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Phylogenetic relationships among the clownfish-hosting sea anemones

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Abstract

The clownfish-sea anemone symbiosis has been a model system for understanding fundamental evolutionary and ecological processes. However, our evolutionary understanding of this symbiosis comes entirely from studies of clownfishes. A holistic understanding of a model mutualism requires systematic, biogeographic, and phylogenetic insight into both partners. Here, we conduct the largest phylogenetic analysis of sea anemones (Order Actiniaria) to date, with a focus on expanding the biogeographic and taxonomic sampling of the 10 nominal clownfish-hosting species. Using a combination of mtDNA and nuDNA loci we test 1) the monophyly of each clownfish-hosting family and genus, 2) the current anemone taxonomy that suggests symbioses with clownfishes evolved multiple times within Actiniaria, and 3) whether, like the

clownfishes, there is evidence that host anemones have a Coral Triangle biogeographic origin. Our phylogenetic reconstruction demonstrates widespread poly- and para-phyly at the family and genus level, particularly within the family Stichodactylidae and genus *Stichodactyla*, and suggests that symbioses with clownfishes evolved minimally three times within sea anemones. We further recover evidence for a Tethyan biogeographic origin for some clades. Our data provide the first evidence that clownfish and some sea anemone hosts have different biogeographic origins, and that there may be cryptic species of host anemones. Finally, our findings reflect the need for a major taxonomic revision of the clownfish-hosting sea anemones.

1. Introduction

Symbiosis often confers novel abilities or characteristics in at least one partner, can lead to adaptive radiation, and contributes meaningfully to the biodiversity within ecosystems. The clownfish-sea anemone symbiosis is an icon of tropical coral reefs of the Indo-West Pacific and is perhaps the most recognizable example of symbiosis on the planet. The complexity of the clownfish-sea anemone symbiosis has attracted a great deal of popular and scientific attention and has been used as a model system to explore adaptive radiation, mutualism, specialism versus generalism, micro- and macro-evolution, animal behavior, social group structure and population dynamics, competition, venom resistance, host choice, larval dispersal and recruitment, biogeography, sex determination, and climate change among others (e.g. Almany et al., 2007; Beldade et al., 2017; Buston, 2004; Buston et al., 2007; Camp et al., 2016; Casas et al., 2018; Fautin, 1991; Hayashi et al., 2019; Huebner et al., 2012; Litsios & Salamin, 2014; Litsios et al., 2012, 2014a, 2014b; Mebs, 2009; Miyagawa-Kohshima et al., 2014; Ollerton et al., 2007; Schmitt & Holbrook, 2003; Szczebak et al., 2013). The charismatic nature of the relationship

between anemones and clownfishes have made both constituents among the most heavily collected and sought after animals in the ornamental aquarium trade and species of conservation concern (Jones et al., 2008; Scott et al., 2014; Shuman et al., 2005; Rhyne et al., 2017; Wabnitz, 2003).

Our evolutionary understanding of this symbiosis, however, comes almost entirely from studies of clownfishes. Currently, there are 30 described species of clownfishes (or anemonefishes), which form the reciprocally monophyletic subfamily Amphiprioninae of the damselfish family Pomacentridae (e.g. Cooper et al., 2009; Litsios et al., 2012, 2014a; Rolland et al., 2018). Mutualism with sea anemones is believed to have been present in the common ancestor of all clownfishes (Litsios et al., 2012), which is estimated to have evolved ~12 mya in the Coral Triangle. The majority of clownfish diversity is the result of a recent adaptive radiation to a symbiotic lifestyle, with 25 of the 30 species having evolved within the last 5 mya (Litsios et al., 2012). Host specificities of clownfishes to sea anemones are also well resolved and span the host specialist-generalist continuum (e.g. Fautin, 1986; Fautin, 1991; Fautin & Allen, 1992; Litsios et al., 2012; Litsios et al., 2014a). Clownfish morphology and patterns of host specificity support the hypothesis that clownfishes have adapted to ecological niches associated with anemone hosts (Litsios et al., 2012).

Unlike the clownfishes, their sea anemone hosts have been poorly represented in systematic and phylogenetic studies, obscuring their biogeographic origin and patterns of diversification that have bearing on our interpretation of how the symbiosis has evolved. Broadly, sea anemones (Cnidaria: Anthozoa: Actiniaria) are a diverse group of benthic anthozoans that are found in every major marine habitat. Contrary to the generally observed pattern of hyperdiversity in the tropics, anemone diversity peaks in temperate marine

ecosystems, where they often constitute the dominant benthic macrofauna (Fautin et al., 2013). The ecological success of sea anemones can be partly attributed to the diverse symbioses they form. This is particularly true for the clownfish-hosting anemones, which receive protection, nitrogen (via fish excrement), and increased gas transfer from their clownfish symbionts (Cleveland et al., 2011; Dunn, 1981; Roopin et al., 2008; Szczbak et al., 2013).

According to Fautin (2013), there are 10 species of sea anemone hosts distributed throughout the range of the clownfish symbiosis, a span that encompasses coral reef habitats from the Northern Red Sea through the Central Pacific Ocean (Dunn, 1981; Fautin, 1991; Fautin & Allen, 1992). Eight of the 10 nominal anemone species have widespread distributions and broadly overlapping biogeographic ranges (Fautin & Allen, 1992). All belong to the anemone superfamily Actinioidea, but are not united in a single taxon that exclusively contains species symbiotic with clownfishes (Dunn, 1981; Fautin, 1991; Fautin & Allen, 1992). The 10 host species are presently interpreted to belong to three families (Actiniidae, Stichodactylidae, and Thalassianthidae) and encompass five genera (*Cryptodendrum*, *Entacmaea*, *Heteractis*, *Macrodactyla*, and *Stichodactyla*). The genera *Cryptodendrum* and *Entacmaea* are monospecific, while four host species are described as belonging to *Heteractis* and three to *Stichodactyla* (reviewed by Dunn, 1981). Two species are described to belong to the genus *Macrodactyla*, with only *M. doreensis* hosting clownfish.

Some disagreement exists regarding the status of the genus *Heteractis*, and there is confusion regarding the familial assignment of *Entacmaea*. England (1988) argued that because the type species for the genus *Heteractis* (*H. aurora*) harbors macrobasic amastigophore nematocysts (*p*-mastigophores A with looped tubule *sensu* Gusmão et al., 2018), this excludes *H. crista*, *H. magnifica*, and *H. malu* from the genus. England (1998) thus resurrected *Radianthus*

as a valid genus and reinstated the family Heteractidae to include *H. aurora*, *R. crisper*, *R. magnifica*, and *R. malu*, removing these two genera from the family Stichodactylidae. England's work on the taxonomy of *Heteractis* was ignored for years and is not currently recognized in commonly used databases (e.g. Fautin 2013, WoRMS). Further, England (1987) listed *E. quadricolor* as belonging to the family Stichodactylidae (rather than Actiniidae), noting that the basitrichs of the tentacles and column are different in Actiniidae and Stichodactylidae, but was not definitive about its final placement and was again broadly overlooked. Finally, an additional taxonomic issue with the clownfish-hosting anemones is that the generic sea anemone name *Macrodactyla* Haddon, 1898 is a junior homonym of a coleopteran genus (*Macrodactyla* Harris in Hitchcock, 1833), and the two anemone species included have been referred as belonging within the actiniid genus *Condylactis*, although without discussion or explanation (see Fautin, 2016).

