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Historical biogeography and speciation in the reef fish genus *Haemulon* (Teleostei: Haemulidae)

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ABSTRACT

The high biodiversity of tropical marine hotspots has long intrigued evolutionary biologists and biogeographers. The genus *Haemulon* (grunts) is one of the most important (numerically, ecologically, and economically) reef fish groups in the New World and an excellent candidate to test hypotheses of speciation and diversity generation in the Greater Caribbean, the richest Atlantic biodiversity hotspot, as well as the eastern Pacific. To elucidate the phylogenetic relationships among the species of *Haemulon*, we obtained a combined total of 2639 base pairs from two mitochondrial genes (cytochrome *b* and cytochrome oxidase I), and two nuclear genes (TMO-4C4 and RAG2) from all nominal species. Parsimony, Maximum likelihood, and Bayesian analyses resulted in a well-resolved phylogeny with almost identical topologies. Previous phylogenetic hypotheses based on adult morphology, such as the close relationship among *H. aurolineatum, H. boschmae*, and *H. striatum* were not supported, whereas others using developmental characters, such as the relationship between *H. plumieri* and *H. sciurus*, were confirmed. Our data also indicate that the populations of the nominal *H. steindachneri* from the two sides of the Isthmus of Panama are genetically divergent at the species level in each ocean, and that the boga, *Inermia vittata* (family Inermiidae), belongs in the genus *Haemulon*. This evidence implies that there are 21 valid species of *Haemulon*, two more than previously recognized. The Amazon barrier and the Isthmus of Panama seem to have played roles in allopatric speciation of *Haemulon*. However, the majority of sister species pairs have completely overlapping distributions, indicating that vicariance is not the only process driving speciation in this genus. We conclude that both vicariance between biogeographic provinces, and ecological mechanisms of speciation within provinces contribute to species richness in the genus *Haemulon*.

1. Introduction

Harboring more than 800 species of fishes, the Greater Caribbean is the most diverse marine biogeographic province of the New World tropics (Briggs, 1974; Rocha, 2003; Floeter et al., 2008). Two major vicariant events have influenced the Greater Caribbean marine fauna in the last 10 million years (Myr). First, the late Miocene establishment of the Amazon river outflow effectively separated the shallow water fauna of the Caribbean from that further south in Brazil (Rocha, 2003; and references therein). Second, the Pliocene rise of the Isthmus of Panama, approximately 3.1 Myr ago (Coates and Obando, 1996) is the geological event responsible for major changes in the New World tropical fauna, not only because it separated the Caribbean and tropical eastern Pacific marine faunas, but also because it brought drastic environmental and oceanographic changes to the region (Jackson et al., 1997; O'Dea et al., 2007). Those vicariant events, combined with the large environmental variation along the New World tropics and the relative simplicity of the Atlantic Ocean (compared to the much more complex Indo-Pacific), make this an ideal area to study evolution and speciation in the marine realm.

The genus *Haemulon* (family Haemulidae) represents an excellent group to study evolution and speciation in the New World tropics. It contains 19 nominal species distributed throughout the tropical eastern Pacific and western Atlantic. The evolutionary history of these species was likely influenced by both the Isthmus of Panama and the Amazon barrier. Because they attain relatively large size and their adult stages are easy to identify visually, their alpha taxonomy is well resolved. Some species live in mid-water and feed on plankton while most live near the bottom and feed on macrobenthic organisms after daily feeding migrations from the reef to soft bottom and vegetated communities (Parrish, 1989). *Haemulon* species display high

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morphological diversity as adults (e.g., *H. macrostomum* and *H. striatum*), yet share extremely similar early developmental stages (Courtenay, 1961) that are often visually separable only by use of complex differences in pigmentation and squamation patterns (Hong, 1977; Lindeman and Richards, 2005). Fishes of this genus numerically dominate shallow tropical reefs in the Caribbean and eastern Pacific (schools reaching the thousands), and comprise one of the most important reef fishes in the region due to their abundance, fishery value, and trophic importance as predators and prey (Meyer et al., 1983; Lindeman and Toxey, 2002; Ferreira et al., 2004).

Fifteen species of *Haemulon* are found in the western Atlantic, and five in the eastern Pacific with one nominally shared by both regions (Lindeman, 1986; Rocha and Rosa, 1999). A variety of hypotheses have been proposed regarding relationships among some groups of species within the genus, most based on general body shape and ecological preferences. These hypotheses placed planktivore species with slender bodies together, with benthic feeding deep-bodied species in other groups (Courtenay, 1961, 1965; Davis, 1967; Lindeman, 1984). However, despite their abundance, economic importance, trophic significance, and ecomorphological diversification, no comprehensive phylogenetic analysis of the genus has been conducted.

Here we present and discuss the results of a phylogenetic analysis of all 19 nominal species of the genus *Haemulon* based on two mitochondrial (cytochrome *b* and cytochrome oxidase I) and two nuclear (TMO 4c4 and recombination-activating gene 2) genes. Specifically, we address the following questions: How did the Amazon and the Isthmus of Panama barriers influence speciation in *Haemulon*? Is the geminate species status of three pairs of *Haemulon* separated by the Isthmus of Panama (Jordan, 1908; Thomson et al., 1979) supported by molecular data? Are the previous morphological hypotheses of natural groups within *Haemulon* (Courtenay, 1961, 1965; Davis, 1967; Lindeman, 1984) supported by molecular data? What can new information on the complex phylogeny and biogeography of *Haemulon* contribute to the sympatric versus allopatric speciation debate?

2. Materials and methods

2.1. Specimen collection

Specimens of all 19 nominal species of the genus *Haemulon* (four tropical and subtropical eastern Pacific species, 14 tropical western Atlantic species and one species that occurs in both oceans) were collected while scuba diving or snorkeling using a pole-spear, or obtained from colleagues (Table 1). Due to overall morphological similarity to *Haemulon*, the boga (*Inermia vittata*, family Inermiidae) was also included in the analysis. Four species of the genus *Anisotremus* were included: the Atlantic *A. surinamensis* and *A. virginicus*, and the Pacific *A. interruptus* and *A. taeniatus*. Those four species are closely related to *Haemulon* (L. Rocha, M. Santiago, and K. Carpenter, unpublished) and thus comprise an appropriate outgroup. Gill, muscle, and/or fin tissue were preserved in the field in 1.5 ml tubes containing either a saturated DMSO solution or 95% ethanol.

At least two (but as many as 10) specimens usually from geographically separated locations were sequenced for each species investigated. Voucher specimens were deposited at the Florida Museum of Natural History (UF), the Smithsonian Institution (USNM), or the Scripps Institute of Oceanography (SIO) fish collections. When the maintenance of voucher specimens was not possible, photographs of the specimens (Table 1) were archived and are available upon request.

