of substitutions along terminal branches, Quartet Puzzling was used to reconstruct maximum likelihood trees (ML), estimate likelihood distances and to map likelihoods (*Puzzle*, version 4.0, Strimmer and von Haeseler, 1996). Quartet Puzzling results were

checked against a full maximum likelihood analysis of a reduced data set, comprising only 7 OTUs and representing a single individual from each major clade.

The basic concordance of topologies across gene regions permitted genetic distances between individual taxa and between clades to be compared across all gene regions examined. A single individual was used to represent each clade for distances calculated between multi-taxa clades. Kendall's coefficient of concordance (Zar, 1984) was used to assess the significance of the rank order of genetic distance comparisons across genes.

Transition and transversion distances were plotted against patristic distance by gene region to investigate if saturation of substitutions was occurring, and if so, at what level of divergence. The patristic distance matrix was generated using the program TreeDis, version 2.0 (Weiller, 1998; http://life.anu.edu.au/molecular/software/tredis/) and the pairwise ML distances were linearized using *Sequencer 5.1.1* (Kessing, 2000; http://nmg.si.edu/Sequencer.html). The constancy of nucleotide substitutions across lineages was evaluated using the two-cluster and branch-length tests of Takezaki et al. (1995) (http://www.cib.nig.ac.jp/dda/ntakezak/ntakezak-j.html).

3. Results

3.1. Molecular characterization

Table 2 presents comparative data for five mitochondrial and one nuclear-encoded gene or gene regions sequenced for selected belonids and outgroup families (Hemiramphidae and Scombridae). The three mitochondrial protein coding genes began with the typical start codon ATG and terminated with TAA (note that the 3′ end of the cyt *b* gene was not sequenced). No termination codons were present in the intervening sequence. Nucleotide frequencies for ATPase genes were not significantly different across taxa, whereas cyt *b* base frequencies were different (χ^2, $p = 0.0342$). Specifically, *S. marina* group and *Belone* were found to have significantly different cyt *b* frequencies of cytosine and thymine compared to *Tylosurus*, the IWP *Strongylura* species, and *S. notata*. ATPase8, ATPase6, and cyt *b* had the following Ts/Tv ratios: 4.99, 4.71, and 3.98 (Table 2).

Nucleotide frequencies for 12S and 16S were not significantly different across taxa. The overall Ts/Tv ratios were 2.51 and 2.44 for the two regions (Table 2). We observed more than a twofold greater substitution rate in loops relative to stems. There was no significant difference in nucleotide frequencies within ribosomal sub-regions among taxa; however, base composition was markedly different between stems and loops (Table 2).

For CK, we typically amplified two products, one approximately 1000 bp and the other about 500 bp. The larger CK amplification product probably represented a duplicated exon (Murphy et al., 1996; Palumbi, 1996). After preliminary sequencing, we chose to use the larger CK fragment because it possessed more phylogenetic information in its additional intron and permitted us to examine a full intervening exon in addition to the two partial flanking exons. Although limited sequence information exists for CK in fishes, we obtained 84–86% similarity to CK from rainbow trout, *Oncorhynchus mykiss* (Teleostei: Salmonidae) (GenBank Accession No. X53859; Garber et al., 1990), and a 70% match with the mouse, *Mus musculus*, CK gene (GenBank Accession No. M74149; van Deursen et al., 1992). Using these alignments we determined the linear arrangement of the belonid exon/intron positions for the approximately 1000 bp CK fragment amplified by the CK1 and CK2 primers. Our data included parts of exons 2 and 3, a putative duplication of the complete exon 2, and

Table 2
Phylogenetic and molecular characterization of belonid and outgroup mitochondrial and nuclear creatine kinase b gene

Gene regions	Individuals sampled	Number of base pairs	Variable sites	Parsimony informative	A	C	G	T	Ts/Tv	gamma, α
Protein coding	27	1652	842 (590)[a]	661 (493)[a]						
ATPase8	56	168	103	84	0.350	0.288	0.095	0.267	4.993	0.521
ATPase6	56	684	362	310	0.276	0.295	0.115	0.314	4.705	0.278
CytB	28	800	354	282	0.261	0.291	0.144	0.304	3.983	0.225
Ribosomal total	27	1005	329 (162)[a]	229 (146)[a]						
12S (total)	43	429	159	110	0.308	0.256	0.218	0.219	2.508	0.191
12S (loops)	43	191	104	79	0.434	0.229	0.140	0.198	3.228	1.548
12S (stems)	43	163	44	26	0.190	0.263	0.297	0.250	5.527	1.673
16S (total)	27	576	210	138	0.306	0.235	0.219	0.241	2.442	0.183
16S (loops)	27	324	164	116	0.402	0.216	0.160	0.222	2.430	1.486
16S (stems)	27	227	47	23	0.180	0.268	0.312	0.240	3.528	4.979
CK (total)	17	1042	185 (96)[a]	137 (35)[a]	0.269	0.219	0.246	0.267	1.917	∞ (1728)
CK (exons)	17	482	88	29	0.274	0.229	0.270	0.227	2.774	∞
CK (introns)	17	560	309	131	0.264	0.210	0.225	0.302	1.665	∞

[a] Numbers of sites in parentheses are for transversions only.

intervening introns 2 and 3. The variation in α (gamma shape parameter) between the mitochondrial and nuclear genomes (Table 2) suggested differences in the mode of substitution between the two classes of genes. CK had an infinitely large α indicating a relatively homogenous pattern of substitution, whereas αs observed for different mitochondrial genes were less than one (0.183–0.521) suggesting considerable heterogeneity in the distribution of nucleotide substitutions within mtDNA genes.

To gain insight into the saturation of transition substitutions we plotted uncorrected genetic distance "*p*" values for each gene against patristic distance (Figs. 2A and B). We used a logarithmic model to plot transition distances for each individual gene against patristic distances for the combined mtDNA data. The curves reached an asymptote for the individual protein-coding genes at distances equal to 0.10–0.15 indicating saturation of transitions beyond these distances (Fig. 2A). Plots for the ribosomal sequences were asymptotic, indicating that transition substitutions are saturated (Fig. 2A). Although we have only presented plots of each gene or gene region against patristic distances, very similar plots were obtained when these genes were analyzed relative to total CK substitutions. The relationship of the mitochondrial transversion and CK time proxies is linear ($r^2 = 0.706$) as shown in Fig. 2B. The r^2 value is depressed because CK does not begin accumulating substitutions until 2.5 mitochondrial transversions have been registered. As in Fig. 2A autocorrelation should also be kept in mind when interpreting the r^2 value for the regression presented in Fig. 2B.

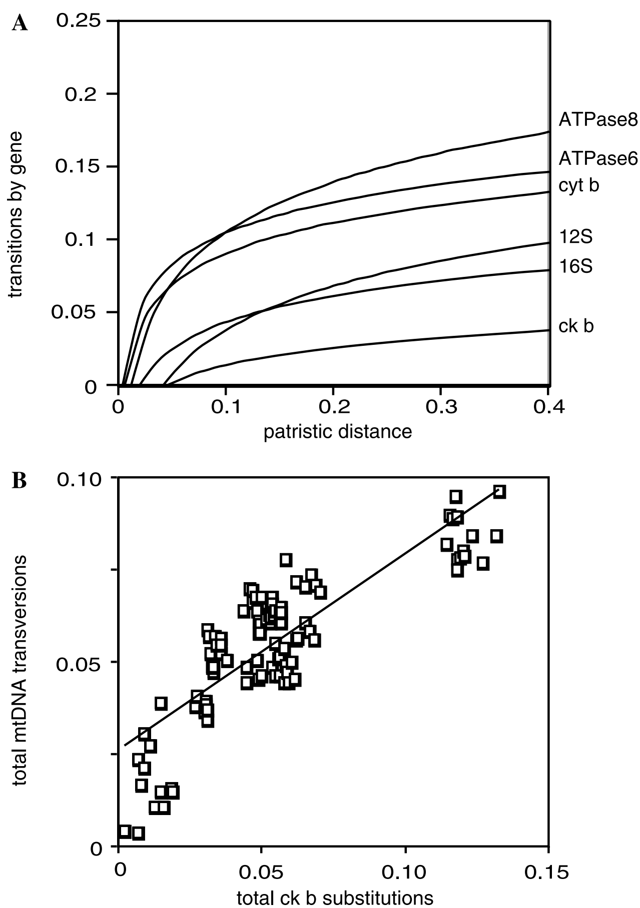

Fig. 2. Genetic distance graphs based on uncorrected measures of nucleotide substitution (*p*) for belonid mitochondrial and creatine kinase genes against patristic distance. (A) Ts distances for each gene plotted against patristic distances for the combined mitochondrial genes. (B) Total mtDNA Tv distance plotted against total CK substitutions (Ts + Tv).

3.2. Phylogenetic analysis

Generally speaking, our phylogenetic analyses of the EA/EP belonids and associated taxa typically identified consistent phylogenetic relationships among species whether using gene regions alone or in combination, different methods of analysis, and different weighting schemes (Figs. 3–5). For example, we used the LogDet (Steel, 1994) model in order to take into account nonstationary nucleotide composition observed for cyt *b*, as well as the parameter-rich Tamura–Nei (Tamura and Nei, 1993) and General Time Reversible (Yang, 1994) models selected by MODELTEST (Posada and Crandall, 1998) as the best models for the mtDNA protein coding data and the combined data. Although the assumptions underlying each substitution model and parsimony are different, the phylogenetic hypotheses resulting from each class of analysis were largely congruent.

