ORIGINAL ARTICLE

Phylogeography, population structure and evolution of coral-eating butterflyfishes (Family Chaetodontidae, genus *Chaetodon*, subgenus *Corallochaetodon*)

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ABSTRACT

Aim This study compares the phylogeography, population structure and evolution of four butterflyfish species in the *Chaetodon* subgenus *Corallochaetodon*, with two widespread species (Indian Ocean – *C. trifasciatus* and Pacific Ocean – *C. lunulatus*), and two species that are largely restricted to the Red Sea (*C. austriacus*) and north-western (NW) Indian Ocean (*C. melapterus*). Through extensive geographical coverage of these taxa, we seek to resolve patterns of genetic diversity within and between closely related butterflyfish species in order to illuminate biogeographical and evolutionary processes.

Location Red Sea, Indian Ocean and Pacific Ocean.

Methods A total of 632 individuals from 24 locations throughout the geographical ranges of all four members of the subgenus *Corallochaetodon* were sequenced using a 605 bp fragment (cytochrome *b*) of mtDNA. In addition, 10 microsatellite loci were used to assess population structure in the two widespread species.

Results Phylogenetic reconstruction indicates that the Pacific Ocean *C. lunulatus* diverged from the Indian Ocean *C. trifasciatus* approximately 3 Ma, while *C. melapterus* and *C. austriacus* comprise a cluster of shared haplotypes derived from *C. trifasciatus* within the last 0.75 Myr. The Pacific *C. lunulatus* had significant population structure at peripheral locations on the eastern edge of its range (French Polynesia, Johnston Atoll, Hawai'i), and a strong break between two ecoregions of the Hawaiian Archipelago. The Indian Ocean *C. trifasciatus* showed significant structure only at the Chagos Archipelago in the central Indian Ocean, and the two range-restricted species showed no population structure but evidence of recent population expansion.

Main conclusions Patterns of endemism and genetic diversity in *Corallochaetodon* butterflyfishes have been shaped by (1) Plio-Pleistocene sea level changes that facilitated evolutionary divergences at biogeographical barriers between Indian and Pacific Oceans, and the Indian Ocean and Red Sea, and (2) semi-permeable oceanographic and ecological barriers working on a shorter time-scale. The evolution of range-restricted species (Red Sea and NW Indian Ocean) and isolated populations (Hawai'i) at peripheral biogeographical provinces indicates that these areas are evolutionary incubators for reef fishes.

Keywords
biogeography, *Chaetodon austriacus*, *Chaetodon lunulatus*, *Chaetodon melapterus*, *Chaetodon trifasciatus*, microsatellites, mtDNA, reef fish, speciation

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INTRODUCTION

How do new species with high dispersal potential arise in an aquatic medium? The Indo-Pacific reef fishes have two biogeographical traits that inform this issue. First, the biodiversity of fishes and other coral-associated species peaks at the central Indo-Australian Archipelago, where Indian and Pacific Ocean faunas overlap (Blum, 1989; Gaither & Rocha, 2013). Second, the highest endemism is in peripheral regions at the ends of the range, including the Red Sea and Hawai'i (Randall, 1998). Evidence supporting genetic differentiation in peripheral biogeographical regions comes from both peripheral locations, which are the western and eastern limits for numerous Indo-Pacific species (DiBattista *et al.*, 2013; Eble *et al.*, 2015). Phylogeographical studies indicate that new species are arising in both the peripheral regions and the biodiversity centre (Bowen *et al.*, 2013). However, few studies have focused on diversification in the Red Sea and north-western (NW) Indian Ocean.

The well-resolved phylogeny of butterflyfishes (family Chaetodontidae), has made this group an appropriate model for understanding the evolution of reef fishes (Fessler & Westneat, 2007; Cowman & Bellwood, 2013; Hodge *et al.*, 2014). Butterflyfishes embody the primary biogeographical patterns outlined above, with greatest diversity in the Indo-Australian Archipelago and highest endemism in peripheral areas. The Red Sea and adjacent Gulf of Aden has 32% endemism in butterflyfishes, compared to 13% in Hawai'i and < 10% elsewhere in the Indo-Pacific (Randall, 2007; DiBattista *et al.*, 2015a). Understanding how the highest levels of endemism arose far from the center of diversity remains an enigma. Biogeographical barriers at these locations may have created isolated populations or endemic species depending on the divergence time (Briggs & Bowen, 2013).

Among butterflyfishes, the subgenus *Corallochaetodon* contains four corallivorous species that have mostly parapatric distributions with narrow areas of overlap on the range edges (Fig. 1). *Chaetodon lunulatus* Quoy & Gaimard, 1824 occurs throughout the Pacific Ocean from Hawai'i and the Tuamotu Islands westward to Indonesia and the eastern Indian Ocean (Christmas Island), while *Chaetodon trifasciatus* Park, 1797 is distributed in the Indian Ocean from Indonesia and Christmas Island to East Africa, but is not known from the Red Sea (Allen *et al.*, 1998). *C. lunulatus* and *C. trifasciatus* may be Indian-Pacific Ocean sister species that diverged during Plio-Pleistocene sea level changes that created the transient Sunda Shelf Barrier (Hsu *et al.*, 2007). *Chaetodon melapterus* Guichenot, 1863 is restricted to the Arabian Gulf, Gulf of Oman, Gulf of Aden and the southern Red Sea, while *Chaetodon austriacus* Rüppell, 1836 occurs predominantly in the northern and central Red Sea (Zekeria *et al.*, 2005), with rare records in the southern Red Sea and adjacent Arabian Sea (DiBattista *et al.*, 2015a). It is

