Phylogenetic relationships among the clownfish-hosting sea anemones

Benjamin M. Titus[1, 2, *], Charlotte Benedict[3], Robert Laroche[4], Luciana C. Gusmão[1], Vanessa Van Deusen[1], Tommaso Chiodo[2], Christopher P. Meyer[5], Michael L. Berumen[6], Aaron Bartholomew[7], Kensuke Yanagi[8], James D. Reimer[9, 10], Takuma Fujii[11], Marymegan Daly[12], Estefanía Rodríguez[1,2]

[1]Division of Invertebrate Zoology, American Museum of Natural History, New York, NY, USA

[2]Sackler Institute for Comparative Genomics, American Museum of Natural History, New York, NY, USA

[3]Department of Biological Sciences, Auburn University, Auburn, AL, USA

[4]Department of Biology, University of Houston, Houston, TX, USA

[5]Department of Invertebrate Zoology, National Museum of Natural History, Smithsonian Institution, Washington DC, USA

[6]Division of Biological and Environmental Science and Engineering, Red Sea Research Center, King Abdullah University of Science and Technology, Thuwal, Saudi Arabia

[7]Gulf Environments Research Institute, American University of Sharjah, Sharjah, United Arab Emirates

[8]Coastal Branch of Natural History Museum and Institute, Chiba, Kastsuura, Chiba, Japan

[9]Molecular Invertebrate Systematics and Ecology Laboratory, Department of Biology, Chemistry, and Marine Sciences, Faculty of Science, University of the Ryukyus, Nishihara, Okinawa, Japan

[10]Tropical Biosphere Research Center, University of the Ryukyus, Nishihara, Okinawa, Japan

[11]International Center for Island Studies, Kagoshima University, Kagoshima, Japan

[12]Department of Evolution, Ecology, and Organismal Biology, The Ohio State University, Columbus, OH, USA

*Corresponding author: bentitus3@gmail.com; btitus@amnh.org

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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 *Sticodactyla*, 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 and famous 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 taxonomically described to belong to a reciprocally monophyletic clade with a common ancestor that was 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 specimen for the genus *Heteractis* (*H. aurora*) harbors macrobasic amastigophore nematocysts (*p*-mastigophores A with looped tubule *sensu* Gusmão et al., 2018), this excludes *H. crispa, 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. crispa, 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) considered and 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. England (1987) made no conclusion regarding the placement of *E. quadricolor* and thus it remains widely accepted as part of the family Actiinidae. 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 (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 widespread confusion regarding how many species of host anemones there actually are, and an abundance of species descriptions. 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, 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 Actiinidae. 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. 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 most exhaustive 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 (Table1; 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 -20ºC. 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 μL PCR product, 2 μL ExoSAP-IT, and 1 μL FastAP. Clean up reactions were carried out under the following conditions: 37ºC for 15 min, 85ºC for 15 min, and hold at 4ºC. Cycle sequencing reactions were carried out using 5 μL 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. 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 the Gblocks: Maximum number of contiguous non-conserved positions, 8; Minimum length of a block, 5; Gap positions allowed. Gblocks 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. 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 a likelihood ratio test of tree topologies (SH test) between the initial unconstrained analysis above, and a separate analysis where the 10 clownfish hosting species were constrained to a monophyletic group within a monophyletic Actinioidea. The SH test was 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 in order to include all sequence data that may have otherwise been excluded by Gblocks in our larger dataset in an attempt to improve phylogenetic resolution within each group and explore 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. doorenensis*, and 3) all members of *E. quadricolor*.

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. doorenensis,* 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

249 with *S. gigantea* and *S. helianthus* (Figure 2; Figure S3). Our in-depth ML analysis of this clade
250 did not recover this relationship but instead reflected that all species from the Indo-Pacific (*S.*
251 *gigantea*, *S. haddoni*, *S. mertensi*, *H. magnifica*, *C. adhaesivum*, and the genus *Thalassianthus*)
252 are more closely related to each other than they are to the Atlantic *S. helianthus* (Figure 3).
253 Interestingly, our analyses recovered the family Thalassianthidae, which includes
254 *Cryptodendrum* and *Thalassianthus*, to be nested within a well-supported clade that included all
255 members of the genus *Stichodactyla* and *H. magnifica* (Figures. 2 & 3; Figure S3). We refer to
256 this clade as "Stichodactylina" or "true carpet anemones" throughout the remainder of the
257 manuscript. The species *S. helianthus* appears to have diverged early in the evolutionary history
258 of Stichodactylina, which split the clade into Atlantic and Indo-Pacific sister lineages (Figures 2
259 & 3; Figure S3).
260 Other than *H. magnifica*, the species currently ascribed to the genus *Heteractis sensu*
261 Dunn (1981) (or to *Heteractis* and *Radianthus sensu* England (1988)), formed a moderately
262 supported clade, along with *M. doreensis*. This clade is within the superfamily Actinioidea and
263 distant to the Stichodactylina (Figure 2; Figure S3). This group of *Heteractis* and *Macrodactyla*
264 species shared a well-supported node with *Phymanthus loligo* deeper in the tree (Figure 2 & 4;
265 Figure S3). Unexpectedly, our analyses find three distinct groupings of individuals identified as
266 *H. aurora, H. crispa, H. malu,* and *M. doreensis* separated by relatively long branches, but with
267 little nodal support for those groupings. Further, these groupings do not correspond to any
268 current sea anemone taxonomy, as individuals identified as members of each nominal species
269 were found within each cluster (Figure 2 & 4; Figure S3). Although this clade included *M.*
270 *doreensis* and excluded *H. magnifica*, it does appear to partially support England's (1988)
271 distinction between these species and members of Stichodactylidae. We refer to this clade as

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

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

289 Likelihood ratio tests in RAxML show that our unconstrained analysis of Actiniaria is a
290 significantly better topology than a topology that constrains all clownfish-hosting lineages to a
291 monophyletic group [$D(LH) = -709.74$, $p < 0.01$]. Thus, based on our unconstrained Actiniaria-
292 wide analysis, we conclude that symbiosis with clownfishes has evolved at least three times
293 within sea anemones (Figure 2; Figure S3); once within the lineage currently construed as *E.*
294 *quadricolor*, once within the true carpet anemones (Stichodactylina) after *S. helianthus* diverged

