Phylogenomics reveals coincident divergence between giant host sea anemones and the clownfish adaptive radiation.

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The mutualism between clownfishes (or anemonefishes) and their giant host sea anemones are among the most immediately recognizable animal interactions on the planet and have attracted a great deal of popular and scientific attention [1-5]. However, our evolutionary understanding of this iconic symbiosis comes almost entirely from studies on clownfishes— a charismatic group of 28 described species in the genus *Amphiprion* [2]. Adaptation to venomous sea anemones (Anthozoa: Actiniaria) provided clownfishes with novel habitat space, ultimately triggering the adaptive radiation of the group [2]. Clownfishes diverged from their free-living ancestors 25-30 MYA with their adaptive radiation to sea anemones dating to 13.2 MYA [2, 3]. Far from being mere habitat space, the host sea anemones also receive substantial benefits from hosting clownfishes, making the mutualistic and co-dependent nature of the symbiosis well established [4, 5]. Yet the evolutionary consequences of mutualism with clownfishes have remained a mystery from the host perspective. Here we use bait-capture sequencing to fully resolve the evolutionary relationships among the 10 nominal species of clownfish-hosting sea anemones for the first time (Figure 1). Using time-calibrated divergence dating analyses we calculate divergence times of less than 25 MYA for each host species, with 9 of 10 host species having divergence times within the last 13 MYA (Figure 1). The clownfish-hosting sea anemones thus diversified coincidently with clownfishes, potentially facilitating the clownfish adaptive radiation, and providing the first strong evidence for co-evolutionary patterns in this iconic partnership.

We assembled a phylogenomic dataset for sea anemones that included representative samples from the 10 clownfish-hosting species (Table S1), which have recently undergone a major nomenclatural revision [5]. Because the clownfish-hosting species have been previously

recovered to belong to three clades that are hypothesized to have evolved symbiosis with clownfishes independently [4, 5], we included >50 species of sea anemones across Order Actiniaria to re-evaluate this hypothesis using genomic data (Table S1). Genomic DNA from newly collected samples was extracted, quantified, and prepared for sequencing using bait-capture probes for Class Anthozoa targeting ultra-conserved element (UCE) and exon loci [6]. Final genomic libraries were sequenced using 150bp paired-end sequencing on an Illumina NovaSeq. After sequencing, exon and UCE loci were assembled and extracted from raw sequence data following [6, 7].

Phylogenetic relationships were reconstructed using both maximum likelihood and coalescent-based species tree approaches [8, 9] (see Supplemental Methods). We employed multiple divergence dating analyses to convert phylogenetic trees into ultrametric chronograms and estimate divergence times for the clownfish-hosting sea anemones for the first time [8] (see Supplemental Methods). Minimum and maximum root ages for Actiniaria were set to 424-608 MYA, previously calculated from fossil-calibrated phylogenomic analyses of Anthozoa [7]. Our analyses recovered fully supported phylogenies with nearly identical topologies across Actiniaria (Figure 1A; S1A, S1B). We resolved the 10 described host anemone species to belong to three clades that do not share recent common ancestors—Clades Stichodactylina, Heteractina, and *Entacmaea*—confirming that symbiosis with clownfishes has evolved at least three times independently in sea anemones [4] (Figure 1A). Divergence times for all host anemone species were calculated at less than 25 MYA, with 9 of 10 host species having divergence times between 6.9-11.6 MYA—fully coincident with diversification times calculated for the clownfish adaptive radiation [2, 3] (Figure 1, S2).