We present analytical results based on four data sets in order to emphasize different aspects of the molecular systematics of New World belonids and their close relatives, or the relative phylogenetic information content of the different gene classes that we employed in our study. (1) ATPase8,6 results are provided because we used this gene combination to assay the largest number of individuals ($n = 56$; Table 1), including a modest phylogeographic appraisal for some species. (2) For 25 individuals (Table 1) we sequenced additional mitochondrial genes (2647 bp in total) to provide increased phylogenetic resolution at this locus. (3) Nuclear-encoded CK data (1042 bp) was sequenced for 18 individuals (Table 1), representing the principal taxa, in order to have an independent hypothesis of belonid relationship to compare to the mtDNA gene tree. (4) Finally we combined nuclear and mitochondrial data for 15 individuals (Table 1) in order to present a combined data analysis.

The ATPase8,6 data were largely congruent with the currently accepted α-taxonomy for the *S. marina* species group (Collette and Berry, 1965), and all species were genetically distinct and represented by reciprocally monophyletic clusters of mtDNA haplotypes. This

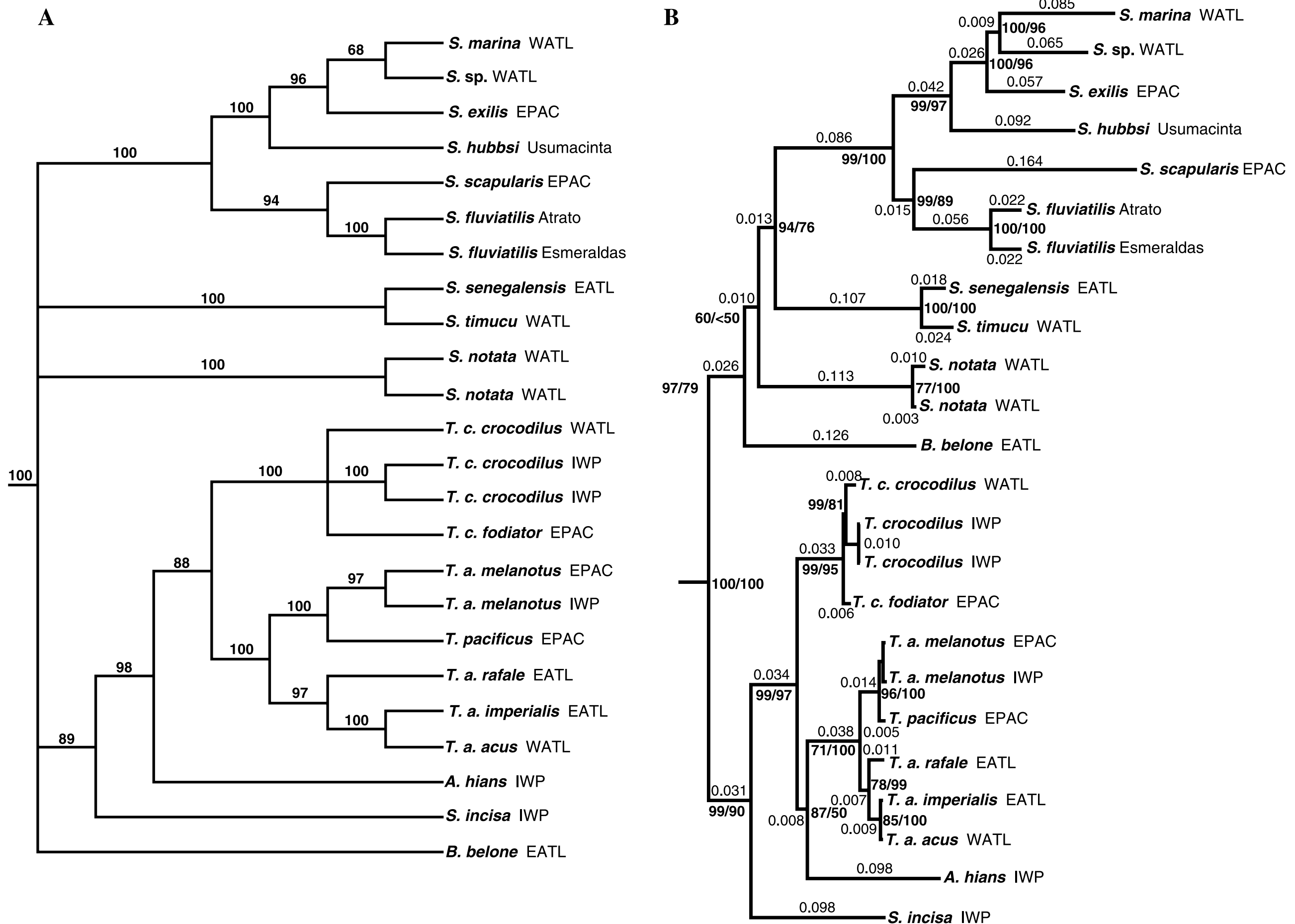

Fig. 3. Parsimony and maximum-likelihood trees for belonids and outgroup taxa based on the combined mtDNA data (2647 bp). Outgroups not shown are *H. unifasciatus* and *Sc. tritor*. Location abbreviations for marine taxa are as in Table 1: eastern Atlantic (EATL), western Atlantic (WATL), eastern Pacific (EPAC), and Indo-West Pacific (IWP). (A) The single bootstraped MP tree (50% majority rule) resulting from a heuristic search of the combined mtDNA data. Bootstrap values (based on 1000 replications) are shown in bold. The tree indices were TL = 3641, CI = 0.474, HI = 0.526, and RI = 0.628. (B) A quartet-puzzling maximum likelihood tree based on GTR distance estimates, invariable site and gamma shape parameters with 10,000 iterations. Genetic distance estimates are presented along the branches when possible, and reliability values are indicated in bold. Neighbor-joining analysis resulted in the same tree with somewhat different bootstrap values (based on 1000 replicates). Where confidence estimates differed, the reliability values are followed by the bootstrap values, with the two values separated by a slash (/).

monophyly permitted us to represent each *Strongylura* species by a single individual in subsequent analyses. Geographic structure was apparent in the named subspecies of *Tylosurus* (see below), although larger sample sizes would be required to confirm phylogeographic patterns. Putative *S. marina* samples were collected from coastal Brazil, but the genetic divergence between these individuals and all named *Strongylura* species and other belonid genera suggested that we have discovered a new species.

The inclusion of *Tylosurus* species enhanced our analyses of belonid molecular evolutionary rates, and also permitted an assessment of their molecular systematics. Geographic differences among *Tylosurus* have been characterized morphologically, resulting in described subspecies (Collette and Parin, 1970). In our analysis *Tylosurus acus* formed two principal mtDNA clades, one Pacific and one Atlantic (Fig. 3). Additional geographic structure was observed in this species and generally conformed to the subspecies taxonomy in use. However, in the Atlantic, *T. a. acus* from Bermuda and *T. a. imperalis* from the Mediterranean were genetically indistinguishable, while *Tylosurus a. rafale* from the Gulf of Guinea was distinct from the above amphi-Atlantic form. The subspecies taxonomy of *Tylosurus crocodilus* also was reflected by mtDNA differences.

Although the ATPase data were phylogenetically informative at higher taxonomic levels we turn to the combined mtDNA data to address these relationships. Partition homogeneity tests did not reject the null hypothesis of homogeneity of substitution across the data partitions representing the five mitochondrial genes ($p = 0.44$), nor for protein-coding versus ribosomal genes ($p = 0.33$). Results based on different

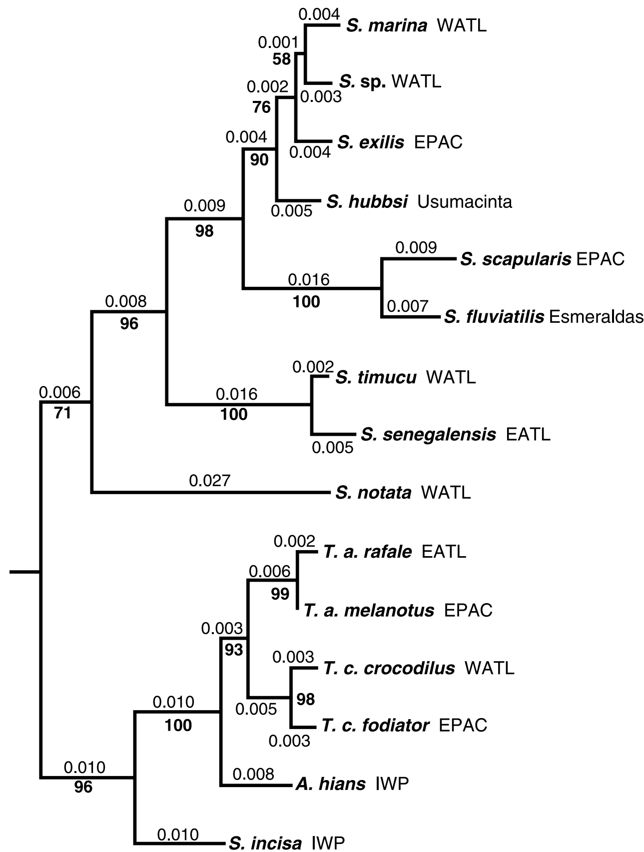

Fig. 4. Maximum-likelihood tree for belonids and outgroup taxa based on the creatine kinase b (CK) data (1042 bp). Outgroup not shown is *H. unifasciatus*. ML tree based on GTR model, invariable site and gamma shape parameters with indels treated as missing. Genetic distance estimates are provided above the branches and bootstrap values (based on 1000 replicates) are below.

