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Causes and consequences of natural hybridisation among coral reef butterflyfishes (*Chaetodon*: Chaetodontidae)

Thesis submitted by

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For the Degree of Doctor of Philosophy in the College of Science and Engineering of James Cook University and AIMS@JCU.

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STATEMENT ON THE CONTRIBUTION OF OTHERS

This thesis is primarily composed of collaborative work with Dr. Lynne van Herwerden, Prof. Morgan Pratchett, Dr. Jean-Paul Hobbs and Dr. Line Bay. Experimental design, data collection, laboratory work and data analyses were primarily conducted by me. My collaborators provided intellectual guidance, assistance with field work, technical instruction and editorial assistance in the preparation of manuscripts. Aside from standardised formatting for the thesis, all chapters have been presented as published.

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DECLARATION OF ETHICS

The research presented here was conducted within the guidelines for research ethics outlined in the *James Cook University Policy on Experimentation Ethics, Standard Practices and Guidelines* (2001), and the *James Cook University Statement and Guidelines on Research Practice* (2001).

This project was approved by James Cook University Animal Ethics Committee and conducted under Permits A1757 and A1951.

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This thesis is dedicated to our children, Roberto and Gaia, to whom I wish a fulfilling life in a world as beautiful as ours. I love you.

ABSTRACT

Natural hybridisation is the successful interbreeding of individuals from different populations, distinguishable through one or more heritable characters, and is a widespread phenomenon in the plant and animal kingdoms. The development of hybridisation theories has largely been based on studies in terrestrial and freshwater ecosystems. Hybridisation was traditionally considered rare and unimportant in marine systems and therefore received little attention. Recently however, there has been a surge of reported marine hybrids, particularly in corals and reef fishes. The ecological promoters and evolutionary and adaptive consequences of reef fish hybridisation are yet to be thoroughly evaluated. Butterflyfishes (f. Chaetodontidae) form a disproportionate number of hybrids and therefore represent an appropriate model group to investigate hybridisation in reef fishes. This thesis examines the causes and consequences of hybridisation in reef fishes and focuses on butterflyfishes (genus *Chaetodon*) at Christmas Island (Indian Ocean), a global hotspot for reef fish hybridisation. The aims of this thesis were to i) review the incidence and ecological/behavioural precursors of hybridisation in reef fishes, while providing a tentative framework for conducting studies within hybrid zones; ii) develop a microsatellite toolkit for species of the *Chaetodon* genus; iii) compare the ecology, behaviour and population genetics of hybridising sister species of butterflyfishes in order to, not only provide a snapshot of the evolutionary consequences of hybridisation in this group, but also determine which processes are likely to promote it; iv) use a comparative life history approach to determine the fitness of butterflyfish hybrids relative to their parental species.

Chapter 1 reviews the current knowledge of hybridisation with a focus on marine fishes. Hybridisation was found to be highly prevalent in marine fish, despite previous assertions of rarity, and showed a taxonomic as well as latitudinal bias. Further, the current marine fish hybridisation literature was found to be largely lacking ecological and behavioural data, in contrast with freshwater counterparts, therefore highlighting the need for a framework

to fill the data gap in order to better accompany the wealth of genetic data produced in the assessment of hybridisation.

The development of a molecular toolkit, necessary for the rest of our investigations, is presented in Chapter 2. Twenty microsatellite loci were developed using 454 sequencing, to apply to the population genetic analysis of the *Chaetodon guttatissimus* × *C. punctatofasciatus* complex. This was done to facilitate direct comparison of the genetic underpinnings of hybridisation in this group to those of another previously studied group (*C. trifasciatus* and *C. lunulatus*), for which species-specific microsatellite loci had been designed and used.

Chapter 3 uses the molecular toolkit and experimental framework outlined in the previous chapters to examine hybridisation between two butterflyfish sister species, *Chaetodon guttatissimus* and *C. punctatofasciatus*. The largely overlapping spatial and dietary ecologies of these species favour heterospecific encounters. Lack of assortative mating and local rarity of *C. punctatofasciatus* promote the formation of heterospecific breeding pairs. Analyses of mtDNA and microsatellite DNA were consistent with the hybrid status of the intermediately coloured hybrids. Maternal contribution to hybridisation in this complex was bidirectional, and introgression by *C. punctatofasciatus* mtDNA was detected in *C. guttatissimus* individuals within and beyond the hybrid zone (almost 1000 km to the west), potentially indicating a Pacific invasion of an Indian Ocean species genome. The comparisons drawn with previous work on hybrdising *Chaetodon trifasciatus* and *C. lunulatus* showed that, despite being driven by similar factors, hybridisation in reef fishes can have varying evolutionary consequences, possibly due to the magnitude of the genetic distance between hybridising species.

Chapter 4 evaluates hybrid fitness in both *Chaetodon* hybridising groups presented in the previous study. Histology confirmed the reproductive viability of hybrids, and liver lipid analyses showed that hybrid condition was not different from parental species. Further, otolith data highlighted no difference in growth rate and maximum length between hybrids and

parents. According to the fitness-related traits measured here, *Chaetodon* hybrids are as fit as their parents, and unlikely to promote the formation of a hybrid swarm. However, sufficient fitness and rapid transfer of genetic material between species allow persistence of hybrids within the suture zone, where they positively contribute to genetic diversity.

The cases of hybridisation studied here appear to be initiated by similar ecological and behavioural settings, albeit showing different genetic consequences. Determining this was possible through the use of a comprehensive approach, which combined molecular analyses and extensive field observations. Further, the apparent lack of differences in fitness between hybrids and parental species points at the persistence of hybrid individuals within the Indo-Pacific suture zone, where they may continue to contribute positively to genetic diversity. The role of hybridisation in evolution and adaptability had been appreciated in terrestrial and freshwater systems, and this thesis shows that hybridisation can have a role in maintaining reef fish diversity. The studies presented here constitute a comprehensive overview of the relevance of hybridisation for reef fishes and may be a stepping stone toward ascertaining its role in the evolution and adaptation of new species in such a diverse group.

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GENERAL INTRODUCTION

Hybridisation definition and theory

Hybridisation is the crossing of genetically distinct taxa, distinguishable through one or more heritable characters. More than 25% of plants and 10% of animals hybridise (Mallet 2005), and the occurrence of natural hybridisation varies spatially, temporally and taxonomically. Some taxonomic groups tend to hybridise much more than expected (Mallet 2005) and hybridisation is concentrated in narrow geographic regions termed "hybrid zones" (Barton & Hewitt 1985). Hybrid zones can exist inside overlapping sympatric ranges (van Herwerden *et al.* 2006) or where two recently diverged, allopatric sister species come into secondary contact (Hewitt 2000).

Harrison (1990) defined hybrid zones as "windows on evolutionary processes", but his view is not one that has been unanimously shared. Natural hybridisation has historically been considered a rare and unimportant phenomenon, particularly because it challenges the biological species paradigm (Abbott *et al.* 2013). Interestingly, the perceived rarity of natural hybridisation caused it to be considered unimportant, whereas rare fitness-increasing mutations have been considered milestones for adaptive evolution (Arnold 2006). Despite this traditional view of natural hybridisation, there is mounting evidence in the scientific literature indicating that studying hybridisation through ecological observations and molecular genetic approaches should be considered fundamental to understanding speciation and evolution (Abbott *et al.* 2013).

Hybridisation is most successful between closely related species (e.g. secondary contact between recently diverged taxa) which often have overlapping ecologies that favour contact, thus increasing the chances of interbreeding (Mallet 2005). Some ecological traits common to hybridising species include: habitat and dietary overlap, which increase heterospecific encounters (Arnold 1997); localised rarity of one or both species, which, because of the lack of conspecific partners, will facilitate heterospecific mating (Hubbs 1955)

and lack of assortative mating, which favours the formation of heterospecific breeding groups (McMillan *et al.* 1999).

Recent research has revealed that natural hybrids are more common than first thought, and hybridisation can have substantial evolutionary and adaptive significance – see Abbott *et al.* (2013) and references therein -. Natural hybridisation can even lead to unpredictable and rapid evolutionary consequences (in a few decades) and increase the genetic diversity of a population and its adaptability to novel environments (Grant & Grant 2002; Grant & Grant 2008; Anderson *et al.* 2009). Additionally, hybrids have the potential to occupy unexploited ecological niches, and through reproductive isolation become new species (Seehausen 2004). Hybridisation can also contribute significantly to the loss of biodiversity through extinction by introgression (Rhymer & Simberloff 1996) or reverse speciation (Taylor *et al.* 2006). Introgression occurs when the genome of one species is invaded by genetic material of another species through repeated hybrid backcrosses (Arnold 1997). Fertile F_1 hybrid offspring must successfully interbreed with one (or both) parental species for introgression to occur (Arnold 1997). Introgressed individuals resulting from these crosses are often phenotypically indistinguishable from one of the parent species, despite the presence of genetic material of the other (Yaakub *et al.* 2006).

Long-term ecological observations, coupled with modern molecular genetic analyses, have allowed researchers to investigate the directionality and evolutionary consequences of hybridisation, often with striking results (Grant & Grant 2002; Anderson *et al.* 2009). Hybridisation is termed unidirectional when males of one species can successfully interbreed with females of another, but not vice versa (Birkhead & Balen 2007). Conversely, bidirectional hybridisation occurs when both sexes in both species can successfully interbreed (Steeves *et al.* 2010). For example, the bidirectional gene exchange between domesticated dogs and North American wolves has been deemed a mechanism of primary importance in the evolution of melanism (i.e. dark coat colour) in the wolf population (Anderson *et al.* 2009). Dark coats were selected against in the wild wolf populations, adapted to live in the tundra

(Anderson *et al.* 2009). The higher genetic diversity of the domesticated dogs – maintained by human selection – flourished in the wild wolf population, re-introducing historically lost melanism (Anderson *et al.* 2009). The authors argued that, despite this being a result of anthropogenic intervention, North American wolves would potentially benefit from melanism, as available tundra habitat declines in the face of global warming (Anderson *et al.* 2009).

Hybridisation can also have rapid and significant positive effects on wild populations on isolated archipelagos, where the influence of human activity is minimal (Grant & Grant 2002). The unidirectional hybridisation in Darwin finches of genus *Geospiza* at the Galapagos Islands, for example, mediated the morphological changes in beak shape of one of the parental species, resulting in increased adaptability to novel food sources (Grant & Grant 2002). Following an El Niño Southern Oscillation (ENSO) event, the primary food source of *G. scadens* became unavailable, resulting in high mortality of females of this species (Grant & Grant 2002). Consequentially, males interbred with *G. fortis* females – which had beak shapes better suited for a wider array of food sources – resulting in hybrids that were better adapted to the novel environment (Grant & Grant 2002). Furthermore, long-term monitoring of the Darwin finches at the Galapagos Islands, has revealed that introgressive hybridisation occurs periodically, resulting in hybrids that are better adapted to some environmental conditions than their parents, increasing the overall genetic diversity and allowing for the persistence of these isolated species in a time of environmental change (Grant & Grant 2008).

Most of the theory on natural hybridisation has been developed from terrestrial and freshwater ecosystems, where the process has been well documented. Hybridisation in marine systems is known to occur in a diverse array of groups including – but not limited to – algae, molluscs, corals (Willis *et al.* 2006), crustaceans and fishes (Gardner 1997). Many cases of documented marine hybridisation involve commercially important species such as Atlantic salmon (Garcia de Leaniz & Verspoor 1989; Ayllon *et al.* 2004), Atlantic cod (Ruzzante *et al.* 2000; Nielsen *et al.* 2003), flatfishes (Fujio 1977), menhadens (Dahlberg 1969), groupers (van

Herwerden *et al.* 2002; Frisch & van Herwerden 2006; van Herwerden *et al.* 2006), abalone (Klinbunga *et al.* 2003), blue mussels (Rawson *et al.* 1996; Rawson *et al.* 1999), snow crabs (Smith *et al.* 2005) and cetaceans (Spilliaert *et al.* 1991). Although the evolutionary consequences of hybridisation have been studied in terrestrial (Grant & Grant 2002) and freshwater (Taylor *et al.* 2006) species, the consequences of hybridisation in marine species are largely unknown - but see (Vollmer & Palumbi 2002)-. Because marine species have life history traits, such as lengthy planktonic larval stages (Victor 1986), that are different to species in other systems (Carr *et al.* 2003) it can not be assumed that the evolutionary consequences of hybridisation in marine species will be the same as those already found for terrestrial and freshwater species.

Hybridisation in the marine environment

Within the marine environment hybridisation studies have traditionally focussed on temperate species (Garcia de Leaniz & Verspoor 1989; Ruzzante *et al.* 2000; Nielsen *et al.* 2003; Ayllon *et al.* 2004; Smith *et al.* 2005). Hybridisation in tropical seas has been comparatively understudied, possibly due to the traditional view that hybridisation is most common at high latitudes (Hubbs 1955). In fact, it was proposed that the lack of reported hybridisation from high diversity tropical systems (e.g. coral reefs) indicated that hybridisation is not important in the evolution of these communities (Hubbs 1955). However, more recent research has shown extensive hybridisation in scleractinian corals - see reviews by van Oppen and Gates (2006); Willis *et al.* (2006) -, and has ascribed significant roles to hybridisation in the evolution of this group, including speciation by hybridisation (Vollmer & Palumbi 2002). While hybridisation is important to corals, coral reefs support an enormous diversity of invertebrate and vertebrate species and the role of hybridisation in the evolution of this diversity is unknown, but warrants investigation, particularly in a time of increased anthropogenic impacts on these communities.

Hybridisation occurs in fishes more than any other vertebrate taxon (Hubbs 1955; Allendorf & Waples 1996) and coral reef fishes constitute the most diverse vertebrate communities on earth. Hybridisation in this group has traditionally been considered rare and unimportant (Hubbs 1955), however, there has been a recent surge of reported hybrids in the reef fish literature (Yaakub *et al.* 2006; Hobbs *et al.* 2009). Moreover, genetic approaches are increasingly yielding evidence that hybridisation is a widespread phenomenon in marine fishes and might have played a significant role in their speciation (Lacson & Nelson 1993; Lacson 1994; Lacson & Clark 1995; Kuriiwa *et al.* 2007; Litsios & Salamin 2014). However, reef fish hybridisation studies that couple genetic and quantitative ecological data are rare - but see Frisch and van Herwerden (2006); Yaakub *et al.* (2006); Marie *et al.* (2007) -. The combined use of these techniques is important because it allows determination of both the ecological processes responsible for the onset of hybridisation, and the genetic and evolutionary consequences of hybridisation.

Known marine hybrid zones include the Florida peninsula (Avise 2000), the Baltic Sea (Johannesson & Andre 2006) and the Indo-Pacific border (Hobbs *et al.* 2009). According to Hobbs *et al.* (2013) 90% of hybridising butterflyfishes occur at four specific geographical locations: southern Japan, Hawaii, Papua New Guinea-Micronesia and the Eastern Indian Ocean. Christmas and Cocos (Keeling) Islands lie at this latter location, and are a recognised reef fish hybrid hotspot where Indian and Pacific Ocean reef fish sister species come into secondary contact at the edges of their respective distributions (Hobbs *et al.* 2009). The high number of reef fish hybrids from Christmas and Cocos (Keeling) Islands makes these locations a unique laboratory to further investigate hybridisation in this group (Hobbs *et al.* 2009; Hobbs & Allen 2014). Conspicuously colourful butterflyfishes of the family Chaetodontidae form a disproportionately high number of hybrids (Allen *et al.* 1998; Kuiter 2002; Yaakub *et al.* 2006). Forty four of 130 species, more than a third, hybridise (Hobbs *et al.* 2013), including at least eight at Christmas Island (Hobbs & Allen 2014).

The Chaetodontidae are characterised by striking colour patterns (Allen *et al.* 1998), making the intermediate colouration of the hybrids easily identifiable in the field (Hobbs *et al.* 2013). The Chaetodontidae are a relatively young reef fish family (Bellwood *et al.* 2010), in which recently diverged, allopatric sister species are not uncommon (Blum 1989). Many of these sister species have come into secondary contact, setting the scene for hybridisation (McMillan *et al.* 1999; Montanari *et al.* 2012). Moreover, the dietary overlap shown by some species in this family (Pratchett 2005), together with habitat overlap, can increase the frequency of heterospecific encounters (favouring hybridisation). In synergy, these characteristics of the Chaetodontidae render butterflyfish a suitable model organism for hybridisation studies in reef fishes. Further, butterflyfishes are significantly affected by reef degradation (Pratchett *et al.* 2004; Pratchett *et al.* 2006b), possibly due to the high incidence of corallivory in this group (Cole *et al.* 2008). Hybridisation can result in increased adaptability to novel environments following natural disturbance (Grant & Grant 2002) and may potentially be beneficial to butterflyfishes in a time when coral reefs are undergoing significant habitat changes.

Thesis objectives and structure

In order to evaluate the importance of hybridisation in reef fishes, its causes and consequences were evaluated in butterflyfishes of genus *Chaetodon.* This thesis i) reviewed the incidence and ecological/behavioural precursors of hybridisation in reef fishes, while providing a tentative framework for conducting studies within hybrid zones; ii) developed a microsatellite toolkit for species of the *Chaetodon* genus; iii) compared ecology, behaviour and genetics of 2 hybridising species pairs of genus *Chaetodon* at the Indo-Pacific marine suture zone; iv) examined the fitness of butterflyfish hybrids relative to their parental species.

Chapter 1

To determine the state of knowledge of hybridisation in marine fishes Chapter 1 presents a review of the literature, focussing on ecology and behaviour within fish hybrid zones. Following the identification of patterns of taxonomic and geographical distribution of hybridisation in marine fishes, the chapter focusses on ecological/behavioural precursors of hybridisation often reported in the freshwater literature, allowing direct comparisons and identification of knowledge gaps. Most current literature on marine fish hybridisation has a strong genetic focus, with little or no quantitative data about the ecology and behaviour of hybridising species. Therefore, the resulting proposed framework suggests that future studies should conduct ecological and behavioural observations within hybrid zones, to identify processes most relevant to overcoming pre-zygotic barriers to reproductive isolation. This will advance our understanding of the adaptive and evolutionary relevance of hybridisation in marine fishes and provide insights into the maintenance of reproductive isolation and the process of speciation in the marine environment.

Chapter 2

Determining the evolutionary consequences of hybridisation requires molecular analyses. The combination of mitochondrial and microsatellite DNA represents a useful approach, as it can show patterns of hybridisation and introgression at both the evolutionary and contemporary timescales. Chapter 2 uses 454 sequencing to develop twenty microsatellite loci for two hybridizing sister species of butterflyfish: the spot-band butterflyfish (*Chaetodon punctatofasciatus*) and peppered butterflyfish (*C. guttatissimus*), which are widely distributed in the Western Pacific and Indian Ocean, respectively. All loci were genotyped in samples collected from Christmas Island (minimum sample size was 16 individuals per population) and were polymorphic. Albeit developed specifically for the work contained in the rest of this thesis, these markers may prove useful for population genetic analyses in other closely related species of genus *Chaetodon*.

Chapter 3

In order to assess causes and consequences of hybridisation in reef fishes, Chapter 3 evaluates the ecology, behaviour and genetics of hybridisation between pair-forming, corallivorous butterflyfishes *Chaetodon guttatissimus* and *C. punctatofasciatus* at Christmas Island, a recognised hotspot for marine hybridisation. Findings are further compared to those from a previous study of hybridisation between *C. trifasciatus* and *C. lunulatus*, two species that also hybridise at Christmas Island but are five-fold more divergent. Following the framework outlined in Chapter 1, underwater visual censuses (UVCs) were used to assess habitat use of parental species and hybrids, to ascertain levels of overlap. Dietary preferences of these obligate corallivores were recorded using 3-minute direct observations, to address similarities or differences between species and hybrids. Abundance of taxa was also recorded during UVCs to determine if heterospecific pair formation is a result of rarity of conspecific mates. Mitochondrial DNA at the cyt-*b* locus was used to show patterns of historical hybridisation and maternal contribution, and microsatellite nuclear DNA analyses helped determine the current extent of population admixture. The comparison to the *C. trifasciatus* group from the same suture zone was useful in highlighting the role of genetic distance between parental species in shaping the consequences of reef fish hybridisation.

Chapter 4

Fitness is multidimensional and often measured using traits directly relating to lifetime reproductive success. To determine if hybrid butterflyfishes are more or less fit than their parent species, Chapter 4 uses a multidisciplinary approach to examine fertility, body condition and growth in *Chaetodon* hybrids from Christmas Island. Qualitative histology was used to confirm viability of *Chaetodon* hybrids. Liver lipid content analysis, based on hepatocyte vacuolation as a proxy, was used for body condition comparisons between hybrids and parental species, because liver is an important energy storage organ in fish (Tocher 2003). Von Bertalanffy growth curves were fit to size-at-age data to ascertain differences (if any) in growth rates and asymptotic length between hybrids and parent species. Comparison of these

traits between parents and hybrids indicates that *Chaetodon* hybrids have similar fitness to their parental species and are likely to persist within the suture zone.

General discussion and conclusions

The studies presented in this thesis represent the most comprehensive assessment of reef fish hybridisation to date. This section of the thesis summarises the findings of individual Chapters and attempts to bring them together to draw generalised conclusions in the context of broader hybridisation theory and literature. The hybridisation scenarios examined here find ground in similar ecological and behavioural contexts but have different molecular consequences. Despite these apparent discrepancies in maternal inheritance and introgression, no detectable difference was found in fitness-related traits between hybrids and parents, indicating that hybrids are likely to persists within the suture zone, but not to supplant their respective parental populations. The inherent rarity of hybrids may contribute to the limited magnitude of their effect over evolutionary and adaptive trajectories of their populations of origin. Nonetheless, both hybrid populations examined here fulfil a very important role in maintaining genetic diversity within the Indo-Pacific suture zone, and this may well increase the adaptive capacity of their parental populations in the face of environmental change.

CHAPTER 1: THE IMPORTANCE OF ECOLOGICAL AND BEHAVIOURAL DATA IN STUDIES OF HYBRIDISATION AMONG MARINE FISHES

1.1 Abstract

Natural hybridisation is a widespread phenomenon, particularly well documented in terrestrial and freshwater ecosystems, where it has been ascribed substantial evolutionary and adaptive relevance. Hybridisation has received comparatively less attention in marine systems, though there has been a recent surge of reported marine hybrids, particularly among corals and fishes. This review summarises the current knowledge of hybridisation in marine fishes, with a focus on ecological and behavioural factors that may play a role in hybridisation processes. Rarity of one or both parental species within the hybrid zone, overlap in habitat use, dietary overlap and the breakdown in assortative mating appear to have a role in facilitating hybridisation. Despite this, most of the recent literature on marine fish hybridisation has a strong genetic focus, with little or no quantitative information about the ecological and behavioural factors that initiate or facilitate hybridisation. Future studies should attempt to gather ecological and behavioural data from hybrid zones, thus teasing out which processes are most relevant to overcoming pre-zygotic barriers to reproductive isolation. Not only will this advance our understanding of the adaptive and evolutionary relevance of hybridisation in marine fishes, but it will also provide unique insights into the maintenance of reproductive isolation and the process of speciation in the marine environment. [1]

1.2 Introduction

This Chapter is published in the journal *Reviews in Fish Biology and Fisheries*: Montanari SR, Hobbs J-PA, Pratchett MS, Herwerden L (2016) The importance of ecological and behavioural data in studies of hybridisation among marine fishes. *Reviews in Fish Biology and Fisheries*, 1-18.

Natural hybridisation is the interbreeding of individuals from two genetically distinct species or populations resulting in viable offspring (Arnold 1997). Natural hybridisation has historically been considered infrequent and of limited ecological or evolutionary relevance, mainly because it challenges the biological species paradigm (Mallet 2005). Interestingly, the presumed rarity of natural hybridisation caused it to be marginalised as unimportant (Mayr 1963). In contrast, rare fitness-increasing mutations have been recognised as milestones for adaptive evolution (Arnold 2006). There is increasing recognition that natural hybridisation can have substantial evolutionary and adaptive significance, increasing or decreasing adaptive capacity and species diversity (Arnold 2006; Abbott *et al.* 2013). To date, more than 25% of plants and 10% of animals have been reported to hybridise, but the true proportion of hybridising species is likely to be higher due to difficulties in detecting hybrids (Mallet 2005).

In just a few decades natural hybridisation can lead to evolutionary novelty (Budd & Pandolfi 2010) and increase the adaptability of a population to changing environments (Grant & Grant 2002; Anderson *et al.* 2009). Furthermore, hybrids have the potential to occupy unexploited ecological niches (Seehausen 2004), and through subsequent reproductive isolation become new species (Smith *et al.* 2003; Verheyen *et al.* 2003; Seehausen 2004). Hybridisation can also contribute significantly to the loss of biodiversity through extinction (Rhymer & Simberloff 1996) or reverse speciation (Seehausen 2006; Taylor *et al.* 2006). Whatever the ultimate outcome, it is clear that hybridisation can play an important role in adaptation and evolution of species.

Most of the research and understanding of natural hybridisation comes from terrestrial and freshwater ecosystems, where there is reportedly a high incidence of hybridisation (Arnold *et al.* 1993; Cruzan & Arnold 1993; Carney *et al.* 1994; Nürnberger *et al.* 1995; MacCallum *et al.* 1998). Traditionally, hybridisation was considered much less common in the marine environment (Hubbs 1955), but was also less well studied. Since 1995, hybridisation has been documented across a broad range of marine plant and animal taxa (Gardner 1997; van Oppen & Gates 2006; Willis *et al.* 2006; Yaakub *et al.* 2006; Hobbs *et al.*

2009; Richards & Hobbs 2015). As for freshwater and terrestrial environments, there appears to be strong taxonomic bias in hybridisation in marine species. Hybridisation is particularly common in marine fishes (Gardner 1997; Yaakub *et al.* 2006), as it is for freshwater fishes (Scribner *et al.* 2000).

The genetic consequences and evolutionary implications of hybridisation in terrestrial and freshwater environments have been widely studied and reviewed (Abbott *et al.* 2013). Numerous molecular studies have also demonstrated the range of genetic outcomes of hybridisation in marine species - reviewed by Richards and Hobbs (2015). However, less attention has been given to the proximal factors responsible for the breakdown in reproductive isolation that leads to hybridisation. Despite a lack of experimental evidence, a number of ecological and behavioural factors have been suggested to increase the incidence of hybridisation in marine fishes. These include: geographic co-occurrence of recently diverged sister taxa that come into secondary contact (McMillan & Palumbi 1995; Mallet 2005); external fertilisation (Hubbs 1955); the breakdown of assortative mating (McMillan *et al.* 1999); overlapping spatial or dietary ecologies that increase heterospecific encounters (van Herwerden *et al.* 2006; Yaakub *et al.* 2006; Marie *et al.* 2007; Yaakub *et al.* 2007; Montanari *et al.* 2012; Montanari *et al.* 2014; Gainsford *et al.* 2015). Local rarity of one or both parental species (Gosline 1948; Randall *et al.* 1977; Frisch & van Herwerden 2006; van Herwerden *et al.* 2006; Marie *et al.* 2007; Hobbs & Allen 2014; Montanari *et al.* 2014) can further favour interbreeding because of the lack of conspecific partners.

Recent reviews have addressed the topic of hybridisation in marine fishes, either to summarise the current knowledge of its consequences and our ability to differentiate it from alternative hypotheses (Richards & Hobbs 2015), or to examine in detail its occurrence in a specific taxon (Hobbs *et al.* 2013). Here we would like to bring the attention to an aspect of hybridisation that has been, in our opinion, overlooked: that is, the ecological and behavioural mechanisms that initiate hybridisation. To this effect, we: i) revise estimates of the incidence of hybridisation in marine fishes; ii) provide a summary of the most commonly reported

ecological and behavioural factors that facilitate hybridisation among marine fishes; iii) identify areas of study where ecological and behavioural data are needed in marine hybridisation studies; and, therefore, iv) suggest an approach for gathering such data, which are necessary to complement the wealth of genetic information currently available. Further, we argue that complementing genetic studies with ecology and behaviour can, not only shed light on the processes that initiate the formation of mixed social groups (thereby setting the scene for hybridisation), but also help characterise the role of hybrid fish taxa within hybrid zones.

