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## Short Communication

Molecular phylogenetics and evolution of *Holacanthus* angelfishes (Pomacanthidae)Yvette Alva-Campbell<sup>a</sup>, Sergio R. Floeter<sup>b</sup>, D. Ross Robertson<sup>c</sup>, David R. Bellwood<sup>d</sup>, Giacomo Bernardi<sup>a,\*</sup><sup>a</sup> Department of Ecology and Evolutionary Biology, University of California, Santa Cruz, CA 95060, USA<sup>b</sup> Departamento de Ecologia e Zoologia, Universidade Federal de Santa Catarina, Florianópolis SC, 88010-970, Brazil<sup>c</sup> Smithsonian Tropical Research Institute, Balboa, Panama<sup>d</sup> Australian Research Council Centre of Excellence for Coral Reef Studies, and School of Marine and Tropical Biology, James Cook University, Qld 4811, Australia

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## ABSTRACT

The angelfish genus *Holacanthus* includes seven species in the tropical Eastern Pacific and Atlantic. In this study we performed an analysis on all species, the closely related regal angelfish, and the Cortez angelfish, using four mitochondrial and one nuclear marker. Our results support a monophyletic *Holacanthus*. The Indo-Pacific regal angelfish, *Pygoplites diacanthus*, was found to be the closest relative to *Holacanthus*. We found a split into two clades with divergences that were consistent with the rise of the Isthmus of Panama. An internally calibrated molecular clock thus placed the origin of *Holacanthus* to ~10.2–7.6 million years ago.

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## 1. Introduction

The angelfish genus *Holacanthus* (family Pomacanthidae) is relatively small with only seven recognized species. *Holacanthus* resides in the tropical portion of three discrete biogeographic regions: the Eastern Atlantic, the Western Atlantic, and the tropical Eastern Pacific (Fig. 1). The West African angelfish, *Holacanthus africanus*, is the only species found along the tropical Eastern Atlantic coastline, in the Gulf of Guinea (São Tomé) and the Cape Verde Islands. Within the Western Atlantic, three species occur on coral and rocky reefs, *H. tricolor*, *H. bermudensis*, and *H. ciliaris*. The rock beauty, *H. tricolor*, and the queen angelfish, *H. ciliaris* have the widest distributions, from Bermuda to Brazil, where different color morphs of *H. ciliaris* can be found on the NE coast and at isolated offshore islands (Luiz, 2003; Feeley et al., 2009). The blue angelfish, *H. bermudensis* has a more restricted distribution, from Bermuda, along the entire Atlantic coast of the US from North Carolina south, and throughout the Gulf of Mexico (Smith-Vaniz et al., 1999). Atlantic *Holacanthus* have traditionally been divided into two separate subgenera, *Angelichthys*, which comprises *H. africanus*, *H. bermudensis*, and *H. ciliaris*, and *Holacanthus* which comprises only *H. tricolor* (Allen et al., 1998; Debelius et al., 2003).

Within the tropical Eastern Pacific, *H. passer*, *H. clarionensis*, and *H. limbaughii* occur on rocky and coral reefs, (Robertson and Allen, 2008) (Fig. 1). The king angelfish, *H. passer*, has the widest distribution, from central Baja California, Mexico, through the Gulf of California south to northern Peru, plus the Revillagigedo (its abundance there is unclear), Cocos, Malpelo, and the Galapagos Islands. Two insular endemic species, the Clipperton angelfish *H. limbaughii*, and the Clarion angelfish, *H. clarionensis*, are found at Clipperton Atoll and the Revillagigedo Islands, respectively. Small numbers, most likely vagrants, of *H. clarionensis* are found at the southern tip of Baja California, while there is a single record of that species at Clipperton Island (Allen and Robertson, 1997). There are no records of *H. limbaughii* from any site other than Clipperton, or of *H. passer* at that island. Traditionally, these three species have been grouped in a single subgenus, *Plitops* (Allen et al., 1998; Debelius et al., 2003).