2.2. DNA extraction, PCR amplification, and sequencing

Total genomic DNA was extracted from tissue samples using the Qiagen DNeasy tissue kit following the manufacturer's protocol. Segments from two mitochondrial DNA (mtDNA) genes, cytochrome *b* (CytB) and cytochrome oxidase I (COI), and two nuclear genes, TMO 4c4 and recombination-activating gene 2 (RAG2) were amplified for all species. Primers used for amplification and sequencing are listed in Table 2. Each PCR had a total volume of 25 μl, containing between 1 and 2 μl (10–20 ng) of purified DNA, 2.5 μl of 10× reaction buffer, 1.5 μl of 8 mM pre-mixed dNTPs, 2.5 mM of $MgCl_2$, 0.25 μM of each primer and one unit of *Taq* DNA polymerase (Promega, Madison, WI). Cycling parameters for the mtDNA were as follows: initial denaturation at 94 °C for 2 min; 35 cycles of 94 °C for 45 s, 50 °C for 45 s, 72 °C for 55 s; and a final extension at 72 °C for 2.5 min. Cycling for amplification of the TMO gene was identical to the mtDNA except for a 54 °C annealing temperature. The RAG2 gene was amplified by a PCR consisting of an initial denaturation at 94 °C for 2 min followed by two sets of cycles: first, nine cycles of 94 °C for 45 s, 56 °C for 45 s, 72 °C for 55 s; second, 29 cycles of 94 °C for 45 s, 53 °C for 45 s, 72 °C for 1 min 45 s; and a final extension at 72 °C for 5 min. Excess oligonucleotide primers in all cases were removed through simultaneous incubation of PCR product with exonuclease I and shrimp alkaline phosphatase (USB Corp., Cleveland, OH).

Double stranded cycle sequencing reactions were conducted using dye-labeled terminators (ABI Prism technology) and the amplification primers. The resulting products were separated on an ABI 3700 DNA sequencer (Applied Biosystems, Inc., Foster City, CA) in DNA sequencing facilities at the Smithsonian Tropical Research Institute and the University of Hawaii. All samples were sequenced in the forward and reverse directions to verify nucleotide designations.

2.3. Phylogenetic analyses

Sequences were trimmed to the size of the smallest fragment to maintain consistency of the data, and aligned using the software Sequencher 4.6 (Gene Codes). No indels were detected. All sequences are deposited in GenBank (Table 1). Modeltest 3.06 (Posada and Crandall, 1998) was used to determine which model of DNA evolution best fit the data based on the Akaike Information Criterion, which selected the Tamura and Nei (1993) model, with a proportion of invariable sites of 0.657 and a gamma distribution with a shape parameter $\alpha = 2.196$ (TrN + I + G) for the mtDNA dataset; the model chosen for the nuclear DNA dataset was Kimura (1980) 2-parameter genetic distance, with a gamma distribution and $\alpha = 0.124$ (K80 + G).

The utility of the mtDNA sequences for deeper phylogenetic branches was evaluated by means of a saturation analysis: the number of substitutions (transitions, transversions and the three codon positions) was plotted against the corrected sequence divergence (model suggested by Modeltest). When saturation is present the plotted curve flattens with increasing genetic distance. A partition homogeneity (congruence) test was carried out on PAUP* 4.0b10 (Swofford, 2002) to determine if the mtDNA and nDNA genes can be combined in the phylogenetic analysis. The test indicated significant heterogeneity ($p = 0.04$) in the dataset including all taxa, but when *Haemulon bonariense* was excluded, the combination of the mtDNA and nDNA datasets was homogeneous ($p = 0.91$); consequently, this species was excluded in all analyses of combined data (see Section 3).

Bayesian phylogenetic analyses were performed with MrBayes 3.1 (Ronquist and Huelsenbeck, 2003). The two gene partitions (mtDNA and nDNA) were assigned separate parameters for models of sequence evolution chosen with Modeltest, except for the

Table 1
Locality, sample sizes (*N*), voucher information (when available), and GenBank accession numbers for sequences used in the analyses

Species	Locality (*N*)	Voucher	GenBank Accession No.
Atlantic species			
Haemulon album	Venezuela (2)	Photo	EU697498, EU697523, EU697549, EU697573
Haemulon aurolineatum	Brazil (2)	Photo	EU697502, EU697528, EU697553, EU697578
	Florida (3)	N/A	
	St. Croix (2)	Photo	
Haemulon bonariense	Venezuela (6)	Photo	EU697522, EU697529, EU697554, EU697579
Haemulon boschmae	Venezuela (3)	UF 160777	EU697503, EU697530, EU697555, EU697580
Haemulon carbonarium	Bahamas (3)	N/A	EU697504, EU697531, EU697556, EU697581
	Bermuda (2)	UF 119735	
	St. Croix (2)	Photo	
Haemulon chrysargyreum	St. Croix (2)	UF 160070	EU697505, EU697532, EU697557, EU697582
	Venezuela (2)	Photo	
Haemulon flavolineatum	Bahamas (5)	Photo	EU697507, EU697534, EU697559, EU697584
	Belize (2)	Photo	
	St. Croix (3)	UF 159904	
Haemulon macrostomum	Belize (3)	Photo	EU697509, EU697536, EU697561, EU697586
Haemulon melanurum	St. Croix (2)	UF 160119	EU697511, EU697538, EU697563, EU697588
	Venezuela (2)	Photo	
Haemulon parra	Belize (2)	Photo	EU697512, EU697539, EU697564, EU697589
	Venezuela (2)	Photo	
Haemulon plumieri	Bahamas (2)	N/A	EU697513, EU697540, EU697565, EU697590
	Brazil (2)	Photo	
	St. Croix (3)	UF 160128	
Haemulon sciurus	Belize (2)	Photo	EU697514, EU697541, EU697566, EU697591
	Bermuda (2)	Photo	
Haemulon squamipinna	Brazil (4)	USNM 342004	EU697517, EU697544, EU697569, EU697594
Haemulon steindachneri	Brazil (3)	Photo	EU697518, EU697545
	Venezuela (6)	UF 160239	EU697520, EU697547, EU697570, EU697595
Haemulon striatum	Panama (3)	UF 146644	
Inermia vittata	Belize (5)	USNM 349224	EU697508, EU697535, EU697560, EU697585
Pacific species			
Haemulon flaviguttatum	Panama (3)	Photo	EU697506, EU697533, EU697558, EU697583
	Mexico (4)	Photo	
Haemulon maculicauda	Panama (3)	Photo	EU697510, EU697537, EU697562, EU697587
Haemulon scudderi	Galapagos (3)	N/A	EU697515, EU697542, EU697567, EU697592
	Panama (3)	Photo	
Haemulon sexfasciatum	Mexico (3)	SIO 03-115	EU697516, EU697543, EU697568, EU697593
Haemulon steindachneri	Panama (3)	Photo	EU697519, EU697546, EU697571, EU697596
	Mexico (3)	Photo	
Outgroup taxa			
Anisotremus interruptus	Panama (2)	Photo	EU697499, EU697525, EU697550, EU697574
Anisotremus surinamensis	Florida (2)	Photo	EU697500, EU697526, EU697575
Anisotremus virginicus	Florida (2)	Photo	EU697524, EU697552, EU697577
Anisotremus taeniops	Panama (2)	Photo	EU697501, EU697527, EU697551, EU697576

The four accession numbers correspond to the CytB, COI, TMO-4C4, and RAG2 sequences, respectively.