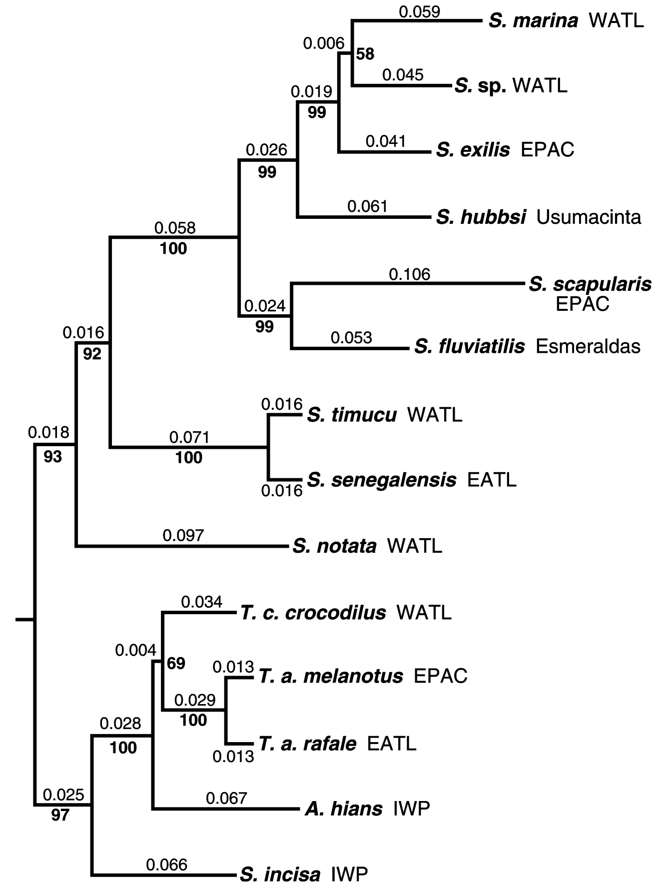

Fig. 5. Combined mtDNA and CK maximum likelihood tree for the *Strongylura marina* group and outgroup species. ML tree based on GTR model, invariable site and gamma shape parameters. Genetic distance estimates are provided above the branches and bootstrap values (based on 1000 replicates) are below.

combinations of the mtDNA data (e.g., protein coding, ribosomal and ribosomal stems and loops) were largely congruent with those reported here for the combined mtDNA data. Only the position of *Ablennes* differed in the MP and ML trees based on the combined mtDNA data (Fig. 3)

Two distinct belonid superclades were consistently identified in the ML analysis of the combined mtDNA (as was the case in all gene-by-gene analyses), though parsimony analysis failed to resolve basal relationships (Figs. 3A and B). One superclade included all of the *S. marina* group species and *Belone belone*. The second superclade included all *Tylosurus* species, *Ablennes hians*, and IWP *Strongylura*. Within the *S. marina* species group, the closest interspecific relationship is amphi-Atlantic, between, *S. timucu* and *S. senegalensis* (Fig. 3A). Although the eastern Atlantic *S. senegalensis* was hypothesized to be the basal member of the *S. marina* species group (Helfman et al., 1997), its close sister relationship with the western Atlantic *S. timucu* was unanticipated. This sister relationship was well supported in all analyses (bootstrap 100). All phylogenetic analyses identified a derived New World *S. marina* group: *S. marina*, *S.* sp. (Brazil), *S. exilis*, *S. hubbsi*, *S. fluviatilis*, and *S. scapularis*. In this group a trans-isthmian relationship is indicated for the eastern Pacific *S. exilis* with the western Atlantic sister pair of *S. marina* and *S.* sp. (Brazil). Whereas the ATPase8,6 data were unable to resolve the trichotomy (bootstrap value <50) formed between these three species, the combined data indicated that *S. exilis* is the sister species to the western Atlantic pair, suggesting an intra-ocean speciation event. The freshwater *S. hubbsi* from the Usumacinta drainage of Guatemala and Mexico, was sister to the aforementioned group of three species, which together formed one subclade within the derived New World clade. The second New World subclade comprised the eastern Pacific *S. scapularis* and the trans-Andean freshwater *S. fluviatilis*. Within *S. fluviatilis* distinct lineages were identified from the Atrato and Esmeraldas Rivers, Caribbean and Pacific drainages, respectively.

A quartet puzzling maximum likelihood (ML) approach, utilizing a gamma substitution rate heterogeneity model, resulted in a well-resolved tree of the belonid clades (Fig. 3B), indicating the two aforementioned super-clades with reliability values of 98 or greater. The ML results did not support the basal position of the amphi-Atlantic species pair *S. senegalensis* and *S. timucu* within the *S. marina* species group. The internal branch between *Belone* and *S. notata* was not well supported (rely value 55), however, the position of *Belone* as basal to the *S. marina* species group was well supported (rely value 98). Although the heuristic parsimony analysis (1000 bootstrap replicates) provided strong support for the intra-clade relationships identified in the ML tree, the parsimony tree, when branches with low bootstrap value (<50) were collapsed, provided no support for the inter-clade relationships that form a basal polytomy (Fig. 3A). Within the second super-clade the ML tree collapsed (rely 68) *Ablennes* into a trichotomy with *T. acus* and *T. crocodilus*, whereas parsimony analysis supported (bootstrap 87) the monophyly of the *T. acus* and *T. crocodilus* species with *Ablennes* as a sister taxon, meeting morphological expectations (Boughton et al., 1991; Collette and Berry, 1965). The IWP *S. incisa* consistently appears at the base of this clade (bootstrap value 100, rely value 99) indicating that the genus *Strongylura* is polyphyletic.

When *Strongylura* was constrained to be monophyletic, the resulting tree was 12 steps longer than the MP tree. We also used the KH test (Kishino and Hasegawa, 1989) to determine that the likelihood score (18927.76) for a tree constraining *Strongylura* to be monophyletic was significantly worse than the MP tree (18874.93) ($p = 0.0020$). We also forced *Belone* to its hypothetical basal position within the Belonidae (Collette and Berry, 1965) and maintained the monophyly of *Strongylura*. This tree required 41 additional steps and its likelihood score (18932.96) was significantly greater than that of the best MP tree (18879.52) ($p = 0.0011$).

The third data set used to assess belonid relationships was the nuclear-encoded CK gene providing an independent gene phylogeny (Fig. 4) to compare to the mtDNA gene tree. The ML and MP CK trees were perfectly congruent with one another and largely congruent with the ML combined mtDNA tree (Fig. 3B). In parsimony analysis (MP trees not shown) indels were treated in two ways resulting in the same tree topology, with different bootstrap values (based on 1000 replicates for each treatment) and tree indices. Indels treated as a fifth base pair had the following tree indices: $TL = 414$, $CI = 0.90$, $HI = 0.10$, and $RI = 0.90$. When indels where treated as missing the tree had the following indices: $TL = 287$, $CI = 0.90$, $HI = 0.10$, and $RI = 0.90$. The CK data resolved the placement of *Ablennes* as sister to *Tylosurus*. *Belone* is absent in the CK analysis due to failed attempts to amplify and sequence CK for this species. The rely and bootstrap estimates of confidence for the CK phenogram and cladogram were generally high. As shown in Fig. 4, indels scored as a fifth base resulted in improved confidence estimates as compared to indels treated as missing. Analysis of the intron data alone resulted in the same tree pictured in Fig. 4, whereas the exon-based tree presented spurious relationships among some taxa, possibly resulting from the very small number of parsimony informative characters ($n = 29$).

The fourth data set combined all mtDNA and nuclear data for 15 individuals based on the outcome of a partition homogeneity test ($p = 1.0$), in which we treated the CK data as one partition and the combined mitochondrial genes as a second partition. Analyses of these data produced the ML tree pictured in Fig. 5. The ML and MP CK trees were perfectly congruent with one another. The MP tree (not shown) resulted from a heuristic search of the data with the following tree indices: $TL = 3127$, $CI = 0.56$, $HI = 0.44$, and $RI = 0.55$. Topology of the tree produced by the combined data is consistent with those generated from the full mtDNA (25 individuals) and CK data, respectively (Figs. 3 and 4).

3.3. Genetic distance assessments

General Time Reversible (GTR) genetic distances between pairs of taxa were compared to assess their relative values across mtDNA protein-coding genes, ribosomal genes and the nuclear-encoded CK gene. The individuals chosen for comparison were selected to represent a hierarchically ordered set of taxa in an effort to evaluate genetic distance at different levels of phylogenetic separation, and included pairs of subspecies, sister species, species groups, and genera. Additional taxa were chosen to represent transisthmian and amphi-Atlantic geographic relationships. The rank order of genetic distances was mtDNA protein-coding genes > mtDNA ribosomal genes > CK. Between the three mtDNA protein-coding genes, ATPase6 and cyt *b* provided similar estimates of genetic distance, while ATPase8 was less consistent. ATPase6 and cyt *b* were both represented by similar numbers of nucleotides, whereas the ATPase8 gene is only 168 nucleotides in length. Thus the lack of consistent distance estimates returned by ATPase8 probably reflects the small size of the nucleotide sample.

The 12S and 16S mtDNA ribosomal genes demonstrated a substitution rate roughly 2–5 times slower than the mtDNA protein-coding genes. As reported previously for fishes (Ortí and Meyer, 1997), mtDNA 12S demonstrated an average substitution rate in belonids somewhat lower than 16S. Mitochondrial DNA ribosomal distances tended to be greater than protein-coding transversion distances.

CK distance analyses were based on the entire sequence excluding indels; thus the nuclear distance measures did not account for indel variation. In general, the CK exon regions were conserved across taxa, whereas the intron regions provided a greater number of phylogenetically informative characters either as nucleotide base substitutions or indels. Presence and absence of indels were easily discerned as synapomorphies. Distance measures were lowest for the CK gene in all species level and higher clade comparisons. Significant concordance of the rank order of distance comparisons between hierarchically ordered taxa across genes was indicated by Kendall's coefficient of concordance, $W = 0.887$ (Zar, 1984). The equivalent χ^2 for the value of W obtained from the belonid data was 48.79, permitting us to reject the hypothesis of no rank order among the genes ($\chi^2_{0.001,11} = 31.26$).

