

## AN INVESTIGATION OF THE GLOBAL POPULATION STRUCTURE OF THE MARLINSUCKER (*REMORA OSTEOCHIR*) INFERRED FROM MITOCHONDRIAL CONTROL REGION DNA SEQUENCE ANALYSIS

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### ABSTRACT

Nucleotide sequences from the hypervariable mitochondrial control region were used to investigate phylogeographic structuring in the marlinsucker, *Remora osteochir* (Cuvier, 1829). Complete DNA sequences were isolated from 71 individuals collected from seven geographically distant sample locations (5 Atlantic and 2 Pacific). Analyses of molecular variance (AMOVA) and  $\Phi_{st}$  resolved significant levels of population structuring among collections from the Atlantic and Pacific oceans, whereas negligible levels of population structuring were resolved among collections from within the Atlantic or Pacific oceans. Cluster analysis of haplotypes based upon inter-haplotype divergences resolved two distinct evolutionary lineages, one composed of haplotypes that only occurred in the Atlantic, and one composed of haplotypes that occurred in both Atlantic and Pacific samples. Observations are consistent with vicariant isolation of Atlantic and Pacific marlinsucker, followed by geologically recent secondary colonization of the Atlantic from the Indian Ocean. Congruence between patterns of genetic differentiation between marlinsucker and their istiophorid hosts, particularly blue marlin, *Makaira nigricans* Lacépède, 1802, and sailfish, *Istiophorus platypterus* (Shaw, 1792), highlights the possibility that this symbiont's phylogeography is ultimately governed by host dispersal.

The family Echeneidae (remoras) is comprised of eight putative species, which exhibit unique symbiotic interactions with a diverse group of marine organisms. Facilitated by a modified dorsal fin in the form of a transversely laminated cephalic suction disc, these fishes readily attach to an array of hosts, including bony fishes, sharks, rays, marine mammals, and reptiles. Most echeneids attach to the external surface of their hosts, although some species are known to enter the gill cavities (Cressey and Lachner, 1970). Possible benefits of this behavior include protection from predators, access to food resources (e.g., ecto-parasites, food scraps), increased reproductive chances, and free transportation (i.e., lower energy expenditure; O'Toole, 2002). It has been shown that the remoras exhibit commensal, mutualistic and potentially parasitic interactions with their marine hosts (Strasburg, 1959, 1962; Cressey and Lachner, 1970; O'Toole, 2002). Assuming negligible ill effects to the host, the remora-host association is considered commensal on the basis of the factors noted above. However, the work of Strasburg (1959, 1967) and Cressey and Lachner (1970) demonstrated that this association is often mutualistic given the relative contribution of parasitic copepods (specifically family Caligidae) to the remora's diet.

Independent of the type of symbiotic interaction, it is evident that host-association is an important behavioral element in the life history of the remoras. The relative importance of host association, and the degree of host reliance during development from larvae through adult are topics of great interest. The degree to which host ecology might affect the ecology of the symbiont is completely unknown. This last ques-

tion is particularly intriguing in the light of the work of Strasburg (1959), Cressey and Lachner (1970), and others who have demonstrated host specificity in this family. In his summary of available host/remora occurrence records published between 1959 and 1996, O'Toole (2002) reported that remoras exhibit a considerable range in both the degree of host specificity and free-swimming behavior. Specifically, the members of the subfamily Echeneiinae, which are found in a coral-reef habitat, demonstrate generalized host association patterns and elevated free-swimming behavior, particularly as adults. In contrast, the Remorinae, which are found in the pelagic environment, exhibit moderate to highly specific host association patterns, and low to moderate levels of free-swimming behavior. Collectively, these characteristics indicate that host ecology may directly influence the ecology of the symbiont. Specifically, population structuring within species of the Remorinae that exhibit a high degree of host specificity and depressed free-swimming behavior could be greatly affected, or driven by distribution and movement patterns of their hosts.

The marlinsucker, *Remora osteochir* (Cuvier, 1829), a member of the Remorinae is an ideal candidate for contrasting host and symbiont population patterns. This "pelagic specialist" preferentially associates with istiophorid billfishes and the relative importance of parasitic copepods in the diet of the marlinsucker suggests that the relationship is mutualistic (Cressey and Lachner, 1970; O'Toole, 2002). Of 495 host occurrence records, 483 (97.6%) involved istiophorid billfishes (O'Toole, 2002). The remaining 2.4% of host association records involve swordfish, *Xiphias gladius* (Linnaeus, 1758), shortfin mako shark, *Isurus oxyrinchus* (Rafinesque, 1810), wahoo, *Acanthocybium solandri* (Cuvier, 1832), dolphinfish, (*Coryphaena* sp.), ocean sunfish, *Mola mola* (Linnaeus, 1758), and fishing gear (bait, buoys). Population genetic structure in istiophorid billfishes has been noted between, and in some cases within ocean basins (reviewed in Graves and McDowell, 2003). Based upon studies utilizing allozymes, mtDNA restriction fragment length polymorphism (RFLP) patterns, mtDNA, anonymous single copy nuclear DNA (ascnDNA) sequences, and nuclear microsatellites, it has been demonstrated that both blue marlin (*Makaira nigricans*, Lacépède, 1802) and sailfish (*Istiophorus platypterus* Shaw, 1792) exhibit significant levels of genetic partitioning between Atlantic and Indo-Pacific oceans. In both blue marlin and sailfish, two distinct mtDNA evolutionary lineages have been described: one lineage that is restricted to the Atlantic, and a second "ubiquitous" lineage composed of both Atlantic and Pacific individuals. Significant heterogeneity has also been noted among sailfish collections within the Indo-Pacific (McDowell, 2002). Based upon analyses of both allozymes and mtDNA RFLP patterns (Graves and McDowell, 1994, 2003) and mitochondrial DNA sequences and nuclear microsatellites (McDowell and Graves, 2008) highly significant levels of population genetic structure within the Indo-Pacific have been inferred in striped marlin [*Kajikia audax* (Philippi, 1887)]. Given their highly specific host-association pattern and depressed level of free-swimming behavior, it is plausible that *R. osteochir* exhibit similar patterns of geographic partitioning to that of their istiophorid hosts. To date, symbioses-dependent structuring has not been explored in these (or any other) marine fishes.

To test the null hypothesis that a single, panmictic population of *R. osteochir* is distributed through Atlantic and Indo-Pacific oceans, DNA sequence analyses of the hyper-variable mitochondrial-encoded control region were performed. Intraspecific variation within and among samples collected from geographically distant locations within the Atlantic and Pacific oceans was used to infer population structure within

this species. A comparison of the population genetic patterns between hosts and symbiont provides insight into the factors potentially affecting remora phylogeography.