295 in the Atlantic, and once within Heteractina (Figure 2; Figure S3). Based on our phylogenetic
296 reconstruction, we identify the genus *Thalassianthus*, within Stichodactylina, as the only group
297 where symbiosis with clownfishes has been lost (Figures 2 & 3; Figure S3).
298
299 **4. Discussion**
300 Our phylogenetic reconstruction of Actiniaria provides the first in-depth molecular investigation
301 into the evolution of the clownfish-hosting sea anemones, shedding light on their systematics,
302 diversity and biogeographic origin. This perspective has been lacking in broader studies of
303 clownfish evolution (e.g. Litsios et al., 2014a). Perhaps unsurprisingly, we recover widespread
304 poly- and para-phyly among the actiniarian families and genera to which the 10 host anemone
305 species are assigned. Instead, we identify three higher-level clades (Stichodactylina, Heteractina,
306 and *E. quadricolor*) where we infer symbioses with clownfishes have evolved independently.
307 Below, we discuss the taxonomic problems our data have revealed at the family, generic, and
308 species levels within the clownfish-hosting sea anemones. We follow with discussion of how our
309 results impact our understanding of how clownfish-sea anemone symbiosis has evolved.
310 Family-level taxonomy within Actinioidea is messy, with no evidence of monophyly for
311 most groups (Rodríguez et al. 2014; Larson & Daly 2016; Daly et al. 2017). The clownfish-
312 hosting anemones are no exception to this general pattern. As outlined above, the clownfish
313 hosting sea anemones are traditionally recognized by Fautin (1991, 2013, 2016) as belonging to
314 three families. These include Actiniidae (*Entacmaea* and *Macrodactyla*), Stichodactylidae
315 (*Stichodactyla* and *Heteractis*), and Thallasiandthidae (*Cryptodendrum*). England (1988)
316 proposed four families, splitting *Heteractis* into Heteractidae, and further suggesting that
317 Heteractidae includes *Heteractis,* with the single species *H. aurora,* and *Radianthus*, for the

318 species commonly referred to as *H. crispa, H. magnifica,* and *H. malu*. Our molecular data
319 provide some evidence for a clade that aligns with Stichodactylidae (our Stichodactylina) and for
320 some aspects of the proposed Heteractidae, but the membership of these groups in our trees is
321 not identical to their traditionally recognized taxonomic composition. Further, Stichodactylina
322 also includes what is traditionally recognized as family Thalassianthidae (see Daly & Fautin
323 2019). In the broader phylogenetic picture, Actiniidae is paraphyletic, consisting of all
324 Actinioidea not assigned to a more exclusive group.

325 The generic and familial taxonomic problems are most significant in *Heteractis* and
326 *Stichodactyla*. Our molecular results do not support maintaining the current taxonomy of Fautin
327 (1991) but are also inconsistent with the proposal of England (1988). In partial agreement with
328 England (1988), we find that *H. aurora, H. crispa,* and *H. malu* do not from a monophyletic
329 familial relationship with the members of the genus *Stichodactyla*, and thus partially support his
330 proposal for the designation of a family Heteractidae. However, we find that *H. magnifica* is
331 more closely related to species in *Stichodactyla* (and to *Cryptodendron* and *Thalassianthus*) than
332 it is to the other species of *Heteractis*. Further, contra the proposal of England (1988) that it is
333 distinct from the other species of the genus, *H. aurora* cannot be differentiated from *H. crispa*
334 and *H. malu*, and thus, our molecular data do not support the resurrection of the genus
335 *Radianthus* based on the presence of *p*-mastigophores A with a looped tubule in the cnidom of
336 *H. aurora*. The nematocysts that were observed by England in *H. aurora* may be interpreted as
337 being an immature stage of the *p*-mastigophores A present in all the species of *Heteractis*
338 (Gusmão et al., 2018). Specimens of *M. doreensis* were also recovered to form a close
339 relationship with *H. aurora*, *H. crispa*, and *H. malu*. This relationship between *Macrodactyla*
340 and species of *Heteractis* contradicts the decision of Fautin (2016) to move these species to

341 *Condylactis* to correct the nomenclature issue with the generic name *Macrodactyla*.
342 *Macrodactyla* is quite distinct, phylogenetically, from the representative of *Condylactis* in our
343 analyses (*C. gigantea*), which groups as sister to one Caribbean species of *Phymanthus* (Figure
344 2; Figure S3). Taken together, the relationships between *H. aurora*, *H. crispa*, *H. malu,* and *M.*
345 *doreensis* form our higher-level Heteractina clade.
346 Our data also highlight generic and familial taxonomic problems of Fautin's and
347 England's genus *Stichodactyla* and family Thalassianthidae. The nesting of the Thalassianthidae
348 within *Stichodactyla*, coupled with the relationships among members of the genus *Stichodactyla*
349 and *H. magnifica* renders *Stichodactyla* paraphyletic. *Heteractis magnifica* and all other Indo-
350 Pacific members of *Stichodactyla, Cryptodendrum*, and *Thalassianthus* share a more recent
351 common ancestor than they do with the Atlantic *S. helianthus*. *Stichodactyla mertensii* Brandt,
352 1835 is the type species of the genus *Stichodactyla* and thus, *S. helianthus* (Ellis, 1768) should
353 likely be designated as belonging to a different genus according to the Principle of Priority (Art.
354 23, ICZN 1999). Similarly, *H. magnifica* should be placed in a different genus in concert with a
355 revision of *Stichodactyla*. Together, all members of *Stichodactyla*, *Cryptodendrum,*
356 *Thalassianthus*, and *H. magnifica* form our Stichodactylina clade.
357 At the species level, our data reveal that only *C. adhaesivum* and *E. quadricolor* were
358 recovered as monophyletic with high support. Within our Heteractina clade, the pattern of
359 relationships we find for the samples of *H. aurora*, *H. crispa, H. malu*, and *M. doreensis* are
360 difficult to interpret. Although there appears to be a moderate amount of genetic variation within
361 Heteractinia, the major nodes within the clade are all poorly supported and do not form species-
362 specific units (Figure 4). It is possible that the repeated occurrence of subclades identified as *H.*
363 *aurora* and *H. crispa* and *H. malu* suggest that these names encompass several as-yet