The rise of the Central American Isthmus separated many populations of marine organisms, with the final closure of the Isthmus of Panama producing geminate pairs of similar-looking species (Jordan, 1908). *Holacanthus* has been described as a potential candidate, with *H. passer* and *H. bermudensis* being the likely geminates (Thomson et al., 2000). In a molecular (mtDNA) study of angelfishes that included four *Holacanthus* species, Bellwood et al. (2004) proposed a phylogenetic hypothesis for the group. That analysis indicated that *H. bermudensis* and *H. passer* were sister species (thus potentially breaking up the subgenera *Plitops* and *Angelichthys*). In addition, that study, which included a thorough representation of most angelfish genera, indicated that the regal

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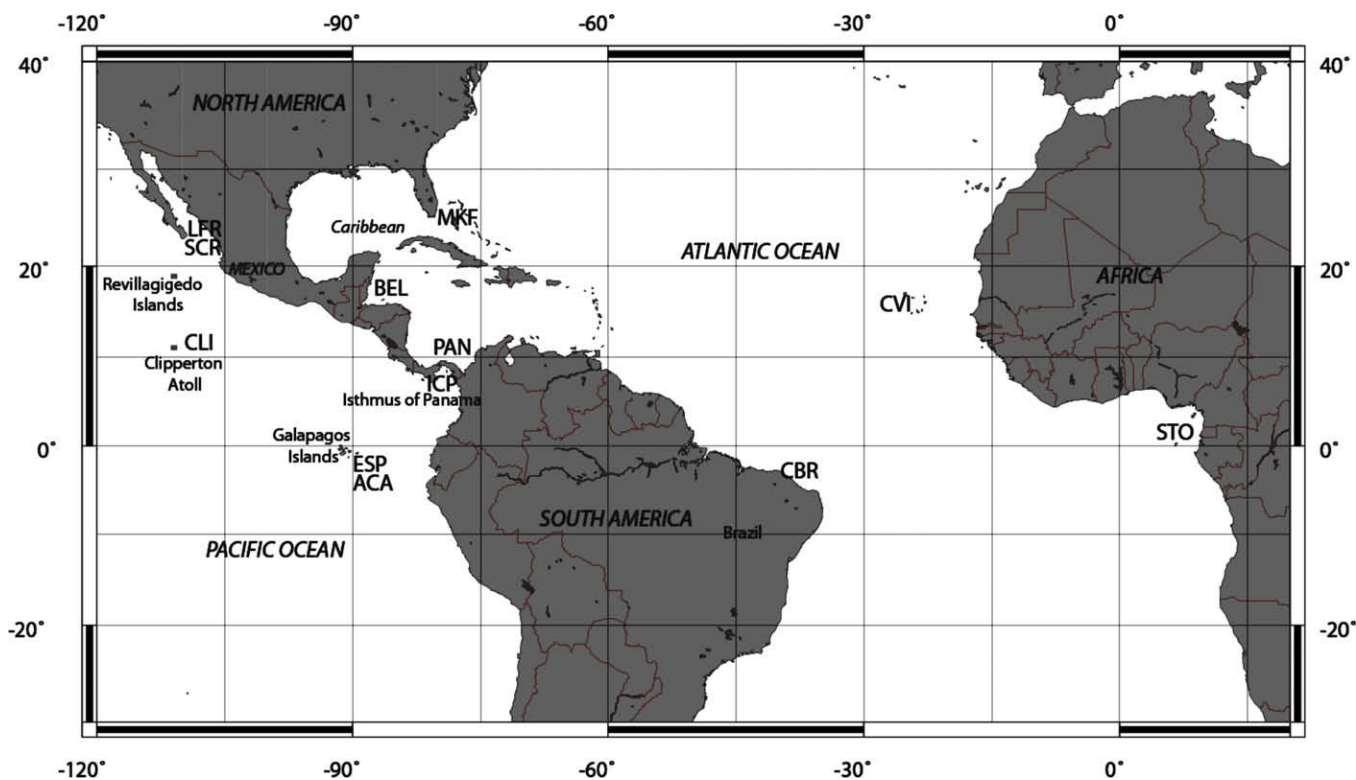


Fig. 1. Distribution map and sampling locations of *Holacanthus*. Labels refer to sampling locations described in Table 1 (map created by OMC, Martin Weinelt).

angelfish, *Pygoplites diacanthus*, the sole member of its genus, is the closest relative of the genus *Holacanthus*, raising the possibility of it being a basal *Holacanthus* (Bellwood et al., 2004), or potentially well within *Holacanthus*, thus disrupting its monophyletic status. Although *Pygoplites diacanthus* was originally described as *Holacanthus diacanthus* (Bleeker, 1857), an early morphological phylogenetic analysis by Shen and Liu (1978) indicated that *Pygoplites* is sister to *Apolemichthys*. A later allozyme comparison done by Chung and Woo (1998) then placed *Pygoplites* closer to *Pomacanthus* than to *Holacanthus* (see Fig. 1 from Bellwood et al., 2004). However, neither of the two molecular studies incorporated sampling of all seven members of *Holacanthus*.

