



Short Communication

Tempo and mode of speciation in *Holacanthus* angelfishes based on RADseq markers [☆]Juliette Tariel ^{a,b}, Gary C. Longo ^a, Giacomo Bernardi ^{a,*}^a Department of Ecology and Evolutionary Biology, University of California Santa Cruz, Santa Cruz, CA 95060, USA^b Ecole Normale Supérieure de Lyon, 15 parvis René Descartes, 69342 Lyon, France

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ABSTRACT

In this study we estimated the timing of speciation events in a group of angelfishes using 1186 RADseq markers corresponding to 94,880 base pairs. The genus *Holacanthus* comprises seven species, including two clades of Panama trans-Isthmian geminates, which diverged approximately 3–3.5 Mya. These clades diversified within the Tropical Eastern Pacific (TEP, three species) and Tropical Western Atlantic (TWA, two species) which our data suggest to have occurred within the past 1.5 My in both ocean basins, but may have proceeded via different mechanisms. In the TEP, speciation is likely to have followed a peripatric pathway, while in the TWA, sister species are currently partially sympatric, thus raising the possibility of sympatric speciation. This study highlights the use of RADseq markers for estimating both divergence times and modes of speciation at a 1–3 My timescale.

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1. Introduction

Speciation in marine fishes is similar to speciation in terrestrial organisms, in that it is generally subjected to a number of factors including behavior, demography, drift, and selection (Bernardi, 2013; Coyne and Orr, 2004). It is, however, unique in regards to dispersal, because marine fishes predominantly exhibit a bipartite life history with sedentary adults and a dispersive pelagic larval phase (Leis, 1991). This phase, which lasts from a few days to several months, often results in high levels of gene flow, which in turn counter genetic separation between populations, a premise for speciation (Selkoe and Toonen, 2011). Thus, dispersal potential and speciation mode have been linked in the past, and indeed, clear cases of sympatric speciation are very rare in marine fishes (Bernardi, 2013; Bowen et al., 2013; Crow et al., 2010; Rocha and Bowen, 2008). In contrast, as for other systems, allopatric speciation is presumed to be relatively common, with a classic example represented by the trans-Isthmian geminate species (Jordan, 1908). There, marine organisms, including about 40 species of fish, were separated by the closure of the Isthmus of Panama. The date of the closure itself has recently been debated, based on new geological and molecular data, arguing for an old closure time (Bacon

et al., 2015a, 2015b; Coates and Stallard, 2013; Jackson and O'Dea, 2013; Lessios, 2015; Marko et al., 2015), rather than the classically accepted 3.1–3.5 Mya (Coates and Obando, 1996; Collins, 1996; Craig et al., 2004; Knowlton, 1993; Lessios, 2008; Marko, 2002; Thomson et al., 2000). Genetic divergence between geminate species varies, and this variation is in part due to variability in substitution rates and in part due to ecological and evolutionary characteristics of specific taxa that determine the actual time of separation between geminates (Knowlton and Weigt, 1998; Lessios, 2008). In general, however, there is remarkable consistency in divergence (time and rate) among geminate taxa, as is the case for the angelfish genus *Holacanthus* (Alva-Campbell et al., 2010; Lessios, 2008).

As mentioned above, approximately 40 species of fish are considered geminates. In some cases, such as for *Holacanthus* angelfishes, sister clades (rather than sister species) were found to be geminates (Alva-Campbell et al., 2010). Indeed, a phylogeny of the entire *Holacanthus* genus (seven species), based on one nuclear and four mitochondrial molecular markers showed that the geminate sister clades included *H. bermudensis* and *H. ciliaris* in the Tropical Western Atlantic (TWA), and *H. passer*, *H. clarionensis*, and *H. limbaughi* in the Tropical Eastern Pacific (TPE) (Alva-Campbell et al., 2010). The divergence time of geminate *Holacanthus* clades, based on fossil calibrated trees (and not the closure of the Isthmus of Panama), was estimated to have occurred between 0 and 4 Mya, a date that is consistent with the classical estimate of the closure, between 3.1 and 3.5 Mya (Bellwood

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* Corresponding author at: Department of Ecology and Evolutionary Biology, University of California Santa Cruz, 100 Shaffer Road, Santa Cruz, CA 95060, USA.

E-mail address: bernardi@ucsc.edu (G. Bernardi).

et al., 2004). Taken together, these data indicate that speciation of the trans-Isthmian species occurred after the closure of the Isthmus of Panama, within each ocean basin.