364 undescribed species (Figure 4). Specimens of *M. doreensis* were also recovered in several of the
365 clades within Heteractina. Two possible hypotheses may explain the patterns we see here: 1) *H.*
366 *crispa, H. malu,* and *M. doreensis* are superficially similar in appearance (Figure 1), share
367 similar habitat space, and could easily be misidentified in the field when these species co-occur.
368 Multiple sequences in our analyses come from GenBank (Table 1) and thus it is possible that
369 misidentification could have led to the patterns we observed here. We believe the likelihood for
370 misidentification within our dataset is low, as the newly generated data for this study came from
371 individuals that were collected, photographed in the field, and positively identified by the authors
372 of this study directly. Although we identified anemones in the field following Fautin and Allen
373 (1992), recent contributions to sea anemone systematics keep pointing to high levels of
374 morphological convergence and the necessity of reevaluating the traditionally used
375 morphological characters and their phylogenetic signal (e.g. Daly et al., 2017 Lauretta et al.,
376 2014, Rodríguez et al., 2012, 2014). A more likely possibility is that 2) these genetic markers
377 simply cannot resolve the relationship between these taxa at this level, and/or incomplete lineage
378 sorting is driving the observed pattern among species. New, high resolution genomic methods,
379 such as bait-capture approaches targeting ultra conserved elements (e.g. Quattrini et al., 2018),
380 are needed to clarify these shallow relationships.

381 Within Stichodactylina we are unable to resolve species-level support for *S. gigantea, S.*
382 *haddoni*, and *S. mertensii* (Figure 3). Samples of *S. haddoni* and *S. mertensii* do intermingle
383 within a fairly well supported group but we do not recover species-level resolution. *Stichodactyla*
384 *haddoni* and *S. mertensii* are described as occupying distinct ecological habitats, with *S. haddoni*
385 occurring exclusively on sandy bottoms and *S. mertensii* occurring exclusively on coral-
386 dominated reefs attached to hard substrata. Interestingly, we repeatedly recover *H. magnifica* and

387 *S. gigantea* within the same group as well (Figures 2 and 3), but with little support. However,
388 like *S. haddoni* and *S. mertensii*, *H. magnifica* and *S. gigantea* also occupy distinct habitats.
389 *Stichodactyla gigantea* is described to occupy shallow sandy-bottom habitats (while *S. haddoni*
390 is described to occur at greater depths), and *H. magnifica* occupies coral-dominated reef habitat
391 attached directly to hard substrata similarly to *S. mertensii*. Like the Heteractina above, targeted
392 phylogenetic analyses of Stichodactylina using highly resolving genomic markers should
393 accompany morphological revisions to resolve hierarchical relationships within the clade, and
394 explore whether there have been repeated ecological speciation events within the group.
395 Although our data show *E. quadricolor* to be monophyletic, the branch lengths and
396 topological support within our focused reconstruction of this species (Figure 5) suggest that this
397 grouping may be a species complex. The ((Red Sea, Maldives), Tonga) topological relationship
398 is a classic Indian Ocean/Pacific Ocean biogeographic pattern found at both the intra- and
399 interspecific levels in the phylogeographic literature (reviewed by Bowen et al., 2016). However,
400 co-occurring samples from Japan also belong to both of these well-supported clades (Figure 5),
401 and provide further evidence these may represent cryptic species. The long branch linking
402 samples from the United Arab Emirates with the rest of the Indo-Pacific can also likely be
403 construed as a third cryptic lineage. Counterintuitively, the ability of the current suite of genetic
404 markers we used in this study to resolve hierarchical relationships within *E. quadricolor* may
405 provide the strongest support for the distinctiveness of these lineages. Anthozoan mtDNA is
406 largely uninformative at the intraspecific-level due to its slow rate of evolution (e.g. Daly et al,
407 2010; Shearer et al., 2002), and the combination of mtDNA and nuDNA markers used here are
408 regularly unable to differentiate between species within the same genus (e.g. Daly et al., 2017;
409 Grajales & Rodríguez, 2016; Larson & Daly, 2016; Rodríguez et al., 2014). However, sympatric

410 species within the genus *Exaiptasia* have been delimited previously using these markers
411 (Grajales & Rodríguez, 2016), and taken together, we provide the first preliminary evidence that
412 there may be more than 10 species of clownfish-hosting sea anemones. As *E. quadricolor* hosts
413 the greatest number of clownfish species throughout the Indo-Pacific (Fautin & Allen, 1992), the
414 presence of extensive undescribed cryptic anemone species within this group could have wide
415 ranging implications for our understanding of clownfish host associations, their symbiotic
416 specialist/generalist designations, and to the degree to which these mutualistic relationships are
417 nested (e.g. Ollerton et al., 2007).

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

422 Although we reject the current taxonomy of the clownfish-hosting sea anemones, we fail
423 to reject the hypothesis that symbiosis with clownfishes evolved multiple times within
424 Actiniaria. While it could be argued that our analysis cannot distinguish between a single or
425 multiple origins of symbiosis with clownfishes, our likelihood ratio test convincingly refutes a
426 model with only a single origin. Thus, we interpret our phylogenetic reconstruction to show that
427 symbiosis with clownfishes evolved, minimally, three times within Actiniaria— all within the
428 superfamily Actinioidea. We identify these independent origins as 1) within Stichodactylina,
429 after *S. helianthus* diverged in the Tropical Western Atlantic, 2) in Heteractina, and 3) in the *E.*
430 *quadricolor* species complex. We identify *Thalassianthus* as a possible lineage in which
431 symbiosis with clownfishes was lost. This interpretation is made with the important caveat that
432 clownfishes would have had to have been symbiotic with the common ancestor of these clades,

433 with this relationship being retained as these lineages further diversified. If diversification of all
434 10 nominal anemone hosts pre-dates the onset of their symbiosis with fishes, then we would
435 interpret the symbiosis to have evolved independently within each host species (i.e. 10
436 independent evolutionary origins).

