A MULTI-DISCIPLINARY EVALUATION OF THE HYBRID ANEMONEFISH *AMPHIPRION LEUCOKRANOS*: BEHAVIOUR SHAPING EVOLUTIONARY OUTCOMES OF HYBRIDIZATION

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STATEMENT OF CONTRIBUTION OF OTHERS

A number of collaborators and colleagues have contributed in various ways to this thesis and the subsequent manuscripts which are published, submitted or in preparation for submission. A summary of these contributions are listed below.

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GENERAL ABSTRACT

Hybridization is an evolutionarily significant and common occurrence in nature. Interspecific breeding between closely related taxa challenges established phylogenies and questions what constitutes a species; where hybridization may drive rapid evolution of taxa and provide a source of evolutionary novelty to ecosystems. Despite the enhanced study of hybridization in recent time, a clear understanding of the mechanisms which initiate and maintain hybridization remains limited. Hybrid zones are ideal for testing theoretical questions regarding speciation and elucidating patterns of variation among species. For many taxa, reproductive success is skewed towards large, socially dominant individuals within groups, and reproductive traits and mating systems are expected to influence the outcomes of hybridization and gene exchange between hybridizing species. In this thesis, I addressed how ecology and behaviour contribute to maintenance and persistence of the *Amphiprion leucokranos* hybrid zone. This research employed a combination of ecological, phylogenetic and population genetic assays, as well as an observational study and behavioural experiment to test key concepts of the importance of hybridization to evolution of species.

A suite of 42 novel and published microsatellite markers were developed and tested in Chapter 2 to facilitate investigation into the relatedness of taxa within the hybrid zone. The influence of ecology and behaviour on the outcomes of hybridization were tested in Chapter 3, addressing how habitat use and relative size differences of parent species and hybrids drive patterns of gene exchange. Findings confirmed *A. leucokranos* to be the hybrid of closely related *A. chrysopterus* and *A. sandaracinos*, and subsequently verified that behavioural isolation, habitat use and species-specific size differences dictates the direction and degree of back-crossing and subsequent introgression. Hybridization took place exclusively with *A. chrysopterus* in the dominant female rank and the smaller *A. sandaracinos* in the sub-dominant male rank, based on mtDNA cytochrome *b* and multiple nDNA microsatellite loci. Overlap in habitat, depth and host anemone use was found, with hybrids intermediate to parents and co-habitation in over 25% of anemones sampled. Hybrids, intermediate in body size, colour and pattern, were classified 55% of

the time as morphologically first generation hybrids relative to parents, whereas 45% of hybrids were more like *A. sandaracinos*, suggesting back-crossing. Unidirectional introgression of *A. chrysopterus* mtDNA into *A. sandaracinos* via hybrid back-crosses was found, with larger female hybrids and small male *A. sandaracinos* mating. Chapter 4 elucidated the mechanisms driving and maintaining hybridization through investigating whether social and ecological factors facilitating hybridization varied across the hybrid zone, and if this influenced gene flow and introgression regionally. Findings revealed that the relative frequency and size disparities of parent species drive regional ecological patterns and gene flow among taxa, where species integrity is maintained despite extensive mixed species group cohabitation and back-crossing. Conspecific groups were most common in Kimbe Bay (65%) where parent species relative frequency was similar. Mixed species groups dominated the Solomon Islands (82%), with larger *A. chrysopterus* found over 1.5 times more often than smaller *A. sandaracinos*. Hybrid phenotypes were highly variable across the hybrid zone, reflecting extensive back-crossing among hybrids and parent species relative to region. nDNA microsatellites defined two genetic clusters in the hybrid zone that represent parent species, despite ongoing back-crossing. Pure parent species size and relative frequency explained the existing genetic structure throughout the hybrid zone, again reflecting the characteristic size-based dominance behaviour of anemonefish. Subsequently, hybrids were directly compared to pure species when queuing within mixed groups for reproductive breeding positions in Chapter 5, addressing a significant gap in hybridization studies where hybrid inferiority is often assumed. Here, the persistence of species barriers in the face of hybridization and back-crossing was investigated, and it was demonstrated that hybrids are not always inferior to pure species, particularly when a predetermined factor such as maximum size influences dominance within a group. Hybrids positively changed rank faster and held dominant ranks more often than the smaller parent species, *A. sandaracinos*, indicating a fitness advantage to hybrids in the context of size-based hierarchical anemonefish breeding queues. Ultimately, hybrid only groups displayed courting behaviours associated with reproduction just as early and frequently as pure parent species. Finally, the finding of ‘queue jumping’ by larger *A. chrysopterus* highlighted the significance of abundance disparities and relative size differences to this hybridization event. This novel

