Cophylogenetic analysis of the relationship between anemonefish *Amphiprion* (Perciformes: Pomacentridae) and their symbiotic host anemones (Anthozoa: Actiniaria)

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ABSTRACT
The association between anemonefish and anemone is a classical example of mutualism in coral reefs. Although mutualism is probably the key innovation that triggered the adaptive radiation of anemonefish into a wide range of habitats, the coevolutionary history between the groups has not been thoroughly tested in a phylogenetic framework. We examined the evolutionary history of the association via distance-based (Parafit and PACo) and event-based methods (Core-PA, Jane). Mitochondrial DNA sequences (COI mtDNA, Cytb, 16S rDNA and 12S rDNA) were used to reconstruct the phylogenies of tropical *Amphiprion* species and their host anemones by using maximum likelihood with best-fit models selected. Neither distance-based analyses nor event-based analyses revealed global significant congruence between the phylogenies of the hosts and the symbionts, and thus no evidence for coevolution between anemone-anemonefish. However, at the individual pair level, the fish showed some dependence on anemone hosts. Even though living in close association and benefiting from each other, the change of genetic composition of one species (anemonefish) does not always evolve in response to changes in the other (anemones). These findings expand our understanding of the pattern and the role of evolutionary events to allow a better prediction of the future of the anemonefish-anemone relationship.

Introduction

Mutualism is a symbiotic relationship in which organisms of different species benefit from their interactions. An iconic example of mutualism is the interaction between sea anemones and anemonefish in coral reefs. Twenty-eight species of anemonefish in the genus *Amphiprion* and *Premnas* are found to cohabit with only 10 species of anemones (Families: Actiniidae, Stichodactylidae and Thalassianthidae). Anemonefish are obligate symbionts, whereas the host anemone species are also found living without the fish. The anemonefish live with immunity among the venomous host anemones. In this association, the benefits for the anemonefish include protection from predators (Fautin 1991), removal of external parasites (Allen 1972), gaining additional nutrients from anemone's tentacle, and increase in reproductive fitness (through egg protection) (Allen 1972; Saenz-Agudelo et al. 2011). In exchange, host anemones exhibit enhanced survivorship compared to those without symbionts (Godwin and Fautin 1992). The defensive territorial

behaviour of anemonefish may reduce predation on the tentacles by specialized feeders such as butterflyfish *Chaetodon fasciatus* (Fautin 1991; Fautin and Allen 1997; Porat and Chadwick-Furman 2004). The presence of anemonefish has been positively associated with higher growth and reproduction of their anemone host (Schmiege et al. 2017). By fanning the anemone host at night, anemonefish appear to supplement oxygen for their host, which increases metabolism of both partners and releases a large amount of waste products, as dissolved ammonia and phosphorus, which the anemone assimilates (Porat and Chadwick-Furman 2004; Porat and Chadwick-Furman 2005; Szczebak et al. 2013).

Anemonefish are long-lived, with a lifespan of more than 30 years. This is twice as long as other damselfish species, and up to six times longer than other marine fish of a comparable size (Holbrook and Schmitt 2005). Some individual anemones have been documented to live as long as 100 years (Holbrook and Schmitt 2005). Mutualism is likely a highly profitable

strategy for both partners, especially for resident anemonefish.

The pattern of host usage differs among anemonefish species; some species, such as *Amphiprion clarkii*, can live with up to ten species of anemone (generalists), while other species, such as *Amphiprion ocellaris* live with a few species of anemone (specialists), and some, such as *Amphiprion frenatus*, only live with one anemone species (extreme specialists) (Table I) (Fautin and Allen 1997). Generalists and specialists can coexist in the same ecological niches due to the differences in host and habitat utilization (Litsios et al. 2014).

The symbiont–host relationship between anemonefish and anemones is likely to have evolved 10 million years ago, with origin of diversification in the Central Indo-Pacific area (Litsios et al. 2014). The relationship probably began with anemonefish competing with related damselfish to occupy anemones as a predation refuge. Over time the relationship evolved from a close behavioural association to an obligate association with full contact with the anemones' stinging tentacles (Holbrook and Schmitt 2004; Burke and Nedosyko 2016). The evolution of the morphology and behaviour of anemonefish led to their specialization as symbiont (Elliott et al. 1999; Litsios et al. 2012; Litsios et al. 2014). In contrast to the studies of behaviour and ecology, the evolutionary aspect of the fish and anemone symbiosis are poorly understood.

Table I. Patterns of host usage by different species of anemonefish (Fautin and Allen 1997).

Anemonefish species	Actiniidea	Stichodactilidea	Stichodactyla			Heteractis				Thalas sianthidae	Total anemone associates
	Entac maea	Macro dactyla								Crypto dendrum	
	EQ	MD	SM	SH	SG	HM	HC	HA	HM	CA	
Amphiprioninea											
Genus *Premnas*											
P. biaculeatus	X										1
Genus *Amphiprion*											
sub-genus Actinicola											
A. ocellaris			X		X	X					3
A. percula					X	X	X				3
sub-genus Paramphiprion											
A. polymnus		X		X			X				3
A. latezonatus	X						X				2
A. sebae				X							1
sub-genus Phalerebus											
A. akallopisos			X			X					2
A. leucokranos											
A. nigripes						X					1
A. pacificus											?
A. perideraion		X			X	X	X				4
A. sandaracinos			X				X				2
sub-genus Amphiprion											
Subcomplex: ephippium											
A. barberi	X						X				2
A. ephippium	X						X				2
A. frenatus	X										1
A. mccullochi	X										1
A. melanopus	X					X	X				3
A. rubrocinctus	X				X						2
Subcomplex: clarkii											
A. akindynos	X		X	X	X	X	X	X			7
A. allardi	X		X					X			3
A. bicinctus	X		X		X	X	X	X			6
A. chagosensis	X							X			1
A. chrysogaster		X	X	X		X	X				5
A. chrysopterus	X		X	X		X	X	X			6
A. clarkii	X	X	X	X	X	X	X	X	X	X	10
A. fuscocaudatus			X								1
A. latifasciatus			X								1
A. omanensis	X			X							2
A. tricinctus	X		X	X			X	X			5
Total fish associates	16	4	12	8	7	11	14	7	1	1	

Notes: **Generalists** are found with more than three anemone species, **specialists** are found with two or three species, and **extreme specialists** are found in association with only one anemone species. Twenty seven species of anemonefish (six species from current study and 21 species retrieved from GenBank), and eight host anemone species (EQ, MD, SM, SH, SG, HM, HC, HA and CA) were included in this study. EQ: *Entacmaea quadricolor*; MD: *Macrodactyla doreensis*; SM: *Stichodactyla mertensi*; SH: *Stichodactyla haddoni*; SG: *Stichodactyla gigantea*; HM: *Heteractis magnifica*; HC: *Heteractis crispa*; HA: *Heteractis aurora*; CA: *Cryptodendrum adhaesium*.

Cophylogeny is a robust phylogenetic systematic approach aimed at reconstructing the relationships among groups of ecologically linked taxa, based on their phylogenetic information (Baudet et al. 2015; Martínez-Aquino 2016). In the past two decades, cophylogeny has been used in studies of various ecological interactions, such as plant-insect mutualism, host–parasite and even cultural inheritance (Legendre et al. 2002; Tehrani et al. 2010; Cruaud et al. 2012). The methods for cophylogenetic studies can be divided into two main categories: Distance-based (statistical) methods and event-based methods (Filipiak et al. 2016; Martínez-Aquino 2016), which have been widely applied to search for associations between hosts and their symbionts, such as filarial worm hosts with their symbiotic bacteria; sloth hosts with their symbiont algae; plant hosts with their symbiotic flies and nematodes (Nelson et al. 2014; Lefoulon et al. 2016; Fountain et al. 2017).

Following recent studies of anemonefish and anemones (Litsios et al. 2012; Litsios et al. 2014; Rolland et al. 2018; Nguyen et al. 2019; Titus et al. 2019), we explored the evidence for potential coevolution in this host-symbiont system. We used a molecular phylogenetic approach, based on mitochondrial markers, to test if the relationship between anemone and anemonefish represents a coevolved system. We also assessed the relative importance of different evolutionary events in the evolutionary history of the fish and the anemones.