Within each clade, we resolve species level relationships for the first time among host taxa. Our analyses calculate the oldest host species, *Stichodactyla mertensii*, to have diverged from its most recent common ancestor 24.9 MYA (95% CI = 21-45.5 MYA; Figure 1, S2). Interestingly, our phylogenetic reconstruction places *S. mertensii* as sister to a clade that contains the non-host anemone *S. tapetum* and members of Family Thalassianthidae. This relationship is fully supported across all analyses making the giant carpet anemones in genus *Stichodactyla*, the largest host anemones, a paraphyletic group (Figure 1C). The remaining carpet anemones, *S. haddoni* and *S. gigantea*, are recovered as sister species that diverged 6.9 MYA (95% CI = 4.2-10.1 MYA) and which form a deeper clade with the magnificent anemone *Radianthus magnifica* dated to 20 MYA (95% CI = 14-27.4 MYA; Figure 1, S2). Although *R. magnifica* is currently considered a single species, our geographic sampling reveals diversity and divergence times within this species that are comparable to the species level divergence seen between *S. haddoni* and *S. gigantea*. We find at least two well resolved sub-clades of *R. magnifica* splitting Indian and Pacific Ocean samples into distinct phylogeographic lineages dated to 7 MYA (95% CI = 4.6-10.1 MYA; Figure 1, S2).

Similarly, the bubble-tip anemone *Entacmaea quadricolor* is recognized as a single widespread species but has been hypothesized to be a cryptic species complex [4]. Our data support this hypothesis, as divergence times between geographic lineages of *E. quadricolor* date to 7.1 MYA (95% CI = 4.2-11.1 MYA; Figure 1, S2). Finally, we date divergence times among the four nominal species within Heteractina (*Heteractis aurora, R. crispa, R. malu, R. doreensis*) to 11.6 MYA (95% CI = 7.8-16.3 MYA; Figure 1, S2). Relationships among species are less well supported within Heteractina, and we recover a sister relationship between *H. aurora* and *R. doreensis* for the first time (Figure 1, S1A, S1B). Like *E. quadricolor* and *R. magnifica,* we find

undescribed diversity within *R. crispa*, with lineages from the Arabian Peninsula diverging from the rest of the Indo-West Pacific 9.8 MYA (95% CI = 6.7-14.1 MYA; Figure 1, S2), followed by geographic lineages from Japan, Palau, Australia, and Moorea all variously dated between 5-7 MYA. Interestingly, we find *R. malu* as a distinct lineage, but nested within the broader *R. crispa* complex, casting doubt on the taxonomic status of this putative species (Figure 1, S1, S2).

Mutualisms are tightly linked ecological interactions that are expected to impact the formation and distribution of species level biodiversity among constituent partners [10]. Our data are consistent with these expectations The ecological setting that led to the evolution of the clownfish-sea anemone symbiosis has been a long-standing mystery. Following divergence from their free-living relatives, over 15 million years passed before clownfishes adaptively radiated to sea anemones throughout the Indo-West Pacific. Why? Our findings suggest that most of the host anemone diversity hadn't evolved yet. We hypothesize that the coincident diversification between host anemones and clownfishes beginning ~13 MYA ultimately facilitated the clownfish adaptive radiation. Our results provide the first strong evidence for co-evolutionary patterns between clownfishes and their host sea anemones, and point to the importance of understanding host sea anemone biology for a comprehensive understanding of this model mutualism.

Supplemental Information

Supplemental information contains one table, two figures, detailed methods, and author contributions.

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Declaration of Competing Interest

We declare we have no competing interests.