GTR distance values distinguishing *Strongylura* and belonid species were generally high (Fig. 3B) in comparison to values typically found in other fishes (Banford et al., 1993; Bermingham et al., 1997b; Meyer, 1993). The lowest interspecific distances were observed between *S. timucu* and *S. senegalensis*: 0.058 and 0.041 for the mtDNA protein-coding genes ATPase6 and cyt *b*, respectively; 0.012 and 0.025 for 12S and 16S genes respectively; and 0.008 for CK. More phylogenetically divergent lineages are represented by the comparisons between *S.* sp. (Brazil) and *T. acus melanotus*: 0.697 and 0.424 for ATPase6 and cyt *b*, respectively; 0.216 and 0.198 for 12S and 16S, respectively; and 0.077 for CK.

All data were tested for molecular rate homogeneity using the two-cluster and branch-length tests (Takezaki et al., 1995). The CK data were determined to be mostly clock-like, however, there were significant differences between two clusters: *S. incisa* versus the *Tylosurus* + *Alblennes* clade, and *S. scapularis* and *S. fluviatilis* versus *S. marina* + *S.* sp. + *S. exilis* + *S. hubbsi*. When the mitochondrial data were added to the CK data, additional clusters and branches failed the respective tests. The two-cluster test indicated that the *S. notata* clade was evolving at a significantly faster rate than *S. marina* group and *Tylosurus* clades ($z = 2.620558$, $p = 99.12$). The branch-length test indicated that *S. marina* and *S. scapularis* had a significantly higher root to tip distance than the average ($z = 2.368137$, $p = 98.18$). Although we did not undertake the iterative analysis required to find a fully linearized tree, our data urge caution when interpreting molecular clock based estimates for *S. marina* and *S. scapularis* speciation dates.

4. Discussion

We follow a sequence in which we first establish the strengths and weaknesses of independent mtDNA and nuclear markers for phylogenetic and temporal inference of belonid relationships. In this context our results supported confident interpretation of the systematic relationships and temporal divergence among Atlantic, eastern Pacific, and New World freshwater *Strongylura* species. In turn, the robust phylogenetic inference for the *S. marina* species group was used to evaluate the EP/EA biogeographic model of marine diversification.

4.1. Molecular resolution of relationships

We have presented the results of belonid phylogenetic analyses based on mtDNA protein-coding, mtDNA ribosomal and nuclear CK genes. It is important to note that molecular characters representing each of the three classes of molecular data diagnosed the individual belonid species included in this study. Furthermore, the three classes of molecular information provided consistent estimates of belonid phylogeny, but different numbers of phylogenetically informative characters and different levels of resolution. For example, the mtDNA protein coding genes (ATPase8,6 and cyt *b*) were the most useful data for determining the phylogenetic relationships of closely related species, and were consistent with the nuclear-encoded CK gene for inferring relationships at the base of the belonid tree.

Aspects of the phylogenetic analysis based on the combined mtDNA data deserve specific comment. Transition substitutions in mtDNA protein-coding genes were saturated at genetic distances corresponding to the base of the belonid tree (Fig. 2A); however, we observed no saturation of transversion substitutions in the mtDNA protein-coding genes. Likewise we observed no saturation of mtDNA ribosomal transitions or transversions, suggesting that the lack of resolution, at the base of the belonid tree generated from these data, does not owe to saturation alone. Given the slower rate of nucleotide substitution in the ribosomal genes, we were somewhat surprised that this class of molecular data performed worse than protein-coding genes in resolving the base of the belonid tree. For example, increasing the mtDNA nucleotide sample (from the initial 842 bp provided by ATPase8,6) resulted in elevated support for the monophyly of the *S. marina* superclade (bootstrap 51–92), but it was the addition of the cyt *b* data, not the mtDNA ribosomal data, that accounted for the increase. Furthermore, lower substitution rates in teleost fishes' mtDNA ribosomal genes (Ortí and Meyer, 1997) had led us to anticipate increased performance at intermediate and deeper levels in the belonid tree. Two observations probably reconcile our empirical results. First, we sampled roughly three times the number of parsimony informative nucleotides representing protein-coding genes as compared to the 12S and 16S genes (Table 2).

Nucleotide sample size alone is not a powerful explanation, however, in light of our CK results. We observed about half the number of phylogenetically informative CK sites than in the mitochondrial protein-coding genes, but dramatically improved tree statistics. Second, and probably most importantly, the complex secondary structure of ribosomal genes creates ambiguities in alignment and phylogenetic analysis. Whereas synapomorphic indels within nuclear introns were easily discerned and aligned, indels within ribosomal loops tended to be unruly and could not be unambiguously aligned.

4.2. Systematic relationships

The Belonidae have been difficult to analyze within a rigorous phylogenetic context, due to a paucity of useful morphological characters (Boughton et al., 1991), hence relationships have been based on overall morphological similarity (Berry and Rivas, 1962; Collette and Berry, 1965; Collette et al., 1984). Thus, to determine the utility of the *S. marina* species group for addressing Rosen's EP/EA biogeographic track, we had to first assess the monophyly of the group. Our phylogenetic sampling included *Belone*, which is posited to be a basal belonid owing to the possession of several generalized morphological features. *Ablennes hians* and *Tylosurus* represented additional outgroups and increased the number of transisthmian and amphi-Atlantic sister taxa comparisons. The Indo-West Pacific *S. incisa* provided a geographic outgroup within *Strongylura*.

Generally speaking, the molecular data support the classical view regarding the species comprising the *S. marina* species group (Cressey and Collette, 1970), however, the combined mtDNA and nuclear CK results did not unequivocally support the inclusion of *S. notata* within the *S. marina* species group. In both the combined ML and parsimony mtDNA analyses (Figs. 3A and B) *S. notata* stems from a basal polytomy with *Belone* (bootstrap support <50), suggesting that *S. notata* may represent a Tethyan relic predating the *S. marina* radiation. The eastern Atlantic *S. senegalensis* was hypothesized to be the basal member of the *S. marina* group (Collette, pers. commun.), however, the close sister relationship of *S. senegalensis* with the western Atlantic *S. timucu* was unanticipated. This relationship was well supported (bootstrap 100) in all analyses, and raises a question that our phylogenetic analysis does not answer. Namely, was the ancestor of this sister-pair an eastern, western or amphi-Atlantic species? This uncertain geographic placement of the *S. timucu* ancestor raises possibility that *Belone* represents the basal eastern Atlantic member of the clade, a hypothesis that suggests that the common ancestor had a distribution spanning the Atlantic Ocean.

4.3. Chronology of speciation

In addition to confident interpretation of species branching order, independent molecular markers permit a more robust assessment of branching times. The consistent ratios of the mtDNA and CK genetic distances suggest that the different divergence values observed across the belonid tree reflect different dates of speciation rather than grossly different rates of molecular evolution along particular lineages (Bermingham and Lessios, 1993; Vawter and Brown, 1986). In addition, the branch-length test of Takezaki et al. (1995) did not reveal large departures from the average root-to-tip distance, although significant differences for some gene regions and some taxa serve as a strong reminder that the following discussion regarding the chronology of belonid diversification is speculative.

The relatively large mtDNA genetic distances between most belonid species begs the question of whether the species are relatively old or simply characterized by an accelerated rate of molecular evolution. For comparison, the EP/EA *Sc. regalis* group (Banford et al., 1993) evidenced much smaller mtDNA genetic distances between closely related species than those observed between *Strongylura* sister species. In our comparisons of interspecific and interclade distances for the mitochondrial and nuclear genomes, whether contrasting closely related or highly divergent species pairs, we generally observed nuclear CK distances about one order of magnitude less than their mitochondrial counterpart. This is similar to the observed ratio for *Scomberomorus* (Banford et al., 1993) and indicates that if molecular evolution is accelerated in the belonids, the rate increase affects both the mitochondrial and nuclear genomes.

Our comparison of mtDNA and CK genetic divergence indicated that the relative timing of belonid speciation events was congruent across the two markers. To provide estimates of the absolute time of separation, we calibrated the belonid mtDNA and CK clocks using two pairs of transisthmian sister taxa (so-called geminates; Jordan, 1908): *T. c. fodiator* and *T. c. crocodilus*, and *T. pacificus* and *T. a. acus*. The two geminate belonid pairs are separated by genetic distances that range from 0.030 to 0.054 for mtDNA protein coding genes and 0.004 to 0.006 for CK. (Owing to the variation in molecular evolutionary rate observed for ATPase8, we averaged only ATPase6 and cyt *b* distances for chronological estimates based on mtDNA protein-coding genes.) These differences in genetic divergence across geminate belonid pairs may reflect different dates of separation. The alternative, different rates of molecular evolution, would require the correlated molecular evolution of the unlinked mtDNA and CK markers (Bermingham and Lessios, 1993; Bermingham et al., 1997b; Knowlton et al., 1993).