Materials and methods

Taxonomic sampling

We have included a total of 55 sequences of anemones representing 10 anemonefish hosting anemone species, eight of which were newly acquired in this study (Table II); the others were obtained from previously published datasets (e.g. Daly et al. 2008; Daly et al. 2010; Rodríguez et al. 2014; Titus et al. 2019). We also included 27 of 28 sequences of anemonefish, of which six species of the *Amphiprion* genus found in Khanh Hoa and Ninh Thuan (Astakhov 2002; Astakhov et al. 2016) are new to this study (Table II). Anemones ($n = 16$) and anemonefish ($n = 107$) were collected by divers from Nha Trang Bay, Khanh Hoa province and Phu Quy Beach, Ninh Thuan province in central Vietnam between November 2014 to April 2017 (Table II). The anemones were selected based on the presence of anemonefish at the time of collection. All individuals were identified using relevant taxonomic references (Dunn 1981; Fautin and Allen 1997; Fautin 2008). The fish were identified based on Allen (1975) and Allen et al. (2005). Vouchers of specimens preserved in formalin were deposited at the Museum of Oceanography Institute, Nha Trang City, Vietnam, and the sequences were reserved in GenBank (Table II).

We further included 14 sequences of non-symbiotic anemone species from the family Actinidea (Daly et al. 2008; Emblem et al. 2014; Rodríguez et al. 2014; Larson and Daly 2016).

DNA extraction, PCR amplification, and sequencing

Anemone genomic DNA was isolated from tentacles or column tissue using the Quiagen DNAeasy® kit. Fish genomic DNA was isolated from muscle tissue using the Thermo Dream Taq DNA Polymerase kit. Manufacturer protocols were followed in both cases. For anemones, three mitochondrial markers: 12S ribosome DNA (12S rDNA), 16S ribosome DNA (16S rDNA) and cytochrome c oxidase subunit 1 (COI mtDNA) were amplified. For anemonefish, the chosen mitochondrial markers were 16S rDNA, Cytochrome b (Cytb) and COI mtDNA.

The primers used for amplifying the genetic markers are listed in Table III. For the anemone's 12S rDNA and anemonefish's 16S rDNA, amplifications were performed using published procedures (Chen and Yu 2000; Palumbi et al. 2002). The primers for 16S rDNA and COI mtDNA in the anemones and for Cytb and COI mtDNA in the anemonefish were developed in this study (Table III). For newly developed primers, amplifications were implemented under the following cycling conditions: initial denaturation at 95°C for 3 min, followed by 35 cycles at 94°C for 30s, annealing for 30s (at 55°C for 16S rDNA, 48°C for the COI mtDNA gene of the anemones; 53°C for both Cytb and COI mtDNA genes of anemonefish). The final extension step was at 72°C for 5 min before the samples were cooled to 4 °C.

Sequence reactions were performed by 10 μl of cleaned PCR product with a dye-labeled dideoxy terminator using the same primers as in the PCR reactions. Forward and reverse sequences were assembled in Sequencer v4.1.4 (GeneCodes, Ann Arbor, Michigan). The Basic Logical Alignment Search Tool (BLAST, https://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to search for identical sequences. Sequences were aligned by Clustal Multiple Alignment, Bioedit v7.2.4 (Hall 1999) and edited by eye. All sequences have been deposited in GenBank (Table II).

Table II. Taxonomic information and GenBank accession numbers for the taxa included in this study.

Family	Genus	Species	Locality	Voucher/Isolate	12S rDNA	16S rDNA	Cytb	COI mtDNA
Actinidae	*Actinia*	*tenebrosa*	New Zealand	isolate TEN	KT852045	KT852111		
	Anthopleura	*artemisia*	Kodiak Island, Alaska	isolate KOD1	KT852015	KT852081		
	Anthostella	*stephensoni*	Unknown		JQ810719	JQ810721		
	Aulactinia	*vancouverensis*	Kodiak Island, Alaska	isolate KOD5	KT852019	KT852085		
	Aulactinia	*verrucosa*	Unknown		EU190723	EU190766		
	Bolocera	*sp.* GB	East Sea	BZ-2016	KU507297	KU507297		KU507297
	Bolocera	*tuediae* GB	Tromso, Norway	NOR1	HG423145	HG423145		HG423145
	Entacmaea	***quadricolor***	**Khanh Hoa, Vietnam (This study)**	**HQ1_KH**	**MH725833**	**MH718497**		**MH727172**
	Entacmaea	*quadricolor*	Japan		MK519401	MK51945		
	Entacmaea	*quadricolor*	Japan		MK519402	MK519457		
	Entacmaea	*quadricolor*	Maldives		MK519404	MK519458		
	Entacmaea	*quadricolor*	Saudi Arabia		MK522405	MK519459		
	Entacmaea	*quadricolor*	Tonga		MK519406	MK519460		
	Entacmaea	*quadricolor*	Tonga		MK519407	MK519461		
	Entacmaea	*quadricolor*	United Arab Emirates		MK519408	MK519462		
	Entacmaea	*quadricolor*	United Arab Emirates		MK519409	MK519463		
	Entacmaea	*quadricolor*	United Arab Emirates		MK519410	MK519464		
	Epiactis	*fermaldi*	USA: Washington State	isolate EFER	KT852005	KT852068		Not available
	Epiactis	*japonica*	Japan: Shinori, Hokkaido	isolate SA5	KT852040	KT852105		Not available
	Epiactis	*georgiana*	Antarctica: Antarctic peninsula	isolate EPIG	KT852007	KT852070		Not available
	Macrodactyla	***doreensis***	**Khanh Hoa, Vietnam (This study)**	**HQ2_KH**	**MH725834**	**MH718498**		**MH727173**
	Macrodactyla	*doreensis*	Unknown		EU190739	EU190785		
	Glyphoperidium	*bursa* GB	USA	AMNH/ Gly	KJ482923	KT852076		Not available
	Isosicyonis	*striata*	Antarctica	AMNH	KR051006	KR051006		KR051006
	Oulactis	*muscosa*	New Zealand		KT852033	KT852097		
	Urticina	*columbiana*	Monterey Bay - USA		U91753		U91613	
	Urticina	*coriacea* GB	USA		U91752			U91615
Stichodactyli dea	***Stichodactyla***	***haddoni***	**Khanh Hoa, Vietnam (This study)**	**HQ3_KH**	**MH725835**	**MH718499**		**MH727174**
	Stichodactyla	*haddoni*	Philippine	*S_haddoni*_PhilShad1	MK519435	MK519491		
	Stichodactyla	***gigantea***	**Khanh Hoa, Vietnam (This study)**	**HQ4_NT**	**MH725836**	**MH718500**		**MH727175**
	Stichodactyla	*gigantea*	Malaysia	Kudat 1	KR812363	KR812343		Not available
	Stichodactyla	*gigantea*	Unknown		EU190747	EU190793		Not available
	Stichodactyla	***mertensii***	**Khanh Hoa, Vietnam (This study)**	**HQ5_NT**	**MH725837**	**MH718501**		**MH727176**
	Stichodactyla	*mertensii*	Lawrence, USA	SMER	KC812141	KC812163		Not available
	Stichodactyla	*mertensii*	Maldives	*S_mertensii*_MDSM1	MK519440	MK519496		
	Stichodactyla	*mertensii*	Maldives	*S_mertensii*_MDSM2	MK519441	MK519497		
	Stichodactyla	*mertensii*	Maldives	*S_mertensii*_MDSM3	MK519442	MK519498		
	Stichodactyla	*mertensii*	Maldives	*S_mertensii*_MDSM4	MK519443	MK519499		
	Stichodactyla	*sp.* GB	Malaysia	Gazumbo	KR812372	KR812353		Not available
	Heteractis	***aurora***	**Vietnam (This study)**	**HQ6_NT**	**MH725838**	**MH718502**		**MH727177**
	Heteractis	*aurora*	Unknown	isolate HR2	KC812139	KC812160		Not available
	Heteractis	*aurora*	Unknown		EU190729	EU190773		Not available
	Heteractis	*aurora*	Maldives	*H_aurora*_MDHA1	MK519412	MK519467		
	Heteractis	*aurora*	Maldives	*H_aurora*_MDHA2	MK519413	MK519468		
	Heteractis	*aurora*	Maldives	*H_aurora*_MDHA3	MK519414	MK519469		
	Heteractis	*aurora*	Maldives	*H_aurora*_MDHA4	MK519415	MK519470		
	Heteractis	***crispa***	**Vietnam (This study)**	**HQ7_NT**	**MH725839**	**MH718503**		**MH727178**
	Heteractis	*crispa*	Japan	CRISP	KC812140	KC812161		
	Heteractis	*crispa*	Japan	*H_crispa*_K002HC	MK519416	MK519471		
	Heteractis	*crispa*	Japan	*H_crispa*_K051HC	MK519417	MK519472		
	Heteractis	*crispa*	Japan	*H_crispa*_K054HC	MK519418	MK519473		
	Heteractis	*crispa*	Palau	*H_crispa*_PLHC516	MK519419	MK519474		
	Heteractis	*crispa*	Palau	*H_crispa*_PLHC624	MK519421	MK519476		
	Heteractis	*crispa*	Palau	*H_crispa*_PLHC635	MK519422	MK519477		
	Heteractis	*crispa*	Saudi Arabia	*H_crispa*_RSA10HC	MK519423	MK519478		
	Heteractis	*crispa*	Saudi Arabia	*H_crispa*_S210HC	MK519424	MK519479		

(Continued)

Table II. Continued.