Inclusion and Diversity

We support inclusive, diverse, and equitable conduct of research

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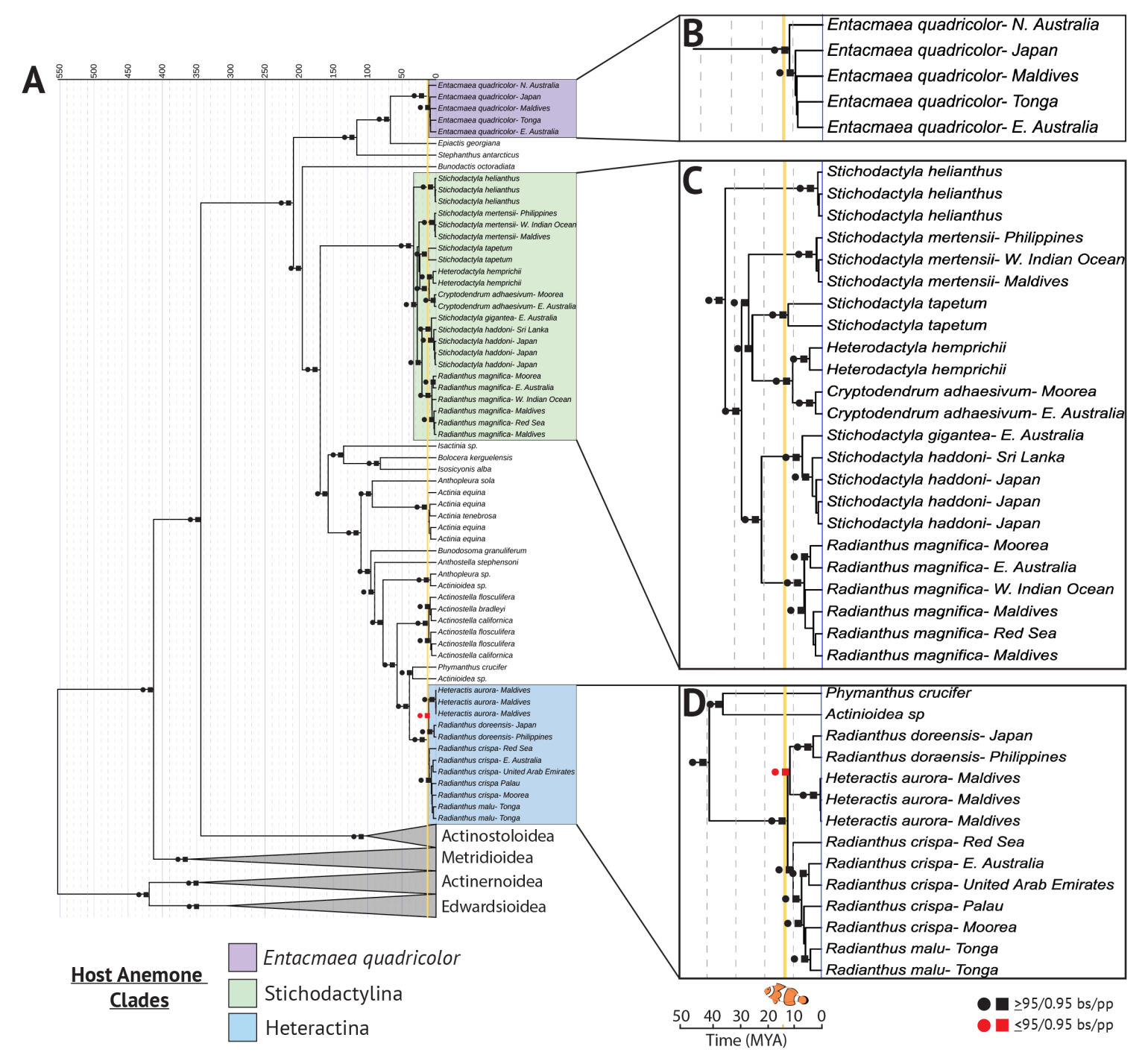
Figure 1. Diversification of the clownfish-hosting sea anemones was coincident with the clownfish adaptive radiation. A) Time-calibrated maximum likelihood cladogram of Order Actiniaria based on 328 ultra-conserved element and exon loci (75% data occupancy matrix). The three clades containing the clownfish hosting sea anemones are highlighted reflecting the multiple evolutionary origins of symbiosis with clownfishes within superfamily Actinioidea. B-D) Detailed time-calibrated maximum likelihood cladograms of *Entacmaea quadricolor*, Clade Stichodactylina, and Clade Heteractina, respectively. In all panels, the orange line denotes the beginning of the clownfish adaptive radiation 13 MYA. Sea anemone superfamilies Actinostoloidea, Metridioidea, Actiniernoidea, and Edwardsioidea are collapsed for clarity.


