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Reconciling molecules and morphology: Molecular systematics and biogeography of Neotropical blennies (*Acanthemblemaria*)

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ABSTRACT

Neotropical reef fish communities are species-poor compared to those of the Indo-West Pacific. An exception to that pattern is the blenny clade Chaenopsidae, one of only three rocky and coral reef fish families largely endemic to the Neotropics. Within the chaenopsids, the genus *Acanthemblemaria* is the most species-rich and is characterized by elaborate spinous processes on the skull. Here we construct a species tree using five nuclear markers and compare the results to those from Bayesian and parsimony phylogenetic analyses of 60 morphological characters. The sequence-based species tree conflicted with the morphological phylogenies for *Acanthemblemaria*, primarily due to the convergence of a suite of characters describing the distribution of spines on the head. However, we were able to resolve some of these conflicts by performing phylogenetic analyses on suites of characters not associated with head spines. By using the species tree as a guide, we used a quantitative method to identify suites of correlated morphological characters that, together, produce the distinctive skull phenotypes found in these fishes. A time calibrated phylogeny with nearly complete taxon sampling provided divergence time estimates that recovered a mid-Miocene origin for the genus, with a temporally and geographically complex pattern of speciation both before and after the closure of the Isthmus of Panama. Some sister taxa are broadly sympatric, but many occur in allopatry. The ability to infer the geography of speciation in *Acanthemblemaria* is complicated by extinctions, incomplete knowledge of their present geographic ranges and by wide-spread taxa that likely represent cryptic species complexes.

1. Introduction

Reef communities harbor the greatest marine fish diversity of any oceanic ecosystem (Sale, 2002). Biodiversity of reef fishes is highest in the Indo-West Pacific and decreases longitudinally to the east and west, with the Neotropics being species-poor in comparison (Bellwood and Wainwright, 2002; Briggs, 1974; MacPherson et al., 2009; Mora et al., 2003). A major exception to this pattern is the Blennioidei, a group of small, bottom-dwelling rocky and coral reef fishes. Blennies are a species-rich group composed of six families. Of those, the Labrisomidae, the Dactyloscopidae, and the Chaenopsidae are the only reef fish families entirely or largely endemic to the New World (Bellwood and Wainwright, 2002; Hastings, 2009).

1.1. Acanthemblemaria

Acanthemblemaria (Metzelaar, 1919) is the most species-rich genus of chaenopsids, as well as one of the most species-rich genera of Neotropical blennies (Hastings, 2009; Hastings and Springer, 2009b). All members in the genus are small (~1.2–3.5 cm standard length) and are obligate dwellers of vacated invertebrate holes on shallow (<1–~22 m) rocky and coral reefs (Stephens, 1963). As currently recognized, *Acanthemblemaria* includes 22 species, 10 in the Tropical Eastern Pacific and 12 in the Tropical Western Atlantic (Hastings, 2009). Since the comprehensive treatment of the family Chaenopsidae by Stephens (1963), more named species have been added to *Acanthemblemaria* than to any other chaenopsid genus. Much of this growth has been due to the recognition that several species with broad distributions contain cryptic, often allopatric taxa (Hastings and Robertson, 1999a; Hastings and Springer, 2009a,b; Lin and Galland, 2010).

The generic name *Acanthemblemaria* comes from the Greek *Akanthos-*, or thorn. The name is apt, as *Acanthemblemaria* blennies are typified by the presence of spinous processes on the frontal bones (Metzelaar, 1919; Smith-Vaniz and Palacio, 1974; Stephens,

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1963). Morphological characters related to head spination represent the majority of the characters used to infer the interspecific relationships in the group (Hastings, 1990). Recent molecular phylogenies of the genus (Eytan, 2010; Lin and Hastings, 2011) recovered *Acanthemblemaria* as monophyletic, but also recovered conflicts with the morphological hypothesis of Hastings (1990), where taxa with clear affinities based on cranial morphology were not closely related in the molecular phylogeny.

The characters used for inferring phylogenetic relationships must be independent of one another (Kluge, 1989). Suites of morphological characters that evolve in concert violate this dictate. Such correlated evolution is most likely to occur when a set of characters underlie a functionally adaptive phenotype or common developmental pathway (Emerson and Hastings, 1998). Such suites of correlated characters can mislead phylogenetic analyses because they track adaptive history instead of phylogeny (Holland et al., 2010; McCracken et al., 1999) or because they are developmentally linked to other characters (Schlosser and Wagner, 2004; West-Eberhard, 2003). In practice, it is difficult to determine the underlying nature of character correlations. This is because a suite of characters that are highly correlated with one another are expected to produce the same result as a suite of independent characters with good phylogenetic signal: strong support for a given clade (Shaffer et al., 1991).

Here we test whether the homoplastic morphological characters related largely to head spination in *Acanthemblemaria* are correlated with one another independent of the phylogeny, and if accounting for that correlation can reconcile the molecular and morphological hypotheses for the genus. We reconstruct the species tree of the genus *Acanthemblemaria* using five nuclear markers and employ Bayesian relaxed clock divergence dating to determine the age of the group and timing of speciation among the members of the genus. We also examine the historical biogeography of the genus, with the aim of elucidating the geography of speciation in the group. Of particular interest is whether speciation in this clade has occurred primarily between ocean basins on either side of the Isthmus of Panama, or within the basins themselves.

2. Materials and methods

2.1. Taxon sampling

Between one and five individuals from 16 of the 22 named *Acanthemblemaria* species, as well as one undescribed species and four outgroup taxa, chosen based on Hastings (1990) and Almany and Baldwin (1996), were included in the study (Table S1). Where possible, individuals were sampled from more than one population. Of the taxa included, six are putative trans-isthmian geminates (Hastings, 1990; Hastings and Springer, 1994), with two geminate pairs in the ingroup and one in the outgroup. Whole fishes were stored individually in 95% ethanol or salt-saturated DMSO at −80 °C. Voucher specimens for some species are present in the SIO Marine Vertebrate Collection and photo vouchers from freshly collected specimens of others have been submitted to the Dryad repository.

2.2. DNA extraction, PCR and sequencing

DNA was extracted using the Qiagen (Valencia, CA) QIAMP DNA Minikit. The polymerase chain reaction (PCR) was performed to amplify five genetic markers (Table 1): nuclear protein-coding genes recombination-activating gene 1 (*rag1*), titin-like protein (*TMO4C4*), melanocortin 1 receptor (*MC1R*), SH3 and PX domain containing three gene (*SH3PX3*), and intron V from nuclear α-tropomyosin (*atrop*). PCR amplification of the full-length *rag1* molecule was not possible for some taxa. In these cases, a set of internal primers were developed for the study and used to amplify *rag1*.

Amplicons were purified with a Strataprep PCR Purification Kit (Stratagene, La Jolla, CA) or directly sequenced without cleanup in both directions on an ABI 3100 or 3130 XL automated sequencer with 1/8 reactions of BigDye Terminators (V3.1, Applied Biosystems) with the amplification primers, or internal primers as indicated in Table 1.

2.3. Sequence alignment and model selection

Sequences for the four protein-coding genes were aligned using MUSCLE (Edgar, 2004) as implemented in Geneious v3.6 (Drummond et al., 2007). The α-tropomyosin sequences, which contained numerous gaps, were aligned in BAli-Phy v2.0.1 (Suchard and Redelings, 2006) using the GTR substitution model, gamma distributed rate variation, and the default indel model. BAli-Phy was run four times to ensure concordance among runs. All the samples of the Markov chain taken before convergence, as determined by stationarity in the Markov chain, which was visualized in Tracer v1.5 (Rambaut and Drummond, 2010), were discarded as burnin. The consensus alignment from the run with the highest posterior probability was used for subsequent analyses and all positions with posterior probabilities less than 0.95 were discarded.

Ten different partitioning strategies were evaluated for both the species tree and concatenated analyses. These partitioning strategies ranged from treating all genes as a single partition, to each gene and codon position given its own partition (Table 2). Models of sequence evolution for each strategy were determined using jModelTest (Posada, 2008) and the AIC, while partitioning strategies were determined using 2 ln Bayes factors (Kass and Raftery, 1995) with the modification of Suchard et al. (2001), implemented in Tracer v1.5.

2.4. Bayesian species tree and divergence dating analyses

2.4.1. Species tree estimation

Species tree analyses were conducted using the ∗BEAST package in BEAST v1.5.4 (Heled and Drummond, 2010). Sequences were grouped by nominal species for the analyses. Trees and clocks were unlinked among all genes, with each gene region dated using the uncorrelated log normal distribution (UCLD) (Drummond et al., 2006) and the calibrations detailed below. The datasets were run twice for 100,000,000 generations, sampling every 5000. Convergence onto the posterior distribution for the estimated topology was assessed using the "compare" and "cumulative" functions in Are We There Yet? (AWTY) (Nylander et al., 2008). Convergence onto the posterior distribution for parameter estimates was assessed by effective sample size (ESS) values greater than 250, as determined in Tracer v1.5 (Rambaut and Drummond, 2010). A time-calibrated phylogeny of the concatenated dataset was also constructed in BEAST, using the same calibrations and run conditions as for the species tree.