## MATERIALS AND METHODS

**SAMPLE COLLECTION.**—Samples ( $n = 71$ ) of marlinsucker were collected between August, 2002 and April, 2005 by pelagic longline or recreational capture of their istiophorid hosts from seven broad geographic provinces within the marlinsucker's geographic range (Fig. 1): the western North Atlantic (WNA;  $n = 6$ ), the Gulf of Mexico/Caribbean Sea (GOM;  $n = 15$ ), the western Equatorial Atlantic (WEA;  $n = 13$ ), the eastern Equatorial Atlantic (EEA;  $n = 12$ ), the central North Atlantic (CNA;  $n = 5$ ), the western South Pacific (WPAC;  $n = 8$ ), and the eastern Equatorial Pacific (EPAC;  $n = 12$ ). Due to logistic constraints, it was not feasible to sample throughout the entire marlinsucker range (e.g., Indian Ocean), although care was made to sample in as wide a range of locations as possible.

Upon capture, whole specimens were placed on ice or immediately frozen. Tissue samples for genetic analyses were taken and stored in either tissue storage buffer (Seutin et al., 1991) or 95% ethanol. Individuals were identified and photographed in the laboratory. Voucher specimens are held at the Virginia Institute of Marine Science (VIMS) or the National Museum of Natural History (USNM).

**DNA EXTRACTION AND PCR AMPLIFICATION.**—Total genomic DNA was extracted from 0.03–0.10 g skeletal and/or heart muscle following the methods of Sambrook and Russell, 2001, ethanol precipitated, and resuspended in 0.1X TE buffer, pH 8.0. Complete double-stranded nucleotide sequences from the hypervariable mitochondrial control region were amplified following standard polymerase chain reaction (PCR) methodology using *Taq* PCR Core reagents (Qiagen Corp. Valencia, CA) with published universal PCR primers DloopK (5' AGCTCAGCGCCAGAGCGC CGGTC TTGTAAA 3'; Lee et al., 1995), DloopL (5' AGTAA-GAGCCACCATCAGT 3'; Lee et al., 1995), 1CD-Loop(H1) (5' TTGGGTTTCTCGTAT-GACCG 3'; Cronin et al., 1993) and the echeeneoid specific primer DloopR1 (5' GCRGATACT-TGCATGTCTAART 3'; this study). Each 25  $\mu$ l PCR reaction contained the following: ap-

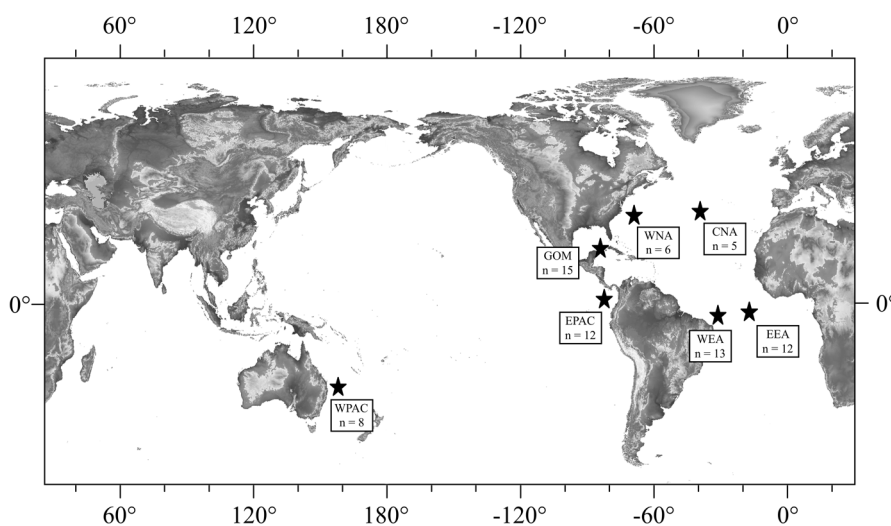


Figure 1. Collection locations and sample sizes of marlinsucker analyzed in this study. Locations include the western North Atlantic (WNA), Gulf of Mexico/Caribbean Sea (GOM), western Equatorial Atlantic (WEA), eastern Equatorial Atlantic (EEA), central North Atlantic (CNA), the western South Pacific (WPAC), and the eastern Equatorial Pacific (EPAC).

proximately 5–25 ng purified gDNA, 2.5  $\mu$ l 10X PCR reaction buffer (Tris-Cl, KCl,  $(\text{NH}_4)_2\text{SO}_4$ , 1.5 mM  $\text{MgCl}_2$ ; pH 8.7), 0.5  $\mu$ l 10 mM dNTP mix (dATP, dCTP, dGTP, dTTP, 10 mM each), 0.125  $\mu$ l *Taq* DNA polymerase (5 units  $\mu\text{l}^{-1}$ ), 0.5  $\mu$ l bovine serum albumin (BSA) (10 mg  $\text{ml}^{-1}$ ), and 10 pmoles of each primer. Negative (no DNA) control reactions were set up alongside experimental reaction mixtures to confirm that contamination via extraneous DNA did not occur. PCR amplification conditions consisted of an initial denaturation of 4 min at 94 °C, followed by 35 cycles of 1 min at 94 °C, 1 min at 50 °C, and 1.5 min at 72 °C, followed by a final extension of 5 min at 72 °C (with minor exceptions). All PCR amplifications were performed using an MJ Research PTC-200 thermocycler (Watertown, MA). PCR products were purified by column filtration (QIAquick PCR Purification, Qiagen Corp., Valencia, CA), or by using EXOSAP (USB Scientific, Cleveland, OH) prior to DNA sequencing.

Purified PCR products were sequenced in forward and reverse directions using BigDye Terminator v3.1 Cycle Sequencing reagents (Applied Biosystems, Warrington, UK) with minor modifications of the manufacturer's recommendations. Sequencing reactions were composed of 10–50 ng template DNA, 0.25  $\mu$ l sequencing primer, 0.25  $\mu$ l BigDye master mix, 1  $\mu$ l 5 $\times$  reaction mix and milli-Q water to a final volume of 5  $\mu$ l. Cycle sequencing conditions consisted of an initial denaturation of 1 min at 96 °C, followed by 25 cycles of 10 s at 96 °C, 5 s at 50 °C, and 4 min at 60 °C. Primers used for cycle sequencing were identical to primers used in original PCR amplification reactions. Amplification products were electrophoresed using an ABI 3100 or ABI 3130 DNA sequencer. Results were analyzed using Sequencing Analysis 5.1.1 software (Applied Biosystems, Warrington, UK). Standard chromatogram format (SCF) curves from multiple sequence files were created using Sequencher 3.0 (Gene Codes Corp., Ann Arbor, MI). Preliminary alignments of consensus sequences were generated using the Clustal W algorithm in MacVector 7.2 (Accelrys Inc., San Diego CA), with default parameters and adjusted by eye. Sequence characteristics including base composition and number of substitutions (as well as relative contribution by transitions, transversions, and insertion/deletions (indels) were calculated in Arlequin 2.0.4 (Schneider et al., 2000). A different haplotype designation was given to each unique DNA sequence.