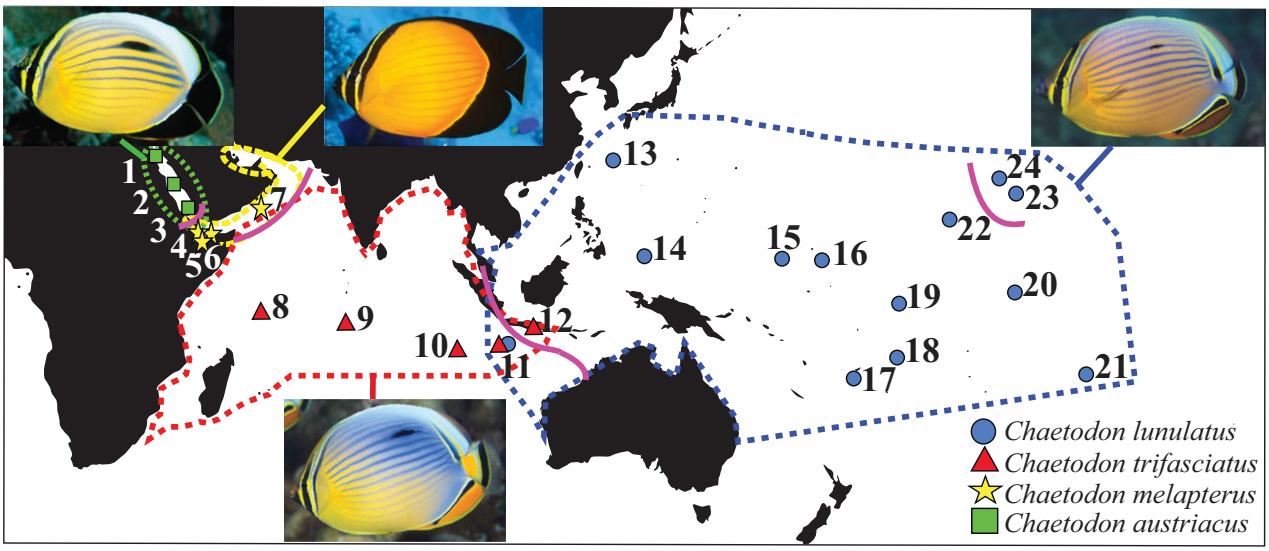

Figure 1 Distribution map of *Chaetodon* subgenus. *Corallochaetodon* (redrawn from Blum, 1989). *Chaetodon lunulatus* (blue, widespread Pacific Ocean), *C. trifasciatus* (red, widespread Indian Ocean), *C. austricaus* (green, largely restricted to the northern and central Red Sea; but see DiBattista *et al.*, 2015a) and *C. melapterus* (yellow, restricted to the southern Red Sea through the Arabian Gulf). The known geographical range of each species is outlined with a dotted line and solid pink lines represent known marine biogeographical barriers (Hsu *et al.*, 2007) that influence the genetic partitions and evolution of *Corallochaetodon.* Sample locations are shown with species-specific coloured symbols and numbers that correspond to the following location names: 1. Jazirat Baraqan, 2. Yanbu, 3. Al Lith, 4. Obock, 5. Bay of Ghoubbet, 6. Maskali, 7. Oman, 8. Seychelles, 9. Diego Garcia, 10. Cocos (Keeling) Islands, 11. Christmas Island, 12. Indonesia, 13. Okinawa, 14. Palau, 15. Pohnpei, 16. Marshall Islands, 17. Fiji, 18. American Samoa, 19. Kanton Island, 20. Kiribati, 21. Mo'orea, 22. Johnston Atoll, 23. Main Hawaiian Islands, 24. Northwestern Hawaiian Islands. Sample sizes for each location are presented in Table 1. Photo credits: L.A. Rocha for *C. austriacus*, T. Sinclair-Taylor for *C. lunulatus*, *C. trifasciatus* and *C. melapterus*.

Table 1 Sample size and molecular diversity indices for *Chaetodon lunulatus*, *C. trifasciatus*, *C. melapterus* and *C. austriacus* based on mtDNA cytochrome *b* sequence data (significant Fu's *Fs* values are in bold, $P < 0.02$). For *C. trifasciatus*, specimens from the eastern Indian Ocean (Cocos-Keeling Islands and adjacent Christmas Island) were pooled to increase statistical power as they were indistinguishable in preliminary analyses.

Location	*N*	Number of haplotypes	Haplotype diversity ($h \pm$ SD)	Nucleotide diversity ($\pi \pm$ SD)	Fu's *Fs*
C. lunulatus					
Christmas Island	6	4	0.867 ± 0.129	0.005 ± 0.004	0.235
American Samoa	15	5	0.714 ± 0.081	0.005 ± 0.003	1.400
Fiji	30	10	0.602 ± 0.104	0.004 ± 0.003	−1.925
Kanton Island	15	5	0.695 ± 0.109	0.004 ± 0.003	0.953
Marshall Islands	29	8	0.727 ± 0.057	0.005 ± 0.003	0.909
Mo'orea	32	8	0.669 ± 0.086	0.005 ± 0.003	−0.040
Okinawa	8	4	0.643 ± 0.184	0.004 ± 0.003	0.730
Pohnpei	30	10	0.782 ± 0.065	0.005 ± 0.003	−0.569
Kiribati	22	3	0.589 ± 0.066	0.004 ± 0.003	4.628
Palau	26	2	0.471 ± 0.063	0.004 ± 0.002	6.684
Johnston Atoll	31	2	0.516 ± 0.024	0.004 ± 0.003	7.635
MHI	33	2	0.504 ± 0.034	0.004 ± 0.003	7.642
NWHI	161	13	0.452 ± 0.048	0.001 ± 0.001	−0.507
C. trifasciatus					
Diego Garcia	29	8	0.672 ± 0.074	0.001 ± 0.001	**−4.538**
Seychelles	21	9	0.795 ± 0.077	0.088 ± 0.044	9.843
Christmas Island	14	7	0.802 ± 0.094	0.010 ± 0.006	0.959
Indonesia	5	3	0.700 ± 0.218	0.002 ± 0.002	0.061
C. melapterus					
Maskali	17	5	0.353 ± 0.353	0.001 ± 0.001	**−2.527**
Obock	29	7	0.778 ± 0.584	0.001 ± 0.001	**−3.754**
Bay of Ghoubbet	15	1	0.000 ± 0.000	0.000 ± 0.000	na
Oman	34	9	0.631 ± 0.507	0.001 ± 0.001	**−7.615**
C. austriacus					
Al Lith	10	2	0.200 ± 0.154	0.000 ± 0.000	na
Jazirat Baraqan	10	6	0.844 ± 0.103	0.002 ± 0.002	**−3.127**
Yanbu	10	7	0.866 ± 0.107	0.001 ± 0.001	**−1.404**

MHI, Main Hawaiian Islands; NWHI, Northwestern Hawaiian Islands.

unknown if the two range-restricted species (*C. melapterus* and *C. austriacus*) arose independently, and whether they evolved from the widespread Indian Ocean species *C. trifasciatus*, as current geographical distributions would indicate. Thus, the subgenus *Corallochaetodon* provides the opportunity to determine how the speciation of butterflyfishes in peripheral locations (*C. melapterus* and *C. austriacus*) compares to that in the center of diversity (*C. lunulatus* and *C. trifasciatus*).

This study is motivated by four primary questions. First, what is the evolutionary history of the subgenus *Corallochaetodon*? Second, what are the geographical patterns of genetic diversity within and between species? Third, what is the population structure (as revealed by mtDNA) of all four species across their geographical ranges? Fourth, what is the fine-scale population structure (as revealed by microsatellite DNA) in the two widespread species (*C. lunulatus* and *C. trifaciatus*), and is there evidence of peripheral speciation? These genetic patterns can illuminate the origins of marine biodiversity, and the measures that would conserve building blocks of future biodiversity.