2.4.2. Divergence dating

Priors on the time to most recent common ancestor (TMRCA) for two species pairs separated by the Isthmus of Panama were specified. The first species pair considered, *Acanthemblemaria betinensis* and *Acanthemblemaria exilispinus*, occur in <1 m of water and are restricted to areas close to the Isthmus (Hastings, 2009). These distributions suggest that their progenitor was split close to the final closure of the Isthmus. The calibration was given an exponential prior with a mean of 7 million years and a zero

Table 1
Primer sets and PCR conditions used in this study.

Marker	Primer name	Primer sequence	References
ATROP	ATROP-L	GAG TTG GAT CGC GCT CAG GAG CG	Hickerson and Cunningham (2005)
	ATROP-H	CGG TCA GCC TCC TCA GCA ATG TGC TT	Hickerson and Cunningham (2005)
RAG1	RAG1Of2	CTG AGC TGC AGT CAG TAC CAT AAG ATG T	Taylor and Hellberg (2005)
	RAG1F.4.27	AGCTGTAGTCAGTAYCACAARATG	This study
	RAG1S2F	CCG AGA AGG CTG TAC GTT TCT CTT	Taylor and Hellberg (2005)
	RAG1S1R	CCT GCC AGC ACA GAA ACA GAC ATA	Taylor and Hellberg (2005)
	RAG1R1.539.519	CAG GAC AGT TCT GAG TTT GGC	This study
	RAG1F3.519.539	GCC AAA CTC AGA ACT GTC CTG	This study
	RAG1S2R	CATTACCGGCTTGAGCTTCATCCT	Taylor and Hellberg (2005)
	RAG1F4.1129.1148	ATGAATGGGAACTTTGCCCG	This study
	RAG1S3F	GCT CAT GAG GCT CTA TAT TCA GAT G	Taylor and Hellberg (2005)
	RAG1Or2	CTG AGT CCT TGT GAG CTT CCA TRA AYT T	Taylor and Hellberg (2005)
SH3PX3	SH3PX3_F461	GTATGGTSGGCAGGAACYTGAA	Li et al. (2007)
	SH3PX3_R1303	CAAACAKCTCYCCGATGTTCTC	Li et al. (2007)
TMO4C4	TMO-F2	GAKTGTTTGAAAATGACTCGCTA	Near et al. (2004)
	TMO-R2	AAACATCYAAMGATATGATCATGC	Near et al. (2004)
MC1R	MC1RFor	ATGGAAATGACCAACRGGTCCYTGC	This study
	MC1RRev	CARGGTTYTMCGCAGCTCCTGGC	This study
	MC1RF477	TCCAGCATCCTCTTCATCG	This study
	MC1RR243	AGCATACCTGGGTGAACGTC	This study
	MC1RR907	CGTAAATGAGCGGGTCGATGA	This study
	MC1RR649	TATGAAGGTAGAGCACCGC	This study

PCR conditions
RAG1, *TMO4C4*, *SH3PX3*, *MC1R*: One cycle of 94 °C for 2 min, 50 °C for 90 s, 72 °C for 2 min followed by 38 cycles of 94 °C for 45 s, 50 °C for 1 min, and 72 °C for 90 s, and a final cycle of 94 °C for 40 s, 50 °C for 1 min, and 72 °C for 10 min.
ATROP: One cycle of 94 °C for 2 min, 62 °C for 1:30, 72° for 2 min followed by 38 cycles of 94 °C for 45 s, 62 °C for 1 min, and 72 °C for 45 s, and a final cycle of 94 °C for 45 s, 62 °C for 1 min, and 72 °C for 10 min.

Table 2
Partitioning strategies evaluated for this study.

Model	Name	Partition description	Number of partitions
1	FULL	All included nucleotide positions	1
2	SNMAT	SRD06 model for nDNA, mtDNA, α-trop concatenated	3
3	SNMIE	SRD06 model for nDNA, mtDNA, α-trop intron and exon	4
4	GENES	Partitioned by gene region, α-trop concatenated	5
5	NMAT	nDNA by codon, mtDNA by codon, α-trop concatenated	5
6	NMIE	nDNA by codon, mtDNA by codon, α-trop intron and exon	6
7	SGAT	SRD06 model for each locus, α-trop concatenated	9
8	SGIE	SRD06 model for each locus, α-trop intron and exon	10
9	GCAT	Each locus by codon position, α-trop concatenated	13
10	GCIE	Each locus by codon position, α-trop intron and exon	14

offset of 3.1 million years. This prior represents the most recent possible split for the geminates at the close of the Isthmus, but allows for a split prior to the closure, although with decreasing probability back in time.

The second pair of geminates considered, *A. rivasi* and *A. castroi*, have a Galápagos–Caribbean distribution (Hastings, 2009). While the most recent possible split between these two would have been the closure of the Isthmus and the earliest possible split the rise of the Galapagos (at most 17 million years ago; Werner and Hoernle, 2003), the split most probably occurred between those dates. A truncated normal prior for the split time of *A. rivasi* and *A. castroi* was specified. A minimum offset of 3.1 million years, representing the most recent possible split for the species pair, was used. The mean and standard deviation were set at 10 and 3.52, respectively, which gave a 95% confidence interval of 3.1 and 16.9 million years for the prior. The third pair of geminates, the outgroup taxa *Ekemblemaria myersi* and *E. nigra*, were not used to calibrate a molecular clock because the *E. myersi* specimen used in this study was not collected in Panama, but further north.

2.5. Analysis of morphological data

A modified version of the morphological matrix from Hastings (1990) was analyzed. *Acanthemblemaria stephensi* and *A. atrata* were not sampled for the species tree analyses, as tissues were not available, and were removed from the matrix. Three taxa were added to the morphological matrix (*A. n. sp.*, *Protemblemaria bicirrus*, and *Cirriemblemaria lucasana*) and scored for the set of 60 characters from Hastings (1990). The new matrix was analyzed in a Bayesian framework using MrBayes v.3.1.2 (Ronquist and Huelsenbeck, 2003) and the Mkv model for morphological data (Lewis, 2001). In MrBayes all characters were set as variable and unordered, save for three that were ordered in Hastings (1990): character 2 (number of spines on the nasal rami (excluding AFO process)), character 3 (process on the nasal bones anterior to the first anterofrontal sensory pore (AFO process)), and character 7 (anterolateral extent of the frontal ridge). The MrBayes analyses were run twice with four heated chains (temp = 0.1) for 10,000,000 generations, sampling every 1000. Convergence onto the posterior distribution for the model parameters and topology was assessed using ESS

values in Tracer v1.5 (Rambaut and Drummond, 2010), and the "compare" and "cumulative" functions in AWTY (Nylander et al., 2008), respectively.

2.6. Identification of correlated incongruent morphological characters

The method of Holland et al. (2010) was used to identify morphological characters that are incongruent with the molecularly-derived *Acanthemblemaria* phylogeny and correlated with one another because of homoplasy. Following Holland et al. (2010) we constructed a matrix of "excess" distances between each pair of morphological characters (the excess distance is the parsimony score of the two characters taken together minus the parsimony score of each character taken individually). Zeros in the matrix indicate compatible pairs of splits. The dissimilarity matrix was visualized in SplitsTree4 (Huson and Bryant, 2006). UPGMA was used to identify a maximal clique of compatible characters. This is heuristic in that it is not guaranteed to find the maximum clique, so the procedure was repeated 100 times with different random orderings of the characters. The size of the largest clique was compared to those found for 100 shuffled alignments created following the second shuffling procedure described by Holland et al. (2010). This procedure creates shuffled alignments that have the same parsimony score on the sequence-based trees as the unshuffled morphological data. Each of the 100 shuffles was based on a different tree from the posterior distribution of the species tree analysis. This gave a null distribution of clique sizes that allowed us to assess if the maximal clique found in the unshuffled data was larger than would be expected by chance conditional on the level of agreement between the morphological characters and the sequence-based trees. If the clique is larger than expected by chance this is interpreted as evidence for convergent evolution in the morphological data.

3. Results

3.1. Molecular data, partitioning strategy, and convergence criteria

The five nuclear gene regions were successfully amplified in all taxa for a total alignment length of 3790 bp. The lengths of the aligned sequences, as well as the proportion of variable and parsimony informative sites for each marker, can be found in Table 3. All sequences have been submitted to GenBank with accession numbers JN897037-JN897271. Using 2 ln Bayes factors, the GCIE partitioning strategy was selected. For each of the analyses (time-calibrated species and concatenated trees, and the morphological tree) convergence diagnostics (AWTY results and ESS values >250) indicated that convergence onto the posterior distribution had occurred.

3.2. The species tree estimate for Acanthemblemaria yielded a well-supported phylogeny but it was in significant conflict with the morphological hypothesis

3.2.1. Comparison of species tree with Hastings (1990)

The Bayesian species tree estimate yielded a well-resolved topology with 13 of 19 nodes supported by Bayesian posterior probability (BPP) values greater than 0.95 (Fig. 1A). However, many of the well-supported nodes conflicted with the morphological hypothesis of Hastings (1990) (Fig. 1B) and the Bayesian estimate of the morphological data inferred in this study (Fig. 1C).

As in Hastings (1990), *Acanthemblemaria* was recovered as monophyletic in the species tree analysis, here with high support (BPP = 1.0) (Fig. 1A). Hastings' phylogeny was highly nested, showing a progression from *A. chaplini* and *A. greenfieldi* at the base of the tree, through the Caribbean *Acanthemblemaria* taxa, to the "*hancocki* species group" at the crown (Fig. 1B). In the ∗BEAST species tree, two major clades, here denoted as Clade I and Clade II, were recovered with high support (Fig. 1A). Each of these clades contained a pair of transisthmian sister species, both of which were recovered with BPP of 1.0. Neither of these transisthmian pairs was basal to the other taxa in their respective clades. The relationships of each of these two pairs of geminate taxa to the other members of their respective clades received high support, but for both there was less than 0.95 posterior support (Fig. 1A).