Variables in the life history of belonids and the geological events associated with the rise of the Isthmus of Panama, cause considerable uncertainty in our efforts to tie the divergence of geminate *Strongylura* and *Tylosurus* to particular biogeographic events. For example, a greater capacity for dispersal by large pelagic *Tylosurus* in contrast to the more inshore habits of the smaller *Strongylura*, may have resulted in periodic movement by *Tylosurus* around the Cape of Good Hope as has been suggested for other tropical marine organisms (Baldwin et al., 1998). Environmental changes occurring over the mid-Miocene to Pleistocene development of the Panama isthmus may have provided at least four distinct opportunities for allopatric speciation (Bermingham et al., 1997b; Knowlton and Weigt, 1998; Knowlton et al., 1993). The first opportunity would have coincided with the low sea level stand in the mid-Miocene at 10.5 mya (Haq et al., 1987), the second in the late Miocene with extensive shoaling and emergence of Central American isthmian landmasses (7–5 mya; Bermingham and Martin, 1998; Coates and Obando, 1996), the third with the celebrated Pliocene completion of the Central American terrestrial isthmian corridor (3.1–2.8 mya; Coates et al., 1992; Keigwin, 1978), and the fourth with an early Pleistocene marine breach of the Central American Isthmus in the area of central Panama (2 mya; Coates and Obando, 1996; Cronin and Dowsett, 1996). Geological evidence for a Pleistocene breach of the Panama Isthmus is controversial, but historical biogeographic component analysis presented by Bermingham and Martin (1998) also suggests the extinction of some freshwater fish mtDNA lineages roughly corresponding in time and space with a putative breach. Furthermore, a geminate pair of inshore marine fishes, *Abudefduf concolor* and *A. taurus*, demonstrate low molecular divergence suggesting either a reduced mtDNA evolutionary rate or a recent split compared to many other presumed geminate fishes (Bermingham et al., 1997b; Lessios et al., 1995).

If we consider the most closely related transisthmian pair, *T. c. fodiator* and *T. c. crocodilus*, to have been separated by the Pliocene completion of the isthmus, our calibration of the belonid mtDNA protein gene clock is 1.0% per million years (0.030/3 my), and the mtDNA ribosomal and CK clocks have only just begun keeping time. If, on the other hand, we date this divergence to the breach of the isthmus, our calibration is 1.4%. This is similar to the average mtDNA calibration reported by Bermingham et al. (1997b) for a number of geminate species pairs and to a Pliocene-based calibration of the *T. pacificus* and *T. a. acus* divergence. The mtDNA genetic distance between this pair is 0.054 and provides a calibration of 1.8% divergence per million years for mtDNA protein-coding genes and 0.2% per million years for both the ribosomal and CK genes. Though with weak support the WATL–IWP sister relationship of *T. crocodilus* subspecies (Fig. 3) suggests periodic movement by *T. crocodilus* around the Cape of Good Hope.

4.4. Vicariance and dispersal across the EP/EA biogeographic track

The biogeographic model underlying Rosen's (1975) EP/EA distributional tracks predicts that eastern Atlantic species ought to be basal owing to the vicariant separation of the New World and eastern Atlantic faunas by the Cenozoic opening of the South Atlantic Ocean (ca. 65–95 mya; Pittman et al., 1993). The eastern Atlantic *Belone belone* is basal, suggesting support for Rosen's EP/EA model, and paralleling our previous finding that the eastern Atlantic *Sc. tritor* was the basal member of the EP/EA *Sc. regalis* group (Banford et al., 1993). In general, however, our findings and those of others (Lessios et al., 1999, 2000; Muss et al., 2001; Scheltema, 1973, 1995) do not fully support Rosen's hypothesis for relationships of New World to eastern Atlantic faunas and the timing of these events. Specifically as an example, the relationship of *S. timucu* and *S. senegalensis* indicates a very recent separation of trans-Atlantic species and includes the possibility of dispersal playing a central role. Ultimately we must conclude that Rosen's model is an oversimplification of the complex biogeographic histories across the EP/EA region.

If we apply a mtDNA belonid-specific clock calibrated by the closure of the Central American isthmus, or one based on multiple transisthmian pairs of taxa, we obtain a basal western Atlantic eastern Atlantic belonid divergence during the Oligocene to mid-Miocene (32–10 mya). The estimated time of the basal separation in EP/EA belonids corresponds to that reported for *Scomberomorus* (Banford et al., 1993) and fits well with geologically dated estimates for the closure of the Tethys Sea (Berggren, 1978; Rögl, 1998; Vrielynck et al., 1997). The timing of the phylogenetic separation of the basal species in these groups also matches fossil evidence indicating a turnover in the composition of faunal assemblages precipitated by the changing oceanographic conditions at Tethys closure (Berggren, 1978; Néraudeau, 1994; Rögl, 1998; Scheltema, 1995; Vrielynck et al., 1997). For example, otolith evidence from fossil teleosts reveals a strong similarity between the Miocene and present-day Caribbean faunas and a departure from the Paleogene composition of these fish communities (Nolf and Stringer, 1992; Nolf and Aguilera, 1998). However, the departure is not complete and the fossil evidence indicates that some Paleogene relicts remain in the modern Caribbean, providing some support for our suggestion that the phylogenetically divergent *S. notata* and *Sc. cavalla* represent remnants of a Tethyan fauna.

Thus, the branching order for belonids (Fig. 5) and *Scomberomorus* (Banford et al., 1993), but not the early

Tertiary or Cretaceous branching time, meet the predictions of Rosen's vicariant model for the EP/EA. Our data also support a vicariant model for transisthmian speciation, but again the timing of separation is called into question by the molecular data. If the *Tylosurus* transisthmian pairs are used to calibrate the belonid clock, our data indicate that the eastern Pacific *S. exilis* and its western Atlantic sisters, *S. marina* and *S.* sp., separated prior to the completion of the Isthmian barrier. The early separation of this transisthmian clade is consistent with the late Miocene shoaling and emergence of islands across the nascent isthmus (Coates and Obando, 1996). Furthermore, this early biological signal of the rising isthmus is echoed by the timing of the first mammalian migrations between the two continents (Stehli and Webb, 1985), the initial invasion of primary freshwater fishes into Central America from South America (Bermingham and Martin, 1998), and the separation of some transisthmian *Alpheus* shrimp (Knowlton et al., 1993) and marine fishes (Bermingham et al., 1997b).

The most closely related pair of *Strongylura* species (0.055) was *S. timucu* and *S. senegalensis* and we presume they separated approximately 3 mya ago, roughly coincident with the rise of the Panamanian Isthmus and the resulting change in ocean currents (Haug and Tiedemann, 1998). As noted previously, our analyses do not permit us to resolve the geographic placement of the *S. timucu* ancestor. An amphi-Atlantic ancestor would argue for continued gene flow across the Atlantic Ocean until very recently (see Muss et al., 2001), whereas a western Atlantic ancestor would presuppose that the genus *Strongylura* went extinct or was never present in the eastern Atlantic prior to or coincident with the recent trans-Atlantic dispersal event suggested by the molecular data.

A particularly interesting feature of belonid biogeography is the number of hypothesized invasions into freshwater suggested by beloniform marine ancestry (Collette et al., 1984; Lovejoy and Collette, 2001). Presently, there are four freshwater belonid genera in the Neotropics, of which only *Strongylura* includes both marine and freshwater species. Our phylogenetic analysis identifies two independent freshwater invasions; *S. hubbsi* in the Usumacinta (Atlantic slope) drainage of Guatemala and Mexico, and *S. fluviatilis* in the Choco region of northwestern South America and along the Pacific slope of Colombia and Ecuador. This conclusion is supported by a log-likelihood test statistically rejecting a single invasion of freshwater ($-\ln L$ 20790.483 vs. 20919.527; $p < 0.0001$). *S. fluviatilis* is sister to the southerly-distributed *S. scapularis*, whereas *S. hubbsi* is sister to a more northerly *Strongylura* clade. Genetic distance data suggested that the *S. hubbsi* lineage diverged in the late Miocene. It is of biogeographic note that the Usumacinta River ichthyofauna includes 18 species with marine ancestry. This river system drains an eroded carbonate and limestone karst formation (Miller, 1966; Myers, 1966; West et al., 1969) and uplift of this formation potentially created an isolated high ionic habitat permitting marine fish to gradually shift to freshwater tolerance.

The isolation of freshwater *S. fluviatilis* was contemporaneous with that of *S. hubbsi* (Fig. 5). Moreover, our divergence dates of freshwater *Strongylura* across the Americas, are remarkably consistent with Lovejoy et al.'s (1998) timescale for the isolation of the freshwater stingrays (Potamotrygonidae) in northern South America. The biological evidence for marine invasions and isolation on the American continents is strongly supported by geological data. Haq et al.'s (1987) sea-level curves indicate a marine incursion in the early Miocene (around 14 mya) followed by an extreme sea level low (−100 m) beginning 10.5 mya. Furthermore, palynological and sedimentological data evidence the isolation of Lake Maracaibo (northern South America) and its gradual shift from marine to freshwater 8–10 mya (Hoorn, 1993). Freshwater environments in the Choco region of northwest Colombia were also developing at this time (Duque-Caro, 1990a,b) and presumably provided the opportunity for the invasion of the *S. fluviatilis* lineage. Subsequently, the divergence of the transandean forms of *S. fluviatilis* along the Atlantic (Atrato River, Colombia) and Pacific (Esmeraldas Region, Ecuador) slopes can be dated to 3 mya using the belonid molecular clock, coincident with completion of the isthmian corridor.

Our phylogenetic analysis of the *S. marina* and *Sc. regalis* species groups permits some preliminary conclusions regarding the EP/EA biogeographic track as a focus for studies of marine speciation. Because the track includes earth history events operating at different spatial and temporal scales and a large array of species with different life histories, it offers the opportunity to investigate the interplay of extrinsic and intrinsic factors in shaping marine diversity. For example, our data suggest that both the closure of the Tethys Sea and the rise of the Panama Isthmus caused the formation of new species. Nonetheless, these earth history events do not sufficiently account for New World *Strongylura* and *Scomberomorus* diversity. The two groups contain a single eastern Atlantic species each, six vs. three western Atlantic species and three vs. two eastern Pacific species for *Strongylura* and *Scomberomorus*, respectively. This finding promotes the view that dispersal and/or within-region vicariance played roles in the diversification of the western Atlantic and eastern Pacific marine faunas. Our data and that of previous workers (Briggs, 1974, citations therein, and 1985) identify the depauperate eastern Atlantic as either an area of low speciation or high extinction. The latitudinal restriction of tropical water masses (Loder et al., 1998) and narrow continental shelf width (<30 km in EA to ~300 km in WA;

Ajao and Houghton, 1998; Castro and de Miranda, 1998) mark the eastern Atlantic as a region with reduced area and heterogeneity as compared to either the western Atlantic or eastern Pacific.