To date, clownfish-hosting anemone species have only been described morphologically and have not been subjected to extensive molecular investigation. Anemones have simple body plans, no hard parts, and few diagnostic morphological characters, making traditional taxonomic descriptions challenging. Extensive phenotypic variation in living specimens makes field identification challenging, often making species identifications in the published literature unreliable (Dunn, 1981). Historically, this has led to an abundance of species descriptions and widespread confusion regarding how many species of host anemones there actually are. For example, there are over 60 synonymized names for *E. quadricolor* alone in the World Registry of Marine Species (Daly & Fautin, 2019). These issues with traditional actiniarian taxonomy have rendered many non-clownfish hosting anemone genera para- or polyphyletic upon molecular phylogenetic investigation (e.g. Daly et al., 2017; Rodríguez et al., 2014). However,

no phylogenetic study has included representatives from each of the 10 nominal host species (e.g. Daly et al., 2008, 2017; Rodríguez et al., 2014), and species that have been examined were often limited to a single individual sample per species, leaving their broader phylogenetic placement, taxonomy, species delimitation, and biogeography untested. Based on the currently accepted sea anemone taxonomy, it is expected that symbiosis with clownfishes evolved independently at least three times—once in each of the three families in which clownfish-host anemones are currently described. However, if England's (1988) re-description of the family Heteractidae is supported, symbiosis with clownfishes would likely be expected to have evolved a fourth time, and possibly a fifth if *Entacmaea* does not belong within Actinidae. Thus, thorough sampling and sequencing efforts are critical to shed light on the evolutionary history of these anemones and the clownfish symbiosis broadly, as well as to provide a more comprehensive understanding of actinarian diversity and evolution. Here, we conducted the largest phylogenetic analysis of Actiniaria to date, and tested 1) the monophyly of each clownfish-hosting family and genus, 2) the current anemone taxonomy that suggests symbiosis with clownfishes evolved multiple times within Actiniaria, and 3) examined if the clownfish hosting anemones, like their clownfish symbionts, have a biogeographic origin in the Coral Triangle.

2. Material and Methods

2.1. Taxonomic sampling

Representatives from each of the 10 nominally described clownfish hosting sea anemones were included in this study (Figure 1). Data were acquired from a combination of field collected tissue samples, museum holdings, the aquarium trade, and GenBank (Table 1). Field collected tissue

samples were collected by hand using SCUBA and preserved in 95% EtOH or RNAlater. We included a total of 59 individual samples across the 10 clownfish hosting anemone species, 43 of which were newly acquired for this study (Table 1). Where possible, we included samples from both the Indian and Pacific Ocean basins for nominal clownfish hosting anemones. Sampling effort was more extensive for *E. quadricolor* and *H. crispa* and we include multiple sample localities across their geographic ranges (Table 1). We also generated new sequence data for *S. helianthus*, a non-clownfish hosting sea anemone and the only member of the genus *Stichodactyla* native to the Tropical Western Atlantic (Table 1). Additional actinioidean in-group samples were obtained from previously published datasets (e.g. Daly et al., 2017; Rodríguez et al., 2014; Table S1). In total, our analyses included 157 individuals from 91 species across the superfamily Actinioidea (Table 1; Table S1). We further included 89 species representing the other four anemone superfamilies Metridioidea, Actinostoloidea, Edwardsioidea, and Actinernoidea (Table S1). Two non-actiniarian samples from Corallimorpharia and Zoantharia were included as outgroups (Table S1). The final dataset of 256 individuals across 180 anemone species represents the largest phylogenetic analysis of actiniarian diversity to date.

2.2. DNA extraction, PCR, and sequencing

Genomic DNA was extracted using the DNeasy Blood and Tissue Kits (QIAGEN Inc.) and stored at -20°C. All DNA extractions were standardized to ~20ng/μL, and template DNA was amplified from genomic samples using published primers and standard PCR techniques. We targeted three mitochondrial (partial 12S rDNA, 16S rDNA, and CO3) and two nuclear (18S rDNA, and partial 28S rDNA) gene markers for phylogenetic reconstruction. Primer sequences and PCR run conditions can be found elsewhere (Daly et al., 2008; Geller & Walton 2001;

Gusmão & Daly, 2010). All PCR products were cleaned using ExoSAP-IT (Thermo Fisher) and FastAP (Thermo Fisher) enzyme reactions. Each PCR clean up used 10 μ L PCR product, 2 μ L ExoSAP-IT, and 1 μ L FastAP. Clean up reactions were carried out under the following conditions: 37°C for 15 min, 85°C for 15 min, and hold at 4°C. Cycle sequencing reactions were carried out using 5 μ L purified PCR product at a concentration of 25 ng of PCR product for every 200 base pairs of length. Cycle sequence products were cleaned using the Sephadex G-50 (Sigma-Aldrich) spin-column protocol. Samples were sequenced using traditional Sanger-based capillary electrophoresis on an ABI 3730 at the American Museum of Natural History (AMNH). Forward and reverse sequences were assembled in Sequencher v. 4.9 (Gene Codes Corporation, Ann Arbor, MI) and compared (via BLAST) against the nucleotide database of GenBank to determine whether the target organism was sequenced, rather than an endosymbiotic dinoflagellate. Intraspecific variation in DNA sequences were verified by examining nucleotide quality scores and chromatograms. Newly generated sequences have been deposited in GenBank (Table 1; Table S1).

2.3. *Phylogenetic analyses*

Newly assembled sequences were aligned for each locus separately using MAFFT v7.394 (Katoh & Standley, 2013) on the CIPRES Science Gateway Portal (Miller et al., 2010) under the following alignment parameters: Strategy, L-INS-i; Scoring matrix for nucleotide sequences, 200PAM/k=2; Gap opening penalty, 1.53; Offset value, 0.1; Max iterate, 1000; Retree, 1. Additionally, multiple sequence alignments for each locus were analyzed using Gblocks v0.91 (Castresana, 2000) to remove poorly aligned and/or mutationally saturated divergent regions. The following parameters were used in Gblocks: Maximum number of contiguous non-

conserved positions, 8; Minimum length of a block, 5; Gap positions allowed. Gblock analysis for the 28s gene further included: Allow smaller final blocks; Allow less strict flanking positions. The complete Gblocks sequence alignments for each locus were concatenated into a final super matrix and analyzed using PartitionFinder2 (Lanfear et al., 2016) to identify the best partitioning scheme and best fit model of nucleotide evolution under the corrected Akaike Information Criterion (AIC).