Table 2
Primers used for amplification and sequencing (* indicates slight modification from the sequence in the original publication)

Gene	Primer name	Primer sequence 5′–3′	Reference
Cytochrome *b*	L14725	GTG ACT TGA AAA ACC ACC GTT G	Meyer (1993)
	H15573	AAT AGG AAG TAT CAT TCG GGT TTG ATG	Meyer (1993)
COI	BOL-F1	TCA ACY AAT CAY AAA GAT ATY GGC AC	*Ward et al. (2005)
	BOL-R1	ACT TCY GGG TGR CCR AAR AAT CA	*Ward et al. (2005)
TMO-4c4	TMO-F1	GAA AAG AGT GTT TGA AAA TGA	Streelman and Karl (1997)
	TMO-R1	CAT CGT GCT CCT GGG TGA CAA AGT	Streelman and Karl (1997)
RAG2	RAG2-F1	GAG GGC CAT CTC CTT CTC CAA	Lovejoy (1999)
	RAG2-R3	GAT GGC CTT CCC TCT GTG GGT AC	Lovejoy (1999)

proportion of invariant sites and gamma, which were estimated during the run. Preliminary runs were performed to monitor the fluctuating value of the posterior probabilities of the Bayesian trees. Preliminary runs were performed to monitor the fluctuating value of the likelihoods of the Bayesian trees, and all parameters appeared to reach stationarity before 250,000 generations (log-likelihood scores of sample points against generation time were plotted using Microsoft Excel and stationarity was achieved when the log-likelihood values of the sample points reached a stable equilibrium value). The Markov chain analysis was run for 20 million generations, sampling trees every 100 generations. To allow an additional range of security, we have chosen to run the analyses employing six simultaneous chains that started with a random tree. A burn-in period, in which the initial 10,000 trees were discarded, was adopted and the remaining tree samples were used to generate a 50% majority-rule consensus tree.

The posterior probability of each clade is provided by the percentage of trees identifying the clade; posterior probabilities equal

to and above 0.95 are considered significant supports (Huelsenbeck and Ronquist, 2001). In addition, PAUP* was used to conduct maximum parsimony (MP) and maximum likelihood (ML) phylogenetic analyses. Trees were constructed through heuristic searches using tree bisection-reconnection (TBR) branch swapping, and their support was estimated by 10,000 (MP) and 500 (ML) bootstrap replicates using default settings in PAUP*. Equal weight was given to all sites.

3. Results

3.1. Sequence data and divergences

Mitochondrial and nuclear DNA sequences were obtained for all nominal species of the genus *Haemulon*, and in most cases from locations across the species range (Table 1). In most cases, no variation or only single point mutations were detected among populations within species and a consensus sequence was generated to represent the species, except for *H. steindachneri* (Brazilian, Caribbean, and eastern Pacific populations were analyzed separately as this was the only species that showed high intra-specific variation).

The mtDNA dataset consists of 1428 bp, corresponding to the combination of 750 bp of the CytB gene and 678 bp of the COI gene. The nuclear DNA dataset consists of 1211 bp, corresponding to the combination of 503 bp of the TMO 4c4 protein coding region and 708 bp of the RAG2 gene. Overall transition to transversion ratio (Ti/Tv) in the mtDNA was 3.29 and overall base frequencies were 22% A, 32% C, 18% G, and 28% T. Nuclear DNA overall Ti/Tv ratio was 2.72 and base frequencies were 26% A, 26% C, 24% G, and 24% T. Double peaks at some positions suggested that a few sites in the nDNA sequences were heterozygous. These positions were coded with the IUB letter corresponding to the two-base ambiguity, and no three of four base peaks were observed. Saturation in transitions and third codon positions was observed in CytB, but that was attributable to the differences between *Haemulon* and the outgroup, *Anisotremus*. When the outgroup was removed from the CytB dataset, no saturation was observed. COI showed no evidence of saturation and may be useful in family level phylogenies of this group; nDNA also did not show any signs of saturation (graphs not shown but available from LAR upon request).

Corrected sequence divergences between sister species were 2.1–14.4% in the mtDNA and 0.16–1.2% in the nDNA (Fig. 1 and Table 3); average distances to outgroup were 44.5% in the mtDNA and 4.2% in the nDNA, and maximum ingroup distances were 36.5% and 5.3% of mtDNA and nDNA, respectively. The only portion of the tree where there was significant conflict between the mtDNA and nDNA was the position of the Caribbean species *H. bonariense* (Fig. 1). The mtDNA of this species is almost identical to that of *H. parra* but its nDNA places it in a well supported group also composed of the eastern Pacific *H. scudderi* and *H. sexfasciatum*. This indicates a transfer of mtDNA (introgression) from *H. parra* to *H. bonariense* probably via a recent hybridization event. Due to this indication of hybridization, *H. bonariense* was excluded from the Bayesian phylogenetic analyses of the combined mitochondrial and nDNA datasets.