In the Tropical Eastern Pacific, the king angelfish, *H. passer* is widely distributed, from Baja California, Mexico, to the Galapagos Islands, Ecuador, and mainland Peru. In contrast, the Clipperton angelfish, *H. limbaughi*, is restricted to Clipperton Atoll, and the Clarion Angelfish, *H. clarionensis*, is mainly found at the Revillagigedos Archipelago (with a few vagrants found at the southern tip of Baja California and Clipperton Atoll), (Allen and Robertson, 1994), lending to the idea that peripatric speciation may have been involved in this group (Alva-Campbell et al., 2010) (Fig. 1). In the Tropical Western Atlantic, the Queen Angelfish, *H. ciliaris*, and the Blue Angelfish, *H. bermudensis*, exhibit extensive overlap in the central part of their range. The range of *H. bermudensis*, however, extends further to the north, including Bermuda, and the range of *H. ciliaris* extends further to the south to northern Brazil (Fig. 1). Thus, *Holacanthus* angelfish provide an opportunity to understand the timing of speciation for each ocean basin within a relatively narrow geographic and time range.

In order to better understand the tempo and mode of speciation of this group of fishes, a high-resolution phylogenetic hypothesis, that would provide finer timescale resolution compared to previously published phylogenies, was needed. The use of a large number of genome-wide molecular markers (RAD seq) has successfully been used for phylogenetic purposes before in cichlids (Cichlidae) and surferperches (Embiotocidae) (Longo and Bernardi, 2015; Wagner et al., 2013). Here we use that approach to better understand the tempo and mode of speciation of *Holacanthus* geminate species within each ocean basin, by estimating the timing of speciation events using a fine-scale approach based on hundreds of RADseq markers.

2. Materials and methods

2.1. Sampling

The proposed phylogeny includes all seven species for the angelfish genus *Holacanthus* (Pomacanthidae). Sampling and locality of individuals were described previously (Alva-Campbell et al., 2010). Briefly, *H. africanus*, *H. tricolor*, *H. bermudensis*, *H. ciliaris*, *H. limbaughi*, and *H. passer*, were collected in Cape Verde Islands,

Panama and Brazil, Florida, Florida and Brazil, Clipperton, and Mexico, respectively. *Holacanthus clarionensis* was obtained from the Aquarium of the Pacific in Long Beach, California. When possible, two individuals per species were used, which was the case for all species except for *H. africanus* and *H. clarionensis* where only one individual for each species was available.

2.2. DNA extractions

Fin clip tissue samples were stored in 95% ethanol and DNA was extracted using DNeasy Blood & Tissue kits (Qiagen) according to manufacturer's protocol. We constructed RAD libraries using a variation of the original protocol with restriction enzyme SbfI (Baird et al., 2008; Longo and Bernardi, 2015; Miller et al., 2007, 2012). Individually barcoded samples were sequenced in a single lane on an Illumina HiSeq 2000 at the Vincent J. Coates Genomics Sequencing Laboratory at UC Berkeley.

2.3. Quality filtering and marker discovery

Raw 100 bp reads were trimmed to 92 bp on the 3' end, quality filtered, and then split according to the 6 bp unique barcode using custom Perl scripts (Miller et al., 2012). Sequences were dropped if the product of quality scores for their 92 bases was below 80%. The barcode (6 bp) and restriction site residue (6 bp) were then removed from the 5' end, resulting in a final sequence length of 80 bp.

We used the software program Stacks version 1.29 (Catchen et al., 2011, 2013) to identify orthologous sequences among *Holacanthus* taxa. We first ran the program *denovo_map.pl*, which runs all three Stacks components in a pipeline (i.e., *ustacks*, *cstacks*, and *sstacks*). We set a minimum stack depth (-m) of three, a maximum of three mismatches per loci for each individual (-M), and allowed up to seven mismatches when building catalog loci (-n). We then ran the Stacks program *populations* to generate output files for input into downstream phylogenetic programs. Due to high coverage across individuals, we increased the minimum stack depth (-m) to eight in *populations* runs. We created a stringent dataset by setting -p at 7, which means all *Holacanthus* species must retain the marker and with -r set to 100%, meaning that every individual in each species must retain the marker. Full sequence RAD markers of each individual were exported for downstream

