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REPORT

# Hybridisation among groupers (genus *Cephalopholis*) at the eastern Indian Ocean suture zone: taxonomic and evolutionary implications

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**Abstract** Hybridisation is a significant evolutionary process that until recently was considered rare in the marine environment. A suture zone in the eastern Indian Ocean is home to numerous hybridising sister species, providing an ideal opportunity to determine how hybridisation affects speciation and biodiversity in coral reef fishes. At this location, hybridisation between two grouper (Epinephelidae) species: *Cephalopholis urodeta* (Pacific Ocean) and *C. nigripinnis* (Indian Ocean) was investigated to determine the genetic basis of hybridisation and to compare the ecology and life history of hybrids and their parent species. This approach aimed to provide insights into the taxonomic and evolutionary consequences of hybridisation. Despite clear phenotypic differences, multiple molecular markers revealed hybrids, and their parent species were genetically homogenous within and (thousands of kilometres) outside of the hybrid zone. Hybrids were at least as fit as their parent species (in terms of growth, reproduction, and abundance) and were observed in a broad range of intermediate phenotypes. The two species appear to be interbreeding at Christmas Island due to inherent biological and ecological compatibilities, and the lack of genetic structure may be explained by three potential scenarios: (1) hybridisation and introgression; (2) discordance between morphology and genetics; and (3) incomplete lineage sorting. Further molecular analyses are necessary to discriminate these scenarios. Regardless of which applies, *C. urodeta* and *C. nigripinnis* are unlikely to evolve in reproductive isolation as they cohabit where they are common (Christmas Island) and will source congeneric mates where they are rare (Cocos Keeling Islands). Our results add to the growing body of evidence that hybridisation among coral reef fishes is a dynamic evolutionary factor.

**Keywords** Christmas Island · Cocos (Keeling) Islands · Epinephelidae · Hybrid fitness · Introgression · Coral reef fish

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## Introduction

The biological species concept is the most commonly used definition of a species—it states that a species is distinguishable through one or more heritable traits and does not interbreed or produce viable offspring with another species (Mayr 1982). Hybridisation challenges this concept and occurs when two different species or populations interbreed and produce viable hybrid offspring. In the natural environment, hybridisation is common (at least 25% of plant and 10% of animal species hybridise) and it is considered

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an important evolutionary factor in shaping biodiversity (Arnold 1997; Mallet 2005). Hybridisation is often prevalent at biogeographic borders (termed suture zones) where multiple sister species come into secondary contact (Barton and Hewitt 1985). While hybridisation is common in terrestrial and freshwater environments (Mallet 2005; Scribner et al. 2000), it has historically been considered rare and unimportant in the marine environment (Hubbs 1955; Arnold 1997). In coral reef ecosystems, hybridisation has most commonly been studied in scleractinian corals (Wolstenholme et al. 2003; Willis et al. 2006; Richards and Hobbs 2015), with application of modern genetic methods revealing further hybridising corals (Carlon and Lippe 2011; Richards and van Oppen 2012). Emerging evidence suggests that hybridisation may also play an under appreciated role in the evolution of coral reef fishes (Hobbs and Allen 2014; DiBattista et al. 2015; Montanari et al. 2016). The prevalence of hybrid coral reef fishes provides the opportunity to determine the causes and consequences of hybridisation in the world's most diverse vertebrate assemblages.

Several theories have been proposed to explain the causes of natural hybridisation in marine fishes. For example, closely related species (Hobbs et al. 2013; DiBattista et al. 2015), which frequently share a number of ecological, morphological, and inherent reproductive compatibilities (Montanari et al. 2014), often hybridise. Species that come into secondary contact after the removal of biogeographic barriers (e.g., due to sea level changes) are also likely to hybridise (Hobbs et al. 2009). Rare species may choose to mate with closely related species when the chances of finding conspecific mates are low (Marie et al. 2007; Hobbs et al. 2009), while ecologically similar species that use the same habitat may interbreed due to an increased chance of heterospecific encounters (van Herwerden and Doherty 2006; Yaakub et al. 2006; Montanari et al. 2014). Fish can also hybridise without the intention of both parental species, through "sneak mating" (Frisch and van Herwerden 2006; van Herwerden et al. 2006) or the accidental mixing of gametes when multiple species spawn in the same location at the same time (Hubbs 1955; Campton 1987).

The outcomes of hybridisation can be determined through comparing the fitness of hybrids to their respective parent species, where individual fitness is defined as an organism's ability to survive and reproduce (Mills and Beatty 1979). Some reef fish hybrids are less fit than their parent species, displaying infertility (Yaakub et al. 2007), lower abundance, and lower genetic diversity (Montanari et al. 2012). Other hybrids, however, can be fertile and considered equally as fit as one of their parent species (Arnold and Hodges 1995; Marie et al. 2007). Hybrid vigour can also occur, where hybrids display greater fitness than parent species, as seen in some terrestrial hybrids (Arnold and Martin 2010).

Molecular analyses are useful for studying hybridisation and introgression (exchange of genetic material between species), and have demonstrated that the evolutionary consequences of hybridisation can vary between cases. For example, hybrids may be fertile and backcross with their parent species (van Herwerden et al. 2006; Marie et al. 2007), which can result in bidirectional introgression (Yaakub et al. 2006) and, if extensive, merging of genomes and reverse speciation (Taylor et al. 2006). Similarly, species radiation can occur, a process by which hybrid lineages become new species by assortative mating and exploiting new or unoccupied niches not used by their parent species (e.g., cichlids: Seehausen 2004) thereby increasing the overall species richness. Coral reef systems contain the most diverse vertebrate communities in the world, and the prevalence of hybridisation in this ecosystem raises the question as to what role hybridisation has played in the origins and maintenance of modern coral reef biodiversity (Richards and Hobbs 2015).

Groupers are a functionally and economically important family of coral reef fishes, yet little is known about hybridisation within this group. Only two natural epinephelid hybrids have been confirmed using genetics (Bostrom et al. 2002; Frisch and van Herwerden 2006; van Herwerden et al. 2006), and a third has been identified based on morphology (Randall and Justine 2008). *Cephalopholis urodeta* (a Pacific Ocean species) and *C. nigripinnis* (an Indian Ocean species) are thought to hybridise based on reports of fishes with intermediate colouration at Christmas Island and the Cocos (Keeling) Islands in the eastern Indian Ocean (Hobbs and Allen 2014). This region represents a narrow area of overlap in the ranges of these largely allopatric sister species (Fig. 1). The region is the largest of five known marine suture zones (Hobbs et al. 2013; DiBattista et al. 2015), where many Indian and Pacific Ocean sister species come into secondary contact and hybridise. *Cephalopholis urodeta* and *C. nigripinnis* are clearly recognisable based on their unique colouration (Heemstra and Randall 1993; Fig. 1), and phylogenetic studies using a range of mitochondrial and nuclear makers reveal that they are genetically distinct species (Craig and Hastings 2006).