mechanism appears to have provided a fitness opportunity for one hybridizing taxon and may be the mechanism which originally facilitated hybridization between *A. chrysopterus* and *A. sandaracinos.*

This thesis represents an important contribution to our understanding of how hybridization persists through time and provides insight into the ecological and evolutionary outcomes for hybridizing taxa. Overall, this research highlights the importance of ecology and behaviour to the consequences of hybridization, driving patterns of gene flow and introgression across the hybrid zone. Findings suggest the coral reef fish hybrid *A. leucokranos* may differentiate from pure parent taxa in time, emphasizing the importance of protection for hybrids that may contribute to the biodiversity of coral reef systems. Despite challenging established phylogenies and fundamental ideas of the purity of 'true species', hybridization may hold value in conserving biodiversity, particularly on coral reefs in global decline. Conservation management must consider evolutionary theory and legislate for the protection of hybrid taxa on a case-by-case basis to effectively manage future biodiversity challenges in a changing climate.

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CHAPTER 1: General introduction

Natural hybridization was once thought to be rare and insignificant to evolution (Mayr, 1942, Dowling and Secor, 1997, Coyne and Orr, 2004). Increased interest in the study of ecology and evolution and the utilization of molecular tools have revealed hybridization to be common (Mallet, 2005) and produce genetically distinct offspring which may contribute to speciation events through introgression (Abbott et al., 2013, Meier et al., 2017). Furthermore, despite classical views of reduced fitness of hybrid offspring compared to pure species, hybrids are not always inferior to pure species and may have intrinsic fitness benefits compared to parent taxa (Arnold, 1997, Arnold et al., 2001, Burke and Arnold, 2001). Hybridization most often occurs between closely related species (Seehausen, 2004) on secondary contact due to range expansion or disturbance (Abbott et al., 2003, Johannesen et al., 2006, Zinner et al., 2009), where one species is relatively rare (Currat et al., 2008). The facilitation of gene exchange between closely related but distinct groups of taxa has the potential to introduce evolutionary novelty within a system faster than known mutation rates (Grant and Grant, 1994, Kunte et al., 2011, Meier et al., 2017). Barriers to gene exchange may breakdown resulting in merging of taxa (Taylor et al., 2006, Kleindorfer et al., 2014) or species integrity maintained through strengthening of gene exchange barriers (Wu, 2001, Via, 2009, Servedio and Noor, 2003) in the face of hybridization. However, among nominal hybrids identified, parent species are often unknown and the mechanisms that promote hybridization and explain patterns of introgression are poorly understood (Abbott et al., 2013). The study of young hybrid taxa therefore allows contemporary insights into potential speciation or reverse speciation events in motion within contact zones of closely related taxa; where rapid adaptive radiations may be underway (Seehausen, 2004, Gourbiere and Mallet, 2010, Price and Bouvier, 2002, Meier et al., 2017).

Hybridization is concentrated in regions termed hybrid zones, showcasing varied genetic, morphological and ecological patterns which result from interspecific reproduction, in which novel ecological opportunities may arise (Abbott et al., 2013). These regions are thought to be the greatest resource for investigating hybridization and the patterns of variation among species. Often, hybrid