Maximum Likelihood (ML) phylogenetic analyses were conducted using RAxML v8.2.10 as implemented on the CIPRES Science Gateway Portal. Analyses were conducted using four partitions as determined by PartitionFinder: CO3, 12S/16S, 18S, and 28S. For each partition we implemented a GTR+G+I model of nucleotide substitution as determined by PartitionFinder analyses. Clade support was analysed using rapid bootstrapping with a subsequent ML search, and we let RAxML halt bootstrapping automatically using MRE-based bootstrapping criterion. In addition, we conducted gene tree phylogenetic analyses (all mtDNA, 18S, and 28S) to explore possible discordance and incomplete lineage sorting among our loci. Each of the three gene tree analyses were conducted in RAxML as above.

RAxML analyses recovered the 10 nominal clownfish hosting species to belong to three clades (see Results). However, poor nodal support across the backbone of the superfamily Actinioidea could not resolve the relationship among these clades and other clades within Actinioidea, and thus, was unable to distinguish between one and three independent evolutionary origins of symbiosis with clownfishes. Thus, we conducted likelihood ratio tests of tree topologies (SH test) between the initial unconstrained analysis above (i.e. three independent evolutionary origins of symbiosis with clownfishes), and separate analyses where the 10 clownfish hosting species were constrained to a single monophyletic group or into two

monophyletic groups (i.e. two independent evolutionary origins of symbiosis with clownfishes). The rationale behind the SH tests were to statistically test for one, two, or three independent evolutionary origins of symbiosis with clownfishes. We constrained the topology of the clownfish hosting species four ways: 1) all clownfish hosting species were constrained to a monophyletic group within a monophyletic Actinioidea, 2) all members of family Thalassianthidae, genus *Stichodactyla*, and genus *Heteractis* (i.e. two evolutionary origins of symbiosis with clownfishes; clades Stichodactylina + Heteractina; see Results), 3) all members of family Thalassianthidae, genus *Stichodactyla*, *H. magnifica*, and *Entacmaea quadricolor* (i.e. two evolutionary origins of symbiosis with clownfishes; clades Stichodactylina + *Entacmaea*; see Results), and 4) all members of *E. quadricolor*, *H. aurora*, *H. crispa*, *H. malu*, and *M. doorensis* (i.e. two evolutionary origins of symbiosis with clownfishes; clades *Entacmaea* + Heteractina). The SH tests were conducted using RAxML on the CIPRES Science Gateway Portal.

Finally, we conducted separate RAxML analyses for each anemone clade in which our larger Actiniaria-wide analyses inferred an independent evolutionary origin of symbiosis with clownfishes (see Results). These analyses were conducted at shallower evolutionary levels to include sequence data that may have otherwise been excluded by Gblocks in our larger dataset, with the goal of improving phylogenetic resolution within each group and exploring potential biogeographic signal. Data were concatenated, partitioned, and analyzed in RAxML as above. The three analyses included 1) all members of the family Thalassianthidae, genus *Stichodactyla*, and *H. magnifica*, 2) all members of *H. aurora*, *H. crispa*, *H. malu*, and *M. doorensis*, and 3) all members of *E. quadricolor*.

Bayesian phylogenetic analyses (e.g. BEAST, MrBayes) were not included in our final results due to an inability to obtain MCMC chain convergence, similar to previous studies (e.g. Rodríguez et al., 2014), and likely owing to the size and lack of signal in our dataset.

3. Results

Concatenated Gblocks alignments resulted in a final data matrix of 5,887 base pairs for the full actiniarian-wide analysis. Alignments for lower level analyses of 1) all members of the family Thalassianthidae, genus *Stichodactyla*, and *H. magnifica*, 2) all members of *H. aurora*, *H. crispa*, *H. malu*, and *M. doorensis*, and 3) *E. quadricolor* resulted in data matrices of 6,941, 7,085, and 6,871 base pairs respectively.

Maximum Likelihood phylogenetic analyses in RAxML recovered each anemone superfamily, and hierarchical relationships among superfamilies, as described by Rodríguez et al (2014), but with varying degrees of nodal support (Figure S1). Like Rodríguez et al. (2014), we obtained inconsistent nodal support across the backbone of Actiniaria, which is common with this suite of phylogenetic markers. Relationships within superfamilies Actinernoidea, Actinostoloidea, Edwardsioidea, and Metridioidea were also broadly reflective of those recovered by Rodríguez et al. (2014; Figure S2).

Within Actinioidea, our ML analyses recovered the family Stichodactylidae (as currently accepted, including *Stichodactyla* and *Heteractis*), as polyphyletic (Figure 2; Figure S3). Only the magnificent anemone *H. magnifica* was recovered in the clade that included members of the genus *Stichodactyla*. Our analyses also find the genus *Stichodactyla* to be paraphyletic (Figure 2; Figure S3). In our full analysis, specimens of *S. mertensii* and *S. haddoni* appeared to share a more recent common ancestor with *C. adhaesivum*, *Thalassianthus aster*, and *T. hemprichii* than

with *S. gigantea* and *S. helianthus* (Figure 2; Figure S3). Our in-depth ML analysis of this clade did not recover this relationship but instead reflected that all species from the Indo-Pacific (*S. gigantea*, *S. haddoni*, *S. mertensi*, *H. magnifica*, *C. adhaesivum*, and the genus *Thalassianthus*) are more closely related to each other than they are to the Atlantic *S. helianthus* (Figure 3). Interestingly, our analyses recovered the family Thalassianthidae, which includes *Cryptodendrum* and *Thalassianthus*, to be nested within a well-supported clade that included all members of the genus *Stichodactyla* and *H. magnifica* (Figures. 2 & 3; Figure S3). We refer to this clade as “Stichodactylina” or “true carpet anemones” throughout the remainder of the manuscript. The species *S. helianthus* appears to have diverged early in the evolutionary history of Stichodactylina, which split the clade into Atlantic and Indo-Pacific sister lineages (Figures 2 & 3; Figure S3).