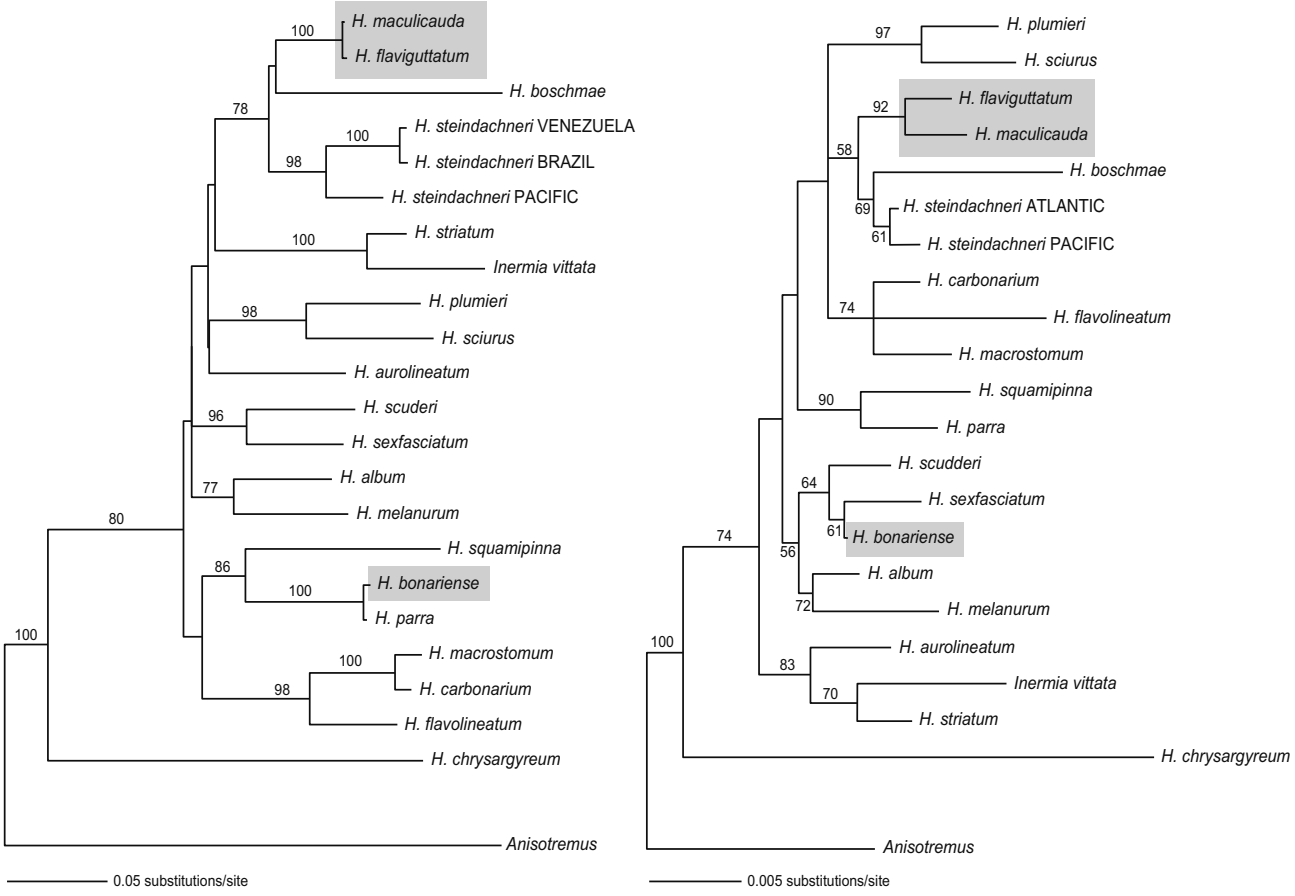

Fig. 1. Maximum likelihood analysis of the mtDNA (left; cytochrome *b* and COI) and nuclear DNA (right; RAG2 and TMO-4C4) datasets for all nominal species of the genus *Haemulon*. Numbers on branches refer to the bootstrap support calculated from 500 replicates assuming model parameters values estimated from Modeltest (see Section 2). The two instances where mtDNA introgression is suspected (see text) are identified by gray shading.

Table 3
Genetic divergences between sister species of *Haemulon* on two mitochondrial and two nuclear genes

Species pairs	Uncorrected *p* distance				Corrected distance			
	CytB	COI	RAG2	Tmo	CytB	COI	RAG2	Tmo
H. steindachneri P/*H. steindachneri* A	0.049	0.061	0.003	0.001	0.057	0.080	0.003	0.001
H. flaviguttatum/*H. maculicauda*	0.004	0.002	0.006	0.006	0.004	0.002	0.006	0.006
H. carbonarium/*H. macrostomum*	0.023	0.017	0.008	0.004	0.024	0.018	0.009	0.004
H. scudderi/*H. sexfasciatum*	0.084	0.061	0.011	0.004	0.115	0.082	0.012	0.004
H. album/*H. melanurum*	0.079	0.072	0.012	0.004	0.105	0.098	0.014	0.004
H. parra/*H. squamipinna*	0.113	0.076	0.011	0.008	0.186	0.113	0.012	0.008
H. plumieri/*H. sciurus*	0.087	0.075	0.009	0.010	0.114	0.107	0.011	0.010
I. vittata/*H. striatum*	0.057	0.064	0.011	0.010	0.070	0.083	0.012	0.011

3.2. Phylogenetic analyses

Results from the maximum likelihood analysis are presented in two trees (Fig. 1), one for mtDNA and the other for nDNA (separate because they include *H. bonariense*); the topology generated by the maximum parsimony analysis was identical. The Bayesian analysis resulted in a 95% credible set of 1087 trees, and the consensus tree was congruent with both maximum parsimony and maximum likelihood topologies. The consensus of the 480,000 trees sampled from 20 million generations of the Markov chain is presented in Fig. 2.

Monophyly of the genus *Haemulon* is well supported, even when representatives of all genera of Haemulidae are included in the analysis (L. Rocha, M. Santiago, and K. Carpenter, unpublished).