While hybridisation is prevalent among many fish species in the eastern Indian Ocean suture zone, little is known of the genetic, ecological, and biological basis of this phenomenon. Therefore, we examined (1) whether *C. urodeta* and *C. nigripinnis* hybridise (and introgress) and how this affects their taxonomic status, (2) whether *C. urodeta*, *C. nigripinnis*, and their hybrids are ecologically different (in terms of abundance and habitat use), and (3) whether *C. urodeta*, *C. nigripinnis*, and their hybrids

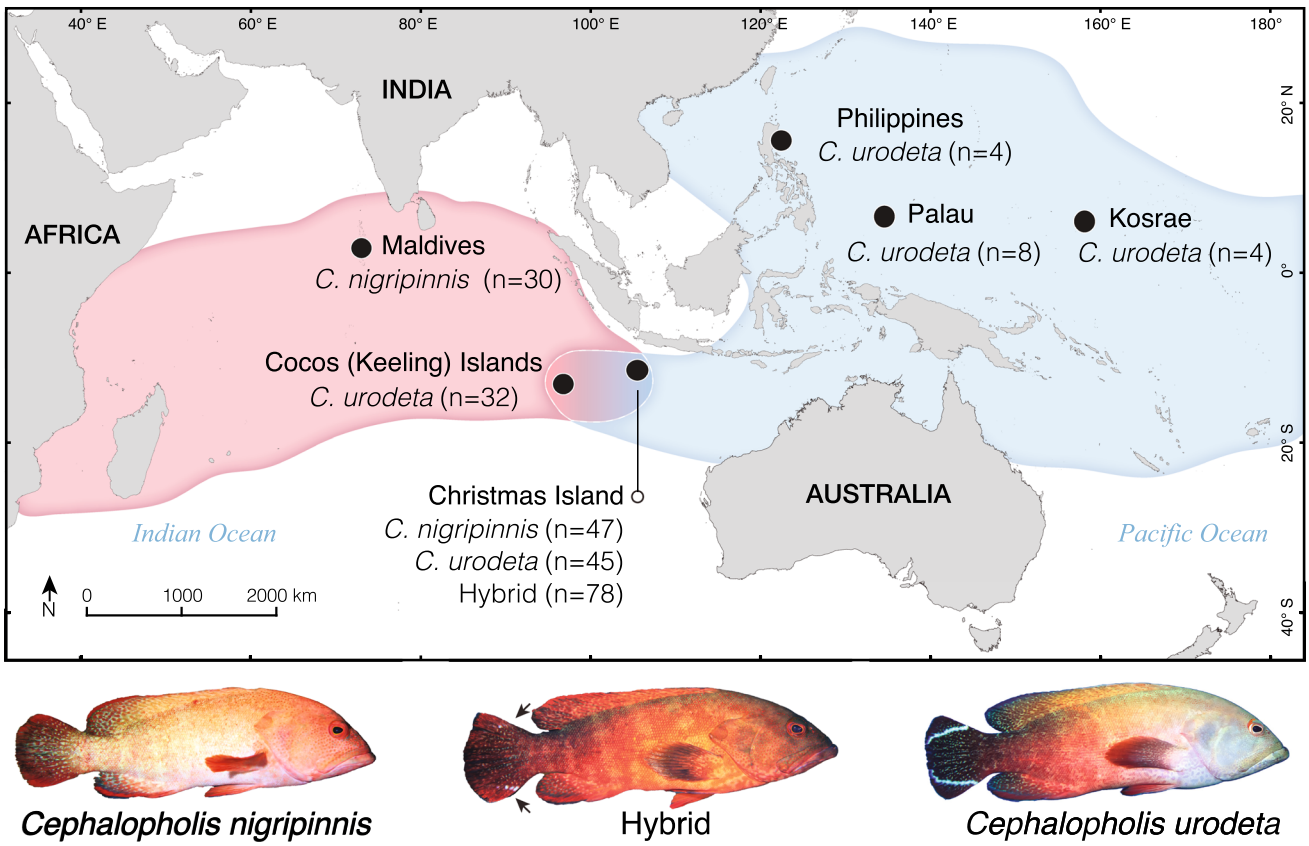



**Fig. 1** Distribution and sampling locations of *Cephalopholis nigripinnis* (*red*) and *C. urodeta* (*blue*) redrawn from Heemstra and Randall (1993). *Photo inset* shows *C. nigripinnis* (*left*), *C. urodeta* (*right*), and their putative hybrid (*centre*). *Arrows* indicate caudal fin markings used to identify putative hybrids

display differences in aspects of fitness (growth and reproduction).

## Methods and materials

### Experimental design

*Study location and sampling*

Underwater visual surveys and specimen collection were conducted at the Cocos (Keeling) Islands (herein referred to as the Cocos Islands) and Christmas Island during April–May 2014. The islands are located in the eastern Indian Ocean approximately 1500 and 2100 km north-west of the Australian coastline, respectively (Fig. 1). *Cephalopholis nigripinnis* and putative hybrids could not be collected from the Cocos Islands as they were rare at this location. Specimens from distant populations in the Indian Ocean (*C. nigripinnis*) and Pacific Ocean (*C. urodeta*) were also collected to make genetic comparisons to individuals within the hybrid zone (Fig. 1).

*Hybridisation and taxonomy*

*Colouration* Following existing taxonomic classification (Heemstra and Randall 1993; Craig and Hastings 2006), individuals with white bars on their caudal fin that converged posteriorly were classified as *C. urodeta*, fish without white bars were classified as *C. nigripinnis*, and fish that displayed intermediate phenotypes (e.g., white spots or dashes) were considered putative hybrids (Fig. 1). Subsequent analyses (visual surveys, genetics, reproduction, population size structure, and growth) were based on these classifications. For the remainder of this paper, putative hybrids will be referred to as "hybrids", while *C. urodeta*, *C. nigripinnis*, and hybrids are collectively referred to as "the three groups".

*Genetics* Genetic analyses were used to determine (1) whether *C. urodeta* and *C. nigripinnis* are hybridising, (2) the degree and direction of gene flow (introgression) between species, and (3) the taxonomic status of *C. urodeta* and *C. nigripinnis*. To address these points, the genetic relationships between the three groups within the hybrid

zone were compared to populations of the parent species outside of the hybrid zone (Fig. 1).

The cytochrome-c oxidase subunit-I (COI) mitochondrial DNA (mtDNA) region was sequenced. To test for congruence, a subset of individuals were also sequenced at intron 1 of the S7 ribosomal protein DNA region (see Electronic Supplementary Materials, ESM, Table S1 for full list of forward and reverse primers). Tissue samples ($n = 207$) were stored in 70% ethanol solution, and DNA was extracted following the "HotShot" method (Meeker et al. 2007). Polymerase chain reaction (PCR) and sequencing were conducted following standard procedures; detailed protocols can be found in ESM.