Other than *H. magnifica*, the species currently ascribed to the genus *Heteractis sensu* Dunn (1981) (or to *Heteractis* and *Radianthus sensu* England (1988)), formed a moderately supported clade, along with *M. doreensis*. This clade is within the superfamily Actinioidea and distant to the Stichodactylina (Figure 2; Figure S3). This group of *Heteractis* and *Macroactyla* species shared a well-supported node with *Phymanthus loligo* deeper in the tree (Figure 2 & 4; Figure S3). Unexpectedly, our analyses find three distinct groupings of individuals identified as *H. aurora*, *H. crispa*, *H. malu*, and *M. doreensis* separated by relatively long branches, but with little nodal support for those groupings. Further, these groupings do not correspond to any current sea anemone taxonomy, as individuals identified as members of each nominal species were found within each cluster (Figure 2 & 4; Figure S3). Although this clade included *M. doreensis* and excluded *H. magnifica*, it does appear to partially support England’s (1988) distinction between these species and members of Stichodactylidae. We refer to this clade as

“Heteractina”, in reference to England’s designation, throughout the rest of the manuscript. In-depth phylogenetic analyses of Heteractina did not provide further resolution among species in this clade (Figure 4).

The individuals of bubble tip anemone *E. quadricolor* formed a highly supported clade, which makes it, together with the adhesive anemone *C. adhaesivum*, one of only two clownfish hosting species to form well-supported monophyletic lineages in our actinarian-wide analysis (Figure 2; Figure S3). No members of Actinioidea formed a well-supported sister relationship to *E. quadricolor*, although the relationship between *E. quadricolor* and a clade of Southern Hemisphere brooding anemones from the genus *Epiactis* would be worth further exploring with genomic data (Figure 2; Figure S3). Intraspecific branch lengths within *E. quadricolor* exceeded those typically found at the intraspecific levels in other species (Figure 2; Figure S3), and our shallow evolutionary analyses recovered well-supported hierarchical relationships that correspond to biogeographic patterns (Figure 5). We recovered two well-supported clades within *E. quadricolor*, one that encompasses samples from the Red Sea, Maldives, and Japan, and a second that includes samples from Tonga and Japan (Figure 5). Samples from the United Arab Emirates appear to have diverged early and likely represents a third lineage, although this node was not well supported (Figure 5).

Likelihood ratio tests in RAxML show that our unconstrained analysis of Actiniaria is a significantly better topology than a topology that constrains all clownfish-hosting lineages to a single monophyletic group [$D(LH) = -709.74$, $p < 0.01$], or topologies that constrain the clownfish-hosting lineages into pairs of clades: Stichodactylina + Heteractina [$D(LK) = -634.58$, $p < 0.01$], Stichodactylina + *Entacmaea* [$D(LK) = -487.76$, $p < 0.01$], and *Entacmaea* + Heteractina [$D(LK) = -288.36$, $p < 0.01$]. Thus, based on our unconstrained Actiniaria-wide

analysis, we conclude that symbiosis with clownfishes has evolved at least three times within sea anemones (Figure 2; Figure S3); once within the lineage currently construed as *E. quadricolor*, once within the true carpet anemones (Stichodactylina) after *S. helianthus* diverged in the Atlantic, and once within Heteractina (Figure 2; Figure S3). Based on our phylogenetic reconstruction, we identify the genus *Thalassianthus*, within Stichodactylina, as the only group where symbiosis with clownfishes has been lost (Figures 2 & 3; Figure S3).

Like previous studies (e.g. Rodríguez et al. 2014; Larson & Daly 2016; Daly et al. 2017) gene tree analyses of Actiniaria provided no additional resolution into the evolutionary relationships among the clownfish anemones, nor any insight into possible sources of gene tree discordance (Figures S4-S6).

4. Discussion

Our phylogenetic reconstruction of Actiniaria provides the first in-depth molecular investigation into the evolution of the clownfish-hosting sea anemones, shedding light on their systematics, diversity and biogeographic origin. This perspective has been lacking in broader studies of clownfish evolution (e.g. Litsios et al., 2014a). Perhaps unsurprisingly, we recover widespread poly- and para-phyly among the actiniarian families and genera to which the 10 host anemone species are assigned. Instead, we identify three higher-level clades (Stichodactylina, Heteractina, and *E. quadricolor*) where we infer symbioses with clownfishes have evolved independently. Below, we discuss the taxonomic problems our data have revealed at the family, generic, and species levels within the clownfish-hosting sea anemones. We follow with discussion of how our results impact our understanding of how clownfish-sea anemone symbiosis has evolved.

Family-level taxonomy within Actinioidea is messy, with no evidence of monophyly for most groups (Rodríguez et al. 2014; Larson & Daly 2016; Daly et al. 2017). The clownfish-hosting anemones are no exception to this general pattern. As outlined above, the clownfish hosting sea anemones are traditionally recognized by Fautin (1991, 2013, 2016) as belonging to three families. These include Actiniidae (*Entacmaea* and *Macrodactyla*), Stichodactylidae (*Stichodactyla* and *Heteractis*), and Thallasianthidae (*Cryptodendrum*). England (1988) proposed four families, splitting *Heteractis* into Heteractidae, and further suggesting that Heteractidae includes *Heteractis*, with the single species *H. aurora*, and *Radianthus*, for the species commonly referred to as *H. crispa*, *H. magnifica*, and *H. malu*. Our molecular data provide some evidence for a clade that aligns with Stichodactylidae (our Stichodactylina) and for some aspects of the proposed Heteractidae, but the membership of these groups in our trees is not identical to their traditionally recognized taxonomic composition. Stichodactylina also includes what is traditionally recognized as family Thallasianthidae (see Daly & Fautin 2019). In the broader phylogenetic picture, Actiniidae is paraphyletic, consisting of all Actinioidea not assigned to a more exclusive group.

The generic and familial taxonomic problems are most significant in *Heteractis* and *Stichodactyla*. Our molecular results do not support maintaining the current taxonomy of Fautin (1991) but are also inconsistent with the proposal of England (1988). In partial agreement with England (1988), we find that *H. aurora*, *H. crispa*, and *H. malu* do not form a monophyletic familial relationship with the members of the genus *Stichodactyla*, and thus partially support his proposal for the designation of a family Heteractidae. However, we find that *H. magnifica* is more closely related to species in *Stichodactyla* (and to *Cryptodendron* and *Thalassianthus*) than it is to the other species of *Heteractis*. Further, contra the proposal of England (1988) that it is

distinct from the other species of the genus, *H. aurora* cannot be differentiated from *H. crispa* and *H. malu*, and thus, our molecular data do not support the resurrection of the genus *Radianthus* based on the presence of *p*-mastigophores A with a looped tubule in the cnidom of *H. aurora*. The nematocysts that were observed by England in *H. aurora* may be interpreted as being an immature stage of the *p*-mastigophores A present in all the species of *Heteractis* (Gusmão et al., 2018). Specimens of *M. doreensis* were also recovered to form a close relationship with *H. aurora*, *H. crispa*, and *H. malu*. This relationship between *Macrodactyla* and species of *Heteractis* contradicts the decision of Fautin (2016) to move these species to *Condylactis* to correct the nomenclature issue with the generic name *Macrodactyla*. *Macrodactyla* is quite distinct, phylogenetically, from the representative of *Condylactis* in our analyses (*C. gigantea*), which groups as sister to one Caribbean species of *Phymanthus* (Figure 2; Figure S3). Taken together, the relationships between *H. aurora*, *H. crispa*, *H. malu*, and *M. doreensis* form our Heteractina clade.