We focus our comparison on fishes because hybridisation in this group is common and relatively well studied (Hubbs 1955; Allendorf & Waples 1996; Gardner 1997; Yaakub *et al.* 2006). Where possible, we include first reports of each hybrid exclusively, and separately cite further work if necessary. This review does not examine physiological and genetic factors implicated in hybridisation: although these have a recognized role in hybridisation, they come into play after the ecological and behavioural factors have initiated the hybridisation process (Figure 1). Ecological and behavioural factors can also determine the genetic outcome of hybridisation events (Gainsford *et al.* 2015), however this review will focus on how these factors initiate hybridisation. We have excluded anadromous fishes, some genera of which hybridise extensively - e.g. *Oncorhynchus,* (Ostberg *et al.* 2004), because these hybridisation events occur in freshwater streams. Finally, this review focuses only on natural instances of hybridisation, even though anthropogenic influences can cause fish species to hybridise (Scribner *et al.* 2000; Taylor *et al.* 2006).

Hybrid fishes have historically been identified in the field through the observation of aberrant colour patterns, often deemed intermediate between those of the putative parent species (Randall 1956). This approach is still used today, and numerical predictions of hybrid colour patterns (Miyazawa *et al.* 2010), as well as genetic confirmation of the hybrid status of the intermediate individuals (DiBattista *et al.* 2012; Bernardi *et al.* 2013; Coleman *et al.* 2014; Montanari *et al.* 2014; Gainsford *et al.* 2015) have confirmed its validity. Given the universal

genetic confirmation of suspected hybrids across a wide range of fishes, we include in this review hybrids that have been reported based on morphology and colouration, but are yet to be genetically confirmed. As is the case in terrestrial and freshwater systems (Mallet 2005), hybrid identification based on phenotypes is likely to significantly underestimate the true incidence of hybridisation. Many more hybrid marine fishes are likely to be detected through genetic analyses, as evidenced by the unintentional discoveries of hybrids in phylogenetic studies (Kuriiwa *et al.* 2007).

1.3 Hybridisation in fishes

1.3.1 Historical notes

Naturally occurring hybrid marine fishes have been reported in the scientific literature since the end of the nineteenth century. Holt (1883), for example, reported probable hybrids between two common flatfishes, turbot (*Scophthalmus maximus*) and brill (*S. rhombus*). Since then, at least 111 hybrids, involving 173 marine fish species, have been reported (Table 1). In contrast with the broad temporal distribution of reported occurrences of hybrid freshwater fishes (Scribner *et al.* 2000), the majority (74%) of marine fish hybrids have been reported since 1990. The reported number of naturally hybridising marine fish species has more than doubled since the seminal review by Gardner (1997). This suggests that incidence of hybridisation among marine fishes was previously underappreciated due to limited research on this topic, but the recent plethora of reported marine fish hybrids provides a timely opportunity to compare processes of hybridisation between marine and freshwater fishes.

1.3.2 Taxonomic distribution and genetic relatedness

There is apparent taxonomic bias in the incidence of hybridisation among marine fishes. The families Chaetodontidae and Pomacanthidae account for a combined total of almost 40% of all marine fishes reported to hybridise (Figure 2). Even within the Chaetodontidae, there are considerable biases between clades in the proportion of species that hybridise (Hobbs *et al*. 2013). Of the remaining 26 families of marine fish involved in

hybridisation, 12 (Acanthuridae, Clupeidae, Gadidae, Haemulidae, Hexagrammidae, Labridae, Pleuronectidae, Pomacentridae, Scophthalmidae, Sebastidae, Serranidae and Siganidae) had more than one reported natural hybrid (Figure 2). Taxonomic bias in hybridisation is also evident in freshwater fishes (Scribner *et al.* (2000): 139 reported hybrids, involving 168 species across 19 families (not including cichlids), two of which, the Cyprinidae (40%) and Centrarchidae (20%), accounted for the most hybrids.

Some genetic factors may explain taxonomic bias in hybridisation incidence. For example, the reported extensive chromosome conservatism of chaetodontids (Molina *et al.* 2013) may increase genetic compatibility of hybridising species. Families that contain a high proportion of recently diverged species may also be more prone to hybridise when they come into contact. More than 90% of marine and 68% of freshwater hybrid fishes were congeneric (Scribner *et al.* 2000). Intergeneric hybrids represent the minority and are confined to particular families of marine and freshwater fishes: the Pleuronectidae (87%) and Cyprinidae (96%), respectively (Scribner *et al.* 2000). These genetic patterns to hybridisation suggest there may be a divergence threshold beyond which the ability to hybridise is lost, as shown in terrestrial species (Mallet 2005).

Sufficient genetic relatedness is a *condicio sine qua non* for the successful production of viable hybrids (Mallet 2007). Although it is difficult to summarise the genetic distance between hybridising species, mainly because authors have used different molecular markers in their studies, some examples can provide insights into the range of distances within which hybridisation is successful. In the Chaetodontidae, species diverging as little as 0.7% (McMillan & Palumbi 1995) and as much as 5% at the same mitochondrial marker (cytochrome *b*), have been shown to hybridise successfully and produce viable offspring (McMillan *et al.* 1999; Montanari *et al.* 2012). Similarly, in the Labridae, hybridisation occurs between species (Yaakub *et al.* 2006; Yaakub *et al.* 2007), with reported genetic distances ranging from <2% (Bernardi *et al.* 2004) to >5.5% (Barber & Bellwood 2005). Divergences in the order of 1% (at the molecular markers of focus) are commonly reported for hybridising

fishes in some families (e.g. Acanthuridae (Marie *et al.* 2007) and Serranidae (van Herwerden *et al.* 2006; Craig & Hastings 2007)). In contrast, menhadens of genus *Brevoortia* have been reported to hybridise (Hettler 1968; Dahlberg 1969) despite being as much as 20% divergent (Anderson 2007). Further, reported intergeneric crosses in flatfishes (Norman 1934) involve species as far apart as >25% (Verneau *et al.* 1994), however the latter examples represent a minority of the hybridisations reviewed here (Table 1). Examination of families with a high incidence of hybridisation and reliable published phylogenies reveals that hybridisation is prevalent between species and their closest relative: for example, 63% of cases for the Chaetodontidae (Littlewood *et al.* 2004; Fessler & Westneat 2007) and 42% for Pomancanthidae (Hodge *et al.* 2013; Gaither *et al.* 2014) (Table 1). Thus, it is clear that marine fishes have a propensity to hybridise that spans across a wide range of genetic distances, however hybridisation is most prevalent among closely related species.

1.3.3 Latitudinal distribution

The plethora of recent studies demonstrating hybridisation in coral reef fishes (Richards & Hobbs 2015) disproves the traditional view that hybridisation is rare on coral reefs (Hubbs 1955). Indeed, the majority (almost 70%) of marine fish hybrids have been reported from tropical waters (Table 1). This contrasts with the latitudinal distribution of hybrid fishes in freshwater, where over 90% of the crosses are either temperate or subtropical (Scribner *et al.* 2000). It is not clear whether there is an underlying reason for this apparent latitudinal bias in marine fish hybrid formation, or whether it is merely a reflection of the higher number of species in the tropics, or greater sampling effort and accessibility to shallow tropical reefs. However, the fact that hybridisation is most prevalent in a high diversity system raises the key question as to whether hybridisation has contributed to this diversity, as is the case for African cichlids (Seehausen 2004), which is a topic worthy of further investigation using molecular approaches.

1.4 Ecology of natural hybridisation in fishes

In freshwater fishes, the importance of the ecology of the parent species in facilitating hybridisation has been well documented (Scribner *et al.* 2000); this may also be true for marine fishes. Ecological factors implicated in 48% of freshwater fish hybridisation events were grouped into three categories: 1) rarity of parental species; 2) spatial overlap in habitat use by the parental species; 3) habitat loss, range expansion, limited spawning habitat and unspecified natural factors (Scribner *et al.* 2000). Only one natural hybridisation event in freshwater fish implicated a role for both rarity and overlap in habitat use.

Despite the recent surge in reported cases of natural hybridisation in marine fishes, the majority of studies lack quantitative data on the role of ecological factors (Table 1). Ecological factors are quantified in only 24% of hybrid cases, while circumstantial evidence or hypothetical statements are presented in 22% of cases (Table 1). Where ecological factors were implicated, rarity of one or both parent species was indicated as the primary factor promoting hybridisation in 81% of the reports on hybrid marine fishes (Table 1). In the remaining cases, habitat use overlap was invoked 54% of the time, often in combination with diet overlap. Dietary overlap has received some attention for facilitating marine fish hybridisation (15% of reported cases), always in association with another ecological driver (Table 1). Specific evidence for each of these factors (rarity of parental species, habitat overlap and dietary overlap) is discussed in turn.

1.4.1 Rarity of parental species

Rarity of the putative parental species has been indicated as a facilitator of hybridisation in marine fish since the early work of Hubbs (1955). Intuitively, a lack of conspecific partners increases the chance that an individual will mate with a heterospecific partner (Hubbs 1955). For hybridising marine species, rarity of parent species has been reported in tropical (24 cases), temperate (nine cases) and subtropical (one case) waters (Table 1) (Gosline 1948; Hettler 1968; Fujio 1977; Ayling 1980; Roques *et al.* 2001; Crow *et al.* 2010; Miralles *et al.* 2014; Mirimin *et al.* 2014). Most studies on the hybridisation of fishes do not however, explicitly consider the local abundance of putative parent species

(Feddern 1968; Fischer 1980; Frisch & van Herwerden 2006; Yaakub *et al.* 2006; Marie *et al.* 2007; Maruska & Peyton 2007; Hobbs *et al.* 2009; Montanari *et al.* 2012; Coleman *et al.* 2014; Montanari *et al.* 2014; DiBattista *et al.* 2015), which makes it difficult to comprehensively assess the importance of mate scarcity in the hybridisation of fishes.

Hybridisation in marine fishes is particularly prevalent at the intersection of biogeographic regions, where species often come into contact with sister species (Hobbs *et al.* 2009; Hobbs & Allen 2014; DiBattista *et al.* 2015; Richards & Hobbs 2015). A notable hotspot for hybridisation is Christmas Island (Indian Ocean), where there is overlap of Pacific and Indian Ocean fauna (Hobbs & Salmond 2008). Of the 681 fish species that have been reported at Christmas Island, 286 (42%) are considered rare (< 2 individuals per 3000 m^2) (Hobbs *et al.* 2014), which may promote high levels of hybridisation at this location. Moreover, at least 80% (12 out of 15) of the putative parental species of commonly observed hybrid fishes recorded from this location are rare (Hobbs & Allen 2014). Species are often rare at the extremes of their geographical ranges, but parental rarity can facilitate hybridisation even when the hybrid zone is more central to the species' ranges (Hettler 1968).

In some instances, extreme rarity of a hybridising species results from vagrants (Hobbs *et al.* 2013). Species not known to hybridise within their normal geographic ranges may hybridise as vagrants. Vagrant fishes, straying from their distributional ranges, may hybridise with allopatric sister species or endemics (Severns & Fiene-Severns 1993; Maruska & Peyton 2007; Craig 2008). Parental rarity has also been shown to favour hybridisation at several spatial scales, from individual coral heads to entire sections of the reef (Feddern 1968). The range of magnitudes and spatial scales at which rarity can play a role in initiating hybridisation in tropical marine fishes requires further investigation (Epifanio & Nielsen 2000). Although hybridisation is reportedly common when one parent species is rare, there are several instances where both parent species are common (Hobbs *et al.* 2009; Hobbs *et al.* 2013; Coleman *et al.* 2014). Thus factors other than rarity of a parent species may also play a role in hybridisation.

The lack of a conspecific partner appears to promote hybridisation across the range of mating systems exhibited by marine fishes including: pair spawning, haremic and mass spawning. Hybridisation has been reported in monogamous anemonefishes (Gainsford *et al.* 2015) and pair-forming butterflyfishes (Hobbs *et al.* 2013; Montanari *et al.* 2014). Pygmy angelfishes tend to spend their lives in harems, and hybridising species are often observed in heterospecific harems and interbreeding (Moyer *et al.* 1983; Hobbs *et al.* 2009; Hobbs & Allen 2014). Some species only form harems during spawning times, and these species may also hybridise (Frisch & van Herwerden 2006). For mass-spawning species, Gosline (1948) suggested that congeneric mating might occur where there are too few of one species to initiate reproductive behaviour. Thus, in a range of mating systems, species are deliberately choosing to mate with another species.

Rarity has also been reported to act in synergy with some degree of niche overlap (either spatial or dietary) between the two parent species to breakdown reproductive isolation - e.g. *Hypoplectrus* spp., (Fischer 1980) - (Table 1). In another case of serranid hybridisation, rarity of one species was reported to promote hybridisation in synergy with habitat overlap at several locations along a latitudinal gradient (Frisch & van Herwerden 2006). The concomitant effect of rarity of the parental species and habitat overlap has also been shown to favour hybridisation in the Labridae (Yaakub *et al.* 2006), Acanthuridae (Figure 3) (Marie *et al.* 2007) and Chaetodontidae (Montanari *et al.* 2012; Hobbs *et al.* 2013; Montanari *et al.* 2014) (Table 1). Further ecological assessment of reef fish hybrid zones is required to quantify the relative importance of ecological factors promoting hybridisation. In particular, to determine if local rarity of one or both parental species has a role in initiating hybridisation, abundance surveys should be routinely conducted in the context of hybridisation studies (Figure 1). This type of survey is inexpensive and time efficient, especially when combined with necessary sampling for genetic analyses, and it can further provide a direct estimate of hybrid prevalence. The presence of marine suture zones (Remington 1968), biogeographic borders where multiple species pairs come into secondary contact and hybridise (Hobbs *et al.*

2009), provides the opportunities to investigate the relative contribution of ecological promoters of hybridisation across multiple taxa in the same setting.

1.4.2 Niche overlap

Even if species co-occur within the same geographic location, inter-specific reproduction will be conditional on some level of niche overlap, such that interbreeding individuals co-occur in the same space concurrently. For extreme habitat-specialists, such as anemone fishes or coral-dwelling fishes, inter-breeding species must co-habit the same specific habitat type (Gainsford *et al.* 2015). Anemone fishes have species-specific preferences for their host anemones, and the control of this limiting resource can lead to strong interspecific competition (Gainsford *et al.* 2015). To cohabit an anemone, two species must have the same preference and also be willing to disregard interspecific competition (Gainsford *et al.* 2015). As such, hybridisation among anemonefishes may be somewhat constrained by species-specific use of different microhabitats (Gainsford *et al.* 2015). For more generalist or wide-ranging species, niche overlap may be structured by depth distributions or large-scale habitat preferences. This is important because changes in habitat availability, due to either acute disturbances or sustained degradation of natural ecosystems (Mullen *et al.* 2012), may bring species together that normally occupy very distinct habitats, thereby facilitating hybridisation ((Yaakub *et al.* 2006).

Despite being a necessary precursor to hybridisation, habitat overlap has been articulated only in 20 cases of marine fish hybridisation, almost 28% of which contained no indication of another ecological driver acting in synergy with habitat overlap to facilitate the hybrid formation process (Table 1) (Nichols 1918; Norman 1934; Schultz & Smith 1936; Yaakub *et al.* 2006; Yaakub *et al.* 2007; Mullen *et al.* 2012; Gainsford *et al.* 2015). Fisheries catch and observational data indicated an overlap in the depth range and substrate use of the hybridising species in early works on flatfishes (f. Pleuronectidae) (Nichols 1918; Norman 1934; Schultz & Smith 1936). More recently, in their molecular genetic assessment of a hybrid between the two Caribbean wrasses *Halichoeres garnoti* and *H. bivittatus*, Yaakub *et*

al. (2007) indicated that this hybridisation event might have been driven mainly by habitat use overlap in concomitance with synchronous spawning events (Table 1).

Habitat overlap can increase the likelihood of hybridisation in marine fishes at several spatial scales (Feddern 1968). In hamlets (f. Serranidae), species with broad, fully overlapping depth distributions have been found to hybridise just as readily as species that only share a narrow depth range (Fischer 1980). Broadly overlapping habitats have been shown to promote hybridisation between the menhadens *Brevoortia patronus* and *B. smithi* (f. Clupeidae) (Hettler 1968), as well as the surgeonfishes *Acanthurus achilles* and *A. nigricans* (f. Acanthuridae) (Randall 1956). Conversely, in another case of hybridisation between the surgeonfishes *Acanthurus leucosternon* and *A. nigricans,* the species involved shared a very narrow depth range, where they were also observed foraging together, indicating a possible dietary overlap (Marie *et al.* 2007). *Acanthurus nigricans* is able to setup and defend territories, but may also form roving schools (Marie *et al.* 2007). These are often multispecies assemblages, not formed to defend a resource (Figure 3). Having the same dietary preferences aids in keeping the multispecies groups together, because individuals share a common goal (Figure 3). Avoiding territorial defence and moving in a roving school may, therefore, create opportunities for hybridisation (Marie *et al.* 2007). Habitat overlap can favour hybridisation even when two species have markedly different distributions on a reef: for instance if one species occurs exclusively on the reef flat, where it encounters individuals of the second species straying from their normal reef crest habitat (Yaakub *et al.* 2006). Habitat modifications (e.g. breakwater structures) can lead to hybridisation because species occupying discrete depth zones and habitats come into close proximity and interact (Kimura & Munehara 2010).

Aside from spatial overlap and co-occurrence of inter-breeding species, the capacity to hybridise may also be facilitated by the timing of reproduction (Schultz & Smith 1936; Frisch & van Herwerden 2006). Regardless of the reproductive mode, spatial and temporal overlap of spawning events facilitate hybridisation in compatible species (van Herwerden & Doherty

2006). In 73% of the reported cases, parental habitat overlap was said to be a factor for marine fish hybridisation in synergy with parental rarity, dietary overlap or a combination of the two. In all but two cases (Gosline 1948; Rao & Lakshmi 1993), the authors included quantitative data to illustrate habitat overlap (Randall 1956; Feddern 1968; Hettler 1968; Fischer 1980; Frisch & van Herwerden 2006; Yaakub *et al.* 2006; Marie *et al.* 2007; Montanari *et al.* 2012; Mullen *et al.* 2012; Hobbs *et al.* 2013).

If inter-breeding species co-occur, it does not seem necessary that they also exploit the same dietary resources. However, high levels of dietary overlap may increase encounters between potential heterospecific partners (Grant & Grant 2002). Conversely, very high levels of dietary overlap may lead to levels of inter-specific competition (Blowes *et al.* 2013) that may reinforce reproductive isolation. To assess if this is the case, where possible, competitive interactions between potentially hybridising species should be recorded from the hybrid zone (Figure 1). Among the Chaetodontidae, Hobbs *et al.* (2013) suggested that specialist coral-feeding species were less likely to hybridise than generalist feeders, which may well reflect strong inter-specific competition among species that are coral-feeding specialists. Even so, dietary overlap is suggested to be an important facilitator of hybridisation for at least seven pairs of marine fishes, always in combination with another ecological process (Table 1). In all of these studies, the diets of the putative parents were deemed essentially the same (Randall 1956; Feddern 1968; Fischer 1980; Montanari *et al.* 2012; Montanari *et al.* 2014). Generalist corallivorous butterflyfishes of genus *Chaetodon* come into contact frequently as they are feeding on the same resources and hybridise (Montanari *et al.* 2012; Montanari *et al.* 2014). Some Chaetodontids are territorial and defend food resources, but these two pairs of hybridising sister species are willing to share the same food source, instead of competitively excluding one another (Montanari *et al.* 2012; Montanari *et al.* 2014). Analogous to the research on terrestrial species that identified a threshold for successful hybridisation as less than 10% genetic divergence (that is, > 90% overlap) between parent species, documentation of habitat and dietary preference data within the hybrid zone (Figure 1) would be helpful in

determining the degree of niche overlap that is required for successful hybridisation in marine fishes. Field-based experiments that involve manipulating the amount of food or habitat resources available could be used to identify these thresholds.

1.5 Behaviour of hybridising marine fishes

Most marine fishes spawn gametes resulting in external fertilization and interbreeding between species can occur accidently or deliberately. Accidental hybridisation occurs when two species mate homospecifically at the same time and place and the gametes from different species inadvertently mix, resulting in fertilisation and viable offspring. Accidental hybridisation may be common in other marine groups – e.g. corals (Willis *et al.* 2006), however only three studies explicitly implicate its role in marine fish hybridisation (Gosline 1948; Frisch & van Herwerden 2006; Yaakub *et al.* 2007). For marine fishes, multi-species spawning aggregations do exist (Heyman & Kjerfve 2008; Karnauskas *et al.* 2011) and although accidental hybridisation has occasionally been suggested, no conclusive evidence has been provided (Frisch & van Herwerden 2006; Yaakub *et al.* 2007).

Deliberate interbreeding has been more commonly reported for hybridising marine fishes (16 studies in Table 1). Although this can occur through the deliberate choice of one species and not the other - e.g. sneak spawning (Frisch & van Herwerden 2006), more commonly reported is the formation of heterospecific social groups where both species choose to interbreed. For example, pygmy angelfishes form harems and two species may accept each other in the harem and choose to interbreed (Moyer *et al.* 1983; Hobbs & Allen 2014). Similarly, hybridising butterflyfishes are often observed as a long-lasting heterosexual breeding pair (Hobbs *et al.* 2013; Montanari *et al.* 2014). In damselfishes that lay demersal eggs, not only does hybridisation represent a deliberate choice by both species during courtship and spawning, but it also represents a deliberate choice by a male to care for, and guard, the eggs of another species (Maruska & Peyton 2007; Gainsford *et al.* 2015). Thus, hybridisation in many marine fishes is due to a breakdown in assortative mating through

deliberate choices made by both parent species. These choices may be influenced by local conditions (e.g. a lack of conspecific partners). It is therefore important to carefully document the abundance and temporal stability of mixed social groups (Figure 1), to determine how and to what extent their offspring will influence the hybrid zone population.

1.6 Conclusions and future directions

Natural hybridisation among marine fishes has been underestimated and perhaps overlooked until very recently. In terrestrial and freshwater systems, by contrast, hybridisation is recognised as being not only highly prevalent, but also important in speciation (Seehausen 2004), extinction (Rhymer & Simberloff 1996) and adaptability to novel environments (Grant and Grant 2002). The literature on marine fish hybrids has been dominated by studies documenting hybrids, or more recently, determining the genetic consequences of hybridisation. Much less research has focused on determining the causes of hybridisation. This review has identified the ecological and behavioural processes that have most frequently been ascribed a role in the initiation of hybridisation in marine fishes, and highlighted a general lack of quantitative ecological and behavioural data from within fish hybrid zones. Understanding how ecological and behavioural processes enable species to overcome the barriers to reproductive isolation (e.g. assortative mating) will prove useful in contextualizing the consequences of hybridisation in the marine environment.

Despite being widely acknowledged (Albert *et al.* 2006), the need for quantitative ecological and behavioural data is rarely met in marine fish hybridisation studies (but see (Frisch & van Herwerden 2006; Yaakub *et al.* 2006; Marie *et al.* 2007; Montanari *et al.* 2012; Montanari *et al.* 2014; DiBattista *et al.* 2015; Gainsford *et al.* 2015). Figure 1 provides a framework for gathering ecological and behavioural data at the critical steps in initiating hybridisation and overcoming pre-zygotic barriers to reproductive isolation. Mate choice experiments would be required to test which factors are most important to the breakdown in assortative mating. Surprisingly, there has been a lack of mate choice experiments on

hybridising marine fishes, and the approach used in McMillan *et al.* (1999) illustrates how to test the role of mate choice in the breakdown in assortative mating.

As described above, niche overlap (particularly habitat/microhabitat and diet) has been identified in several hybrid reports as a factor increasing heterospecific encounters between potentially hybridising species. *In situ* surveys documenting habitat and dietary preferences of parental species (Figure 1) can help to quantify the degree of such overlap. Quantification would in turn provide a means to differentiate between species that rarely come into contact within the hybrid zone - leading to rare, evolutionarily irrelevant hybridisation events (Yaakub *et al.* 2007) – and species that, conversely, spend most of their lives together in heterospecific social groups, thereby producing a large number of viable hybrid offspring with rapid evolutionary and adaptive consequences (Taylor *et al.* 2006).

Field studies that document key ecological and behavioural factors (Figure 1) are required to identify the proximal cause for hybridisation in marine fishes. Previous work on hybridising Galapagos finches provides an example of a successful approach. Careful documentation of abundance (and diet) through time enabled the authors to show that hybrid numbers rapidly fluctuated in response to resource availability, resulting in the persistence of the population in times of scarcity (Grant & Grant 2002; Grant *et al.* 2005; Grant & Grant 2008). Similarly, it was thanks to abundance data that the mechanism underlying mixed pair formation was elucidated, namely the choice to mate with more abundant heterospecific partners in response to conspecific rarity mediated by a lack of food resources (Grant & Grant 2002). Further, careful documentation of the ecology and behaviour of hybridising and non-hybridising relatives within the hybrid zone is required to tease out which factors are most important to initiating hybridisation. Similarly, comparisons between ecological conditions inside and outside a hybrid zone will help determine what facilitates hybridisation between sympatric species in some parts of the range but not elsewhere. It is also important to test predictions by considering ecological similarities among closely related species that do not hybridise, despite opportunities to interbreed when co-occurring. Finally, experiments involving the

manipulation of abundance, availability of adult mates, amount and type of food or habitat, could be used on suitable species (e.g. anemonefish) to determine the relative importance of different ecological and behavioural factors in facilitating hybridisation. Determining what conditions cause hybridisation in marine fishes is critical to understanding how marine fishes achieve reproductive isolation and thus initiate the speciation process. Finally, elucidating the causes of hybridisation is necessary to predicting how changing environmental conditions will affect hybridisation.

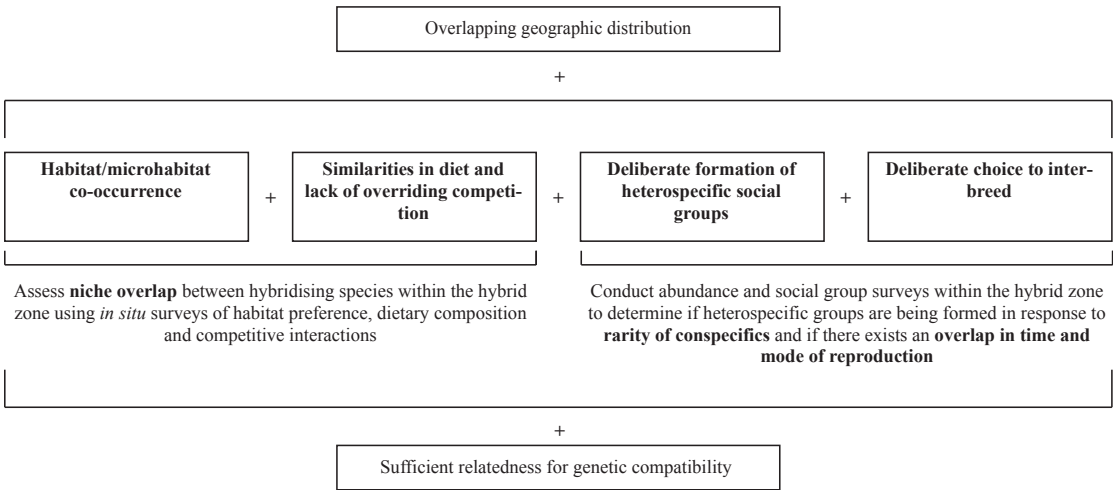

Figure 1.1. Provisional framework for examining the initiation of deliberate hybridisation in marine fishes. Biogeographical distribution and ecology/behaviour lay the foundations for hybridisation to occur, before genetic compatibility determines the outcome of hybridisation. Although overlapping geographic distributions is all that is required to initiate hybridisation, ecological (e.g. niche overlap) and behavioural factors increase the likelihood of hybridisation occurring. Highlighted in bold are the aspects of marine fish hybridisation for which data are largely lacking.