**POPULATION GENETIC ANALYSES.**—Estimates of molecular diversity within each collection, including haplotype diversity ( $h$ ) and nucleotide sequence diversity ( $\pi$ ) (Nei, 1987), were calculated using Arlequin. Divergence between populations was estimated using pairwise nucleotide sequence divergence ( $\delta$ ). Nucleotide sequence divergence values were used to generate an unweighted pair group method with arithmetic mean (UPGMA) (Sneath and Snokal, 1973) tree in PAUP\* 4.0b4 (Swofford, 1999) to visualize phylogeographic patterns within this species. Sequence divergence values were used to evaluate geographic structuring of molecular variance. Hierarchical analysis of molecular variance (AMOVA) was used to partition variation between ocean basins, among collections within ocean basins, and among individuals within collections. Population pairwise  $\Phi_{st}$  values (Excoffier et al., 1992), which are analogous to Wright's F-statistics (Wright, 1978), were calculated and used as a proxy of gene flow. All analyses were performed using the Tamura-Nei model (Tamura and Nei, 1993). For AMOVA and  $\Phi_{st}$  calculations, the probability of significance was assessed using 10,000 permutations of the data. Neutrality tests were performed to infer violation from the neutral theory of molecular evolution (Kimura, 1983). Mismatch distributions were generated using the graphing function in Microsoft Excel using absolute pairwise differences between haplotypes. Demographic parameters including  $\tau$ ,  $\theta_0$ ,  $\theta_1$ , Harpending's raggedness index (Harpending et al., 1993), Tajima's D (Tajima, 1989), and Fu's  $F_s$  (Fu, 1997) were estimated in Arlequin using 10,000 replicates.

Interclade nucleotide divergence values were used to estimate time ( $T$ ) since separation and putative secondary colonization of Atlantic marlinsucker using a simple back-calculation based on nucleotide sequence divergence ( $\delta$ ) and an estimated rate of mutation ( $\mu$ ), using the equation  $T = \delta/2\mu$ . A mutation rate of 3.6% per million years, which was estimated using control region variation in amphi-Panamic geminate pairs of snook (Donaldson and Wilson, 1999), was implemented in all analyses. This mutation rate estimate falls between the com-

monly cited whole mtDNA mutation rate of 2% per million years (Brown et al., 1979), and an empirical estimate. The empirical estimate is based on the transition (Ts)/transversion (Tv) ratio of 4.3 estimated in a 300bp region encompassing the hypervariable 5' end of the marlinsucker control region, which suggests a "moderate" mutation rate (~5% per million years; McMillan and Palumbi, 1997). However, these values should be considered approximate as the estimated range of mutation in control region varies widely among bony fishes; estimates have ranged from 2.2–4.5%/MY between lineages for east African cichlids (Sato et al., 2003) to as high as 33%–100%/MY for Indo-Pacific butterflyfishes (McMillan and Palumbi, 1997).

## RESULTS

**SEQUENCING CHARACTERISTICS.**—Complete mitochondrial control region sequences were collected from 71 individuals of *R. osteochir* (GenBank accession numbers FJ348267–FJ348337), and ranged from 952 to 959 base pairs (bp) in length. A final alignment of 971 bp exhibited 223 polymorphic sites (23%), which included 160 transitions, 59 transversions, and 32 indels. The relative contribution of each nucleotide was approximately 32.9% A, 20.8% C, 13.8% G, and 32.5% T; indicating an A-T bias. Sequence variability was unequally distributed across the control region. The 5' (tRNA<sup>Phe</sup>) region contained the most polymorphism, although additional highly variable segments were spread throughout the gene region. Seventy-one unique haplotypes were observed in the 71 samples examined. The absolute number of differences between haplotypes ranged from 2 to 62 (mean = 34.34, SE = 0.26) and nucleotide sequence divergence between haplotypes ranged from 0.21 to 6.71% (mean = 3.49%, SE = 0.02; Table 1).

**POPULATION GENETIC STRUCTURE.**—High levels of haplotype ( $h$ ) and nucleotide sequence diversity ( $\pi$ ) were estimated at each of the seven collection locations (Table 1). Given that 71 unique haplotypes were found, haplotype diversity was unity at all locations. Nucleotide sequence diversity ranged from 1.95 to 3.76% (overall mean = 3.67%, SE = 0.03%). The lowest level of sequence diversity was found in the CNA collection, which coincidentally had the smallest sample size ( $n = 5$ ). The mean number of pairwise differences between individuals collected in this region was also low (18.6) compared to the other collection locations (mean = 26.3). The highest level of diversity was found in the GOM collection, in which the mean number of pairwise differences between haplotypes was 36.1. Within the Atlantic, net nucleotide sequence divergence between collection locations ranged from zero to 0.82% (GOM:CNA; Table 2). In the Pacific, average net nucleotide sequence divergence between eastern and western collection locations was –0.10%. Between Atlantic and Pacific collections, the lowest net nucleotide sequence divergence was found between CNA and WPAC (0.00%), and the highest level of divergence was found between GOM and WPAC (0.89%).

Population pairwise  $\Phi_{st}$  analyses resolved significant levels of population differentiation between Atlantic and Pacific collections, as well as one comparison within the Atlantic (Table 2). Elevated  $\Phi_{st}$  values (0.114–0.211,  $P < 0.05$ ) were estimated between all Atlantic and Pacific collections, with the exception of the CNA:WPAC and CNA:EPAC pairs, which had  $\Phi_{st}$  values that were negative and non-significant ( $P > 0.5409$ ). Pairwise  $\Phi_{st}$  values among Atlantic sampling locations ranged from –0.055 to 0.171. All values were non-significant, except between CNA and GOM, where a  $\Phi_{st}$  of 0.171 ( $P = 0.024$ ) was estimated. No evidence of structure was noted between eastern and western Pacific collections ( $\Phi_{st} = -0.025$ ,  $P = 0.800$ ).

Table 1. Genetic Diversity Indices of *Remora osteochir* collections. Bolded values are significant ( $P < 0.01$ ). Location abbreviations are as follows: western South Pacific, WPAC; eastern Equatorial Pacific, EPAC; central North Atlantic, CNA; western North Atlantic, WNA; Gulf of Mexico/Caribbean Sea, GOM; eastern Equatorial Atlantic, EEA; western Equatorial Atlantic, WEA.