### *Ecology*

*Abundance* *Cephalopholis urodeta*, *C. nigripinnis*, and their hybrids co-occur at the Cocos Islands and Christmas Island; determining whether *C. urodeta* and/or *C. nigripinnis* are rare may explain why they are hybridising, as rare species are more likely to hybridise because they are unable to find conspecific partners (Marie et al. 2007). Furthermore, if one of the three groups is more abundant, then it is assumed to be more successful than the other groups in that environment.

The abundance (number of individuals per 250 $m^2$) of each group was estimated through underwater visual census (UVC) conducted on SCUBA. Four sites were surveyed on the outer reef at the Cocos Islands, and five sites were surveyed on the outer reef at Christmas Island (ESM Fig. S1). For each site, a total of six replicate 50 × 5 m transects were conducted at two depths (5 and 20 m). The number and total length (TL, to the nearest 10 mm) of individuals in each of the three groups were recorded.

*Habitat use* Depth distribution and microhabitat use were measured to determine (1) whether the parent species coexist in similar habitats and are therefore more likely to hybridise and (2) whether hybrids use a different habitat to the parental species. To compare microhabitat use between the three groups, point intercept transects were undertaken. The frequency of eleven microhabitat categories (ESM Table S2) was recorded at 1-m intervals along each 50-m UVC transect. Live corals were classified according to morphology, as the spatial complexity of the habitat type is more important to predatory reef fish than the species identity of the coral (Almany 2004).

### *Biology*

*Growth* Growth characteristics of *C. urodeta*, *C. nigripinnis*, and their hybrids were compared to determine whether hybrids have different growth rates (which may be indicative of fitness differences), as seen in some aquaculture crosses (James et al. 1999). Individual fish were collected across the full size range for each of the three groups. Estimates of the age of individual fish were obtained by counting annual growth rings on thin transverse sections of the sagittal otoliths (following Chan and Sadovy 2002; ESM Fig. S2). Otoliths were processed following Taylor and McIlwain (2010) with two separate, blind reads conducted on each otolith to quantify the precision of counts. Seventeen otoliths with inconsistent readability (defined as a difference >2 yr between reads) were excluded from analyses.

*Reproduction* *Cephalopholis* species often form haremic social groups controlled by a large dominant male (Shpigel and Fishelson 1991; Liu and Sadovy 2005). These large males have greater mating opportunities, and their proportion in the population of parent species and hybrids was investigated as an indicator of reproductive success. Specimens were caught and sexed through histological examination of gonads. The size range of each sex was then compared to population length frequency distributions from UVC surveys, to infer the proportion of males in each group. Gonads were fixed in a 4% formalin solution and preserved in 70% ethanol before being stained, sectioned, and sexed following Nakai and Sano (2002).

## Data analyses

### *Hybridisation and taxonomy*

DNA sequences were aligned, edited, and trimmed in Geneious Pro version 4.8. Nuclear S7 sequences were phased using the Bayesian program PHASE version 2.1 (Stephens and Donelly 2003), contained within the program DnaSP version 5.0 (Librado and Rozas 2009). A single run in PHASE (100,000 iterations) with a burn-in of 10,000 iterations was able to resolve the majority of alleles with >80% certainty; those resolved with <80% certainty were excluded from further analyses. All sequences were deposited on GenBank (accession numbers: KU064284-KU064687).

COI haplotypes and phased S7 alleles were collapsed into unique sequences for each locus using the online tool Fabox (www.users-birc.au.dk). Alleles were then imported into jModelTest version 1.0.1 (Posada 2008), which was used in combination with an Akaike Information Criterion (AIC) to determine the best nucleotide substitution model for the data prior to analyses in ARLEQUIN version 3.0 (Excoffier et al. 2005). For COI, the HKY+G model was selected with a gamma parameter of 0.1. For S7, the TPM3uf+I+G model was selected with a gamma parameter of 0.01.

To determine the phylogenetic relationship between sequences, two separate median-joining networks were created for COI and S7. Networks were developed using NETWORK version 4.5.1.0 (www.fluxus-engineering.com/network_terms.htm) with median-joining algorithm and default settings according to Bandelt et al. (1999).

To test for overall differences in genetic diversity between populations, haplotype ($h$) and nucleotide diversity ($\pi$), indices were calculated in ARLEQUIN and used in subsequent AMOVA analyses to generate global $\Phi_{ST}$ values. Where global $\Phi_{ST}$ values were significant ($P < 0.05$), pairwise $\Phi_{ST}$ values were calculated through nonparametric permutations ($n = 99{,}999$). Pairwise tests were corrected for multiple comparisons according to Narum (2006). Separate analyses were performed for COI and S7 sequences. Fu's $Fs$ values were also calculated in ARLEQUIN to test neutrality assumptions; significance was tested at 99,999 permutations.

To calculate the degree and direction of gene flow between *C. urodeta* and *C. nigripinnis,* migration rates ($nm$: where $n$ is effective population size and $m$ is migration rate) were calculated with the Bayesian MCMC search strategy implemented in the software MIGRATE-N version 3.6 (Beerli and Palczewski 2010) using both genetic loci (COI and S7). We ran two replicates, each with four heated chains, and a single chain of one million steps with a 10% burn-in (*C. nigripinnis*, $n = 44$; *C. urodeta*, $n = 66$). Posterior distributions were generated using the Metropolis–Hastings algorithm. The default settings for priors were used with a full migration matrix model for an initial run and then constrained (or expanded) for the final run. MCMC chain convergence was confirmed with unimodal, normally distributed priors for all four parameters. Estimates of the effective number of immigrants per generation were calculated by multiplying final values of $\theta$ and $m$ (Beerli 2009). Given the difference in mutation rate and inheritance mode of mitochondrial COI and nuclear S7, we have chosen not to compare absolute parameter estimates between markers, but instead compare the directionality of gene flow within each marker.

## Ecology

*Abundance* To compare abundance between the three groups, abundance data from UVC surveys at Christmas Island were analysed in a three-factor sampling design. The factors were species group (fixed, 3 groups), depth (fixed, 2 levels: 5 and 20 m), and site (random, 5 sites) using permutational multivariate analyses of variance (PERMANOVA; Anderson et al. 2008) in PRIMER version 6 (Clarke and Gorley 2006). Statistical analyses were not necessary to detect differences in abundance at the Cocos Islands, because only a single *C. nigripinnis* and hybrid were observed there.

*Habitat use* To compare habitat use where the three groups co-occur (Christmas Island), two analyses were performed. Firstly, depth (5 and 20 m) and site were included in a three-factor PERMANOVA design. Secondly, a distance-based linear model (DistLM) was calculated using point intercept transect data and abundance data in PERMANOVA+ (Anderson et al. 2008) in PRIMER (Clarke and Gorley 2006). These analyses selected microhabitat types that best explained the variation in species abundance.