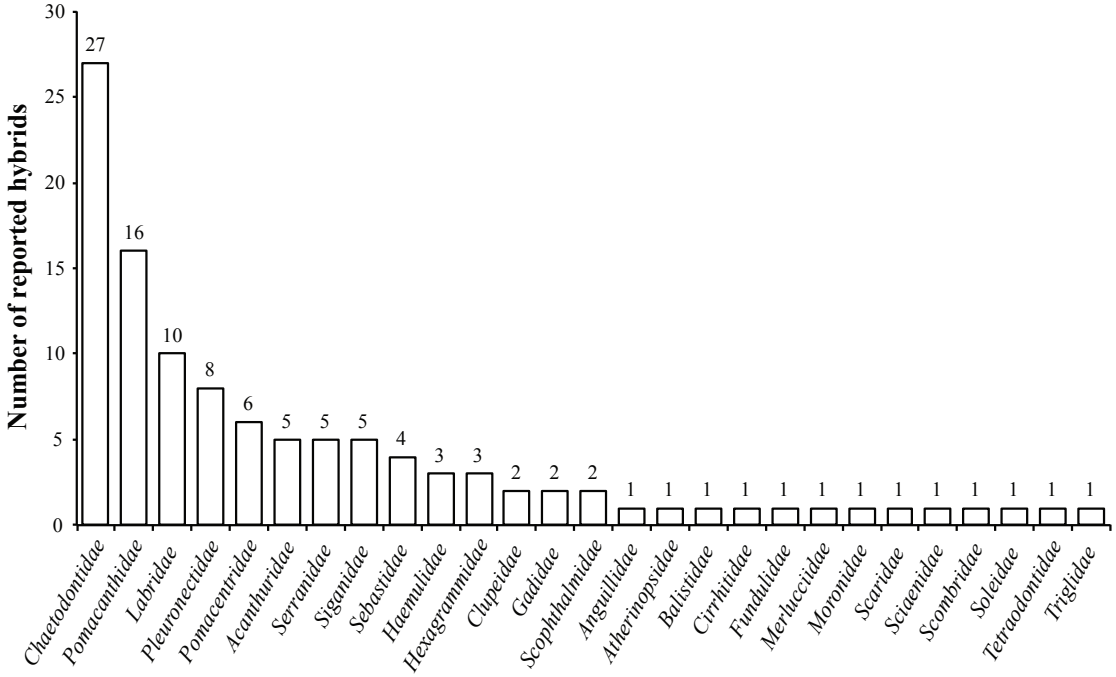

Figure 1.2. Number of hybrid marine fishes grouped by family. Almost 40% of the reported hybrids belong to families Chaetodontidae and Pomacanthidae, two taxa characterised by striking colour patterns and that receive disproportionately high attention from SCUBA divers and aquarium enthusiasts.

Figure 1.3. *Acanthurus leucosternon* (A) in a multispecies roving school with *A. nigricans* (B). Hybridisation between these two species at Christmas Island is mediated by rarity of one parent species and some degree of niche overlap (habitat and diet) (Marie *et al.* 2007).

Table 1.1. Naturally hybridising marine fish species ordered by family. Locations where the hybrids were reported from; general climatic pattern of the waters where the hybridising species are found: tropical (Tr), subtropical (ST) or temperate (Te); ecological factor having a role in the hybridisation, as suggested by the author(s): rarity of one or both parental species (R), overlapping habitat use (H) or dietary overlap (D) of the putative parents and other (O); quantitative ecological/behavioural data are available in the selected hybrid report (Q); circumstantial, anecdotal or hypothetical evidence is provided (C); no ecological/behavioural data are present in the hybrid report (N/A). Sources are mostly first reports: where more than one reference is provided, the more recent studies have further evaluated the same hybrids.

Family	Species 1	Species 2	Location	Climate	Factor	Ecology/behaviour	Source
Acanthuridae	*Acanthurus leucosternon*	*Acanthurus nigricans*	Christmas Is.	Tr	R, H	Q	Marie *et al.* (2007)
Acanthuridae	*Acanthurus japonicus*	*Acanthurus nigricans*	Taiwan	Tr		N/A	Randall and Frische (2000)
Acanthuridae	*Acanthurus achilles*	*Acanthurus nigricans*	Phoenix Is.	Tr	H, D	Q	Randall (1956)
Acanthuridae	*Naso elegans*	*Naso lituratus*	Christmas Is.	Tr	R	Q	Hobbs *et al.* (2009)
Acanthuridae	*Acanthurus lineatus*	*Acanthurus sohal*	Socotra Archipelago	Tr	R	Q	DiBattista *et al.* (2015)
Anguillidae	*Anguilla anguilla*	*Anguilla rostrata*	Iceland	Te	R	C	Albert *et al.* (2006)
Atherinopsidae	*Menidia menidia*	*Menidia beryllina*	Florida	Te	R, H	C	Gosline (1948)
Balistidae	*Melichthys indicus*	*Melichthys vidua*	Christmas Is.	Tr	R	Q	Hobbs *et al.* (2009)

Chaetodontidae	*Chaetodon argentatus*	*Chaetodon mertensii*	N/A	Tr		N/A	Allen *et al.* (1998)
Chaetodontidae	*Chaetodon argentatus*	*Chaetodon xanthurus*	N/A	Tr		N/A	Kuiter (2002)
Chaetodontidae	*Chaetodon auriga*	*Chaetodon ephippium*	Tuamotu	Tr	R	C	Randall *et al.* (1977)
Chaetodontidae	*Chaetodon auriga*	*Chaetodon fasciatus*	Red Sea	Tr		N/A	Gardner (1997)
Chaetodontidae	*Chaetodon austriacus*	*Chaetodon melapterus*	N/A	Tr		N/A	Allen *et al.* (1998)
Chaetodontidae	*Chaetodon burgessi*	*Chaetodon tinkeri*	N/A	Tr		N/A	Allen *et al.* (1998)
Chaetodontidae	*Chaetodon burgessi*	*Chaetodon flavocoronatus*	N/A	Tr		N/A	Allen *et al.* (1998)
Chaetodontidae	*Chaetodon daedalma*	*Chaetodon nippon*	N/A	Tr		N/A	Allen *et al.* (1998)
Chaetodontidae	*Chaetodon ephippium*	*Chaetodon semeion*	Marshall Is.	Tr	R	C	Randall *et al.* (1977)
Chaetodontidae	*Chaetodon ephippium*	*Chaetodon xanthocephalus*	N/A	Tr	R	N/A	Kuiter (2002)
Chaetodontidae	*Chaetodon guentheri*	*Chaetodon daedalma*	N/A	Tr		N/A	Allen *et al.* (1998)
Chaetodontidae	*Chaetodon guentheri*	*Chaetodon oxycephalus*	N/A	Tr		N/A	Kuiter (2002)
Chaetodontidae	*Chaetodon kleinii*	*Chaetodon unimaculatus*	Marshall Is.	Tr	R	C	Randall *et al.* (1977)
Chaetodontidae	*Chaetodon miliaris*	*Chaetodon tinkeri*	Hawaii	Tr	R	C	Randall *et al.* (1977)
Chaetodontidae	*Chaetodon ornatissimus*	*Chaetodon reticulatus*	N/A	Tr		N/A	Allen *et al.* (1998)
Chaetodontidae	*Chaetodon rafflesii*	*Chaetodon vagabundus*	N/A	Tr		N/A	Kuiter (2002)
Chaetodontidae	*Chaetodon*	*Chaetodon rainfordi*	GBR	Tr	R	C	Randall *et al.* (1977)

	aureofasciatus						
Chaetodontidae	*Chaetodon guttatissimus*	*Chaetodon punctatofasciatus*	Christmas Is.	Tr	H, D, R	Q	Hobbs *et al.* (2009); Montanari *et al.* (2014)
Chaetodontidae	*Chaetodon punctatofasciatus*	*Chaetodon pelewensis*	GBR	Tr		N/A	Randall *et al.* (1977)
Chaetodontidae	*Chaetodon trifasciatus*	*Chaetodon lunulatus*	Christmas Is.	Tr	H, D, R	N/A	Hobbs *et al.* (2009); Montanari *et al.* (2012)
Chaetodontidae	*Chaetodon ornatissimus*	*Chaetodon meyeri*	Palau; Christmas Is.	Tr		N/A	Randall *et al.* (1977)
Chaetodontidae	*Chaetodon auriga*	*Chaetodon lunula*	Red Sea	Tr		N/A	Randall *et al.* (1977)
Chaetodontidae	*Chaetodon miliaris*	*Chaetodon multicinctus*	Hawaii	Tr		N/A	Randall *et al.* (1977)
Chaetodontidae	*Chaetodon ocellatus*	*Chaetodon striatus*	Puerto Rico	Tr	O	C	Clavijo (1985)
Chaetodontidae	*Chaetodon collare*	*Chaetodon lunula*	Socotra Archipelago	Tr	R	Q	DiBattista *et al.* (2015)
Chaetodontidae	*Chaetodon gardineri*	*Chaetodon leucopleura*	Socotra Archipelago	Tr	R	Q	DiBattista *et al.* (2015)
Chaetodontidae	*Chaetodon melapterus*	*Chaetodon trifasciatus*	Socotra Archipelago	Tr	R	Q	DiBattista *et al.* (2015)
Cirrhitidae	*Cirrhitichthys calliurus*	*Cirrhitichthys oxycephalus*	Socotra Archipelago	Tr	R	Q	DiBattista *et al.* (2015)

Clupeidae	*Brevoortia patronus*	*Brevoortia smithi*	Florida	ST	R, H	Q	Hettler (1968)
Clupeidae	*Brevoortia smithi*	*Brevoortia tyrannus*	Florida	ST		N/A	Dahlberg (1969)
Fundulidae	*Fundulus majalis*	*Fundulus similis*	Florida	Te		N/A	Duggins *et al.* (1995)
Gadidae	*Gadus morhua*	*Melanogrammus aeglefinus*	Nova Scotia	Te		N/A	Gardner (1997)
Gadidae	*Gadus morhua*	*Gadus morhua*	Baltic Sea; North Sea	Te		N/A	Nielsen *et al.* (2003)
Haemulidae	*Anisotremus virginicus*	*Anisotremus surinamensis*	Brazil	ST		N/A	Bernardi *et al.* (2013)
Haemulidae	*Haemulon flaviguttatum*	*Haemulon maculicauda*	Panama	Tr		N/A	Rocha *et al.* (2008)
Haemulidae	*Haemulon bonariense*	*Haemulon parra*	Venezuela	Tr		N/A	Rocha *et al.* (2008)
Hexagrammidae	*Hexagrammos octogrammus*	*Hexagrammos otakii*	Japan	Te	R	C	Munehara *et al.* (2000); Crow *et al.* (2010)
Hexagrammidae	*Hexagrammos octogrammus*	*Hexagrammos agrammus*	Japan	Te	R	C	Munehara *et al.* (2000); Crow *et al.* (2010)
Hexagrammidae	*Hexagrammos agrammus*	*Hexagrammos otakii*	Japan	Te	R	C	Munehara *et al.* (2000); Crow *et al.* (2010)
Labridae	*Bodianus pulchellus*	*Bodianus rufus*	Brazil	Tr		N/A	Sazima and Gasparini (1999)
Labridae	*Halichoeres garnoti*	*Halichoeres bivittatus*	Belize, Caribbean	Tr	H	C	Yaakub *et al.* (2007)

Labridae	*Notolabrus celidotus*	*Notolabrus fucicola*	NE New Zealand	Te	R	Q	Ayling (1980)
Labridae	*Notolabrus celidotus*	*Notolabrus inscriptus*	NE New Zealand	Te		N/A	Ayling (1980)
Labridae	*Notolabrus fucicola*	*Notolabrus inscriptus*	NE New Zealand	Te		N/A	Ayling (1980)
Labridae	*Notolabrus fucicola*	*Notolabrus tetricus*	SE Australia	Te		N/A	Russell (1988)
Labridae	*Thalassoma jansenii*	*Thalassoma quinquevittatum*	Coral Sea	Tr	R, H	Q	Yaakub *et al.* (2006)
Labridae	*Thalassoma hardwicke*	*Thalassoma quinquevittatum*	Saipan	Tr		N/A	Myers (1999)
Labridae	*Thalassoma lutescens*	*Thalassoma duperrey*	Johnston Atoll	Tr		N/A	Sale (1991); Lobel (2003)
Labridae	*Thalassoma nigrofasciatum*	*Thalassoma quinquevittatum*	Coral Sea	Tr		N/A	Walsh and Randall (2004)
Merlucciidae	*Merluccius capensis*	*Merluccius paradoxus*	South Africa	Te	R	C	Miralles *et al.* (2014)
Moronidae	*Dicentrarchus labrax*	*Dicentrarchus labrax*	Mar Menor	ST		N/A	Lemaire *et al.* (2005)
Pleuronectidae	*Isopsetta isolepis*	*Parophrys vetulus*	Puget Sound, WA USA	Te		N/A	Garrett (2005)
Pleuronectidae	*Limanda limanda*	*Platichthys flessus*	England	Te		N/A	Norman (1934)
Pleuronectidae	*Limanda limanda*	*Pleuronectes platessa*	England	Te		N/A	Norman (1934)
Pleuronectidae	*Platichthys flessus*	*Pleuronectes platessa*	Baltic Sea; England	Te		N/A	Norman (1934)
Pleuronectidae	*Platichthys stellatus*	*Kareius bicoloratus*	Japan	Te	R	C	Fujio (1977)
Pleuronectidae	*Platichthys stellatus*	*Parophrys vetulus*	Puget Sound, WA	Te	H	Q	Schultz and Smith (1936); Garrett *et al.*

			USA				(2007)
Pleuronectidae	*Pleuronectes platessa*	*Glyptocephalus cynoglossus*	Baltic Sea	Te		N/A	Norman (1934)
Pleuronectidae	*Pseudopleuronectes americanus*	*Limanda ferruginea*	New York, NY USA	Te	H	C	Nichols (1918)
Pomacanthidae	*Apolemichthys xanthurus*	*Apolemichthys trimaculatus*	Seychelles; Maldives	Tr		N/A	Pyle and Randall (1994)
Pomacanthidae	*Centropyge eibli*	*Centropyge flavissima*	Christmas Is.	Tr	R	Q	Pyle and Randall (1994); DiBattista *et al.* (2012)
Pomacanthidae	*Centropyge eibli*	*Centropyge vrolikii*	Indonesia	Tr	R	C	Pyle and Randall (1994); DiBattista *et al.* (2012)
Pomacanthidae	*Centropyge flavissima*	*Centropyge vrolikii*	Marshall Is.; Christmas Is.	Tr	R	Q	Pyle and Randall (1994); DiBattista *et al.* (2012)
Pomacanthidae	*Centropyge loricula*	*Centropyge potteri*	Hawaii	Tr	R	C	Pyle and Randall (1994)
Pomacanthidae	*Centropyge bispinosa*	*Centropyge heraldi*	Philippines	Tr		N/A	Pyle and Randall (1994)
Pomacanthidae	*Centropyge bispinosa*	*Centropyge shepardi*	Guam	Tr	R	C	Pyle and Randall (1994)
Pomacanthidae	*Chaetodontoplus caeruleopunctatus*	*Chaetodontoplus septentrionalis*	Japan	Tr		N/A	Pyle and Randall (1994)
Pomacanthidae	*Chaetodontoplus melanosoma*	*Chaetodontoplus septentrionalis*	Indonesia; Taiwan-S Japan	Tr		N/A	Pyle and Randall (1994)

Pomacanthidae	*Holacanthus bermudensis*	*Holacanthus ciliaris*	Florida	Tr	H, D, R	Q	Feddern (1968)
Pomacanthidae	*Paracentropyge multifasciata*	*Paracentropyge venusta*	Philippines	Tr		N/A	Pyle and Randall (1994)
Pomacanthidae	*Pomacanthus arcuatus*	*Pomacanthus paru*	Laboratory*	Tr		N/A	Pyle and Randall (1994)
Pomacanthidae	*Pomacanthus chrysurus*	*Pomacanthus maculosus*	Kenya	Tr		N/A	Pyle and Randall (1994)
Pomacanthidae	*Pomacanthus maculosus*	*Pomacanthus semicirculatus*	Kenya	Tr		N/A	Pyle and Randall (1994)
Pomacanthidae	*Pomacanthus sexstriatus*	*Pomacanthus xanthometapon*	GBR	Tr		N/A	Pyle and Randall (1994)
Pomacanthidae	*Pomacanthus navarchus*	*Pomacanthus xanthometapon*	Aquarium*	Tr		N/A	Pyle and Randall (1994)
Pomacentridae	*Abudefduf abdominalis*	*Abudefduf vaigiensis*	Hawaii	Tr	R	Q	Maruska and Peyton (2007); Coleman *et al.* (2014)
Pomacentridae	*Acanthochromis polyacanthus*	*Acanthochromis polyacanthus*	GBR	Tr	H	C	Planes and Doherty (1997); van Herwerden and Doherty (2006)
Pomacentridae	*Stegastes planifrons*	*Stegastes leucostictus*	Florida	Tr		N/A	Gardner (1997)
Pomacentridae	*Amphiprion chrysopterus*	*Amphiprion sandaracinos*	PNG	Tr	R, H	Q	Fautin and Allen (1997); Gainsford *et al.* (2015)
Pomacentridae	*Amphiprion bicicntus*	*Amphiprion omanensis*	Scocotra	Tr	R	Q	DiBattista *et al.* (2015)

			Archipelago				
Pomacentridae	*Dascyllus carneus*	*Dascyllus margintus*	Scocotra Archipelago	Tr	R	Q	DiBattista *et al.* (2015)
Scaridae	*Chlorurus perspicillatus*	*Chlorurus sordidus*	Hawaii	Tr		N/A	Randall (2005)
Sciaenidae	*Argyrosomus inodorus*	*Argyrosomus japonicus*	South Africa	Te	R	C	Mirimin *et al.* (2014)
Scombridae	*Scomberomorus commerson*	*Scomberomorus guttatus*	India	Tr	R, H, O	C	Rao and Lakshmi (1993)
Scophthalmidae	*Scophthalmus maximus*	*Scophthalmus rhombus*	Baltic Sea; North Sea	Te	H	C	Holt (1883); Norman (1934)
Scophthalmidae	*Scophthalmus maximus*	*Scophthalmus maximus*	Baltic Sea; North Sea	Te		N/A	Nielsen *et al.* (2004)
Sebastidae	*Sebastes auriculatus*	*Sebastes caurinus*	Puget Sound, WA USA	Te		N/A	Seeb (1998); Buonaccorsi *et al.* (2005)
Sebastidae	*Sebastes auriculatus*	*Sebastes maliger*	Puget Sound, WA USA	Te		N/A	Seeb (1998); Buonaccorsi *et al.* (2005)
Sebastidae	*Sebastes caurinus*	*Sebastes maliger*	Puget Sound, WA USA	Te		N/A	Seeb (1998)
Sebastidae	*Sebastes fasciatus*	*Sebastes mentella*	S Newfoundland	Te	R, H	C	Roques *et al.* (2001)
Serranidae	*Hypoplectrus aberrans*	*Hypoplectrus nigricans*	Panama	Tr	H, D	Q	Fischer (1980)

Serranidae	*Hypoplectrus unicolor*	*Hypoplectrus puella*	Jamaica	Tr	R, H	Q	Fischer (1980)
Serranidae	*Hypoplectrus aberrans*	*Hypoplectrus puella*	Panama	Tr	H, D	Q	Fischer (1980)
Serranidae	*Hypoplectrus puella*	*Hypoplectrus indigo*	Panama	Tr	R, D	Q	Fischer (1980)
Serranidae	*Plectropomus maculatus*	*Plectropomus leopardus*	GBR	Tr	R, H	Q	Frisch and van Herwerden (2006)
Siganidae	*Siganus guttatus*	*Siganus lineatus*	Philippines	Tr		N/A	Kuriiwa *et al.* (2007)
Siganidae	*Siganus doliatus*	*Siganus virgatus*	Philippines	Tr		N/A	Kuriiwa *et al.* (2007)
Siganidae	*Siganus corallinus*	*Siganus puellus*	Palau	Tr		N/A	Kuriiwa *et al.* (2007)
Siganidae	*Siganus fuscescens*	*Siganus canaliculatus*	Japan	ST		N/A	Kuriiwa *et al.* (2007)
Siganidae	*Siganus unimaculatus*	*Siganus vulpinus*	Philippines	Tr		N/A	Kuriiwa *et al.* (2007)
Soleidae	*Solea aegyptiaca*	*Solea senegalensis*	France; Tunisia	ST		N/A	She *et al.* (1987); Ouanes *et al.* (2011)
Tetraodontidae	*Arothron nigropunctatus*	*Arothron mappa*	Christmas Is.	Tr	R	Q	Hobbs *et al.* (2009)
Triglidae	*Prionotus alatus*	*Prionotus paralatus*	Alabama, USA	ST		N/A	McClure and McEachran (1992)

* Not included in the summary calculations and discussion presented here, because the hybridisations were not observed in the wild

CHAPTER 2: ISOLATION AND CHARACTERIZATION OF TWENTY MICROSATELLITE MARKERS FOR THE STUDY OF HYBRIDIZATION IN BUTTERFLYFISH OF THE GENUS *CHAETODON*

2.1 Abstract

Twenty polymorphic microsatellite loci were developed via 454 sequencing for two hybridizing sister species of butterflyfish: the spot-band butterflyfish (*Chaetodon punctatofasciatus*) and peppered butterflyfish (*C. guttatissimus*), which are widely distributed in the Western Pacific and Indian Ocean, respectively. All loci were genotyped in samples collected from Christmas Island: *C. guttatissimus* (n = 25), *C. punctatofasciatus* (n = 17) and hybrids (n = 16). Mean alleles per locus (*Na*) were: 9.05 for *C. guttatissimus*, 9.95 for *C. punctatofasciatus* and 9.45 for hybrids. Observed heterozygosity (*HO*) ranged from 0.00 to 1.00 for *C. guttatissimus*; from 0.08 to 0.88 for *C. punctatofasciatus*; and from 0.19 to 0.94 for hybrids. Most loci conformed to Hardy–Weinberg expectations, were in linkage equilibrium, and did not contain null alleles. These markers will be useful for testing population genetic hypotheses including patterns of hybridization in this pair of butterflyfishes.

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Hybridization is widespread in coral reef fishes, but its consequences are still poorly understood. Uni- or bidirectional parental contributions, and presence or absence of introgression, can all characterize reef fish hybridization (McMillan *et al.* 1999; Yaakub *et al.* 2006; Montanari *et al.* 2012). The relative importance of these processes may be explained by the magnitude of the genetic distance between parental species (Mallet 2005) but this is not known for most species.

Butterflyfishes (f. Chaetodontidae) are a young reef fish family (Cowman & Bellwood 2011) characterized by having high levels of gene flow among geographically isolated populations (Lawton *et al.* 2011b) and a high proportion of hybridizing species (Hobbs *et al.* 2013). The peppered butterflyfish (*Chaetodon guttatissimus*) and the spot-band butterflyfish (*Chaetodon punctatofasciatus*) are allopatric sister species with large geographical ranges spanning the Indian and Western Pacific Oceans (Allen *et al.* 1998). Both taxa occur at Christmas Island (Indian Ocean, Australia) at the edge of their distributions (Hobbs & Salmond 2008) and hybridize at this reef fish suture zone (Hobbs *et al.* 2009).

Another pair of butterflyfishes hybridize at Christmas Island (Montanari *et al.* 2012). Comparisons between these hybridizations will allow to identify patterns of directionality and introgression in reef fish hybridization. To examine the specific consequences of hybridization in the *C. punctatofasciatus – C. guttatissimus* complex, we developed markers for the parental species, rather than attempting cross-amplification of available markers (Lawton *et al.* 2010).

All loci were developed from *C. punctatofasciatus* DNA, extracted using Gentra Puregene (Qiagen). DNA (1 μg) was shotgun sequenced on 12.5% of a Roche GS-FLX (AGRF, Brisbane, Australia) (Gardner *et al.* 2011). All 454 sequencing results were deposited on Dryad (Meglécz *et al.* 2012) with doi:10.5061/dryad.jd183.

A total of 113,361 reads (average sequence length = 348 bp; total GC content = 43.07%) were screened for di-hexanucleotide repeats using the default settings of QDD (Meglécz *et al.* 2010). Microsatellite coverage was 0.03%, within the average obtained for the

Actinopterigii (Meglécz *et al.* 2012). PCR primers with the lowest pair penalty (Meglécz *et al.* 2010) were synthesized for 24 tri-hexanucleotide loci.

Initial testing for amplification and marker diversity was performed on five individuals of each taxon. PCR contained 1 X Type-it Multiplex PCR Master Mix (Qiagen), 20–50 ng template, and 0.2 M each primer. Each tailed forward primer and a reporter primer (5' TET-labeled) were mixed at a 1:4 ratio (total = 0.2 M) for indirect labeling (Shimizu *et al.* 2002). PCRs included an initial denaturation of 95°C for 5 min followed by 28 cycles of 95°C for 30 s, 60°C for 90 s and 72°C for 30 s followed by 30 min at 60°C on a Bio-Rad C1000 Thermal Cycler (Bio-Rad). Genotypes were run on an ABI 3730XL Genetic Analyzer (Applied Biosystems) with a LIZ-500 size standard and scored using Microsatellite Plugin v1.1.0 (Biomatters Ltd.).

Twenty loci reliably amplified and were polymorphic (Table 1). All these were directly fluoro-labeled and genotyped in 58 individuals collected from Christmas Island, using Chelex-extracted DNA: *C. guttatissimus* (n = 25), *C. punctatofasciatus* (n = 17) and hybrids (n = 16). Multiplexing (Table 1) was carried out using PCR conditions described above.

Number of alleles (*Na*), observed (*HO*) and expected (*HE*) heterozygosities and probabilities of departure from Hardy–Weinberg Equilibrium (HWE) were calculated using the R package *adegenet* (Jombart 2008). GENEPOP v4.2 (Rousset 2008) and MICROCHECKER v2.2.3 (van Oosterhout *et al.* 2004) were used to check for linkage disequilibrium and null alleles. CERVUS v3.0 (Kalinowski *et al.* 2007) and COLONY v2.0.4 (Wang 2004) were used to calculate Polymorphic Information Content (PIC) and sibship probabilities.

For *C. guttatissimus HO* ranged from 0.00 to 1.00 and for *C. punctatofasciatus* from 0.08 to 0.88 (Table 1). Mean *Na* was 9.05 ± 0.84 SE and 9.95 ± 0.69 SE, respectively (Table 1). Eight loci departed significantly from HWE in the parental species (Table 1), possibly due to siblings in the sample. All loci departing from HWE showed presence of null alleles (frequency = 0.16-0.48), with the exception of Cpun3 in *C. punctatofasciatus*. Two between-

locus comparisons deviated from linkage equilibrium (Bonferroni-adjusted α = 0.0026; *C. punctatofasciatus*: Cpun6-Cpun7, $p = 0.001$ and Cpun19-Cpun21, $p < 0.001$).

For hybrids, mean *Na* was 9.45 ± 0.77 SE and *HO* ranged from 0.19 to 0.94 (Table 1). Four loci departed significantly from HWE (Table 1): all but Cpun17 displayed evidence of null alleles (frequency = 0.13-0.30), whereas no loci deviated from linkage equilibrium.

Markers reported here are polymorphic, amplify in two sister species of genus *Chaetodon* and will be used for resolving population structure, patterns of hybridization and speciation in this species pair and closely related taxa.

Table 2.1. Primer sequences and characteristics of 20 microsatellite loci: number of alleles (*Na*), observed (H_O) and expected (H_E) heterzygosity, probability of departure from HWE (*p*), Polymorphic Information Content (PIC) and estimated null-allele frequency (NULL). *Chaetodon guttatissimus* (n = 25, GUT), hybrids (n = 16, HYB) and *C. punctatofasciatus* (n = 17, PUN) were all collected from Christmas Island.