*Pygoplites diacanthus* is widely distributed over the entire Indo-central Pacific. This distribution does not indicate whether *Holacanthus* originated either from the ancestor of *Pygoplites* in the TEP after migrating from the central Pacific, or in West Africa, after colonization from the Indian Ocean. Thus, a complete phylogeny of the genus *Holacanthus* is necessary to elucidate the evolutionary history of this genus.

To achieve this goal, we sampled all seven known *Holacanthus* species (including different color morphs), *P. diacanthus* and the Cortez angelfish, *Pomacanthus zonipectus*, a TEP species, as an outgroup, and used four mitochondrial and one nuclear molecular markers. We first established a complete phylogeny of the genus, and then further investigated the status of geminate groups using the more variable mitochondrial control region marker.

## 2. Materials and methods

### 2.1. Collections and DNA samples

Sampling sizes and locations are listed in Table 1. Preservation and DNA extraction protocols followed Bernardi et al. (2008).

Table 1

Collection localities for *Holacanthus* spp., *Pygoplites diacanthus*, and the outgroup, *Pomacanthus zonipectus*. Columns represent the number of individuals included in the study, and the abbreviations used in Figs. 1 and 2.

Species	Sampling site	n	Label
<i>Holacanthus africanus</i>	Cape Verde Islands	1	CVI
	São Tomé	1	STO
<i>Holacanthus bermudensis</i>	Marathon Key, Florida	3	MKF
<i>Holacanthus ciliaris</i>	Turneffe Atoll, Belize	2	BEL
	Regular morph, Ceará State, Brazil	1	CBR1
	Blue morph, Ceará State, Brazil	1	CBR2
	Yellow morph, Ceará State, Brazil	1	CBR3
	Marathon Key, Florida	1	MKF
<i>Holacanthus clarionensis</i>	Aquarium of the Pacific	1	APA
<i>Holacanthus limbaughii</i>	Clipperton Atoll	6	CLI
<i>Holacanthus tricolor</i>	Ceará State, Brazil	1	CBR
	San Blas, Panama	4	PAN
	Marathon Key, Florida	1	MKF
	Isla Contadora, Panama	1	ICP
	Santa Cruz, Mexico	2	SCR
<i>Holacanthus passer</i>	Los Frailes, Mexico	2	LFR
	Espanola, Galapagos, Ecuador	1	ESP
	Santa Cruz, Galapagos, Ecuador	1	ACA
Outgroups			
<i>Pomacanthus zonipectus</i>	Isla Contadora, Panama	1	RCP
<i>Pygoplites diacanthus</i>	Moorea, French Polynesia	3	MOO

### 2.2. PCR amplification and sequencing

Amplifications of 12S rRNA, 16S rRNA, cytochrome *b*, and control region segments were performed using the universal primers 12SAL-12SBH, 16SAR-16SBR, GLUDGL-CB3H, and CRA-CRE, respectively (Palumbi et al., 1991; Lee et al., 1995) with 35 cycles at a denaturation temperature of 94 °C for 30 s, an annealing temperature of 52–54 °C, and an extension of 30 s at 72 °C. Amplification of the 1st intron of the nuclear S7 ribosomal protein used the primers

S7RPEX1F and S7RPEX2R (Chow and Hazama, 1998) followed similar cycles but with an annealing temperature of 56 °C.

After purification of the PCR products, following the manufacturer's protocol (Applied Biosystems, Foster City, CA), sequencing was performed in both directions with the primers used in the PCR amplification on an ABI 3100 automated sequencer (Applied Biosystems, Foster City, CA). In the case of the nuclear marker, heterozygous individuals were found to be very rare, and when present, only one allele was scored per individual (we did not find individuals with more than one heterozygous site, thus making the calling of the two alleles possible).