Clade I was composed of a majority of Eastern Pacific taxa, Clade II of mostly Caribbean taxa. In Clade I, a monophyletic group of taxa that occurs in the Eastern Pacific, with the exception of the geminate *A. rivasi*, was found. This clade, *A. crockeri* + "the *hancocki* species group" (*sensu* Hastings, 1990), was also recovered by

Table 3
Lengths of aligned sequences and the proportion of variable and parsimony informative sites for each marker and partition.

	Included length	% Variable sites (no. variable sites)	% PI sites (no. PI sites)
Gene region			
RAG1	1503 bps	15.17 (228)	11.38 (171)
MC1R	855 bps	16.61 (142)	12.28 (105)
SH3PX3	741 bps	16.87 (125)	12.96 (96)
TMO4C4	411 bps	24.82 (102)	18.98 (78)
α-tropomyosin	280 bps	20.71 (58)	13.93 (39)
TOTAL	3790 bps	17.28 (655)	12.9 (489)
Partition			
RAG1 (1)	501 bps	6.59 (33)	5.39 (27)
RAG1 (2)	501 bps	5.19 (26)	3.39 (17)
RAG1 (3)	501 bps	33.73 (169)	26.55 (133)
MC1R (1)	285 bps	4.91 (14)	3.86 (11)
MC1R (2)	285 bps	2.46 (7)	1.4 (4)
MC1R (3)	285 bps	42.81 (122)	36.14 (103)
SH3PX3 (1)	247 bps	2.43 (6)	2.02 (5)
SH3PX3 (2)	247 bps	1.21 (3)	0.81 (2)
SH3PX3 (3)	247 bps	46.96 (116)	38.06 (94)
TMO4C4 (1)	137 bps	12.41 (17)	8.76 (12)
TMO4C4 (2)	137 bps	5.84 (8)	4.38 (6)
TMO4C4 (3)	137 bps	56.2 (77)	45.99 (63)
α-Tropomyosin (I)	92 bps	56.52 (52)	42.39 (39)
α-Tropomyosin (E)	188 bps	3.19 (6)	1.06 (2)
Total	3790 bps	17.28 (655)	12.9 (489)

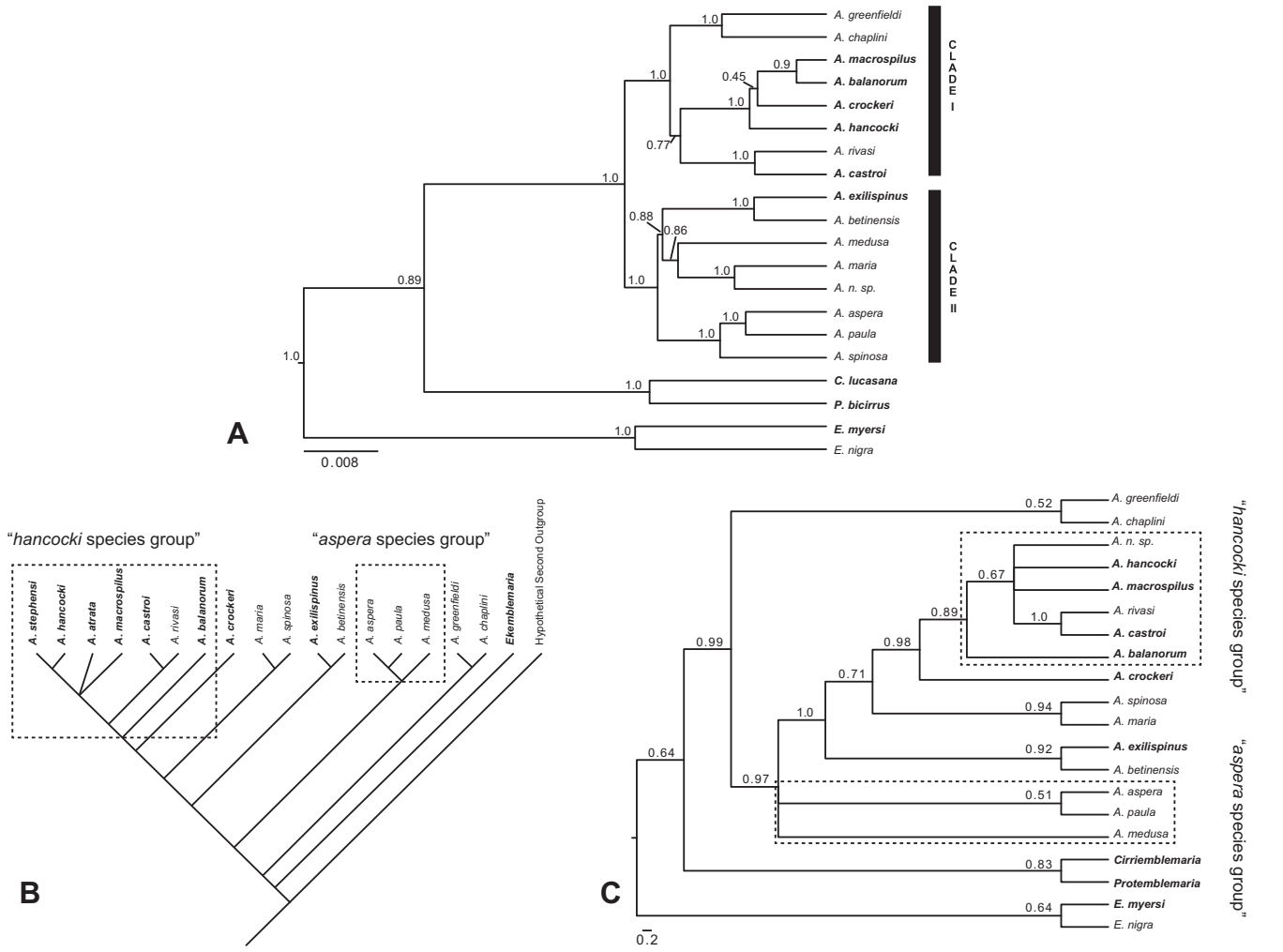

Fig. 1. Molecular and morphological hypotheses of the phylogeny of *Acanthemblemaria*, with Tropical Eastern Pacific (TEP) and Caribbean taxa in bold or normal font, respectively. (A) Bayesian species tree estimated in ∗BEAST. Posterior probabilities are shown at all nodes and branch lengths are in units of substitutions per site. The majority of taxa in Clade I (five versus three) occur in the TEP, while Clade II consists of primarily Caribbean species (seven versus one). (B) Morphological phylogeny inferred using maximum parsimony from Hastings (1990). (C) 50% majority rule consensus tree from the Bayesian estimate of the morphological dataset. Posterior probabilities greater than 0.5 shown at nodes. The "*hancocki*" and "*aspera*" species groups, *sensu* Hastings (1990) are enclosed by boxes.

Hastings. However, the species tree analysis recovered the trans-isthmian geminates *A. castroi* and *A. rivasi* as sister to the remaining species in the clade, with *A. crockeri* nested within the "*hancocki* species group", but with poor support.

In Clade II, the well-supported relationship between the geminate taxa *A. betinensis* and *A. exilispinus* was also recovered in the Hastings (1990) analysis. However, many of the other relationships within Clade II conflicted with the morphological phylogeny. The *A. maria*/*A. spinosa* split was not recovered in the species tree, nor was the "*aspera* species group" of (*A. medusa*, (*A. aspera*, *A. paula*)). Instead, *A. spinosa* was found to be sister to *A. aspera* and *A. paula*, while *A. maria* was sister to the undescribed *Acanthemblemaria* species (not included in Hastings, 1990). *A. medusa*, which was placed as sister to *A. aspera* and *A. paula* in the "*aspera* species group" based on morphological data, was found to be sister to *A. maria* and *A. n. sp.*, albeit with a BPP of 0.86.

3.2.2. Comparison of species tree with Bayesian estimates of morphology

The phylogeny based on Bayesian inference of morphological data closely mirrored the parsimony analysis of Hastings (1990), although support was poor for many of the nodes (Fig. 1C). All the relationships and clades inferred by Hastings were recovered here with the exception of the *hancocki*/*stephensi* split, as the latter taxon was not included in this study. The undescribed *Acanthemblemaria* species, which was not included in Hastings (1990), was recovered here as a member of the "*hancocki* species group". Also, as in Hastings (1990), the morphological tree inferred here was highly nested, with the same progression of taxa.

3.3. Identification of correlated incongruent morphological characters

The median size of the maximal clique within the 60 morphological characters was 24 characters. It is possible to get large cliques of compatible characters by chance, but never as large as the one recovered for the unshuffled data: the shuffled data produced maximal cliques of size 9–22 (median 16). (Recall that the shuffling procedure of Holland et al. (2010) is guaranteed to produce shuffled characters with the same level of incongruence to the molecular trees as the unshuffled characters.) This suggests that convergent evolution has occurred amongst the morphological characters.