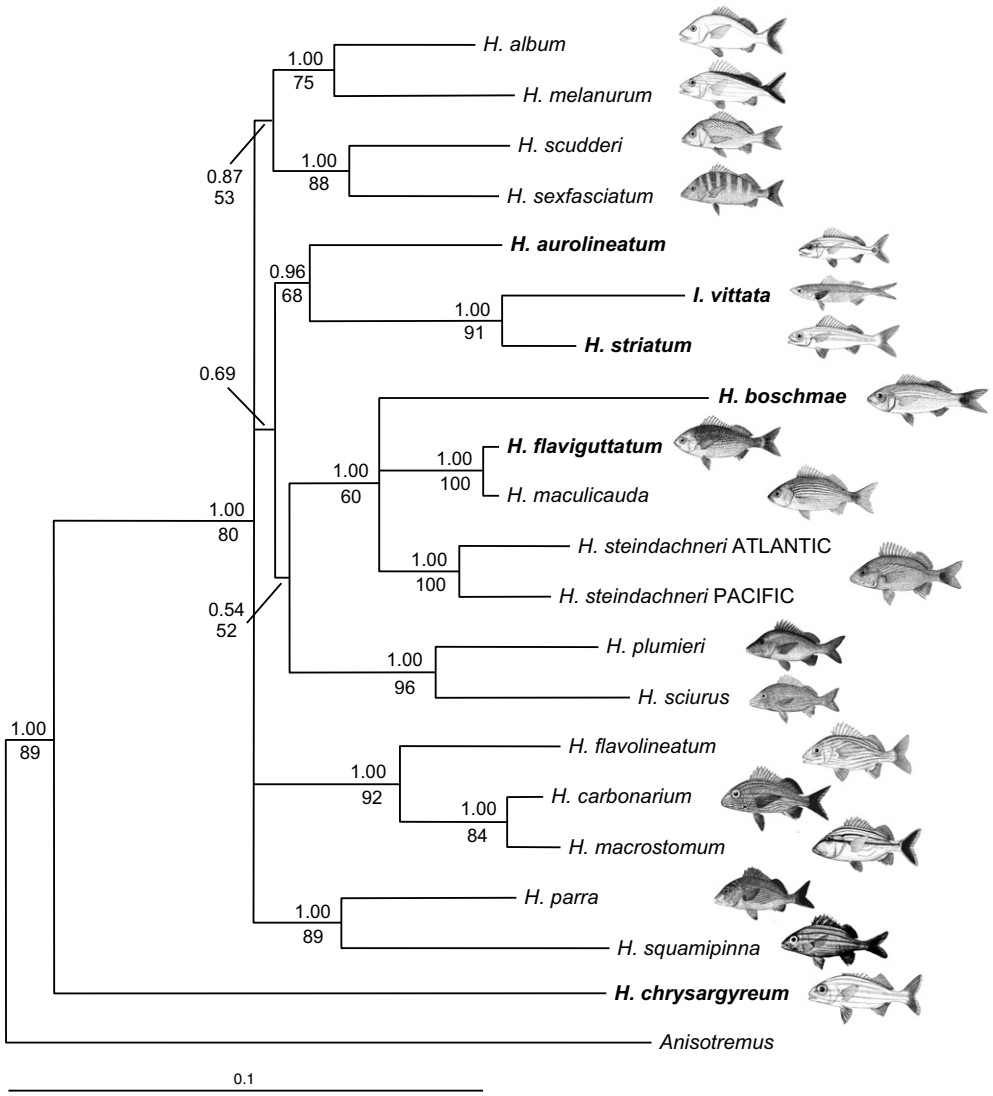

Fig. 2. The 50% majority-rule consensus tree from the Bayesian analysis of the combined cytochrome *b*, COI, RAG2, and TMO-4C4 datasets (total of 2639 base pairs) representing all nominal *Haemulon* species, except *H. bonariense* (see Fig. 1 and text). Numbers above branches correspond to posterior probabilities estimated using the Bayesian approach, and numbers below branches refer to the bootstrap support calculated from the maximum likelihood analysis of 500 sequence replicates assuming model parameters values estimated from Modeltest (see Section 2). Names in bold font represent planktivore species, remaining species are benthic predators.

Table 4
Relationships among western Atlantic *Haemulon* previously proposed in the literature

Group	Courtenay (1961, 1965)	Davis (1967)	Lindeman (1984)
I	*H. album* *H. macrostomum* *H. plumieri* *H. bonariense*	*H. album* *H. macrostomum* *H. plumieri*	*H. plumieri* *H. sciurus*
II	*H. sciurus* *H. melanurum* *H. steindachneri*	*H. sciurus* *H. melanurum* *H. steindachneri* *H. aurolineatum*	
III	*H. parra* *H. flavolineatum* *H. carbonarium*	*H. parra* *H. flavolineatum* *H. carbonarium* *H. bonariense*	*H. flavolineatum* *H. carbonarium* *H. bonariense*
IV	*H. chrysargyreum* *H. striatum* *H. boschmae*	*H. chrysargyreum* *H. striatum* *H. boschmae*	*H. striatum* *H. boschmae*

Group numbers follow Davis (1967). Some species were considered intermediates and not assigned to groups by Lindeman (1984).

Many sister species relationships are also evident from the tree, but many previously proposed groups (Table 4) of species are not well supported.

The boga (*I. vittata*, family Inermiidae), used in our tree initially as an outgroup, is recovered consistently by all four gene sequences within the genus *Haemulon*, in the position of sister species to *H. striatum* (Figs. 1 and 2). In addition to the close relationship between *H. striatum* and *I. vittata*, and the Atlantic and Pacific lineages of *H. steindachneri*, six pairs of sister species were strongly supported in our analyses: (1) *H. squamipinna* and *H. parra*; (2) *H. sciurus* and *H. plumieri*; (3) *H. melanurum* and *H. album*; (4) *H. maculicauda* and *H. flaviguttatum*; (5) *H. scudderi* and *H. sexfasciatum*; and (6) *H. carbonarium* and *H. macrostomum*.

The phylogenetic relationships recovered for *Haemulon* (Figs. 1 and 2) indicate that feeding habits changed several times during the group's evolutionary history. The combined Bayesian analysis supported a clade formed by the planktivores *H. aurolineatum, H. striatum*, and *I. vittata*. Although weakly supported in the mitochondrial tree, this clade makes ecological sense because these three species share a number of adaptations that allows them to spend time high in the water column and feed on plankton (e.g., slender bodies, smaller mouths, more gill rankers). A strong relationship among the group formed by *H. carbonarium, H. flavolineatum*, and *H. macrostomum* was also supported by our analyses. These are deep-bodied, benthic species that feed on macro-invertebrates. However, other well supported groups contain species with both characteristics. For example, a clade that was also well supported in all analysis contains the slender-bodied planktivores *H. flaviguttatum* and *H. boschmae* and the deeper-bodied macro-invertebrate feeders *H. steindachneri* and *H. maculicauda*. Moreover, within this group, *H. maculicauda* (macro-invertebrate feeder) and *H. flaviguttatum* (planktivore) show a sister species relationship.

4. Discussion

4.1. Morphology versus molecules

Although no comprehensive morphology-based phylogeny of the entire genus *Haemulon* has been proposed, several groups and sister species relationships were previously suggested based on morphological and ecological characters (Courtenay, 1961, 1965; Davis, 1967; Lindeman, 1984). Some of the sister species hypotheses were well supported by our analysis, whereas none of the larger groups (Table 4) were supported.

The presence of scales covering the entire pectoral fin along with similar fin ray counts were considered important morphological characters and led Rocha and Rosa (1999) to propose a sister species relationship between the widely distributed *H. parra* and the Brazilian endemic *H. squamipinna*. The molecular phylogeny supports this hypothesis. A strong relationship between *H. plumieri* and *H. sciurus* was proposed by Lindeman (1984), who identified similarities in early-juvenile pigment patterns, fin ray counts and adult head pigmentation. This sister relationship is also supported by our data. However, sister species relationships proposed for the pairs *H. boschmae*/*H. striatum* (Courtenay, 1965), *H. parra*/*H. scudderi* and *H. album*/*H. sexfasciatum* (Jordan, 1908) were not supported by our data. This discrepancy was not surprising because relationships between *H. parra* and *H. squamipinna* and between *H. plumieri* and *H. sciurus* were based on a combination of several morphological and developmental characters, whereas those between the other species were based on superficial similarity.

Three pairs of sister species of *Haemulon* were thought to have been formed by the rise of the Isthmus of Panama: (1) *H. album*/*H. sexfasciatum*; (2) *H. parra*/*H. scudderi*; and (3) *H. steindachneri* (Pacific)/*H. steindachneri* (Atlantic). These three pairs, along with pairs in other genera of fishes were used by Jordan (1908) to formulate the law of geminate species, which provided early evidence for the hypothesis of allopatric speciation supported by Mayr (1942) and other architects of the modern synthesis. Of these three proposed geminate pairs, the only one supported by our phylogeny is that comprised of the two forms of *H. steindachneri*, with species in the other two pairs being distantly related in our analyses. Since we show fixed genetic differences at both the mtDNA and nDNA between the Pacific and Atlantic forms of *H. steindachneri*, we suggest that these should be treated as two distinct species, but defer the formal recognition of such species to a more complete taxonomical study. The recognition of these two forms as different species has already been proposed based on external color differences (Moura, 2003).