### 2.3. Phylogenetic analyses

We used the computer program Clustal V implemented by Sequence Navigator (Applied Biosystems) to align the DNA sequences. Overall, few insertions and deletions (indels) were observed. For 12SrRNA, three indels of one base pair (bp) each were observed, for 16S rRNA six indels accounting for a total of 9 bp, and for S7 fragments, four indels accounting for a total of 14 bp were observed. These indels were removed from the subsequent phylogenetic analyses, yet their inclusion did not change the results. Phylogenetic relationships were assessed by Maximum Likelihood (ML, GARLI software, Zwickl, 2006), Maximum Parsimony (MP, PAUP\* software, Swofford, 2003), and Neighbor-Joining (NJ, PAUP software), methods. For Maximum Likelihood topologies, we conducted 10 independent runs in GARLI, using default settings and the automated stopping criterion, terminating the search when the ln score remained constant for 20,000 consecutive generations. The tree with the highest likelihood was retained and is presented here. MP searches included 100 random addition replicates and TBR branch swapping with the Multrees option. NJ reconstructions used distances based on substitution models obtained with Modeltest (Posada and Crandall, 1998) (HKY + G). Statistical confidence in nodes was evaluated using 2000 non-parametric bootstrap replicates (Felsenstein, 1985) (100 replicates for Maximum Likelihood in GARLI, using the automated stopping criterion set at 10,000 generations). Topological differences were tested using a Shimodaira and Hasegawa test (Shimodaira and Hasegawa, 1999) implemented by PAUP, based on resampling of estimated log-likelihoods tests (RELL, 1000 replicates). Topological differences with or without an enforced molecular clock were tested using a Shimodaira and Hasegawa test (Shimodaira and Hasegawa 1999) implemented by PAUP. Genetic divergence was estimated using distances based on substitution models obtained with Modeltest (HKY + G).

## 3. Results

### 3.1. Sequences

We used two sets of data, one broad set for a phylogenetic analysis and one more restricted set for a detailed analysis of Trans-Isthmian species. The dataset for the broad analysis comprised 2283 aligned base pairs (bp), which included 346, 547, 699 and 692 bp for 12S rRNA, 16S rRNA, cytochrome *b*, and 1st intron of the ribosomal protein S7 fragments, respectively. The dataset for the restricted analysis, based on the mitochondrial control region, was a set of 21 individuals that were representative of Trans-Isthmian geminate species (see Fig. 2B). This dataset comprised 410 aligned base pairs.

### 3.2. Phylogenetic relationships

#### 3.2.1. Broad phylogeny

Phylogenetic relationships based on each molecular marker were found not to be statistically different from each other, thus

we decided to concatenate all markers (three mitochondrial and one nuclear marker). The three reconstruction methods (ML, MP, and NJ) resulted in identical topologies (Fig. 2A shows the maximum likelihood topology). Using *P. zonipectus* as an outgroup, we found that the regal angelfish, *P. diacanthus* was not a member of *Holacanthus*, a result that is consistent with previous findings (Shen and Liu, 1978; Chung and Woo, 1998; Bellwood et al., 2004). Forcing *Pygoplites* inside *Holacanthus* always resulted in a topology that was significantly worse than what is presented in Fig. 2A (SH test,  $p < 0.05$ ).

The West Africa angelfish, *H. africanus*, from São Tomé were not distinct from Cape Verde Islands individuals, and were found to be basal to the other members of the genus (Fig. 2A). The second species branching off in that tree was the rock beauty, *H. tricolor*. Two clades developed after *H. tricolor* branched off, one that included the Caribbean species *H. ciliaris* and *H. bermudensis*, the other included the TEP species, *H. passer*, *H. clarionensis*, and *H. limbaughi*.

Very little genetic differentiation was found among the extreme localities. For example, individuals from Brazil and the Caribbean of *H. tricolor* were genetically indistinguishable (Fig. 2A).