437 The lack of a fossil record for anemones broadly and unknown mutation rates for the loci
438 we have used here prevents us from producing time-calibrated phylogenetic trees. However,
439 biogeographic patterns can help provide a minimum age for some of our clades. The
440 phylogenetic position of the non-hosting, Tropical Western Atlantic species *S. helianthus* within
441 Stichodactylina suggests that this clade has a Tethyan biogeographic origin, as it is divided into
442 Atlantic and Indo-Pacific lineages (Figures 2 & 3). The final closure of the Tethys Sea, termed
443 the Terminal Tethyan Event (TTE), occurred during the Miocene and has been dated to between
444 12-18 myr (reviewed by Cowman & Bellwood, 2013). Although vicariance between Atlantic and
445 Indo-Pacific lineages has been shown to include numerous pulses of diversification that often
446 pre-date the TTE in other taxonomic groups (Cowman & Bellwood, 2013), this event establishes
447 a minimum potential age on the Stichodactylina and for the adoption of the symbiosis, which is
448 inferred to have occurred at or after the split between *S. helianthus* and the rest of
449 Stichodactylina. Further, within *E. quadricolor*, because anemones from the United Arab
450 Emirates are interpreted as sister to the rest of the Indo-Pacific, we also infer a Tethyan origin
451 and minimum age of 12-18 myr for *E. quadricolor*. Tethyan relic species found around the
452 Arabian Peninsula, such as cowries and opisthobranch molluscs, show similar phylogenetic
453 structure (e.g. Malaquias & Reid, 2009; Meyer, 2003). Interestingly, the root of the clownfish
454 clade is consistently dated between 12-19 myr (e.g. Litsios et al., 2012; Litsios et al., 2014a;
455 Rolland et al., 2018), which broadly coincides with the minimum potential age of the

456 Stichodactylina, the *E. quadricolor* clade and the TTE. The clownfish subfamily, however,
457 shows strong support for a Coral Triangle biogeographic origin, followed by an independent
458 geographical radiation in the Western Indian Ocean ~4 mya (Litsios et al., 2014a). Taken
459 together, it appears probable that the biogeographic setting of diversification differed between
460 clownfish and at least two major lineages of host anemones.

461 Like many obligate mutualisms, the clownfish-sea anemone symbiosis is often described
462 in a manner in which it is presumed that the interacting species have co-evolved to varying
463 degrees. Throughout their ranges, host anemones are rarely unoccupied, and indeed, anemones
464 do not serve as mere habitat space for clownfish. Rather, they receive tangible benefits from their
465 symbiotic partners that allow them to compete and maintain habitat space, ward off predators,
466 and obtain a steady source of nitrogen in an oligotrophic environment (Cleveland et al., 2011;
467 Dunn, 1981; Fautin, 1991; Roopin et al., 2008; Szczebak et al., 2013). The ecological success of
468 the anemone hosts, particularly on low-latitude reefs, clearly relies on clownfish. Whether the
469 diversity and evolutionary history of the host anemones has been similarly driven by their
470 symbiosis with clownfish is less clear. Our ability to discern a Tethyan origin for the
471 Stichodactylina, and possibly *E. quadricolor*, demonstrates that host anemones likely had to
472 expand their geographic ranges from the Tethys Sea across the Indian Ocean and into the Coral
473 Triangle as solitary individuals before establishing symbiosis with clownfishes. Further, if host
474 anemone diversification into Atlantic and Indo-Pacific Ocean basins preceded the TTE, as may
475 be the case in other groups with Tethyan biogeographic histories, it would imply that the
476 majority of host anemone diversity arose before the clownfish radiation. Whether diversification
477 of the clownfishes and host anemones is fully decoupled will require extensive biogeographic
478 sampling across all host anemones. This appears warranted as *E. quadricolor* (and possibly *H.*

479 *magnifica* and others) may be a species complex and host anemones could be far more diverse
480 than we currently recognize. Beyond spatial patterns of diversification, our understanding of
481 actiniarian diversity broadly would stand to benefit from calibrated genomic mutation rates,
482 allowing us to explore temporal patterns of co-diversification as well. These will be valuable
483 avenues for future studies employing highly resolving genomic markers coupled with increased
484 biogeographic sampling for each nominal anemone host lineage.
485

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493

494 **Ethics**

495 Sea anemones and tissue samples were collected under research permits: 30-D/INDIV/2018/27
496 (Maldives) and RE-17-04 and RE-18-07 (Palau). From the United Arab Emirates, tissue samples
497 were collected and exported with official written permission from M. Fathima Al Antubi, head of
498 the Environmental Protection Department, Government of Fujairah, Dibba Municipality.
499 Samples from the Philippines were collected with the support of the Philippines Department of
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Data, code, and materials

All data are available in the Dryad Digital Repository: doi:XXXXX

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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740 **Table 1.** Clownfish-hosting sea anemone species and samples included in this study as part of the broader phylogenetic analysis of
741 Actiniaria. Table includes information on Family, Genus, and Species designations based on the currently accepted taxonomy from
742 Fautin (2013). All species listed belong to superfamily Actinioidea. Locality indicates country of origin for each sample, and Ocean
743 Basin reflects the body of water each locality broadly belongs to. GenBank accession numbers in **Bold** are new to this study. *Denotes
744 *Stichodactyla helianthus* from the Tropical Western Atlantic- a non-clownfish host species.
745