Locus	Primer sequences (5'-3')	Motif	T_a(°C)	*Na* GUT HYB PUN	H_O GUT HYB PUN	H_E GUT HYB PUN	*p* GUT HYB PUN	**PIC** **NULL**	GenBank Accession #
Cpun1[1]	F: **FAM**GTGGAGGCAACAGAACAGGT R: GGCCTTCATCTCACAGCTTC	$(TCA)_8$	60	2 3 7	0.08 0.19 0.39	0.08 0.17 0.67	1.000 0.982 **0.000****	0.31 0.28	KC699732
Cpun2[1]	F: **VIC**CATCAGAGGAAGCGAAGACC R: GCCCTTGAAGCAGTCTGAAG	$(CAA)_8$	60	4 4 4	0.40 0.50 0.56	0.44 0.54 0.53	0.220 0.973 0.984	0.45 0.05	KC699733

Locus	Primer sequences (5'-3')	Motif	T_a(°C)	*Na* GUT HYB PUN	H_O GUT HYB PUN	H_E GUT HYB PUN	*p* GUT HYB PUN	**PIC** **NULL**	GenBank Accession #
Cpun3[2]	F: **FAM**TTCCTCTCCTATCTGACGCC R: TCTGAGCGACAACAATGAGC	$(GGA)_8$	60	6 5 6	0.36 0.37 0.53	0.62 0.65 0.63	**0.007**** 0.033* **0.001****	0.61 0.20	KC699734
Cpun4[1]	F: **PET**GCTTGAGGTTCAACACGGAT R: AAGGAGCTCGCACAAATCAC	$(GTT)_8$	60	11 9 11	0.40 0.31 0.53	0.83 0.83 0.88	**0.000**** **0.001**** **0.008****	0.85 0.35	KC699735
Cpun6[1]	F: **NED**ACCCTTCCCTACATGCTCCT R: TGCACATATGCATTCATCTCC	$(GGA)_{10}$	59	8 9 10	0.79 0.69 0.76	0.79 0.82 0.77	0.285 0.593 0.973	0.78 0.03	KC699736

Locus	Primer sequences (5'-3')	Motif	T_a(°C)	*Na* GUT HYB PUN	H_O GUT HYB PUN	H_E GUT HYB PUN	*p* GUT HYB PUN	**PIC** **NULL**	GenBank Accession #
Cpun7[2]	F: **NED**CGAAGTCACTCTGAACGCTG R: AGTCAACACAGGAGCGACG	$(AGG)_{10}$	60	9 8 8	0.54 0.73 0.50	0.80 0.80 0.81	**0.000**** 0.222 0.050*	0.83 0.19	KC699737
Cpun9[3]	F: **FAM**CACAATGCCAGCAATGATCT R: GCTGAAGTGCAGAATGATGG	$(GAG)_{11}$	59	10 9 8	0.18 0.46 0.35	0.85 0.87 0.80	**0.000**** **0.007**** **0.001****	0.86 0.46	KC699738
Cpun10[2]	F: **VIC**CCTTTAACGAGGCAGCTCAC R: AAGTGAAGTGTTTCACCGGG	$(CAT)_{11}$	60	10 10 12	0.60 0.75 0.71	0.78 0.84 0.87	0.074 0.367 0.026*	0.84 0.12	KC699739

Locus	Primer sequences (5'-3')	Motif	T_a(°C)	*Na* GUT HYB PUN	H_O GUT HYB PUN	H_E GUT HYB PUN	*p* GUT HYB PUN	**PIC** **NULL**	GenBank Accession #
Cpun11[2]	F: **PET**AAGTGCGTCCACATTCAACA R: CAGAGCCAACTCCACACTGA	$(GTT)_{11}$	60	16 16 14	1.00 0.94 0.88	0.91 0.92 0.91	0.400 0.427 0.793	0.93 0.00	KC699740
Cpun12[3]	F: **PET**AGGTGGAGAGCAGAAGCAGA R: GTGTGACAGGTGACCCTCCT	$(GGA)_{12}$	60	6 7 7	0.59 0.73 0.67	0.76 0.80 0.72	0.081 0.138 0.765	0.75 0.09	KC699741
Cpun13[3]	F: **VIC**CGTCGTTAAAGCCCTGAGAG R: TCAGAGGTCAAACTGTCGCA	$(GGA)_{12}$	60	11 9 7	0.29 0.67 0.08	0.88 0.88 0.83	**0.000**** 0.073 **0.000****	0.88 0.48	KC699742

Locus	Primer sequences (5'-3')	Motif	T_a(°C)	*Na* GUT HYB PUN	H_O GUT HYB PUN	H_E GUT HYB PUN	*p* GUT HYB PUN	**PIC** **NULL**	GenBank Accession #
Cpun14[4]	F: **VIC**TCAGCAGCACTCCTCTCATC R: GGTGGAAGACACCAGTGAGAC	$(TCT)_{13}$	59	5 11 13	0.20 0.75 0.72	0.19 0.69 0.88	1.000 0.377 0.878	0.64 0.15	KC699743
Cpun15[3]	F: **NED**CAGCATTTGGCTAGCTTGGT R: TGGCAGCTGATCAGAAATGA	$(TAT)_{13}$	60	6 8 12	0.39 0.80 0.76	0.37 0.69 0.86	0.934 0.778 0.252	0.71 0.10	KC699744
Cpun17[4]	F: **FAM**TGAATGGATGAATGGATGGTT R: CCTGGGAGGAGACAAACAGA	$(ATGG)_{10}$	60	10 13 14	0.92 0.73 0.78	0.86 0.88 0.91	0.171 **0.009**** 0.567	0.89 0.05	KC699745

Locus	Primer sequences (5'-3')	Motif	T_a(°C)	*Na* GUT HYB PUN	H_O GUT HYB PUN	H_E GUT HYB PUN	*p* GUT HYB PUN	**PIC** **NULL**	GenBank Accession #
Cpun18[4]	F: **NED**AACAAAGCTTTCAGGCTCCA R: GTCTGTCCACACGTCACAGG	$(CTGT)_{12}$	60	11 10 9	1.00 0.56 0.56	0.88 0.83 0.83	0.794 0.054 0.104	0.88 0.08	KC699746
Cpun19[5]	F: **VIC**TCCTCTCCATCGTCTCCAAC R: GTTGTAGAGGTGCCATGCAG	$(GTCT)_{12}$	60	11 14 13	0.67 0.62 0.41	0.84 0.86 0.84	0.027* **0.007**** **0.000****	0.86 0.19	KC699747
Cpun20[4]	F: **PET**GGCAACTGGGTTCAGATGAT R: CTGTTCGTCCTTGGATTGCT	$(TGGA)_{13}$	60	15 16 16	0.70 0.79 0.69	0.92 0.91 0.92	0.230 0.307 0.102	0.93 0.13	KC699748

Locus	Primer sequences (5'-3')	Motif	T_a(°C)	*Na* GUT HYB PUN	H_O GUT HYB PUN	H_E GUT HYB PUN	*p* GUT HYB PUN	**PIC** **NULL**	GenBank Accession #
Cpun21[5]	F: **PET**CTCTTCTGACGGACGGTGAT R: TGACTTTCTATTGAGCCGCA	$(GACGT)_{10}$	60	16 12 11	0.76 0.87 0.76	0.90 0.88 0.85	0.751 0.384 0.093	0.90 0.06	KC699749
Cpun22[5]	F: **FAM**GAAGGCTGTGCTGACACTGA R: GAGTTTGAAGCCGTGTGGAG	$(AGGAC)_{11}$	60	6 7 8	0.6 0.81 0.83	0.75 0.81 0.82	0.505 0.906 0.665	0.79 0.04	KC699750
Cpun23[5]	F: **NED**GACAGAGCGATGTGCTATGG R: AGGTCCCTCAGCAAGGAGAT	$(GGAGA)_{13}$	60	8 9 9	0.00 0.62 0.47	0.86 0.86 0.81	**0.000**** 0.087 0.038*	0.88 0.41	KC699751

[1,2,3,4,5] Multiplex plate allocation; * $p < 0.05$; ** $p < 0.0167$ after sequential Bonferroni correction (highlighted in bold).

CHAPTER 3: DOES GENETIC DISTANCE BETWEEN PARENTAL SPECIES INFLUENCE OUTCOMES OF HYBRIDISATION AMONG CORAL REEF BUTTERFLYFISHES?

3.1 Abstract

Christmas Island is located at the overlap of the Indian and Pacific Ocean marine provinces, and is a hotspot for marine hybridisation. Here we evaluate the ecological framework and genetic consequences of hybridisation between butterflyfishes *Chaetodon guttatissimus* and *Chaetodon punctatofasciatus*. Further, we compare our current findings to those from a previous study of hybridisation between *Chaetodon trifasciatus* and *Chaetodon lunulatus*. For both species groups, habitat and dietary overlap between parental species facilitate frequent heterospecific encounters. Low abundance of potential mates promotes heterospecific pair formation and the breakdown of assortative mating. Despite similarities in ecological frameworks, the population genetic signatures of hybridisation differ between the species groups. Mitochondrial and nuclear data from *C. guttatissimus* × C. *punctatofasciatus* (1% divergence at cyt *b*) show bidirectional maternal contributions and relatively high levels of introgression, both inside and outside the Christmas Island hybrid zone. In contrast, *Chaetodon trifasciatus* × *C. lunulatus* (5% cyt *b* divergence) exhibit unidirectional mitochondrial inheritance and almost no introgression. Back-crossing of hybrid *C. guttatissimus* × *C. punctatofasciatus* and parental genotypes may eventually confound species-specific signals within the hybrid zone. In contrast, hybrids of *C. trifasciatus* and *C. lunulatus* may coexist with and remain genetically distinct from the parents. Our results, and comparisons with hybridisation studies in other reef fish families, indicate that genetic distance between hybridising species may be a factor influencing outcomes of hybridisation in reef fish, which is consistent with predictions from terrestrially-derived hybridisation theory.

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3.2 Introduction

Hybridisation is often a significant evolutionary force that can erode genetic diversity in natural populations (Abbott *et al.* 2013), but can also contribute to creating and maintaining genotypic novelty (Seehausen 2004; Mallet 2007; Nolte & Tautz 2010; Abbott *et al.* 2013). Hybridisation challenges the assumptions of the biological species concept: to provide a suitable framework for the interpretation of natural hybridisation (Frankham *et al.* 2012), we define species as separate 'genotypic clusters' that remain stable in the face of gene flow (Mallet 2007). Hybridisation can increase genotypic variation, which may be significant in enhancing adaptation to altered or novel environments - e.g. Darwin finches (Grant & Grant 2002) -. Further, hybridisation can have significant effects on evolution through the formation of hybrid swarms - e.g. sticklebacks (Taylor *et al.* 2006) - and stable hybrid lineages, which coexist in sympatry with parental species - e.g. sparrows (Hermansen *et al.* 2011) -. Albeit well understood in terrestrial and freshwater systems, the role of hybridisation in shaping the evolution of marine organisms remains, with a few exceptions - e.g. corals (Willis *et al.* 2006) - , in need of thorough evaluation.

Several ecological and behavioural processes promote natural hybridisation (Willis *et al.* 2013). Closely related species often share similar ecological niches (habitat, diet) and this can increase the frequency of heterospecific encounters - e.g. fire-bellied toads (MacCallum *et al.* 1998) -. Species in low abundance may choose to mate with close relatives when conspecific partners are not available, thus rarity of one or both species within the contact zone might result in the formation of heterospecific social groups - e.g. (Grant & Grant 2008) -, the breakdown of assortative mating (Arnold 1997), and hybridisation. Through ecological observations, the abovementioned studies have identified conditions that favour hybridisation in terrestrial systems but quantitative ecological data are scarce in the marine hybridisation literature (Montanari *et al.* 2012).

Studies have shown a negative correlation between frequency of hybridisation and evolutionary divergence (Edmands 2002; Mallet 2005, 2007): genetic distance, with some

exceptions (Edmands 2002), is considered a good predictor of reproductive isolation (Singhal & Moritz In press). Further, interspecific gene flow mediated by hybridisation (introgression) can occur between species with varying levels of divergence, but appears to be strongest in more closely related species (Mallet 2005). The evolutionary proximity of the parental species facilitates hybridisation because closely related species are more likely to be genetically compatible, and therefore capable of producing viable hybrids (Mallet 2005). Conversely, if divergence is too extensive, successful hybridisation might not be possible due to genetic incompatibility (Mallet 2005; Abbott *et al.* 2013). Geographic locations where hybridisation is most prevalent are ideal to investigate the outcomes of hybridisation in taxa with varying degrees of relatedness, because these narrow areas allow controlling for environmental variation that may influence patterns of hybridisation (Avise 2000).

Suture zones are geographic locations where hybrid zones naturally cluster (Swenson & Howard 2004) and were defined by Remington (1968) as "[bands] of geographic overlap between major biotic assemblages, including some pairs of species or semispecies which hybridise in the zone". In terrestrial suture zones the extent of divergence and reproductive isolation between hybridising species can vary greatly and influence the evolutionary consequences of hybridisation (Moritz *et al.* 2009): here we propose to test this terrestrially derived notion in marine species.

The best known tropical marine suture zone is located at the Indo-Pacific biogeographic border, in the eastern Indian Ocean (Hobbs *et al.* 2009). Here the fish fauna is characterised by an admixture of Indian and Pacific Ocean taxa (Hobbs & Salmond 2008). Typically allopatric sister species make secondary contact at this border, where they form the highest number of reef fish hybrids reported from any marine location (Hobbs *et al.* 2009). Christmas Island, Australia, is an oceanic seamount located on the Indo-Pacific biogeographic border (Allen *et al.* 2007), and its reefs provide a unique location to apply terrestrially derived theory to test ecological frameworks and evolutionary consequences of hybridisation in a tropical marine suture zone.

Butterflyfishes inhabit coral reefs worldwide, are dependent on live coral for food (Cole *et al.* 2008) and shelter (Wilson *et al.* 2013), readily respond to changes in reef environments (Pratchett *et al.* 2008) and thus are ideal candidates to examine effects of hybridisation on adaptation (Grant & Grant 2002). Butterflyfishes are well known for their propensity to hybridise, with more than 50% of species in the family involved in heterospecific pairing and/or interbreeding (Hobbs *et al.* 2013). Hybrids occur mostly along zones where major biogeographic provinces overlap (Hobbs *et al.* 2013), including at least eight butterflyfish species that form hybrids at Christmas Island (Hobbs *et al.* 2009; Hobbs *et al.* 2013). Chaetodon butterflyfishes are an ideal system to investigate reef fish hybridisation because many species are monogamous (Yabuta 1997; Pratchett *et al.* 2006a). Further, even though there may be instances where hybrids go undetected (Hobbs *et al.* 2013), butterflyfish hybrids are generally easy to recognise through intermediate colouration (McMillan *et al.* 1999; Montanari *et al.* 2012; Hobbs *et al.* 2013).

In a previous study of hybridisation between *Chaetodon trifasciatus* and *C. lunulatus* at Christmas Island (Montanari *et al.* 2012), we hypothesised that the magnitude of divergence between hybridising parents might influence patterns of introgression in reef fishes based on comparisons of our results to those from the literature (incorporating several geographic locations and reef fish families). By examining hybridisation between *Chaetodon guttatissimus* Bennett, 1832 and *Chaetodon punctatofasciatus* Cuvier, 1831 at the Indo-Pacific marine suture zone, the present study allows us to control for taxon- and location-specific factors that may influence patterns of introgression in reef fishes. Therefore, the aims of this paper are to: (i) determine the ecological and behavioural context of hybridisation between C. guttatissimus and C. punctatofasciatus by assessing abundance, spatial and dietary overlap, and breeding pair formation in parental species and hybrids; (ii) investigate the genetic mechanisms and evolutionary consequences of hybridisation between these species through analyses of mitochondrial (mt) and nuclear microsatellite DNA; (iii) discuss similarities and differences in ecology, genetics and potential evolutionary trajectories of *C.*

guttatissimus × *C. punctatofasciatus* and *C. trifasciatus* × *C. lunulatus* (Montanari et al. 2012) at the Indo-Pacific suture zone. Specifically we evaluate whether genetic distance between hybridising species influences maternal inheritance and introgression in tropical marine fish.

3.3 Material and Methods

3.3.1 Study location and species

This study was conducted in October-November 2010 at Christmas Island, Australia, in the northeastern Indian Ocean (10°25'-10°34'S, 105°32'-105°42'E) (Figure 1, inset). The peppered butterflyfish, C. guttatissimus (Figure 2A), is wide-ranging in the Indian Ocean, occurring from the East coast of Africa to the Indo-Pacific biogeographic border at Christmas and Cocos (Keeling) Islands (Allen *et al.* 1998). The spot-band butterflyfish, C. punctatofasciatus (Figure 2A), is distributed throughout the Western Pacific Ocean, from Indonesia to the Line Islands and from the Ryukyu Islands to the Great Barrier Reef (Allen *et al.* 1998). Christmas Island is the edge of the respective distributions of these butterflyfishes (Allen *et al.* 1998; Hobbs & Salmond 2008), which form heterospecific pairs at this location (Hobbs et al. 2009) (Figure 2A). Importantly, putative hybrids with colouration intermediate to C. guttatissimus and C. punctatofasciatus (Figure 2B) are seen at Christmas Island.

3.3.2 Hybrid zone ecology

Abundance, depth distribution, and diet surveys

To assess the abundance of all taxa, underwater visual censuses (UVCs) were conducted at nine sites along the accessible coasts (Figure 1). In face of the relative rarity of the focal species, transect size was increased (Thompson 2004) during additional abundance surveys along the north coast (Figure 1). Transect length varied (ranging from 162.1 m to 580.5 m), but all data were standardised, with densities presented as the number of fishes per 3000 m^2. Surveyors swam unidirectionally along depth contours while towing a body board fitted with a Global Positioning System (GPS) receiver. The total area sampled for each of 14

replicate transects was calculated based on independent measures of each GPS track (see Additional Material and Methods in Supporting Information). T-tests were used to assess significant differences in abundance between parental species and hybrids.

To assess depth distribution of the parent species and hybrids, the depth at which individual fishes were first sighted was recorded during UVCs (n = 30 individuals for all taxa). Depth data were examined using a one-way analysis of variance (ANOVA), comparing the mean depth occupied by parent species and hybrids.

In situ three-minute feeding observations – following Pratchett (2005) - were conducted for all individuals recorded during the depth distribution UVCs. To examine dietary overlap between parent species and hybrids, we recorded the number of bites taken from different benthic prey or substrates. Prey items included predominantly scleractinian corals that were categorised based on genus and growth form *sensu* Montanari *et al.* (2012). Dietary composition was analysed using a multivariate analysis of variance (MANOVA), comparing the proportion of bites taken from each prey category by the parents and hybrids. Feeding rates (number of bites over three minutes) were compared between parents and hybrids using a one-way ANOVA, to further identify differences (if any) in feeding behaviour.

Pairing behaviour surveys

During UVCs along the north coast, pair composition was recorded to determine the frequency of assortative pairing behaviour in the C. guttatissimus group. Pairings were noted for all focal fishes encountered, regardless of whether both partners were within the transect area, and therefore included in the abundance counts. Unpaired fishes were small (< 70 mm TL) and most likely juveniles. For each parent species and hybrids, expected pairing frequencies were calculated by multiplying the proportional observed abundances by the number of paired individuals, and observational data were analysed for departures from expectations using a χ^2-test.

3.3.3 Hybrid zone genetics

Sampling and DNA extraction

Samples of C. guttatissimus (n = 25), C. punctatofasciatus (n = 18) and C. guttatissimus × C. punctatofasciatus hybrids (n = 16) were collected within the Christmas Island hybrid zone. Chaetodon guttatissimus samples from outside the hybrid zone were collected at Cocos (Keeling) Islands (n = 18) and Zanzibar (n = 1). Similarly, putative purebred C. punctatofasciatus were collected from the Marshall Islands (n = 7) and Guam (n = 1) in the Pacific Ocean. Individual fish were speared whilst SCUBA diving and fin clips were preserved in 80% ethanol for later genetic analysis. Purebred parental species from locations as far as 7500 km away from the hybrid zone were useful in phylogenetic analyses, to tease apart species-specific genetic signals from the signal obtained from the hybrid zone. Chaetodon citrinellus from Lizard Island were used to root all phylogenetic analyses described below (Fessler & Westneat 2007). DNA was extracted from fin clips using 5% Chelex-100 (Walsh *et al.* 1991).

MtDNA sequences and microsatellite genotypes

Mitochondrial cytochrome (cyt) b primers (McMillan & Palumbi 1995), previously utilised in hybridisation studies of Chaetodon butterflyfishes (Montanari *et al.* 2012), were used to amplify 566 bp of the cyt b gene in all samples. Sequences from Montanari *et al.* (2012) were also used to redraw the relevant haplotype network. Polymerase chain reactions (PCR), PCR evaluation, product purification, sequencing, alignment and manual editing were conducted as described in Montanari *et al.* (2012). Twenty microsatellite markers developed for C. punctatofasciatus (Montanari *et al.* 2013) were used to further examine hybridisation in the C. guttatissimus × C. punctatofasciatus group. PCR and genotyping were performed as described in Montanari *et al.* (2013).

Phylo- and population genetic analyses

In order to identify species-specific and hybridisation signals, phylogenetic relationships were inferred using four approaches: Neighbour-Joining (NJ), Maximum

Parsimony (MP), Bayesian Inference (BI) and Maximum Likelihood (ML). All phylogenetic model parametrisations and cyt b haplotype network constructions were done following Montanari *et al.* (2012) to allow direct comparisons and minimise model-related variation (see Additional Material and Methods in Supporting Information). Population genetic analyses followed the same protocols as described in Montanari *et al.* (2012) and did not include Zanzibar C. guttatissimus (n = 1) or Guam C. punctatofasciatus (n = 1), due to small sample sizes (see Additional Material and Methods in Supporting Information).

Microsatellite genotypes were partitioned into clusters assuming an admixture model with independent allele frequencies between populations, using STRUCTURE v2.3.4 (Pritchard *et al.* 2000). Each value of k (set from 1 to 10) was independently evaluated 20 times, with 1,500,000 iterations following a 100,000-long burn-in (Gilbert *et al.* 2012). The best fit model was chosen with the Evanno method (Evanno *et al.* 2005) implemented in STRUCTURE HARVESTER v0.6.93 (Earl & vonHoldt 2012) and values of Δk plotted and presented as Supporting Information. Admixture coefficients (Q), averaged over the 20 independent runs, were visualised by means of a barplot with credibility regions for $k = 2$ (corresponding to the parental species irrespective of geographic origin). Posterior probabilities, based on microsatellite genotypes, of individuals belonging to six classes (pure parental species, F1 or F2 hybrids and backcrosses in either direction) were calculated using NEWHYBRIDS (Anderson & Thompson 2002). Populations outside the hybrid zone were designated as pure parental species as prior information and the chain was run for 1,500,000 iterations, after 150,000 burn-ins. Probabilities were subsequently averaged at population level. A discriminant analysis of principal components (DAPC) (Jombart *et al.* 2010) was run on all loci to investigate the relationship between the sampled populations.

The STRUCTURE, NEWHYBRIDS and DAPC analyses described above were also run on genotypes from Montanari *et al.* (2012) and added as Supporting Information. By choosing a number of PCs equal to the number of individuals divided by three and a number of DA eigenvectors corresponding to the number of populations minus one in both analyses,

the genotypic variability retained in DAPC was similar between the two hybridising butterflyfish groups. This allowed direct comparisons, thus highlighting key differences in the evolutionary consequences of hybridisation in the two Chaetodon species groups at the Christmas Island suture zone.

3.4 Results

3.4.1 Hybrid zone ecology

Abundance, depth distribution and diet

Hybrid C. guttatissimus × C. punctatofasciatus were relatively common at Christmas Island (2 ± 0.47 SE individuals per 3000 m^2) and at least as abundant as the least common parental species, C. punctatofasciatus (2 ± 0.47 SE) ($t_{(26)}$ = 0.42, p = 0.68) (Table 1). Chaetodon guttatissimus was significantly more abundant (40 ± 4.5 SE) than C. punctatofasciatus ($t_{(13)}$ = 8.32, p < 0.0001) (Table 1).

The ecology (specifically habitat use and dietary composition) of *C. guttatissimus*, *C. punctatofasciatus* and their hybrids was very similar. There was no significant difference in depth distribution between C. guttatissimus (average depth 16.6 m ± 0.49 SE) and C. punctatofasciatus (15.5 m ± 0.49 SE) ($F_{(1, 89)}$ = 3.14, p = 0.08) (Table 1). The parental species occupied relatively narrow, largely overlapping, depth ranges (Table 1). The depth distribution of the hybrids (16.2 m ± 0.50 SE) was not statistically different from that of either parent species ($F_{(2, 29)}$ = 1.47, p = 0.235). Similarly, dietary composition was not significantly different between parent species (Pillai's $Trace_{(34)}$ = 0.51, p = 0.085) (Table 1). Both parental species most frequently fed on encrusting Montipora and massive Porites, which are among the most common coral genera at Christmas Island. The hybrids fed largely on the same prey as their parental species (Pillai's $Trace_{(34)}$ = 0.51, p = 0.085) (Table 1). The feeding rates (number of bites per three minute observation) of parent species and hybrids were not significantly different ($F_{(2, 29)}$ = 2.03, p = 0.14).

Pairing behaviour

The relative number of individuals that paired with conspecifics, heterospecifics or hybrids, was generally proportional to the abundance of these individuals (Figure 3A, Table 1). The pairing behaviour of C. punctatofasciatus did not significantly deviate from the frequencies expected based on abundances ($\chi^2_{(2, n = 30)} = 2.89$, $p > 0.24$), indicating that this species is pairing non-assortatively under these conditions (Figure 3A, Table 1). Chaetodon guttatissimus appeared to actively choose to pair heterospecifically (disassortative mating) ($\chi^2_{(2, n = 264)} = 14.91$, $p < 0.001$), but this may be a statistical artefact of the large sample size for this species (Figure 3A). Hybrids were never observed paired together, and formed pairs with the parental species non-assortatively ($\chi^2_{(2, n = 26)} = 3.25$, $p > 0.19$) (Figure 3A). This indicates that hybrids are likely choosing partners based on their prevalence rather than phenotype.

3.4.2 Hybrid zone genetics

Five hundred and sixty-six base pairs (bp) of the mitochondrial cyt b region were resolved for a total of 86 individuals in the C. guttatissimus group. The alignment contained 92 parsimony informative sites and identified 49 discrete haplotypes (Figure 3B). Twenty microsatellite loci reliably amplified and were scored in 83 individuals: one C. punctatofasciatus from Christmas Island was excluded due to > 20% missing data. Population level tests showed significant departures from HWE in 26 of 100 tests after sequential Bonferroni correction ($\alpha = 0.01$) (Table S1). Eighteen (69%) of these HWE departures were concentrated at five loci (Cpun3, 4, 7, 9 and 13) (Table S1). Null alleles contributed to departures from HWE in all abovementioned loci. Chaetodon punctatofasciatus from Christmas Island had the most private alleles (17) compared to all other taxa in this group (Table S1).

Phylogenetic relationships

Congruent phylogenetic relationships were inferred with four methodologies (NJ, MP, BI and ML) and a clear separation between the two parental clades was strongly supported by

all analyses (Figure 3B). Six fixed nucleotide changes (1% divergence at cyt *b*) separated the two parental species, despite evidence of some interspecific mtDNA exchange (Figure 3B). All C. punctatofasciatus individuals and three of 44 individuals (7%) identified in the field as C. guttatissimus based on colouration were contained in a single clade (Figure 3B). Two of these individuals were from Cocos (Keeling) Islands, outside the hybrid zone of Christmas Island (Figure 3B). Hybrids in the C. guttatissimus group shared haplotypes with both parental clades, indicating a bidirectional maternal contribution to hybridisation (Figure 3B). This contrasts with the C. trifasciatus group, where redrawn haplotype relationships from Montanari *et al.* (2012) show 5% divergence between the parent species at cyt *b*, and all hybrids occur in only one of the two parental clades (unidirectional maternal contribution - Figure S1B).