For DistLM analyses, a resemblance matrix of abundance between sites and species groups was created using the zero-adjusted Bray–Curtis coefficient (Clarke et al. 2006). Depth was not included in this model as its effect on abundance was nonsignificant (see results, habitat use). To select the most parsimonious model, the DistLM was conducted using the BEST (both backward and forward) selection procedure and the AIC selection criteria (Bozdogan 1987). Variables chosen to represent the model were plotted using a distance-based redundancy analysis (dbRDA). Spearman rank correlations of the densities of each species group to the dbRDA axes were also calculated. Prior testing of correlations between habitat variables revealed turf algae were strongly correlated (>70%) with massive and encrusting corals (ESM Table S3), and so these coral variables were excluded from subsequent analyses.

## Biology

*Growth* Growth characteristics of each group were modelled using the von Bertalanffy growth function (VBGF). The VBGF is described by the equation: $Lt = L\infty\ \{1 - \exp\ [-K\ (t - t0)]\}$; where $L_t$ = mean total length of fish of age $t$; $L_\infty$ = asymptotic mean total length; $K$ = is a rate constant that determines the rate at which $L_t$ approaches $L_\infty$; $t$ = age of the fish; and $t_o$ = the hypothetical age at which the mean length is zero if it had always grown in a manner described by the VBGF. Growth rates were compared following methods described by Rhodes et al. (2011). Ellipsoidal 95% bivariate confidence intervals were plotted around estimates of the growth coefficient ($K$) and the mean asymptotic total length ($L_\infty$) for each group. Overlapping ellipses were considered statistically similar.

*Reproduction* Data on the size range of sexes (from histology) were used to infer the proportion of males in each group. Population length frequency distributions of fish observed during UVC were compared through multiple

Kolmogorov–Smirnov tests to determine whether one group had a greater proportion of large fish, which are likely to be males. Only four mature females were observed, these individuals were combined with immature females under the category "females".

## Results

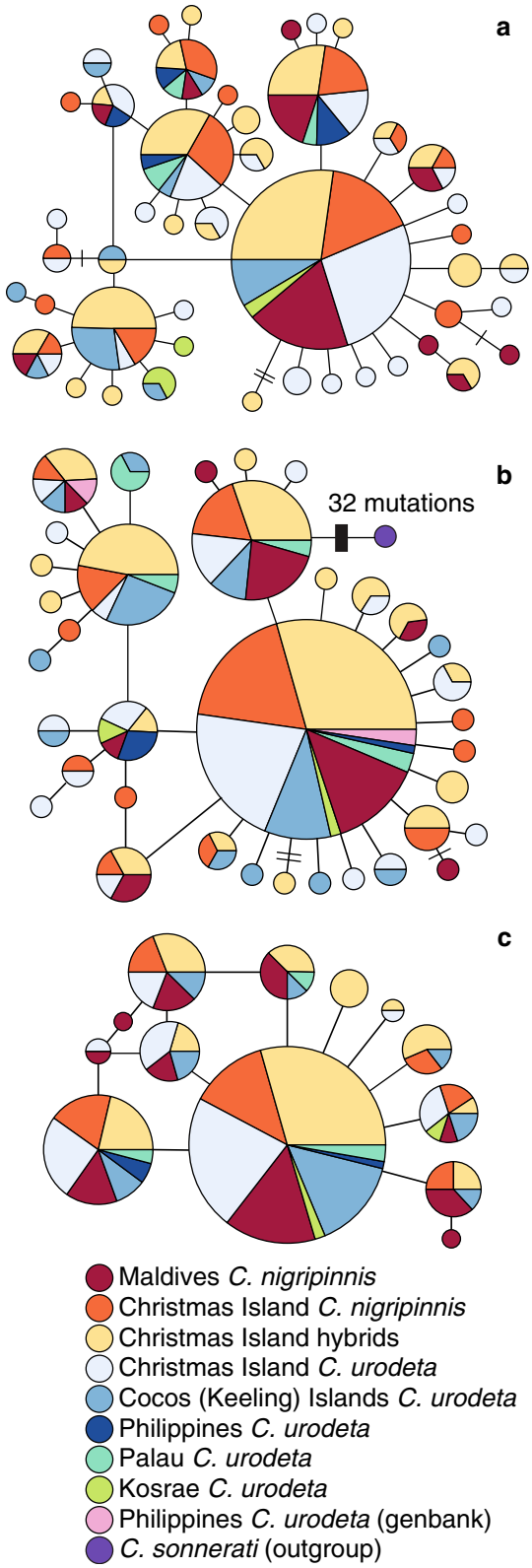
### Hybridisation and taxonomy

From 248 samples, 235 amplified successfully at COI, while 173 amplified at S7. Both markers were unable to distinguish *C. urodeta*, *C. nigripinnis*, and their hybrids (Fig. 2), despite the inclusion of parent species populations sampled approximately 6500 and 3000 km outside the hybrid zone, respectively (Fig. 1).

For the nuclear gene S7, AMOVA revealed no genetic differences between populations (overall $\Phi_{ST} = -0.002$, $P = 0.506$), with 100% of genetic variation observed within groups. For the mtDNA COI gene, however, AMOVA revealed significant differences in population diversity (overall $\Phi_{ST} = 0.012$, $P = 0.042$), although only 1% of the variation was observed between groups. Subsequent population pairwise $\Phi_{ST}$ tests for COI showed genetic homogeneity among all populations with the exception of Maldives *C. nigripinnis* vs. Kosrae–Palau–Philippines *C. urodeta* and Maldives *C. nigripinnis* vs. Cocos Islands *C. urodeta* (Table 1). Pairwise $\Phi_{ST}$ tests for S7 were similar among all population comparisons (Table 1).

Haplotype diversity and nucleotide diversity at COI was comparable among populations, ranging from 0.714 ($\pi = 0.002$; $F_S = -4.97$) to 0.912 ($\pi = 0.003$; $F_S = -11.01$) and from 0.002 ($h = 0.714$; $F_S = -4.97$) to 0.003 ($h = 0.912$; $F_S = 11.01$), respectively (Table 2). Genetic diversity did not appear to be affected by sample size, which ranged from 14 to 69 (Table 2). Tests for sequence neutrality showed negative and significant Fu's *Fs* values for all populations; values ranged from −3.14 ($h = 0.901$; $\pi = 0.003$) to −13.11($h = 0.784$; $\pi = 0.003$) (all $P < 0.02$), suggesting that all populations may have undergone recent expansion.