Locality	No. of Samples	No. of Haplotypes (Clade I, Clade II)	Haplotype diversity (h) $\pm$ SE	Nucleotide diversity (B) $\pm$ SE	Mean no. of pairwise differences $\pm$ SE	Fu's Fs	$\tau$ (95% C.I.)	$\theta_0$ (95% C.I.)	$\theta_1$ (95% C.I.)	Harpending's s r
<b>By ocean basin</b>										
Atlantic	51	51	1.00	0.0346 $\pm$ 0.0005	33.4449 $\pm$ 0.4150	<b>-24.151</b>	44.561 (27.127–50.953)	0.011 (0–9.042)	74.879 (41.138–379.146)	0.002
Pacific	20	20	1.00	0.0273 $\pm$ 0.0010	26.2574 $\pm$ 0.8720	<b>-6.221</b>	23.204 (16.316–40.760)	7.014 (0–23.437)	163.447 (70.376–99.999.00)	0.007
<b>By clade</b>										
Clade I	24	24	1.00	0.0246 $\pm$ 0.0008	23.6058 $\pm$ 0.6475	<b>-9.313</b>	31.893 (18.498–39.252)	0.002 (0–7.497)	58.837 (33.113–473.212)	0.007
Clade II	47	47	1.00	0.0244 $\pm$ 0.0004	23.6273 $\pm$ 0.3217	<b>-24.152</b>	18.498 (13.279–32.859)	7.656 (0–25.146)	282.812 (77.265–99.999.00)	0.003
<b>By sampling location</b>										
WPAC	8	8 (0,8)	1.00	0.0244 $\pm$ 0.0026	23.3430 $\pm$ 2.1769					
EPAC	12	12 (0,12)	1.00	0.0297 $\pm$ 0.0019	28.5012 $\pm$ 1.6532					
CAN	5	5 (0,5)	1.00	0.0195 $\pm$ 0.0039	18.6392 $\pm$ 3.1627					
WNA	6	6 (3,3)	1.00	0.0358 $\pm$ 0.0054	34.2698 $\pm$ 4.5137					
GOM	15	15 (9,6)	1.00	0.0376 $\pm$ 0.0019	36.0587 $\pm$ 1.6239					
EEA	12	12 (5,7)	1.00	0.0359 $\pm$ 0.0023	34.5391 $\pm$ 1.9948					
WEA	13	13 (7,6)	1.00	0.0332 $\pm$ 0.0020	31.8297 $\pm$ 1.6836					
Overall	71	71	1.00	0.0367 $\pm$ 0.0003	26.2574 $\pm$ 0.2998					

Table 2. Genetic relationships among marlinsucker (*Remora osteochir*) collections based on mitochondrial control region sequences. Above the diagonal are corrected nucleotide sequence divergence ( $\delta$ ) values between collections, and below the diagonal are pairwise  $\Phi_{st}$  values. P-values < 0.05 are noted with one asterisk, < 0.01 with two asterisks.

Locality	CNA	WEA	EEA	GOM	WNA	EPAC	WPAC
CNA	–	0.007	0.0039	0.008*	0.004	0.000	–0.000
WEA	0.161	–	–0.001	–0.001	–0.002	0.007**	0.008**
EEA	0.084	–0.019	–	–0.000	–0.001	0.005**	0.005**
GOM	0.171*	–0.041	–0.008	–	–0.001	0.007**	0.009**
WNA	0.105	–0.055	–0.035	–0.033	–	0.004*	0.005*
EPAC	–0.009	0.180**	0.130**	0.175**	0.114*	–	–0.001
WPAC	–0.029	0.210**	0.1230*	0.205**	0.15985*	–0.025	–

Analyses of molecular variance demonstrated significant genetic heterogeneity between Atlantic and Pacific collections. When haplotypes were binned into Atlantic and Pacific groups, a highly significant proportion (14.88%) of the variance was calculated between ocean basins ( $P = 0.0001$ ; Table 3). When haplotypes were binned into collections nested within ocean basins (Atlantic  $n = 5$ , Pacific  $n = 2$ ), 14.66% of the variance was due to variation between ocean basins ( $P < 0.0001$ ). A small but significant amount of the variance (0.64%,  $P < 0.001$ ) was accounted for by differences between collections within ocean basins. However, when CNA was removed from the analysis, 18.34% ( $P < 0.0001$ ) of the variance was due to variation between ocean basins and no variance was accounted for by differences between collections within ocean basins.

CLADES.—The UPGMA tree generated from pairwise nucleotide sequence divergence values resolved two distinct lineages: one lineage (Clade I) was composed of only Atlantic specimens, whereas the other lineage (Clade II) was composed of both Atlantic and Pacific samples (Fig. 2). Clade I included 24 haplotypes and was represented in four of the five Atlantic sampling locations with the following frequencies: WNA 0.5; GOM 0.6; WEA 0.54, and EEA 0.42. No Clade I haplotypes were found in the CNA sample. Clade II included 47 haplotypes and was represented in all seven sampling locations. When all haplotypes were considered, roughly equal estimates of nucleotide sequence diversity were calculated for Clades I and II (2.46 and 2.44%,

Table 3. Analyses of molecular variance among collections of marlinsucker.

Source of variation	% variation	Fixation indices	P-value
Among oceans	14.88	$\Phi_{ST} = 0.1488$	0.0001
Within oceans	85.12		
Among oceans	14.66	$\Phi_{CT} = 0.1466$	< 0.0001
Among samples within oceans	0.64	$\Phi_{SC} = 0.0075$	0.0008
Within samples	84.7	$\Phi_{ST} = 0.14662$	0.0018
Among clades	39.66	$\Phi_{ST} = 0.3966$	< 0.0001
Within clades	60.34		
Among Clade I samples	–2.760	$\Phi_{ST} = -0.0276$	0.6745
Within Clade I samples	102.760		
Among Atlantic Clade II samples	–1.56	$\Phi_{ST} = -0.0156$	0.6445
Within Atlantic Clade II samples	101.56		
Among host populations	–2.500	$\Phi_{ST} = -0.0250$	0.9053
Within host populations	102.500		