Population genetic structure

Cytochrome *b* haplotype (h) and nucleotide (π) diversities, as well as gene diversity based on microsatellites (1-Q inter) within the Christmas Island hybrid zone were high for all taxa in the C. guttatissimus group (Table S2). The AMOVA fixation index for mtDNA cyt b was $\Phi_{st} = 0.48$ $p < 0.0001$. Microsatellites indicated a clear separation between parental species and hybrids and had raw $F_{st} = 0.038$, $p < 0.0001$, $D_{est} = 0.115$ and ENA corrected values that were comparable to raw values, indicating low confounding effects from null alleles (Table S4). Nearly all pairwise F_{st} tests were significant for mitochondrial and nuclear markers, and this was further confirmed with D_{est} (Table S3). Genetic structure was evident between parental species irrespective of geographic location (Tables S3). Analyses of cyt b did not detect significant intra-specific structure between populations of either *C. guttatissimus* or C. punctatofasciatus (Table S3A). Microsatellites indicated weak intra-specific structure between C. guttatissimus populations, but not between C. punctatofasciatus populations (Table S3B), possibly due to small sample size of the Marshall Island population. The hybrid population significantly differed from all other populations (Table S3).

Mitochondrial data indicated that hybrids are less differentiated from the parental species within the hybrid zone (Table S3A). However, microsatellite data showed that the hybrids are less differentiated from *C. punctatofasciatus* within the hybrid zone, but more from *C. guttatissimus* (Table S3B).

STRUCTURE identified two clusters (Figure S2A): some admixture was detected between parental species (Figure 3D), both outside and within the hybrid zone - consistent with previous mtDNA and microsatellite analyses. Interestingly, the *C. guttatissimus* population from Cocos (Keeling) showed a slightly higher level of admixture with *C. punctatofasciatus* than the Christmas Island population (Figure 3D). Most notably, the *C. punctatofasciatus* population in the contact zone showed greater levels of admixture than *C. guttatissimus* (Figure 3D), but with high levels of variability in the estimates. The hybrids' intermediacy was evident compared to both parental species, particularly *C. guttatissimus* (Figure 3D). Two clusters were also identified in the *C. trifasciatus* group (Montanari et al. 2012) (Figure S2B): this dataset shows lower levels of parental admixture (particularly in the *C. lunulatus* populations) and hybrid intermediacy is clear in this group (Figure S1D). In both butterflyfish groups however, STRUCTURE lacks the resolution to reliably detect backcrossing and hybrid classes (possibly as a result of the small sample sizes and limited number of molecular markers).

NEWHYBRIDS assigned over 95% of *C. guttatissimus* individuals to their pure species, in both populations of origin (Figure 4). As also suggested in STRUCTURE, the Cocos (Keeling) population had a somewhat greater probability of introgression than the Christmas Island population (Figure 4). The hybrids were clearly intermediate and were mostly either assigned to *C. guttatissimus* or designated as F2 hybrids (Figure 4). Likewise, a similarly high probability of being F2 hybrids (almost 30%) was assigned to the Christmas Island population of *C. punctatofasciatus,* consistent with the suggested pattern of introgression observed in the STRUCTURE analysis (Figures 3D and 4). This contrasts with the *C. trifasciatus* group (Montanari *et al.* 2012), in which both parental species were

assigned to their respective pure clusters with >92% probability irrespective of geographic location (Figure S3). The hybrids in this group had a range of probabilities of being assigned to either parental cluster, F1, F2 or either backcross (10-25%) (Figure S3). Moreover, approximately 60% of assignments were to inter-parental crosses, the remainder being to pure parental clusters (Figure S3). In both NEWHYBRIDS analyses, the standard deviation around the mean posterior probabilities was negligible for all taxa, except for the hybrids, underlining the uncertainty associated with assigning these intermediate individuals.

DAPC examined the relationship between clusters, predefined as combinations of taxon and geographic location (Figure 3C). The hybrid population was distinct from all others and hybrid genotypes were intermediate between parental species' genotypes (Figure 3C). Little partitioning was evident between populations of the same species (Figure 3C), consistent with other analyses. Chaetodon guttatissimus × C. punctatofasciatus hybrids occupied a broad parameter space close to their parental clusters and confidence ellipses were shared in seven of 16 individuals (Figure 3C). In contrast, microsatellite data from Montanari *et al.* (2012), presented in a re-parametrised DAPC (see Material and Methods), show almost no overlap of hybrid and parental genotypes in the *C. trifasciatus* group (Figure S1C).

3.5 Discussion

3.5.1 Hybrid zone ecology

Chaetodon guttatissimus and C. punctatofasciatus as well as C. lunulatus and C. trifasciatus (Montanari *et al.* 2012) have come into secondary contact at the tropical marine suture zone of Christmas Island. Our results highlight several ecological factors that are likely to contribute to the propensity of these species to hybridise. Some degree of habitat overlap is a necessary precursor to hybridisation in sexual vertebrates - e.g. Bombina toads (Vines *et al.* 2003) -. The distributions of C. guttatissimus, C. punctatofasciatus and their hybrids largely overlap at Christmas Island: all taxa occupy sites with similar exposure (north coast) and have

relatively narrow and consistent depth ranges. Habitat overlap was also reported between C. lunulatus and C. trifasciatus (Montanari *et al.* 2012) and has been documented for a large number of hybridising marine fishes (Nichols 1918; Norman 1934; Schultz & Smith 1936; Gosline 1948; Randall 1956; Feddern 1968; Hettler 1968; Fischer 1980; Rao & Lakshmi 1993; Frisch & van Herwerden 2006; Yaakub *et al.* 2006; Marie *et al.* 2007; Yaakub *et al.* 2007; Hobbs *et al.* 2013). Such overlap increases the chance of heterospecific encounters between hybridising butterflyfishes at the Indo-Pacific suture zone.

Chaetodon guttatissimus, C. punctatofasciatus, C. trifasciatus and C. lunulatus are relatively specialised obligate corallivores (Cole *et al.* 2008), and their feeding mode has been confirmed through both gut content analyses (Harmelin-Vivien 1989; Sano 1989) and direct observations (Pratchett 2005). This study and data from Montanari *et al.* (2012) indicated that, in each hybridising group, the two parental species and their respective hybrids fed on the same suite of coral prey. Further, gut content analyses and direct feeding observations in tropical marine fishes belonging to the Acanthuridae (Randall 1956), Pomacanthidae (Feddern 1968) and Serranidae (Fischer 1980) showed, in all cases, that the diets of hybridising parents and hybrids were essentially the same. In synergy with overlap in habitat use, dietary overlap further increases encounter probability between hybridising butterflyfishes at Christmas Island.

Rarity of conspecific mates is considered a promoting factor in hybridisation among terrestrial organisms - e.g. Darwin finches (Grant & Grant 2002) - and reef fishes (Randall *et al.* 1977; Pyle & Randall 1994; van Herwerden *et al.* 2002; Maruska & Peyton 2007; Hobbs *et al.* 2009). Although C. guttatissimus is relatively common at Christmas Island, its sister taxon, C. punctatofasciatus, is rare. At Christmas Island, C. punctatofasciatus occurs in densities 40 – 100 times lower than those found at locations near the centre of its distribution range - e.g. Indonesia and Palau (Findley & Findley 2001) -. The local rarity of C. punctatofasciatus may explain why many of these individuals are found in heterospecific pairs. Chaetodon trifasciatus and C. lunulatus are both rare at Christmas Island (Montanari *et*

al. 2012) and their abundances are one to three orders of magnitude lower compared to any other location for which abundance data are available (Adrim & Hutomo 1989; Findley & Findley 2001; Pratchett *et al.* 2004; Pereira & Videira 2005; Pratchett *et al.* 2006b).

The frequency of heterospecific pair formation was proportional to the abundance of parent species in both the *C. guttatissimus* and *C. trifasciatus* (Montanari *et al.* 2012) hybrid groups at Christmas Island. This supports the hypothesis that rare species (and hybrids) are forming heterospecific pairs based on encounter rates and that a rare species (or hybrid) will choose a partner based on availability rather than the phenotypic identity of the individual. A breakdown in assortative mate choice has been reported for other pair forming Chaetodon butterflyfishes that are known to hybridise (McMillan *et al.* 1999; Hobbs *et al.* 2013). The parent species and hybrids examined in this study and in Montanari *et al.* (2012) belong to subgenera thought to be exclusively monogamous (Pratchett *et al.* 2006a; Craig *et al.* 2010), and indeed examination of the gonads of heterospecific pairs at Christmas Island revealed that these pairs always comprised a mature male and a mature female (Hobbs unpublished data). Therefore, the observed heterospecific breeding pairs are likely producing the hybrids seen at Christmas Island. Overall, our observations indicate that the ecological and behavioural processes that set the scene for hybridisation are similar across Chaetodon butterflyfish hybrid groups at Christmas Island and probably explain the onset of hybridisation in pair-forming butterflyfishes elsewhere (Hobbs *et al.* 2013).

3.5.2 Hybrid zone genetics

Mitochondrial and nuclear DNA analyses confirmed hybridisation in both the C. guttatissimus and C. trifasciatus (Montanari *et al.* 2012) groups. However, despite similarities in the ecological context of hybridisation in the two complexes, the genetic mechanisms are clearly different. In C. guttatissimus - C. punctatofasciatus, which are 1% divergent at cyt b as measured in this study, hybrids shared mtDNA with both parental clades, indicating bidirectional maternal contribution to hybridisation, a mode previously reported in reef fishes (McMillan *et al.* 1999; van Herwerden & Doherty 2006). This is consistent with field

observations of heterospecific pairs in which females were identified as either C. guttatissimus or C. punctatofasciatus, but in contrast with unidirectional mitochondrial inheritance in C. trifasciatus × C. lunulatus, 5% divergent at cyt b (Montanari *et al.* 2012). In previous studies of reef fish hybridisation, most or all hybrids reportedly shared haplotypes with the more abundant parental species, suggesting sneak mating by males of the rare species with females of the common species, likely due to rarity of conspecifics (van Herwerden *et al.* 2006; Yaakub *et al.* 2006; Marie *et al.* 2007). In both cases of Chaetodon hybridisation examined here and in Montanari *et al.* (2012), hybrids shared most (or all) haplotypes with the rarest of contributing parents. Although this could be an artifact of small samples sizes (inherent to hybridisation studies, where hybrid taxa are often rare), females of the rare parent species appear to actively choose to mate with males of the more abundant sister species, probably due to the lack of conspecific males. In order to discriminate whether these results are consistent with female-mediated partner choice (Wirtz 1999), or represent selection against offspring that result from the opposite cross, further enquiry should be directed toward hybrid fitness in Chaetodon butterflyfish.

Mitochondrial introgression was detected in Christmas Island C. guttatissimus individuals, which shared haplotypes with C. punctatofasciatus. This supports backcrossing of hybrid females with C. guttatissimus males. Microsatellite analyses also showed nuclear introgression in either direction, but mostly toward *C. punctatofasciatus*. The detection of both mtDNA and nDNA introgression in this group is perhaps not surprising, given the close genetic proximity of the parent species (Mallet 2005). Introgressed individuals were all identified as pure parents based on colouration, indicating that assessment of hybrid abundance based on colouration alone can lead to underestimation (Hobbs *et al.* 2013). Some "purebred" C. guttatissimus from Cocos (Keeling) Islands also had C. punctatofasciatus mtDNA and nDNA even though hybrids have never been observed at this location. Larval dispersal from Christmas to Cocos (Keeling) Islands (facilitated by westward flowing surface currents) might explain the presence of these individuals (Yaakub *et al.* 2006; Craig 2008).

Previous studies of reef fish hybridisation showed that gene flow between the parent species was either bidirectional or directed from the abundant maternal species to the rare paternal species (McMillan *et al.* 1999; van Herwerden *et al.* 2006; Yaakub *et al.* 2006; Marie *et al.* 2007). Unidirectional mtDNA introgression - or lack thereof, as in the C. trifasciatus group (Montanari *et al.* 2012) – indicates that a partial barrier to gene flow is still present, perhaps due to assortative mating or selection against hybrids (Rhymer & Simberloff 1996). Assortative mating is unlikely, because our observations indicate that, in both groups, pairs are formed bidirectionally, and hybrids pair with either parental species, providing the opportunity for backcrossing. Further, the admixture detected in nDNA shows that the historic hybridisation suggested by the mtDNA introgression is ongoing, and that hybrids are still contributing to interspecific gene flow.

An alternative interpretation of our detection of mtDNA and nDNA introgression between C. guttatissimus and C. punctatofasciatus is incomplete lineage sorting. Recent and robust phylogenies of the Chaetodontidae based on two mtDNA and rRNA markers unequivocally partition the two sister species, suggesting that the lineages have sorted completely (Littlewood *et al.* 2004; Hsu *et al.* 2007). Moreover, our phylogenetic analyses have shown that C. guttatissimus and C. punctatofasciatus populations sampled from locations most distant from the hybrid zone have distinct, species-specific mtDNA haplotypes. However, detection of introgressed individuals outside the hybrid zone points to possible incomplete lineage sorting, because allopatric populations of these species show some degree of admixture, irrespective of the geographic distance between them. To discriminate between this scenario and introgressive hybridisation, further studies should include more samples across the distribution ranges of these species, and apply genotyping-by-sequencing techniques to increase resolution.

3.5.3 Consequences of hybridisation

Contrary to what has been observed in the Solomon Islands – Papua New Guinea hybrid zone involving C. punctatofasciatus and *C. pelewensis* (McMillan *et al.* 1999) and in

another hybridising fish, Acanthurus leucosternon, at Christmas Island (Marie *et al.* 2007), the introgressive hybridisation between C. guttatissimus and C. punctatofasciatus is not strong enough to swamp species-specific signals. Although this pattern could be the result of chance given the small sample size, our data suggest that divergence between C. guttatissimus and C. punctatofasciatus is decreasing within the hybrid zone and gene flow mediated by the hybrids appears to be ongoing. Persistence of hybrids and introgressed individuals at Christmas and Cocos (Keeling) Islands may eventually confound species signals in the C. guttatissimus hybrid group, resulting in a hybrid swarm *sensu* Taylor *et al.* (2006). Alternatively, the presence of novel genotypes (and the high genetic diversity) in the hybrid population at Christmas Island may one day enable hybrids to exploit niches not occupied by parent species. This process was documented in terrestrial - Geospiza Darwin finches (Grant & Grant 2002) - and freshwater environments - cichlids (Seehausen 2004) -, and can lead to the formation of new species (Seehausen 2004). Long term monitoring of the reef fish suture zone at Christmas Island (Hobbs *et al.* 2009; Arnold & Martin 2010), through regular assessment of hybrid prevalence and genotypic make up across a wide range of taxa, could further elucidate the ecological and evolutionary relevance of hybridisation in reef fishes.

The scenario emerging from the C. trifasciatus hybrid group (Montanari *et al.* 2012) appears different to that of the *C. guttatissimus* group. Lack of introgression, evident both in mtDNA and microsatellites, and unidirectional mtDNA inheritance in the *C. trifasciatus* group indicate that interspecific gene flow mediated by hybrids is minimal at Christmas Island. Even though failure to detect significant levels of introgression in this group could be due to sample size, the sample sizes in the two groups were similar, leading us to expect similar power of detection in both hybridising groups. Interestingly, in the *C. trifasciatus* group, a Zanzibar individual identified in the field as *C. trifasciatus* showed almost 2% divergence at cyt *b* from its putative species clade (Montanari *et al.* 2012): this could be a rare backcross with a hybrid formed between *C. trifasciatus* and other members of Corallochaetodon that occur in that area (e.g. *C. melapterus* – B. Bowen, pers. comm.). This

needs further work to be confirmed, but if found to be true could indicate that barriers to gene flow are permeable in Corallochaetodon, despite the apparent lack of backcrossing at Christmas Island (Montanari *et al.* 2012).

The rarity of both parent species and hybrids in the *C. trifasciatus* group may prevent detection of introgression and bidirectional maternal contribution at Christmas Island (Montanari *et al.* 2012). In this group, the measured 5% divergence at cyt b (Montanari *et al.* 2012) appears to be large enough to generate genotypic novelty in the form of a persistent sympatric hybrid taxon, albeit small enough to warrant successful hybridisation (Mallet 2005). Nuclear microsatellite DNA data were particularly informative for this group, confirming the hybrids' status as hybrids rather than aberrant colourations of *C. lunulatus*, a possibility not ruled out by previous mtDNA analyses (Montanari *et al.* 2012). Microsatellites further showed that hybrid genotypes are intermediate and different to those of the parent species, even within the hybrid zone, thus maintaining their genotypic identity despite extensive ecological, behavioural and reproductive contact with parental species. Hybrid genotypes or hybrid species sometimes colonise environments distinct to those of their parents, as observed for example in cichlids and sculpins (Seehausen 2004; Nolte *et al.* 2006). However, sympatric hybrid coexistence with parental forms does occur – sparrows (Hermansen *et al.* 2011); swallowtail butterflies (Kunte *et al.* 2011) -, and this could be the case for C. trifasciatus × C. lunulatus hybrids at Christmas Island.

The apparent negative interaction between extent of divergence and introgression highlighted in this study finds further validation when data from other hybridising reef fishes are examined. As noted in Montanari *et al.* (2012) for example, in the Solomon Islands, hybridisation between *C. punctatofasciatus* and *C. pelewensis* (McMillan *et al.* 1999), divergent by 0.7% at cyt *b* (McMillan & Palumbi 1995), results in extensive bidirectional introgression (McMillan *et al.* 1999). This interaction holds true even in families other than the Chaetodontidae. In the Labridae, bidirectional introgression was detected in hybridising *Thalassoma jansenii* and *T. quinquevittatum* (Yaakub *et al.* 2006), divergent by less than 2%

at cyt *b* (Bernardi *et al.* 2004). Conversely, in *Halichoeres garnoti* and *H. bivittatus,* divergent by >5.5% based on three mtDNA markers (Barber & Bellwood 2005), hybridisation did not result in introgression (Yaakub *et al.* 2007). In the Acanthuridae, hybridisation between *Acanthurus leucosternon* and *A. nigricans*, 1% divergent at mtDNA COI, was introgressive and bidirectional (Marie *et al.* 2007). In hybridising Serranids *Plectropomus leopardus* and *P. maculatus,* 1% divergent based on two nuclear and two mtDNA markers (Craig & Hastings 2007), hybridisation was highly introgressive, but the maternal contribution was unidirectional (van Herwerden *et al.* 2006).

Further inquiry should be aimed at evaluating the relative importance of divergence levels in shaping the evolutionary outcomes of reef fish hybridisation, and to test whether reef fish have a threshold of divergence beyond which their ability to hybridise is lost, as suggested for terrestrial species (Mallet 2005). Given their position at the Indo-Pacific marine suture zone, Christmas and Cocos (Keeling) Islands could provide an ideal location for these future studies. Further, application of genomic tools may identify adaptive genes that are differentiated between hybridising reef fish species, which will provide insights into adaptation and selection for hybrid genotypes in environments that are novel compared to those inhabited by the parental species outside the hybrid zone.

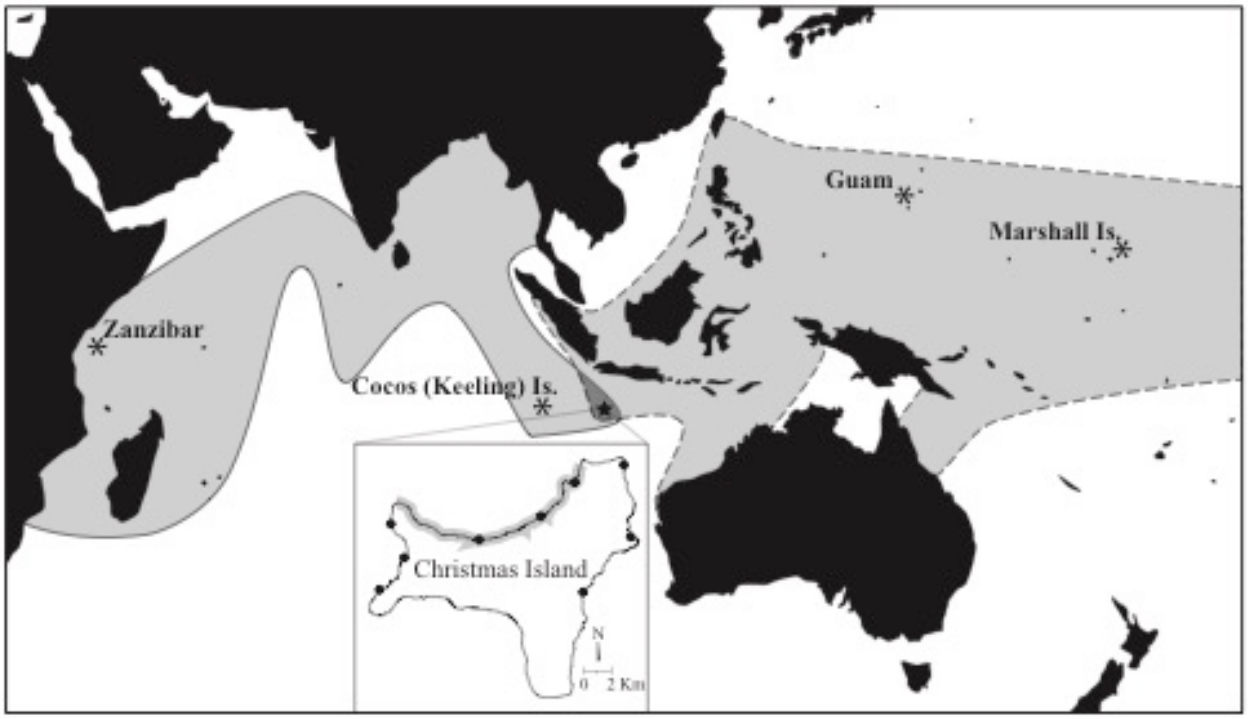

Figure 3.1. Map showing the distribution of *Chaetodon guttatissimus* (solid line) and *C. punctatofasciatus* (dashed line), in the Indian and Pacific Oceans, respectively. Asterisks represent sampling locations outside the Christmas Island hybrid zone (detailed sample sizes are given in Material and Methods). The star symbol identifies the position of Christmas Island within the area of overlap (darker shade of grey) between the two species. Inset shows details of the Christmas Island study sites used for the distribution surveys (black circles) and north coast area covered during the GPS-assisted surveys (thicker grey line).

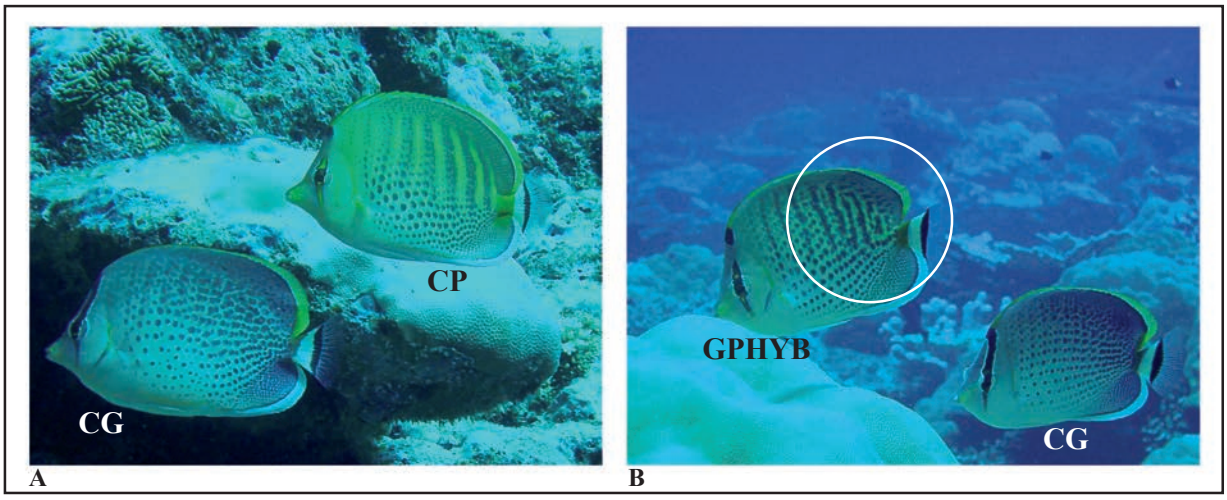

Figure 3.2. A) Chaetodon guttatissimus (CG) and *C. punctatofasciatus* (CP) observed in a heterospecific pair at Christmas Island. **B)** A hybrid (GPHYB) of this species complex, paired with *C. guttatissimus* (CG) at Christmas Island: the circle highlights the distinguishing maze-like dorsal pattern (cf the clear, straight lines of *C. punctatofasciatus* in photograph A). Maze-like patterns, such as these, have been shown to be characteristic of natural fish hybrids (Miyazawa *et al.* 2010).

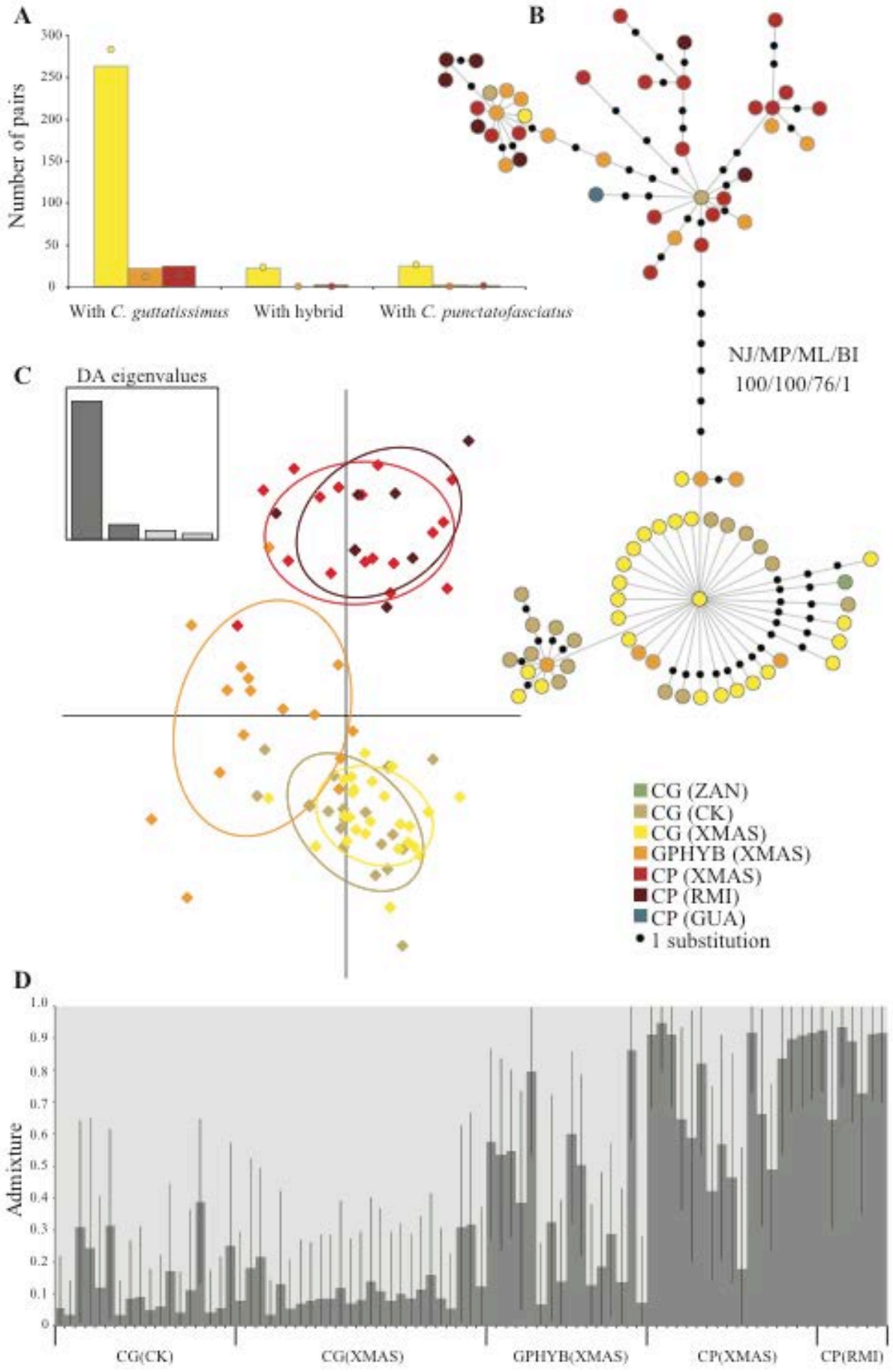
A
Number of pairs
300
250
200
150
100
50
0
With C. guttatissimus
With hybrid
With C. punctatofasciatus
B
NJ/MP/ML/BI
100/100/76/1
C
DA eigenvalues
CG (ZAN)
CG (CK)
CG (XMAS)
GPHYB (XMAS)
CP (XMAS)
CP (RMI)
CP (GUA)
1 substitution
D
Admixture
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
CG(CK)
CG(XMAS)
GPHYB(XMAS)
CP(XMAS)
CP(RMI)

Figure 3.3. A) Pairing frequencies of *C. guttatissimus* (yellow), *C. guttatissimus* × *C. punctatofasciatus* hybrids (orange) and *C. punctatofasciatus* (red). All three taxa are colour-coded according to the legend below. Bars represent observed pairings from Christmas Island, and dots represent expected pairing frequencies based on observed taxon abundance. Observed pairing does not statistically deviate from expectations, indicating that taxa are pairing non-assortatively. B) MST showing haplotype relationships in the C. guttatissimus group. Each circle represents one individual and is colour-coded for taxon and geographic origin. Each black dot on connecting branches represents one substitution (bp). Bootstrap support values for phylogenetic relationships inferred by NJ, MP, ML and posterior probabilities from BI are shown for the partition between the two major clades in the species group. **C)** Scatterplot of DAPC (Jombart et al. 2010) performed on 20 microsatellite loci for five populations of the C. guttatissimus group. Populations are shown by colours and 95% inertia ellipses, squares represent individual genotypes. Axes show the first two discriminant functions, and eigenvalues the genetic information retained by discriminant functions. **D)** Barplot of STRUCTURE admixture coefficients based on 20 microsatellite loci in five populations of the C. guttatissimus group. Bars represent individuals, black lines are 90% credibility regions, and subdivisions show the genotypic admixture between clusters (k = 2, representing the parent species). Colour coding as well as taxon and geographic location abbreviations are valid throughout all panels: CG = C. guttatissimus; CP = C. punctatofasciatus; GPHYB = C. guttatissimus × C. punctatofasciatus; CK = Cocos (Keeling) Islands; GUA = Guam; RMI = Republic of Marshall Islands; XMAS = Christmas Island; ZAN = Zanzibar.