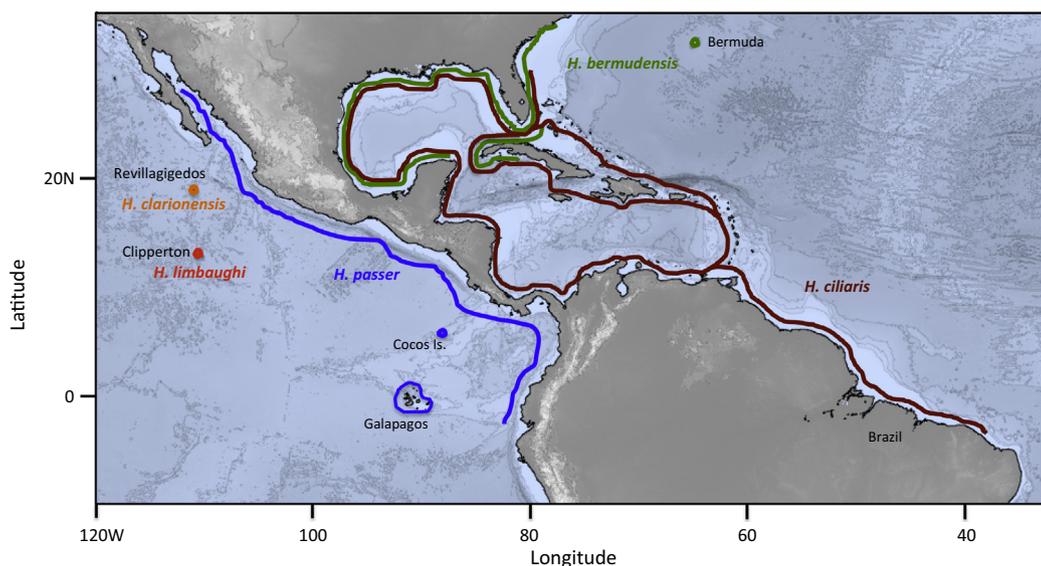


Fig. 1. Range distribution of geminate clades of *Holacanthus* species.

analyses. The quality filtered sequences are deposited at the National Center for Biotechnology Information short-read archive (accession no. SRP065950).

2.4. Phylogenetic inference

We constructed concatenated supermatrices using complete RAD sequences (80 bp) for each individual (with a consensus sequence at polymorphic sites using IUPAC ambiguity codes) with FASconCAT-G (Kück and Longo, 2014).

We used both maximum likelihood methods as implemented in PhyML v.3.0 (Guindon et al., 2010) and Bayesian phylogenetic inference as implemented in MrBayes v.3.2.2 (Ronquist et al., 2012) to assess relatedness within *Holacanthus*. For MrBayes analyses we used the GTR + Γ + 1 model and selected a Markov Chain Monte Carlo (MCMC) search algorithm with a chain length of 1,000,000 using four chains with a sampling frequency of 1000. In PhyML we selected the GTR model of sequence evolution, six substitution rate categories, set the initial tree to random, and performed 100 bootstrap replicates. GTR substitution models were used with the concatenated supermatrices due to the complex evolutionary history resulting from concatenating hundreds of RADseq loci. Phylogenetic trees and corresponding support values were visualized using FigTree v1.4.0 (Rambaut, 2014). The retention of orthologous RAD markers across taxa decreases with increasing divergence time (Longo and Bernardi, 2015; Rubin et al., 2012), which makes the use of divergent outgroups difficult. Previous phylogenies, however, indicate that the focal group of this study (the geminate clades) is well within the genus *Holacanthus* (Alva-Campbell et al., 2010), so we used the basal taxon *H. africanus* to root the tree.

2.5. Estimating divergence times

Divergence times were estimated using standard models of evolution implemented in BEAST v. 1.8.1 (Drummond et al., 2012). We used a lognormal relaxed clock-model, in combination with a birth–death (BD) prior for rates of cladogenesis (Drummond and Rambaut, 2007) with a GTR + G model of substitution. A user specified tree was provided based on the congruent PhyML and MrBayes trees. One run was conducted with 10 million generations each, with sampling every 1000 generations. A time tree was obtained using TreeAnnotator v1.7.5 (Drummond et al., 2012).

We used two priors with normal distributions as calibration points, with the minimum and maximum bounds implemented with the 95th percentile of the distribution. We used an internal calibration point based on the closure of Panama at 3.1–3.5 Mya (mean at 3.25 Mya) for the geminate clades and 6 Mya for all TWA and TEP species based on prior findings estimates (Alva-Campbell et al., 2010).

3. Results and discussion

3.1. RAD markers and phylogenetic results

Our approach yielded 1186 loci, which produced 1320 phylogenetically informative SNPs (concatenated supermatrix was 94,880 bp).