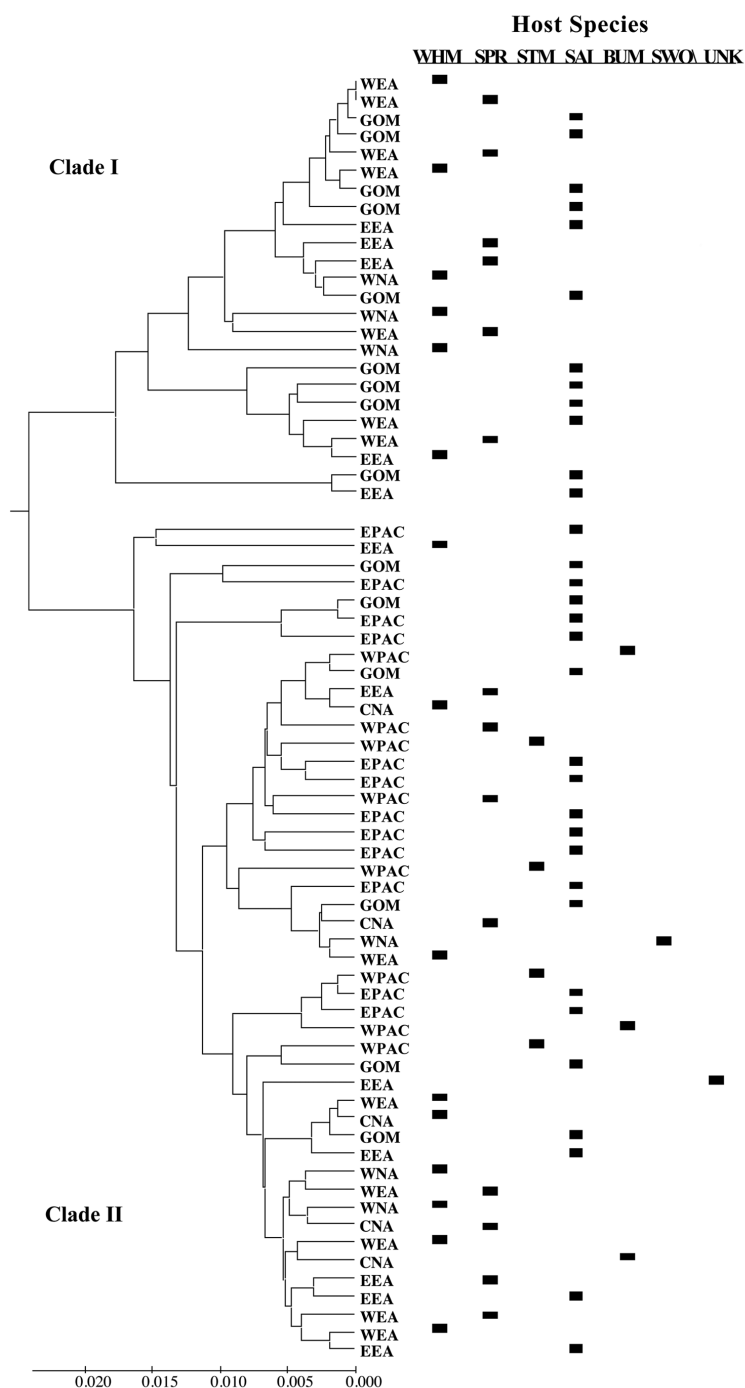


Figure 2. Unrooted UPGMA tree of complete mitochondrial control region DNA sequences amplified from marlinsucker. Hosts from which the marlinsuckers were collected, including white marlin (WHM), spearfish (SPR), striped marlin (STM), sailfish (SAI), blue marlin (BUM), swordfish (SWO), and unknown (UNK), are noted in the columns to the right of the tree. Location abbreviations are as follows: western South Pacific, WPAC; eastern Equatorial Pacific, EPAC; central North Atlantic, CNA; western North Atlantic, WNA; Gulf of Mexico/Caribbean Sea, GOM; eastern Equatorial Atlantic, EEA; western Equatorial Atlantic, WEA.

respectively). The average corrected nucleotide sequence divergence between clades was 2.30% (4.75% uncorrected), and a  $\Phi_{st}$  of 0.485 ( $P < 0.0001$ ) was estimated.

Based upon clade organization, three subgroups were defined to further evaluate the level of differentiation between haplotypes: Atlantic-I (Clade I haplotypes found in the Atlantic), Atlantic-II (Clade II haplotypes found in the Atlantic) and Pacific (Clade II haplotypes found in the Pacific). Corrected nucleotide sequence divergence between Atlantic-II:Pacific groups was estimated at 0.11%, whereas divergence between both Atlantic-I:Atlantic-II and Atlantic-I:Pacific pairs was nearly twenty times as great (2.31 and 2.35%, respectively). Within the Atlantic, neither Atlantic-I nor Atlantic-II haplotypes demonstrated significant genetic differentiation by sampling location. Considering only Atlantic-I haplotypes,  $\Phi_{st}$  values among Atlantic locations ranged from  $-0.070$  to  $0.035$  ( $P \gg 0.05$ ).  $\Phi_{st}$  values among Atlantic-II haplotypes in the Atlantic ranged from  $-0.126$  to  $0.061$  ( $P \gg 0.05$ ).

**EVOLUTIONARY HISTORY.**—Significant differences were observed in the mismatch distributions estimated from pairwise comparisons of marlinsucker haplotypes. Overall, a bimodal mismatch distribution was resolved when Atlantic and Pacific haplotypes were binned together. When binned by clade, Clade I haplotypes demonstrated a ragged, multimodal distribution, whereas Clade II haplotypes exhibited a broad unimodal distribution. Estimates of  $\tau$  differed significantly by haplotype binning strategy: when binned by ocean basin, the lowest values of  $\tau$  were estimated in the Pacific. When samples were binned by clade the lowest values of  $\tau$  were estimated in Clade II (Table 1). Large differences between  $\theta_0$  and  $\theta_1$  were noted in all combinations of marlinsucker haplotypes. Harpending's raggedness index was non-significant ( $P > 0.08$ ) for all binned haplotype classes, while Fu's  $F_s$  values were all significantly negative ( $-6.221$  to  $-24.151$ ;  $P < 0.008$ ), consistent with an ancient demographic expansion within all clades and ocean basins (Table 1). Corrected nucleotide sequence divergence ( $\delta$ ) values between Pacific and Atlantic-I haplotypes (0.02353) suggests that cladogenesis occurred approximately 0.33 million years ago. Putative recolonization was estimated to occur 16,000 yrs ago using corrected nucleotide sequence divergence ( $\delta$ ) estimates between Atlantic-II and Pacific haplotypes (0.00114).

**HOST ASSOCIATION.**—No evidence of structuring in the Atlantic was resolved when marlinsucker samples were grouped by istiophorid host (Fig. 2). Since only one blue marlin and one swordfish-marlinsucker pair were observed, only three host classes were specified (white marlin, WHM; sailfish, SAI; and spearfish, SPR). Population pairwise  $\Phi_{st}$  values in all pairwise comparisons were negative and non-significant (Table 3). Analyses of molecular variance (AMOVA) resolved 100% of the variance within collections.