Haplotype and nucleotide diversity were also comparable among sites based on S7. For all six populations, haplotype and nucleotide diversity ranged from 0.620 ($\pi = 0.004$; $F_S = -4.65$) to 0.749 ($\pi = 0.005$; $F_S = -4.93$) and from 0.003 ($h = 0.636$; $F_S = -1.26$) to 0.005 ($h = 0.749$;



◀**Fig. 2** Median-joining network based on **a** 623 base pairs (bp) of mitochondrial cytochrome-c oxidase subunit-I (COI; $n = 235$), **b** the same dataset supplemented by *Cephalopholis urodeta* ($n = 4$; GenBank accession numbers: FJ583012.1, FJ583013.1, FJ583014.1, and FJ583015.1) and *C. sonnerati* outgroup ($n = 2$; GenBank accession numbers: DQ107916.1 and DQ107927.1) sequences from GenBank trimmed to a common length of 528 bp, and **c** 241 bp of nuclear ribosomal protein S7 DNA ($n = 173$) sequence data from *C. urodeta*, *C. nigripinnis*, and their hybrids sampled across the Indo-Pacific region. Each *circle* represents a haplotype while its size is proportional to its total frequency. *Branches* and *black cross-bars* represent a single nucleotide change unless otherwise noted

$F_S = -4.93$), respectively (Table 2). Genetic diversity was not affected by sample size which ranged from 14 to 69 (Table 2). Tests for neutrality revealed significant negative values for four out of six populations (Table 2), with Fu's $F_S$ ranging from $-1.26$ ($h = 0.636$; $\pi = 0.003$) to $-5.25$ ($h = 0.644$; $\pi = 0.004$) (all $P < 0.02$).

The effective number of immigrants per generation at nuclear S7 was similar in both directions (*C. urodeta* to *C. nigripinnis* = 20.28; *C. nigripinnis* to *C. urodeta* = 29.82).

**Table 1** Matrix of population pairwise $\Phi_{ST}$ values ($P$ values in parentheses) for mitochondrial cytochrome-c oxidase subunit-I (COI; top) and intron 1 of the S7 ribosomal protein (bottom) for *Cephalopholis urodeta*, *C. nigripinnis*, and their hybrids

| COI ($n = 248$) | CI—hybrid | CI—*C. nigripinnis* | CI—*C. urodeta* | CKI—*C. urodeta* | IO—*C. nigripinnis* |
|---|---|---|---|---|---|
| CI—hybrid | | | | | |
| CI—*C. nigripinnis* | 0.009 (0.802) | – | | | |
| CI—*C. urodeta* | 0.004 (0.587) | 0.012 (0.907) | – | | |
| CKI—*C. urodeta* | 0.006 (0.237) | 0.018 (0.122) | 0.027 (0.053) | – | |
| IO—*C. nigripinnis* | 0.024 (0.055) | 0.020 (0.099) | 0.018 (0.086) | 0.048 (0.019)* | – |
| PO—*C. urodeta* | 0.016 (0.199) | 0.020 (0.187) | 0.035 (0.085) | 0.169 (0.210) | 0.136 (> 0.001)** |
| S7 ($n = 169$) | CI—hybrid | CI—*C. nigripinnis* | CI—*C. urodeta* | CKI—*C. urodeta* | IO—*C. nigripinnis* |
| CI—hybrid | | | | | |
| CI—*C. nigripinnis* | >0.001 (0.400) | – | | | |
| CI—*C. urodeta* | 0.020 (0.129) | 0.019 (0.608) | – | | |
| CKI—*C. urodeta* | 0.003 (0.462) | 0.026 (0.842) | 0.014 (0.606) | – | |
| IO—*C. nigripinnis* | 0.026 (0.100) | 0.006 (0.315) | 0.006 (0.505) | 0.006 (0.457) | – |
| PO—*C. urodeta* | 0.019 (0.261) | 0.033 (0.683) | 0.004 (0.366) | 0.004 (0.371) | 0.023 (0.265) |

Location abbreviations: Christmas Island (CI), Cocos (Keeling) Islands (CKI), Maldives (IO), Palau + Kosrae + Philippines (PO)

* Significant values at $P < 0.05$; ** significant values at $P < 0.0015$ (corrected for multiple tests as per Narum 2006)

**Table 2** Molecular diversity indices based on mitochondrial cytochrome-c oxidase subunit-I (COI; top) and intron 1 of the nuclear ribosomal protein S7 (bottom) sequence data from *Cephalopholis urodeta*, *C. nigripinnis*, and hybrids sampled from the Indo-Pacific region

| Population | $N$ | $H_N$ | $H_U$ | Haplotype diversity ($h \pm$ SD) | Nucleotide diversity ($\pi \pm$ SD) | Fu's $F_S$ |
|---|---|---|---|---|---|---|
| COI | | | | | | |
| CI—hybrid | 69 | 20 | 7 | 0.865 ± 0.030 | 0.00325 ± 0.00205 | −12.32* |
| CI—*C. nigripinnis* | 40 | 15 | 6 | 0.865 ± 0.040 | 0.00310 ± 0.00200 | −8.58* |
| CI—*C. urodeta* | 46 | 18 | 5 | 0.784 ± 0.061 | 0.00293 ± 0.00191 | −13.11* |
| CKI—*C. urodeta* | 29 | 16 | 6 | 0.912 ± 0.035 | 0.00364 ± 0.00229 | −11.01* |
| IO—*C. nigripinnis* | 29 | 10 | 3 | 0.714 ± 0.083 | 0.00229 ± 0.00260 | −4.97* |
| PO—*C. urodeta* | 14 | 8 | 1 | 0.901 ± 0.058 | 0.00339 ± 0.00225 | −3.14* |
| S7 | | | | | | |
| CI—hybrid | 67 | 10 | 1 | 0.644 ± 0.0630 | 0.00380 ± 0.00395 | −5.25* |
| CI—*C. nigripinnis* | 32 | 6 | 0 | 0.684 ± 0.0759 | 0.00426 ± 0.00326 | −1.48 |
| CI—*C. urodeta* | 50 | 8 | 0 | 0.636 ± 0.0689 | 0.00366 ± 0.00290 | −3.55* |
| CKI—*C. urodeta* | 31 | 8 | 0 | 0.620 ± 0.0972 | 0.00352 ± 0.00285 | −4.65* |
| IO—*C. nigripinnis* | 40 | 10 | 2 | 0.749 ± 0.0637 | 0.00500 ± 0.00362 | −4.93* |
| PO—*C. urodeta* | 12 | 4 | 0 | 0.636 ± 0.1277 | 0.00308 ± 0.00275 | −1.26 |

Location abbreviations: Christmas Island (CI), Cocos (Keeling) Islands (CKI), Maldives (IO), Palau + Kosrae + Philippines (PO)

* Significant Fu's $F_S$ values at $P < 0.02$; $N$ = sample size; $H_N$ = number of haplotypes; $H_U$ = number of unique haplotypes