#### 3.2.2. Trans-Isthmian phylogeny

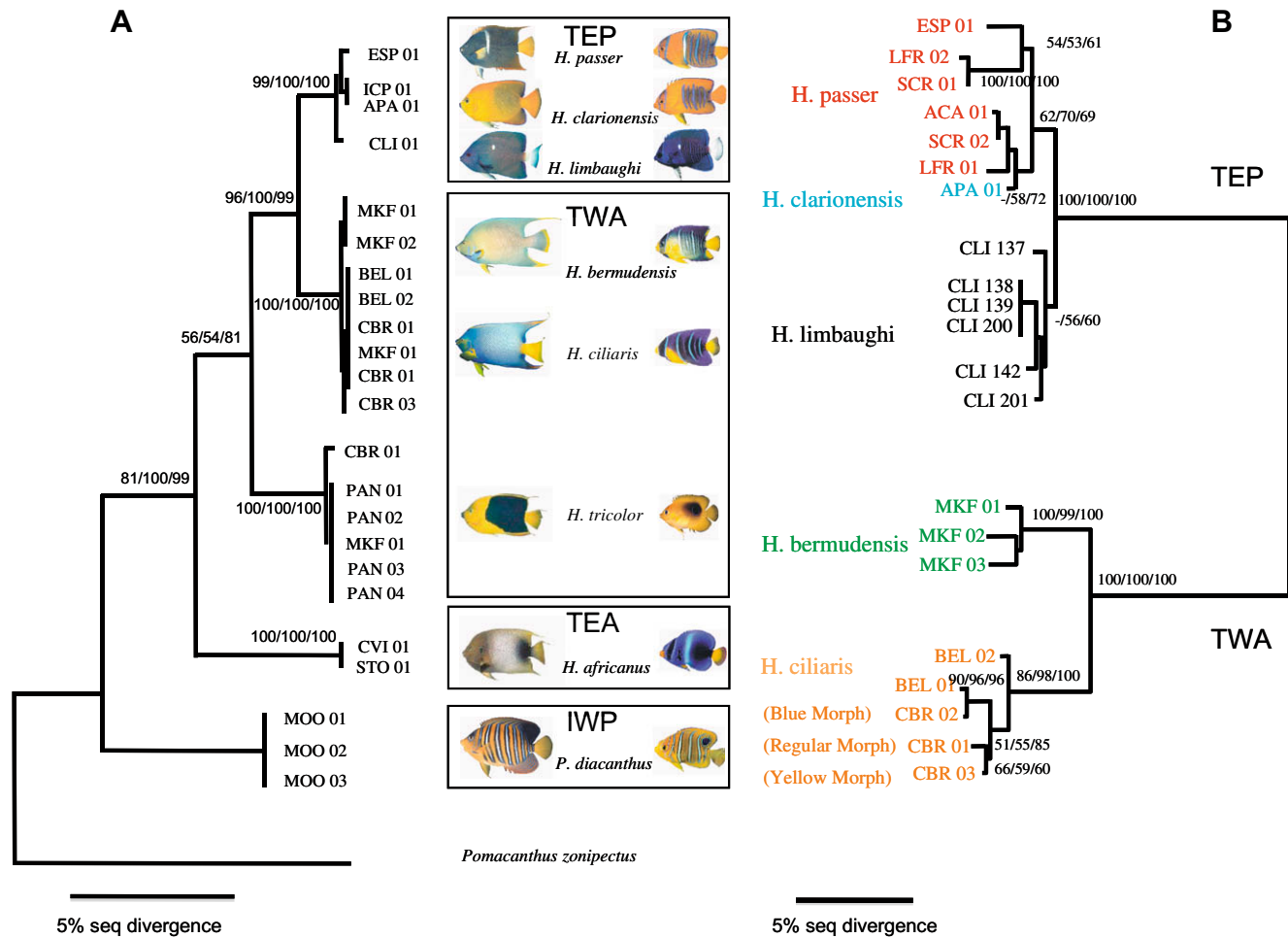
In order to obtain a fine-scale phylogeny for the group of species that occur across the Isthmus of Panama, we decided to use more individuals and the faster mitochondrial control region as a molecular marker. Results obtained with the control region are consistent with results of the wider analysis presented above (Fig. 2B). In the TEP, one clade included *H. passer* and *H. clarionensis*. *Holacanthus clarionensis* did not separate in its own lineage. No coloration or morphological characters indicated that the sequenced individual was a hybrid, yet further work is necessary to determine the genetics of this species. The *passer-clarionensis* clade was sister to all Clipperton angelfish, *H. limbaughi*, which formed a monophyletic group. Considering its very restricted geographic range (Clipperton Atoll) and its known small population size (Robertson, 2001 estimated that there were ~60,000 adults there in 1998), *Holacanthus limbaughi* exhibited a remarkable genetic diversity (four haplotypes out of six individuals).