Our data also highlight generic and familial taxonomic problems of Fautin's and England's genus *Stichodactyla* and family Thalassianthidae. The nesting of the Thalassianthidae within *Stichodactyla*, coupled with the relationships among members of the genus *Stichodactyla* and *H. magnifica* renders *Stichodactyla* paraphyletic. *Heteractis magnifica* and all other Indo-Pacific members of *Stichodactyla*, *Cryptodendrum*, and *Thalassianthus* share a more recent common ancestor than they do with the Atlantic *S. helianthus*. *Stichodactyla mertensii* Brandt, 1835 is the type species of the genus *Stichodactyla* and thus, *S. helianthus* (Ellis, 1768) should likely be designated as belonging to a different genus according to the Principle of Priority (Art. 23, ICZN 1999). Similarly, *H. magnifica* should be placed in a different genus in concert with a

revision of *Stichodactyla*. Together, all members of *Stichodactyla*, *Cryptodendrum*, *Thalassianthus*, and *H. magnifica* form our Stichodactylina clade.

At the species level, our data reveal that only *C. adhaesivum* and *E. quadricolor* were recovered as monophyletic with high support. Within our Heteractina clade, the pattern of relationships we find for the samples of *H. aurora*, *H. crista*, *H. malu*, and *M. doreensis* are difficult to interpret. Although there appears to be a moderate amount of genetic variation within Heteractinia, the major nodes within the clade are all poorly supported and do not form species-specific units (Figure 4). It is possible that the repeated occurrence of subclades identified as *H. aurora* and *H. crista* and *H. malu* suggest that these names encompass several as-yet undescribed species (Figure 4). Specimens of *M. doreensis* were also recovered in several of the clades within Heteractina. Two possible hypotheses may explain the patterns we see here: 1) *H. crista*, *H. malu*, and *M. doreensis* are superficially similar in appearance (Figure 1), share similar habitat space, and could easily be misidentified in the field when these species co-occur. Multiple sequences in our analyses come from GenBank (Table 1) and thus it is possible that misidentification could have led to the patterns we observed here. We believe the likelihood for misidentification within our dataset is low, as the newly generated data for this study came from individuals that were collected, photographed in the field, and positively identified by the authors of this study directly. Although we identified anemones in the field following Fautin and Allen (1992), recent contributions to sea anemone systematics keep pointing to high levels of morphological convergence and the necessity of reevaluating the traditionally used morphological characters and their phylogenetic signal (e.g. Daly et al., 2017 Laretta et al., 2014, Rodríguez et al., 2012, 2014). A more likely possibility is that 2) these genetic markers simply cannot resolve the relationship between these taxa at this level, and/or incomplete lineage

sorting is driving the observed pattern among species. New, high resolution genomic methods, such as bait-capture approaches targeting ultra conserved elements (e.g. Quattrini et al., 2018), are needed to clarify these shallow relationships.

Within Stichodactylina we are unable to resolve species-level support for *S. gigantea*, *S. haddoni*, and *S. mertensii* (Figure 3). Samples of *S. haddoni* and *S. mertensii* do intermingle within a fairly well supported group but we do not recover species-level resolution. *Stichodactyla haddoni* and *S. mertensii* are described as occupying distinct ecological habitats, with *S. haddoni* occurring exclusively on sandy bottoms and *S. mertensii* occurring exclusively on coral-dominated reefs attached to hard substrata. Interestingly, we repeatedly recover *H. magnifica* and *S. gigantea* within the same group as well (Figures 2 and 3), but with little support. However, like *S. haddoni* and *S. mertensii*, *H. magnifica* and *S. gigantea* also occupy distinct habitats. *Stichodactyla gigantea* is described to occupy shallow sandy-bottom habitats (while *S. haddoni* is described to occur at greater depths), and *H. magnifica* occupies coral-dominated reef habitat attached directly to hard substrata similarly to *S. mertensii*. Like the Heteractina above, targeted phylogenetic analyses of Stichodactylina using highly resolving genomic markers should accompany morphological revisions to resolve hierarchical relationships within the clade, and explore whether there have been repeated ecological speciation events within the group.

Although our data show *E. quadricolor* to be monophyletic, the branch lengths and topological support within our focused reconstruction of this species (Figure 5) suggest that this grouping may be a species complex. The ((Red Sea, Maldives), Tonga) topological relationship is a classic Indian Ocean/Pacific Ocean biogeographic pattern found at both the intra- and inter-specific levels in the phylogeographic literature (reviewed by Bowen et al., 2016). However, co-occurring samples from Japan also belong to both of these well-supported clades (Figure 5), and

provide further evidence these may represent cryptic species. The long branch linking samples from the United Arab Emirates with the rest of the Indo-Pacific can also likely be construed as a third cryptic lineage. Counterintuitively, the ability of the current suite of genetic markers we used in this study to resolve hierarchical relationships within *E. quadricolor* may provide the strongest support for the distinctiveness of these lineages. Anthozoan mtDNA is largely uninformative at the intraspecific-level due to its slow rate of evolution (e.g. Daly et al., 2010; Shearer et al., 2002), and the combination of mtDNA and nuDNA markers used here are regularly unable to differentiate between species within the same genus (e.g. Daly et al., 2017; Grajales & Rodríguez, 2016; Larson & Daly, 2016; Rodríguez et al., 2014). However, sympatric species within the genus *Exaiptasia* have been delimited previously using these markers (Grajales & Rodríguez, 2016), and taken together, we provide the first preliminary evidence that there may be more than 10 species of clownfish-hosting sea anemones. As *E. quadricolor* hosts the greatest number of clownfish species throughout the Indo-Pacific (Fautin & Allen, 1992), the presence of extensive undescribed cryptic anemone species within this group could have wide ranging implications for our understanding of clownfish host associations, their symbiotic specialist/generalist designations, and the degree to which these mutualistic relationships are nested (e.g. Ollerton et al., 2007).

In summary, our data conclusively reject the current taxonomy of the clownfish-hosting sea anemones. Major revisionary work at the familial, generic, and specific level is required to align taxonomy of the clownfish-hosting anemones with the phylogenetic results we present here.