MATERIALS AND METHODS

Sample collection

Tissue (fin clips or gill filament) were obtained from specimens collected using polespears whilst SCUBA diving at 24 locations across the Indo-Pacific (including the Red Sea) from 2005 to 2013 (*C. lunulatus* $N = 438$, *C. trifasciatus* $N = 69$, *C. melapterus* $N = 95$, *C. austriacus* $N = 30$) (Table 1). *Chaetodon lunulatus* was intensively sampled in the Hawaiian Archipelago to assess connectivity across this 2600 km island chain. All tissues were preserved in a saturated salt dimethyl sulfoxide (DMSO) solution (Seutin *et al.*, 1991). DNA was extracted using a 'HOTSHOT' protocol (Meeker *et al.*, 2007), and aliquots were stored at −20 °C.

Mitochondrial DNA sequencing

A 605 bp segment of mtDNA cytochrome *b* (cyt *b*) gene was resolved for specimens of each species. Details of the PCR methodology are available in Appendix S1 and Waldrop

(2014). The cyt *b* data comprises a single locus but offers the advantages of haploid inheritance, lack of recombination, comparison to existing studies and availability of universal primers for efficient production of sequence data. Unique mtDNA cyt *b* haplotypes are deposited in GenBank under accession numbers KP241594 to KP241672.

Phylogenetic relationships

Phylogenetic relationships were examined among the four species by constructing neighbour-joining (NJ), maximum-likelihood (ML) and maximum-parsimony (MP) trees from the cyt *b* haplotypes of all individuals (PAUP*, Swofford, 2003; implemented in GENEIOUS PRO 6.0.6, Drummond *et al.*, 2010; and MEGA 5.2.2, Tamura *et al.*, 2011). Bootstrap support values were calculated using default settings with 10,000 replicates in both packages. A single *Chaetodon vagabundus* Linnaeus, 1758 sample (Genbank accession numbers: JF458006) was used to root trees. For simplicity, a subset of unique haplotypes was used to create the final tree. An unrooted network of haplotypes was also assembled using a median-joining algorithm and default settings in NETWORK 4.5.1.0 (Bandelt *et al.*, 1999). Molecular clock rate is provisionally estimated at 2% per Myr (between lineages) for the cyt *b* gene (Bowen *et al.*, 2001; Reece *et al.*, 2011). Evolutionary distances among lineages were calculated with the Tamura-Nei model and 1,000 bootstrap replicates in MEGA.

Population structure for mtDNA

An Akaike's information criterion (AIC) test in JMODELTEST 2.1.3 (Posada, 2008) was used to determine the best nucleotide substitution model for each species. The HKY model (Hasegawa *et al.*, 1985) was selected for *C. lunulatus*, *C. trifasciatus* and *C. austriacus*, and TrN+G (Tamura & Nei, 1993) was selected for *C. melapterus*. The TrN+G is the only one of these models available in ARLEQUIN 3.5.1.3 (Excoffier *et al.*, 2005) analytical software and was selected for all phylogeographical inferences. ARLEQUIN was used to calculate haplotype (*h*) and nucleotide diversity (π), Fu's *Fs* test of neutrality (Fu, 1997) and to apply an analysis of molecular variance (AMOVA; Excoffier *et al.*, 1992) to test for the patterns of population structure; tests were run for each species separately. Samples with $N < 5$ were excluded from all population-level analyses and pooled into their respective larger sampling locations to provide adequate statistical power. Hawaiian specimens of *C. lunulatus* were subdivided into the Main Hawaiian Islands (MHI, high islands) and Northwestern Hawaiian Islands (NWHI, low islands and atolls) to test for genetic structure within the archipelago. *C. trifasciatus* specimens from the eastern Indian Ocean (Cocos-Keeling Islands and adjacent Christmas Island) were pooled to increase statistical power as they were indistinguishable in preliminary analyses.

Population structure – microsatellites

Microsatellite primers were designed for *C. lunulatus* by Lawton *et al.* (2010, 2011). Here the widespread *C. lunulatus* and *C. trifasciatus* were genotyped at 10 loci (see Table S1.1 in Appendix S1 in Supporting Information). The range-restricted *C. melapterus* and *C. austriacus* were not genotyped because large samples were not available, finances were limited and cross-species applications can be complicated by allele dropout, homoplasy and other problems (see Selkoe & Toonen, 2006). Details of PCR amplifications are available in Appendix S1 and Waldrop (2014). Initially, specimens from Hawai'i were separated into individual sampling locations by island. However, mtDNA data revealed a genetic break between the MHI and NWHI concordant with a multi-species connectivity study (Toonen *et al.*, 2011). For subsequent analyses, Hawai'i was partitioned into two groups; MHI and NWHI. However, a full comparison among Hawaiian sample sites is provided in Table S2.1 in Appendix S2.

For each locus, the mean number of alleles (N_A), observed (H_O) and expected (H_E) heterozygosities, departure from Hardy–Weinberg equilibrium (HWE) and linkage disequilibrium (LD) were assessed with GENEPOP 4.2 (Raymond & Rousset, 1995). MICRO-CHECKER 2.2.3 was used to identify null alleles and excessive stutter peaks (Van Oosterhout *et al.*, 2004), and significance levels for multiple comparisons were adjusted using the sequential Bonferonni correction. GENODIVE 2.0b23 (Meirmans & van Tienderen, 2004) was used to estimate population structure for each species. STRUCTURE 2.3.4 was used to assign individuals to distinct genetic clusters (populations) without presumption of predefined geographical locations (Pritchard *et al.*, 2000). The most likely number of clusters was identified based on the probability of $K = 1$ to $K = 12$ or $K = 1$ to $K = 4$ for *C. lunulatus* and *C. trifasciatus* respectively. Analyses were repeated five times and averaged. Each replicate run consisted of 1,000,000 Markov chain Monte Carlo (MCMC) repetitions, a burn-in of 10,000 iterations and assumed correlated allele frequencies with admixed populations (as per DiBattista *et al.*, 2012). STRUCTURE HARVESTER 0.6.93 was used to determine most likely value of *K* following Evanno *et al.* (2005) to visualize likelihood values and the number of groups that best fit the data (Earl & von Holdt, 2012).

RESULTS

Phylogenetic relationships

The authors recognize the limitations of a single-locus phylogeny, and so here we provide the mtDNA results as an initial hypothesis of relationships among the four species. All tree-building methods used to analyse the mtDNA cyt *b* fragment (605 bp) produced nearly identical tree topologies with bootstrap support values for species level relationships

of 80–100% (Fig. 2). The primary feature of this phylogeny is a bifurcation with $d = 0.06$ sequence divergence between Pacific Ocean *C. lunulatus* and the Indian Ocean *C. trifasciatus*. The two range-restricted species, *C. melapterus* and *C. austriacus*, are more closely related to the Indian Ocean species ($d = 0.015$). However, they did not form monophyletic groups, and share the most common haplotype (Fig. 2). The relationship within the subgenus *Corallochaetodon* is apparent in the parsimony network (Fig. 3), where Pacific Ocean *C. lunulatus* and Indian Ocean *C. trifasciatus* are separated by 28 diagnostic nucleotide substitutions, and the *C. melapterus–C. austriacus* cluster is separated from *C. trifasciatus* by three diagnostic nucleotide substitutions.