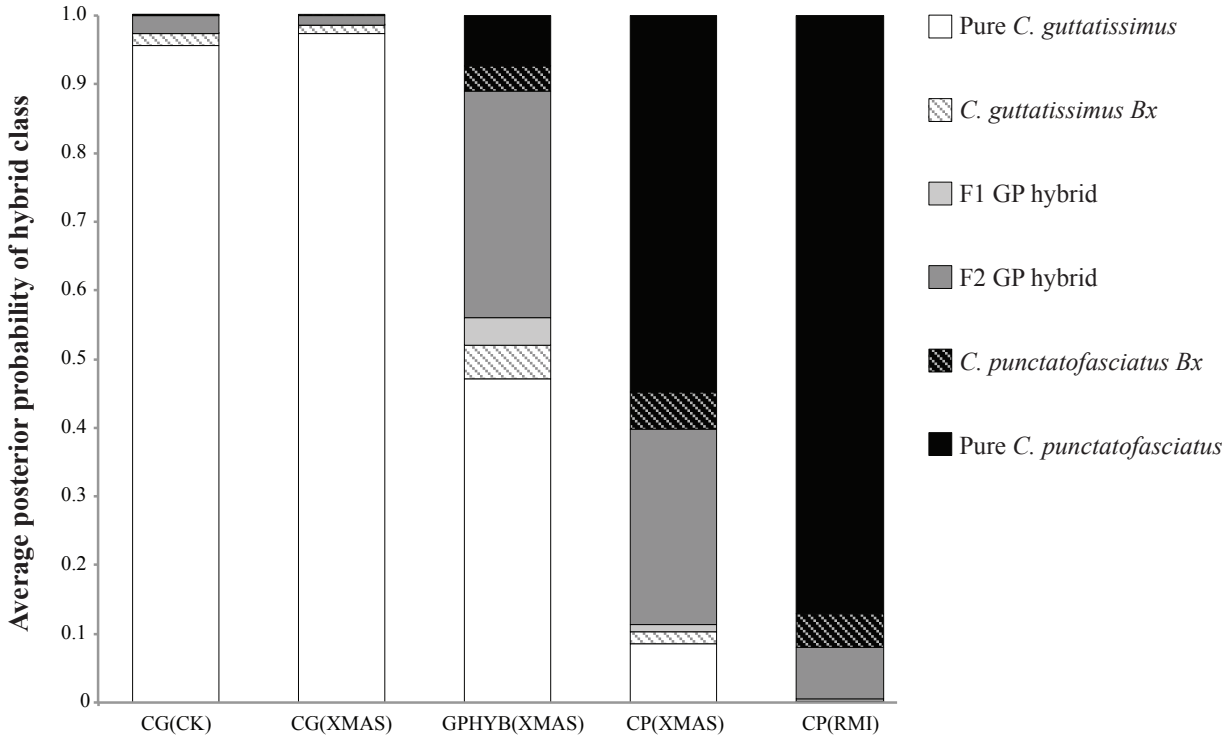

Figure 3.4. Posterior probabilities, based on microsatellite data, of individuals of the *C. guttatissimus* group belonging to six classes: pure parental species, F1 or F2 hybrids and backcrosses (*Bx*) in either direction. Individual data were averaged within population of origin. Colour codes for the six classes are given in the legend. Each bar represents one population and is designated by species and geographic location (for sample sizes refer to Material and Methods). Abbreviations: CG = *C. guttatissimus*; CP = *C. punctatofasciatus*; GPHYB = *C. guttatissimus* × *C. punctatofasciatus*; CK = Cocos (Keeling) Islands; RMI = Republic of Marshall Islands; XMAS = Christmas Island.

Table 3.1. Qualitative summary of ecological and behavioural conditions conducive to hybridisation in two pairs of allopatric *Chaetodon* sister species in secondary contact at the Christmas Island suture zone in the Indo-Pacific. Data for the *C. trifasciatus* group are summarised from Montanari *et al.* (2012) and presented here for comparison.

	***C. guttatissimus* group**	***C. trifasciatus* group**
Parental species abundance	One parent rare (2 individuals per 3000 m^2)	Both parents rare (< 2 individuals per 3000 m^2)
Hybrid abundance	As abundant as rare parent	Rarer than both parents
Parental depth distribution	Range: 13 - 17 m; largely overlapping (> 93%)	Range: 5 - 8 m; largely overlapping (> 98%)
Hybrid depth distribution	Overlapping (> 99%) with parents	Overlapping (83%) with parents
Parental species diet	Generalist corallivores; largely overlapping (> 73%)	Generalist corallivores; largely overlapping (> 77%)
Hybrid diet	Generalist corallivore; overlapping (> 76%) with parents	Generalist corallivore; overlapping (>81%) with parents
Parental species pairing behaviour	Non-assortative	Non-assortative
Hybrid pairing behaviour	Pairing with both parents; non-assortative	Pairing with both parents; non-assortative

3.6 Supporting Information

3.6.1 Additional Material and Methods

Abundance surveys

The GPS tracks recorded during the survey swims were divided into deep and shallow transects and independently measured, excluding distances covered to reach initial depth and those swum whilst moving between deep and shallow water transects. Deep (10 to 25 m) transects were 10 m wide and 323.56 m long on average (average transect area 3,235.64 m^2). Shallow (3 to 9 m) transects were 20 m wide (due to low abundances, and made possible by high visibility and flat topography) and average length was 314.76 m (average transect area 6,295.14 m^2).

Phylo- and population genetic analyses

NJ and MP algorithms were implemented in Mega 4(Tamura *et al.* 2007), BI was run through the Mr. Bayes plug-in for Geneious Pro v5.3.3 (Biomatters Ltd.) (Huelsenbeck & Ronquist 2001) and ML analysis was performed using Garli v0.95 (Zwickl 2006) and Bootscore v3.11 (Sukumaran 2007). The Maximum Composite Likelihood method (Tamura *et al.* 2004) with 1000 Bootstrap replicates was used to compute the NJ tree. All gaps and missing data were pairwise deleted. The overall shortest tree was selected from 10 independent MP analyses. A Close-Neighbour-Interchange algorithm (Nei & Kumar 2000), with search level 2 (Eck & Dayhoff 1966; Nei & Kumar 2000) and initial trees inferred by random addition (10 replicates), was used to obtain the tree. All gaps and missing data were discarded. The JC69 substitution model (Jukes & Cantor 1969) - selected using jModelTest v0.1.1 (Guindon & Gascuel 2003; Posada 2008) - was used in the BI analysis, which was Monte Carlo simulated on four Markov chains (100,000 generations each) (MCMC), sampling trees every 100 generations. The consensus tree was computed using the 1000 best post-burn-in trees, applying a 50% majority rule. Ten independent ML analyses were run and the resulting best trees checked for consistent topology. A consensus tree based on the best

topology obtained was computed following 100 bootstrap-replicated ML analyses. Cyt b haplotypes were assembled in a minimum-spanning tree (MST) using Hapstar v0.6 (Prim 1957; Excoffier *et al.* 2005) and the support values for the two main clades from the abovementioned phylogenetic analyses were indicated on the MST.

Haplotype (h) and nucleotide (π) diversities were calculated for all populations in Arlequin v3.1 (Excoffier *et al.* 2005). Intra-population gene diversity based on microsatellite loci (1-Q Inter) was computed using option five of web-based GENEPOP v4.2 (Rousset 2008). Analyses of molecular variance (AMOVA) and pairwise F_{st}, performed in Arlequin with 1000 permutations, were used to assess cyt *b* spatial heterogeneity. Number of alleles (N_a), observed (H_O) and expected (H_E) heterozygosities and probabilities of departure from HWE were calculated using the R package *adegenet* (Jombart 2008). MICROCHECKER v2.2.3 (van Oosterhout *et al.* 2004) was used to check for null alleles. Excluding Null Alleles (ENA) corrected estimates of population structure, associated with null allele frequencies, were calculated in Freena (Chapuis & Estoup 2007). Missing data were regarded as null homozygotes. Smogd v1.2.5 (Crawford 2010) was used to calculate estimators of actual differentiation (D_{est})(Jost 2008).

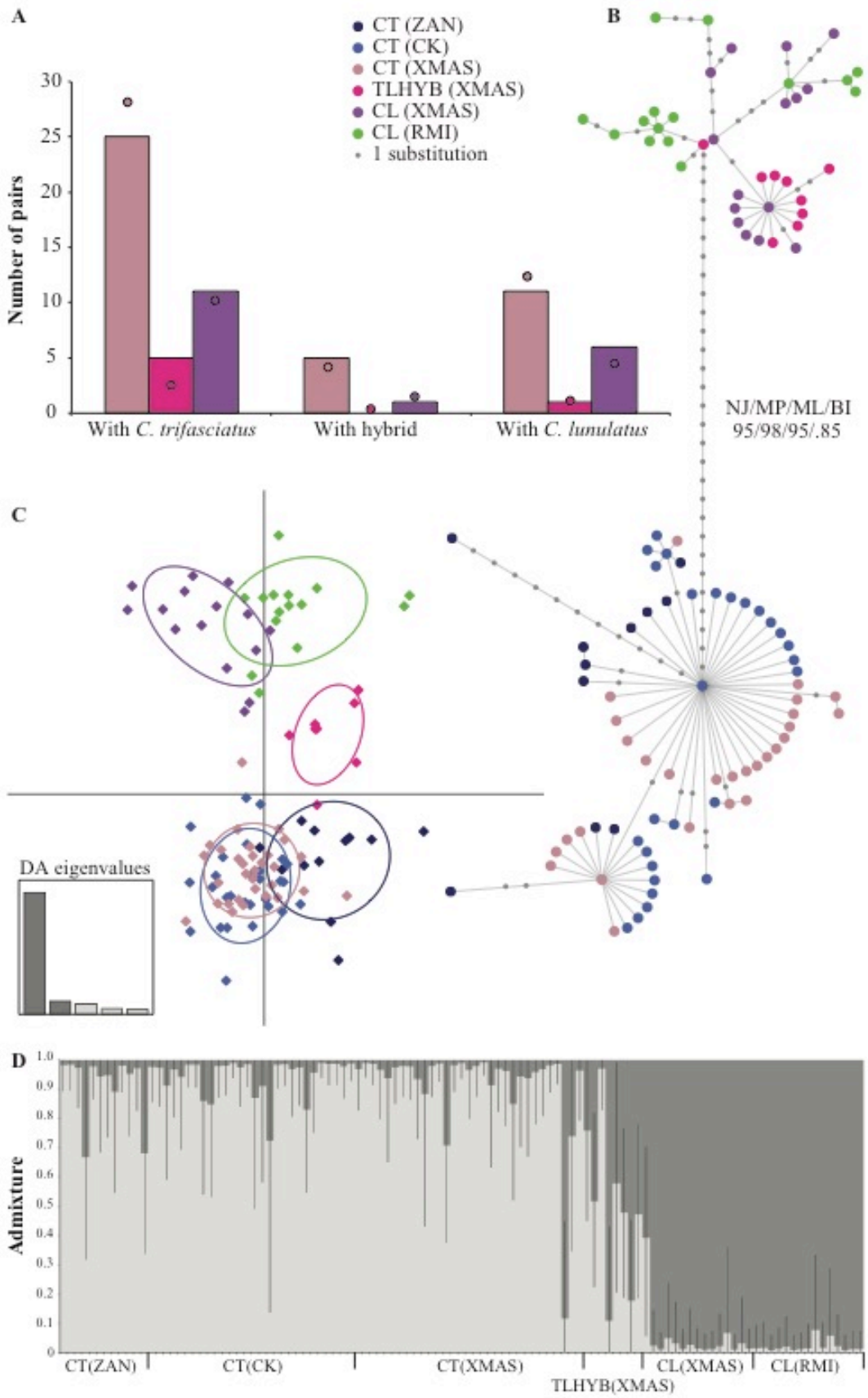

Figure 3.S1. A) Pairing frequencies of *C. trifasciatus* (light pink), *C. trifasciatus* × *C. lunulatus* hybrids (dark pink) and *C. lunulatus* (purple). All three taxa are colour-coded according to the legend above. Bars represent observed pairings from Christmas Island, and

dots represent expected pairing frequencies based on observed taxon abundance. Data are redrawn from Montanari *et al.* (2012). Observed pairing does not statistically deviate from expectations of random mating, indicating that taxa are pairing non-assortatively. B) MST showing haplotype relationships in the C. trifasciatus group (redrawn from Montanari *et al.* (2012). Each circle represents one individual and is colour-coded for taxon and geographic origin. Each grey dot on connecting branches represents one substitution (bp). Bootstrap support values for phylogenetic relationships inferred by NJ, MP, ML and posterior probabilities from BI are shown for the partition between the two major clades in the species group. **C)** Scatterplot of DAPC (Jombart *et al.* 2010) performed on 12 microsatellite loci for six populations of the C. trifasciatus group (redrawn from Montanari *et al.* (2012)). Populations are shown by colours and 95% inertia ellipses, squares represent individual genotypes. Axes show the first two discriminant functions, and eigenvalues the genetic information retained by discriminant functions. **D)** Barplot of STRUCTURE admixture coefficients based on 12 microsatellite loci in six populations of the C. trifasciatus group (Montanari *et al.* 2012). Bars represent individuals, black lines are 90% credibility regions, and subdivisions show the genotypic admixture between clusters (k = 2, representing the parent species). Colour coding as well as taxon and geographic location abbreviations are valid throughout all panels: CL = C. lunulatus; CT = C. trifasciatus; TLHYB = C. trifasciatus × C. lunulatus; CK = Cocos (Keeling) Islands; RMI = Republic of Marshall Islands; XMAS = Christmas Island; ZAN = Zanzibar.

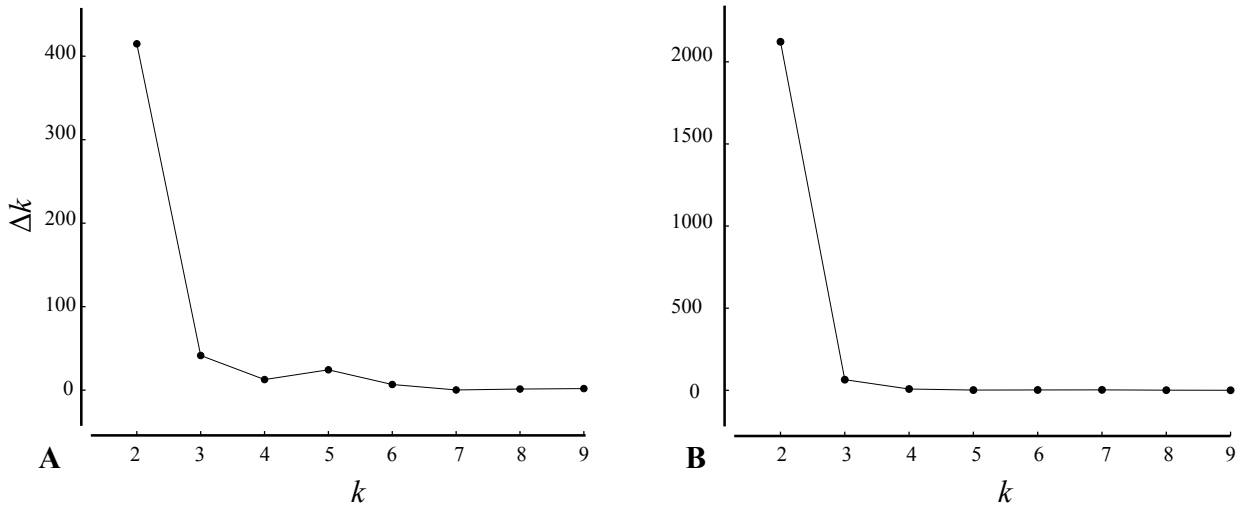

Figure 3.S2. Δ*k* plots (Evanno *et al.* 2005) from STRUCTURE HARVESTER (Earl & vonHoldt 2012) showing a sharp decline in the rate of change of the log probability of data for values of $k > 2$. Data are based on: **A)** 20 microsatellite loci genotyped in 84 individuals of the *C. guttatissimus* group; **B)** 12 microsatellite loci genotyped in 109 individuals of the *C. trifasciatus* group (Montanari *et al.* 2012). In both groups $k = 2$ clusters were chosen as the best-fit model.

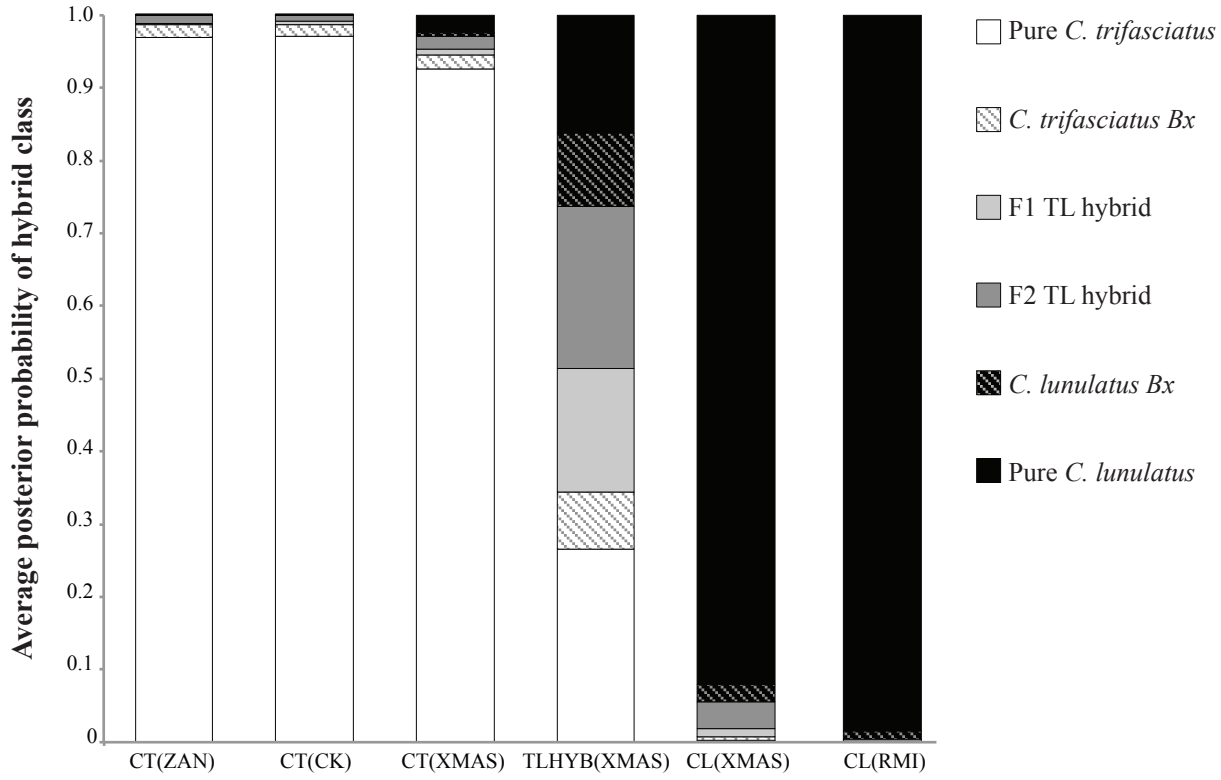

Figure 3.S3. Posterior probabilities, based on microsatellite data from Montanari *et al.* (2012), of individuals of the *C. trifasciatus* group belonging to six classes: pure parental species, F1 or F2 hybrids and backcrosses (*Bx*) in either direction. Individual data were averaged within population of origin. Colour codes for the six classes are given in the legend. Each bar represents one population and is designated by species and geographic location (for sample sizes refer to Montanari *et al.* (2012)). Abbreviations: CT = C. trifasciatus; CL = C. lunulatus; TLHYB = C. trifasciatus × C. lunulatus; CK = Cocos (Keeling) Islands; RMI = Republic of Marshall Islands; XMAS = Christmas Island; ZAN = Zanzibar.

Table 3.S1. Summary statistics for 20 microsatellite loci (Montanari *et al.* 2013) used to genotype the *C. guttatissimus* group: for each adequately sampled population we report sample size (n), number of alleles (N_a), private alleles (P_a), average inbreeding coefficient (F_{IS}), observed (H_O) and expected (H_E) heterozygosities, and probability of departure from HWE (p). * significance at $p < 0.05$; ** significance at $p < 0.01$ after sequential Bonferroni correction (highlighted in bold)

Locus	Cocos (Keeling) Islands C. guttatissimus (n = 18)	Christmas Island C. guttatissimus (n = 25)	Christmas Island Hybrids (n = 16)	Christmas Island C. punctatofasciatus (n = 17)	Marshall Islands C. punctatofasciatus (n = 7)
Cpun1	$N_a = 3$ $P_a = 0$ $H_O = 0.11$ $H_E = 0.11$ $F_{IS} = -0.01$ p = 1.00	$N_a = 2$ $P_a = 0$ $H_O = 0.08$ $H_E = 0.08$ $F_{IS} = -0.02$ p = 1.00	$N_a = 3$ $P_a = 0$ $H_O = 0.18$ $H_E = 0.17$ $F_{IS} = -0.04$ p = 0.98	$N_a = 7$ $P_a = 3$ $H_O = 0.39$ $H_E = 0.67$ $F_{IS} = 0.49$ p < 0.01**	$N_a = 4$ $P_a = 0$ $H_O = 0.43$ $H_E = 0.70$ $F_{IS} = 0.41$ p = 0.37
Cpun2	$N_a = 4$ $P_a = 0$ $H_O = 0.50$ $H_E = 0.42$ $F_{IS} = -0.18$ p = 1.00	$N_a = 4$ $P_a = 0$ $H_O = 0.40$ $H_E = 0.44$ $F_{IS} = 0.12$ p = 0.22	$N_a = 4$ $P_a = 1$ $H_O = 0.50$ $H_E = 0.54$ $F_{IS} = 0.11$ p = 0.97	$N_a = 4$ $P_a = 1$ $H_O = 0.55$ $H_E = 0.53$ $F_{IS} = 0.02$ p = 0.98	$N_a = 3$ $P_a = 0$ $H_O = 0.28$ $H_E = 0.54$ $F_{IS} = 0.48$ p = 0.11
Cpun3	$N_a = 5$ $P_a = 0$ $H_O = 0.61$ $H_E = 0.71$ $F_{IS} = 0.15$ p = 0.01**	$N_a = 6$ $P_a = 0$ $H_O = 0.36$ $H_E = 0.62$ $F_{IS} = 0.44$ p < 0.01**	$N_a = 5$ $P_a = 0$ $H_O = 0.37$ $H_E = 0.65$ $F_{IS} = 0.45$ P = 0.03*	$N_a = 6$ $P_a = 1$ $H_O = 0.53$ $H_E = 0.63$ $F_{IS} = 0.19$ p < 0.01**	$N_a = 4$ $P_a = 0$ $H_O = 0.28$ $H_E = 0.57$ $F_{IS} = 052$ p = 0.06
Cpun4	$N_a = 7$ $P_a = 0$ $H_O = 0.61$ $H_E = 0.77$ $F_{IS} = 0.21$ p < 0.01**	$N_a = 11$ $P_a = 1$ $H_O = 0.40$ $H_E = 0.83$ $F_{IS} = 0.53$ p < 0.01**	$N_a = 9$ $P_a = 0$ $H_O = 0.31$ $H_E = 0.83$ $F_{IS} = 0.64$ p < 0.01**	$N_a = 11$ $P_a = 2$ $H_O = 0.53$ $H_E = 0.87$ $F_{IS} = 0.42$ p < 0.01**	$N_a = 4$ $P_a = 0$ $H_O = 0.71$ $H_E = 0.63$ $F_{IS} = -0.15$ p = 0.10