Although we reject the current taxonomy of the clownfish-hosting sea anemones, we fail to reject the hypothesis that symbiosis with clownfishes evolved multiple times within

Actiniaria. While it could be argued that our analysis cannot distinguish between a single or multiple origins of symbiosis with clownfishes, our likelihood ratio test convincingly refutes a model with only a single origin. Thus, we interpret our phylogenetic reconstruction to show that symbiosis with clownfishes evolved, minimally, three times within Actiniaria— all within the superfamily Actinioidea. We identify these independent origins as 1) within Stichodactylina, after *S. helianthus* diverged in the Tropical Western Atlantic, 2) in Heteractina, and 3) in the *E. quadricolor* species complex. We identify *Thalassianthus* as a possible lineage in which symbiosis with clownfishes was lost. This interpretation is made with the important caveat that clownfishes would have had to have been symbiotic with the common ancestor of these three clades, with this relationship being retained as these lineages further diversified. If diversification of all 10 nominal anemone hosts pre-dates the onset of their symbiosis with fishes, then we would interpret the symbiosis to have evolved independently within each host species (i.e. 10 independent evolutionary origins).

The lack of a fossil record for anemones broadly and unknown mutation rates for the loci we have used here prevents us from producing time-calibrated phylogenetic trees. However, biogeographic patterns can help provide a minimum age for some of our clades. The phylogenetic position of the non-hosting, Tropical Western Atlantic species *S. helianthus* within Stichodactylina suggests that this clade has a Tethyan biogeographic origin, as it is divided into Atlantic and Indo-Pacific lineages (Figures 2 & 3). The final closure of the Tethys Sea, termed the Terminal Tethyan Event (TTE), occurred during the Miocene and has been dated to between 12-18 myr (reviewed by Cowman & Bellwood, 2013). Although vicariance between Atlantic and Indo-Pacific lineages has been shown to include numerous pulses of diversification that often pre-date the TTE in other taxonomic groups (Cowman & Bellwood, 2013), this event establishes

a minimum potential age on the Stichodactylina and for the adoption of the symbiosis, which is inferred to have occurred at or after the split between *S. helianthus* and the rest of Stichodactylina. Further, within *E. quadricolor*, because anemones from the United Arab Emirates are interpreted as sister to the rest of the Indo-Pacific, we also infer a Tethyan origin and minimum age of 12-18 myr for *E. quadricolor*. Tethyan relic species found around the Arabian Peninsula, such as cowries and opisthobranch molluscs, show similar phylogenetic structure (e.g. Malaquias & Reid, 2009; Meyer, 2003). Interestingly, the root of the clownfish clade is consistently dated between 12-19 myr (e.g. Litsios et al., 2012; Litsios et al., 2014a; Rolland et al., 2018), which overlaps with the minimum potential age of the Stichodactylina, the *E. quadricolor* clade and the TTE. The clownfish subfamily, however, shows strong support for a Coral Triangle biogeographic origin, followed by an independent geographical radiation in the Western Indian Ocean ~4 mya (Litsios et al., 2014a). Taken together, it appears probable that the biogeographic setting of diversification differed between clownfish and at least two major lineages of host anemones.

Throughout their ranges host anemones are rarely unoccupied, and indeed, anemones do not serve as mere habitat space for clownfish. Rather, they receive tangible benefits from their symbiotic partners that allow them to compete and maintain habitat space, ward off predators, and obtain a steady source of nitrogen in an oligotrophic environment (Cleveland et al., 2011; Dunn, 1981; Fautin, 1991; Roopin et al., 2008; Szczebak et al., 2013). The ecological success of the anemone hosts, particularly on low-latitude reefs, clearly relies on clownfish. Whether the diversity and evolutionary history of the host anemones has been similarly driven by their symbiosis with clownfish is less clear. A Tethyan origin for the Stichodactylina, and possibly *E. quadricolor*, demonstrates that host anemones likely had to expand their geographic ranges from

the Tethys Sea across the Indian Ocean and into the Coral Triangle as solitary individuals before establishing symbiosis with clownfishes. Further, if host anemone diversification into Atlantic and Indo-Pacific Ocean basins preceded the TTE, as may be the case in other groups with Tethyan biogeographic histories, it would imply that the majority of host anemone diversity arose before the clownfish radiation. Whether diversification of the clownfishes and host anemones is fully decoupled will require extensive biogeographic sampling across all host anemones. This appears warranted as *E. quadricolor* (and possibly *H. magnifica* and others) may be a species complex and host anemones could be far more diverse than we currently recognize. Beyond spatial patterns of diversification, our understanding of actinarian diversity broadly would stand to benefit from calibrated genomic mutation rates, allowing us to explore temporal patterns of co-diversification as well. These will be valuable avenues for future studies employing highly resolving genomic markers coupled with increased biogeographic sampling for each nominal anemone host lineage.

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Ethics

Sea anemones and tissue samples were collected under research permits: 30-D/INDIV/2018/27 (Maldives) and RE-17-04 and RE-18-07 (Palau). From the United Arab Emirates, tissue samples were collected and exported with official written permission from M. Fathima Al Antubi, head of the Environmental Protection Department, Government of Fujairah, Dibba Municipality. Samples from the Philippines were collected with the support of the Philippines Department of Agriculture, Bureau of Fisheries and Aquatic Resources, and the National Fisheries Research and Development Institute of the Philippines under permits GP-0085-15 and GP-0077-14, and are loaned to MD via Materials Transfer Agreement between her and the California Academy of Sciences. Sampling in Saudi Arabia was conducted under KAUST's authority in cooperation with the relevant governmental agencies. No permits were required to collect sea anemones in Japan.

Competing interests

We declare we have no competing interests

Author contributions

BMT conceived the project, collected tissue samples and field data, performed laboratory benchwork and sequencing, performed analyses, and wrote the paper. CB, RL, LCG, VVD, and TC performed laboratory bench-work and sequencing, performed analyses, and critically revised the paper. CPM, MLB, AB, KY, JDR, TF, MD collected tissue samples and field data, and critically revised the paper. ER conceived the project and critically revised the paper.

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Table 1. Clownfish-hosting sea anemone species and samples included in this study as part of the broader phylogenetic analysis of Actiniaria. Table includes information on Family, Genus, and Species designations based on the currently accepted taxonomy from Fautin (2013). All species listed belong to superfamily Actinioidea. Locality indicates country of origin for each sample, and Ocean Basin reflects the body of water to which each locality broadly belongs. GenBank accession numbers in **Bold** are new to this study. *Denotes *Stichodactyla helianthus* from the Tropical Western Atlantic, a non-clownfish host species.