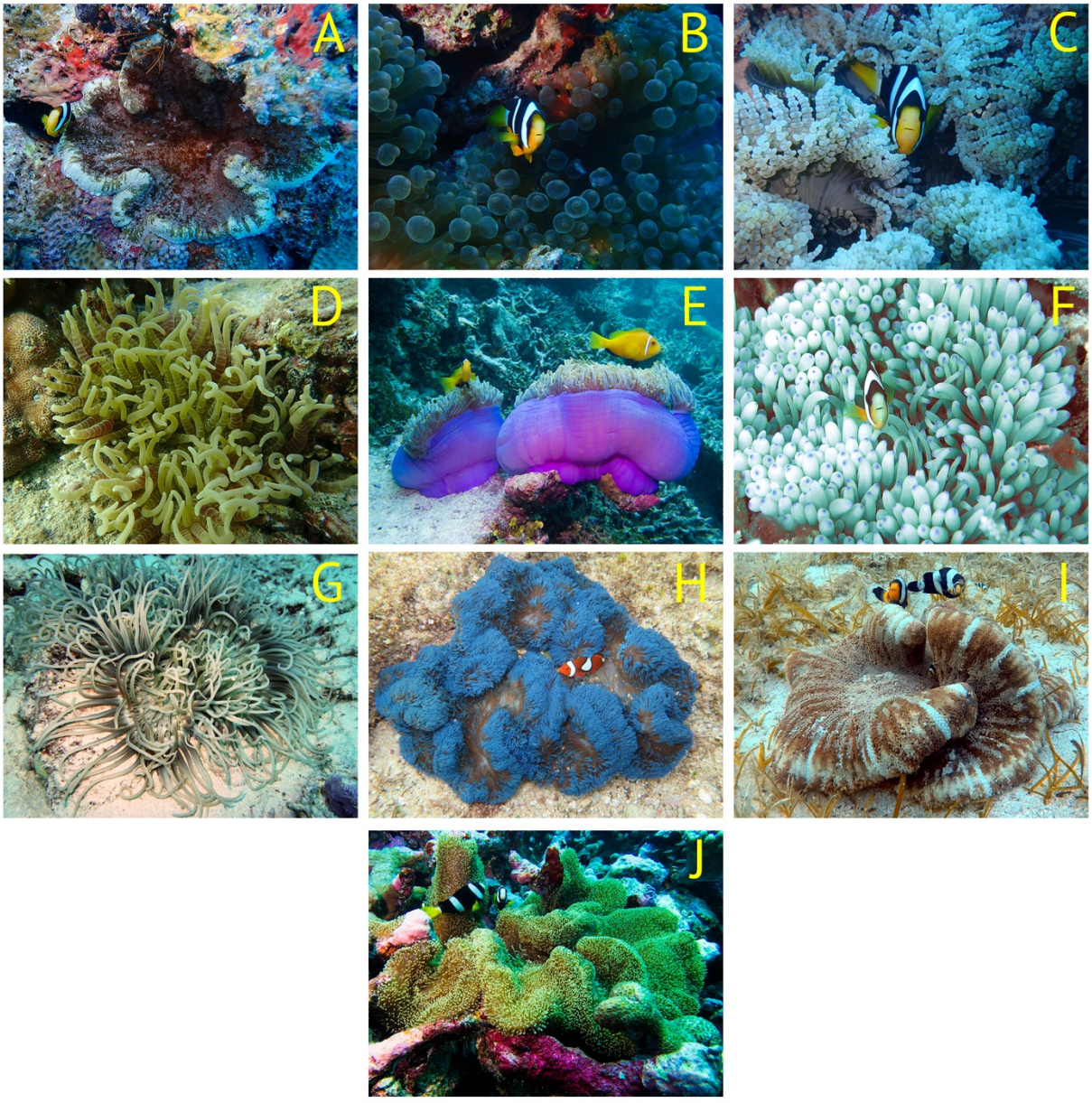
Family	Genus	Species	Locality	Ocean Basin	CO3	12S	16S	18S	28S
Actiniidae	*Entacmaea*	*quadricolor*	Japan	West Pacific	**MK522439**	**MK519401**	**MK519456**	-	-
Actiniidae	*Entacmaea*	*quadricolor*	Japan	West Pacific	**MK522440**	**MK519402**	**MK519457**	**MK519571**	-
Actiniidae	*Entacmaea*	*quadricolor*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

Stichodactylidae	*Heteractis*	*crispa*	United Arab Emirates	Gulf of Oman	**MK522466**	**MK519427**	**MK519482**	-	-
Stichodactylidae	*Heteractis*	*crispa*	United Arab Emirates	Gulf of Oman	**MK522467**	**MK519428**	**MK519483**	-	**MK519652**
Stichodactylidae	*Heteractis*	*crispa*	United Arab Emirates	Gulf of Oman	**MK522468**	**MK519429**	**MK519484**	-	**MK519653**
Stichodactylidae	*Heteractis*	*magnifica*	Unknown	Unknown	KJ482988	EU190732	EU190777	-	JK483093
Stichodactylidae	*Heteractis*	*magnifica*	Unknown	Unknown	GU473361	-	-	EU190862	EU190821
Stichodactylidae	*Heteractis*	*magnifica*	Unknown	Unknown	KC812231	-	KC812162	KC812187	-
Stichodactylidae	*Heteractis*	*magnifica*	Maldives	Central Indian	**MK522469**	**MK519430**	**MK519485**	**MK519575**	**MK519656**
Stichodactylidae	*Heteractis*	*magnifica*	Maldives	Central Indian	**MK522470**	**MK519431**	**MK519486**	**MK519576**	**MK519657**
Stichodactylidae	*Heteractis*	*magnifica*	Maldives	Central Indian	**MK522471**	**MK519432**	**MK519487**	**MK519577**	**MK519658**
Stichodactylidae	*Heteractis*	*magnifica*	Maldives	Central Indian	**MK522472**	**MK519433**	**MK519488**	**MK519578**	**MK519659**
Stichodactylidae	*Heteractis*	*magnifica*	Maldives	Central Indian	**MK522473**	**MK519434**	**MK519489**	**MK519579**	**MK519660**
Stichodactylidae	*Heteractis*	*magnifica*	Palau	West Pacific	**MK522459**	**MK519420**	**MK519475**	**MK519580**	**MK519655**
Stichodactylidae	*Heteractis*	*malu*	Tonga	Southwest Pacific	**MK522474**	-	-	-	-
Stichodactylidae	*Heteractis*	*malu*	Tonga	Southwest Pacific	**MK522475**	-	**MK519490**	-	-
Stichodactylidae	*Stichodactyla*	*gigantea*	Unknown	Unknown	GU473347	EU190747	-	EU190873	EU190835
Stichodactylidae	*Stichodactyla*	*gigantea*	Unknown	Unknown	KC812232	-	-	KC812188	-
Stichodactylidae	*Stichodactyla*	*gigantea*	Unknown	Unknown	KY789299	-	EU190793	-	-
Stichodactylidae	*Stichodactyla*	*haddoni*	Philippines	Coral Triangle	**MK522476**	**MK519435**	**MK519491**	**MK519583**	**MK519662**
Stichodactylidae	*Stichodactyla*	*haddoni*	Unknown	Unknown	KC812233	-	FJ417090	FJ417089	-
Stichodactylidae	*Stichodactyla*	*helianthus**	Panama	Tropical Western Atlantic	**MK522477**	**MK519436**	**MK519492**	**MK519563**	**MK519667**
Stichodactylidae	*Stichodactyla*	*helianthus**	Panama	Tropical Western Atlantic	**MK522478**	**MK519437**	**MK519493**	**MK519564**	**MK519668**
Stichodactylidae	*Stichodactyla*	*helianthus**	Panama	Tropical Western Atlantic	**MK522479**	**MK519438**	**MK519494**	-	**MK519669**
Stichodactylidae	*Stichodactyla*	*helianthus**	Panama	Tropical Western Atlantic	**MK522480**	**MK519439**	**MK519495**	-	**MK519670**
Stichodactylidae	*Stichodactyla*	*mertensii*	Unknown	Tropical Western Atlantic	KC812234	KC812141	KC812163	KC812189	-
Stichodactylidae	*Stichodactyla*	*mertensii*	Maldives	Central Indian	**K522481**	**MK519440**	**MK519496**	**MK519584**	**MK519663**
Stichodactylidae	*Stichodactyla*	*mertensii*	Maldives	Central Indian	**K522482**	**MK519441**	**MK519497**	**MK519585**	**MK519664**
Stichodactylidae	*Stichodactyla*	*mertensii*	Maldives	Central Indian	**K522483**	**MK519442**	**MK519498**	**MK519586**	**MK519665**
Stichodactylidae	*Stichodactyla*	*mertensii*	Maldives	Central Indian	-	**MK519443**	**MK519499**	**MK519587**	**MK519666**
Thalassianthidae	*Cryptodendrum*	*adhaesivum*	Maldives	Central Indian	**K522484**	--	**MK519500**	**MK519565**	**MK519638**
Thalassianthidae	*Cryptodendrum*	*adhaesivum*	Unknown	Unknown	KC812235	KC812142	KC812163	KC812190	KC812214
Thalassianthidae	*Cryptodendrum*	*adhaesivum*	Unknown	Unknown	KC812236	KC812143	KC812165	KC812191	KC812215
Thalassianthidae	*Cryptodendrum*	*adhaesivum*	Unknown	Unknown	KC812237	KC812144	KC812166	KC812192	KC812216