Family	Genus	Species	Locality	Voucher/Isolate	12S rDNA	16S rDNA	Cytb	COI mtDNA
	Heteractis	*crispa*	Saudi Arabia	*H_crispa*_S224HC	MK519425	MK519480		
	Heteractis	*crispa*	Tonga	*H_crispa*_TGHC3	MK519426	MK519481		
	Heteractis	*crispa*	United Arab Emirates	*H_crispa*_UAEHC02	MK519427	MK519482		
	Heteractis	*crispa*	United Arab Emirates	*H_crispa*_UAEHC04	MK519428	MK519483		
	Heteractis	*crispa*	United Arab Emirates	*H_crispa*_UAEHC06	MK519429	MK519484		
	Heteractis	***malu***	**Vietnam (This study)**	**HQ8_NT**	**MH725840**	**MK519471**		**MH727179**
	Heteractis	*magnifica*	Unknown		EU190732	EU190777		
	Heteractis	*magnifica*	Maldives	*H_magnifica*_7iHM	MK519430	MK519485		
	Heteractis	*magnifica*	Maldives	*H_magnifica*_8iHM	MK519431	MK519486		
	Heteractis	*magnifica*	Maldives	*H_magnifica*_11iHM	MK519432	MK519487		
	Heteractis	*magnifica*	Maldives	*H_magnifica*_3iHMpp	MK519433	MK519488		
	Heteractis	*magnifica*	Maldives	*H_magnifica*_23iHMpp	MK519434	MK519489		
	Heteractis	*magnifica*	Palau	*H_magnifica*_PLHM616	MK519420	MK519475		
Metridiidae	*Metridium*	*senile*	USA	AMNH	NC000933	NC000933		NC000933
Thalassianthidae	*Cryptodendrum*	*adhaesivum*	Unknown	isolate CRYP	KC812164	KC812142		
Pomacentridae	*Amphiprion*	*akallopisos*	Thailand			NC030590	NC030590	NC030590
	Amphiprion	*akindynos*	Australia	GA032		KF264151		KF264273
	Amphiprion	*allardi*	Comoros	GA033		KF264152	KU176398	
	Amphiprion	*barberi*	Fiji	GA071		KF264153		KF264275
	Amphiprion	*bicinctus*	Unknown			NC_016701	NC_016701	NC_016701
	Amphiprion	*chagosensis*	The United Kingdom	GA098		KF819364		KF819381
	Amphiprion	*chrysogaster*	Mauritius	GA077		KF264155		KF264277
	Amphiprion	*chrysopterus*	French Po	GA040		KF264128		KF264280
	Amphiprion	***clarkii***	**Khanh Hoa, Vietnam (This study)**	**KC1B_KH**		**MH718491**	**MH727180**	**MH727166**
	Amphiprion	*clarkii*	Thailand			AB979449	AB979449	AB979449
	Amphiprion	*clarkii*	China			NC023967	NC023967	NC023967
	Amphiprion	*ephippium*	Thailand: Krabi			AB979272	AB979272	AB979272
	Amphiprion	***frenatus***	**Khanh Hoa, Vietnam (This study)**	**KC4F_KH**		**MH718494**	**MH727182**	**MH727168**
	Amphiprion	*frenatus*	Unknown	AF20131222A		KJ833752	KJ833752	KJ833752
	Amphiprion	*latezonatus*	Australia	GA023		KF264165		KF264287
	Amphiprion	*latifasciatus*	Madagascar	NBE0191		JF457235	JF434737	JF457905
	Amphiprion	*latifasciatus*	Madagascar	GA083		KF264166		KF264288
	Amphiprion	*leucokranos*	Madagascar	GA066		KF264167		KF264289
	Amphiprion	*mccullochi*	Australia	GA056		KF264168		KF264290
	Amphiprion	*melanopus*	Indonesia	GA012		KF264169		KF264291
	Amphiprion	*nigripes*	Sri Lanka	GA055		KF264170		KF264292
	Amphiprion	***ocellaris***	**Khanh Hoa, Vietnam (This study)**	**KC6_KH**		**MH718496**	**MH727185**	**MH727171**
	Amphiprion	*ocellaris*	Unknown			NC009065	NC009065	NC009065
	Amphiprion	*ocellaris*	Thailand			AB979697	AB979697	AB979697
	Amphiprion	*omanensis*	Oman	GA051		KF264173		KF264295
	Amphiprion	*pacificus*	Fiji	GA069		KF264174		KF264296
	Amphiprion	***sandaracinos***	**Khanh Hoa, Vietnam (This study)**	**KC2Y_KH**		**MH718492**	**MH727181**	**MH727167**
	Amphiprion	*sandaracinos*	Papua New Guinea	GA037		KF264183		KF264306
	Amphiprion	*sandaracinos*	Indonesia	GA018		KF264183		KF264305
	Amphiprion	*percula*	Thailand			AB979450	AB979450	AB979450
	Amphiprion	***perideraion***	**Khanh Hoa, Vietnam (This study)**	**KC3_KH**		**MH718493**	**MH727183**	**MH727169**
	Amphiprion	*perideraion*	Unknown	AP20140328A		KJ833753	KJ833753	KJ833753
	Amphiprion	***polymnus***	**Khanh Hoa, Vietnam (This study)**	**KC5B_KH**		**MH718495**	**MH727184**	**MH727170**
	Amphiprion	*polymnus*	Unknown			KJ101554	KJ101554	KJ101554
	Amphiprion	*rubrocinctus*	Australia	GA070		KF264182		KF264304
	Amphiprion	*sebae*	Thailand: Krabi			AB979696	AB979696	AB979696
	Amphiprion	*tricinctus*	Marshall Islands	GA058		KF264308		KF264308
	Premnas	*biaculeatus*	Thailand: Chonburi			LC089001	LC0809001	LC089001
	Chrysiptera	*rollandi*	New Caledonia/ Australia: Queensland			AY098629	AY208573	KP195013

Note: GenBank accession numbers in **Bold** are new to this study.

Phylogenetic analyses

The markers ranged in length from 424 to 812 base pairs (bp) in the anemone species, and from 505 to 1077 bp in the fish species (Table III). Sequences of each marker of both anemone and anemonefish were matched against the NCBInr database using *blastn* algorithm (Altschul et al. 1990). Reference sequences for phylogenetic analysis were selected among the

Table III. Primers used for amplifying the genetic markers of anemonefish and anemones in this study.

Marker	Primer source	Annealing temperature	Sequences of Primer	Marker aligned length (bp)
1. Anemone				
12S rDNA	Chen and Yu (2000)	50–55°C	**ANTMTSSU-F** 5′-AGCCACACTTTCACTGAAACAAGG-3′	812
			ANTMTSSU-R 5′-GTTCCCYYWCYCTYACYATGTTACGAC-3′	
16S rDNA	Newly developed	55°C	**16S rDNA_ANF**: 5′GGTATGAATGGCGTCACGAAGG 3′	424
			16S rDNA_ANR: 5′CCACACTAAGATGACGGGTCAC 3′	
COI mtDNA	Newly developed	48°C	**COI MTDNAANE-F**: 5′-GGTACTATGTTAGGGGACGAC-3′	472
			COI MTDNAANE-R: 5′-CCACACAAATAANGGGAGTC-3′	
2. Anemonefish				
16S rDNA	Palumbi et al. (2002)	48°C	**16S rDNAar**: 5′- CGCCTGTTTATCAAAAACAT- 3′	505
			16S rDNAbr: 5′- CCGGTCTGAACTCAGATCACGT- 3′	
Cytb	Newly developed	53°C	**CytbF**: 5′-CATCAGACATTGGTAGAG-3′	1077
			CytbR:5′-CGTCTGCAATTAGGAGTCAG-3′	
COI mtDNA	Newly developed	53°C	**COI MTDNAF**: 5′-GCAGAATTAAGCCAACCAGG-3′	590
			COI MTDNAR: 5′-GGTTTCGGTCNGTTAAGAGC-3′	

best hits based on their identity percentage. Combined datasets showed higher resolution and support values than any of the single datasets alone (Daly et al. 2010; Rodríguez et al. 2014; Titus et al. 2019). Therefore, a combined dataset of the three markers 16S rDNA, COI mtDNA and Cytb mtDNA were used for the fish phylogeny, and the combined dataset of the three markers 16S rDNA, 12S rDNA and COI mtDNA were used for the anemone phylogeny.