## DISCUSSION

Both the level of polymorphism and the magnitude of divergence noted between haplotypes of the marlinsucker, *R. osteochir*, indicate that the control region contained sufficient genetic variation to infer population heterogeneity in this species. The mitochondrial control region of the marlinsucker displayed sequence characteristics similar to those examined in other fish taxa. The size of the non-coding region (952–959bp) is slightly larger than that noted in swordfish (835bp; Rosel and Block, 1996), bluefin tuna (820–860bp; Carlsson et al., 2004), wahoo (889–894bp; Garber et al., 2005) and sailfish (839–855bp; McDowell, 2002), but falls within the

range noted in Percidae (888–1223bp; Faber and Stepien, 1997). Consistent with the observations of Saccone et al. (1999), average nucleotide composition was biased towards adenine and thymine, which together accounted for 65.4% of the total nucleotide usage. As with the mtDNA of other fishes (reviewed in Meyer, 1993), there was a transitional bias. A transition:transversion ratio of 2.8 was estimated over the entire control region, which is consistent with observations in brook charr, *Salvelinus fontinalis* (Mitchill, 1814) (2.3; Bernatchez and Danzmann, 1993) and red drum, *Sciaenops ocellatus* (Linnaeus, 1766) (3.4; Seyoum et al., 2000). Nucleotide sequence variability was not equally distributed across the entire control region. The area of highest variability was located in the 5' (tRNA<sup>Pro</sup>) region, which is congruent with observations in swordfish (Rosel and Block, 1996; Reeb et al., 2000), white sturgeon, *Acipenser transmontanus* Richardson, 1836 (Brown et al., 1993), wahoo (Garber et al., 2005), and ninespine sticklebacks of the genus *Pungitius* (Takahashi and Goto, 2001). Overall, the level of nucleotide polymorphism (23%) across the entire marlinsucker control region is comparable to that seen in partial control region sequences analyzed from red snapper, *Lutjanus campechanus* (Poey, 1860) (22%; Garber et al., 2004), and red drum (22%; Seyoum et al., 2000) and slightly lower than the degree of polymorphism observed in swordfish (30%; Alvarado-Bremer et al., 1995) and albacore tuna (35%; Viñas et al., 2004).

All sampling locations displayed extremely high levels of haplotype diversity and moderate levels of nucleotide diversity. Analyses of control region sequences from other cosmopolitan pelagic fishes including swordfish (Alvarado-Bremer et al., 1996; Rosel and Block, 1996), bluefin tuna (Carlsson et al., 2004, 2007; Alvarado-Bremer et al., 2005), albacore tuna (Viñas et al., 2004), and wahoo (Garber et al., 2005), have also revealed high levels of haplotype diversity ( $h \geq 0.99$ , all studies). Population-wide nucleotide diversity estimates in marlinsucker (1.95–3.76% at seven sampling locations, 3.67% overall), are also consistent with observations in other species, including swordfish (3.45%; Rosel and Block, 1996), bluefin tuna (1.5% Carlsson et al., 2004; 4.3%; Alvarado-Bremer et al., 2005), albacore tuna (5.4%; Viñas et al., 2004), and wahoo (5.3%; Garber et al., 2004).

On the basis of elevated haplotype and mean nucleotide sequence diversities across all sample collections, (with the exception of the CNA collection, which shows a moderate level of nucleotide diversity) it is unlikely that either Atlantic or Pacific populations were recently colonized. High  $h$  and high  $\pi$  patterns are indicative of a long time interval since the split from a common ancestral haplotype (Hallerman, 2003).

The most intriguing result of the present study was the resolution of two distinct marlinsucker mtDNA lineages: Clade I composed of samples restricted to the Atlantic, and Clade II composed of both Atlantic and Pacific samples. These observations are congruent with observations in Atlantic populations of blue marlin (Graves and McDowell, 1995; Buonaccorsi et al., 2001), sailfish (Graves and McDowell, 1995; McDowell, 2002), bigeye tuna (Chow et al., 2000; Durand et al., 2005; Martinez et al., 2006) and swordfish (Alvarado-Bremer et al., 1996, 2005; Rosel and Block, 1996). In this study, nearly equal numbers of Clade I and Clade II haplotypes were resolved overall (24 Clade I, 27 Clade II), although a homogeneous distribution of haplotypes by sampling location was not observed. Within the Atlantic, the relative contribution of Clade II haplotypes at each sampling location ranged from 40% to 100%, although no significant geographic pattern to the haplotype distribution was found.

Potential explanations for the presence of two mitochondrial lineages in highly vagile, cosmopolitan fishes such as swordfish, billfish, and tunas have been offered by a number of authors. The most frequently used explanation in fishes (and perhaps the most logical explanation for the patterns seen in marlinsucker) involves vicariant isolation during the Pleistocene and subsequent secondary contact (Graves and McDowell, 1995; Chow et al., 2000; Buonaccorsi et al., 2001; Alvarado-Bremer et al., 2005; Durand et al., 2005; Martinez et al., 2006). Three vicariant events that occurred during the last 20 million yrs have been postulated to be responsible for the isolation between tropical and subtropical marine fishes of the Atlantic and Pacific: the closure of the Tethys Seaway (15–20 mya), the rise of the Isthmus of Panama (3.1–3.5 mya) and the development of the Benguela upwelling system off South Africa (2.0–2.5 mya) (Bermingham et al., 1997; Bowen et al., 2001; Rocha et al., 2005). Recent studies have shown that the Benguela upwelling is somewhat permeable, and that contact between Atlantic and Indian Ocean fauna has occurred since its inception (Vermeij and Rosenberg, 1993; Graves and McDowell, 1995, 2003; Chow et al., 2000; Buonaccorsi et al., 2001; McDowell, 2002; Peeters et al., 2004; Durand et al., 2005; Martinez et al., 2006). Secondary contact involving unidirectional migration across the Benguela upwelling barrier during warm interglacial periods has been used to explain the presence of two separate mtDNA lineages in a number of pelagic fishes in the Atlantic (Chow et al., 2000; Buonaccorsi et al., 2001; McDowell, 2002; Graves and McDowell, 2003; Durand et al., 2005; Martinez et al., 2006). During the last 2 million yrs, glacial-interglacial cycles have occurred on the order of every 100,000 yrs (with multiple transient heating/cooling events in between), suggestive of numerous potential inter-ocean contact periods between allopatric pelagic fishes of the Atlantic and Pacific (Fennel, 1999; Petit et al., 1999; Peeters et al., 2004; Rocha et al., 2005).<sup>-</sup>