**Table 3** MIGRATE results calculated with the Bayesian MCMC search strategy based on mitochondrial cytochrome-c oxidase subunit-I (COI; top) and intron 1 of the nuclear ribosomal protein S7 (bottom) sequence data from *Cephalopholis nigripinnis* and *C. urodeta* from the Indian Ocean and Pacific Ocean

| Parameter | 2.5% CI | 97.5% CI | Median | Mean |
|---|---|---|---|---|
| COI | | | | |
| $\theta_1$ | 0.000 | 0.016 | 0.008 | 0.008 |
| $\theta_2$ | 0.061 | 0.398 | 0.225 | 0.225 |
| $m_{2->1}$ | 0.000 | 2320.0 | 550.0 | 787.5 |
| $m_{1->2}$ | 2326.7 | 8853.3 | 4956.7 | 5224.9 |
| S7 | | | | |
| $\theta_1$ | 0.003 | 0.033 | 0.011 | 0.014 |
| $\theta_2$ | 0.002 | 0.025 | 0.008 | 0.010 |
| $m_{2\rightarrow1}$ | 418.0 | 3000.0 | 2237.0 | 2021.5 |
| $m_{1\rightarrow2}$ | 992.0 | 3000.0 | 2337.0 | 2210.7 |

1 = *C. urodeta*; 2 = *C. nigripinnis* $\theta$ = effective population size; $m$ = migration rate; CI = confidence interval

For the mtDNA COI gene, migration rates were several times higher from *C. urodeta* to *C. nigripinnis* (176.93) than from *C. nigripinnis* to *C. urodeta* (42.9). For the former, lower limits on posterior probabilities were close to zero; this implies either low levels of introgression or recent introgression – given that recently introgressed populations will not be in migration–drift equilibrium (full results in Table 3).

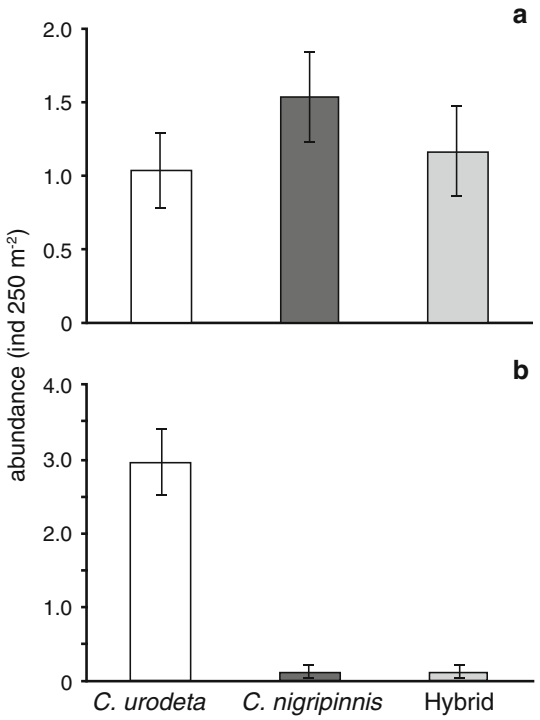



**Fig. 3** Mean abundance (±SE) of *Cephalopholis urodeta*, *C. nigripinnis*, and their hybrids across all sites and depths surveyed using UVC at **a** Christmas Island and **b** Cocos (Keeling) Islands

## Ecology

### *Abundance*

Individuals with *C. nigripinnis* colouration and *C. urodeta* colouration were observed at both the Cocos Islands and Christmas Island. Hybrid individuals were identified at both locations and exhibited a range of intermediate colour patterns (ESM Fig. S3). At Christmas Island, *C. urodeta*, *C. nigripinnis*, and their hybrids all exhibited similar abundances ($F = 1.020$, $df = 2$, $P = 0.398$; Fig. 3a), with mean abundances of 1.03 (±0.26 SE), 1.53 (±0.30 SE), and 1.17 (±0.32 SE) individuals per 250 m$^2$, respectively. At the Cocos Islands, *C. urodeta* were far more abundant than *C. nigripinnis* and their hybrids (Fig. 3b), with mean abundances of 2.96 (±0.45 SE), 0.13 (± 0.09 SE), and 0.13 (±0.09 SE) individuals per 250 m$^2$, respectively.

### *Habitat use*

All three groups showed similar spatial patterns in abundance at Christmas Island, with no significant interaction between species and depth ($F = 0.157$, $df = 2$, $P = 0.854$), or between species and sites ($F = 1.091$, $df = 8$, $P = 0.377$). For all three groups, relative abundance was higher in shallow (5 m) waters compared to deeper (20 m) waters (Fig. 4a); however, this result was not significant ($F = 5.665$, $df = 1$, $P = 0.085$). Overall abundance varied significantly between sites ($F = 18.52$, $df = 4$, $P > 0.001$), with Ethel Beach in particular supporting higher densities for all three groups (Fig. 4b).

The best model identified by the DistLM analyses included the microhabitats of turf algae, rubble, and calcareous algae which explained 34% of the total variation in abundance of each species group ($R^2 = 0.36$, $AIC = 210.43$). The first two dbRDA axes accounted for 76 and 20% of the variation in the fitted model. All three groups were abundant in turf algae microhabitats, which were strongly correlated with dbRDA axis 1 (Fig. 5). Modest differences in habitat use can be explained by dbRDA axis 2, which revealed that *C. nigripinnis* are more abundant in bare rubble and calcareous algae microhabitats (Fig. 5). Habitat complexity (measured following Wilson et al. 2007) did not vary significantly among sites (data not shown).

## Biology

### *Growth*

For all three groups, a total of 170 otoliths were processed of which 153 were used in growth analyses. Growth coefficients ($K$) and asymptotic lengths ($L_\infty$) for *C. urodeta*

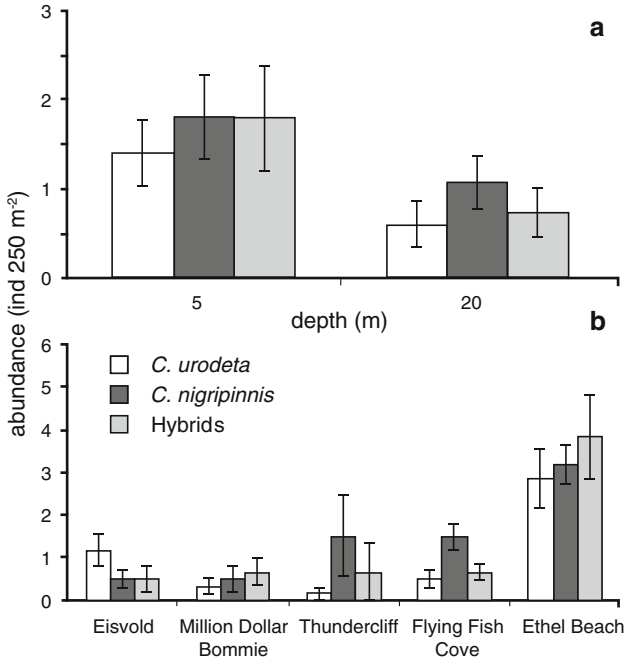



**Fig. 4** Mean abundance (±SE) of *Cephalopholis urodeta*, *C. nigripinnis*, and their hybrids at **a** depths and **b** sites surveyed using UVC at Christmas Island