746

747

748 **Figures**



749

750 **Figure 1.** Representative images of the 10 species of clownfish-hosting sea anemones. A)
751 *Cryptodendrum adhaesivum*, B) *Entacmaea quadricolor*, C) *Heteractis aurora*, D) *Heteractis*
752 *crispa*, E) *Heteractis magnifica*, F) *Heteractis malu* (photo credit: Matthew Lee), G)
753 *Macrodactyla doreensis*, H) *Stichodactyla gigantea* (photo credit: Anne Hoggett), I)
754 *Stichodactyla haddoni* (photo credit: Singgih Afifa Putra), J) *Stichodactyla mertensii*. Photo of
755 *M. doreensis* by Lyle Vail, used under creative commons license attribution 3.0 unported license
756 http://creativecommons.org/licenses/by/3.0/. Photos A-E, and J by B. Titus.
757
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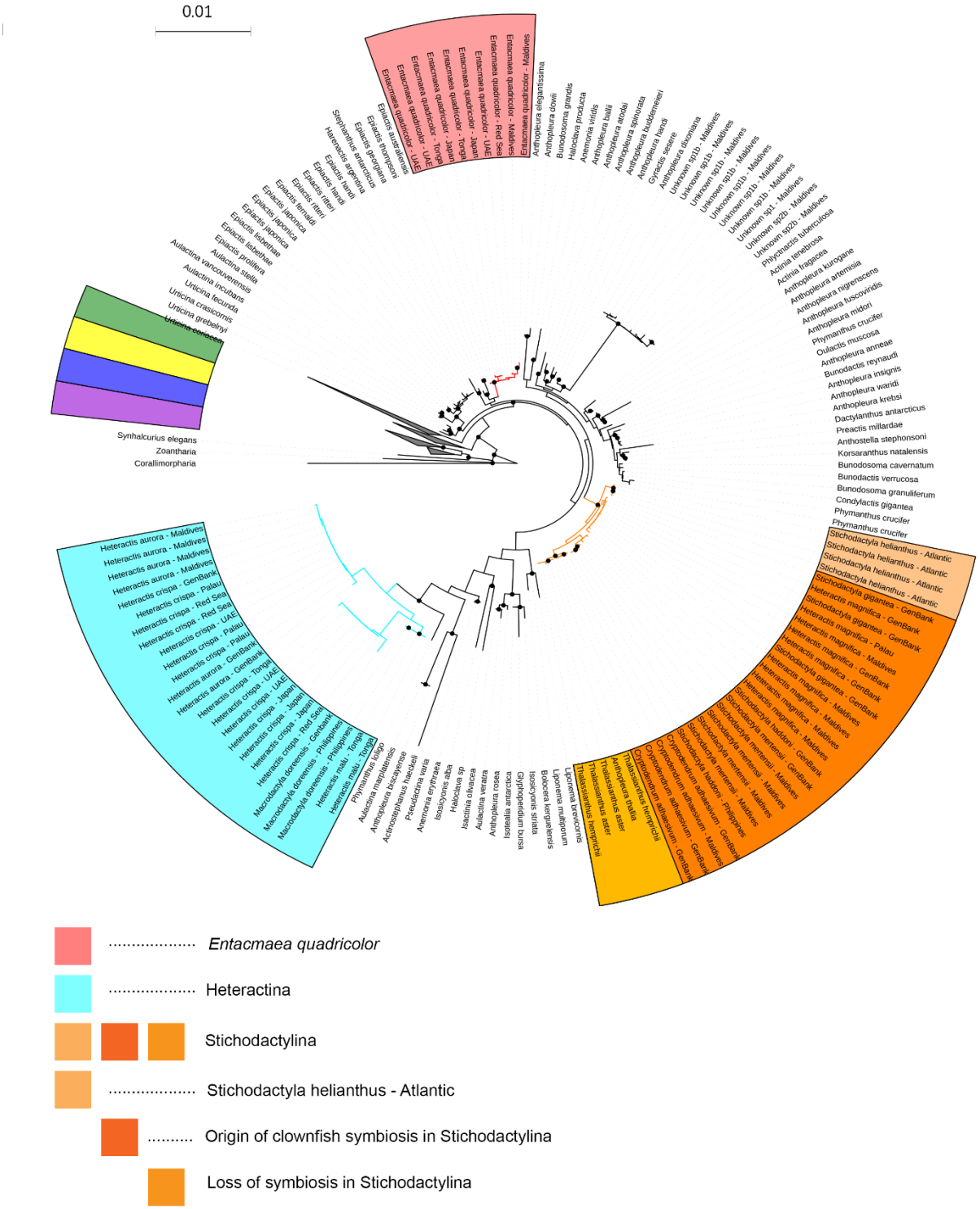


760
761 **Figure 2.** Phylogenetic reconstruction of Actiniaria. Tree resulting from Maximum Likelihood
762 (ML) analysis in RAxML of concatenated dataset (CO3, 12S, 16S, 18S, 28S). Tree reflects
763 relationships within anemone superfamily Actinioidea, with remaining superfamilies
764 Actinernoidea (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 *Macrodactyla 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 ≥ 70.