The West Atlantic clade included *H. bermudensis* and *H. ciliaris*, which formed two reciprocally monophyletic clades. Here, again little intra-specific diversity was found. Different *H. ciliaris* color morphs from NE Brazil (namely 'blue face' and 'yellow face' morphs collected along Ceará State coast) and the Caribbean are very similar (sequences divergence was less than 1%).

### 3.3. Genetic divergence and temporal divergence

A molecular clock could not be rejected for either datasets (Shimodaira and Hasegawa test,  $p = 1.00$  for the first dataset and  $p = 1.00$  for the second dataset). In the case of the mitochondrial control region, average pairwise sequence divergence between the Western Atlantic and TEP clades was 18.7%. A thorough review performed by Lessios (2008) on multiple species of geminate pairs of marine fish found that the species that most likely separated due to the rise of the Isthmus of Panama exhibited a divergence range of 9.7–22%. These values are consistent with the idea that the rise of the Isthmus may have coincided with the divergence of the two Trans-Isthmian *Holacanthus* clades.

When analyzing cytochrome *b* sequences, which are widely used for timing divergences in fishes, the average sequence divergence between the Western Atlantic and the TEP clade was approximately 4.5%. This value again falls within the range of other geminate fish pairs (using the same gene region) that are most likely to have been separated at the closure of the Isthmus of Panama (3.3–4.8%, Lessios, 2008).



**Fig. 2.** (A) Molecular phylogeny of *Holacanthus* spp. and *Pygoplites diacanthus* based on mitochondrial and nuclear molecular markers (12SrRNA, 16S rRNA, cytochrome *b*, 1st intron of ribosomal protein S7) using Maximum Likelihood (Maximum Parsimony, and Neighbor-Joining reconstructions resulted in the same topology). *Pomacanthus zonipectus* was used as an outgroup. Labels are described in Table 1. Bootstrap support (or consensus value) is shown when above 50%, for the three methods used, ML, NJ, and MP, in that order. Percent sequence divergence is represented on the scale bar. (B) Molecular phylogeny of five species of *Holacanthus* that reside in the TEP and TWA based on 410 bp of the mitochondrial control region using Maximum Likelihood. Labels are described in Table 1. Bootstrap support (or consensus value) is shown when above 50%, for the three methods used, ML, NJ, and MP, in that order. Percent sequence divergence is represented on the scale bar.

The average cytochrome *b* sequence divergence between *H. africanus* and the rest of the *Holacanthus* clade was approximately 12.1%, the divergence between *Pygoplites diacanthus* and *Holacanthus* species was 14.7%. These two values provide the boundaries to estimate the age of the genus *Holacanthus*. Using the calculated rate of divergence based on the Trans-Isthmian geminate clades, this suggests that *Holacanthus* appeared approximately 10.2–7.6 million years ago.

## 4. Discussion

### 4.1. Phylogenetic relationships, evolutionary history and biogeographic patterns

The genus *Holacanthus* comprises only seven species, yet it offers unique possibilities in testing biogeographic, ecological and evolutionary hypotheses. The overall monophyly of the genus is confirmed here. Considering the thorough investigation presented by Bellwood et al. (2004), it is unlikely that other unsampled angelfish species would fall within *Holacanthus*. *Pygoplites diacanthus* was found to be very close and basal to *Holacanthus*. Keeping *Pygoplites* in its own monospecific genus or considering it a basal *Holacanthus* is more of a semantic issue at this stage.

Our data indicate that the genus *Holacanthus* likely originated approximately 10.2–7.6 mya. Since the basal species is found in Western Africa, *Holacanthus* may have originated from an Indian Ocean invasion, a scenario that has been observed in other groups, including pygmy angelfishes (Bowen et al., 2006; Rocha et al., 2005b). These dates, however, are different from the proposed timeframe of the split between *P. diacanthus* and *Holacanthus* by Bellwood et al. (2004): 13 Mya (minimum age) or even 23 Mya, which would lead to a Tethyan separation hypothesis (see proposed scenarios in Floeter et al., 2008). In any case, the first split (or invasion with further speciation) within *Holacanthus* is likely to have occurred between the Eastern Atlantic and the Indian Ocean. In contrast, an invasion via the Central American Seaway prior to the closure of the Isthmus of Panama, such as proposed for the wrasse genus *Halichoeres* (Barber and Bellwood, 2005; Floeter et al., 2008) is a less likely scenario for *Holacanthus* as the TEP species (subgenus *Plitops*) are relatively recent and not the immediate sisters to *Pygoplites*.