Family	Genus	Species	Locality	Ocean Basin	CO3	12S	16S	18S	28S
Actiniidae	<i>Entacmaea</i>	<i>quadricolor</i>	Japan	West Pacific	MK522439	MK519401	MK519456	-	-
Actiniidae	<i>Entacmaea</i>	<i>quadricolor</i>	Japan	West Pacific	MK522440	MK519402	MK519457	MK519571	-
Actiniidae	<i>Entacmaea</i>	<i>quadricolor</i>	Maldives	Central Indian	MK522441	MK519403	-	MK519566	MK519639
Actiniidae	<i>Entacmaea</i>	<i>quadricolor</i>	Maldives	Central Indian	MK522442	MK519404	MK519458	MK519567	MK519644
Actiniidae	<i>Entacmaea</i>	<i>quadricolor</i>	Saudi Arabia	Red Sea	MK522443	MK519405	MK519459	MK519568	MK519643
Actiniidae	<i>Entacmaea</i>	<i>quadricolor</i>	Tonga	Southwest Pacific	MK522444	MK519406	MK519460	MK519569	MK519645
Actiniidae	<i>Entacmaea</i>	<i>quadricolor</i>	Tonga	Southwest Pacific	MK522445	MK519407	MK519461	MK519570	MK519646
Actiniidae	<i>Entacmaea</i>	<i>quadricolor</i>	United Arab Emirates	Gulf of Oman	MK522446	MK519408	MK519462	MK519572	MK519640
Actiniidae	<i>Entacmaea</i>	<i>quadricolor</i>	United Arab Emirates	Gulf of Oman	MK522447	MK519409	MK519463	MK519573	MK519641
Actiniidae	<i>Entacmaea</i>	<i>quadricolor</i>	United Arab Emirates	Gulf of Oman	MK522448	MK519410	MK519464	-	MK519642
Actiniidae	<i>Macroactyla</i>	<i>dorensis</i>	Philippines	Coral Triangle	MK522449	MK519411	MK519465	MK519581	MK519661
Actiniidae	<i>Macroactyla</i>	<i>dorensis</i>	Philippines	Coral Triangle	MK522450	-	MK519466	MK519582	-
Actiniidae	<i>Macroactyla</i>	<i>dorensis</i>	Unknown	Unknown	GU473342	EU190739	EU190785		KJ483049
Stichodactylidae	<i>Heteractis</i>	<i>aurora</i>	Maldives	Central Indian	MK522451	MK519412	MK519467	-	-
Stichodactylidae	<i>Heteractis</i>	<i>aurora</i>	Maldives	Central Indian	MK522452	MK519413	MK519468	-	-
Stichodactylidae	<i>Heteractis</i>	<i>aurora</i>	Maldives	Central Indian	MK522453	MK519414	MK519469	-	MK519647
Stichodactylidae	<i>Heteractis</i>	<i>aurora</i>	Maldives	Central Indian	MK522454	MK519415	MK519470	-	-
Stichodactylidae	<i>Heteractis</i>	<i>aurora</i>	Unknown	Unknown	KC812229	KC812139	KC812160	KC812184	-
Stichodactylidae	<i>Heteractis</i>	<i>aurora</i>	Unknown	Unknown	-	EU190729	EU190773	-	-
Stichodactylidae	<i>Heteractis</i>	<i>aurora</i>	Unknown	Unknown	-	-	-	KC812185	-
Stichodactylidae	<i>Heteractis</i>	<i>crispa</i>	Unknown	Unknown	KC812230	KC812140	KC812161	KC812186	-
Stichodactylidae	<i>Heteractis</i>	<i>crispa</i>	Japan	West Pacific	MK522455	MK519416	MK519471	-	MK519648
Stichodactylidae	<i>Heteractis</i>	<i>crispa</i>	Japan	West Pacific	MK522456	MK519417	MK519472	-	MK519649
Stichodactylidae	<i>Heteractis</i>	<i>crispa</i>	Japan	West Pacific	MK522457	MK519418	MK519473	-	MK519650
Stichodactylidae	<i>Heteractis</i>	<i>crispa</i>	Palau	West Pacific	MK522458	MK519419	MK519474	-	-
Stichodactylidae	<i>Heteractis</i>	<i>crispa</i>	Palau	West Pacific	MK522460	MK519421	MK519476	-	-
Stichodactylidae	<i>Heteractis</i>	<i>crispa</i>	Palau	West Pacific	MK522461	MK519422	MK519477	-	-
Stichodactylidae	<i>Heteractis</i>	<i>crispa</i>	Saudi Arabia	Red Sea	MK522462	MK519423	MK519478	-	MK519654
Stichodactylidae	<i>Heteractis</i>	<i>crispa</i>	Saudi Arabia	Red Sea	MK522463	MK519424	MK519479	-	-
Stichodactylidae	<i>Heteractis</i>	<i>crispa</i>	Saudi Arabia	Red Sea	MK522464	MK519425	MK519480	-	-
Stichodactylidae	<i>Heteractis</i>	<i>crispa</i>	Tonga	Southwest Pacific	MK522465	MK519426	MK519481	MK519574	MK519651
Stichodactylidae	<i>Heteractis</i>	<i>crispa</i>	United Arab Emirates	Gulf of Oman	MK522466	MK519427	MK519482	-	-
Stichodactylidae	<i>Heteractis</i>	<i>crispa</i>	United Arab Emirates	Gulf of Oman	MK522467	MK519428	MK519483	-	MK519652
Stichodactylidae	<i>Heteractis</i>	<i>crispa</i>	United Arab Emirates	Gulf of Oman	MK522468	MK519429	MK519484	-	MK519653
Stichodactylidae	<i>Heteractis</i>	<i>magnifica</i>	Unknown	Unknown	KJ482988	EU190732	EU190777	-	JK483093