Genetic diversity

Haplotype diversity within each species was moderate to high (*C. lunulatus* $h = 0.45$ to 0.87; *C. trifasciatus* $h = 0.67$ to 0.80; *C. melapterus* $h = 0.00$ to 0.78; *C. austriacus* $h = 0.20$ to 0.87; Table 1). For the species with the largest geographical range (*C. lunulatus*), haplotype diversity was highest at the peripheral location on the western edge of its range (Christmas Island), and was generally lowest at peripheral locations on the eastern edge of its range (Johnston Atoll, MHI, NWHI). For *C. trifasciatus*, haplotype diversities are similar across the range. In the two range-restricted species (*C. melapterus*, and *C. austriacus*), haplotype diversity was lower at one sampled location (Table 1). Nucleotide diversity was low for all species (*C. lunulatus* $\pi = 0.001$ to 0.005; *C. trifasciatus* $\pi = 0.001$ to 0.088; *C. melapterus* $\pi = 0.000$ to 0.001; *C. austriacus* $\pi = 0.000$ to 0.002; Table 1), indicating a cluster of closely related haplotypes within each species.

For the two widespread species, only one of the 17 sample locations was significant for Fu's *Fs* (*C. trifasciatus* at Diego Garcia). For the two range-restricted species, tests for Fu's *Fs* could only be conducted on samples from five locations and all produced significant negative values: *C. melapterus* at Maskali, Obock and Oman; *C. austriacus* at Jazirat Baraqan and Yanbu (Table 1).

Population structure (mtDNA)

Significant population structure was observed in *C. lunulatus* (overall $\Phi_{ST} = 0.27$; $P < 0.001$). In comparisons among sample locations, 30 of 78 pairwise comparisons were statistically significant ($P < 0.05$; Table 2). Five locations accounted for all the significant comparisons: Fiji with six of 12 significant comparisons, Johnston Atoll with three of 12 significant comparisons, Mo'orea (French Polynesia) with 12 of 12 significant comparisons, MHI with five of 12 significant comparisons and the NWHI with 12 of 12 significant comparisons (Table 2). Within the Hawaiian Archipelago, there were 13 of 28 significant comparisons among sample locations (see Table S2.1 in Appendix S2). All of the significant comparisons were among the three southernmost sampled locations (Hawai'i Island, O'ahu and French Frigate Shoals) and the most northern sample location (Kure Atoll).

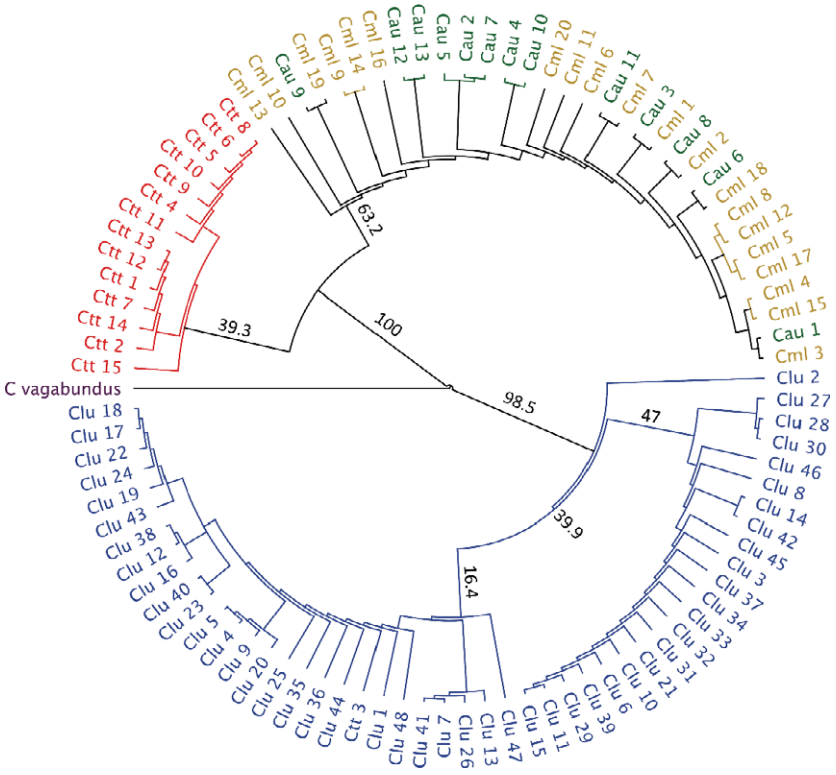

Figure 2 Neighbour-joining tree based on mtDNA cytochrome *b* sequences, highlighting the relationship between sister species in *Chaetodon* subgenus *Corallochaetodon* (bootstrap values shown based on 1000 replicates). For simplicity, only a representative subset of specimens is shown. Maximum-likelihood and maximum-parsimony trees yielded the same topology among species. *Chaetodon vagabundus* is used as an outgroup (Genbank accession number JF458006). *C. lunulatus* = Clu, *C. trifasciatus* = Ctt, *C. melapterus* = Cml and *C. austriacus* = Cau.