In addition, one emerging species was detected in our analysis. The mtDNA of the Brazilian and Venezuelan populations of *H. steindachneri* are separated by a 0.7% sequence divergence in the combined mtDNA dataset. These two populations are separated by the 2300 km wide Amazon river barrier. Even though their divergence is smaller than that of some surgeonfishes and wrasses influenced by the same barrier (Rocha et al., 2002, 2005; Rocha, 2004) and there is no difference in morphology or the nuclear genes analyzed, their genetic uniqueness at the mtDNA supports the view that these populations should be regarded as distinct evolutionary units and not as a single, widely distributed panmictic species, and be managed accordingly (Rocha et al., 2007).

4.2. The status of I. vittata

Inermia vittata (Poey, 1860), which is currently placed in the family Inermiidae (Johnson, 1980; Lindeman, 2005), clusters within the genus *Haemulon*. This phylogenetic position is supported by all four genes in our analysis. Our specimens of *Inermia* were obtained from two different collections 11 years apart: a Smithsonian Institution expedition to Belize in 1992 and an expedition also to Belize by Peter Wainright (University of California, Davis) in 2003. We examined photographic records of *Inermia* specimens collected in 2003, and a voucher collected during the Smithsonian expedition (USNM 349224). Tissues of the later specimen are also deposited at the University of Kansas tissue collection (KU328 and KU329). Detailed examination of specimens and photographs led us to conclude that the specimens that we sequenced are undoubtedly *I. vittata*, eliminating the possibility of misidentification. We conclude that the species *I. vittata* (Poey, 1860) should now be treated as *Haemulon vittatum* (Poey, 1860).

The close morphological relationship of *Inermia* to the Haemulidae led to the erection of the superfamily Haemuloidea, consisting of the Haemulidae and Inermiidae (Johnson, 1980). The two monotypic inermiid genera, *I. vittata* and *Emmelichthyops atlanticus* were united because of their morphological shared unique characters, including highly protrusible jaw systems, and were considered to be derived from the Haemulidae (Johnson, 1980). Unfortunately, we were not able to obtain tissue samples of *E. atlanticus*, and cannot evaluate its taxonomic position based on molecular data. Available information suggests that *E. atlanticus* should be provisionally placed in the family Emmelichthyidae (Heemstra and Randall, 1977) pending additional phylogenetic analyses, and the family Inermiidae should no longer be considered valid. The recognition of *I. vittata* as belonging to the genus *Haemulon* and the possible specific status of the Atlantic form of *H. steindachneri* indicates that there are at least 21 valid species of *Haemulon*, two more than previously recognized. The family Inermiidae should no longer be considered valid. Several unresolved nomenclatural issues regarding the status of *H. steindachneri* and other *Haemulon* species are being treated elsewhere (R. Moura and K. Lindeman, personal communication).

The apparent discordance between morphological and molecular phylogenetics observed in *Inermia* is not unique among fishes, there are at least three similar examples: (1) the semi-pelagic species *Paranthias furcifer* and *P. colonus* have long been classified in a unique genus within the groupers, however, recent molecular phylogenetic evidence shows that the benthic *Cephalopholis fulva* (with a typical grouper morphology) is much more closely related to *Paranthias* than to any other similar grouper in the genus *Cephalopholis* (Craig and Hastings, 2007); (2) the fusiliers (family Caesionidae) resemble *Inermia* in body shape, general morphology and ecology, with their distinctive appearance being used to justify their position as a family or subfamily, however, a recent molecular phylogenetic study revealed that they are nested within the snapper genus *Lutjanus*, which, similarly to *Haemulon*, have deep bodies and live near the bottom (Miller and Cribb, 2007); (3) the bird wrasses of the genus *Gomphosus* (Labridae) were classified in their own genus because they possess an extremely elongated snout (resembling a humming bird beak), however, the genus is nested within the more diverse wrasse genus *Thalassoma* (Bernardi et al., 2004).

What all these examples have in common are seemingly rapidly evolved and often extreme morphological adaptations that are associated with rapid ecological shifts (e.g., a change from benthic predation to planktivory in the cases of *Inermia, Paranthias*, and Caesionidae). Thus, the unique morphology of *I. vittata* among other *Haemulon* can be explained by natural selection rapidly driving morphology during a shift to pelagic habits. Its small and protrusible mouth, pointed head, fusiform (spindle-shaped) body, smaller scales, keels on caudal peduncle and reduced dorsal-fin elements all translate into adaptations that allow it to better exploit pelagic environments. The key insight from our and other recent phylogenetic surveys is that morphological adaptations can evolve rapidly within widespread genera with otherwise relatively homogeneous morphological and ecological attributes.

4.3. Introgressive hybridization

Discordance among gene trees can indicate introgressive hybridization (Sang and Zhong, 2000). Our phylogenetic analyses demonstrate two instances of probable hybridization between species of *Haemulon*. First, *H. flaviguttatum* has an mtDNA sequence almost identical to that of *H. maculicauda* (~0.3% corrected sequence divergence, or 4 mutations in 1428 base pairs), whereas their nuclear DNA is very different (~0.6% corrected sequence divergence), with divergence levels compatible with that of other sister species in the genus (ranging between 0.16% and 1.2% in other pairs, Table 3). The most probable explanation for this observation is an ancient divergence combined with a more recent transfer if mtDNA from one species to the other via an introgressive hybridization event. When a female first generation (F1) hybrid is fertile and back crosses with its male parental species, it transfers the mtDNA from one species to the other. Here we hypothesize that female hybrids successfully crossed with the male parental species and transferred the mtDNA from one species to the other. Several factors (including selection and sterility) may limit nuclear DNA introgression, however, mtDNA introgression seems to be common in hybridization among coral reef fishes (van Herwerden et al., 2006; Yaakub et al., 2006). As pointed out by McCafferty et al. (2002), larger sample sizes from different geographical locations may reveal the direction of the mtDNA transfer, but such an analysis falls outside the scope of the present paper.

The second case of possible hybridization comes from the discordant position of *H. bonariense* in the mtDNA and nDNA trees. In the mtDNA tree, *H. bonariense* is very close to *H. parra*, whereas in the nDNA tree these two species are distantly related. The mtDNA divergence between *H. parra* and *H. bonariense* (~0.5%) is below that of other species pairs (~2.1–14.4%) in the genus, whereas their nDNA divergence (~1.3%) is above that of other species pairs (~0.16–1.2%, Table 3). In this case, because the species involved in the probable hybridization event are not sisters, and because we can infer with a high degree of certainty that *H. bonariense* is closely related to *H. scudderi* and *H. sexfasciatum* based on the nDNA tree, it appears that the specimen of *H. bonariense* that we sequenced contained mtDNA that originated from *H. parra*.