Cpun6	$N_a = 9$ $P_a = 0$ $H_O = 0.67$ $H_E = 0.79$ $F_{IS} = 0.16$ $p = 0.03$*	$N_a = 8$ $P_a = 0$ $H_O = 0.79$ $H_E = 0.79$ $F_{IS} = 0.01$ $p = 0.28$	$N_a = 9$ $P_a = 0$ $H_O = 0.68$ $H_E = 0.82$ $F_{IS} = 0.19$ $p = 0.59$	$N_a = 10$ $P_a = 2$ $H_O = 0.76$ $H_E = 0.77$ $F_{IS} = 0.04$ $p = 0.97$	$N_a = 4$ $P_a = 0$ $H_O = 0.71$ $H_E = 0.63$ $F_{IS} = -0.15$ $p = 0.77$
Cpun7	$N_a = 6$ $P_a = 0$ $H_O = 0.44$ $H_E = 0.78$ $F_{IS} = 0.43$ $p < 0.01$**	$N_a = 9$ $P_a = 0$ $H_O = 0.52$ $H_E = 0.80$ $F_{IS} = 0.35$ $p < 0.01$**	$N_a = 8$ $P_a = 1$ $H_O = 0.73$ $H_E = 0.84$ $F_{IS} = 0.18$ $p = 0.22$	$N_a = 8$ $P_a = 0$ $H_O = 0.50$ $H_E = 0.81$ $F_{IS} = 0.45$ $p = 0.05$*	$N_a = 6$ $P_a = 1$ $H_O = 0.43$ $H_E = 0.87$ $F_{IS} = 0.53$ $p < 0.01$**
Cpun9	$N_a = 8$ $P_a = 0$ $H_O = 0.33$ $H_E = 0.85$ $F_{IS} = 0.61$ $p < 0.01$**	$N_a = 10$ $P_a = 1$ $H_O = 0.18$ $H_E = 0.85$ $F_{IS} = 0.72$ $p < 0.01$**	$N_a = 9$ $P_a = 0$ $H_O = 0.46$ $H_E = 0.87$ $F_{IS} = 0.44$ $p < 0.01$**	$N_a = 8$ $P_a = 0$ $H_O = 0.35$ $H_E = 0.80$ $F_{IS} = 0.58$ $p < 0.01$**	$N_a = 4$ $P_a = 0$ $H_O = 0.43$ $H_E = 0.71$ $F_{IS} = 0.42$ $p = 0.03$*
Cpun10	$N_a = 11$ $P_a = 0$ $H_O = 0.66$ $H_E = 0.76$ $F_{IS} = 0.13$ $p = 0.27$	$N_a = 10$ $P_a = 0$ $H_O = 0.60$ $H_E = 0.79$ $F_{IS} = 0.25$ $p = 0.07$	$N_a = 10$ $P_a = 0$ $H_O = 0.75$ $H_E = 0.86$ $F_{IS} = 0.13$ $p = 0.36$	$N_a = 12$ $P_a = 1$ $H_O = 0.70$ $H_E = 0.87$ $F_{IS} = 0.21$ $p = 0.03$*	$N_a = 7$ $P_a = 0$ $H_O = 0.43$ $H_E = 0.86$ $F_{IS} = 0.52$ $p < 0.01$**
Cpun11	$N_a = 16$ $P_a = 1$ $H_O = 1.00$ $H_E = 0.94$ $F_{IS} = -0.06$ $p = 0.87$	$N_a = 16$ $P_a = 3$ $H_O = 1.00$ $H_E = 0.93$ $F_{IS} = -0.07$ $p = 0.41$	$N_a = 16$ $P_a = 0$ $H_O = 0.94$ $H_E = 0.95$ $F_{IS} = 0.01$ $p = 0.82$	$N_a = 14$ $P_a = 0$ $H_O = 0.88$ $H_E = 0.93$ $F_{IS} = 0.06$ $p = 0.34$	$N_a = 9$ $P_a = 0$ $H_O = 1.00$ $H_E = 0.91$ $F_{IS} = -0.10$ $p = 0.63$
Cpun12	$N_a = 7$ $P_a = 0$ $H_O = 0.78$ $H_E = 0.80$ $F_{IS} = 0.03$ $p = 0.46$	$N_a = 6$ $P_a = 0$ $H_O = 0.59$ $H_E = 0.76$ $F_{IS} = 0.17$ $p = 0.08$	$N_a = 7$ $P_a = 0$ $H_O = 0.73$ $H_E = 0.80$ $F_{IS} = 0.10$ $p = 0.14$	$N_a = 7$ $P_a = 0$ $H_O = 0.67$ $H_E = 0.72$ $F_{IS} = 0.06$ $p = 0.76$	$N_a = 5$ $P_a = 1$ $H_O = 0.57$ $H_E = 0.72$ $F_{IS} = 0.22$ $p = 0.18$

Cpun13	N_a = 10 P_a = 0 H_O = 0.61 H_E = 0.84 F_{IS} = 0.28 p < 0.01**	N_a = 11 P_a = 1 H_O = 0.28 H_E = 0.88 F_{IS} = 0.61 p < 0.01**	N_a = 9 P_a = 1 H_O = 0.66 H_E = 0.88 F_{IS} = 0.16 p = 0.07	N_a = 7 P_a = 0 H_O = 0.08 H_E = 0.83 F_{IS} = 0.58 p < 0.01**	N_a = 4 P_a = 0 H_O = 0.14 H_E = 0.71 F_{IS} = 0.81 p < 0.01**
Cpun14	N_a = 8 P_a = 1 H_O = 0.50 H_E = 0.44 F_{IS} = -0.14 p = 1.00	N_a = 5 P_a = 0 H_O = 0.20 H_E = 0.19 F_{IS} = -0.04 p = 1.00	N_a = 11 P_a = 0 H_O = 0.75 H_E = 0.69 F_{IS} = -0.05 p = 0.37	N_a = 13 P_a = 1 H_O = 0.72 H_E = 0.88 F_{IS} = 0.16 p = 0.88	N_a = 5 P_a = 0 H_O = 0.86 H_E = 0.82 F_{IS} = -0.04 p = 0.17
Cpun15	N_a = 3 P_a = 0 H_O = 0.22 H_E = 0.37 F_{IS} = 0.40 p = 0.16	N_a = 6 P_a = 1 H_O = 0.39 H_E = 0.37 F_{IS} = -0.09 p = 0.93	N_a = 8 P_a = 2 H_O = 0.80 H_E = 0.69 F_{IS} = -0.15 p = 0.78	N_a = 12 P_a = 2 H_O = 0.76 H_E = 0.85 F_{IS} = 0.13 p = 0.25	N_a = 8 P_a = 0 H_O = 0.71 H_E = 0.91 F_{IS} = 0.23 p = 0.02*
Cpun17	N_a = 13 P_a = 0 H_O = 0.83 H_E = 0.89 F_{IS} = 0.07 p = 0.39	N_a = 10 P_a = 0 H_O = 0.92 H_E = 0.86 F_{IS} = -0.05 p = 0.17	N_a = 13 P_a = 2 H_O = 0.73 H_E = 0.88 F_{IS} = 0.18 p < 0.01**	N_a = 14 P_a = 1 H_O = 0.78 H_E = 0.91 F_{IS} = 0.19 p = 0.56	N_a = 10 P_a = 0 H_O = 0.83 H_E = 0.97 F_{IS} = 0.15 p = 0.18
Cpun18	N_a = 13 P_a = 1 H_O = 0.89 H_E = 0.91 F_{IS} = 0.02 p = 0.44	N_a = 11 P_a = 1 H_O = 1.00 H_E = 0.88 F_{IS} = -0.11 p = 0.79	N_a = 10 P_a = 0 H_O = 0.56 H_E = 0.83 F_{IS} = 0.35 p = 0.06	N_a = 9 P_a = 0 H_O = 0.56 H_E = 0.83 F_{IS} = 0.32 p = 0.10	N_a = 9 P_a = 0 H_O = 0.86 H_E = 0.91 F_{IS} = 0.06 p = 0.08
Cpun19	N_a = 11 P_a = 0 H_O = 0.61 H_E = 0.88 F_{IS} = 0.31 p = 0.02*	N_a = 11 P_a = 0 H_O = 0.67 H_E = 0.84 F_{IS} = 0.22 p = 0.03*	N_a = 14 P_a = 3 H_O = 0.62 H_E = 0.86 F_{IS} = 0.30 p < 0.01**	N_a = 13 P_a = 1 H_O = 0.41 H_E = 0.84 F_{IS} = 0.53 p < 0.01**	N_a = 8 P_a = 0 H_O = 0.96 H_E = 0.90 F_{IS} = 0.05 p = 0.73

Cpun20	N_a = 17 P_a = 1 H_O = 0.83 H_E = 0.85 F_{IS} = 0.13 p < 0.01**	N_a = 15 P_a = 1 H_O = 0.69 H_E = 0.92 F_{IS} = 0.24 p = 0.23	N_a = 16 P_a = 1 H_O = 0.78 H_E = 0.91 F_{IS} = 0.14 p = 0.31	N_a = 16 P_a = 1 H_O = 0.69 H_E = 0.92 F_{IS} = 0.26 p = 0.10	N_a = 9 P_a = 2 H_O = 0.86 H_E = 0.91 F_{IS} = 0.06 p = 0.16
Cpun21	N_a = 15 P_a = 2 H_O = 0.83 H_E = 0.94 F_{IS} = 0.11 p = 0.37	N_a = 16 P_a = 1 H_O = 0.76 H_E = 0.90 F_{IS} = 0.17 p = 0.75	N_a = 12 P_a = 0 H_O = 0.87 H_E = 0.88 F_{IS} = 0.03 p = 0.38	N_a = 11 P_a = 2 H_O = 0.76 H_E = 0.85 F_{IS} = 0.13 p = 0.09	N_a = 7 P_a = 2 H_O = 0.71 H_E = 0.86 F_{IS} = 0.18 p = 0.10
Cpun22	N_a = 10 P_a = 2 H_O = 0.94 H_E = 0.81 F_{IS} = -0.17 p = 0.21	N_a = 6 P_a = 0 H_O = 0.64 H_E = 0.75 F_{IS} = 0.17 p = 0.50	N_a = 7 P_a = 0 H_O = 0.81 H_E = 0.81 F_{IS} = 0.03 p = 0.90	N_a = 8 P_a = 0 H_O = 0.83 H_E = 0.82 F_{IS} = -0.02 p = 0.66	N_a = 7 P_a = 0 H_O = 0.86 H_E = 0.86 F_{IS} = 0.00 p = 0.92
Cpun23	N_a = 10 P_a = 2 H_O = 0.67 H_E = 0.88 F_{IS} = 0.24 p < 0.01**	N_a = 8 P_a = 1 H_O = 0.00 H_E = 0.86 F_{IS} = 0.42 p < 0.01**	N_a = 9 P_a = 1 H_O = 0.62 H_E = 0.86 F_{IS} = 0.03 p = 0.09	N_a = 9 P_a = 0 H_O = 0.47 H_E = 0.81 F_{IS} = 0.44 p = 0.04*	N_a = 7 P_a = 0 H_O = 0.57 H_E = 0.85 F_{IS} = 0.35 p = 0.17

Table 3.S2. Sample sizes, cyt b number of haplotypes (nh), haplotype (h) and nucleotide (π) diversities and intra-population gene diversity based on 20 microsatellite loci (1-Q Inter). Data are presented for all adequately sampled populations of the C. guttatissimus group (total n = 84). Guam and Zanzibar populations were not included due to small sample size (each n = 1).

Population	n	nh	h	π	**1-Q Inter**
Cocos Is. C. guttatissimus	18	14	0.95	0.007	0.747
Christmas Is. C. guttatissimus	25	19	0.93	0.004	0.726
Christmas Is. Hybrid	16	15	0.99	0.011	0.804
Christmas Is. C. punctatofasciatus	18	14	0.97	0.006	0.840
Marshall Is. C. punctatofasciatus	7	7	1	0.008	0.828

Table 3.S3. Pairwise population comparisons in the C. guttatissimus group: A) Φ_{st} (lower diagonal) generated from 566 bp of mitochondrial cyt b gene, and corresponding p values (upper diagonal); B) F_{st} (with respective p values computed over 1023 permutations) (lower diagonal) and harmonic mean of D_{est} (Jost 2008) (upper diagonal) based on 20 microsatellite loci. Significant comparisons are highlighted in bold. CG = C. guttatissimus; CP = C. punctatofasciatus and GPHYB = C. guttatissimus × C. punctatofasciatus hybrids; XMAS = Christmas Island; CK = Cocos (Keeling) Islands; RMI = Marshall Islands.

A

	CG (CK)	CG (XMAS)	GPHYB (XMAS)	CP (XMAS)	CP (RMI)
CG (CK)		p = 0.168	p < 0.001	p < 0.001	p < 0.001
CG (XMAS)	0.022		p < 0.001	p < 0.001	p < 0.001
GPHYB (XMAS)	0.416	0.313		p < 0.001	p < 0.001
CP (XMAS)	0.696	0.618	0.166		p = 0.177
CP (RMI)	0.705	0.667	0.187	0.056	

B

	CG (CK)	CG (XMAS)	GPHYB (XMAS)	CP (XMAS)	CP (RMI)
CG (CK)		0.009	0.053	0.116	0.163
CG (XMAS)	0.012 p = 0.036		0.065	0.093	0.162
GPHYB (XMAS)	0.024 p < 0.001	0.024 p < 0.001		0.052	0.105

CP (XMAS)	0.068 $p < 0.001$	0.065 $p < 0.001$	0.030 $p < 0.001$		0.001
CP (RMI)	0.096 $p < 0.001$	0.101 $p < 0.001$	0.055 $p < 0.001$	0.017 $p = 0.288$	

Table 3.S4. Raw and ENA corrected (Chapuis & Estoup 2007) population differentiation and estimator of actual differentiation (Jost 2008) (D_{est}) presented locus-by-locus and as a mean over all loci. All values were significant within the 95% confidence interval. Results based on allele frequencies of 20 microsatellite loci in the *C. guttatissimus* group.

LOCUS	Raw	ENA corrected	D_{est}
Cpun01	0.155	0.215	0.094
Cpun02	0.009	0.006	0.012
Cpun03	0.019	0.015	0.016
Cpun04	0.024	0.037	0.268
Cpun06	0.020	0.024	0.137
Cpun07	0.040	0.035	0.202
Cpun09	0.036	0.039	0.397
Cpun10	0.032	0.034	0.213
Cpun11	0.008	0.008	0.119
Cpun12	0.024	0.025	0.116
Cpun13	0.021	0.030	0.253
Cpun14	0.229	0.237	0.470
Cpun15	0.204	0.190	0.482
Cpun17	0.006	0.006	0.092
Cpun18	0.020	0.015	0.206
Cpun19	0.010	0.008	0.021
Cpun20	0.001	0.003	0.144
Cpun21	0.023	0.023	0.267
Cpun22	0.011	0.010	0.019
Cpun23	0.050	0.057	0.367
Average over 20 loci	0.038	0.042	0.115

CHAPTER 4: NATURALLY OCCURRING HYBRIDS OF CORAL REEF BUTTERFLYFISHES HAVE SIMILAR FITNESS COMPARED TO PARENTAL SPECIES

4.1 Abstract

Hybridisation can produce evolutionary novelty by increasing fitness and adaptive capacity. Heterosis, or hybrid vigour, has been documented in many plant and animal taxa, and is a notable consequence of hybridisation that has been exploited for decades in agriculture and aquaculture. On the contrary, loss of fitness in naturally occurring hybrid taxa has been observed in many cases. This can have negative consequences for the parental species involved (wasted reproductive effort), and has raised concerns for species conservation. This study evaluates the relative fitness of previously documented butterflyfish hybrids of the genus *Chaetodon* from the Indo-Pacific suture zone at Christmas Island. Histological examination confirmed the reproductive viability of *Chaetodon* hybrids. Examination of liver lipid content showed that hybrid body condition was not significantly different from parent species body condition. Lastly, size at age data revealed no difference in growth rates and asymptotic length between hybrids and parent species. Based on the traits measured in this study, naturally occurring hybrids of *Chaetodon* butterflyfishes have similar fitness to their parental species, and are unlikely to supplant parental species under current environmental conditions at the suture zone. However, given sufficient fitness and on going genetic exchange between the respective parental species, hybrids are likely to persist within the suture zone.

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4.2 Introduction

Natural hybridisation was once considered rare and unimportant (Mayr 1963), but a large and increasing body of literature suggests that this process may be critically important for both adaptation and speciation (Abbott *et al.* 2013). Importantly, natural hybridisation can play a role in the formation of new species if it produces novel genotypes that outperform their parental species or persist in previously unoccupied niches (Arnold 1997). Conversely, hybridisation can contribute to the loss of biodiversity through extinction (Rhymer & Simberloff 1996) or reverse speciation (Seehausen 2006; Taylor *et al.* 2006). The evolutionary consequences and implications of hybridisation are largely dependent upon the extent to which hybrids interact with their parent species (e.g., differential habitat use, assortative mating) and individual fitness.

Heterosis (commonly referred to as hybrid vigour) (Shull 1948) is a notable consequence of hybridisation and has been exploited for decades in agriculture and aquaculture. Hybrids of many plant and animal species exhibit increased vigour (e.g., faster growth, larger size, and higher reproductive output) and can be more stress tolerant relative to either parental species (Bartley *et al.* 2000; Chen 2013). However, the mechanistic underpinnings of heterosis are only just beginning to emerge, and involve the complex interplay of epigenetic modification of gene regulation (Chen 2013) and environmental selection for novel genotypes (Burke & Arnold 2001). In at least some instances, hybrid genotypes experience marked loss of fitness relative to their parental species, which is commonly attributed to meiotic irregularities or genetic incompatibility (Burke & Arnold 2001). In the extreme, hybrids may be sterile or non-viable (Schilthuizen *et al.* 2011). However, the fitness of hybrids is influenced by both endogenous (environment-independent) and exogenous (environment-specific) selective processes (Burke & Arnold 2001). Where genetic incompatibility is not an issue (Abbott *et al.* 2013), exogenous selection enables hybrid genotypes to outperform their parental counterparts in at least some situations and environments (Burke & Arnold 2001).

Natural hybridisation has been particularly well studied among terrestrial and freshwater species (Arnold *et al.* 1993; Cruzan & Arnold 1993; Carney *et al.* 1994; MacCallum *et al.* 1998). Herein, the prevalence of hybridisation (largely apparent from genetic analyses that reveal high levels of introgression) shows that postzygotic barriers to inter-breeding among recently diverged species are rarely complete, but may be permeable in time or space (Abbott *et al.* 2013). Hybridisation can therefore provide an additional (and potentially major) source of genetic variation, contributing to adaptive radiation in highly diverse or changing environments (Seehausen 2004; Riginos & Cunningham 2007). Recent pulses in the incidence of "natural" hybridisation are widely attributed to anthropogenic degradation or disruption of natural ecosystems, such as translocation of species and fragmentation of habitats (Allendorf *et al.* 2001; Hoffmeister *et al.* 2005). Hybridisation among some wild species would not have occurred naturally and is leading to extensive genetic mixing and effective extinction of one or both parental species (Allendorf *et al.* 2001). However, genetic variation through hybridisation may also yield novel genotypes and expedite adaptation, thereby ensuring species persistence in the face of changing environmental conditions (Anderson *et al.* 2009; Arnold & Martin 2010).

The prevalence and importance of hybridisation has not been appreciated in marine systems until very recently (Gardner 1997; Richards & Hobbs 2015). Given the very high diversity and relatively recent divergence of species in some marine habitats (e.g., coral reefs), it is little surprise that hybridisation is highly prevalent among marine species (Mallet 2001; Yaakub *et al.* 2006; Bowen *et al.* 2013; Montanari *et al.* 2016). Hybridisation is particularly apparent in narrow and specific geographic areas, where regional biotas intersect at biogeographic borders or suture zones (Remington 1968; Hobbs *et al.* 2013; Hobbs & Allen 2014). As shown in other ecosystems, taxonomic bias in the occurrence of hybridisation is also evident among marine species: hybridisation is particularly prevalent among coral reef fishes, especially butterflyfishes (family Chaetodontidae) and angelfishes (family Pomacanthidae) (Pyle & Randall 1994; Allen *et al.* 1998; Yaakub *et al.* 2006; Hobbs *et al.*

2013; Hobbs *et al.* 2014). Accordingly, there has been disproportionate research attention given to the molecular and ecological factors that promote hybridisation in these groups (DiBattista *et al.* 2012; Montanari *et al.* 2012; DiBattista *et al.* 2013; Montanari *et al.* 2014; DiBattista *et al.* 2015; Montanari *et al.* 2016). However, the evolutionary implications of hybridisation in coral reef fishes are not yet well understood.

The purpose of this study was to explicitly test for variation in fitness of documented hybrids relative to parental species for coral reef butterflyfishes (*Chaetodon*: Chaetodontidae). Fitness is ultimately a measure of individual reproductive success and is the average contribution to the next generation gene pool by individuals of a particular genotype. Directly measuring fish reproductive success in the wild can prove impractical in the absence of long-term mark-and-recapture studies coupled with parentage analysis. In the case of *Chaetodon* hybrids, fertility has been either anecdotally reported or inferred through the detection of introgression (Montanari *et al.* 2012; Montanari *et al.* 2014). Some differences in growth rates and longevity have been reported in one other case of tropical reef fish hybridisation: *Cephalopholis* groupers at Christmas and Cocos (Keeling) Islands (Payet *et al.* 2016). Further, increased growth rates, particularly during early life-history stages, are associated with enhanced survivorship, faster maturation, and greater female fecundity at a given age, thereby representing a useful proxy for fitness (Taylor *et al.* 2012). The aims of this paper were to compare fitness between parental species and naturally occurring butterflyfish hybrids of genus *Chaetodon* based on: 1) reproductive output, measured as relative gonad mass; 2) body condition, inferred from hepatocyte vacuolation; and 3) growth, inferred from size at age relationships.

4.3 Materials and Methods

4.3.1 Study sites and species

Sampling was conducted between July 2008 and November 2013 at Christmas Island, Australia (10.4475° S, 105.6904° E). All samples used in the fertility and body condition

analyses described below were collected over 2 weeks between November 15th, 2013 and November 28th, 2013, in order to minimise differences due to yearly or seasonal variation (Table 1). The study was undertaken in accordance with the Committee of Animal Ethics of James Cook University of North Queensland (AEC Approval Number: A1757). All fishes were speared on SCUBA and immediately euthanized by severing the first postcranial trunk vertebra, in accordance with the permit above. This study focussed on two hybridising butterflyfish groups, for which detailed genetic analyses have confirmed the status of hybrids and parental species (Montanari *et al.* 2014). Despite some between-group differences in mitochondrial inheritance and introgression rates, hybridisation appears to be on going in both groups, and the hybrids display no obvious differences in ecology or behaviour relative to their parental species (Montanari *et al.* 2014). To date however, nothing is known about the fitness of these hybrids and whether they are likely to persist in the wild. Total length (TL) was measured to the closest mm and each fish was weighed (after blotting) on electronic scales to the closest mg. Livers and gonads were extracted and weighed to the closest mg, and stored in 4% buffered formaldehyde for histological examination. Otoliths were extracted, rinsed in ethanol and preserved dry for size at age analysis.

4.3.2 Fitness measurements

Fertility

To confirm that hybrid fishes were fertile, we undertook a qualitative histological assessment of female and male gonads for all taxa. Preserved gonads were processed using an automatic tissue processor (Intelsint – EFTP) with ascending grades of ethanol, three changes of absolute ethanol, and cleared in xylene followed by three changes of paraplast wax. Tissues were then embedded using a Shandon Histocentre 3 embedding centre, and blocks were cut at 5µm using a Micron rotary microtome. Slides were dried at 60ºC, then manually stained with Mayer's Haematoxylin and Young's eosin/erythrosine, and mounted in DPX (Woods & Ellis 1994). Each slide was viewed under transmitted light with a compound microscope, and three haphazardly chosen sections photographed at 400x using an Olympus DP21 system to

provide evidence of hybrid fertility (e.g. presence of gametocytes). Further, relative gonadal mass, or gonadosomatic index (GSI) (Barber & Blake 2006), was calculated for all individuals in each taxon, and used as a proxy for reproductive output. Fishes used in these analyses were all paired at the time of collection, indicating they had reached sexual maturity (Pratchett *et al.* 2006a). Butterflyfish are thought to spawn year-round under ideal conditions (Yabuta & Berumen 2013) and the assumption that all specimens were reproductively synchronised, with similarly developed gonads, was deemed reasonable. The *C. trifasciatus* hybridising group was data deficient, and therefore not included in formal statistical comparisons. For the *C. guttatissimus* group, one-way analysis of variance (ANOVA) was used to evaluate the effect of taxon on GSI, separately for each gender.

Body condition

To provide a measure of general body condition, livers were prepared for histological examination following the methods described above for gonads. Hepatocyte vacuolation was used as a proxy for liver lipid content and body condition (Theilacker 1978; Hoey *et al.* 2007). We recorded the proportion of 42 points that intercepted vacuolated hepatocytes (Pratchett *et al.* 2001) using a grid superimposed on each photograph in ImageJ (Schneider *et al.* 2012). Generalised linear models assuming a binomial distribution (Warton & Hui 2010) were used, for each hybrid group separately, to determine the effect of taxon on hepatocyte vacuolation.

Aging

To determine the age of specimens, sagittal otoliths were embedded in an epoxy resin block and a transverse section (approximately 400 μm) was cut from each using a Buehler low-speed saw to expose the otolith core (Secor *et al.* 1990). Individual sections were mounted on glass microscope slides with thermoplastic cement and polished with 1200-grit wet-dry sanding paper (Secor *et al.* 1990). Each section was viewed under transmitted light with a dissecting microscope for annual increments and a compound microscope for daily

increments. Where possible, the number of presumed daily or annual increments was counted along the dorsal axis, as the increments were generally more distinct in this region.

Size at age

Von Bertalanffy growth functions (VBGFs) (von Bertalanffy 1938) were fitted to length at age data, separately for each taxon. Unconstrained least-squares estimates of the VBGF parameters L_∞ (asymptotic length), K (growth rate) and t_0 (theoretical time at length 0) were generated using R function *nls* (Bates & Watts 1988). The effect of taxon on VBGFs was determined by assessing the degree of overlap of the 95% confidence intervals around the VBGF parameter estimates.

4.4 Results and discussion

4.4.1 Fertility

Mature hybrid females and males had normally developed gonads, similar to those of the parental species, showing all stages of oocyte and spermatocyte development respectively (Figure 1). GSI did not vary significantly between hybrids and parental species in either females or males of the *C. guttatissimus* group (Figure 2). Differences in GSI between sexes were clear in all taxa and variation around the median was high for all sex/taxon combinations (Figure 2). GSIs of hybrid females and males were no different to those of their parent species of the same sex ($F_{(2,26)} = 0.59$, $p = 0.56$ and $F_{(2,24)} = 0.88$, $p= 0.43$), respectively (Figure 2).

4.4.2 Body condition

Hepatocyte vacuolation was not influenced by taxon in either hybrid group (Figure 3). In both groups, within-taxon variability in liver lipid content was high (Figure 3). In the *C. guttatissimus* group, median hepatocyte vacuolation was generally low and ranged from 12% to 26% (Figure 3A). Hybrid *C. guttatissimus* × *C. punctatofasciatus* had similar levels of liver lipids compared to their parent species ($z_{(33)} = 0.50$, $p =0.62$). In the *C. trifasciatus* group, median hepatocyte vacuolation had a broader range from 10% to 48% (Figure 3B) and

hybrids were not significantly different from their parental species ($z_{(7)} = 0.55$, p =0.58) from the suture zone, potentially confounded by small sample size.

4.4.3 Size at age

There was no difference in asymptotic length for parental versus hybrid individuals in either species group (Figure 4). Average L_∞ estimates were consistent with observed maximum lengths: *C. guttatissimus* 104.66 mm, *C. guttatissimus* × *C. punctatofasciatus* hybrids 105.71 mm, *C. punctatofasciatus* 104.41 mm, *C. trifasciatus* 139.79 mm, *C. trifasciatus* × *C. lunulatus* hybrids 146.52 mm and *C. lunulatus* 143.91 mm. The 95% confidence intervals of estimates showed a high degree of overlap between parent species and hybrids in both groups (Figure 4). This suggests marginal differences in asymptotic length (L_∞), growth rate (K) and theoretical time at length 0 (t_0), between hybrids and parental species in each respective group. This indicates that hybrid taxa in both groups grow at a similar rate to their parent species within the suture zone.

This study indicates that inter-specific breeding across two distinct species groups of *Chaetodon* butterflyfishes results in viable hybrid offspring. Naturally occurring hybrids of *Chaetodon* butterflyfishes considered here (*C. guttatissimus* × *C. punctatofasciatus* and *C. trifasciatus* × *C. lunulatus*) have similar condition to their respective parental species from the suture zone in at least three distinct fitness related traits including fecundity, body condition, and growth. Heterosis or decreased fitness have been documented in some hybrid teleost fishes (e.g. salmonids, minnows, barramundi) (McGinnity *et al.* 2003; Rosenfield *et al.* 2004; Cancela *et al.* 2010) and Payet *et al.* (2016) found some possible differences in longevity and growth in hybrid groupers. Here we explicitly test for increased vigour following interspecific breeding of wild tropical reef fishes, by examining several fitness-associated traits.