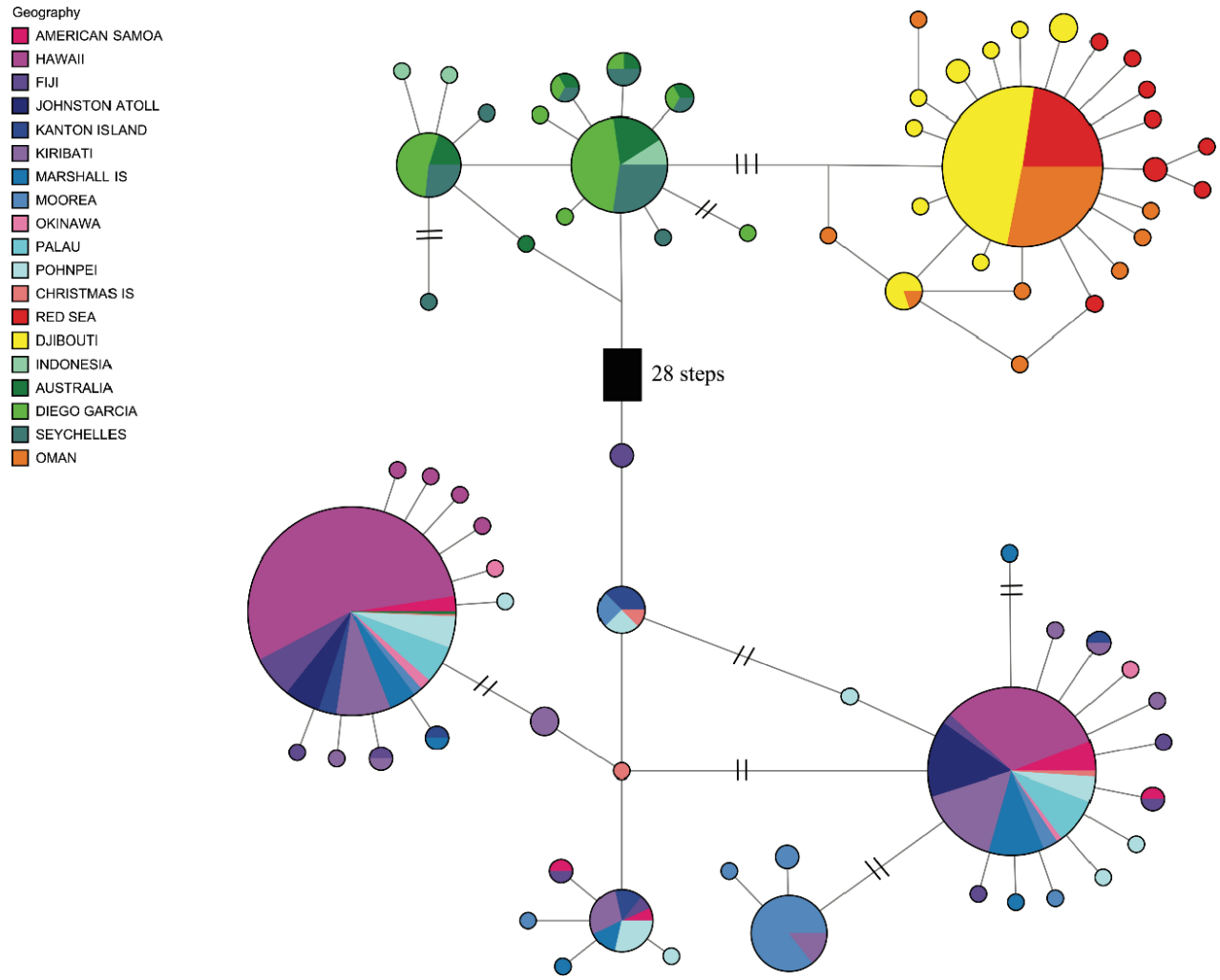

Figure 3 Statistical parsimony network for *Chaetodon lunulatus* (pink, purple, blue shades), *C. trifasciatus* (green shades), *C. melapterus* (yellow and orange) and *C. austriacus* (red) based on mtDNA cytochrome *b* sequences. The area of each circle is proportional to the abundance of the respective haplotype: small circles indicate rare or unique haplotypes and the largest circle indicate the most common haplotype observed in 286 sampled individuals. Black bars and black branches represent a single mutation (unless otherwise noted) and colours indicate haplotype sampling location (see the key).

No significant structure overall or significant pairwise comparisons were detected among four locations in *C. trifasciatus* (Φ_{ST} = 0.01; P = 0.50), four locations in *C. melapterus* (Φ_{ST} = 0.01; P = 0.16), or three locations in *C. austriacus* (Φ_{ST} = 0.04; P = 0.21) (Table 3). However, *C. melapterus* and *C. austriacus* were significantly isolated at a population level (Φ_{ST} = 0.06; P = 0.001). Notably, we did not sample *C. melapterus* in the Arabian Gulf and along the Somalian coastline due to logistical limitations; additional sampling in these regions could change conclusions about population structure.

Population structure (msatDNA) within *C. lunulatus* and *C. trifasciatus*

Significant population structure was also detected for *C. lunulatus* using msatDNA (F_{ST} = 0.05, P = 0.001). The msatDNA results were similar to of mtDNA with most of the significant pairwise comparisons involving locations on the eastern edge of the geographical range: Johnston Atoll, Mo'orea, MHI and the NWHI. Microsatellite allele frequencies were significantly different in 49 of 91 comparisons for *C. lunulatus* (Table 4; see also Table S2.1 in Appendix S2).

For *C. lunulatus*, STRUCTURE identified mean probabilities as being highest at K = 3 (Fig. 4), which was verified using STRUCTURE HARVESTER (see Fig. S2.1 in Appendix S2). One widespread population spanned locations from the western range edge (Christmas Island) eastward to Kiribati in the central Pacific Ocean. The second population was comprised predominately of individuals from isolated locations on the eastern range edge: Johnston Atoll, MHI and the NWHI. The third population was largely restricted to the NWHI.

Table 2 Matrix of population pairwise Φ_{ST} values (above diagonal) and associated *P*-values (below diagonal) based on 605 bp of mtDNA cytochrome *b* sequence data from *Chaetodon lunulatus*. Significant *P*-values are indicated in bold ($P < 0.05$). All negative Φ_{ST} values were adjusted to 0.

Location	Christmas Island	American Samoa	Fiji	Kanton Island	Marshall Island	Mo'orea	Okinawa	Pohnpei	Kiribati	Palau	Johnston Atoll	MHI	NWHI
Christmas Island	–	0	0.097	0.012	0	**0.284**	0.107	0	0	0.084	0.006	0.003	**0.597**
American Samoa	0.568	–	**0.105**	0.095	0	**0.286**	**0.074**	0	0	0.040	0	0	**0.507**
Fiji	0.108	**0.036**	–	0	**0.086**	**0.478**	0	0.024	0.022	0.000	**0.083**	**0.162**	**0.114**
Kanton Island	0.333	0.081	0.477	–	0.079	**0.470**	0	0	0.031	0.040	0.105	**0.178**	**0.245**
Marshall Islands	0.414	0.973	**0.036**	0.099	–	**0.307**	0.050	0	0	0.023	0	0	**0.431**
Mo'orea	**0.036**	**<0.001**	**0.000**	**0.000**	**0.000**	–	**0.463**	**0.370**	**0.371**	**0.431**	**0.342**	**0.298**	**0.757**
Okinawa	0.234	**0.036**	0.847	0.387	0.189	**<0.001**	–	0.008	0	0	0.037	0.125	**0.099**
Pohnpei	0.658	0.387	0.144	0.423	0.369	**<0.001**	0.252	–	0	0.010	0.016	**0.055**	**0.332**
Kiribati	0.324	0.514	0.126	0.216	0.640	**<0.001**	0.306	0.667	–	0	0	0.017	**0.335**
Palau	0.252	0.198	0.324	0.126	0.234	**<0.001**	0.396	0.207	0.559	–	0.003	0.068	**0.228**
Johnston Atoll	0.324	0.450	**0.018**	0.063	0.577	**<0.001**	0.108	0.189	0.631	0.432	–	0	**0.405**
MHI	0.279	0.550	**0.009**	**0.018**	0.423	**<0.001**	0.099	**0.045**	0.342	0.108	0.622	–	**0.509**
NWHI	**0.009**	**<0.001**	**0.009**	**<0.001**	**<0.001**	**<0.001**	**0.018**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	–

The msatDNA data revealed low but significant population structure for *C. trifasciatus* ($F_{ST} = 0.003$, $P = 0.03$). Microsatellite allele frequencies were significantly different in three of six comparisons (Table 5), between Diego Garcia and all the other sampled locations (Seychelles, Christmas Island and Indonesia). Microsatellite statistics for each location and both species are provided in Table S2.2 in Appendix S2. STRUCTURE identified mean probabilities as being highest at $K = 2$ (Fig. 5), which was consistent with the results from STRUCTURE HARVESTER (see Fig. S2.2 in Appendix S2), indicating isolation of Diego Garcia but no distinction of samples from the east (Christmas Island, Indonesia) and west (Seychelles) of this remote location in the Chagos Archipelago. Overall, there was no consistent evidence for departure from HWE, linkage disequilibrium or null alleles across all sampled locations in both species.