The concern of sample contamination obviously needs to be addressed in both cases. We tried to eliminate this possibility by analyzing multiple specimens from all species involved in potential hybridization in two different molecular laboratories, the Naos laboratory of Smithsonian Tropical Research Institute in Panama and the Hawaii Institute of Marine Biology in Oahu, Hawaii. The tissue samples went through the entire process, from DNA extraction to sequencing in both labs. In addition, the mtDNA of the potential hybrids contains unique mutations and are not 100% identical, indicating that hybridization is historical and not ongoing and eliminating the possibility of mislabeling or mixture of tissues or tubes. Morphological analyses have identified many reef fish hybrids (e.g., Randall et al., 1977; Pyle and Randall, 1994), and several cases have recently been confirmed by genetic analyses (van Herwerden et al., 2002; Yaakub et al., 2006), indicating that hybridization in marine fishes is not rare.

4.4. Speciation in Haemulon

The biogeography of *Haemulon* is interesting in that many closely related sister species pairs have either completely or partially overlapping distributions (Fig. 3). The only exception is *H. steindachneri*, in which populations (or species) are found on either side of the Isthmus of Panama, and the Atlantic species is divided by the Amazon outflow barrier. Often, the pairs with partially overlapping distributions contain one species with a relatively narrow distribution and the other with a wider distribution that covers and exceeds that of its sister. The tropical Atlantic contains the following pairs: (1) *H. squamipinna* restricted to Brazil and its sister *H. parra* in Brazil and the Caribbean; (2) *H. sciurus* restricted to the Caribbean and *H. plumieri* in Brazil and the Caribbean; (3) *I. vittata* restricted to the Caribbean and *H. striatum* in Brazil and the Caribbean; (4) *H. melanurum* from NE Brazil to the Caribbean and *H. album* in the Caribbean and Brazilian offshore islands; (5) the group formed by *H. carbonarium*, *H. flavolineatum*, and *H. macrostomum* with overlapping distributions in the Caribbean. In the eastern Pa-

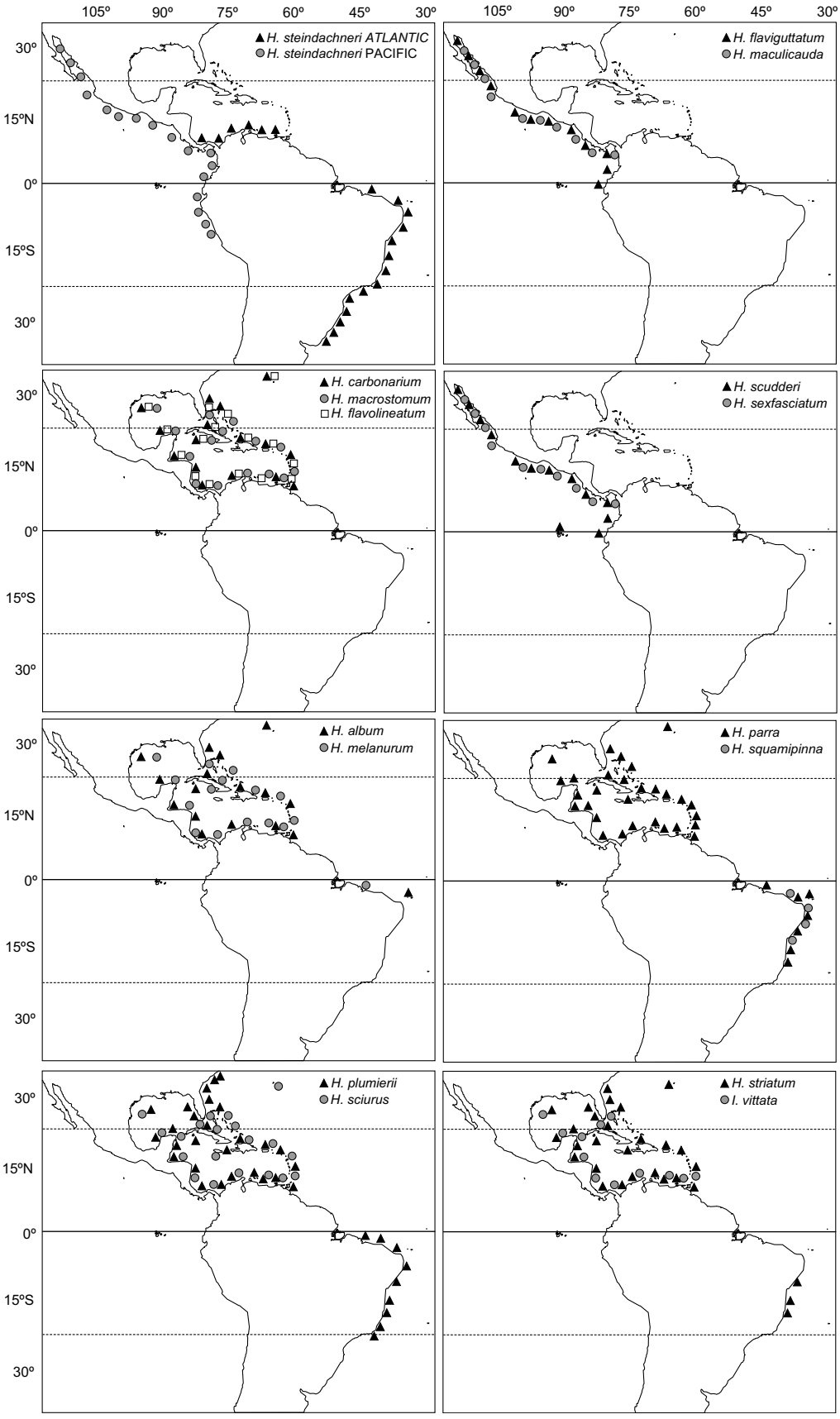

Fig. 3. Distribution maps of closely related species of *Haemulon*.

cific, a strong sister species relationship is recovered for two pairs: (1) *H. maculicauda* and *H. flaviguttatum*, and (2) *H. sexfasciatum* and *H. scudderi*. Both of these pairs have completely overlapping distributions from Mexico to Colombia and Ecuador.

The overlapping distribution of closely related species pairs in *Haemulon* raises a series of interesting questions: Are these cases of speciation without geographical isolation (sympatric speciation)? Do different components of the species pairs occupy different ecological niches? Are there biological characters among the species of *Haemulon* that facilitate sympatric speciation?