Stichodactylidae	<i>Heteractis</i>	<i>magnifica</i>	Unknown	Unknown	GU473361	-	-	EU190862	EU190821
Stichodactylidae	<i>Heteractis</i>	<i>magnifica</i>	Unknown	Unknown	KC812231	-	KC812162	KC812187	-
Stichodactylidae	<i>Heteractis</i>	<i>magnifica</i>	Maldives	Central Indian	MK522469	MK519430	MK519485	MK519575	MK519656
Stichodactylidae	<i>Heteractis</i>	<i>magnifica</i>	Maldives	Central Indian	MK522470	MK519431	MK519486	MK519576	MK519657
Stichodactylidae	<i>Heteractis</i>	<i>magnifica</i>	Maldives	Central Indian	MK522471	MK519432	MK519487	MK519577	MK519658
Stichodactylidae	<i>Heteractis</i>	<i>magnifica</i>	Maldives	Central Indian	MK522472	MK519433	MK519488	MK519578	MK519659
Stichodactylidae	<i>Heteractis</i>	<i>magnifica</i>	Maldives	Central Indian	MK522473	MK519434	MK519489	MK519579	MK519660
Stichodactylidae	<i>Heteractis</i>	<i>magnifica</i>	Palau	West Pacific	MK522459	MK519420	MK519475	MK519580	MK519655
Stichodactylidae	<i>Heteractis</i>	<i>malu</i>	Tonga	Southwest Pacific	MK522474	-	-	-	-
Stichodactylidae	<i>Heteractis</i>	<i>malu</i>	Tonga	Southwest Pacific	MK522475	-	MK519490	-	-
Stichodactylidae	<i>Stichodactyla</i>	<i>gigantea</i>	Unknown	Unknown	GU473347	EU190747	-	EU190873	EU190835
Stichodactylidae	<i>Stichodactyla</i>	<i>gigantea</i>	Unknown	Unknown	KC812232	-	-	KC812188	-
Stichodactylidae	<i>Stichodactyla</i>	<i>gigantea</i>	Unknown	Unknown	KY789299	-	EU190793	-	-
Stichodactylidae	<i>Stichodactyla</i>	<i>haddoni</i>	Philippines	Coral Triangle	MK522476	MK519435	MK519491	MK519583	MK519662
Stichodactylidae	<i>Stichodactyla</i>	<i>haddoni</i>	Unknown	Unknown	KC812233	-	FJ417090	FJ417089	-
Stichodactylidae	<i>Stichodactyla</i>	<i>helianthus</i> *	Panama	Tropical Western Atlantic	MK522477	MK519436	MK519492	MK519563	MK519667
Stichodactylidae	<i>Stichodactyla</i>	<i>helianthus</i> *	Panama	Tropical Western Atlantic	MK522478	MK519437	MK519493	MK519564	MK519668
Stichodactylidae	<i>Stichodactyla</i>	<i>helianthus</i> *	Panama	Tropical Western Atlantic	MK522479	MK519438	MK519494	-	MK519669
Stichodactylidae	<i>Stichodactyla</i>	<i>helianthus</i> *	Panama	Tropical Western Atlantic	MK522480	MK519439	MK519495	-	MK519670
Stichodactylidae	<i>Stichodactyla</i>	<i>mertensii</i>	Unknown	Tropical Western Atlantic	KC812234	KC812141	KC812163	KC812189	-
Stichodactylidae	<i>Stichodactyla</i>	<i>mertensii</i>	Maldives	Central Indian	K522481	MK519440	MK519496	MK519584	MK519663
Stichodactylidae	<i>Stichodactyla</i>	<i>mertensii</i>	Maldives	Central Indian	K522482	MK519441	MK519497	MK519585	MK519664
Stichodactylidae	<i>Stichodactyla</i>	<i>mertensii</i>	Maldives	Central Indian	K522483	MK519442	MK519498	MK519586	MK519665
Stichodactylidae	<i>Stichodactyla</i>	<i>mertensii</i>	Maldives	Central Indian	-	MK519443	MK519499	MK519587	MK519666
Thalassianthidae	<i>Cryptodendrum</i>	<i>adhaesivum</i>	Maldives	Central Indian	K522484	--	MK519500	MK519565	MK519638
Thalassianthidae	<i>Cryptodendrum</i>	<i>adhaesivum</i>	Unknown	Unknown	KC812235	KC812142	KC812163	KC812190	KC812214
Thalassianthidae	<i>Cryptodendrum</i>	<i>adhaesivum</i>	Unknown	Unknown	KC812236	KC812143	KC812165	KC812191	KC812215
Thalassianthidae	<i>Cryptodendrum</i>	<i>adhaesivum</i>	Unknown	Unknown	KC812237	KC812144	KC812166	KC812192	KC812216

Figure Legend:

Figure 1. Representative images of the 10 species of clownfish-hosting sea anemones. A) *Cryptodendrum adhaesivum*, B) *Entacmaea quadricolor*, C) *Heteractis aurora*, D) *Heteractis crispa*, E) *Heteractis magnifica*, F) *Heteractis malu* (photo credit: Matthew Lee), G) *Macroactyla doreensis*, H) *Stichodactyla gigantea* (photo credit: Anne Hoggett), I) *Stichodactyla haddoni* (photo credit: Singgih Afifa Putra), J) *Stichodactyla mertensii*. Photo of *M. doreensis* by Lyle Vail, used under creative commons license attribution 3.0 license <http://creativecommons.org/licenses/by/3.0/>. Photos A-E, and J by B. Titus.

Figure 2. Phylogenetic reconstruction of Actiniaria. Tree resulting from Maximum Likelihood (ML) analysis in RAxML of concatenated dataset (CO3, 12S, 16S, 18S, 28S). Tree reflects relationships within anemone superfamily Actinioidea, with remaining superfamilies Actinernoidea (purple), Actinostoloidea (yellow), Edwardsioidea (blue), and Metridioidea (green) collapsed for clarity. Legend highlights the three clades where symbiosis with clownfishes has evolved: Red = *Entacmaea quadricolor*, Light Blue = Heteractina (*Heteractis aurora*, *H. crispa*, *H. malu*, and *Macroactyla doreensis*), Oranges = Stichodactylina (*Cryptodendrum adhaesivum*, *H. magnifica*, *Stichodactyla gigantea*, *S. haddoni*, *S. helianthus*, *S. mertensii*, *Thalassianthus aster*, *T. hemprichii*). Within Stichodactylina different hues of orange represent where *S. helianthus* diverged into the Atlantic Ocean, where symbiosis with clownfishes arose in the Indo-West Pacific, and where symbiosis with clownfishes may have been lost in the genus *Thalassianthus*. Black filled circles represent nodes with bootstrap resampling values ≥ 75 .

Figure 3. Partitioned Maximum Likelihood phylogenetic reconstruction of the carpet sea anemone Stichodactylina clade using all available sequence data (CO3; 12S, 16S, 18S, 28S). Orange arrow represents potential origin of symbiosis with clownfishes. Light orange arrow represents potential loss of symbiosis with clownfishes in the genus *Thalassianthus*. Colored nodes reflect bootstrap resampling values ≥ 75 . Pictured are the five host species *Cryptodendrum adhaesivum*, *Heteractis magnifica*, *Stichodactyla gigantea*, *S. haddoni*, and *S. mertensii*.

Figure 4. Partitioned Maximum Likelihood phylogenetic reconstruction of the Heteractina clade using all available sequence data (CO3; 12S, 16S, 18S, 28S). Colored nodes reflect bootstrap resampling values ≥ 75 . Pictured are the four host anemone species *Heteractis aurora*, *H. crispa*, *H. malu*, and *Macroactyla doreensis*.

Figure 5. Partitioned Maximum Likelihood phylogenetic reconstruction of the bubble-tip sea anemone *Entacmaea quadricolor* using all available sequence data (CO3; 12S, 16S, 18S, 28S). Tree reflects samples collected from throughout the biogeographic range of the species. Colored nodes reflect bootstrap resampling values ≥ 75 .

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Graphical abstract

Origins of clownfish symbiosis in host sea anemones

Highlights:

- Phylogeny does not support current taxonomy of clownfish-host anemones
- Three independent origins of symbiosis with clownfishes.
- Evidence for a Tethyan biogeographic origin for two anemone host clades.
- First evidence of cryptic sea anemone species among the clownfish-hosting lineages.
- Major taxonomic revision is needed among clownfish-hosting sea anemones.

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