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

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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 compared to those from 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.

1.1. 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; 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, ...
1963). Morphological characters related to head spination repre-
sent the majority of the characters used to infer the interspecific
relationships in the group (Hastings, 1990). Recent molecular phy-
logenies of the genus (Eytan, 2010; Lin and Hastings, 2011) recov-
ered Acanthemblemaria as monophyletic, but also recovered con-
licts 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 mor-
phological 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 under-
lying nature of character correlations. This is because a suite of
characters that are highly correlated with one another are ex-
pected to produce the same result as a suite of independent char-
acters with good phylogenetic signal: strong support for a given
clade (Shaffer et al., 1991).

Here we test whether the homoplastic morphological char-
acters related largely to head spination in Acanthemblemaria are cor-
related 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 spe-
cies tree of the genus Acanthemblemaria using five nuclear mark-
ers and employ Bayesian relaxed clock divergence dating to de-
termine the age of the group and timing of speciation among
the members of the genus. We also examine the historical bioge-
ography of the genus, with the aim of elucidating the geography
of speciation in the group. Of particular interest is whether speci-
cation 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 out-
group. Whole fishes were stored individually in 95% ethanol or
are restricted to areas close to the Isthmus (Hastings, 2009).
Ten different partitioning strategies were evaluated for both the
species tree and concatenated analyses. These partitioning stra-
tegies 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 stra-
tegies 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. Conver-
gence 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 as-
sessed 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 condi-
tions 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

\[\text{PCR} \]
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 Galápagos (at most 17 million years ago; Werner and Hoenerle, 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 not available, and were removed from the matrix. Three taxa were not sampled for the species tree analyses, as tissues were not available, and were removed from the matrix. The new matrix was analyzed in a Bayesian framework using MrBayes v.3.1.2 (Ronquist and Huelsenbeck, 2000) 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. Convergents onto the posterior distribution for the model parameters and topology was assessed using ESS.

### Table 1

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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
Hastings. However, the species tree analysis recovered the trans-

Hastings geminates A. castroi and A. rivisi 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

taxa 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 unde-

scribed 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 “han-

cocki species group”. Also, as in Hastings (1990), the morpholog-

ical tree inferred here was highly nested, with the same progressions of taxa.

3.3. Identification of correlated incongruent morphological characters

The median size of the maximal clique within the 60 morpho-

logical characters was 24 characters. It is possible to get large cli-

ques of compatible characters by chance, but never as large as the one recovered for the unshuffled data: the shuffled data pro-

duced maximal cliques of size 9–22 (median 16). (Recall that the shuffling procedure of Holland et al. (2010) is guaranteed to pro-

duce 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 morphologi-

cal characters.

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.
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 antero-frontal 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 transisthmiomian gemitines 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 transisthmiomian gemitines 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. balanor um/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 (“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 of A. medusa. (C) Split network after the removal of A. medusa and A. spinosa.
(Böhlike, 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 frontal 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 spinos (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 (Böhlike, 1957; Böhlike 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

### Table 4

<table>
<thead>
<tr>
<th>Character number</th>
<th>Character names and states</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A. Characters supporting A. maria/A. spinosa split</strong></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Lateral supratemporal ridge: spines present medially</td>
</tr>
<tr>
<td>5</td>
<td>Posterior extent of the frontal ridge: to lateral supratemporal ridge</td>
</tr>
<tr>
<td>7</td>
<td>Anterolateral extent of the frontal ridge: confluent with the dorso-posterior margin of the postorbital</td>
</tr>
<tr>
<td>27</td>
<td>Orbital margin of the postorbital: serrations or spines present</td>
</tr>
<tr>
<td>30</td>
<td>Dorso-posterior margin of the postorbital: a row of laterally projecting spines present, contiguous with a row of spines on the frontal wedge</td>
</tr>
<tr>
<td>48</td>
<td>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</td>
</tr>
<tr>
<td><strong>B. Characters incompatible with A. maria/A. spinosa split</strong></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Central area of the frontal wedge: an open swath with no spines or ridges present (maria) OR spines or ridges present (spinosa)</td>
</tr>
<tr>
<td>31</td>
<td>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)</td>
</tr>
<tr>
<td>42</td>
<td>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)</td>
</tr>
<tr>
<td>47</td>
<td>Posterior inner margin of the pelvis: no ossified threads present (spinosa) OR two central threads of bone present (maria)</td>
</tr>
<tr>
<td>56</td>
<td>Modal number of common pores: one (spinosa) OR two or more (maria)</td>
</tr>
<tr>
<td>57</td>
<td>Posterior pair of anterofrontal pores: fused into a single medial pore (spinosa) OR separate (maria)</td>
</tr>
<tr>
<td><strong>C. Characters supporting A. medusa as part of the “aspera species group”</strong></td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>Ventral margin of the lacrimal: three or four blades present</td>
</tr>
<tr>
<td>23</td>
<td>Ventral margin of the lacrimal at the third anterior infraorbital pore: a distinct notch present</td>
</tr>
<tr>
<td><strong>D. Characters incompatible with A. medusa as part of the “aspera species group”</strong></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Anterior margin of the nasal bones: smooth (medusa and aspera) OR spines or serrations present (paula)</td>
</tr>
<tr>
<td>7</td>
<td>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 (SOI) but anterior to the frontal/postorbital juncture (paula)</td>
</tr>
<tr>
<td>8</td>
<td>Central area of the frontal wedge: an open swath with no spines or ridges present (aspera and medusa) OR spines or ridges present (paula)</td>
</tr>
<tr>
<td>44</td>
<td>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)</td>
</tr>
<tr>
<td>45</td>
<td>Hyposal: five: ossified, autogenous (paula) OR unossified or not autogenous (aspera and medusa) (Pleiomorphic condition uncertain)</td>
</tr>
</tbody>
</table>

(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).
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.*). 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
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. cubana, was recently described from Cuba (Garrido and Varela, 2008), A. cubana lives in sympathy 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. 3 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...
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 may 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
range overlap (Fig. 5). The species in the \textit{A. spinosa} (\textit{A. aspera}, \textit{A. paula}) clade had the largest degree of range overlap (Fig. 5A). \textit{A. aspera} and \textit{A. spinosa} co-occur over a large portion of their respective ranges. \textit{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, \textit{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 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 palmenta (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. 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 sympathy. Fine scale habitat segregation may allow for co-existence of these taxa, and warrants further study.

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Appendix A. Supplementary material