Sequences of the anemone and anemonefish markers were then joined by Fasta Alignment joiner online (http://users-birc.au.dk/biopv/php/fabox/alignment_joiner.php#). Both datasets of sequences were aligned and manually reviewed using Bioedit v7.2.4 (Hall 1999). Only specimens with at least two out of three markers available in GenBank were included in the final phylogenetic analyses.

Metridium senile (Metridiidae) was chosen as outgroup for the anemone phylogeny; while *Chrysiptera rollandi*, a member of a separate genus in the damselfish family Pomocentridae was selected as outgroup for the anemonefish phylogeny.

The anemone and fish phylogenies were constructed using Maximum likelihood (ML) as implemented in R-package *phangorn* (Schliep 2011). Prior to running ML, the best fit model of nucleotide substitution was selected based on the Akaike Information Criterion (AIC) option in Modeltest. The program has been implemented in the *ape* package (updated by Paradis and Schliep 2019), and makes Modeltest operational in R environment. Bootstrap values were calculated using 1000 replicates. Tree display and editing were performed in MEGA7 (Kumar et al. 2016). Bayesian analysis (Mr Bayes) was not included in our results due to the failure to obtain MCMC chain convergence for running the anemone phylogeny. The problem has been reported previously by other authors (see Rodríguez et al. 2014; Titus et al. 2019).

Cophylogenetic analyses

In our models, we considered the symbiotic anemonefish to represent the role of symbiont, and the anemones represented the role of the host. Consensus sequences of combined mitochondrial genes were first used for the analysis of the congruence of fish and anemone phylogenies. Two statistical programmes, which have been developed to test the extent of a global hypothesis of coevolution between hosts and their symbionts, Parafit (Legendre et al. 2002) and Procrustean Approach to Cophylogeny (PACo) (Balbuena et al. 2013) were used. Cophylogenies were constructed using Parafit and PACo, in which Parafit focuses on testing random associations between the host and symbiont taxa, while PACo explicitly tests the dependence of the symbiont phylogeny upon the host phylogeny (Filipiak et al. 2016).

Because of the limitation of the cophylogenetic programmes (particularly with event-based methods) to handle large datasets in reasonable time, we pruned the anemone and anemonefish phylogenies, so that each species was represented by single nodes.

Parafit and PACo were both implemented in R. By using the phylogenetic distance matrix and fish-anemone association, these approaches help to test for the global fit and individual host–symbiont associations contributing to the global fit. This fit can be used to infer the congruence between host and symbiont phylogenies. For PACo, each host–symbiont pair was evaluated by a jackknife procedure, with 10,000 permutations, to estimate the squared residuals of each single association and its 95% confidence interval.

To visualize anemone-anemonefish association, a tanglegram was generated from the best ML tree in TreeMap 3.0b (Charleston and Robertson 2002).

An event-based approach was also used to examine the evolutionary history of the anemonefish-anemone

association. The ML best trees of anemonefish and anemone were used as input data, with all non-symbiotic anemone species and outgroups removed from the dataset. An event-cost analysis was performed using Jane 4.0 (Conow et al. 2010) to examine differences in the phylogenetic trees and the set of events that may lead to the coevolution of the host and symbiont. This method also evaluated the role of each event (cospeciation, duplication, duplication and host switching, failure to diverge) in affecting the overall cost of the optimal evolutionary history (Deng et al. 2013; Lauron et al. 2015). Event cost values were set to 0 for cospeciation, and from 0 to 2 for the remaining events, in searching for minimum total costs. One hundred generations and population sizes of 100 were chosen for the genetic algorithm in Jane 4. The statistical significance of the total cost for each cost scheme was tested using the null distribution of cost values based on 100 randomly generated trees. Further analysis, Core-PA (Merkle et al. 2010), which tests for significant evolutionary events (co-speciation, sorting, duplication, and host switching) was also applied. An adaptive cost method that automatically calculated event cost value, using a simplex optimization algorithm, was applied to find the best cost for each event while also searching for a minimum total cost (Merkle et al. 2010). We used 100 randomization of anemone-anemonefish associations to determine if the number of each event type differed significantly from the random associations between the two trees, and whether the reconciliation from the original dataset was more trustworthy than reconciliation from the random instances.

Results

Phylogenies of anemone – host and anemonefish – symbiont

The combined datasets consisted of 1709bp for anemones and 2171bp for anemonefish. The best fit models for anemones and anemonefish were TrN + G + I and GTR + G + I, respectively, which were then used to build the ML trees (Figures 1 and 2). Overall, anemonefish phylogeny was recovered with fairly consistent support values for the *Amphiprion* genus. We obtained varying degrees of nodal support across the backbone of Actinioidae, which also has been indicated in previous studies (Rodríguez et al. 2014; Titus et al. 2019).

Traditional classification has divided the anemonefish hosting sea anemones in three families clustered within the superfamily Actinioidae. These are

Actiniidae (*Entacmaea* and *Macrodactyla*), Stichodactylidae (*Stichodactyla* and *Heteractis*), and Thalassianthidae (*Cryptodendrum*) (Fautin 1991, 2016). Our findings, as previously shown by Titus et al. (2019), do not agree with the current taxonomy. Our ML analyses recovered Stichodactylidae as polyphyletic (Figure 1). The anemone species of *Heteractis* were clustered with genera (e.g. *Macrodactyla*, *Authopleura*, *Aulactina* and *Oulastis*) within the Actiniidae, while both *Heteractis* and *Stichodactyla* nested with *Cryptodendrum* in the Thalassianthidae family. Our analyses also found the genus *Stichodactyla* to be paraphyletic (Figure 1) with *C. adhaesivum* (family Thalassianthidae) was placed in a well-supported clade (97%) within specimens of *S. gigantea*, *S. haddoni*, *S. mertensii* and *H. magnifica*.

In this study, we found no evidence for a monophyletic Actiniidae (Figure 1). In Clade 1, three groupings of individuals identified as *H. aurora*, *H. crispa* and *H. malu* were recovered to be nested within genera (*Macrodactyla*, *Authopleura*, *Aulactinia* and *Oulactis*) of the family Actiniidae. In Clade 2, the 10 specimens of the *E. quadricolor* formed a highly supported monophyletic clade (84%). However, no members of the Actiniidae family were found to have a sister group relationship with *E. quadricolor*.

We observed some geographical variation in the molecular data in these species. Some of the anemone species sequences identified in the present study differed from the sequences of the same species in GenBank (Table SI, supplementary material). For example, our *S. gigantea* and *S. gigantea* from Malaysia (KR812363) were 99.1% identical; and our *S. mertensii* and *S. mertensii* (KC812141, USA) were 99.8% identical. Differences between the same species were also detected when GenBank sequences were compared, such as *H. aurora* KC812140 and *H. aurora* EU190729 (99.1%). Samples of the same species in the same location also showed genetic differences, for example *H. crispa* Japan MK519417 and *H. crispa* Japan MK519418 (99.7%).

Our anemonefish phylogeny comprised 27 out of the 28 currently defined anemonefish species (Ollerton et al. 2007; Allen et al. 2010; Burke and Nedosyko 2016). The extremely rare species *Amphiprion fuscocaudatus* was not included since its sequence was not available in GenBank. *Amphiprion leucokranos* was chosen to represent two natural hybrids *A. leukranos* and *A. thielli* (Ollerton et al. 2007).