DISCUSSION

Phylogenetic relationships

The primary phylogenetic feature of the subgenus *Corallochaetodon* is mtDNA sequence divergence of $d = 0.06$ between Indian Ocean *C. trifasciatus* and Pacific *C. lunulatus*. Based on the conventional molecular clock of 2% per Myr, this corresponds to approximately 3 Myr of separation (see Table S2.3 in Appendix S2) (consistent with Hsu *et al.*, 2007; Bellwood *et al.*, 2010), which is close to the onset of modern glacial cycles at 2.6 to 2.8 Ma (Dwyer *et al.*, 1995; Williams *et al.*, 1997). The shallow Sunda Shelf is exposed during glacial periods with low sea levels, forming land bridges through the Indonesian Archipelago that restricted exchange between the Indian and Pacific Oceans (Randall, 1998; Rocha *et al.*, 2007). This indicates that transient allopatry may have a role in the formation of this species pair, a process that is apparent (or suspected) in other Indian-Pacific species pairs (Gaither & Rocha, 2013).

A divergence time of approximately 3 Myr for *C. trifasciatus* and *C. lunulatus* falls within the range of divergence times (0.3–6.6 Myr) for other Indian and Pacific sister species of reef fishes (Gaither & Rocha, 2013). However, divergence times in other Indian and Pacific Ocean butterflyfish sister species tend to be less (0.3–1.4 Myr) (Fessler & Westneat, 2007; Hsu *et al.*, 2007; Bellwood *et al.*, 2010; DiBattista *et al.*, 2012). Variation in divergence times may be due to a number of factors including: (1) potential differences in mutation rates, (2) the intermittency of the Sunda Shelf Barrier during the Pleistocene due to repeated glacial cycles (i.e. different species pairs diverged at different low sea level stands), and (3) the conditions determining secondary contact and reproductive isolation may have affected species differently.

The range-restricted *C. austriacus* and *C. melapterus* share a common haplotype, and are closely affiliated with *C. trifasciatus* ($d = 0.015$). The divergence between *C. trifasciatus* and the range-restricted species is approximately 0.75 Myr (see Table S2.3 in Appendix S2), which corresponds with Pleis-

Table 3 Matrix of population pairwise Φ_{ST} values (above diagonal) and associated *P*-values (below diagonal) based on 605 bp of mtDNA cytochrome *b* sequence data from *Chaetodon trifasciatus*, *C. melapterus* and *C. austriacus*. All negative Φ_{ST} values were adjusted to 0.

Location	Diego Garcia	Seychelles	Christmas Island	Indonesia
C. trifasciatus				
Diego Garcia	–	0.014	0.027	0
Seychelles	0.268	–	0	0
Christmas Island	0.238	0.961	–	0
Indonesia	0.483	0.769	0.678	–
C. melapterus	Maskali	Obock	Bay of Ghoubbet	Oman
Maskali	–	0.030	0	0.001
Obock	0.108	–	0.022	0.007
Bay of Ghoubbet	0.991	0.270	–	0
Oman	0.459	0.288	0.667	–
C. austriacus	Al Lith	Jazirat Baraqan	Yanbu	
Al Lith	–	0.095	0.028	
Jazirat Baraqan	0.207	–	0	
Yanbu	0.491	0.573	–	

tocene sea level changes that repeatedly isolated the Red Sea region from the Indian Ocean (Fig. 1; Blum, 1989; DiBattista *et al.*, 2013). Furthermore, strong upwelling in the NW Indian Ocean (off the southern Oman coast) may facilitate allopatric divergence between species from the Indian Ocean (e.g. *C. trifasciatus*) and Red Sea to Arabian Gulf region (*C. austriacus* and *C. melapterus*).

While the monophyly of *C. austriacus* and *C. melapterus* could not be corroborated, these two putative species are genetically distinct at a population level ($\Phi_{ST} = 0.06$; $P = 0.001$) indicating either early stages of speciation or distinct colour morphs separated by habitat discontinuities. This finding should be interpreted in light of the relatively recent origins of reef faunas inhabiting the Red Sea (DiBattista *et al.*, 2013; DiBattista *et al.*, 2015c) and Arabian Gulf (Sheppard *et al.*, 2010). Estimated time since divergence is approximately 50 kyr, and was likely initiated by vicariant isolation at the Strait of Bab al Mandab (at the mouth of the Red Sea – Fig. 1). This barrier flooded about 20 ka, and *C. austriacus* and *C. melapterus* now have limited contact in the southern Red Sea (Randall, 1994), a region characterized by changes in environmental conditions (e.g. salinity, temperature, nutrients: Kemp, 1998; Sheppard, 1998) that are reflected in the fish community (Roberts *et al.*, 1992; DiBattista *et al.*, 2015a). Given that *C. austriacus* and *C. melapterus* inhabit different environmental conditions on either side of this area, successful colonisation across this potential barrier may be limited, thereby facilitating divergence. When the two species come into contact, differences in colouration and assortative mating may maintain reproductive isolation (McMillan *et al.*, 1999).

The distribution of all four sister species overlap at their range edges, at (or adjacent to) biogeographical barriers (Fig. 1). In the eastern Indian Ocean, cohabitation and a breakdown in assortative mating between *C. lunulatus* and *C. trifasciatus* at Christmas Island has led to hybridisation (Hobbs *et al.*, 2009; Montanari *et al.*, 2014); however, there has only been limited and localized introgression between the species. In the western Indian Ocean, *C. trifasciatus* and *C. melapterus* hybridise at Socotra, with some evidence of introgression beyond this hybrid zone in Djibouti (DiBattista *et al.*, 2015b). In the southern Red Sea, *C. austriacus* and *C. melapterus* cohabit and potentially hybridise (Randall, 1994; Kuiter, 2002), but the former is considered rare in this understudied region (Righton *et al.*, 1996). This pattern of decreasing hybridisation and introgression with increasing divergence time is consistent with other butterflyfish studies (Montanari *et al.*, 2014). Overall, it appears that Plio-Pleistocene sea level changes have facilitated allopatric speciation in both the butterflyfish centres of diversity (Indonesia) and peripheral areas (Red Sea). Secondary contact and hybridisation could erode species boundaries (Coleman *et al.*, 2014); however, abrupt differences in environmental conditions across areas of secondary contact could facilitate evolutionary divergence.