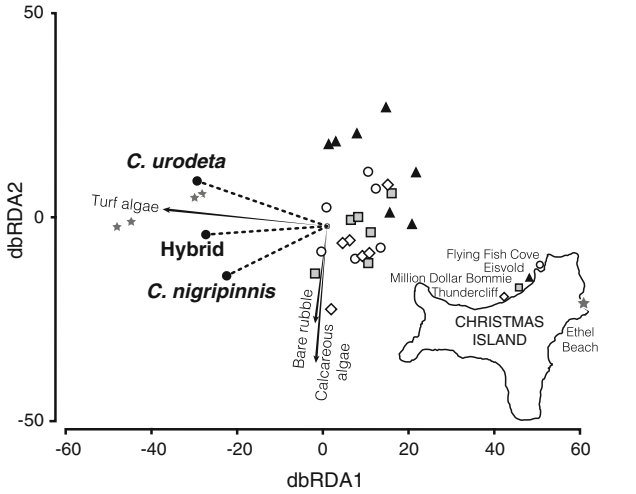



**Fig. 5** Distance-based redundancy analyses (dbRDA) ordination of first and second fitted axis, relating microhabitat type to the abundance of *Cephalopholis urodeta*, *C. nigripinnis*, and hybrids at Christmas Island. *Vectors* indicate the strength and direction of Spearman rank correlations of microhabitat types and species groups to the first and second dbRDA axis. dbRDA *axis 1* explained 75.6% of fitted variation and 27.2% of total variation. dbRDA *axis 2* explained 19.7% of fitted variation and 7.1% of total variation

and *C. nigripinnis* were similar, whereas hybrids exhibited a lower value of $K$ and larger asymptotic lengths (Fig. 6a, b, c). The 95% bivariate confidence ellipses for all three groups overlapped (Fig. 6d); therefore, values of both $K$ and $L_\infty$ were similar. Hybrids exhibited the largest

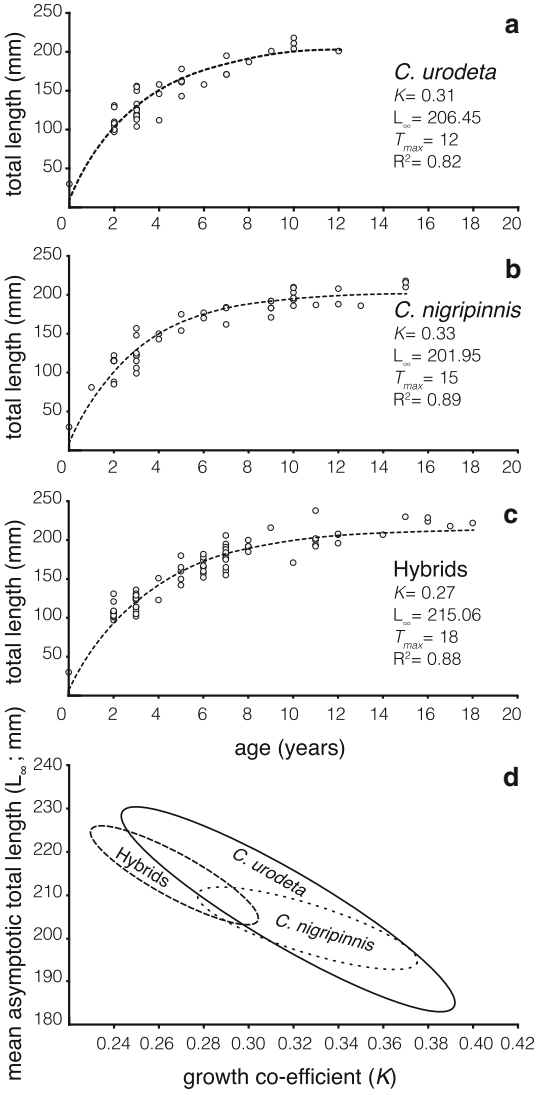



**Fig. 6** Size-at-age data and von Bertalanffy growth functions for **a** *Cephalopholis urodeta* ($n = 38$), **b** *C. nigripinnis* ($n = 45$), and **c** their hybrids ($n = 70$) sampled at Christmas Island. **d** Comparison of growth parameters between the three groups using bivariate 95% confidence *ellipses* surrounding estimates of $K$ and $L_\infty$. Overlapping *ellipses* indicate similar parameters

estimate of maximum age (18 yr) followed by *C. nigripinnis* and *C. urodeta* (Fig. 6a, b, c).

### *Reproduction*

All three groups displayed a sex-biased size structure: Larger fish (TL > 200 mm) were male, smaller fish (TL < 150 mm) were female, and transitional fish, which contained germ cells from both sexes, were intermediate in length (TL = 140–170 mm; for size distributions of sexes see ESM Fig S4). Length frequency distributions based on UVC surveys revealed the size structure of the hybrid

group was similar to that of *C. nigripinnis* ($D = 0.24$, $P = 0.15$) and *C. urodeta* ($D = 0.21$, $P = 0.37$), while *C. nigripinnis* contained a greater proportion of larger individuals than *C. urodeta* ($D = 0.45$, $P < 0.001$; for length frequency distributions see ESM Fig. S5).

## Discussion

Sister species *C. urodeta* and *C. nigripinnis* were genetically similar across all locations sampled in this study. While we do not consider it unusual that the two species are similar where they interbreed (the Cocos–Christmas hybrid zone), it was unexpected to find that populations 9000 km outside of the hybrid zone were genetically indistinguishable. Biological and ecological compatibilities may explain the prevalence of hybrids at the Cocos Islands and Christmas Island where *C. urodeta* and *C. nigripinnis* co-occur, although this does not explain the lack of genetic differentiation between more distant populations.

The results contradict the phylogenetic study by Craig and Hastings (2006), although that study used a single sample from each species and therefore did not account for intraspecific variation. Ariyanti et al. (2015) also detected genetic differences between *C. urodeta* and *C. nigripinnis*, although the results of their study should be interpreted with caution considering the small sample size and problems associated with confirming species identity on GenBank.

The conflict between colouration and genetic identity (and ultimately the taxonomic classification) of *C. urodeta* and *C. nigripinnis* may be explained by three scenarios. Firstly, alternate colour morphs may have evolved faster than genetic differences due to strong selective pressure (e.g., Bowen et al. 2006; Puebla et al. 2007). Secondly, fluctuating Pleistocene sea levels (Hobbs et al. 2009; Gaither and Rocha 2013) may have formed an inconsistent geographic barrier, preventing Pacific and Indian Ocean species from diverging in allopatry, resulting in incomplete lineage sorting. Finally, hybridisation following secondary contact at the Cocos Islands and Christmas Island may have led to introgression and "mixing" of genes beyond the hybrid zone, as reported in other reef fishes (McMillan et al. 1999; Gaither et al. 2011), albeit not at the same spatial scale described here (i.e., 9000 km).