The Amphiprioninae subfamily is traditionally divided, based on morphology, into six complexes,
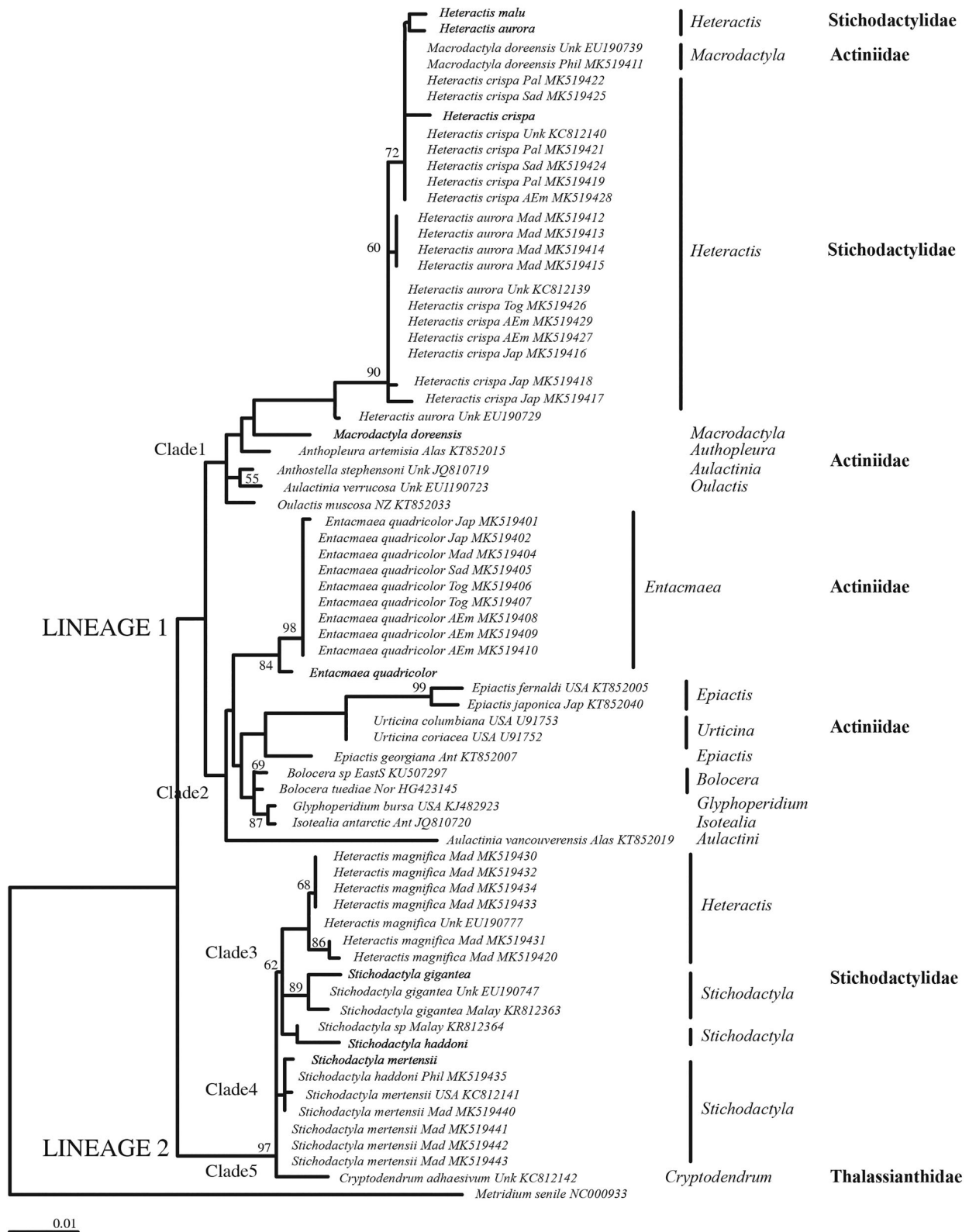
Figure 1. Anemone phylogenetic tree from combined mitochondrial gene dataset (COI mtDNA, 16S rDNA and 12S rDNA) based on 71 sequences of anemones, of which eight sequences are from the current study. The tree corresponds to the best ML tree assuming TrN + G + I model chosen from ModelTest available in *ape* package. Node supports are indicated by bootstrap values (when >50%). Sequences from this study are in bold text. Sequences from GenBank include locations and ID. *Metridium senile* was used as the outgroup. Abbreviations: Unk = Unknown; Phil = Philippine; Pal = Palau; Sad = Saudi Arabia; AEm = United Arab Emirates; Mad = Maldives; Tog = Tonga; Jap = Japan; Alas = Alaska; Malay = Malaysia; Ant = Antarctica; EastS = East Sea; Nor = Norway.

Figure 2. Anemonefish phylogeny tree from combined mitochondrial gene dataset (COI mtDNA, 16S rDNA and Cytb) representing 27 out of 28 species of anemonefish, of which six sequences are from the current study; The tree corresponds to the best ML tree assuming GTR + G + I model chosen from ModelTest available in *ape* package. Node supports are indicated by bootstrap values (when >50%). Sequences from this study are in bold text. Sequences from GenBank include locations and ID. *Chrysiptera rollandi* was used as outgroup. Abbreviations: Unk = Unknown; Mau = Mauritius; UK = The United Kingdom; SiL = Sri Lanka; Mag = Madagascar; Thai = Thailand; Ind = Indonesia; Aus = Australia; FPo = France Po; Marl = Marshall Islands.

including one monophyletic genus *Amphiprion* with two subgenera *Amphiprion* and *Premnas* (Figure S1, supplementary material). Our anemonefish phylogeny identified a moderately supported (75%) monophyletic Amphiprioninae, also consisting of two sub-genera: *Amphiprion* and *Premna* (Figure 2). However, the topology of the morphological classification and our anemonefish phylogeny were not congruent, except concerning the *Premnas biaculeatus* and the *A. ocellaris/Amphiprion percula* complexes.

At the genus level, *Amphiprion* was identified as a paraphyletic taxon, divided into two clades with

Premnas clustered in between. One clade was formed by three subgenera *Amphiprion*, *Phalerebus* and *Paramphiprion*, while the other clade was *Actinicola*. *Actinicola*, which consisted of three specimens of *A. ocellaris* (NC009065; AB979697 and our sample) and *A. percula* (AB979450) with good support (100%), formed as the basal group of the subfamily Amphiprioninae (Figure 2). The sub-genus *Amphiprion* appeared to be polyphyletic, with the sub-genera – complex (*Amphiprion*- SC: *clarkii*, and *Amphiprion*- SC: *ephippium*) recovered not as sister taxa, but nested within members of the sub-genera *Phalerebus*, and *Paramphiprion*. In turn, the

genera *Paramphiprion* and *Phalerebus* were also found to be polyphyletic, as the species nested within members of other subgenera.

At the species level, we compared our six species sequences to sequences of the same species in GenBank. We found the lowest similarity among different specimens of *A. clarkii*, better matches in *A. ocellaris* and 100% similarity for the rest of the species (see Table SII, supplementary material). *Amphiprion pacificus* is nearly identical (99.8%) to both *A. sandaracinos* specimens and placed within subgenus *Phalerebus* (Figure 2, Table SI, supplementary material).

Anemone – anemonefish cophylogeny

Distance-based approach to testing cophylogeny

The anemonefish and anemone phylogenies built on consensus sequences were used to assess phylogenetic congruence.

Based on the Parafit statistic (ParafitGlobal = 0.000687; *P* value = 0.163) and the PACo global goodness-of-fit statistic (observed $m^2 = 0.0456$; *P* value = 0.319), the anemone and anemonefish phylogenies were not significantly congruent at the global level. However, the analysis of individual links, using Parafit, indicated significant congruence in three out of 81 associations (Table IV). Those associations may represent coevolutionary links, based on their small contribution to the residual sum of squares in the Parafit analysis (*P* value < 0.05). In the PACo analysis, on the other hand, inspection of squared residuals indicated 39 of 81 associations that presented low square-residual values and apparently represented co-evolutionary links (Figure 3). The significant links obtained by Parafit also showed a significant contribution in PACo (Figure 3).

Event-based approach to testing cophylogeny

The tanglegram (Figure 4) built from the phylogenetic trees and individual associations between *Amphiprion* species and their anemone hosts showed no obvious congruence between the host anemones and the symbiont fish topologies.

Our optimal trees reconstructed and analysed in Jane 4.0 exploited a total of 54 cost schemes using all possible combinations of vector costs (Table SII). In those schemes, 19 out of 54 scenarios found no significant lower costs than expected by chance (*P* > 0.05, Table SII). In all cases, the number of co-speciation events varied from 0 to 2, between 0 and 6 for duplication and host-switching events, and 22–24 for duplication events. The number of loss events (between 98

and 110) and failure to diverge events (54) are the most numerous across all schemes.