Although hybrid butterflyfishes examined here exhibited similar levels of fecundity (GSI), body condition (hepatocyte vacuolation), and growth (size at age) compared to parental species from the suture zone, it is possible that heterosis or decreased fitness may be

expressed in other traits or environments not evaluated here. Importantly, hybrids of some freshwater fishes (e.g. hybrids of pupfish and minnow, cichlids) exhibit enhanced performance and/or capacity to exploit novel niches that are generally unavailable to parental species (Seehausen *et al.* 2003; Rosenfield *et al.* 2004; Seehausen 2004). Ecological surveys for the *Chaetodon* species groups considered in this study show that hybrids occupy the same habitats and ostensibly use the same resources as their parent species (Montanari *et al.* 2012; Montanari *et al.* 2014). This is not unexpected, given that hybridising species of *Chaetodon* butterflyfishes tend to exhibit striking similarities in their ecology (Hobbs *et al.* 2013), which may well be an important requisite for hybridisation between teleost fishes (Montanari *et al.* 2016). Hybrids may nonetheless have traits that differentiate them from their parental species, and enable increased tolerance of changing environmental conditions or increased occupation of distinct niches not detected here. This would only be apparent from either ongoing monitoring of hybrid prevalence in the field or experimental tests of physiological tolerances.

This study represents a snapshot in time and space of the relative fitness of hybrids and their respective parent species, providing an important reference point. Ongoing monitoring of hybrid prevalence is important, because if hybrids disperse away from the Christmas Island suture zone they may encounter different environmental conditions. It is unknown what the relative fitness of the hybrids would be in these new environments, but hybrid freshwater fishes have been successful in exploiting new environments (Seehausen *et al.* 2003; Seehausen 2004). In addition, environmental conditions are changing throughout all oceans and reefs - including those at Christmas Island (Hobbs 2014) - for a number of reasons, thus the fitness of hybrids compared to parental species may change at the suture zone in the future. For example, rising sea temperatures directly impact reef fish metabolism (Johansen & Jones 2011; Messmer *et al.* 2016) and indirectly impact corallivorous species (such as the butterflyfishes in this study) through thermal bleaching and mortality of corals that are important for food and habitat (Pratchett 2005, 2007; Cole *et al.* 2008; Bellwood *et al.* 2010). Finally, given that hybrids represent a continual source of novel genetic combinations, the

ongoing hybridisation of butterflyfishes at Christmas Island may, in the future, produce hybrids that are fitter than their parent species (Anderson *et al.* 2009). In this study we found hybrids that had similar fitness related traits to parent species at the time of collection at Christmas Island. Further research on these taxa at other times and locations will provide insights into how the relative fitness of hybrids changes with environmental conditions.

Apparent similarities in trait values for hybrid versus parental species of *Chaetodon* butterflyfishes may partly reflect the limited sample sizes, especially in terms of numbers of hybrids sampled (n = 3-13 for *C. trifasciatus* × *C. lunulatus* and n = 10-37 for *C. guttatissimus* × *C. punctatofasciatus*, see also Table 1). Unfortunately, limited sample sizes are an inherent limitation for studies of natural hybridisation, because these taxa are often rare (Thompson 2004). The *C. guttatissimus* group was analysed with a minimum of ten hybrid individuals and showed the same patterns as the *C. trifasciatus* group with a minimum of 3 hybrids. We would expect discrepancy in results between groups if small sample sizes played a major role.

The vigour expressed in some F1 hybrids is often lost in subsequent generations (F2 and/or backcrosses) (McGinnity *et al.* 2003). Distinguishing between pure individuals and later generation backcrosses (F4 or later) can represent a challenge and may not be particularly useful, because the signal of hybridisation is lost (Lavretsky *et al.* 2016). Further, the limited sample size did not allow for the subdivision of individuals into discrete hybrid classes (e.g F1, backcrosses) for the statistical analyses presented here. Both species groups examined here exhibited the full spectrum of hybrid genotypes (e.g. F1, F2 and backcrosses), as indicated by microsatellite data in previous studies (Montanari *et al.* 2014) and subsequently confirmed with whole genome SNP scans (unpublished data). These observations *per se* confirm not only the fertility, but also the viability of *Chaetodon* hybrids, and are corroborated by the histology and GSI data presented here. Hybrids in both groups backcross with either parent species, in frequencies directly proportional to their relative abundance (i.e. non-assortatively) (Montanari *et al.* 2014). They are also infrequently seen in hybrid-hybrid

pairs, suggesting that the production of F2 individuals is a distinct possibility, as evident from genetic analyses (Montanari *et al.* 2014). Indeed, F1 individuals are the least common in both groups (Montanari *et al.* 2014) and hence represent the minority of the hybrids sampled in this study. It seems therefore reasonable to conclude that the loss of fitness frequently reported in subsequent generation hybrids (Huff 2010; Huff *et al.* 2011) does not apply to butterflyfishes of genus *Chaetodon* at Christmas Island, where they hybridise naturally.

4.5 Conclusions

Hybridisation can play an active role in shaping populations and communities, thus impacting biodiversity. One or both parent species in the two *Chaetodon* groups considered here are locally rare (Hobbs *et al.* 2009; Montanari *et al.* 2012; Montanari *et al.* 2014). Hybridisation can be an evolutionarily relevant source of genetic diversity for these species, because the probability of conspecific mating is low (Seehausen 2004). Unlike cases of hybridisation that have anthropogenic causes and consequences that are deemed detrimental to the species involved (Hoffmeister *et al.* 2005; Taylor *et al.* 2006), hybridisation among *Chaetodon* butterflyfishes and other coral reef fishes at Christmas Island (Hobbs & Allen 2014) seems to find its roots in secondary contact between recently diverged sister species (Montanari *et al.* 2012; Montanari *et al.* 2014). The similarity in fitness related traits between butterflyfish hybrids and their parental species supports the likely persistence of hybrids and their potential as sources of novel genetic diversity, adaptability and biodiversity within this isolated geographical location.

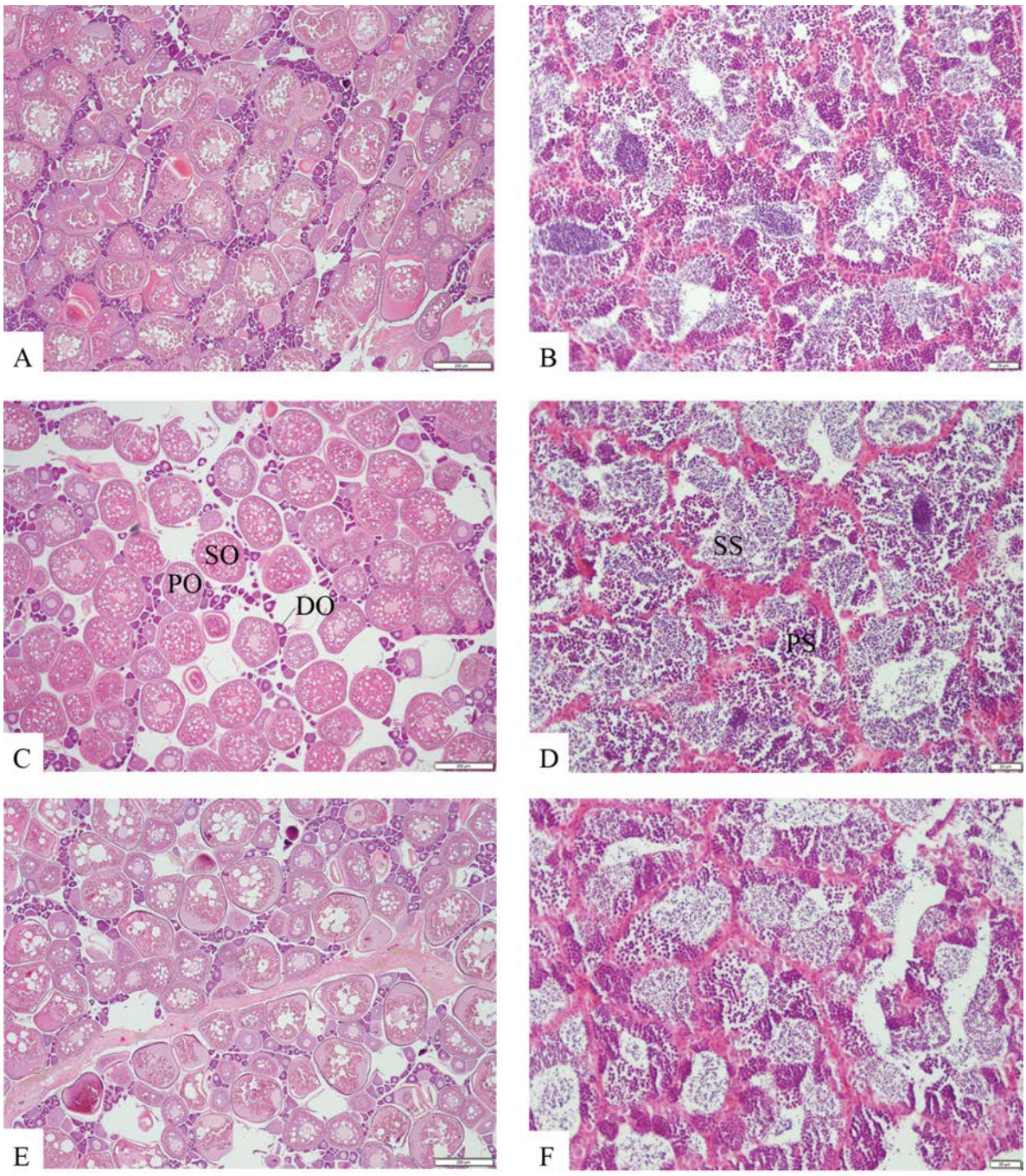

Figure 4.1. Female and male gonads of hybridising *Chaetodon* butterflyfishes. Typical appearance of female (A, C and E) and male (B, D and F) gonads of hybridising *Chaetodon* butterflyfishes from the Christmas Island suture zone. *Chaetodon guttatissimus* (A and B); *C. guttatissimus* × *C. punctatofasciatus* hybrids (C and D); *C. punctatofasciatus* (E and F). Mature hybrids (C and D) of both sexes had normal gametocytes, similar to those of their parental species, at all stages of development. DO: primary oocyte in diplotene stage; PO:

primary oocyte; SO: secondary oocyte; PS: primary spermatocyte; SS: secondary spermatocyte.

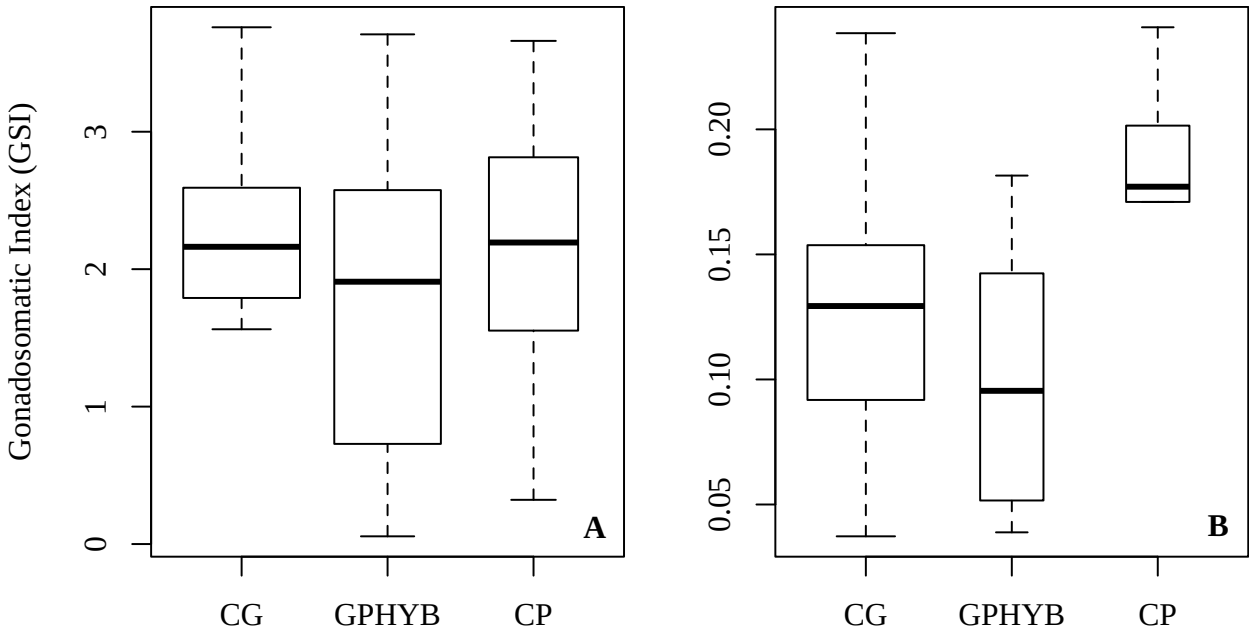

Figure 4.2. Gonadosomatic indices of the *C. guttatissimus* hybrid group at Christmas Island. The width of boxes is proportional to the square root of sample size (see Table 1), for females (A) and males (B). CG: *C. guttatissimus*; GPHYB: *C. guttatissimus* × *C. punctatofasciatus* hybrids; CP: *C. punctatofasciatus*.

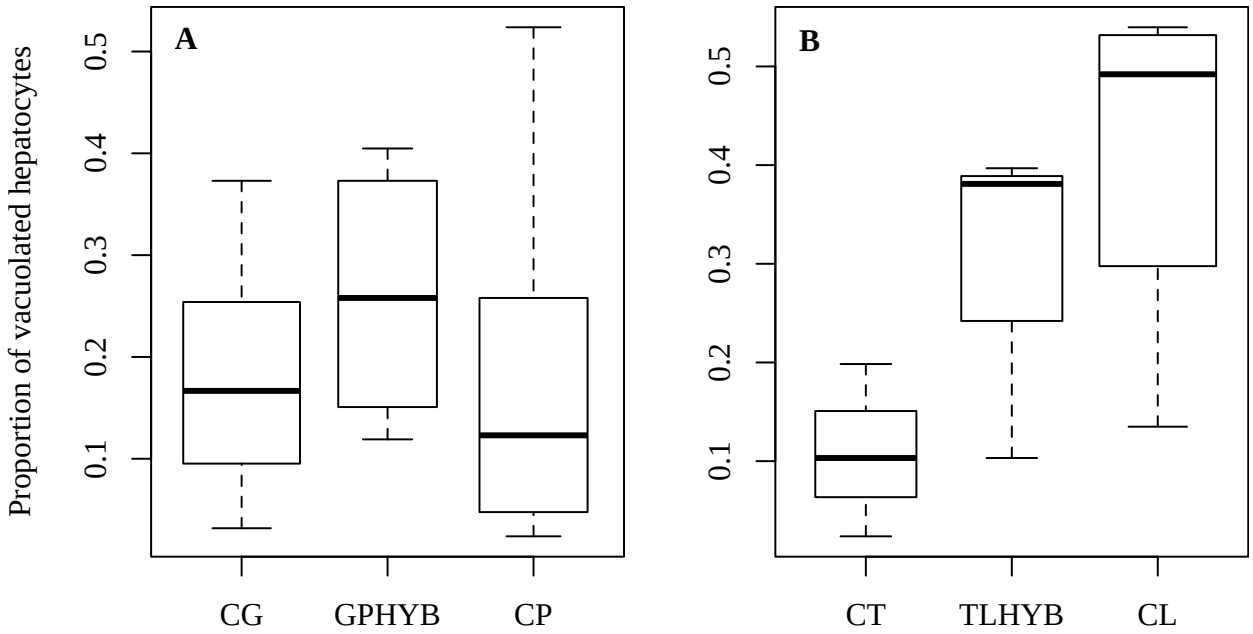

Figure 4.3. Hepatocyte vacuolation in *C. guttatissimus* (A) and *C. trifasciatus* (B) hybrid groups. Solid boxes indicate standard errors and whiskers indicate range (see Table 1 for sample sizes). CG: *C. guttatissimus*; GPHYB: *C. guttatissimus* × *C. punctatofasciatus* hybrids; CP: *C. punctatofasciatus*; CT: *C. trifasciatus*; TLHYB: *C. trifasciatus* × *C. lunulatus* hybrids; CL: *C. lunulatus*.

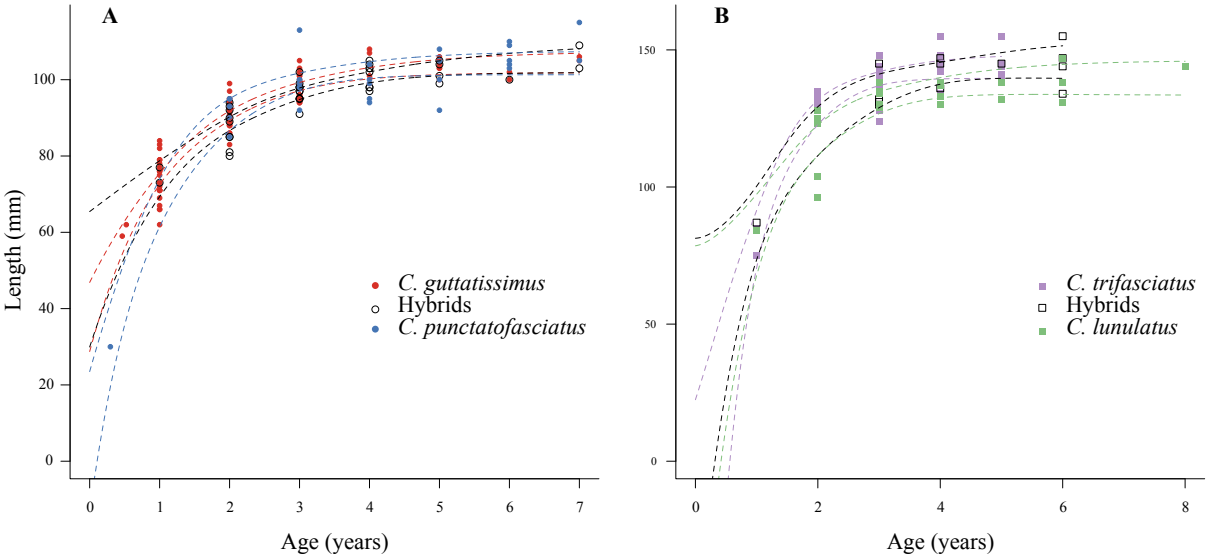

Figure 4.4. Size at age relationships in hybridising *Chaetodon* butterflyfishes at Christmas Island. Von Bertalanffy growth functions fitted to size at age data of all taxa in the *C. guttatissimus* (A) and *C. trifasciatus* (B) hybrid groups. Dots are individual data points and dashed lines are 95% confidence intervals around the fitted models. For sample sizes refer to Table 1.

Table 4.1. Sample sizes for the components of the present study, divided by taxon.

Taxon	Fertility	Body condition	Size at age
Chaetodon guttatissimus	29	14	87
C. punctatofasciatus	12	12	31
***C. guttatissimus* × *punctatofasciatus* hybrids**	15	10	37
C. trifasciatus	4	3	39
C. lunulatus	5	4	23
***C. trifasciatus* × *lunulatus* hybrids**	3	3	13

GENERAL DISCUSSION AND CONCLUSIONS

The studies presented in this thesis are the most comprehensive overview of reef fish hybridisation to date. Using a multidisciplinary approach the work presented here has shone light on marine fish hybridisation, while raising a number of important questions. Review of the current and past marine fish hybridisation literature, presented in Chapter 1, highlighted several knowledge gaps in this field of study, the most important of which were: i) many of the reviewed studies were dated, simplistic reports of odd taxonomic occurrences, for which no data were available other than, occasionally, some meristic analyses; ii) the studies that contained ecological and behavioural information (less than 20 percent) often did so by including data from outside the hybrid zone, limiting the interpretability of those data in terms of the mechanistic underpinnings of hybridisation; iii) genetic data included in the reviewed studies were plentiful, but hard to directly compare, as authors often used a disparate suite of mitochondrial and nuclear markers, precluding the definition of a threshold of divergence after which the ability to hybridise is lost (Mallet 2005). The prevalence of hybridisation in the marine fish realm, coupled with a general lack of ecological and behavioural data from marine hybrid zones, call for more comprehensive studies that should strive to combine direct field-based observations with genetic data. This kind of investigation can lead to deeper conclusions in regards to the evolutionary significance of hybridisation, as shown in seminal studies in the terrestrial and freshwater literature – e.g. Grant *et al.* (2002); Seehausen (2004) -

.

Another interesting question that arose from this work is the apparent latitudinal bias toward tropical marine fish hybridisation. Hubbs (1955) contended that hybridisation at low latitudes is less likely, due to high species richness and general lack of exploitable niches, particularly on coral reefs. The findings presented here are in contrast with these notions and may have their roots in several possible scenarios. The accessibility of shallow, clear tropical waters may today increase our ability to detect hybrids and also, in general, augment the number of studies conducted on tropical marine fauna. Further, the vast majority of the

hybridising tropical marine fishes in the review share striking colour patterns that make them readily identifiable to amateur ichthyologists and aquarium enthusiasts, which may artificially increase the latitudinal bias. Whatever the ultimate cause, recent re-evaluations of biodiversity theory in marine systems (Bowen *et al.* 2013), beg the question of how much influence hybridisation has had in creating and maintaining the staggering levels of diversity we observe in the marine tropical belt.

The choice of taxon for the studies in Chapters 2, 3 and 4 (*Chaetodon*: Chaetodontidae), although not devoid of shortcomings, had several benefits, furthering our understanding of marine fish hybridisation. The butterflyfishes discussed here are pair-forming and monogamous (Pratchett *et al.* 2006a; Yabuta 2007), thus making the distinction between heterospecific social groups easier. In pair-forming, monogamous species of birds, such as *Geospiza* finches (Grant & Grant 2008), the study of hybridisation events has shown that hybridisation can be driven by periodic oscillations in climatic conditions, leading to scarcity of resources. In this regard, our choice of taxon could also lead to ascertain the effects of hybridisation on reef fish populations following climate-driven resource paucity. The species studied here, as well as their respective hybrids, are obligate corallivores (Cole *et al.* 2008), highly dependent on live coral for food and shelter. Monitoring of the hybridising populations of Christmas Island following the dramatic bleaching event of 2016 (Zinke *et al.* 2018), could highlight effects of hybridisation on resource use (if any) as previously done on Darwin finches (Grant & Grant 2002).

When comparing causes and consequences of hybridisation between two different groups of *Chaetodon* butterflyfishes, we found that hybridisation was initiated by similar ecological and behavioural factors, had somewhat dissimilar genetic consequences (e.g. presence/absence of introgression, uni/bi-directional maternal contribution), but we detected similarly low levels of fitness differentiation between hybrids and parents. Following the secondary contact of otherwise allopatric sister species at Christmas Island, along the Indo-Pacific suture zone, these species of butterflyfish readily form heterospecific breeding pairs.

The frequency of pair formation seems driven by the abundance of available partners, indicating a breakdown of assortative mating. Local rarity of conspecifics, as well as overlap in the dietary and spatial ecology of the hybridising parent species (which increases the encounter rate between potential mates), set the scene for heterospecific pair formation. Interestingly, these ecological and behavioural conditions leading to hybridisation have been ascribed a similar role in freshwater fishes (Scribner *et al.* 2000) and terrestrial taxa (Grant *et al.* 2005).

The mitochondrial and microsatellite DNA analyses presented here indicated that, despite having i) the same secondary contact patterns at the Indo-Pacific suture zone, ii) the same social structure (monogamy), and iii) highly similar ecological and behavioural underpinnings for hybridisation (overlap in diet and habitat use, rarity of parental species and non-assortative mating), the two groups of butterflyfish differed in the genetic outcomes of hybridisation. The presence of introgression and bidirectional maternal contribution in the *C. gtttatissimus* group and the contrasting absence of introgression and unidirectional maternal contribution in the *C. trifasciatus* group, in the absence of other detectable differences, lead to the hypothesis that these differences may be a result of the different magnitudes in genetic distance between parent species pairs. This hypothesis found confirmation when data were compared to those available for other reef fish families, allowing some level of generalisation that fit well with general hybridisation theory (Mallet 2001; Whinnett *et al.* 2005; Abbott *et al.* 2013).

Despite apparent similarities with freshwater and terrestrial systems, given the vastly different distribution patterns and life history traits of marine, freshwater and terrestrial organisms, it was reasonable to expect differences in the causes and consequences of hybridisation (Carr *et al.* 2003). Interestingly, the most striking similarities between the study systems presented here and any other terrestrial counterpart were found with flight-capable species - such as butterflies (Whinnett *et al.* 2005) and birds (Grant *et al.* 2005) -, characterised by wide dispersal patterns, somewhat similar to those ascribed to marine

organisms with long pelagic larval duration. The most surprising finding highlighted in the study presented here was the apparent lack of fitness differences between hybrids and parents. Although hybridisation is often thought to have a positive or negative effect on the fitness of hybrid taxa (Pekkala *et al.* 2014), it is not uncommon for hybrids resulting from crosses between recently diverged species to show negligible fitness effects (Grant *et al.* 2005). The study presented in Chapter 4, although limited by small sample sizes, indicated that butterflyfish hybrids have similar fitness to their parents and, given that the Chaetodontidae are a relatively young family (Bellwood *et al.* 2010) characterised by the strong presence of recently diverged sister species - many of which hybridise (Hobbs *et al.* 2013) - it is possible that hybridisation in this family does not carry any significant fitness costs.

It would be of interest to ascertain if such generalisation on the effect of hybridisation on fitness can be made across multiple reef fish families. Having a wider taxonomic sampling range, across, for example, multiple feeding and reproductive strategies, would allow this and also broader generalisations as to what effects hybridisation can or cannot have on marine fish diversity. In the study systems presented here, it is apparent that hybrids will persist in the wild, fulfilling the very important role of genetic exchange through introgression and contributing to the maintenance of biodiversity. This is an effect of hybridisation that has been widely observed in terrestrial (Grant & Grant 2008; Anderson *et al.* 2009) and freshwater (Seehausen 2004) systems, and assessing its presence in reef fishes as a whole would shed light on the role hybridisation plays in the evolution of this group, particularly in light of recent re-evaluations of biodiversity theory in marine systems (Bowen *et al.* 2013).

Lastly, inexpensive and time-efficient genome-wide single nucleotide polymorphism (SNP) screens will prove effective in highlighting crucial aspects of hybridisation at genome level. Introgression is an important consequence of hybridisation and can rapidly accelerate the rate of adaptation and evolution (Abbott *et al.* 2013), however it does not occur at the same rate across genomes. Some markers introgress faster than others, evidence that the influence of hybridisation on diversity and evolution can be subtle (Abbott *et al.* 2013).

Preliminary SNP data from the *Chaetodon guttatissimus* complex indicate that our limited ability to distinguish later generation hybrids (e.g. backcrosses) phenotypically and with the use of microsatellite markers is greatly improved with the use of genome-wide data. The lack of a reliable reference genome for *Chaetodon* limits our power to detect critically important mutations that may be relevant to adaptability. Nonetheless, SNP genome-wide data will hopefully enable us to show that disappearing characteristic hybrid colouration in later generation hybrids is directly linked to genome-wide loss of genetic hybridisation signal (Lavretsky *et al.* 2016). Although the hybridisation signal may be lost within three to four generations of backcrossing (Lavretsky *et al.* 2016), differentiating first generation and backcrossed hybrids with a suitable level of confidence makes genomic data invaluable, particularly if we are to determine how readily hybridisation can influence adaptability. Specifically, genome-wide analyses have enabled identifying genes under selection which lead to direct fitness estimates. For example, the detection of introgressed wolf SNPs in coyote populations lead to the identification of fitness-increasing genes related to body size and skeletal proportions (vonHoldt *et al.* 2016). In the case of butterflyfish hybrids studied here size differences were not detected. However, there may be fitness-related advantages that could become apparent through genomic analyses: for example MHC-mediated parasite resistance as seen in salmonids (Consuegra & Garcia de Leaniz 2008). Hybrid zones are windows to our understanding of evolution and adaptation (Harrison 1990): modern genetic technology combined with careful field observations can generate fresh insights into the role hybridisation has had, presently has and will have in marine fish evolution.

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