Holacanthus species have been included in previous phylogenetic studies. A study based on 12S and 16S mitochondrial markers that included four species of *Holacanthus* found *H. tricolor* to be basal, and then *H. ciliaris* to be sister to a clade formed by *H. passer* and *H. bermudensis* (presumed geminate species) (Bellwood et al., 2004). However, several nodes in this phylogeny were poorly supported, leaving relationships open to future change. A more recent

study based on one nuclear and four mitochondrial markers, included all species of *Holacanthus* and two outgroups (*Pygoplites diacanthus* and *Pomacanthus zonipectus*) (Alva-Campbell et al., 2010). This latter phylogeny was very similar to the phylogeny presented here based on RADseq markers (Fig. 2). Tropical Eastern Pacific species (*H. passer*, *H. limbaughi*, *H. clarionensis*) cluster together and are sister to the Tropical Western Atlantic species (*H. bermudensis*, *H. ciliaris*), *Holacanthus tricolor* being the sister clade to this TEP-TWA cluster, and *H. africanus* being placed as a basal taxon. The main difference between the phylogeny based on RADseq markers and the phylogeny proposed by Alva-Campbell et al. (2010) is in the relative placement of the TEP species. In the Alva-Campbell et al. phylogeny, *H. passer* is sister to *H. clarionensis*, while in the current phylogeny, *H. clarionensis* and *H. limbaughi* are sister species (Fig. 2). However, support in the Alva-Campbell phylogeny for these groupings was low (62–70% bootstrap support), while support for the relationships among TEP species in the RADseq phylogeny was very high (100%).

3.2. Divergence time estimates

Divergence times using RADseq markers and anchored at the closure of the Isthmus of Panama (3.1–3.5 Mya) center the origin of the genus *Holacanthus* around 8.2 Mya, a value that is consistent with two previously published estimates (Alva-Campbell et al., 2010; Bellwood et al., 2004). In turn, the divergence of the geminate clades is centered at approximately 1.7 Mya and 1.4 Mya for the Tropical Western Atlantic and the Tropical Eastern Pacific clades, respectively.

3.3. Speciation within ocean basins

Holacanthus diversification within the TWA and TEP occurred approximately at the same time, around 1.5 Mya. Speciation within each ocean basin seems, however, to have taken very different paths. In the TWA, the blue angelfish, *Holacanthus bermudensis*, exhibits a partial overlapping geographic range with its sister species, the Queen angelfish, *H. ciliaris* (Fig. 1). Our data show that the divergence between these two species occurred early into the history of this clade, as shown, for example, by the divergence of the two *H. bermudensis* samples centered at 1.1 Mya. It is possible, early in the history of this clade, for sympatric speciation to have played a role in the divergence of these species. Yet a requirement for arguing sympatric speciation is the unlikely presence of allopatry (Coyle and Orr, 2004), which in this case can certainly not be excluded. The early stages of sympatric speciation usually prevent hybridization (Coyle and Orr, 2004), yet later secondary contact may exhibit some crosses (Crow et al., 2010). In fact, observations of hybrids between *H. bermudensis* and *H. ciliaris* (usually referred to as *H. townsendi*) are not uncommon (Feddern, 1968).

The situation in the TEP is quite different. There, *H. passer* has a very wide distribution while the other two species are restricted to a small atoll (Clipperton, *H. limbaughi*) or an archipelago (Revillagigedos, *H. clarionensis*). Apparently vagrant *H. clarionensis* are occasionally seen at Clipperton Atoll. Indeed, they were observed in the early 1990s (Allen and Robertson, 1994, 1997), but neither earlier (Baldwin, 1963) nor later (Robertson, personal communication, Béarez and Seret, 2009; Fourrière et al., 2014). This suggests that rare dispersal does occur between the Revillagigedos and Clipperton, consistent with a long pelagic larval duration (approximately 25 days, Thresher and Brothers, 1985), thus providing a mechanism for the origin of *H. limbaughi*, as was suggested in general terms (Robertson, 2001). Results presented here are consistent with a scenario of rare long-range dispersal, with *H. limbaughi* and *H. clarionensis* being sister species as shown in Fig. 2, with their divergence being centered around 1.1 Mya.