Results of the present study support the theory that Atlantic marlinsucker were isolated from their Pacific conspecifics, and subsequently brought back into contact in the relatively recent geological past. Furthermore, these results support the hypothesis that the Atlantic population was secondarily colonized by Indo-Pacific individuals, although it is unclear when these event(s) occurred. This hypothesis is supported by several lines of evidence. Overall, the level of genetic diversity estimated in the Atlantic collections ( $\pi = 0.035$ ) was substantially greater than that estimated in the Pacific collections ( $\pi = 0.027$ ). Two significantly different mitochondrial lineages (Clades I and II) were resolved in the Atlantic ( $\Phi_{st} = 0.485$ ,  $P < 0.0001$ ), only one of which is found in the Pacific. The level of divergence between collections of Atlantic-II (putative Atlantic recolonists) and Pacific haplotypes ( $\delta = 0.11\%$ ,  $P = 0.0055$ ;  $\Phi_{st} = 0.044$ ,  $P = 0.0041$ ) was significantly lower than that measured between collections of Atlantic-I and Pacific haplotypes ( $\delta = 2.35\%$ ,  $P < 0.0001$ ;  $\Phi_{st} = 0.472$ ,  $P < 0.0001$ ). Furthermore, the level of divergence between collections of Atlantic-II and Pacific haplotypes was significantly more than the level of divergence resolved among sampling locations in the Pacific ( $\delta = -0.01\%$ ,  $P = 0.726$ ;  $\Phi_{st} = -0.025$ ,  $P = 0.800$ ). In addition,  $\tau$ ,  $\theta_0$  and  $\theta_1$  values suggest that clade II has undergone a greater demographic expansion, consistent with the secondary colonization, although the confidence intervals for the  $\tau$  values are overlapping (i.e., which clade is older cannot be ascertained with certainty).

Patterns of genetic differentiation between samples of marlinsucker collected from seven locations in the Atlantic and Pacific indicate that marlinsucker exhibit significant levels of population structuring between ocean basins, but negligible geograph-

ic heterogeneity within ocean basins. Both population pairwise fixation indices and hierarchical analyses of variance indicate a significant level of genetic differentiation between marlinsucker collections from different ocean basins.  $\Phi_{st}$  estimates between Atlantic and Pacific ranged from 0.1137 to 0.2105 ( $P < 0.05$ ), with the exception of pairwise comparisons involving CNA. These results are congruent with AMOVA analyses, which estimate a highly significant portion of the total genetic variance partitioned between collections from different ocean basins. When haplotypes were grouped by ocean basin (two collections), 14.88% of the total variance was partitioned between ocean basins. When haplotypes were binned by sampling location within ocean basins (seven collections), 14.66% of the total variance was partitioned between ocean basins indicating that inter-ocean divergence is almost entirely characterized by the relative frequency of the two main clades. These results are consistent with observations in several other cosmopolitan pelagic fishes (Graves and McDowell, 1995; Alvarado-Bremer et al., 1996, 2005; Rosel and Block, 1996; Chow et al., 2000; Buonaccorsi et al., 2001; McDowell, 2002; Durand et al., 2005; Martinez et al., 2006). On the basis of DNA sequence variability in a 300bp fragment of the control region, Rosel and Block (1996) estimated a  $\Phi_{st}$  of 0.153 between Atlantic and Pacific collections of swordfish and AMOVA analyses partitioned 15.6% of the total variance between collections. Using RFLP analysis of whole molecule mitochondrial DNA, McDowell (2002) demonstrated significant levels of genetic partitioning between sailfish collected from different ocean basins. When haplotypes were binned by ocean basin, 32.53% of the total variance was partitioned between samples from different ocean basins, corresponding to an  $\Phi_{st}$  estimate of 0.325. In agreement with the work of Graves and McDowell (1995), Alvarado-Bremer et al. (1996, 2005); Rosel and Block (1996), Chow et al. (2000), Buonaccorsi et al. (2001), McDowell (2002), Durand et al. (2005), and Martinez et al. (2006), the levels of interocean divergence estimated between Atlantic and Pacific marlinsucker samples suggest a significant barrier to contemporary gene flow between ocean basins.

No evidence of geographic structuring was found in marlinsucker collections within the Pacific Ocean. The  $\Phi_{st}$  estimate between eastern and western Pacific collections was negative and non-significant ( $P = 0.7998$ ), consistent with basin-wide genetic homogeneity, although increased sampling effort may alter this conclusion. These results parallel observations in blue marlin, where negligible levels of structuring were resolved within the Pacific using cytochrome *b* DNA sequencing (Finnerty and Block, 1992), allozyme and anonymous single copy nuclear DNA (ascnDNA; Buonaccorsi et al., 1999) and whole mtDNA restriction fragment length polymorphism (RFLP) analyses (Buonaccorsi et al., 2001). In contrast, the results of the present study differ from the observations made in a number of other pelagic fishes with which marlinsucker associate. Significant levels of population structuring within the Pacific Ocean have been described in striped marlin (Graves and McDowell, 1995, 2003; McDowell and Graves, 2008), sailfish (McDowell, 2002), and swordfish (Reeb et al., 2000). Although the results of the present study suggest that a significant amount of gene flow occurs between distant sampling locations, further sampling is required to evaluate these observations with greater statistical power. In this study, samples were collected from only two sampling locations in the Pacific Ocean (eastern Australia and southwestern Panama) and totaled 8 and 12 individuals, respectively. A broader geographic sampling regimen with increased sample sizes will be required to reevaluate the levels of differentiation measured.

The interpretation of the genetic relationships among geographically distant collections in the Atlantic was less clear than that of the Pacific. When Atlantic marlinsucker haplotypes were analyzed by sampling location,  $\Phi_{st}$  estimates in all intra-ocean comparisons except those involving CNA were non significant ( $P > 0.0762$ ), suggesting a lack of genetic structuring between sampling locations. A highly significant amount of genetic differentiation was observed between CNA and GOM marlinsucker collections ( $\Phi_{st} = 0.1707$ ,  $P = 0.0243$ ); however, this comparison was not significant after correction for multiple tests and is most likely a product of the small sample size in the CNA collection ( $n = 5$ ). As such, the interpretation of these data could be flawed due to type 1 error.

To further evaluate intraocean substructuring, AMOVA and  $\Phi_{st}$  analyses were performed by analyzing Atlantic-I and Atlantic-II haplotypes by sampling location. Results of these tests indicate a lack of intraocean structure:  $\Phi_{st}$  values between all Atlantic sampling locations were not significant for either Clade I or Clade II samples; 100% of the variance was partitioned within collections for both Clade I and Clade II haplotypes. These observations do not reject the hypothesis of panmixia, and indicate that a significant amount of gene flow exists among Atlantic sampling locations.