3.4. A. spinosa and A. medusa were responsible for the majority of incongruence between molecules and morphology

The splits network of the morphological tree with the sequence-based tree revealed many areas of agreement between the two trees, as indicated by strictly bifurcating splits, including the entire "*hancocki* species group" clade (Fig. 2A). The two taxa that were responsible for the majority of the conflict between the two trees, as visualized by conflicting networks of splits, were *A. spinosa* and *A. medusa*. For both of these taxa a relatively large number of extra splits had to be traversed to unite them with clades specified by either the morphological or molecular phylogeny. When both of these taxa were removed from the tree, conflicting splits disappeared from the splits networks (Fig. 2C).

3.5. Evidence of a mix of historical and convergent signal for the placement of A. spinosa, but not A. medusa

We investigated the characters responsible for the conflict between the morphological and molecular trees and the source of the incompatible splits. In the case of *A. spinosa*, the *A. maria*/*A. spinosa* split that was recovered from the morphological matrix was supported by six characters (Table 4A). However, six other characters in the morphological matrix were incompatible with the *A. maria*/*A. spinosa* split (Table 4B). When a maximum parsimony (MP) tree was inferred using only these incompatible characters, six most parsimonious trees were found, all supporting the clade (*A. aspera*, (*A. paula*, *A. spinosa*)) (not shown.) This clade was found in the species tree as well, although in the species tree the sister relationship was (*A. spinosa*, (*A. aspera*, *A. paula*)). A single character in the morphological matrix (57; posterior pair of anterofrontal pores fused into a single medial pore) was a synapomorphy for the clade (*A. aspera*, *A. paula*, *A. spinosa*).

The inclusion of *A. medusa* in the "*aspera* species group", which consists of (*A. aspera*, *A. medusa*, *A. paula*), was supported by two characters in the morphological matrix (Table 4C). However, the morphological dataset contained five characters in conflict with the "*aspera* species group" (Table 4D). Unlike the conflicting characters for the *A. maria*/*A. spinosa* split, the maximum parsimony trees inferred from the characters conflicting with the "*aspera* species group" did not recover the clade found in the species tree: (*A. medusa*, (*A. maria*, *A. n. sp.*)). Instead, all the MP trees recovered a clade consisting of *A. aspera*, *A. chaplini*, *A. greenfieldi*, and *A. medusa* and no morphological characters supported the clade found in the species tree.

3.6. Time calibrated phylogenies recovered a mid-Miocene origin for Acanthemblemaria

3.6.1. Species tree

The dated species tree analysis found that *Acanthemblemaria* originated in the mid-Miocene, with a complex pattern of speciation within the genus both before and after the closure of the Isthmus of Panama (Fig. 3 and Table 5). The time to most recent common ancestor (TMRCA) of *Acanthemblemaria* was recovered with a mean of 13.1 mya and lower and upper confidence levels of 7.4 and 20.9 mya, respectively.

Three out of six terminal splits in *Acanthemblemaria* were inferred to have occurred prior to the closure of the Isthmus of Panama. Two of these three ingroup splits were the transisthmian geminates *A. castroi*/*A. rivasi* and *A. betinensis*/*A. exilispinus* with mean split times of 4.6 and 4.2 mya, respectively. The third terminal split prior to the closure of the isthmus, that of *A. chaplini*/*A. greenfieldi*, had a mean divergence date of 8.2 mya, but was not significantly older than either of the geminate taxa. In addition to those three splits, two clades that did not include transisthmian geminates were also found to have split prior to the closure of the isthmus. The (*A. spinosa*, (*A. aspera*, *A. paula*)) clade had a mean TMRCA of 7.7 mya and the (*A. medusa*, (*A. maria*, *A. n. sp.*)) clade had mean divergence time of 8.3 mya (Table 5).

For three pairs of terminal taxa, a split after the closure of the Isthmus of Panama could not be rejected. The mean TMRCA for two of those splits, *A. aspera*/*A. paula* and *A. maria*/*A. n. sp.* were similar, 5.3 and 5.7 mya, with lower confidence limits of 2.63 and 2.76 mya, respectively. In contrast, the third split, *A. balanorum*/*A. macrospilus*, was substantially younger, with a mean inferred divergence time of 2.7 mya. The clade to which those two species belong, (*A. hancocki*, (*A. crockeri*, (*A. balanorum*, *A. macrospilus*))) was also inferred to have diverged after the closure of the Isthmus (3.9 mya, but with a lower confidence limit of 1.9 mya).

3.6.2. Concatenated tree

The time-calibrated estimate of the phylogeny from the concatenated dataset yielded a well-supported phylogeny that was congruent with the species tree, both in topology and support, as well as divergence times of major clades and splits (Fig. 4 and Table 5). For the splits and clades that were shared between the species tree and concatenated analyses (i.e. all interspecific splits) divergence dates were in agreement. However, there was a trend towards older divergence estimates from the species tree analysis compared to the concatenated analysis, although it was not significant (Table 5).

The concatenated analysis revealed substantial divergence times among populations for six nominal species: *A. chaplini*, *A. rivasi*, *A. medusa*, *A. maria*, *A. paula*, and *A. spinosa*, where the mean TMRCA between populations within species was at least 1 mya for all taxa (except *A. rivasi* at 0.97 mya). The most extreme example comes from *A. chaplini*. Individuals sampled from Bocas del Toro, Panama and the Abacos in northwest Bahamas were deeply diverged from *A. chaplini* sampled from New Providence, in the central Bahamas (Fig. 4). The mean TMRCA for the intraspecific split in *A. chaplini* was 5.06 mya, with lower and upper HPDs of 2.77 and 7.95 mya, respectively (Table 5). This split time was significantly older than the one between the *A. chaplini* individuals from Panama and the northwest Bahamas (Table 5 and Fig. 4). As opposed to *A. chaplini*, the other species with substantial intraspecific divergence did not have significantly different split times between populations.

4. Discussion

4.1. Acanthemblemaria – molecules versus morphology

Our phylogenetic reconstruction of the genus *Acanthemblemaria* based on molecular data was in significant conflict with the phylogenetic estimate of the group based on morphological data (Figs. 1 and 2). Our results also conflicted with those from a recent total evidence analysis of relationships within the Chaenopsidae based on one mitochondrial marker, four nuclear markers and 148 morphological characters (Lin and Hastings, 2011). That study sampled fewer species within *Acanthemblemaria* and because of conflicts among genetic markers the morphological signal was dominant within this portion of the chaenopsid phylogeny (Lin and Hastings, 2011, Fig. 6) resulting in a hypothesis of relationships resembling the morphological analysis of Hastings (1990).

Two species were responsible for most of the conflict between the molecular and morphological phylogenies – *A. medusa* and *A. spinosa* (Fig. 2). *A. spinosa* (the "spinyhead blenny") has an elaborate suite of spinous processes on several bones of the head. *A. maria* has the most elaborate spinous processes in the group

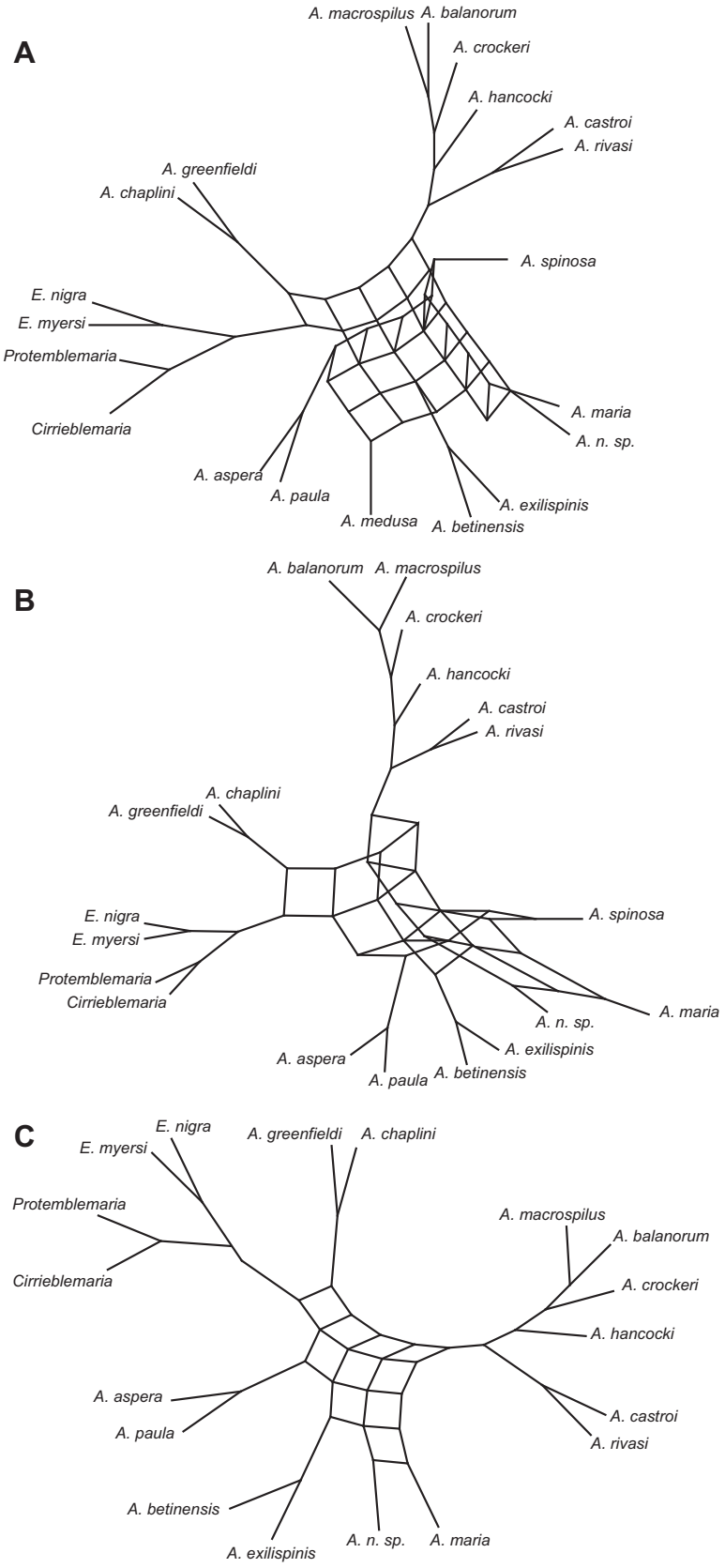

Fig. 2. Split networks of the morphological tree with the species tree. Splits that agreed between the two trees are indicated by strictly bifurcating splits. Conflicting splits are represented as a network of edges. (A) Split network for all taxa. (B) Split network after the removal *A. medusa*. (C) Split network after the removal of *A. medusa* and *A. spinosa*.