Genetic diversity

Although the geographical ranges of the four species in the subgenus *Corallochaetodon* vary by an order of magnitude, there was no obvious relationship between haplotype diversity and range size. Terrestrial studies commonly find low haplotype diversity in range-restricted endemics (Frankham, 1998). However, endemic reef fishes can have population sizes numbering in the millions (Hobbs *et al.*, 2011) and this may explain why they have haplotype diversities similar to widespread species (Eble *et al.*, 2009; Hobbs *et al.*, 2013; Delrieu-Trottin *et al.*, 2014). Excluding the Arabian Gulf, where atypical conditions have resulted in an unusually low abundance and diversity of butterflyfishes (Pratchett *et al.*, 2013), *C. austriacus* and *C. melapterus* are the most common butterflyfish species in their respective ranges (M. L. Berumen &

Table 4 Matrix of population pairwise F_{ST} values (above diagonal) and associated *P*-values (below diagonal) based on microsatellite genotypes for *Chaetodon lunulatus*. Significant *P*-values are highlighted in bold ($P < 0.05$). All negative F_{ST} values were adjusted to 0.

Location	Christmas Island	Indonesia	American Samoa	Fiji	Kanton Island	Marshall Islands	Mo'orea	Okinawa	Pohnpei	Kiribati	Palau	Johnston Atoll	MHI	NWHI
Christmas Island	–	0	0.003	0.001	0.012	0.006	**0.041**	0.010	0.006	0	0.011	**0.084**	**0.032**	**0.090**
Indonesia	0.498	–	0.007	0.002	0.001	0	**0.030**	0.002	0.0	0	0	**0.079**	**0.024**	**0.078**
American Samoa	0.378	0.067	–	**0.009**	0.002	0.006	**0.027**	0.012	**0.010**	0	0.007	**0.082**	**0.037**	**0.075**
Fiji	0.396	0.267	**0.036**	–	0.002	0.002	**0.030**	0.007	0.005	0.000	**0.007**	**0.088**	**0.030**	**0.089**
Kanton Island	0.124	0.411	0.322	0.260	–	0	**0.023**	0.003	0.001	0	0.004	**0.087**	**0.035**	**0.076**
Marshall Islands	0.217	0.706	0.067	0.150	0.772	–	**0.029**	0.005	0.000	0.000	0.002	**0.084**	**0.030**	**0.079**
Mo'orea	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	–	0.056	0.032	0.024	0.029	**0.087**	**0.058**	**0.095**
Okinawa	0.203	0.300	0.089	0.093	0.331	0.116	**<0.001**	–	0.005	0.007	0.005	**0.096**	**0.034**	**0.082**
Pohnpei	0.232	0.676	**0.022**	0.071	0.361	0.531	**<0.001**	0.151	–	0	0.000	**0.085**	**0.029**	**0.081**
Kiribati	0.497	0.744	0.602	0.443	0.779	0.394	**<0.001**	0.109	0.773	–	0	**0.076**	**0.023**	**0.067**
Palau	0.128	0.779	0.072	**0.017**	0.154	0.203	**<0.001**	0.140	0.441	0.554	–	**0.080**	**0.023**	**0.078**
Johnston	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	–	**0.051**	**0.038**
MHI	**0.005**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	–	**0.053**
NWHI	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	–

MHI, Main Hawaiian Islands.

J-.P.A Hobbs, unpublished data). Therefore, the large population sizes of the range-restricted *C. austriacus* and *C. melapterus* would help generate and maintain high haplotype diversity. Nearly all the populations of the two restricted-range species had significant negative Fu's *Fs* values. Therefore, it appears that *C. austriacus* and *C. melapterus* have undergone recent population expansion.

Population structure – mtDNA

Data from the wide-ranging *C. lunulatus* indicate strong population structure, whereas the sister species *C. trifasciatus* showed significant genetic structure only at Diego Garcia (Chagos Archipelago). Data from the two range-restricted species, *C. austriacus* and *C. melapterus*, detected no population structure based on our approach, which may indicate that each represents a single panmictic population. This can be explained by their limited distributions in the NW Indian Ocean, with no apparent biogeographical barriers within each range.

Corallochaetodon mtDNA sequence data revealed that range size was not related to genetic population structure, which is a proxy for realized dispersal ability (Eble *et al.*, 2009). The widespread *C. lunulatus* showed significant population structure at eastern peripheral locations, consistent with known distributional barriers (Blum, 1989; Hsu *et al.*, 2007). The distinction of the Mo'orea population of *C. lunulatus* (Lawton *et al.*, 2011; this study) is concordant with other Pacific Ocean species and may be caused by isolating oceanographic currents (Gaither *et al.*, 2010; Eble *et al.*, 2011). The isolation of Johnston Atoll indicates that the pelagic larval duration (*c.* 35 days: Soeparno *et al.*, 2012) of *C. lunulatus* is insufficient to make the 40–50 day transit to the nearest reef (Hawaiian Archipelago) (Kobayashi, 2006).

Population differentiation between Hawai'i and other Pacific locations has been reported in many other reef fishes (Leray *et al.*, 2010; DiBattista *et al.*, 2011; Gaither *et al.*, 2011; Szabo *et al.*, 2014; Fernandez-Silva *et al.*, 2015). The recurrent trend of genetic distinctness in this region can be attributed to three factors: (1) isolation due to location and oceanographic currents, (2) dispersal characteristics of the fishes and (3) adaptation to environmental conditions in Hawai'i (Hourigan & Reese, 1987). Widespread reef fishes usually exhibit genetic homogeneity within the Hawaiian archipelago (Craig *et al.*, 2007; Eble *et al.*, 2009; Gaither *et al.*, 2010, 2011; DiBattista *et al.*, 2011, 2012; Reece *et al.*, 2011; Ludt *et al.*, 2012); however, the genetic differentiation of *C. lunulatus* across the archipelago (between the low islands of the NWHI and the high volcanic islands of the MHI) is more typical of endemic reef fishes and invertebrates (Eble *et al.*, 2009; Craig *et al.*, 2010; Toonen *et al.*, 2011).

Population structure – msatDNA

Investigation of fine-scale population structure in the two widespread species using msatDNA revealed patterns similar

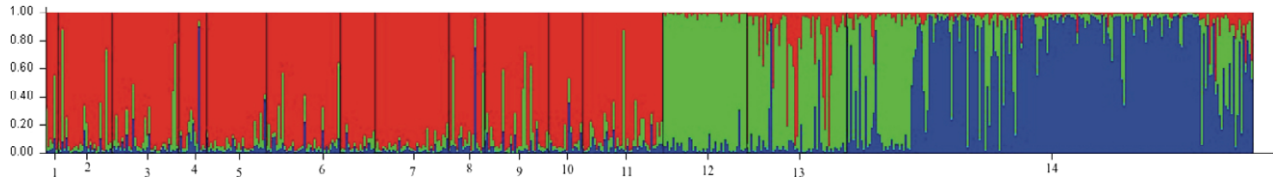

Figure 4 STRUCTURE bar plot for *Chaetodon lunulatus* showing the highest mean probability of $K = 3$. Locations: 1. Christmas Island, 2. Indonesia, 3. Palau, 4. Okinawa, 5. Pohnpei, 6. Marshall Islands, 7. Fiji, 8. American Samoa, 9. Mo'orea, 10. Kanton Island, 11. Kiribati, 12. Johnston Atoll, 13. Main Hawaiian Islands, 14. Northwestern Hawaiian Islands.

to the mtDNA with *C. trifasciatus* exhibiting low structure, whereas *C. lunulatus* had more pronounced structure. For *C. trifasciatus*, the msatDNA differed from mtDNA results in one point –the former support the genetic isolation of Diego Garcia (Chagos Archipelago) in the central Indian Ocean. The population genetic separation of Chagos has been observed in other reef fauna (Gaither *et al.*, 2010; Eble *et al.*, 2011; Vogler *et al.*, 2012) and may be related to seasonal monsoon-driven currents that switch direction between easterly and westerly, possibly limiting larval dispersal to this location (Sheppard *et al.*, 2012).