Given that the coevolution scenarios might depend on good estimations of the set of event cost values (Jane 4.0 analysis), we conducted separate analyses in Core-PA, where the event cost values are automatically estimated. By reconstructing the anemone-anemonefish trees in Core-PA, we found 37 possible solutions, of which 25 were considered the best reconciliations that strictly fit event-cost values to the coevolution event frequencies (q_c<0.25) (Table V). From those, we chose the seven best-fit solutions (with the smallest q_c) to test if coevolution events were more frequent than if they occured randomly. However, none of the seven solutions gave lower q_c values than the q_c values of random instances (p_{qu}>0.05, Table V). None of cospeciation events, or other coevolution events (sorting, duplication, host switch) were more frequent than expected by chance alone ($\boldsymbol{p_{co',>}}/\boldsymbol{p_{co,\geq}}$ > 0.05, Table V). Overall, there was no signal of co-evolution, and no evidence that anemonefish speciation was dependent on speciation of the anemone host.

Discussions

Phylogenies of anemones and anemonefish

Our molecular data reconstructed Stichodactylidae as polyphyletic and Actiniidae as paraphyletic in the superfamily Actinioidea. It also recovered the family Thalassianthidae as a clade nesting within *Stychodactyla*, leaving *Stychodactyla* paraphyletic. The topology of our anemone phylogeny completely agrees with the topology of anemonefish-hosting anemones in the study of Titus et al. (2019). Also in agreement with Titus et al. (2019), our phylogeny indicates significant taxonomic problems, at both family and genus levels, when compared to classical morphological taxonomy of anemonefish-hosting anemones (Dunn 1981; Fautin, 2008).

At the family level, the Thalassianthidae, which includes a single anemonefish-hosting species (*C. adhaesivum*), is one of three families of symbiotic anemones (Dunn 1981; Fautin 1991, 2008). However, the single species *C. adhaesivum* coupled members of the genus *Stichodactyla* and *H. magnifica*, leaving this species as a clade belongings to Stichodactylidea. Therefore, the family Thalassianthidae appeared to be within the family Stichodactylidea, rather than being the third family of anemonefish-hosting anemones. At the genus level, our study showed taxonomic problems in *Stichodactyla* and *Heteractis*. Specimens of *H. magnifica* did not cluster with other *Heteractis*

Table IV. ParaFit analysis of the fish and anemone phylogenies.

Pairs	Anemone species	Anemonefish species	ParafitLink P value
[1]	Entacmaea quadricolor	A. allardi Comoros KF264152	0.341
[2]	Entacmaea quadricolor	A. chagosensis UK KF819364	0.346
[3]	Entacmaea quadricolor	A. bicinctus Unk NC016701	0.331
[4]	Entacmaea quadricolor	A. omanensis Oman KF264173	0.336
[5]	Entacmaea quadricolor	A. ephippium Thai AB979272	0.177
[6]	Entacmaea quadricolor	A. melanopus Ind KF264169	0.202
[7]	Entacmaea quadricolor	A. barberi KF264153	0.197
[8]	Entacmaea quadricolor	A. frenatus	0.186
[9]	Entacmaea quadricolor	A. akindynos KF264151	0.291
[10]	Entacmaea quadricolor	A. mccullochi Aus KF264168	0.294
[11]	Entacmaea quadricolor	A. chrysopterus FPo KF264128	0.662
[12]	Entacmaea quadricolor	A. clarkii	0.852
[13]	Entacmaea quadricolor	A. tricinctus Marl KF264308	0.876
[14]	Entacmaea quadricolor	A. latezonatus Aus KF264165	0.956
[15]	Entacmaea quadricolor	Premnas biaculeatus Thai LC089001	0.979
[16]	Macrodactyla doreensis	A. chrysogaster Mau KF264155	0.313
[17]	Macrodactyla doreensis	A. polymnus	0.336
[18]	Macrodactyla doreensis	A. perideraion	0.581
[19]	Macrodactyla doreensis	A. clarkii	0.898
[20]	Heteractis malu	A. clarkii	0.063
[21]	Heteractis aurora	A. allardi Comoros KF264152	0.095
[22]	Heteractis aurora	A. chrysogaster Mau KF264155	0.092
[23]	Heteractis aurora	A. chagosensis UK KF819364	0.101
[24]	Heteractis aurora	A. bicinctus Unk NC016701	0.063
[25]	Heteractis aurora	A. akindynos KF264151	0.105
[26]	Heteractis aurora	A. chrysopterus FPo KF264128	0.056
[27]	Heteractis aurora	A. clarkii	0.047*
[28]	Heteractis aurora	A. tricinctus Marl KF264308	0.051
[29]	Heteractis crispa	A. polymnus	0.156
[30]	Heteractis crispa	A. bicinctus Unk NC016701	0.084
[31]	Heteractis crispa	A. omanensis Oman KF264173	0.134
[32]	Heteractis crispa	A. ephippiumThai AB979272	0.267
[33]	Heteractis crispa	A. melanopus Ind KF264169	0.300
[34]	Heteractis crispa	A. barberi KF264153	0.342
[35]	Heteractis crispa	A. akindynos KF264151	0.183
[36]	Heteractis crispa	A. sandaracinos	0.205
[37]	Heteractis crispa	A. perideraion	0.273
[38]	Heteractis crispa	A. chrysopterus FPo KF264128	0.102
[39]	Heteractis crispa	A. clarkii	0.061
[40]	Heteractis crispa	A. tricinctus Marl KF264308	0.069
[41]	Heteractis crispa	A. latezonatus Aus KF264165	0.993
[42]	Heteractis crispa	A. percula Thai AB979450	0.990
[43]	Stichodactyla haddoni	A. chrysogaster Mau KF264155	0.351
[44]	Stichodactyla haddoni	A. polymnus	0.426
[45]	Stichodactyla haddoni	A. omanensis Oman KF264173	0.413
[46]	Stichodactyla haddoni	A. akindynos KF264151	0.246
[47]	Stichodactyla haddoni	A. chrysopterus FPo KF264128	0.403
[48]	Stichodactyla haddoni	A. clarkii	0.044*
[49]	Stichodactyla haddoni	A. tricinctus Marl KF264308	0.058
[50]	Stichodactyla haddoni	A. sebae Thai AB979696	0.082
[51]	Stichodactyla gigantea	A. bicinctus Unk NC016701	0.907
[52]	Stichodactyla gigantea	A. akindynos KF264151	0.853
[53]	Stichodactyla gigantea	A. perideraion	0.873
[54]	Stichodactyla gigantea	A. clarkii	0.241
[55]	Stichodactyla gigantea	A. ocellaris	0.049*
[56]	Stichodactyla gigantea	A. percula Thai AB979450	0.061
[57]	Stichodactyla mertensii	A. allardi Comoros KF264152	0.622
[58]	Stichodactyla mertensii	A. bicinctus Unk NC016701	0.407
[59]	Stichodactyla mertensii	A. ephippiumThai AB979272	0.324
[60]	Stichodactyla mertensii	A. melanopus Ind KF264169	0.320
[61]	Stichodactyla mertensii	A. barberi KF264153	0.373
[62]	Stichodactyla mertensii	A. frenatus	0.363
[63]	Stichodactyla mertensii	A. akindynos KF264151	0.151
[64]	Stichodactyla mertensii	A. mccullochi Aus KF264168	0.269
[65]	Stichodactyla mertensii	A. sandaracinos	0.298
[66]	Stichodactyla mertensii	A. akallopisos Thai NC030590	0.269
[67]	Stichodactyla mertensii	A. clarkii	0.169
[68]	Stichodactyla mertensii	A. tricinctus Marl KF264308	0.244
[69]	Stichodactyla mertensii	A. ocellaris	0.458
[70]	Heteractis magnifica Mad MK519431	A. chrysogaster Mau KF264155	0.751

Table IV. Continued.