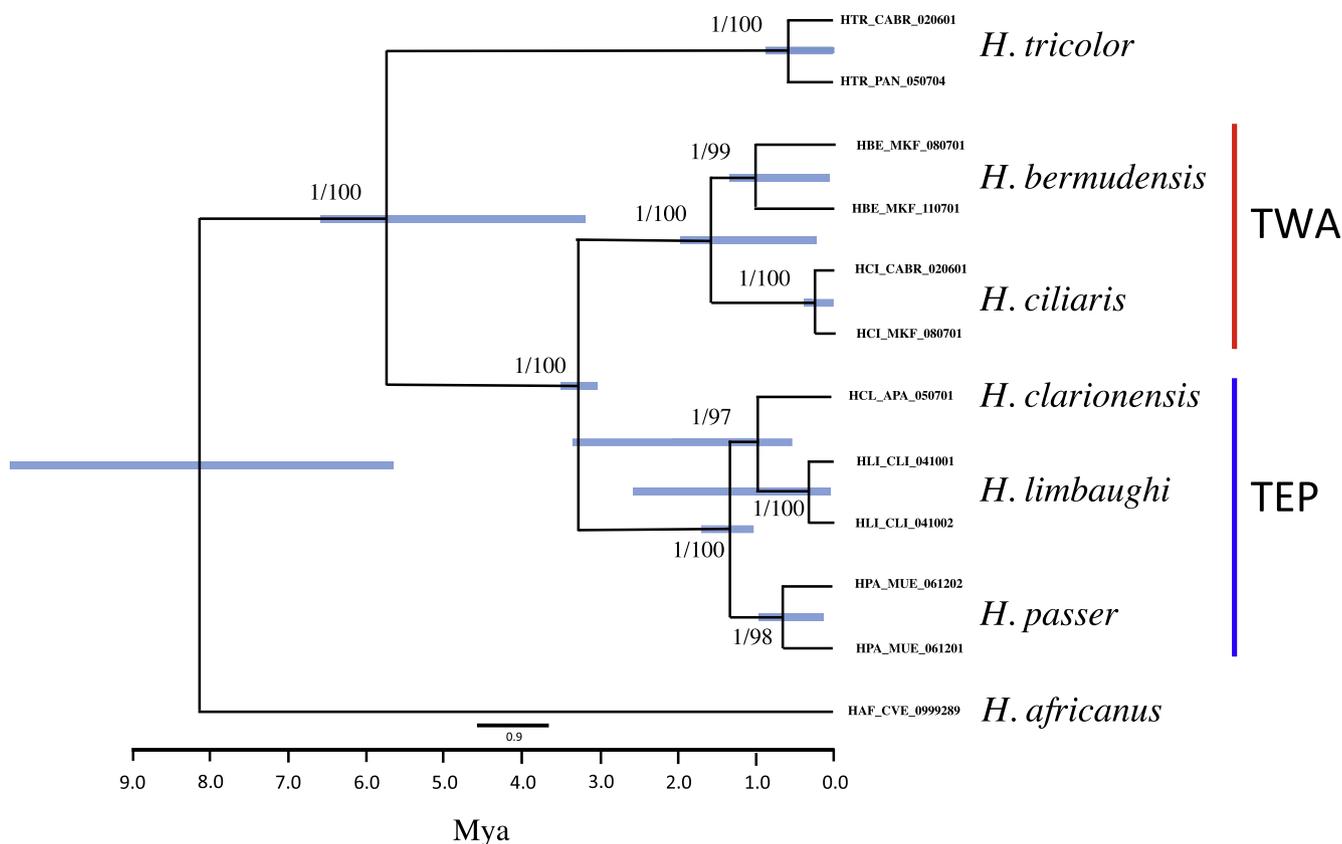


Fig. 2. Time calibrated phylogeny of the angelfish genus *Holacanthus* based on Maximum Likelihood and Bayesian methods using internal time calibration based on the rise of the Isthmus of Panama, 3.1–3.5 Mya. Horizontal blue bars at the nodes represent the 95% confidence intervals for each date estimate. Nodes above the nodes represent confidence estimates based on posterior probability and bootstrap support. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

4. Conclusion

The goal of this study was to estimate the tempo and mode of speciation in trans-Isthmian geminate clades of *Holacanthus* angelfishes based on RADseq markers. As was shown for other angelfishes (Hodge et al., 2013), *Holacanthus* illustrates different speciation modes. Results indicate that speciation within the TEP and TWA occurred approximately 1.5 Mya. Divergence between *H. bermudensis* and *H. ciliaris* within the TWA occurred very early, potentially in sympatry, while divergence in the TEP is likely to have occurred in peripatry, resulting from rare dispersal events at the oceanic island groups of Revillagigedos and Clipperton. Our study highlights the potential use of RADseq markers to estimate fine-scale divergence times and showcases an interesting model of speciation for this group of fishes. When using RADseq markers in a phylogenetic context, orthologous loci need to be used. The variability of orthologous restriction sites limits the use of RADseq markers for phylogenetic reconstructions. Yet, when applicable, as for closely related cichlids (Wagner et al., 2013), the surperch family (Longo and Bernardi, 2015), or for this study the genus *Holacanthus*, RADseq markers prove to be an extremely powerful tool.

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