The genus *Holacanthus* has previously been divided into four subgenera. Our data do not support the subgenus *Angelichthys* as it were originally proposed (*H. africanus*, *H. bermudensis*, *H. ciliaris*). Our data, however, support a separation of species by geographic regions: the Eastern Atlantic species from the Western Atlantic clade, and the Atlantic clade from the TEP clade.

#### 4.2. Modes of speciation

*Holacanthus* affords hypothesis testing due to its presence on both sides of known biogeographic barriers. While *H. bermudensis* and *H. passer* were thought to be Trans-Isthmian geminate species (Bellwood et al., 2004), our study shows that they actually belong to multispecific clades that were separated by the closure of the Isthmus of Panama. The genetic divergence between these clades is similar to other known cases of geminate species (e.g. Domingues et al., 2005; Bernardi et al., 2008; Lessios, 2008 comments about this issue). Thus the rise of the Isthmus of Panama likely preceded the diversification of those clades in both the W Atlantic and TEP. Vicariant speciation is likely to be responsible for the initial divergence of angelfish among these two neotropical clades.

The close relationship of the species within the Caribbean, and within the TEP, and the presence of hybrids within these species complexes (*H. bermudensis* × *H. ciliaris*, *H. passer* × *H. clarionensis*) underscore their evolutionary proximity (Sala et al., 1999). The broad distribution of *H. passer* in the TEP relative to the other two insular species suggests that *H. passer* may have been the ancestral species that gave rise first to *H. limbaughi* and then to *H. clarionensis*. The fact that *H. clarionensis* is almost entirely limited to the Revillagigedo islands, and that *H. limbaughi* only occurs at Clipperton, may suggest that these species arose via peripatric speciation, particularly in the case of the latter, with its extremely isolated population. However, more information is needed on the abundance of *H. passer* at the Revillagigedo Islands; if it is common there then the case for isolation leading to a peripatric origin of *H. clarionensis* weakens. The origin of *H. bermudensis* is more problematic than that of the two insular Caribbean species. Although *H. bermudensis* is effectively isolated at Bermuda (*H. ciliaris* is very rare there, Smith-Vaniz et al. (1999)), its geographic range and population is mostly continental and its sister *H. ciliaris* is common throughout the continental range of *H. bermudensis*. A peripatric origination scenario would have to have *H. bermudensis* originating in Bermuda, whose reef fish fauna has been subject to major recent extinctions during ice ages (Smith-Vaniz et al., 1999). Parapatric speciation, speciation with geographic segregation but ongoing gene flow (see Rocha and Bowen, 2008) provides an alternative mechanism that could account for the origins of *H. bermudensis* as a northern, warm-temperate form separating off from a southern, tropical form (*H. ciliaris*). The distributions of *H. bermudensis* and *H. ciliaris* are similar to those of a labrid for which such parapatric speciation has been proposed (see Rocha et al., 2005a). Parapatric speciation in response to differences between insular and continental environmental conditions could also account for the development of *H. clarionensis* in the Revillagigedo Islands despite ongoing immigration by *H. passer*. Two of the three oldest divergences in *Holacanthus* (the split of *H. africanus* from an apparent Indian Ocean ancestor, the Trans-Atlantic split of *H. tricolor* from *H. africanus* and the division of the two Trans-Isthmian clades) are also consistent with allopatric speciation scenarios. The geographic distributions of the neotropical species that evolved after the closure of the Isthmus of Panama are most consistent with modes of speciation that involve partial or complete allopatry. Therefore at present there seems to be no reason to invoke purely sympatric speciation as a contributor to the evolution of *Holacanthus*.

#### 5. Conclusion

*Holacanthus*, although a small and geographically confined genus (compared to other angelfish genera), joins other angelfishes in providing a very useful model for the study of marine reef fish evolution. The results of our phylogenetic analyses indicate when and how the genus originated, that its seven species include

Trans-Isthmian geminate species complexes, and that speciation likely occurred well after the closure of the Central American Isthmus in both the Caribbean and the tropical Eastern Pacific.

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