zones mark the parapatric boundaries of closely related species, where overlaps in dispersal distance to biogeographical distributions of independently evolved sister taxa facilitate contact. Within the marine environment hybridization is becoming increasingly evident (Willis et al., 2006), particularly along coastal areas (Gardner, 1997), such as coral reefs (Marie et al., 2007, Vollmer and Palumbi, 2002, Yaakub et al., 2006), sub-tidal zones (Koehn, 1991, Rawson and Hilbish, 1995) and in open ocean habitats such as along hydrothermal vents (Johnson et al., 2013, Van Dover et al., 2002, O'Mullan et al., 2001). Suture zones, where hybridization is prolific for many groups of taxa (Remington, 1968, Hewitt, 2000), define hybrid hotspots. In tropical regions, hybridization is concentrated at two coral reef suture zones. The Socotra Archipelago, where the Red Sea, Arabian Sea, western Indian Ocean and greater Indo-Polynesian regions meet is a hot spot for hybridization, particularly in coral reef fishes. Similarly, Christmas Island marks the location where regionally distinct Indian and Pacific Ocean species come into contact and hybridize (Hobbs et al., 2009, Marie et al., 2007, Hobbs and Allen, 2014). Similarly, suture zones are known within the temperate marine environment, regions along the southern Atlantic coast of Florida (Bowen and Avise, 1990, Karl and Avise, 1992) and the Baltic Sea (Johannesen et al., 2006, Riginos and Cunningham, 2005).

Ecological factors associated with hybridization and the evolutionary outcomes for taxa in hybrid zones are complex and dynamic (Abbott et al., 2013), and may act to promote or facilitate hybridization between closely related species. Closely related taxa which overlap biogeographically (McMillan and Palumbi, 1995, Mallet, 2005), often from allopatric distributions, must also make contact on an ecological scale, where behaviour and ecology play a significant role in shaping hybridization event outcomes. Relative local abundance of species (Randall et al., 1977b, Frisch and van Herwerden, 2006, Marie et al., 2007, Hobbs and Allen, 2014, Montanari et al., 2014) and the availability of a shared limited resource (Gainsford et al., 2011, Marie et al., 2007, Montanari et al., 2012, Montanari et al., 2014, van Herwerden et al., 2006, Yaakub et al., 2007, Yaakub et al., 2006) will contribute to how often taxa come into potential reproductive contact and whether a breakdown in assortative mating occurs when pairs and groups form (Montanari et al., 2016, McMillan et al., 1999). These ecological factors are known to be highly variable across hybrid

zones, and as such the causes and consequences associated with species-specific hybridization events may also vary.

Hybridization can result in a diverse range of outcomes over evolutionary time (Abbott et al., 2013, Arnold, 1992, Arnold, 1997, Barton, 2001). Firstly, reinforcement of reproductive isolation may be driven by natural selection against maladaptive hybridization in previously separated populations on secondary contact. This was demonstrated during experimentally induced hybridization in naturally sympatric and allopatric *Drosophila* populations; where allopatric populations evolved similar cuticular hydrocarbons, important in communication and mate choice, to those occurring in sympatry following hybridization, reinforcing mate recognition (Higgie et al., 2000). Reinforcement of pre-mating isolation was also found to be driven by natural selection on frog call, an important trait in mate choice, following hybridization between northern and southern lineages of a common rainforest frog leading to rapid diversification (Hoskin et al., 2005). Alternatively, reverse speciation may occur when two species fuse to become one taxon. For instance, hybridization lead to reverse speciation in sympatric three-spined Sticklebacks in the lakes of British Columbia (Taylor et al., 2006). In this case, benthic and limnetic species were known to live in sympatry, biologically delineated by morphological, ecological and genetic differences maintaining strong reproductive isolation of taxa. Hybridization between previously reproductively isolated species proliferated following the introduction of an exotic species and increased nutrient loading from urban runoff, which resulted in increased turbidity within lakes. In this example, two morphologically and genetically distinct species evolved into a hybrid swarm and then a single, highly variable population over a period of 20 years due to a breakdown of isolating barriers, resulting in a loss of species following hybridization (Taylor et al., 2006). Hybridization may also lead to the generation of new distinct populations of mixed ancestry, thus providing the foundation for diversification and speciation that is reliant on the continued production of fit hybrids in a stable hybrid zone over time. A number of hybridization events are proposed to be the driving force behind the evolution of novel trait combinations in east African Lake Victoria cichlid fishes, which resulted in five species following large-scale adaptive radiation in recent evolutionary time (Keller et al.,

2013). Species are currently differentiated based on varied colouration, feeding ecology, depth distribution, morphological traits and genetic differences which evolved during diversification facilitated by hybridization. In another example, sister cichlid population pairs isolated on separate islands have been found to diversify in an adaptive radiation following two speciation events, where the second event was associated with introgressive interspecific gene flow from an allopatric population which arose earlier (Meier et al., 2017).