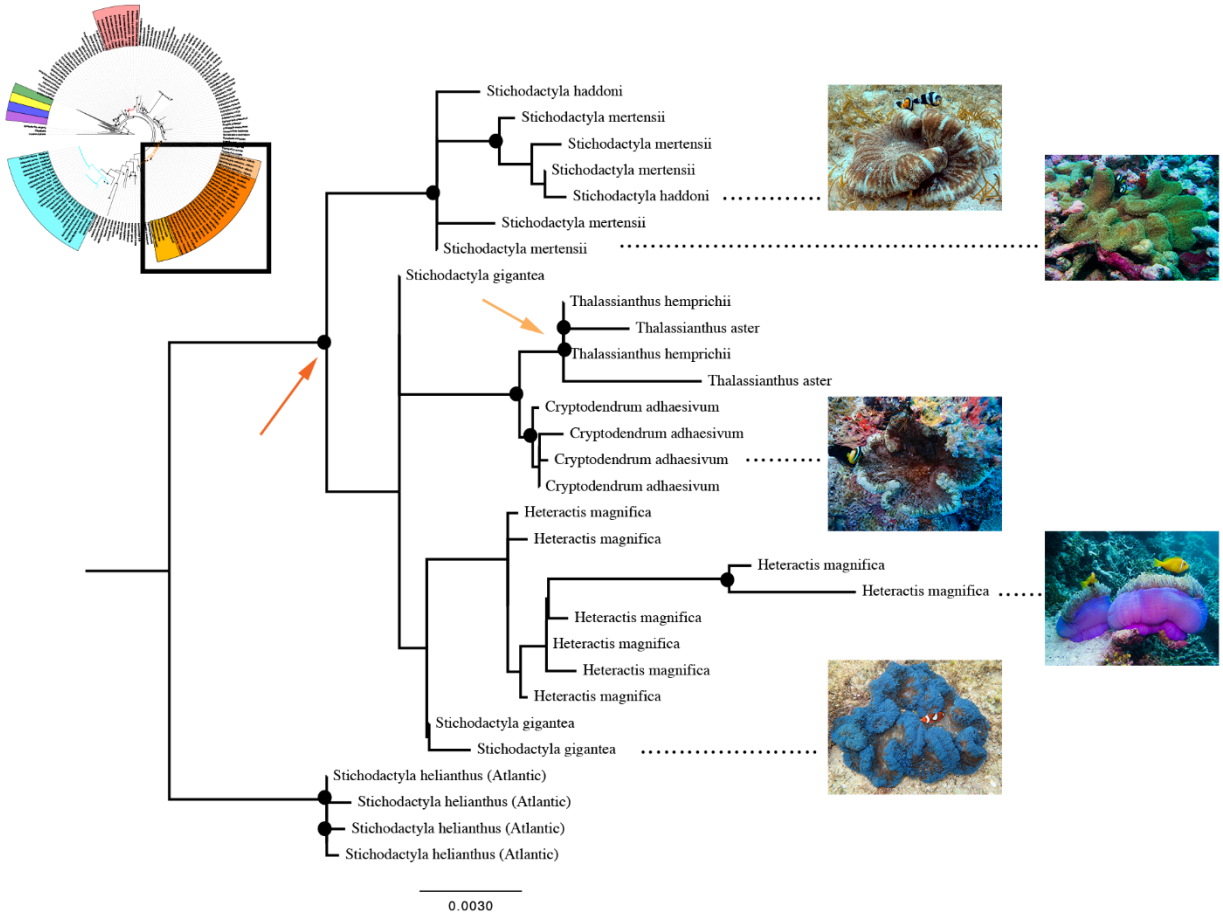
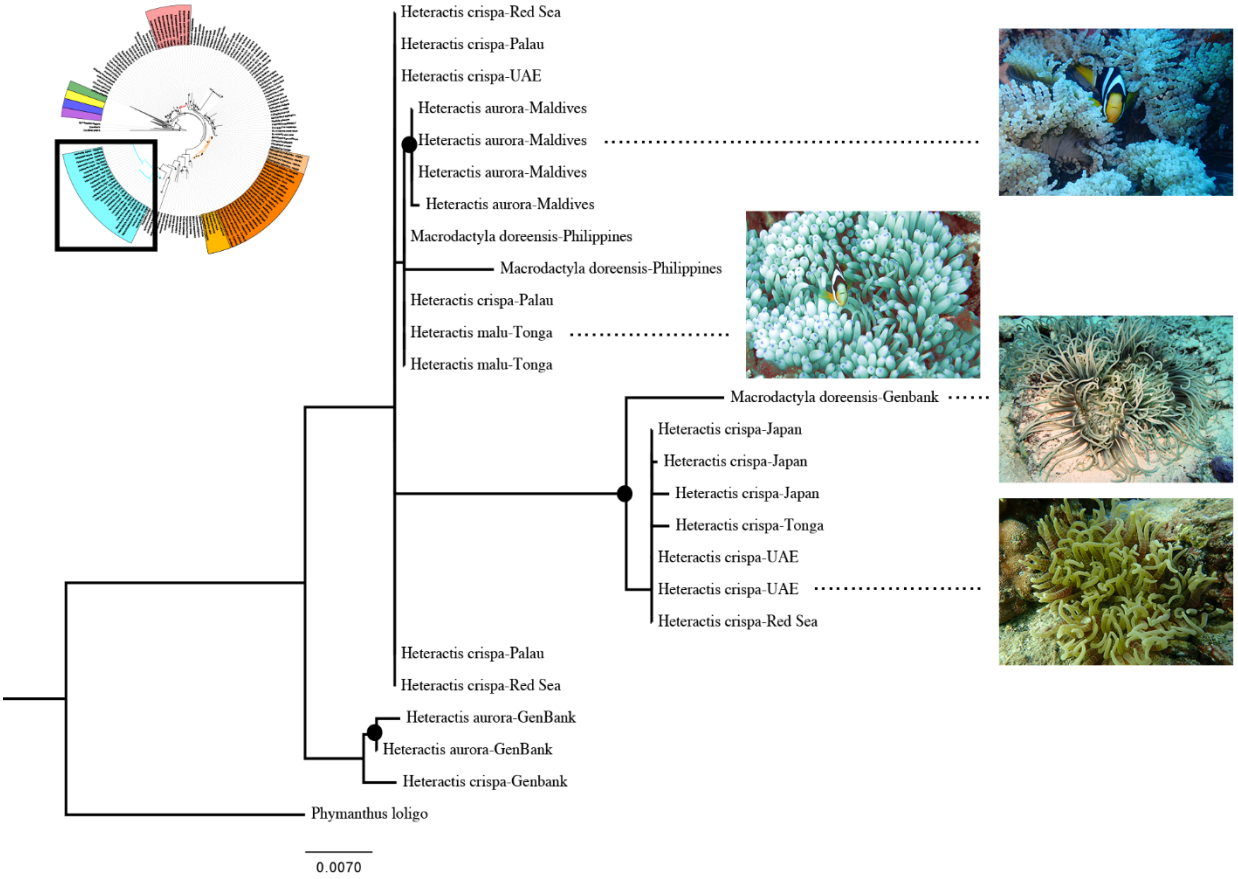