Table 4
(A–D) The list of characters found which support or conflict with the placement of *A. spinosa* and *A. medusa* in the morphological phylogeny. Character numbers, names, and states are from the morphological data matrix used in this study, which was based on Hastings (1990).

Character number	Character names and states
A. Characters supporting A. maria/A. spinosa split	
4	Lateral supratemporal ridge: spines present medially
5	Posterior extent of the frontal ridge: to lateral supratemporal ridge
7	Anterolateral extent of the frontal ridge: confluent with the dorsoposterior margin of the postorbital
27	Orbital margin of the postorbital: serrations or spines present
30	Dorso-posterior margin of the postorbital: a row of laterally projecting spines present, contiguous with a row of spines on the frontal wedge
48	Shape of the proximal dorsal-fin pterygiophores (at the level of the mid-spinous dorsal fin): a single central strut present with a flat sheet of bone both anteriorly and posteriorly
B. Characters incompatible with A. maria/A. spinosa split	
8	Central area of the frontal wedge: an open swath with no spines or ridges present (*maria*) OR spines or ridges present (*spinosa*)
31	Shape of the junction of the circumorbitals: entire, the lacrimal and postorbital both extending to the posterior angle (*spinosa*) OR the postorbital excluded from the posterior angle of the circumorbitals (*maria*)
42	Neural spur, a lateral projection on the anterior portion of the neural arch: present on one to four caudal vertebrae (*spinosa*) OR absent from all caudal vertebrae (*maria*)
47	Posterior inner margin of the pelvis: no ossified threads present (*spinosa*) OR two central threads of bone present (*maria*)
56	Modal number of common pores: one (*spinosa*) OR two or more (*maria*)
57	Posterior pair of anterofrontal pores: fused into a single medial pore (*spinosa*) OR separate (*maria*)
C. Characters supporting A. medusa as part of the "aspera species group"	
21	Ventral margin of the lacrimal: three or four blades present
23	Ventral margin of the lacrimal at the third anterior infraorbital pore: a distinct notch present
D. Characters incompatible with A. medusa as part of the "aspera species group"	
1	Anterior margin of the nasal bones: smooth (medusa and aspera) OR spines or serrations present (paula)
7	Anterolateral extent of the frontal ridge:confluent with the middle of the supraorbital flange, at or anterior to the second supraorbital sensory pore (medusa and aspera) OR confluent with the lateral edge of the supraorbital flange, at or posterior to the first supraorbital sensory pore (SOl) but anterior to the frontal/postorbital juncture (paula)
8	Central area of the frontal wedge: an open swath with no spines or ridges present (aspera and medusa) OR spines or ridges present (paula)
44	Epipleural ribs: present on all precaudal vertebrae (within one before to one after the last precaudal vertebra) (medusa and paula) OR absent from two or more posterior precaudal vertebrae (aspera)
45	Hypural five: ossified, autogenous (paula) OR unossified or not autogenous (aspera and medusa) (Pleisomorphic condition uncertain)

(Böhlke, 1961) and a gross skull morphology similar to *A. spinosa* (Smith-Vaniz and Palacio, 1974). Analyses based on morphological data recovered *A. maria* as the sister species to *A. spinosa*, both in this study (Fig. 1C), and in Hastings (1990) (Fig. 1B). This inferred sister relationship between *A. maria* and *A. spinosa* was, unexpectedly, not reflected in the genetically-based species tree, where *A. spinosa* was recovered as sister to *A. aspera* and *A. paula* (Fig. 1A).

The *A. maria*/*A. spinosa* clade recovered from the analyses of the morphological data was supported by six characters (Table 4A). Five of these come from three bones in the skull: the frontals and the two infraorbitals (Table 4A and Hastings (1990)). These may be functionally constrained in an unknown way, may share a common developmental pathway, or the character states could have been scored erroneously by Hastings (1990).

Six morphological characters were incompatible with an *A. maria* and *A. spinosa* clade (Table 4B). A parsimony analysis of these six characters recovered the clade (*A. aspera*, *A. paula*, *A. spinosa*), which was also found in the species tree analysis (Fig. 1A). However, in contrast to the species tree, the parsimony analysis recovered *A. spinosa* sister to *A. paula*, with *A. aspera* sister to these two taxa (not shown). Only one of those six characters relates to spines (Table 4B) and its state is shared by *A. paula* and *A. spinosa* (Hastings, 1990). Taken together with the convergent character states of skull bones in *A. maria* and *A. spinosa*, this result gives credence to the idea that suites of characters relating to spinous processes have evolved multiple times in *Acanthemblemaria*. These results suggest that although there was strong support in the morphological data for the sister relationship of *A. maria* and *A. spinosa*, there was also some support for the (*A. aspera*, *A. paula*, *A. spinosa*) clade, but it got "outvoted" in the morphological analyses.

In contrast to *A. spinosa*, the placement of *A. medusa* in the morphological analyses does not appear to be caused by convergence. The morphological phylogeny places *A. medusa* sister to *A. aspera* and *A. paula*, in the "*aspera* species group" (Fig. 1A and B, and Hastings, 1990). This group is supported by two synapomorphies, both related to the lacrimal bone (Table 4C). However, more characters did not support the "*aspera* species group" than did; five in total (Table 4D). When parsimony trees were constructed using these five characters, the clade found in the species tree (*A. maria*, *A. medusa*, *A. n. sp.*) was not recovered (not shown). These results show that there was not strong support for the "*aspera* species group" *sensu* Hastings (1990) in the morphological data. However, in contrast to *A. spinosa*, there was little if any support for an alternate placement of *A. medusa*.

Suites of characters can cause substantial errors in phylogenetic analyses based on morphology because they can create the illusion that relationships are supported by more independent characters than is the case. Known suites of correlated characters point to the role of natural selection in the repeated evolution of functionally adaptive phenotypes and/or the role of common developmental mechanisms (Emerson, 1982; Emerson and Hastings, 1998; Holland et al., 2010; McCracken et al., 1999).

The function of the spinous processes on the skull bones of *Acanthemblemaria* is not known. *Acanthemblemaria* blennies spend most of their lives in vacated invertebrate holes (Böhlke, 1957; Böhlke and Chaplin, 1993). As such, the heads of these fishes are frequently the only exposed part of their bodies and thus likely targets for (possibly convergent) selective pressure. Skull morphology does not appear to be important in feeding behavior, nor does it influence predation success (Clarke et al., 2009, 2005; Finelli et al., 2009). There may be selection for skulls that efficiently block the blenny shelters as a means of defense against predators (Lindquist and Kotrschal, 1987). Defense against conspecifics seems more likely, as shelters may be limiting (Hastings and Galland, 2010) and *A. spinosa* individuals can use the head spines to wedge