On coral reefs, where communities of closely related species assemblages co-exist in a dynamic environment (Choat and Bellwood, 1991, Bellwood and Hughes, 2001), conditions may promote hybridization between species and significantly contribute to evolutionary processes for taxa (Rocha et al., 2005, Mallet, 2007, Gardner, 1997, Marie et al., 2007). With the implementation of molecular tools, hybridization on coral reefs is becoming increasingly evident and better studied, particularly in hard corals and coral reef fishes. In coral reef fishes alone, hybridization has been recorded in almost 30% of Pomacanthidae (Pyle and Randall, 1994, Gainsford et al., 2015) and Chaetodontidae (McMillan et al., 1999, Montanari et al., 2012, Hobbs et al., 2013) families to date, and to a lesser extent in the Labridae, Pomacentridae and Serranidae (Yaakub et al., 2006, DiBattista et al., 2016). The evolutionary consequences of hybridization to coral reef fish species varies greatly, with outcomes including uni- and bi-directional gene exchange, with and without the introgression of genetic material of one species into the other via continued hybridization and backcrossing (Bernal et al., In Press). Introgression facilitates rapid diversification and can lead to enhanced taxonomic diversity and enhanced adaptive potential to change, where pre-adapted hybrids may retain genetic variation of parental contributors allowing exploitation of niche environments and resources (Seehausen, 2004, Meier et al., 2017).

Evolutionary outcomes of hybridization often depend on the reproductive traits and mating systems of species involved. As such, including ecological and behavioural assays in hybridization studies is vital to comprehensively understand the species specific mechanisms involved in each event and the evolutionary consequences for hybridizing taxa (Montanari et al., 2016). In coral reef fishes, a variety of mating systems exist, including taxa that live and reproduce in groups, form pairs

and those that associate in polygamous assemblages. Coral trout form haremic groups and have been found to hybridize in some parts of their range. In one study, large male *Plectropomus maculatus* were found at 25% of *Plectropomus leopardus* spawning aggregations, which facilitated gene exchange through sneak mating between species and lead to introgression and subsequent fixation (Frisch and van Herwerden, 2006, van Herwerden et al., 2006). Pair forming butterflyfish have also been implicated in a number of hybridization events. In some cases this was facilitated by non-assortative mating as a result of local species rarity which resulted in uni-directional gene-exchange between species with no introgression, having limited, localized evolutionary consequences for the species involved (Montanari et al., 2012). Finally, Labrids provide an example of hybridization in polygamous assemblages, where sneak mating (or accidental fertilisation) of a gravid female by a male wrasse of a rarer species during spawning aggregations has been documented (Yaakub et al., 2006). This sneak mating behaviour paired with a polygamous mating system has resulted in bi-directional gene exchange and subsequent genetic introgression.

The primary goal of this thesis is to elucidate how ecology and behaviour contribute to maintenance and persistence of a natural hybrid zone, addressing a major gap in the understanding of ecological and evolutionary mechanisms underlying natural hybridization. Research focused on the nominal coral reef fish species *Amphiprion leucokranos* throughout its natural range from Papua New Guinea to the Solomon Islands. Anemonefish though to be involved in the hybridization are vastly different in size, known to share anemone hosts and have recently come back into contact in one biogeographical region with an evolutionarily recent history of disturbance. A phylogeny based on a consensus of three mtDNA genes (cytochrome *b*, 16S rRNA and D-loop) across 28 species of anemonefish found species *A. chrysopterus* and *A. sandaracinos* to be distinct, with the putative hybrid *A. leucokranos* identified as most closely related to *A. chrysopterus* (Santini and Polacco, 2006). This known phylogeny suggests that taxa are closely related and potentially that most often *A. chrysopterus* is the female in this hybridization; however findings are derived from exclusively female-mediated mitochondrial genes and a significant gap exists in comprehensively understanding the relatedness of these taxa.