Estimates of cladogenesis and putative recolonization of the Atlantic by Indo-Pacific marlinsucker are similar to those reported for other pelagic fishes exhibiting this intriguing two-clade mtDNA pattern (Buonaccorsi et al., 2001; McDowell, 2002; Graves and McDowell, 2003; Alvarado-Bremer et al., 2005; Martinez et al., 2006). In the present study, cladogenesis was estimated to have occurred between 0.33 and 0.46 mya. These values are roughly consistent with divergence time estimates between Atlantic and Pacific stocks of blue marlin (0.6 million yrs; Graves and McDowell, 1995; Buonaccorsi et al., 2001), sailfish (0.37–0.66 million yrs; McDowell, 2002), big-eye tuna (0.32–0.43 million yrs; Martinez et al., 2006) and swordfish (0.7–3.0 million yrs; Alvarado-Bremer et al., 2005). These results suggest that contact between Atlantic and Pacific marlinsucker persisted long after the rise of the Isthmus of Panama and the development of the Benguela upwelling system. Based upon  $\tau$  estimates from Atlantic-II haplotypes and the level of divergence measured between Atlantic-II and Pacific haplotypes (0.11%,  $P = 0.0055$ ), the putative recolonization of the Atlantic by Indo-Pacific marlinsuckers could have occurred between 16,000 and 220,000 yrs before present. Although coalescent and divergence-based estimates disagree upon a definitive point in time, these results suggest that Atlantic and Pacific marlinsucker stocks were separated for a period of at least 100,000 yrs prior to secondary contact. Furthermore, the most recent contact between Atlantic and Pacific stocks could have occurred near the end of the Pleistocene. It should be noted, however, that the conversion of genetic distances into geologic time should be approached with caution. Mutation rate heterogeneity has been described at the nucleotide position, gene region, gene, and organismal level (reviewed in Hillis et al., 1996; Avise, 1994) making this methodology somewhat contentious. A more conservative approach would be to use relative, rather than absolute distance. As such, it would appear that secondary colonization occurred in the “relatively recent” geological past. A low, but highly significant level of fixation ( $\Phi_{st} = 0.044$ ,  $P = 0.004$ ) between Atlantic-II and Pacific samples suggests that sufficient time has passed for genetic divergence to develop between these samples. As such, it appears that contemporary gene flow between

Atlantic and Pacific marlinsucker is not occurring, a situation that is similar to that of Atlantic and Indo-Pacific populations of blue marlin and sailfish.

**HOST-SYMBIONT PHYLOGEOGRAPHY.**—The intimate association between marlinsucker and the fishes of the family Istiophoridae has important implications to the ecology of *R. osteochir*. Barriers to genetic homogenization that commonly affect free living (i.e., not host-associating) marine fishes, are likely circumvented by attaching to istiophorids. Tagging data demonstrate that a number of istiophorids are capable of long-distance migration, including cross-ocean excursions (Ortiz et al., 2003). These observations support the hypothesis that barriers to dispersion, such as the eastern Pacific barrier, may not be significant obstacles to the marlinsucker.

Localized spawning and recruitment, which promotes genetic isolation, also does not appear to be a controlling factor in the population structuring of the marlinsucker. Ripe male and female marlinsucker pairs have been observed cohabitating on the same istiophorid host (Cressey and Lachner, 1970; Collette, pers. obs.; Gray, pers. obs.), suggesting the potential for repeated spawning events between cohabitating fishes, and a wide distribution area for their progeny given the highly migratory nature of their hosts. In this study, structuring was not revealed in the Atlantic when marlinsucker were grouped by istiophorid host. This suggests that while marlinsucker selectively associate with istiophorid billfishes generally, they are not selective of a particular species of billfish, at least in the Atlantic. The combination of these factors provides a strong homogenization force that potentially reduces (or prevents) intra-ocean population structuring in the marlinsucker.

Given the stochastic nature of dispersal in cosmopolitan marine fishes (Graves, 1998), it seems plausible that at a maximum, the levels of population structure observed in marlinsucker should reflect the level of structure of the least structured host. Patterns of interocean genetic differentiation observed in marlinsucker are strikingly similar to patterns observed in blue marlin and sailfish (Graves and McDowell, 1995, 2003; Buonaccorsi et al., 2001; McDowell, 2002), and include significant levels of genetic divergence and the presence of two mitochondrial lineages in the Atlantic Ocean. Consistent with observations in white marlin (Graves and McDowell, 2001, 2006), sailfish (McDowell, 2002) and blue marlin (Buonaccorsi et al., 2001), no evidence of intraocean structuring was resolved among Atlantic collections of marlinsucker. Consistent with observations in black marlin (Faltermann, 1999) and blue marlin (Buonaccorsi et al., 1999, 2001), and in contrast to observations in sailfish (McDowell, 2002) and striped marlin (McDowell and Graves, 2008), no evidence of population structuring was resolved among collections in the Pacific. Overall, the patterns of genetic differentiation in marlinsucker most closely resemble the patterns seen in blue marlin, the istiophorid species that exhibits the least amount of intraocean structure (while still occurring in both oceans).

Results of the present study demonstrate clear similarities between host and symbiont phylogeography. It is unclear, however, whether the observed phylogeographic patterns in marlinsucker are a direct result of host ecology, or whether the congruent patterns are the result of parallel evolution that occurred in response to past vicariant events. On one hand, the congruent patterns of genetic differentiation between these species highlight the possible effect that tight linkage between host and symbiont may have upon the geographical distribution of the symbiont. As such, these patterns potentially demonstrate how host natural history may directly affect the evolutionary trajectory of the symbiont. Morphological evidence supports this theo-

ry: the marlinsucker is engineered for host association, not free swimming behavior. The disc in marlinsucker may command up to 40% of the total length and may extend beyond the maximum body width, implying that this species is well equipped to remain attached to highly mobile pelagic hosts such as the istiophorids. Furthermore, given their general body design, marlinsucker are more suited for burst swimming, rather than the continuous, high-speed swimming behavior exhibited by their hosts. Alternatively, since correlation does not denote causation, the congruent phylogeographic patterns between host and symbiont may simply indicate parallels between two divergent groups of pelagic fishes that have been influenced by similar events and processes in the distant past. Most likely however, remora phylogeography is ultimately a product of both of these factors. The patterns of genetic differentiation observed in marlinsucker are likely a result of both host-dependent effects (e.g., habitat preference, migration patterns) as well as parallel evolutionary forces (e.g., vicariant isolation, selection, genetic drift) affecting both the host and symbiont.

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