MsatDNA analyses for *C. lunulatus* were consistent with the mtDNA results in indicating divergent populations at peripheral locations on the eastern range edge: Mo'orea, Johnston Atoll, MHI and NWHI. The majority of the geographical range of *C. lunulatus* is comprised of relatively close islands and reefs throughout the Central-West Pacific; however, the large distance and prevailing currents work against colonisation of Hawai'i and French Polynesia, thus explaining the genetic distinctness of populations at these peripheral locations (Hourigan & Reese, 1987; Gaither *et al.*, 2010). This isolation is the starting point for peripheral speciation, explaining why Hawai'i has one of the highest levels of reef fish endemism in the world (Randall, 2007).

An interesting outcome for *C. lunulatus* is the population separation between the high islands of the MHI and the low islands and atolls of the NWHI; *C. lunulatus* is the first widespread reef fish to show strong population structure across the Hawaiian Archipelago. Part of the explanation may be habitat preference: this species uses sheltered, coral-rich areas and the lack of this habitat between MHI and NWHI may explain the genetic break. Indeed, at the MHI region adjacent to this break (Kaua'i), previous transect data (unpub. data) and our own efforts indicate a near absence of *C. lunulatus*. Another part of the explanation may include Johnston Atoll to the south. Johnston has long been postulated to be a gateway into Hawai'i (Hourigan & Reese, 1987), and STRUCTURE analysis shows an affiliation between Johnston and the MHI, to the exclusion of the NWHI (Fig. 4). This invokes the possibility that Hawai'i was colonized twice, possibly from different sources.

Table 5 Matrix of population pairwise F_{ST} values (above diagonal) and associated *P*-values (below diagonal) based on microsatellite genotypes for *Chaetodon trifasciatus*. Significant *P*-values are highlighted in bold ($P < 0.05$). All negative F_{ST} values were adjusted to 0.

Location	Diego Garcia	Seychelles	Christmas Island	Indonesia
Diego Garcia	–	0.005	0.006	0.012
Seychelles	**0.047**	–	0	0
Christmas Island	**0.013**	0.742	–	0.001
Indonesia	**0.018**	0.496	0.350	–

CONCLUSION

We conclude that Plio-Pleistocene sea level changes have influenced speciation at both the center of diversity and peripheral areas for butterflyfishes of the subgenus *Coral-*

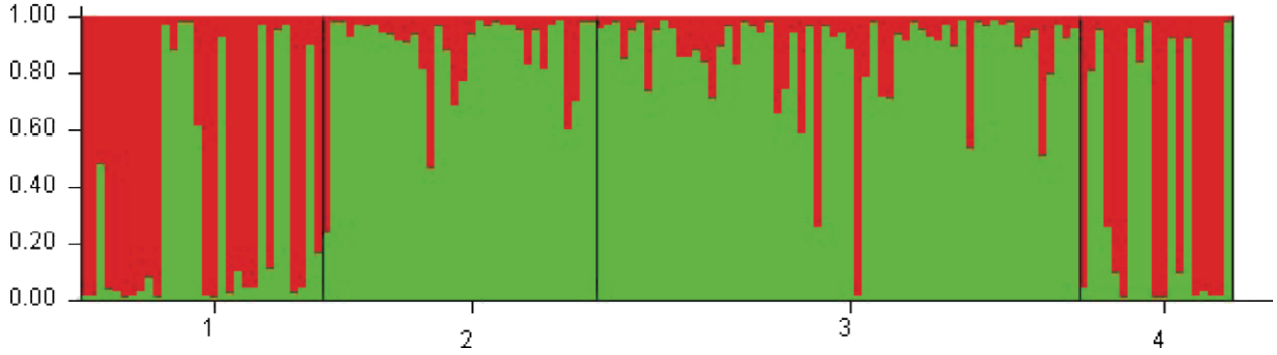

Figure 5 STRUCTURE bar plot for *Chaetodon trifasciatus,* showing the highest mean probability of $K = 2$. Locations: 1. Diego Garcia, 2. Seychelles, 3. Christmas Island, 4. Indonesia.

lochaetodon. Evolutionary divergence among *Corallochaetodon* species may have been initiated along the intermittent biogeographical barriers between Indian and Pacific Oceans, and between the Indian Ocean and Red Sea. Phylogenetic analyses revealed that the two species restricted to the Red Sea to Arabian Sea region are indistinguishable at cyt *b*. Genetic diversity decreases from west to east for the widespread *C. lunulatus*, but there are no patterns for the other three species. The two range-restricted species appear to have undergone recent population expansion and exhibit no population structure, while the widespread Indian Ocean species (*C. trifasciatus*) showed little population structure, which is likely attributed to variable local conditions (e.g. seasonal monsoon currents). Peripheral populations on the eastern range edge of the widespread Pacific species *C. lunulatus* were genetically distinct from populations in the center of the range. The recent evolution of *C. melapterus* and *C. austriacus* in the Red Sea to Arabian Sea region, and genetic distinctness of peripheral populations of the widespread *C. lunulatus,* indicate that such peripheral marine habitats can be engines of biodiversity (Bowen *et al.*, 2013). Thus, peripheral speciation (through isolation and vicariant events) would help explain why the Red Sea and Hawai'i, at opposite extremes of the Indo-Pacific ranges, are endemic hotspots for reef fishes.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Appendix S1 Additional materials & methods.
Appendix S2 Supporting tables & figures.

BIOSKETCH

Ellen Waldrop conducted this research as a M.Sc. thesis project at the University of Hawai'i. The authors are interested in the origins of marine biodiversity and the prudent management of evolutionary lineages.

Author contributions: B.W.B. initiated the research; E.W., J.-P.A.H., J.D.D., L.A.R., R.K.K., M.L.B. and B.W.B. conducted field expeditions and sampling; E.W. and J.D.D. provided genetic data; E.W. analysed the data; E.W., B.W.B., J.P.H. and J.D.D. contributed to the writing; and all authors commented on the final draft.

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