A
550
500
450
400
350
300
250
200
150
100
50
0
Entacmaea quadricolor- N. Australia
Entacmaea quadricolor- Japan
Entacmaea quadricolor- Maldives
Entacmaea quadricolor- Tonga
Entacmaea quadricolor- E. Australia
Epiactis georgiana
Stephanthus antarcticus
Bunodactis octoradiata
Stichodactyla helianthus
Stichodactyla helianthus
Stichodactyla helianthus
Stichodactyla mertensii- Philippines
Stichodactyla mertensii- W. Indian Ocean
Stichodactyla mertensii- Maldives
Stichodactyla tapetum
Stichodactyla tapetum
Heterodactyla hemprichii
Heterodactyla hemprichii
Cryptodendrum adhaesivum- Moorea
Cryptodendrum adhaesivum- E. Australia
Stichodactyla gigantea- E. Australia
Stichodactyla haddoni- Sri Lanka
Stichodactyla haddoni- Japan
Stichodactyla haddoni- Japan
Stichodactyla haddoni- Japan
Radianthus magnifica- Moorea
Radianthus magnifica- E. Australia
Radianthus magnifica- W. Indian Ocean
Radianthus magnifica- Maldives
Radianthus magnifica- Red Sea
Radianthus magnifica- Maldives
Isactinia sp.
Bolocera kerguelensis
Isosicyonis alba
Anthopleura sola
Actinia equina
Actinia equina
Actinia tenebrosa
Actinia equina
Actinia equina
Bunodosoma granuliferum
Anthostella stephensoni
Anthopleura sp.
Actinioidea sp.
Actinostella flosculifera
Actinostella bradleyi
Actinostella californica
Actinostella flosculifera
Actinostella flosculifera
Actinostella californica
Phymanthus crucifer
Actinioidea sp.
Heteractis aurora- Maldives
Heteractis aurora- Maldives
Heteractis aurora- Maldives
Radianthus doreensis- Japan
Radianthus doreensis- Philippines
Radianthus crispa- Red Sea
Radianthus crispa- E. Australia
Radianthus crispa- United Arab Emirates
Radianthus crispa Palau
Radianthus crispa- Moorea
Radianthus malu- Tonga
Radianthus malu- Tonga
Actinostoloidea
Metridioidea
Actinernoidea
Edwardsioidea
B
Entacmaea quadricolor- N. Australia
Entacmaea quadricolor- Japan
Entacmaea quadricolor- Maldives
Entacmaea quadricolor- Tonga
Entacmaea quadricolor- E. Australia
C
Stichodactyla helianthus
Stichodactyla helianthus
Stichodactyla helianthus
Stichodactyla mertensii- Philippines
Stichodactyla mertensii- W. Indian Ocean
Stichodactyla mertensii- Maldives
Stichodactyla tapetum
Stichodactyla tapetum
Heterodactyla hemprichii
Heterodactyla hemprichii
Cryptodendrum adhaesivum- Moorea
Cryptodendrum adhaesivum- E. Australia
Stichodactyla gigantea- E. Australia
Stichodactyla haddoni- Sri Lanka
Stichodactyla haddoni- Japan
Stichodactyla haddoni- Japan
Stichodactyla haddoni- Japan
Radianthus magnifica- Moorea
Radianthus magnifica- E. Australia
Radianthus magnifica- W. Indian Ocean
Radianthus magnifica- Maldives
Radianthus magnifica- Red Sea
Radianthus magnifica- Maldives
D
Phymanthus crucifer
Actinioidea sp
Radianthus doreensis- Japan
Radianthus doraensis- Philippines
Heteractis aurora- Maldives
Heteractis aurora- Maldives
Heteractis aurora- Maldives
Radianthus crispa- Red Sea
Radianthus crispa- E. Australia
Radianthus crispa- United Arab Emirates
Radianthus crispa- Palau
Radianthus crispa- Moorea
Radianthus malu- Tonga
Radianthus malu- Tonga
Host Anemone Clades
Entacmaea quadricolor
Stichodactylina
Heteractina
50 40 30 20 10 0
Time (MYA)
≥95/0.95 bs/pp
≤95/0.95 bs/pp