The difference in species counts across New World *Strongylura* and *Scomberomorus* may be largely accounted for by intrinsic differences in the physiological potential for life in freshwater. More generally, life history characteristics including dispersal are often considered to play a significant role in marine fish speciation (Graves, 1998; Shulman and Bermingham, 1995; Waples, 1987; Waples and Rosenblatt, 1987), although dispersal ability has been argued both to promote and limit the potential for speciation. For *Strongylura*, a long-distance dispersal event may have led to the recent origin of the western Atlantic *S. timucu* and eastern Atlantic *S. senegalensis*, thus accounting for the final difference in species counts between the *S. marina* and *Sc. regalis* groups. Our point is not that our accounting is necessarily correct or that we have correctly identified the causes of speciation in the two species groups. Rather, we wish simply to note that comparative study of EP/EA species within a phylogenetic framework permits strong inference regarding the interplay of earth and life history in the formation of marine species.

Acknowledgments

This research was supported by Smithsonian Institution (SI) and Smithsonian Tropical Research Institute (STRI) graduate fellowships, and National Marine Fisheries Service (NMFS) internships to HB, National Geographic Grant In-Aid of Field Research (#5425-95 to B.B.C. and H.B.), and the STRI Molecular Evolution Program. For sample acquisition we thank: D. Seaver and A. Estes of VIMS; K. Carpenter of ODU, then FAO; L. Parenti of USNM; D. Adams, J. Colvocoresses, and M. Fluck of FMRI; C. Tabit of VIMS now UWG; Doug Graff, Peace Corps, Sao Tome; P. Buckup and H. Seciocios of MNRJ; R. Texeira, Brazil; R. Rosa of UFP. H.B.'s own collections abroad have been assisted and made possible by the logistical support and permission of the following persons and institutions: For collections in San Blas, Panama the permission of the Kuna People (Kuna Yala); marine collections in general in Panama where made possible by Recursos Marinos Panama; E. Anang and Mr. Orfori-Adu of FRUB, Ghana; V. Hyatt of SI put H.B. in contact with the MUCIA group in Ghana who very warmly extended the use of their guest houses; the hospitality of E. Gournis while in Dakar was a special treat; while in Ecuador R. Barriga of EPN, K. Galacatos and N. Lovejoy made H.B.'s trip an enjoyable success. Within our lab group H.B. appreciates the willingness of E.B., J. Hunt (now FMNH), S. McCafferty, and G. Ybazata (now Univ. of Chicago) to be dragged out of the lab for needlefish hunts. The greatest of thanks goes to the entire support staff at STRI's Naos Island Marine Lab, especially Sr. A. Verlarde whom kept our field needs met. L. Kral of UWG kindly provided technical support in the final stages of manuscript preparation. This manuscript benefited from careful review by L. Parenti, M. Vecchione, and two anonymous reviewers. This research is in partial fulfillment of H.B.'s Ph.D. dissertation, SMS/VIMS, College of William and Mary.

Appendix A

All specimens used in this study are listed by identification number (Table 1), species, status of voucher, gene regions sequenced, and each gene region is followed by its GenBank Accession No. For museum acronyms see Leviton et al. (1985).

STRI-4732, *Ablennes hians*, USNM347819; ATPase6&8 AF231583, Cytb AF231639, 12S AF231540, 16S AF231513, CK AF231667.

AB-02-97, *Belone belone*, no voucher; ATPase6&8 AF231584, Cytb AF231640, 12S AF231541, 16S AF231514.

STRI-3096, *Strongylura exilis*, STRI00192; ATPase6&8 AF231585.

STRI-3097, *Strongylura exilis*, STRI00192; ATPase6&8 AF231586, Cytb AF231641, 12S AF231542, 16S AF231515, CK AF231668.

STRI-1485, *Strongylura fluviatilis*, uncataloged Bogata, Colombia; ATPase6&8 AF231587, Cytb AF231642,12S AF231543,16S AF231516.

STRI-1486, *Strongylura fluviatilis*, STRI00193; ATPase6&8 AF231588, 12S AF231544.

STRI-6133, *Strongylura fluviatilis*, uncataloged Quito, Ecuador; ATPase6&8 AF231590, Cytb AF231643, 12S AF231545, 16S AF231517, CK AF231669.

STRI-6134, *Strongylura fluviatilis*, uncataloged Quito, Ecuador; ATPase6&8 AF231589, 12S AF231546.

STRI-6138, *Strongylura fluviatilis*, uncataloged Quito, Ecuador; 12S AF231547.

GU-5-96, *Strongylura hubbsi*, CU; ATPase6&8 AF231591, 12S AF231549.

GU-6-96, *Strongylura hubbsi*, CU; ATPase6&8 AF231592, Cytb AF231644, 12S AF231548, 16S AF231518, CK AF231670.

STRI-4702, *Strongylura incisa*, USNM348248; ATPase6&8 AF231593, Cytb AF231645, 12S AF231550, 16S AF231519.

STRI-4711, *Strongylura incisa*, USNM348249; Cytb AF231646, 12S AF231551, 16S AF231520, CK AF231671.

HB-18, *Strongylura marina*, USNM-pending; ATPase6&8 AF231594, 12S AF231552.

HB-19, *Strongylura marina*, USNM-pending; ATPase6&8 AF231595.
HB-22, *Strongylura marina*, USNM-pending; ATPase6&8 AF231596.
HB-268, *Strongylura marina*, USNM-pending; ATPase6&8 AF231597, Cytb AF231647, 12S AF231553, 16S AF231521, CK AF231672.
HB-270, *Strongylura marina*, USNM-pending; ATPase6&8 AF231598, 12S AF231554.
HB-53, *Strongylura marina*, USNM-pending; ATPase6&8 AF231601, 12S AF231557.
HB-404, *Strongylura* sp., USNM-pending; ATPase6&8 AF231599, 12S AF231555, 16S AF231522, Cytb AF231648, CK AF231673.
HB-408, *Strongylura* sp., USNM-pending; ATPase6&8 AF231600, 12S AF231556, Cytb AF231649.
HB-1, *Strongylura notata*, USNM-pending; ATPase6&8 AF231605.
HB-159, *Strongylura notata*, USNM344599; ATPase6&8 AF231602, Cytb AF231650, 12S AF231558, 16S AF231523.
HB-2, *Strongylura notata*, USNM-pending; ATPase6&8 AF231606, 12S AF231560.
HB-72, *Strongylura notata*, USNM-pending; ATPase6&8 AF231603.
HB-82, *Strongylura notata*, USNM-pending; ATPase6&8 AF231604, Cytb AF231651, 12S AF231559, 16S AF231524, CK AF231674.
STRI-3051, *Strongylura scapularis*, STRI00194; ATPase6&8 AF231607, Cytb AF231652, 12S AF231561, 16S AF231525, CK AF231675.
STRI-6605, *Strongylura senegalensis*, USNM-pending; ATPase6&8 AF231608, Cytb AF231653, 12S AF231562, 16S AF231526, CK AF231676.
STRI-6606, *Strongylura senegalensis*, USNM-pending; ATPase6&8 AF231609, 12S AF231563.
HB-180, *Strongylura timucu*, USNM342925; 12S AF231566.
HB-181, *Strongylura timucu*, USNM342925; ATPase6&8 AF231614.
HB-190, *Strongylura timucu*, USNM342925; ATPase6&8 AF231615, 12S AF231567, CK AF231678.
HB-191, *Strongylura timucu*, USNM342925; ATPase6&8 AF231616, Cytb AF231655, 12S AF231568.
HB-401, *Strongylura timucu*, USNM-pending; ATPase6&8 AF231617, 12S AF231569.
HB-405, *Strongylura timucu*, USNM-pending; ATPase6&8 AF231618, 12S AF231570.
STRI-3817, *Strongylura timucu*, STRI00197; ATPase6&8 AF231610, 12S AF231564.
STRI-3846, *Strongylura timucu*, STRI-00179; ATPase6&8 AF231611.
STRI-4308, *Strongylura timucu*, STRI00196; ATPase6&8 AF231612, Cytb AF231654, 12S AF231565, 16S AF231527, CK AF231677.
STRI-4310, *Strongylura timucu*, STRI00196; ATPase6&8 AF231613.
BBC-11BERM, *Tylosurus acus acus*, USNM337725; ATPase6&8 AF231619, Cytb AF231656, 12S AF231571, 16S AF231528.
BBC-13BERM, *Tylosurus acus acus*, USNM337725; ATPase6&8 AF231620.
GO-1-95, *Tylosurus acus imperalis*, no voucher; ATPase6&8 AF231621, Cytb AF231657, 12S AF231572, 16S AF231529.
GO-2-95, *Tylosurus acus imperalis*, no voucher; ATPase6&8 AF231622.
STRI-4700, *Tylosurus acus melanotus*, USNM348245; ATPase6&8 AF231623, Cytb AF231658, 12S AF231573, 16S AF231530.
STRI-4704, *Tylosurus acus melanotus*, USNM348245; ATPase6&8 AF231624.
STRI-5109, *Tylosurus acus melanotus*, STRI00181; ATPase6&8 AF231625, Cytb AF231659, 12S AF231574, 16S AF231531, CK AF231679.
STRI-5110, *Tylosurus acus melanotus*, STRI00181; ATPase6&8 AF231626.
STRI-6516, *Tylosurus acus rafale*, USNM-pending; ATPase6&8 AF231629, Cytb AF231661, 12S AF231576, 16S AF231533, CK AF231680.
STRI-6536, *Tylosurus acus rafale*, USNM-pending; ATPase6&8 AF231630.
HB-959, *Tylosurus crocodilus fodiator*, STRI00183; ATPase6&8 AF231636, 12S AF231580, 16S AF231537, CK AF231682.
STRI-5111, *Tylosurus crocodilus fodiator*, STRI00184; ATPase6&8 AF231635.
STRI-2579, *Tylosurus crocodilus crocodilus*, STRI00187; ATPase6&8 AF231633.
STRI-3837, *Tylosurus crocodilus crocodilus*, STRI00185; ATPase6&8 AF231632, Cytb AF231663, 12S AF231578, 16S AF231535, CK AF231681.
BBC-4-PHIL, *Tylosurus crocodilus crocodilus*, USNM348292; ATPase6 & AF231634, Cytb AF231664, 12S AF231579, 16S AF231536.
HB-166, *Tylosurus crocodilus crocodilus*, USNM339006; ATPase6&8 AF231631, Cytb AF231662, 12S AF231577, 16S AF231534.
HB-954, *Tylosurus pacificus*, STRI00182; ATPase6&8 AF231628.
HB-955, *Tylosurus pacificus*, STRI00182; ATPase6&8 AF231627, Cytb AF231660, 12S AF231575, 16S AF231532.