Figure 3. Partitioned Maximum Likelihood phylogenetic reconstruction of the carpet sea anemone Stichodactylina clade using all available sequence data (CO3; 12S, 16S, 18S, 28S). Orange arrow represents potential origin of symbiosis with clownfishes. Light orange arrow represents potential loss of symbiosis 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*.

787



788
789 **Figure 4.** Partitioned Maximum Likelihood phylogenetic reconstruction of the Heteractina clade
790 using all available sequence data (CO3; 12S, 16S, 18S, 28S). Colored nodes reflect bootstrap
791 resampling values ≥75. Pictured are the four host anemone species *Heteractis aurora, H. crispa,*
792 *H. malu,* and *Macrodactyla doreensis*.
793

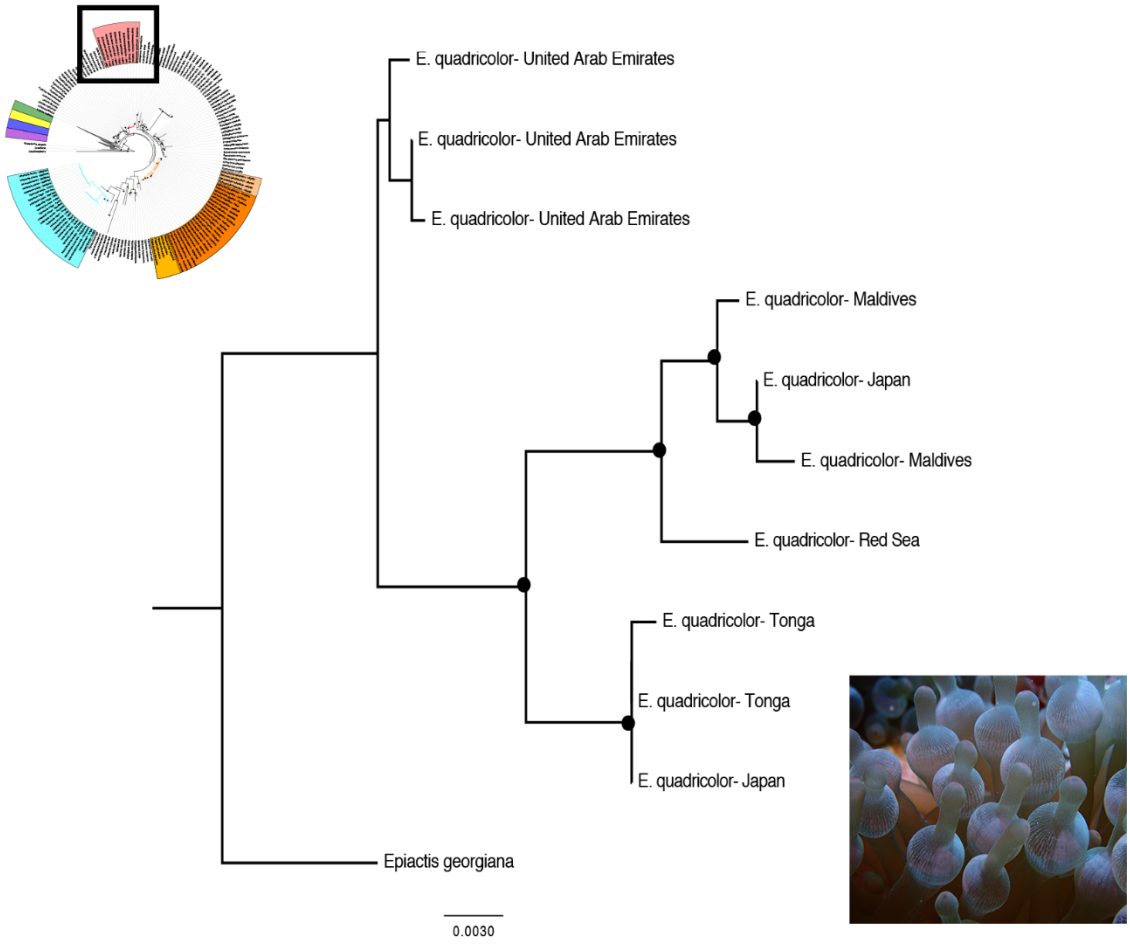


Figure 5. Partitioned Maximum Likelihood phylogenetic reconstruction of the bubble-tip sea anemone *Entacmaea quadricolor* using all available sequence data (CO3; 12S, 16S, 18S, 28S). Tree reflects samples collected from throughout the biogeographic range of the species. Colored nodes reflect bootstrap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