Pairs	Anemone species	Anemonefish species	ParafitLink P value
[71]	*Heteractis magnifica* Mad MK519431	*A. nigripes* SiLKF264170	0.824
[72]	*Heteractis magnifica* Mad MK519431	*A. bicinctus* Unk NC016701	0.668
[73]	*Heteractis magnifica* Mad MK519431	*A. melanopus* Ind KF264169	0.845
[74]	*Heteractis magnifica* Mad MK519431	*A. akindynos* KF264151	0.586
[75]	*Heteractis magnifica* Mad MK519431	*A. perideraion*	0.464
[76]	*Heteractis magnifica* Mad MK519431	*A. akallopisos* Thai NC030590	0.540
[77]	*Heteractis magnifica* Mad MK519431	*A. chrysopterus* FPo KF264128	0.763
[78]	*Heteractis magnifica* Mad MK519431	*A. clarkii*	0.398
[79]	*Heteractis magnifica* Mad MK519431	*A. ocellaris*	0.141
[80]	*Heteractis magnifica* Mad MK519431	*A. percula* Thai AB979450	0.136
[81]	*Cryptodendrum adhaesivum* Unk KC812142	*A. clarkii*	0.170

Notes: Individual link test results are indicated. Values with asterisk (*) indicate a significant association ($P \le 0.05$).
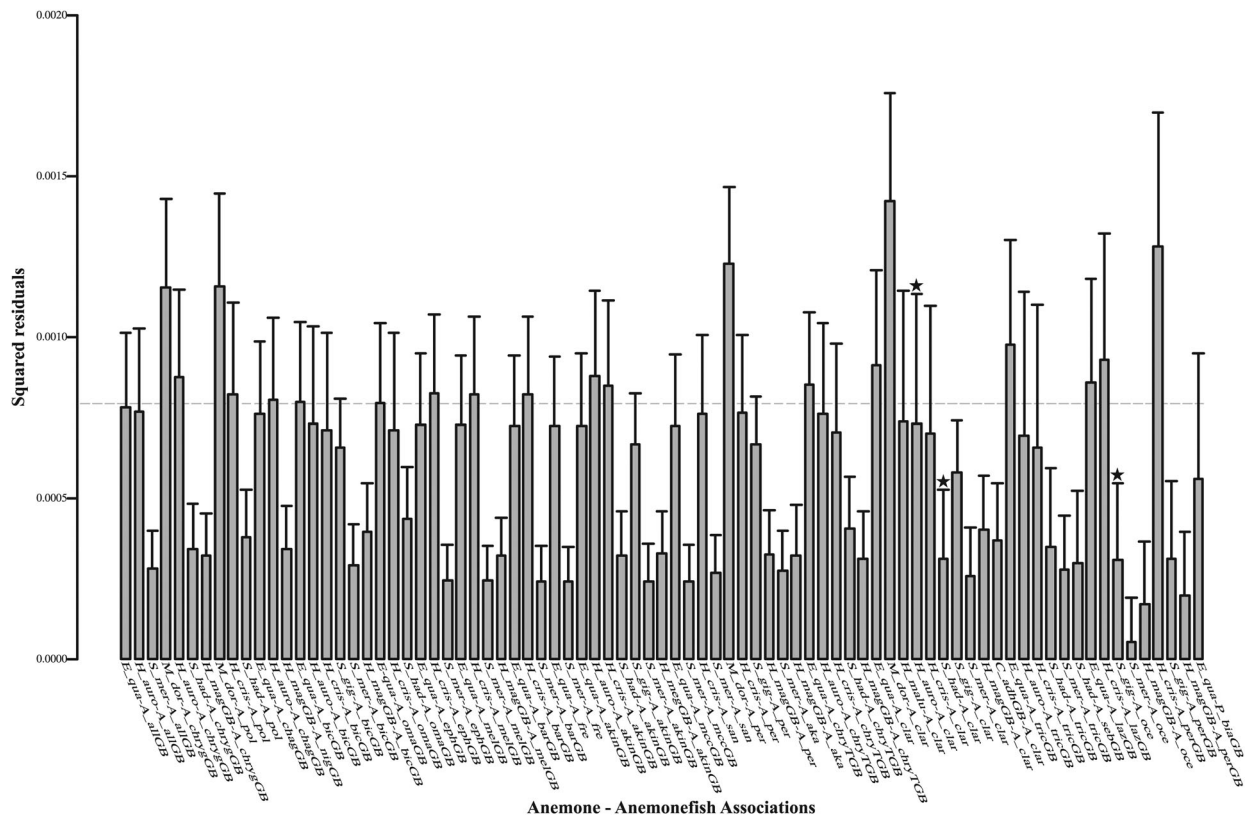

Figure 3. Contribution of individual associations to global phylogenetic congruence of *Amphiprion* symbionts and their anemone hosts. The columns represent the squared residuals of each association contributing to the total sum of squared residuals computed by PACo for generic distances derived from the phylogenies shown in Figures 1 and 2. Jacknifed squared residuals (bars) and upper 95% confidence intervals (error bars). Median host-symbiont associations whose 95% squared residual confidence interval falls above the median value probably represent non-congruent associations. Stars represent links significantly supported ($a < 0.05$) from ParaFitLink, including *H. aurora – A. clarkii*; *S. haddoni – A. clarkii* and *S. gigantea – A. ocellaris*. Abbreviations of Anemone names: Entacmaea (E_qua = *E. quadricolor*); Macrodactyla (M_dor = *M. doreensis*); Stichodactylidae (S_had = *S. haddoni*; S_gia = *S. gigantea*; S_mer = *S. mertensii*); Heteractic (H_auro = *H. aurora*; H_cris = *H. crispa*; H_malu = *H. malu*; H_magGB = *H. magnifica* Mad MK519431); *Cryptodendrum* (C_adhGB = *C. adhaesivum* Unk KC812142). Anemonefish names: A_allGB = *A. allardi* Comoros KF264152; A_latifGB = *A. latifasciatus* Mag JF457235; A_chrygGB = *A. chrysogaster* Mau KF264155; A_pol = *A. polymnus*; A_chagGB = *A. chagosensis* UK KF819364; A_nigGB = *A. nigripes* SiL KF264170; A_bicGB = *A. bicinctus* Unk NC016701; A_omaGB = *A. omanensis* Oman KF264173; A_ephGB = *A. ephippium* Thai AB979272; A_melGB = *A. melanopus* Ind KF264169; A_barGB = *A. barberi* KF264153; A_fre = *A. frenatus*; A_rubGB = *A. rubrocinctus* Aus KF264182; A_akinGB = *A. akindynos* KF264151; A_mccGB = *A. mccullochi* Aus KF264168; A_san = *A. sandaracinos*; A_per = *A. perideraion*; A_aka = *A. akallopisos* Thai NC030590; A_chryTGB = *A. chrysopterus* FPo KF264128; A_leuGB = *A. leucokranos* Mag KF264167; A_clar = *A. clarkii*; A_tricGB = *A. tricinctus* MarI KF264308; A_sebGB = *A. sebae* Thai AB979696; A_lazGB = *A. latezonatus* Aus KF264165; A_oce = *A. ocellaris*; A_perGB = *A. percula* Thai AB979450; P_biaGB = *P. biaculeatus* Thai LC089001.
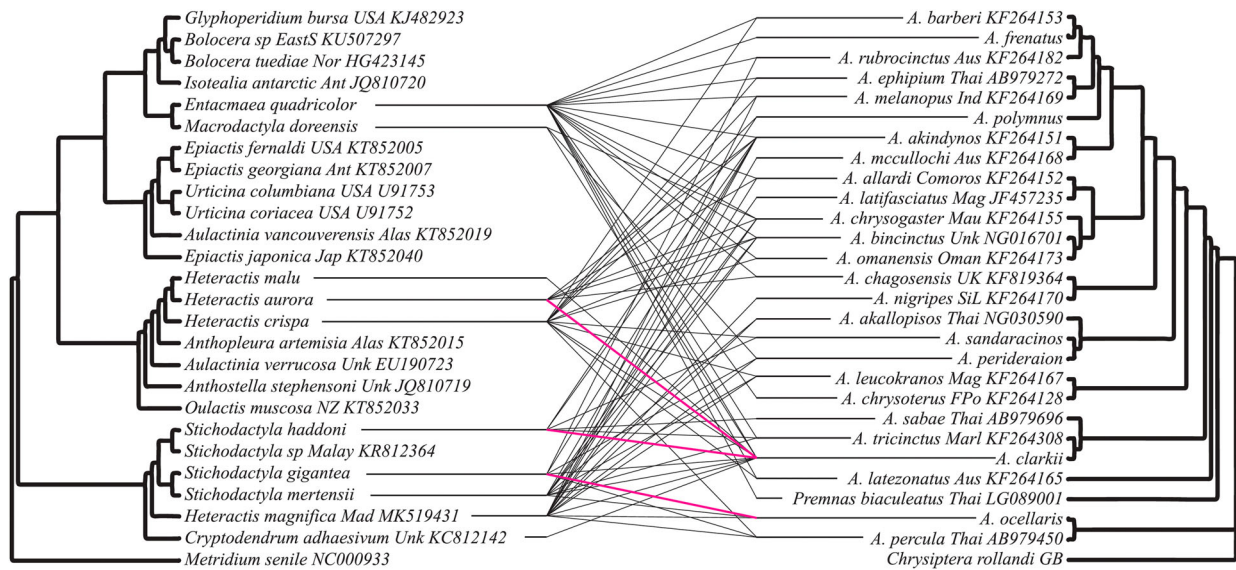
Figure 4. Tanglegram of anemone host species ML tree (left) compared to the anemonefish symbiont species ML tree (right). Trees were reconstructed from the combine datasets of: 12S rDNA-16S rDNA-COI mtDNA genes (anemone) and Cytb-16S rDNA-COI mtDNA genes (anemonefish). Thin lines link host – symbiont associations. Pink lines indicate host-symbiont associations that contribute significantly to the Parafit Global statistics. Grey lines indicate associations that do not contribute significantly to the Parafit Global statistics.