References

Ajao, E.A., Houghton, R.W., 1998. Coastal ocean of equatorial West Africa from 10°N to 10°S, coastal segment (17,E). In: Robinson,

A., Brink, H. (Eds.), The Sea, vol. 11. Wiley, New York, pp. 605–631.

Applied Biosystems, ABI, 1992. SeqEd. DNA Sequence Editor, version 1.0.3.

Baldwin, J.D., Bass, A.L., Bowen, B.W., Clark Jr., W.H., 1998. Molecular phylogeny and biogeography of the marine shrimp *Penaeus*. Mol. Phylogenet. Evol. 10, 399–407.

Banford, H.M., 1998. Biogeography of amphi-Atlantic and amphi-American fishes: the *Scomberomorus regalis* (Scombridae), *Strongylura marina* (Belonidae) and *Hyporhamphus unifasciatus* (Hemiramphidae) species groups. Doctoral thesis, College of William and Mary, USA.

Banford, H.M., Bermingham, E., Collette, B.B., McCafferty, S.S., 1993. Phylogenetic systematics of the *Scomberomorus regalis* (Teleostei: Scombridae) species group: molecules, morphology and biogeography of Spanish mackerels. Copeia, 596–613.

Berggren, W.A., 1978. Recent advances in Cenozoic planktonic foraminiferal biostratigraphy, biochronology, and biogeography: Atlantic Ocean. Micropaleontology 24, 337–370.

Bermingham, E., Lessios, H., 1993. Rate variation of protein and mitochondrial DNA evolution as revealed by sea urchins separated by the Isthmus of Panama. Proc. Natl. Acad. Sci. USA 90, 2734–2738.

Bermingham, E., Seutin, G., Ricklefs, R.E., 1996. Regional approaches to conservation biology: RFLPs, DNA sequences, and Caribbean birds. In: Smith, T., Wayne, R. (Eds.), Molecular Genetic Approaches in Conservation. Oxford University Press, New York, pp. 104–124.

Bermingham, E., Banford, H.M., Martin, A.P., Aswani, V., 1997a. Smithsonian Tropical Research Institute Neotropical Fish Collections. In: Malabarba, L. (Ed.), Neotropical Fish Collections. Museu de Ciencias e Tecnologia, PUCTS, Puerto Alegre, Brazil, pp. 37–38.

Bermingham, E., McCafferty, S.S., Martin, A.P., 1997b. Fish biogeography and molecular clocks: perspectives from the Panamanian Isthmus. In: Kocher, T., Stepien, C. (Eds.), Molecular Systematics of Fishes. Academic Press, San Diego, pp. 113–128.

Bermingham, E., Martin, A.P., 1998. Comparative mtDNA phylogeography of neotropical freshwater fishes: testing shared history to infer the evolutionary landscape of Central America. Mol. Ecol. 7, 499–517.

Berry, F.H., Rivas, L.R., 1962. Data on six species of needlefishes (Belonidae) from the western Atlantic. Copeia, 152–160.

Boughton, D., Collette, B., McCune, A., 1991. Heterochrony in jaw morphology of needlefishes (Teleostei: Belonidae). Syst. Zool. 40, 329–354.

Briggs, J.C., 1974. Marine Zoogeography. McGraw-Hill, New York.

Briggs, J.C., 1985. Species richness among the tropical shelf. Sov. J. Mar. Biol. 11, 295–302.

Castro, B.M., de Miranda, L.B., 1998. Physical oceanography of the western Atlantic continental shelf located between 4°N and 34°S, coastal segment (4,W). In: Robinson, A., Brink, K. (Eds.), The Sea, vol. 11. Wiley, New York, pp. 209–251.

Coates, A.G., Jackson, J.B.C., Collins, L.S., Cronin, T.M., Dowsett, H.J., Bybell, L.M., Jung, P., Obando, J.A., 1992. Closure of the Isthmus of Panama: the near-shore marine record of Costa Rica and Panama. Geol. Soc. Am. Bull. 104, 814–828.

Coates, A.G., Obando, J.A., 1996. The geologic evolution of the Central American Isthmus. In: Jackson, J., Budd, A., Coates, A. (Eds.), Evolution and Environment in Tropical America. University of Chicago Press, Chicago, pp. 21–56.

Collette, B.B., Berry, F.H., 1965. Recent studies on the needlefishes (Belonidae): an evaluation. Copeia 1965, 386–392.

Collette, B.B., Parin, N.V., 1970. Needlefishes (Belonidae) of the eastern Atlantic Ocean. Atlantide Report No. 11.

Collette, B.B., McGowen, G., Parin, N.V., Mito, S., 1984. Beloniformes: development and relationships. In: Moser, H., Richards, W., Cohen, D., Fahay, M., Kendal Jr., A., Richardson, S. (Eds.), Ontongeny and Systematics of Fishes, Am. Soc. Ich. Herp. Special Publ. No. 1, pp. 335–354.

Cressey, R.F., Collette, B.B., 1970. Copepods and needlefishes: a study in host–parasite relationships. Fish. Bull., U.S. 63, 347–432.

Cronin, T.M., Dowsett, H.F., 1996. Biotic and oceanographic response to the Pliocene closing of the Central American Isthmus. In: Jackson, J., Budd, A., Coates, A. (Eds.), Evolution and Environment in Tropical America. University of Chicago Press, Chicago and London, pp. 76–104.

van Deursen, J., Schepens, J., Peters, W., Meijer, D., Grosveld, G., Hendriks, W., Wieringa, B., 1992. Genetic variability of the murine creatine kinase B gene locus and related pseudogenes in different inbred strains of mice. Genomics 12, 340–349.

Duque-Caro, H., 1990a. Neogene stratigraphy, paleoceanography and paleobiography in northwest South America and the evolution of the Panama Seaway. Palaeogeogr., Palaeoclim., Palaeoecol. 77, 203–234.

Duque-Caro, H., 1990b. The Choco Block in the northwestern corner of South America: structural, tectonostratigraphic, and paleogeographic implications. J. S. Am. Earth Sci. 3, 71–84.

Eastman Kodak Co., 1994. Macvector sequence analysis software, version 4.5.2.

Farris, J.S., Kallersjo, S., Kluge, A.G., Bult, C., 1995. Constructing a significance test for incongruence. Syst. Biol. 44, 570–572.

Garber, A., Winkfein, R., Dixon, G., 1990. A novel creatine kinase cDNA whose transcript shows enhanced testicular expression. Biochim. Biophys. Acta 1087, 256–258.

Graves, J.E., 1998. Molecular insights into the population structures of cosmopolitan marine fishes. J. Heredity 89, 427–437.

Haq, B.U., Hardenbol, J., Vail, P.R., 1987. Chronology of fluctuating sea levels since the Triassic. Science 235, 1156–1167.

Haug, G.H., Tiedemann, R., 1998. Effect of the formation of the Isthmus of Panama on Atlantic Ocean thermohaline circulation. Nature 393, 673–676.

Helfman, G.S., Collette, B.B., Facey, D.E., 1997. The Diversity of Fishes. Blackwell Science, USA.

Hoorn, C., 1993. Marine incursions and the influence of Andean tectonics on the Miocene depositional history of northwestern Amazonia: results of a palynostratigraphic study. Palaeogeogr., Palaeoclim., Palaeoecol. 105, 267–309.

Jordan, D.S., 1908. The law of geminate species. Am. Nat. 42, 73–80.

Keigwin Jr., L., 1978. Pliocene closing of the Isthmus of Panama, based on biostratigraphic evidence from nearby Pacific Ocean and Caribbean deep-sea cores. Geology 6, 630–634.

Kessing, B., 2000. Sequencer 5.1.1. Available from <http://nmg.si.edu/Sequencer.html>.

Kishino, H., Hasegawa, M., 1989. Evaluation of the maximum likelihood estimate of the evolutionary tree topologies from DNA sequence data, and the branching order in Hominoidea. J. Mol. Evol. 29, 170–179.

Kocher, T.D., Thomas, W.K., Meyer, A., Edwards, S.V., Pääbo, S., 1989. Dynamics of mitochondrial DNA evolution in animals: amplification and sequencing with conserved primers. Proc. Natl. Acad. Sci. USA 86, 6196–6200.