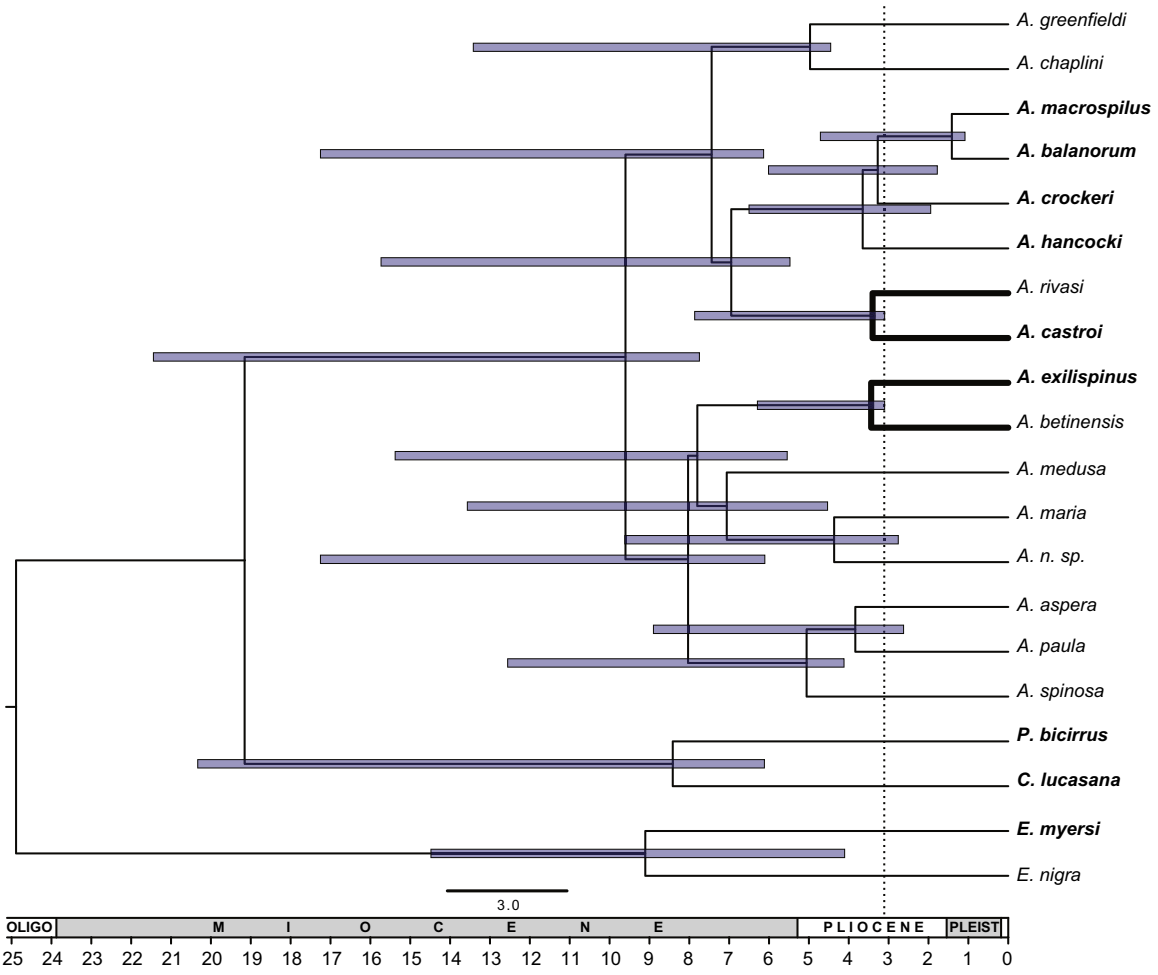

Fig. 3. Time-calibrated species tree, with branch lengths in units of millions of years. Support values for the species tree are the same as those from Figure 1A. Node bars indicate the 95% upper and lower HPDs for node heights. The vertical dashed line indicates the final closure of the Isthmus of Panama, 3.1 mya.

themselves into shelters and temporarily prevent extraction by larger conspecifics (PAH, pers. observ.).

It seems unlikely that *A. maria* and *A. spinosa* are subject to exactly the same selective pressures. *A. maria* occurs in high-energy environments on the reef crest or in shallow water and generally does not live in live or standing dead corals, nor does it shelter in holes high up off the reef substrate (Clarke, 1994; Greenfield, 1981; Greenfield and Johnson, 1990); Eytan and Hellberg, unpub. data). *A. spinosa*, on the other hand, is found in deeper, lower energy sections of the reef, typically in live or standing dead coral not close to the reef substrate (Clarke, 1989, 1994, 1996; Greenfield and Greenfield, 1982; Eytan and Hellberg, unpub. data).

Alternatively, convergence in the skull spines of *A. maria* and *A. spinosa* may have arisen due to a common pattern of heterochrony. All *Acanthemblemaria* species have spinous processes on the frontal bones, but with differences in the degree of spination. A common pathway could underlie the development of spines in all species and different phenotypes arise due to differences in developmental timing. In the case of *A. maria* and *A. spinosa*, hypermorphosis, where there is a delay in the offset of a developmental process, could give rise to the extreme spination found in these species. As suggested by Emerson and Hastings (1998), this could be tested by studying the ontogenetic trajectory of spine development in a number of different *Acanthemblemaria* species to determine the onset and offset of these traits.

4.2. Acanthemblemaria diversity

Our results demonstrate that *Acanthemblemaria* species diversity is presently under-described. The molecular phylogenies inferred in this study supported the inclusion of the undescribed species from Isla Margarita (*A. n. sp.*) as belonging to *Acanthemblemaria* (Figs. 1A and 4). In addition, two other lineages were identified as possible undescribed taxa. The first represents a population of *A. rivasi* from coastal Venezuela. Acero (1984) noted diagnosable differences between *A. rivasi* populations from the southern and southwestern Caribbean and those from Central America, where the species was originally described by Stephens (1970). Acero found that *A. rivasi* individuals from Colombia and Venezuela have significantly different numbers of total dorsal fin and segmented anal fin elements from those in Costa Rica and Panama. In addition, individuals from Venezuela have a pattern of bright blue dots on the head that is less prominent in Central American populations. These meristic and color differences between *A. rivasi* populations, together with the reciprocal monophyly of Venezuelan and Panamanian *A. rivasi* populations based on the concatenated dataset (Fig. 4), the population in coastal Venezuela likely represents an undescribed species.

Another undescribed species, sister to *A. chaplini*, was found in the concatenated phylogeny. *A. chaplini* from New Providence, Bahamas, was recovered as sister to *A. chaplini* individuals from the Abacos in the Bahamas and Panama (Fig. 4). These last two

Table 5
Estimated divergence times for selected nodes in the species tree (top) and concatenated tree (bottom). All times are in millions of years and bold values indicate splits inferred to have occurred prior to the final closure of the Isthmus of Panama, 3.1 mya.

Node	Mean	Lower 95 HPD	Upper 95 HPD
Species tree divergence times			
Root	**29.07**	**16.74**	**47.97**
Acanthemblemaria	**13.15**	**7.75**	**21.44**
A. betinenis/*A. exilispinus*	**4.16**	**3.1**	**6.29**
A. castroi/*A. rivasi*	**4.63**	**3.1**	**7.31**
A. spinosa (*A. aspera*, *A. paula*)	**7.71**	**4.12**	**12.56**
A. chaplini/*A. greenfieldi*	**8.2**	**4.45**	**13.42**
"barnacle blennies"	**9.63**	**5.47**	**15.73**
A. aspera/*A. paula*	5.33	2.63	8.9
Clade I	**10.53**	**6.14**	**17.25**
Clade II	**10.74**	**6.14**	**17.25**
Ekemblemaria myersi/*E. nigra*	**8.46**	**4.12**	**14.46**
Cirriemblemaria lucasana/ *Protemblemaria bicirrus*	**12.08**	**6.04**	**20.44**
A. maria/*A. n. sp.*	5.74	2.76	9.62
A. medusa, (A. maria/*A. n. sp.)*	**8.31**	**4.53**	**13.57**
"hancocki species group"	3.91	1.94	6.5
A. balanorum/*A. macrospilus*	2.71	1.08	4.71
A. crockeri, (A. balanorum/*A. macrospilus)*	3.54	1.78	6.01
A. medusa, A. n. sp., A. maria, A. exiispinus, A. betinensis	**9.6**	**5.54**	**15.38**
Concatenated tree divergence times			
Root	**24.93**	**14.82**	**38.06**
Acanthemblemaria	**11.47**	**7.11**	**17.4**
A. betinenis/*A. exilispinus*	**3.96**	**3.1**	**5.72**
A. spinosa (*A. aspera*, *A. paula*)	**7.17**	**4.25**	**10.98**
"barnacle blennies"	**8.61**	**5.35**	**13.12**
A. aspera/*A. paula*	5.1	2.94	7.99
Clade I	**9.7**	**6.08**	**14.81**
Clade II	**9.4**	**5.79**	**14.24**
Ekemblemaria myersi/*E. nigra*	**7.49**	**3.97**	**11.96**
Cirriemblemaria lucasana/*Protemblemaria bicirrus*	**10.97**	**5.99**	**17.3**
A. maria/*A. n. sp.*	5.46	3.06	8.55
A. medusa, (A. maria/*A. n. sp.)*	7.61	4.56	11.67
"hancocki species group"	3.66	2.01	5.73
A. balanorum/*A. macrospilus*	2.61	1.32	4.16
A. crockeri, (A. balanorum/*A. macrospilus)*	3.2	1.73	5.03
A. medusa, A. n. sp., A. maria, A. exiispinus, A. betinensis	**8.75**	**5.43**	**13.3**
A. paula TMRCA	1.45	0.62	2.46
A. aspera TMRCA	0.58	0.13	1.16
A. spinosa TMRCA	1.61	0.7	2.7
A. maria TMRCA	1.14	0.47	1.96
A. medusa TMRCA	1.39	0.55	2.44
A. cf. chaplini TMRCA	0.72	0.21	1.37
A. rivasi/*A. cf. rivasi*	0.97	0.38	1.71
A. rivasi s.l./*A. castroi*	**4.36**	**3.1**	**6.57**
A. chaplini/*A. cf. chaplini*	5.06	2.77	7.95
A. chaplini s.l./*A. greenfieldi*	**7.64**	**4.54**	**11.74**

were separated from the New Providence individual by a long branch, with a mean TMRCA of 5 my, which was deeper than that of some nominal congeners (Table 2 and Fig. 4). This is despite the much greater distance between the Abacos and Panama (~2000 km) than the Abacos and New Providence (~130 km). The Abacos and New Providence are separated by the deep waters of the Northeast Providence Channel, which may help maintain the deep genetic divergence between the two populations. However, the Caribbean Sea between the Bahamas and Panama is not shallow, discounting the possibility that water depth alone is responsible for the isolation of these lineages.