Anemonefish are group forming coral reef fish with strong hierarchical behaviour dictating the reproductive success of individuals within queues (Buston, 2004). In queues, large and dominant individuals are reproductively more successful than subordinates, where size drives position within the dominance hierarchy (Buston, 2004, Buston and Cant, 2006). Females are largest, followed in size and rank by males, and non-breeding subordinates which are progressively smaller in size. As such, larger individuals within groups have greater reproductive output and the potential to be fitter than smaller individuals as a function of size dominance (Fricke, 1979, Ochi, 1986, Hattori, 1991, Buston, 2003). Anemonefish are also protandrous hermaphrodites (Moyer and Nakazono, 1978), changing from non-breeding subordinates to males and finally females within queues following a higher ranked individual′s removal from groups either naturally or by manipulation. Anemonefish are site attached, with group size driven by anemone host size, and easily manipulated *in situ* (Allen, 1972). The site attachment and strong hierarchical mating behaviour of anemonefish are ideal for monitoring and manipulating groups experimentally to address ecological and evolutionary questions.

In chapter 2 of this thesis, a genetic toolkit was developed to investigate the contemporary relatedness of focal study taxa through characterising and then testing the cross-amplification success of 42 microsatellite markers, including eight novel markers. Subsequent chapters 3 and 4 were extensively informed by the suite of microsatellite markers developed in Chapter 2 and additionally the female-mediated mitochondrial cytochrome *b* gene. Based on known hierarchical behaviour in anemonefish, where dominance is directly related to size of individuals within groups, Chapter 3 explicitly tested how habitat use and relative size differences of parent species and hybrids determine patterns of gene exchange. It was expected that when taxa are in mixed groups, the larger species, *A. chrysopterus,* will always be the female contributor to the hybridization and the smaller species, *A. sandaracinos,* will be the male, due to the size dominance hierarchy that dictates anemonefish queues. Firstly, the ecological scale at which species are in contact, from anemone host co-habitation to relative abundance of taxa across one region of the hybrid zone, was determined based on one hundred anemonefish groups, including pure groups of parent taxa and mixed groups

including putative hybrids. Paired with habitat usage and species size data, molecular data confirmed that hybrid status of *A. leucokranos* derived from the evolutionarily recent hybridization between large *A. chrysopterus* females and small *A. sandaracinos* males. Chapter 4 then assessed 1) whether social and ecological processes that are associated with hybridization including relative abundance and resource use vary across the hybrid zone, and 2) subsequently whether spatial variation in social and ecological factors affect patterns of hybridization and introgression among species. This chapter aimed to elucidate the underlying mechanisms associated with the persistence and maintenance of the *A. leucokranos* hybrid zone and the evolutionary outcomes that might be expected given time. Finally, Chapter 5 experimentally compared hybrids to pure species *in situ*, both in the observational study of queues over time and in manipulative experiments of anemonefish queues. Hybrid theory suggests that hybridization should be selected against through reinforcement of reproductive barriers to maintain species boundaries. Here, hybrid inferiority was assessed in an ecological and behavioural context within a strict dominance hierarchy, testing assumptions of reproductive inferiority often associated with hybrid taxa.

This thesis is distinct among hybridization studies, drawing on a broad suite of techniques to investigate hybrid ecology and evolution where a paucity of studies exists that incorporate multi-disciplinary methodology and *in situ* experimental designs. This thesis employs on a combination of ecological, phylogenetic and population genetic assays, as well as an observational study and behavioural experiment to test key concepts on the importance of hybridization to evolution of species. The study of young hybrid taxa, such as anemonefish, allows for contemporary insights into potential speciation events in motion, and in this way the *A. leucokranos* hybrid zone provides an ideal natural laboratory for studying hybridization and investigating the patterns of variation among species.

CHAPTER 2: Characterisation and cross-amplification success of 42 microsatellite markers in two *Amphiprion* species and a hybrid anemonefish commercially harvested for the ornamental fish trade.