Differentiation in isolation (allopatric speciation) followed by secondary contact is an alternative hypothesis to sympatric speciation frequently used to explain cases in which species pairs have sympatric distributions. Secondary contact seems to be the most likely explanation for the differentiation of two Atlantic pairs, *H. parra*/*H. squamipinna* and *H. plumieri*/*H. sciurus*. Both pairs may have been formed in association with the Amazon barrier, which isolates Brazil and the Caribbean (Briggs, 1974; Rocha, 2003). Once the two components of a pair are formed, dispersal across the Amazon barrier (*H. parra* invading the Brazilian range of *H. squamipinna* and *H. plumieri* invading the Caribbean range of *H. sciurus*) may explain the overlapping distributions (Rocha, 2003). This scenario of speciation followed by dispersal and secondary contact was described as part of the dynamics of the Amazon barrier (Rocha, 2003), and was used to explain a portion of the diversification in western Atlantic parrotfishes (Robertson et al., 2006).

Some of the pairs contain species that occupy different ecological niches, and this may indicate speciation driven by adaptation to novel environments, or ecological speciation (Schluter, 2001; Rocha and Bowen, 2008). The pair *H. flaviguttatum* and *H. maculicauda* in the eastern Pacific is such an example; the former species swims high in the water column, usually dozens of meters away from reefs feeding on plankton, whereas the latter species forms large aggregations very close to reefs and remains close to the bottom feeding on benthic macro-invertebrates (McKay and Schneider, 1995).

The group formed by *H. flavolineatum*, *H. carbonarium*, and *H. macrostomum* may represent another example of ecological speciation. *Haemulon macrostomum* is a solitary species that specializes in feeding on echinoderms, whereas *H. carbonarium* and *H. flavolineatum* often form schools and feed on a diverse array of benthic invertebrates (Lindeman and Toxey, 2002). Thus, it is possible that natural selection for alternate ecological conditions (pelagic versus benthic in the first case and solitary with specialized feeding habits versus more gregarious generalists in the second) has driven evolutionary divergence. Alternatively, but much less likely, allopatric speciation within the Caribbean followed by range expansion and secondary contact could also explain the pattern in this three-species group. However, such isolation has only been demonstrated in cleaner gobies, a group that has very limited dispersal ability (Taylor and Hellberg, 2006). In grunts, a Caribbean-wide genetic survey of *H. flavolineatum* using highly variable microsatellite markers showed that no major genetic discontinuities are present, and that even though there is some evidence for isolation by distance at an ecological time scale, dispersal probably homogenizes Caribbean populations at evolutionary time scales (Purcell et al., 2006).

Even though secondary contact and ecological speciation seem to explain the formation of some of the species pairs, others such as *H. melanurum*/*H. album* and *H. scudderi*/*H. sexfasciatum*, ecologically similar species that occupy very similar geographic ranges, seem to defy explanation. The answer may come from the biology of *Haemulon*. A peculiar characteristic of this group of fishes is that they produce loud grunt-like sounds by grinding their pharyngeal teeth (Burkenroad, 1930); the source of their common name "grunts". Many species in the family inhabit semi-turbid coastal waters, where vision is obscured and the may use distinctive vocalization to find mates. Moreover, there appears to be a positive correlation between hearing ability and sound production in grunts, indicating that they use the sounds for intra-specific communication (Cruz and Lombarte, 2004). Changes in sound production accompanied by changes in female mate preference may provide the means for isolation in sympatry, one of the few sympatric speciation models accepted by Mayr (1942). While this hypothesis seems plausible, presently it is only speculation since spawning (which probably occurs at night) has never been observed in grunts, and the role of sound in species recognition and reproduction also remains unknown.

The only clear example of allopatric speciation in the genus was only recently confirmed. Classic taxonomic assessments of *Haemulon* have considered *H. steindachneri* as a single species comprised by two populations, one in the Atlantic and one in the Pacific, separated for ~3 Myr by the Isthmus of Panama (Courtenay, 1961). However, our analyses show deep genetic divergences between these populations, a result also observed in a study using protein electrophoresis, which revealed a Nei's *D* genetic distance $D = 0.131$ between eastern Pacific and Caribbean populations of *H. steindachneri* (Vawter et al., 1980). These results support a recent morphological re-evaluation of some species of the genus, in which recognition of different species in each side of the Isthmus of Panama is proposed (Moura, 2003). Even though Jordan (1908) suggested that three species pairs were formed by the rise of the Isthmus in *Haemulon*, close relationships between two of those pairs (*H. parra*/*H. scudderi* and *H. album*/*H. sexfasciatum*) were not supported by our analysis, indicating that the Isthmus had a smaller impact on the historical biogeography and speciation of *Haemulon* than previously thought. This observation also stresses the need for complete taxon sampling and thorough phylogenetic analysis before comparisons among putative trans-isthmian sister species are made (Craig et al., 2004).

The Kimura two parameter (K2; Kimura, 1980) COI divergence between the *H. steindachneri* geminate pair of 6.5% is compatible with divergences of other trans-isthmian pairs. This divergence is below that of five other pairs of the genera *Chromis*, *Chaetodon*, *Priacanthus*, *Thalassoma*, and *Ophioblennius*, whose divergences range from 9.35% to 12.37% when using the K2 model, but above that of other pairs, including another pair of the family Haemulidae, *Anisotremus surinamensis* and *A. interruptus*, whose divergence is 1.62% (Bermingham et al., 1997). It has been suggested that trans-isthmian species with lower divergences are those that have a greater affinity for coastal and estuarine waters (Knowlton et al., 1993; Tringali et al., 1999; Bernardi and Lape, 2005). This prediction comes from the geological history of the closure of the Isthmus: before the final closure, the only connections between the Caribbean and the eastern Pacific were coastal shallow areas probably with estuarine conditions (Coates and Obando, 1996). Thus, species that tolerate such conditions would be the last ones to have their populations separated by the rising Isthmus and show the lower sequence divergences (Knowlton and Weigt, 1998). Both *H. steindachneri* and the *Anisotremus* pair fit the profile as they are coastal species often found in low salinity waters (Raz-Guzman and Huidobro, 2002), with juveniles in very shallow water over sandy or rubble bottoms (Lindeman and Toxey, 2002).

4.5. Final remarks

The general incongruence between the relationships proposed here and those proposed based on external morphology, combined with other examples from the literature, indicate that rapid morphological changes associated with ecological adaptations may be a general trend in reef fishes. Among the points of incongruence, we show evidence that the family Inermiidae should no longer be treated as valid, and that one of its only two species, *I. vittata*, should be considered a species of *Haemulon*. In addition, two species pairs previously considered as sisters separated by the Isthmus of Panama are not closely related in our phylogeny.

Finally, the evolutionary history of *Haemulon* also helps us understand the faunal richness of the Greater Caribbean marine biodiversity hotspot. The presence of many sister species pairs with partially or completely overlapping geographical distributions indicates that vicariance is not the only process driving speciation in *Haemulon*. In cases involving the Amazon barrier, it appears that vicariance can act first to separate and create species pairs, but that dispersal and secondary contact are responsible for the ultimate faunal enrichment. However, there are cases involving habitat shifts and a lack of vicariant barriers between sister species, indicating that ecological and/or sympatric speciation may also be possible in this group.

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