Because New Providence is the type locality for *A. chaplini* (Böhlke, 1957), the individuals from the Abacos and Panama should be described as a new species. A species similar to *A. chaplini*, *A. cubana*, was recently described from Cuba (Garrido and Varela, 2008). *A. cubana* lives in sympatry with *A. chaplini* on Cuban reefs and is distinguished from the latter by slight differences in papillae. Given the slight differences between *A. cubana* and *A. chaplini*, it is not clear if the former is a valid species. However, those subtle differences may represent a deeply divergent lineage, such as the one we found in this study. Without further examination it is difficult to determine the validity of *A. cubana*, whether it represents one of the two lineages we have sampled here, or if it belongs to a third, unsampled, lineage. To resolve this, sympatric *A. cubana* and *A. chaplini* individuals should be collected and analyzed genetically.

4.3. Biogeography and timing of speciation in Acanthemblemaria

Our divergence dating recovered a mid-Miocene origin for the genus *Acanthemblemaria* and extant species pairs were found to have diverged both before and after the closure of the Isthmus of Panama (Figs. 3 and 4). In addition, we found that sister taxa had a variety of geographic distributions, from broadly sympatric to completely allopatric (Figs. 5 and Hastings, 2009).

The Isthmus of Panama has long been recognized as a major driver of allopatric marine speciation in the Neotropics (Hastings, 2000, 2009; Jordan, 1908; Knowlton et al., 1993; Lessios, 2008; Lessios et al., 2001). However, its importance in the diversification of reef fishes has not been consistent across groups. Taylor and Hellberg (2005) found that for the Neotropical goby genus *Elacatinus*, the Isthmus of Panama was associated with two splits and that

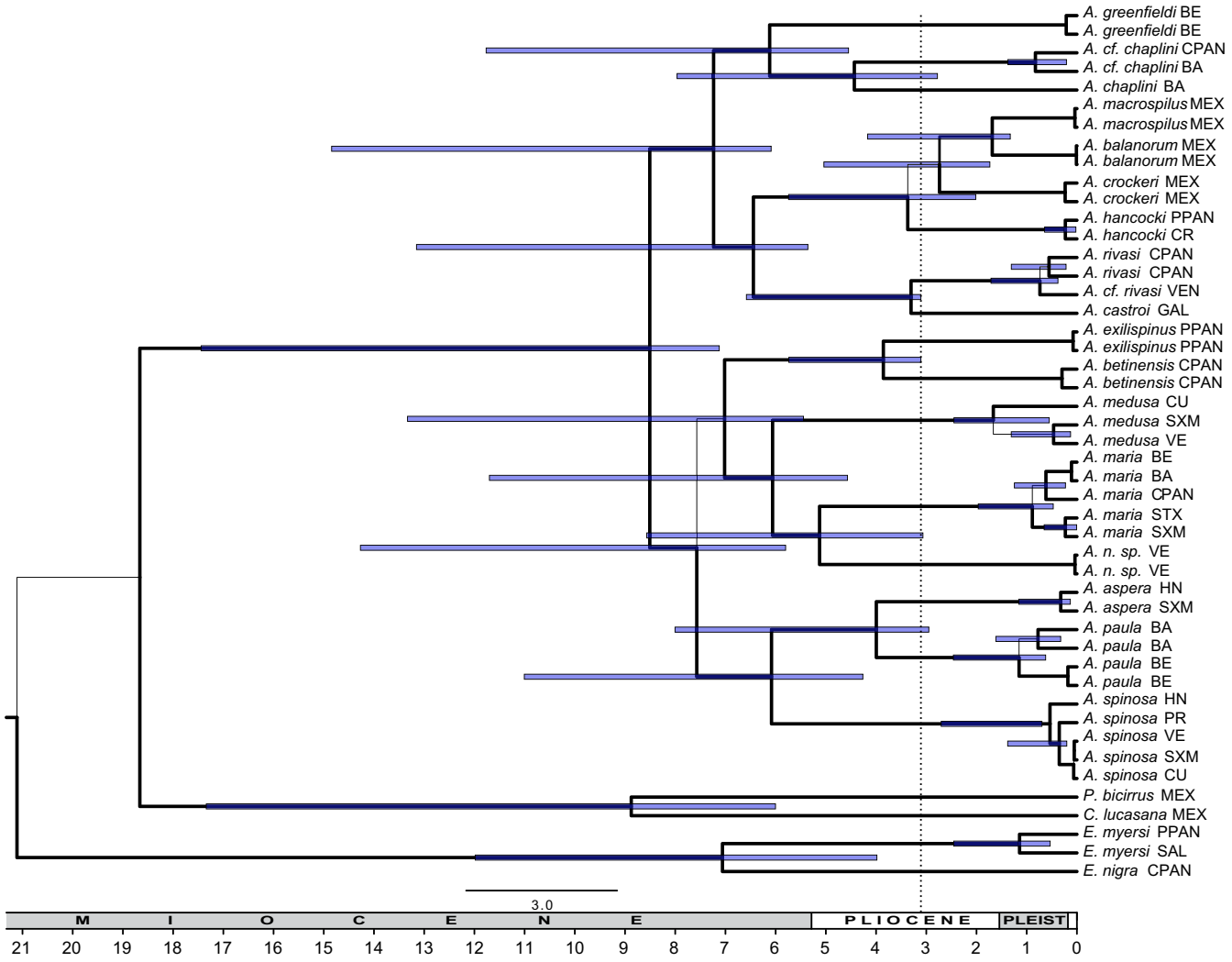

Fig. 4. Time-calibrated Bayesian phylogeny of the concatenated dataset, with branch lengths in units of millions of years. Branches subtending nodes with <0.95 BPP are light; all others are bold. Node bars indicate the upper and lower 95% HPDs for node heights and the vertical dashed line indicates the final closure of the Isthmus of Panama, 3.1 mya. Locality abbreviations are listed after species names and are as follows: BA: Bahamas, BE: Belize, CPAN: Caribbean Panama, CR: Costa Rica, CU: Curaçao, GAL: Galapagos, HN: Honduras, MEX: Pacific Mexico, PPAN: Pacific Panama, PR: Puerto Rico, SAL: El Salvador, STX: St. Croix, SXM: Saint Maarten, VE: Venezuela. All locality information can be found in the Appendix.

no sister taxa were transisthmian geminates. Instead, the *Risor* clade was divided by the Isthmus, as was a basal *Elacatinus* species, which was sister to the rest of the genus. Likewise, Rocha et al. (2008) found that for *Haemulon* grunts there was limited support for the Isthmus playing a role in generating diversity. A single pair of geminate taxa was recovered in their analysis, while two pairs of taxa proposed by Jordan (1908) to be geminates were not. However, they did recover sister clades sundered by the Isthmus (Rocha et al., 2008). Craig et al. (2004) reported findings similar to Rocha et al. and cautioned that inadequate taxon sampling my lead to erroneous conclusions regarding the role of the closure of the Isthmus in recent speciation events.

Our results are similar to these three studies, but with a more complicated pattern. We recovered two pairs of geminate taxa: *A. betinensis* and *A. exilispinus*, and *A. castroi* and *A. rivasi* (Figs. 1, 3 and 4). Both pairs were sister to other clades or pairs of species, and neither was basal in the phylogeny. We also recovered a basal split in Clade I between *A. greenfieldi* and *A. chaplini* and the "*hancocki* species group". Therefore, the Caribbean taxa were not monophyletic. This split in Clade I was quite old, with a mean TMRCA of 10.5 my and 9.7 my, respectively, and matched the TMRCA of Clade II (Table 2). The sister relationship between *A. greenfieldi* and *A. chaplini* and the "*hancocki* species group" was surprising, as they are well separated by morphology and by distribution (Hastings, 1990; Smith-Vaniz and Palacio, 1974). Given the age of this split and difference between these species, Clade I may have been larger in the past, with subsequent extinctions, as suggested by the distributions of *A. chaplini* and *A. greenfieldi* (see below).

Both Taylor and Hellberg (2005) and Rocha et al. (2008) found that the majority of taxa in their studies diversified within ocean basins. However, the geography of speciation differed between *Elacatinus* and *Haemulon*. Taylor and Hellberg (2005) found that Caribbean *Elacatinus* species diversified in allopatry and that sister taxa had either allopatric or micro-allopatric distributions. In contrast, Rocha et al. (2008) found that most sister taxa and closely related species had sympatric distributions.