Table V. Anemone-anemonefish reconstructions from Core-PA.

Solution	Event frequency	Best cost vector (in order: cospeciation, sorting, duplication, and host switch)	*q_c	**p_{co}, > /$p_{co,\geq}$	***p_{qu}
1	(6,17,17,2)	(0.2294; 0.0822; 0.0810; 0.6073)	0.0126889	0.91	0.94
2	(9,15,14,2)	(0.1487; 0.0926; 0.1024; 0.6563)	0.0225076	0.84	0.95
3	(7,28,18,0)	(0.0036; 0.0010; 0.0015; 0.9938)	0.0293712	0.89	0.80
4	(14,30,11,0)	(0.0007; 0.0004; 0.0010; 0.9980)	0.0364755	0.38	0.67
5	(8,11,14,3)	(0.1641; 0.1269; 0.1006; 0.6083)	0.0380134	0.86	0.95
6	(5,13,17,3)	(0.2249; 0.1005; 0.0769; 0.5976)	0.0427939	0.97	0.89
7	(6,12,16,3)	(0.1961; 0.1123; 0.0838; 0.6078)	0.0454717	0.91	0.90

*q_c: best fit of the selected cost vector to the absolute event frequencies of the corresponding solution. **$p_{co,\geq}$: probability that a reconstruction based on a randomized instance leads to more (respectively an equal number or more) cospeciation. ***p_{qu}: probability that a randomized reconstruction leads to a smaller value of q_c.

genera, but formed a well-supported monophyletic clade (97%) with *Stichodaclyla* species (*S. gigantea*, *S. haddoni*) and with *Cryptodendron* (*C. adhaesivum*). Therefore, this species seems to be more closely related to *Stichodactyla* than *Heteractis*. Likewise, *H. crispa*, *H. malu*, and *H. aurora*, rather than forming a monophyletic clade to *Stichodactyla*, seemed to cluster within the Actiniidae. *Macrodactyla* (with a single species *M. doreensis*) was also recovered as several clades within *Heteractis,* the Stichodactylidea family, instead of being a monophyletic genus in Actiniidae family (Dunn 1981; Fautin 1991, 2008).

At the species level, our *E. quadricolor* grouped with other sequences from GenBank as a monophyletic clade with moderate support (84%). Within *Heteractis*, we found it difficult to interpret the relationships between specimens of *H. aurora*, *H. crispa*, *H. malu* and *M. doreensis*. We were also unable to resolve

species-level relation between *S. haddoni* and *S. mertensii* within *Stichodactyla*. Titus et al. (2019) also encountered problems distinguishing these and other anemone species. Furthermore, the moderate genetic variation found in our study, among samples from different geographic localities, were also detected in previous studies (Daly et al. 2008; Titus et al. 2019).

Our anemonefish tree topology supports a monophyletic origin of the subfamily Amphiprioninae, with an early divergence of the *Actinicola* subgenera (*A. ocellaris* and *A. percula)* from the rest of the anemonefish. This is consistent with previous studies (Elliott et al. 1999; Santini and Polacco 2006; Litsios et al. 2012). The tree also suggests that anemonefish might have evolved from specialist ancestors rather than from the generalist species *A. clarkii* (in the *Amphiprion* subgenus) as proposed by the morphology-based

phylogenetic hypothesis (Allen 1972, Figures S1 and S2). This is in agreement with Elliott et al. (1999), who first suggested the specialist ancestor of anemonefish in their study.

The paraphyly of the *Amphiprion* genus, and non-monophyletic topologies of the subgenera *Amphipiron*, *Phalerebus* and *Paramphiprion* were also indicated in previous studies (Jang-Liaw et al. 2002; Litsios et al. 2014; Li et al. 2015). *Premnas biaculeatus* was placed within the *Amphiprion* genus in our phylogeny, as previously proposed (Elliott et al. 1999; Santini and Polacco 2006; Litsios et al. 2012). However, in our ML analysis, *Premnas* was not recovered as the sister group to the *A. ocellaris* + *A. percula* clade, but became the root of another clade (*Amphiprion*, *Paraphiprion* and *Phalerebus*). Our reconstruction is similar to the topology in Santini and Polacco (2006), which does not place *P. biaculeatus* in a basal position to all the other anemonefish.

Our long-branch distances between *A. clarkii* specimens were also identified in other studies (Litsios et al. 2014). In fact, *A. clarkii* specimens clustered by colour rather than by geographic origin (Litsios et al. 2014). Although *A. pacificus* is nearly identical in appearance to *A. akallopisos*, DNA sequencing data from mitochondrial genes suggest a close relationship to *A. sandaracinos*, which was also recovered in the molecular phylogenetic analysis of Allen et al. (2010).

To complete the phylogenies, we added sequences of additional anemonefish and anemone species from GenBank in our analyses (as described in the Material and methods). While doing this we discovered several incidences of possible misidentified species in the GenBank sequences, so these were used with caution. We used mitochondrial genes (combining COI mtDNA + 16S rDNA + 12S rDNA data) in the anemone phylogenetic analyses. At the intraspecific level, the Anthozoan mtDNA, unfortunately, provides a poor phylogenetic resolution due to its slow rate of evolution (Shearer et al. 2005; Daly et al. 2010). This could contribute to the difficulties in differentiating between species within the same genus (Titus et al. 2019). New, high-resolution genomic methods, such as targeted capture and enrichment approaches for ultra-conserved elements would likely improve the phylogenetic resolution to resolve hierarchical relationships between close anemone taxa.

Anemone–anemonefish cophylogeny

The statistical cophylogenetic analysis did not support a global congruence between the phylogenies; although some individual anemonefish-anemone links showed significant congruence, suggesting that coevolution could occur at the level of individual species pairs. Simulations reported by Legendre et al. (2002) suggested that when the global test is not significant, but tests of individual links are, then one might be dealing with a mixed structure containing a co-evolutionary portion and a random portion. Legendre and co-workers also emphasized that only highly significant association should be considered (Legendre et al. 2002). In our case, three out of 81 links were significant, but none of those was highly significant (Table III). We, therefore, choose not to consider those individual links as co-evolutionary links in anemone-anemonefish associations.

The number of co-speciation events indicating in Jane is limited, from 0 up to 2 in all cost schemes, suggesting that co-speciation, if possible, is quite rare in the anemone-anemonefish relationship. Moreover, the relative number of schemes showed no significant cost reconstruction than randomization in Jane 4.0, which suggests the outcome of possible co-evolutionary scenarios may vary according to the cost assignment. However, the best cost schemes that have been found in the Core-PA analysis, did not detect any significant global co-speciation; the signal is largely used to evaluate coevolution (Charleston and Perkins 2006; Doña et al. 2017). These analyses support no coevolution between anemones and anemonefish.

Diversification of the host anemones was independent of their fish symbionts, as indicated by the lack of congruence in the trees in the Parafit analyses. Likewise, the PACo analysis found no significance based on global goodness-of-fit tests, although the presence of almost half of the significant individual pair links suggests that diversification in the anemonefish may be dependent on their hosts. Anemonefish are found only in close association with an anemone host, while these anemones can be found living without a symbiont (Mebs 2009). Thus, it is most likely that the anemonefish group adapted in relation to their anemone hosts while the anemones have evolved independently of the fish. Anemonefish seem to be able to diversify in every colonized area, rather than a single evolution pattern for morphology and life style (Santini and Polacco 2006). Anemonefish also exhibit patterns of ecological adaptive radiation via ecological speciation and likely developed a convergent phenotype correlated to the host-associated ecological niches (Litsios et al. 2012). Titus et al. (2019) also found that anemonefish and anemones differ in their biogeographical origins, and thus the likelihood of co-evolution is low.

Although the relationship is beneficial for both fish and anemone (Mariscal 1970; Fautin 1991; Roopin

et al. 2008; Godinot and Chadwick 2009), it appears that this classical example of a symbiotic interspecies relationship is based solely on dependence of fish upon anemones, with regard to the microevolution. There are even data suggesting that anemonefish feed on the gametes spawned by their anemone hosts (Scott and Francisco 2006), in which case the relationship would be more beneficial for the fish symbionts than for the anemone hosts.

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