It is not uncommon for colouration and genetic identity to conflict in marine reef fishes. For example, a single colour-morph of the ubiquitous damselfish *Dascyllus trimaculatus* is comprised of up to three mtDNA lineages (Bernardi et al. 2002), while differently coloured species of butterflyfish and angelfish show no genetic distinction (McMillan et al. 1999; DiBattista et al. 2012). In some cases, it may take multiple molecular markers to detect differences, and it is possible that *C. urodeta* and *C. nigripinnis* are distinguishable at a marker not used in this study. For example, only three of 16 screened markers were able to detect diagnostic differences between parent species of hybridising damselfishes (Coleman et al. 2014). Wider genome scans for single nucleotide polymorphisms (SNPs) and application of restriction-site-associated DNA sequencing (RAD-Seq) may help identify cryptic differences as seen in populations of surgeonfish (Gaither et al. 2015) and American eels (Pavey et al. 2015). Even though the markers used here have been widely applied in the population genetics of fishes, a more thorough genetic analysis may be necessary to discriminate incomplete lineage sorting from hybridisation.

Regardless of this ambiguity, the spectrum of intermediate hybrid phenotypes at Christmas Island suggests that introgression may account for some degree of genetic mixing, which (according to the MIGRATE analyses at mtDNA COI) is an order of magnitude higher from *C. urodeta* to *C. nigripinnis*. Interestingly, the *C. urodeta* population at Christmas Island contained the highest proportion of small fish which, according to the size-biased sex structure, were more likely to be females. It is possible that *C. urodeta* are reproducing with *C. nigripinnis* mostly as females and passing on their mtDNA disproportionately.

Like many other fishes that hybridise at the Cocos Islands and Christmas Island, both parent species are allopatric to the Indian and Pacific oceans, and are similar in biology and ecology where they overlap, which provides ample opportunities to hybridise. Introgression does not appear to be widespread as pure parent phenotypes are still maintained within and outside of the hybrid zone. Owing to the isolation of the Cocos Islands and Christmas Island, limitations in larval dispersal may prevent genetic mixing with neighbouring parent populations. However, this does not explain how *C. urodeta* and *C. nigripinnis* maintain their unique phenotypes within the hybrid zone if recruits are not arriving from outside populations. Cryptic hybridisation may also explain the maintenance of parental species phenotypes; for example, the genes controlling colour are likely not linked to the few loci tested in this study.

In any case, the likelihood of *C. urodeta* and *C. nigripinnis* evolving reproductive isolation and becoming genetically distinct is low. At the Cocos Islands, abundances of the two species were significantly different; this could reflect differences in habitat use at this location and potentially lead to fewer heterospecific encounters and reproductive isolation as seen in other reef fishes (Rocha et al. 2005). However, a lack of conspecifics may still cause *C. nigripinnis* to seek out and hybridise with *C. urodeta* at this location (e.g., Hobbs et al. 2009; Hobbs and Allen 2014). At Christmas Island, both species use the same microhabitat and have similar abundances across sites and depths; this cohabitation is likely to increase the chances of hybridisation, even if accidental as reported in

other fishes (Campton 1987; Frisch and van Herwerden 2006).

Despite the assumption that many hybrids suffer from fitness disadvantages and shorter lifespan (Arnold and Martin 2010), comparisons here suggest that *C. urodeta* and *C. nigripinnis* hybrids may live longer than their parent species. Factors that promote longevity in fishes include greater tolerance to disease and reduced predation (Reznick et al. 2002), both of which can be products of greater heterozygosity as a result of hybridisation (Arnold and Martin 2010). The hybrids in this study also displayed the slowest growth rates and represented the oldest and largest fish sampled – all characteristics that are directly (Trippel et al. 1997) or indirectly (Thompson and Munro 1978; Reznick et al. 2002) associated with fecundity. While these results may imply a fitness advantage for hybrids, a larger sampling effort would be required to reach more definitive conclusions about potential life-history differences among our study populations. Overall, in terms of age and growth, hybrids displayed fitness that was at least equal to their parent species.

In terms of reproduction, all three groups displayed size-biased patterns of sex: Males were large, females were small, and transitional fish were intermediate in size. This suggests *C. urodeta* and *C. nigripinnis* may be protogynous hermaphrodites that form haremic social groups, as reported for *C. urodeta* in other locations (Donaldson 1995; Nakai and Sano 2002). In these social groups, the position of the dominant male is considered advantageous because, per individual, males have more breeding opportunities than females. Considering this, similarities in the frequency of male hybrids (implied by similar size structures) suggests that hybrids are likely to have an equal reproductive success to their parent species. Hybrids were also equally abundant as both parent species at Christmas Island lived just as long and were able to exploit a similar microhabitat. Therefore, according to the proxies measured in this study, the hybrids at Christmas Island exhibit fitness that is at least equal to their parent species.

Hybridisation is common among reef fishes at the Cocos Islands and Christmas Island (Hobbs et al. 2009; Hobbs and Allen 2014), yet there is no consistent pattern explaining the fitness and potential evolutionary outcomes of this phenomenon. From the various hybrids that have been studied at this location, the outcomes have ranged from hybridisation with no introgression (Montanari et al. 2012), introgression inside the hybrid zone (Marie et al. 2007), and evidence of introgression just beyond the hybrid zone (Montanari et al. 2014). The abundance of later-generation hybrids at Christmas Island implies high levels of gene flow between *C. urodeta* and *C. nigripinnis*, and yet the markers used here may have limited power to detect such events and distinguish between hybridisation and incomplete lineage sorting. Analyses of faster evolving (and diagnostic) microsatellite markers in addition to using newer Bayesian assignment approaches (e.g., Joly et al. 2009; Blanco-Pastor et al. 2012) with wider genome scans (e.g., SNPs, Gaither et al. 2015; RAD-Seq, Pavey et al. 2015) may help detect finer scale differences between species (if they are in fact present) or confirm hybridisation. Sampling populations even further from the hybrid zone (e.g., French Polynesia and the east African coast) may also determine whether the parent species are "pure" at the edges of their geographic range.

*Cephalopholis urodeta* and *C. nigripinnis* appear to be interbreeding at the Cocos Islands and Christmas Island owing to biological and ecological compatibilities. Based on the available evidence, the genetic homogeneity between the sister species may be due to genetic/morphological discordance, incomplete lineage sorting, or hybridisation with subsequent introgression. Regardless of the pathway, *C. urodeta* and *C. nigripinnis* are unlikely to evolve in reproductive isolation as they cohabit where they are common (Christmas Island) and will source congeneric mates where they are rare (Cocos Islands). The causes and consequences of hybridisation in coral reef fishes are not yet well understood and appear to vary among taxonomic families and biogeographic regions. This study supports the concept that hybridisation plays a dynamic role in the evolution of reef fishes.

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