In this study, we found a combination of both patterns. The distributions of sister taxa and sister clades overlapped substantially in some cases, while others were allopatric (Fig. 5). The three Caribbean clades (*A. spinosa*, (*A. aspera*, *A. paula*); *A. medusa*, (*A. maria*, *A. n. sp.*); *A. chaplini*, *A. greenfieldi*) varied in their extent of

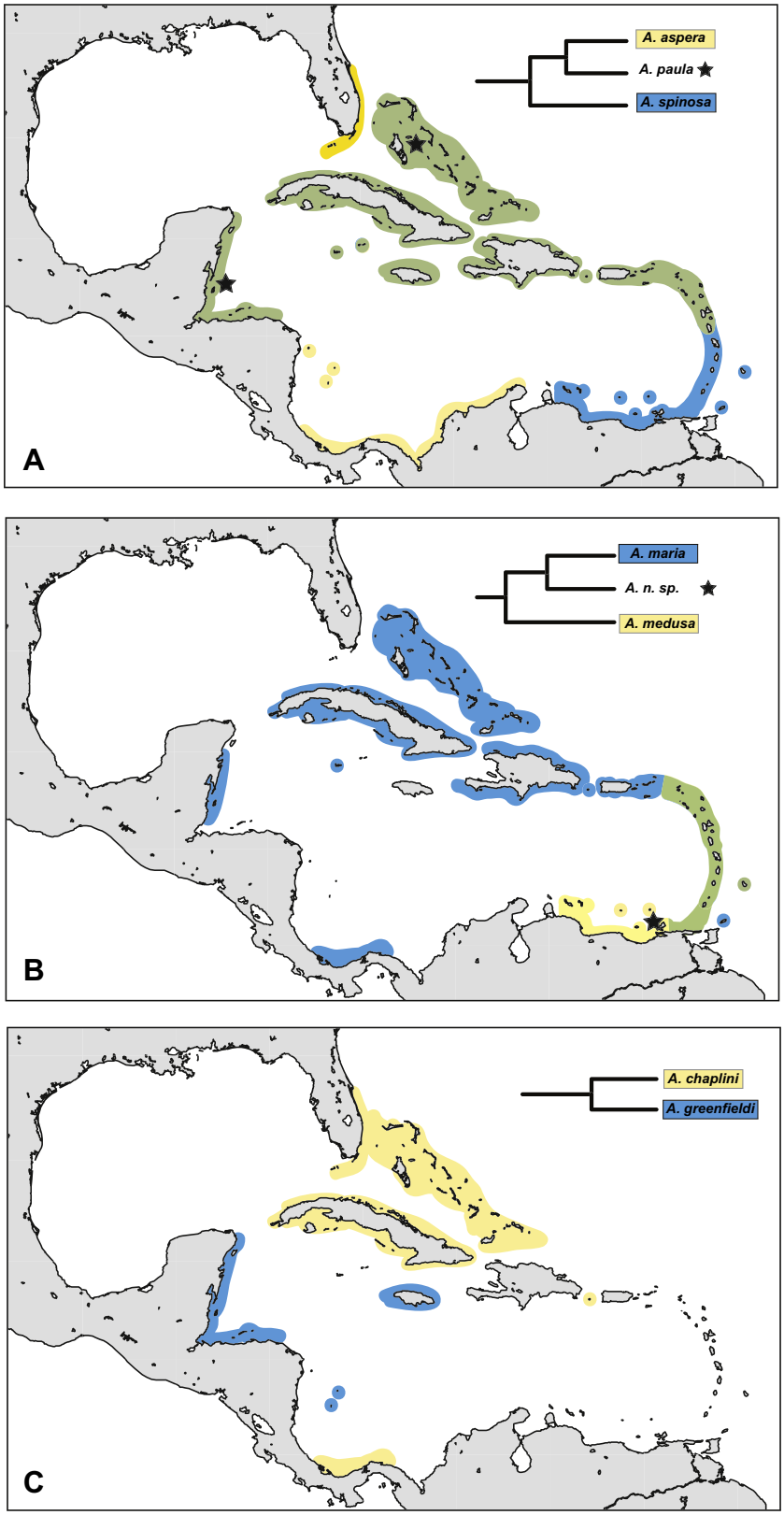

Fig. 5. The confirmed distributions (in yellow, blue, or a star for point localities) and degree of range overlap (in green) for the three Caribbean clades. (A) *A. spinosa*, (*A. aspera*, *A. paula*). (B) *A. medusa*, (*A. maria*, *Acan. n. sp.*). (C) *A. chaplini*, *A. greenfieldi*. Distribution information comes from Smith-Vaniz and Palacio (1974), Dennis et al. (2004, 2005), Hastings and Robertson (1999b), as well as subsequent examination of museum specimens and personal observations by RIE. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

range overlap (Fig. 5). The species in the *A. spinosa*, (*A. aspera*, *A. paula*) clade had the largest degree of range overlap (Fig. 5A). *A. aspera* and *A. spinosa* co-occur over a large portion of their respective ranges. *A. paula* was found in close sympatry with these species in two locations: the Belizean barrier reef and New Providence in the Bahamas. Since its description, *A. paula* has been considered a

micro-endemic species, thought to only occur in a small area in Belize (Hastings, 2009; Johnson and Brothers, 1989). The species is very small (18 mm maximum standard length), lays few eggs (less than five per brood), and is a habitat specialist (Clarke, 1994; Greenfield and Greenfield, 1982; Johnson and Brothers, 1989), giving credence to the idea that its ability to colonize new regions is poor. Here we document a 1500 km range extension for the species, showing that *A. paula*'s distribution is much larger than previously thought.

A. aspera, *A. paula*, and *A. spinosa* demonstrate fine scale habitat partitioning where they co-occur. In Belize, each species is found on a different section of the reef, spanning a depth gradient from ~<1–6 m in *A. paula*, 3–12 m in *A. spinosa*, and 5–22 m in *A. aspera* (Clarke, 1994; Eytan and Hellberg, unpub. data). Where they co-occur, these species partition out the substrate by hole size, coral type, and shelter height, in some cases all co-occurring on the same stand of coral (Clarke, 1994; Eytan and Hellberg, unpub. data). This fine scale partitioning could be an example of ecological character displacement permitting co-existence of closely related species (Bay et al., 2001; Robertson, 1996). Alternatively, these species may have diverged in parapatry with disruptive selection due to competition for shelters driving speciation. However, evidence to support either hypothesis is lacking, and further study is warranted to address this question.

The *A. medusa*, (*A. maria*, *Acan. n. sp.*) clade also had a broad distribution and often overlapping ranges, but sister taxa do not. *A. n. sp*, recovered as sister to *A. maria*, has never been recorded east of Isla Margarita (Ramjohn, 1999), nor has it been recorded as far west as Los Roques, Venezuela (Cervigón, 1991), suggesting that its distribution may be quite restricted, as it is only known from a small area. Additional sampling may change this, though. While its range is close to that of *A. maria*, the two taxa do not overlap, but have abutting distributions (Fig. 5A).

The sister pair of *A. chaplini* and *A. greenfieldi* exist in complete allopatry with disjunct ranges (Fig. 5C). *A. chaplini* is found in Florida and the Bahamas, as well as further south in Panama (Hastings and Robertson, 1999b). Meanwhile, *A. greenfieldi* is found in the central and western Caribbean, in between the two regions where *A. chaplini* is found. A Panama–Florida distributional tract may not be uncommon though, as it has been found in *Elacatinus* gobies (Taylor and Hellberg, 2005, 2006), several peripheral freshwater fishes (Gilmore and Hastings, 1983), and in the coral *Acropora palmata* (Baums et al., 2005). These two species have the oldest divergence time of any *Acanthemblemaria* sister taxa (Figs. 3 and 4, Table 2). It may be that extensive extinctions have occurred since these taxa split, perhaps in the eastern Caribbean or Caribbean coast of South America, resulting in the observed allopatric distributions.

In contrast to the old split between *A. chaplini* and *A. greenfieldi*, the "*hancocki* species group" in the eastern Pacific is a young clade. The mean TMRCA of the included taxa in the group was estimated to be 3.91 or 3.66 my for the species tree and concatenated analyses, respectively (Table 2). However, the lower 95% HPD was as young as 1.9 mya. This suggests diversification of this species group occurred after the closure of the Isthmus of Panama. The sister taxa in this group, *A. macrospilus* and *A. balanorum* occur in sympatry in southern Mexico (Fig. 2.1.2 in Hastings, 2009). In the Gulf of California, *A. balanorum* partially overlaps the range of the recently described sister species of *A. macrospilus* (*A. hastingsi* Lin and Galland, 2010; not included in this study). As in the *A. spinosa*, (*A. aspera*, *A. paula*) clade, where *A. hastingsi* and *A. balanorum* co-occur, they partition out the available habitat along a depth gradient (Lindquist, 1985).

Determining the geography of speciation for any taxonomic group is difficult because current species distributions may not reflect those at the time of speciation (Losos and Glor, 2003). In the case of *Acanthemblemaria*, this is exacerbated by evidence that extinction (Clarke, 1996; Eytan and Hellberg, 2010), poorly known geographic ranges (Dennis et al., 2004, 2005; Hastings and Robertson, 1999b; this study), and the presence of cryptic taxa (Hastings, 2009; Hastings and Springer, 2009a; Lin and Galland, 2010; this study) may be common in this genus.

5. Conclusions

In this study, three lineages were recovered as possible new species, which would bring the membership of the genus to 25 taxa and make *Acanthemblemaria* one of the most species-rich clades of Neotropical reef fishes. We found that some of the head spines characteristic of *Acanthemblemaria* have evolved repeatedly, leading to conflict between the morphological and molecular phylogenies of the group. This was typified by *A. spinosa* and *A. maria*, both of which have elaborate spinous processes, but were not recovered as sister to each other in the molecular phylogenetic analyses. Multiple skull bones appear to have evolved in concert, perhaps due to selection acting on constrained developmental pathways. Bayesian divergence dating found that the genus diverged in the mid-Miocene. A complex pattern of clades was recovered, diverging both before and after the closure of the Isthmus of Panama, almost entirely within present-day ocean basins. While several clades have overlapping ranges, most sister taxa occur in allopatry. The exception was the *A. spinosa*, (*A. aspera*, *A. paula*) clade, which exists in sympatry. Fine scale habitat segregation may allow for co-existence of these taxa, and warrants further study.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2011.09.028.

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