Knowlton, N., Weigt, L.A., 1998. New dates and new rates for divergence across the Isthmus of Panama. Proc. R. Soc. Lond. B 265, 2257–2263.

Knowlton, N., Weigt, L.A., Solørzano, L.A., Mills, D.K., Bermingham, E., 1993. Divergence in proteins, mitochondrial DNA, and reproductive compatibility across the Isthmus of Panama. Science 260, 1629–1632.

Lessios, H.A., Allen, G.R., Wellington, G.M., Bermingham, E., 1995. Genetic and morphological evidence that the eastern Pacific

damselfish *Abudefduf declivifrons* is distinct from *A. concolor* (Pomacentridae). Copeia 1995, 277–288.

Lessios, H.A., Kessing, B.D., Robertson, D.R., Paulay, G., 1999. Phylogeography of the pantropical sea urchin *Eucidaris* in relation to land barriers and ocean currents. Evolution 53, 806–817.

Leviton, A.R., Gibbs Jr., H., Heal, E., Dawson, C.E., 1985. Standards in herpetology and ichthyology: Part I. Standard symbolic codes for institutional resource collections in herpetology and ichthyology. Copeia 1985, 802–832.

Loder, J.W., Boicourt, W.C., Simpson, J.H., 1998. Western ocean boundary shelves, coastal segment (W). In: Robinson, A., Brink, K. (Eds.), The Sea, vol. 11. Wiley, New York, pp. 3–27.

Lopez, J.V., Yuhki, N., Masuda, R., Modi, W., O'Brien, S.J., 1994. *Numt*, a recent transfer and tandem amplification of mitochondrial DNA to the nuclear genome of the domestic cat. J. Mol. Evol. 39, 174–190.

Lovejoy, N.R., Collette, B.B., 2001. Phylogenetic relationships of New World needlefishes (Teleostei: Belonidae) and the biogeography of transitions between marine and freshwater habitats. Copeia 2001, 324–338.

Lovejoy, N.R., Bermingham, E., Martin, A.P., 1998. Marine incursions into South America. Nature 396, 421–422.

Maddison, W.P., Maddison, D.R., 1997. MacClade, version 3.07. Sinauer, Sunderland, MA.

Maniatis, T., Fritsch, E.F., Sambrook, J., 1982. Molecular Cloning. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Meyer, A., 1993. Evolution of mitochondrial DNA in fishes. In: Hochachka, P., Mommsen, (Eds.), Biochemistry and Molecular Biology of Fishes, vol. 2. Elsevier Science, Amsterdam, pp. 1–38.

Miller, R.R., 1966. Geographical distribution of Central American freshwater fishes. Copeia 1966, 773–802.

Murphy, R., Sites, J., Buth, D., Haufler, C., 1996. Proteins: isozyme electorphoresis. In: Hillis, D., Moritz, C., Mable, B. (Eds.), Molecular Systematics. Sinauer, USA, pp. 51–120.

Muss, A., Robertson, D.R., Stepien, C.A., Wirtz, P., Bowen, B.W., 2001. Phylogeography of *Ophioblennius*: the role of ocean currents and geography in reef fish evolution. Evolution 55, 561–572.

Myers, G.S., 1966. Derivation of the freshwater fish fauna of Central America. Copeia 1966, 766–773.

Néraudeau, D., 1994. Hemiasterid echinoids (Echinodermata: Spatangoida) from the Cretaceous Tethys to the present-day Mediterranean. Palaeogeogr., Palaeoclim., Palaeoecol. 110, 319–344.

Nolf, D., Stringer, G.L., 1992. Neogene paleontology in the northern Dominican Republic, 14. Otoliths of teleostean fishes. Bull. Am. Paleon. 102, 41–81.

Nolf, D., Aguilera, O., 1998. Fish otoliths from the Cantaure Formation (early Miocene of Venezuela). Bull. de L'Institut Royal des Sciences Naturelles de Belgique, Sciences de la Terre 68, 237–262.

Ortí, G., Meyer, A., 1997. The radiation of characiform fishes and the limits of resolution of mitochondrial ribosomal DNA sequences. Syst. Biol. 46, 75–100.

Ortí, G., Petry, P., Porto, J., Jégu, M., Meyer, A., 1996. Patterns of nucleotide change in mitochondrial ribosomal RNA genes and the phylogeny of piranhas. J. Mol. Evol. 42, 169–182.

Palumbi, S., 1996. Nucleic Acids II: the polymerase chain reaction. In: Hillis, D., Moritz, C., Mable, B. (Eds.), Molecular Systematics. Sinauer, USA, pp. 205–247.

Palumbi, S., Martin, A., Romano, S., McMillan, W.O., Stice, L., Grabowski, G., 1991. Simple fool's guide to PCR. ver. 2.0. University of Hawaii.

Pittman, W.C.I., Cande, S., LaBrecque, J., Pindell, J., 1993. Fragmentation of Gondwana: the separation of Africa from South America. In: Goldblatt, P. (Ed.), Biological Relationships Between Africa and South America. Yale University Press, New Haven, CO, pp. 15–34.

Posada, D., Crandall, K.A., 1998. MODELTEST: Testing the model of DNA substitution. Bioinformatics 14, 817–818.

Rambaut, A., 1995. Se-Al: Sequence Alignment Program v1.d1. University of Oxford.

von Rögl, F., 1998. Palaeogeographic considerations for Mediterranean and Paratethys Seaways (Oligocene to Miocene). Ann. Naturhist. Mus. Wien 99A, 279–310.

Rosen, D.E., 1975. A vicariance model of Caribbean biogeography. Syst. Zool. 24, 431–464.

Saghai-Maroof, M.A., Soliman, K.M., Jorgensen, R.A., Allard, R.A., 1984. Ribosomal DNA spacerlength polymorphisms in barley: Mendelian inheritance, chromosomal location, and population dynamics. Proc. Natl. Acad. Sci. USA 81, 8014–8019.

Saiki, R.K., Scharf, S., Faloona, F., Mullis, K.B., Horn, G.T., Erlich, H.A., Arnheim, N., 1985. Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. Science 230, 1350–1354.

Scheltema, R.S., 1973. Eastward and westward dispersal of tropical prosobranch larvae across the mid-Atlantic barrier. Malacologia 14, 1–409.

Scheltema, R.S., 1995. The relevance of passive dispersal for the biogeography of Caribbean mollusks. Am. Malacol. Bull. 11, 99–115.

Seutin, G., White, B.N., Boag, P.T., 1990. Preservation of avian blood and tissue samples for DNA analyses. Can. J. Zool. 69, 82–90.

Shulman, M.J., Bermingham, E., 1995. Early life histories, ocean currents, and the population genetics of Caribbean reef fishes. Evolution 49, 897–910.

Steel, M.A., 1994. Recovering a tree from the Markov leaf colorations it generates under a Markov model. Appl. Math. Lett. 7, 19–23.

Stehli, F.G., Webb, S.D. (Eds.), 1985. The Great American Biotic Interchange. Plenum Press, New York.

Strimmer, K., von Haeseler, A., 1996. Quartet Puzzling: a quartet maximum-likelihood method for reconstructing tree topologies. Mol. Biol. Evol. 13, 964–969.

Swofford, D., 1997. PAUP*: Phylogenetic Analysis Using Parsimony (and other methods), version 4.0.0d64, prerelease. Sinauer Associates, Sunderland, MA.

Swofford, D., 1999. PAUP*: Phylogenetic Analysis Using Parsimony (and other methods), version 4.0b2, beta. Sinauer Associates, Sunderland, MA.

Takezaki, N., Rzhetsky, A., Nei, M., 1995. Phylogenetic test of the molecular clock and linearized trees. Mol. Biol. Evol. 12, 823–833.

Tamura, K., Nei, M., 1993. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Mol. Biol. Evol. 10, 512–526.

Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. Clustal W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice. Nucleic Acids Res. 22, 4673–4680.

Vawter, L., Brown, W.M., 1986. Nuclear and mitochodrial DNA comparisons reveal extreme rate variation in the molecular clock. Science 234, 194–196.

Vrielynck, B., Odin, G.S., Dercourt, J., 1997. Miocene palaeogeography of the Tethys Ocean; potential global correlations in the Mediterranean. In: Montanari, A., Odin, G., Coccioni, R. (Eds.), Miocene Stratigraphy: An Integrated Approach. Elsevier Science, Amsterdam, pp. 157–165.

Waples, R.S., 1987. A multispecies approach to the analysis of gene flow in marine shore fishes. Evolution 41, 385–400.

Waples, R.S., Rosenblatt, R.H., 1987. Patterns of larval drift in southern California marine shore fishes inferred from allozyme data. Fish. Bull. 85, 1–11.

Weiller, G.F., 1998. TreeDis Version 2.0. Available from <http://life.anu.edu.au/molecular/software/tredis/>.

West, R.C., Putsy, N.P., Thom, B.G., 1969. The Tabasco lowlands of southeastern Mexico, vol. 70. Tech. Rept. Coastal Studies Institute, Lousiana State University, pp. 33–57.

Yang, Z., 1994. Estimating the pattern of nucleotide substitution. J. Mol. Evol. 39, 306–314.

Zar, J.H., 1984. Biostatistical Analysis. Prentice-Hall, Englewood Cliffs, NJ.

Zhang, D., Hewitt, G.M., 1996. Nuclear integrations: challenges for mitochondrial DNA markers. TREE 11, 247–251.

Zuckerkandl, E., Pauling, L., 1965. Evolutionary divergence and convergence in proteins. In: Bryson, V., Vogel, H. (Eds.), Evolving Genes and Proteins. Academic Press, New York, pp. 97–165.