Supplementary Material Appendix 1 in: Gainsford, A., Jones, G.P., Hobbs, J-P., Heindler, F.M., and van Herwerden, L., (In Prep). Balancing Introgression and species integrity across a coral reef fish hybrid zone.

2.1 Abstract

This study presents cross-amplification success of 42 *Amphiprion* microsatellite loci including 8 novel markers, across individuals from *A. sandaracinos, A. chrysopterus* and the hybrid *A. leucokranos,* which are commercially targeted for the aquaria trade. Analysis revealed 15, 20 and 24 highly polymorphic loci (PIC > 0.5) in the two parent species and hybrid, respectively, for use in population genetic and parentage studies, with 305 unique alleles found overall (ranging from 1 to 13 alleles per locus) with 7 alleles per locus on average. Observed and expected heterozygosities ranged from 0.000 to 1.000 and 0.000 to 0.978, respectively. Significant deviations from Hardy-Weinberg equilibrium were found in eight loci, possibly due to relatedness among samples or the presence of null alleles.

2.2 Introduction

Anemonefish (Pomacentridae) represent a monophyletic clade recently diversified through adaptive radiation linked with hybridization events (Litsios and Salamin 2014). The yellow anemonefish, *Amphiprion sandaracinos* (Allen, 1972), has been shown to hybridize with the orange-fin anemonefish, *Amphiprion chrysopterus*, and hybrid *Amphiprion leucokranos* (Gainsford et al. 2015). Anemonefish are commercially harvested for the ornamental fish trade, where global conservation and management does not include protection for hybrid populations, despite the

importance of hybridization to the evolution and diversification of commercially targeted species (Litsios and Salamin 2014).

The utility of microsatellite markers for investigating ecologically relevant evolutionary processes, such as hybridization in natural populations, can be attributed to the high resolution genetic information they provide. Microsatellites (simple sequence repeats or short tandem repeats) commonly display high levels of polymorphism and allelic diversity, with fast mutation rates (Sunnucks, 2000). When combined microsatellite markers generate unique genetic profiles for individuals within populations, proving to be a highly informative tool for ecological and evolutionary studies, readily applied to evaluate contemporary species to population level connectivity, reproductive behavioural patterns and parentage assignment within a population. Applying this molecular tool will provide insight into contemporary patterns of dispersal and gene flow within and among study taxa to inform conclusions in Chapters 3 and 4.

This study presents cross-amplification success of 42 *Amphiprion* microsatellite loci including the development of 8 novel markers, across individuals from *A. sandaracinos, A. chrysopterus* and the hybrid *A. leucokranos,* commercially targeted for the aquaria trade, to investigate patterns of gene flow among taxa and the evolutionary implications of this for the species involved.

2.3 Methods and Results

Microsatellite marker development followed standard protocols described elsewhere (Gardner et al. 2011), using genomic DNA isolated from *A. sandaracinos,* sampled from Christmas Island, Indian Ocean (10°25’-10°34’S, 105°32-105°42E), and shotgun sequenced on one-sixteenth of a plate using Titanium GS-FLX (454 Roche) instrumentation. Approximately 40.58Mbp of DNA sequence data was obtained from 883,689 sequence reads (average length 292bp), representing approximately 4.2% coverage of an average *Amphiprion* spp. haploid genome (http://www.genomesize.com). Sequences were screened for di- to hexa-nucleotide microsatellites with six or more repeats using default settings of QDD v1.3 as described elsewhere (Holleley and

Geerts 2009), yielding 109 primer pairs. Primer selection included: amplicon length of 90-300bp; 20bp optimal primer length (range 19-25bp); optimal primer melting temperature T_M 60°C (range 58-60°C); pure repeat quality; repeat motifs (tri- and tetra-nucleotides preferred); and reduced penalty values.

Primer pairs for 24 loci were selected for development, and forward primers synthesised to include a lambda tag (5-GGTGGCGACTCCTGGAG-3) at the 5' end, for indirect fluorescent labelling by primer tailing. Testing of amplification success and specificity was carried out on 8 individuals from natural populations of *A. sandaracinos*, *A. chrysopterus,* and hybrid *A. leucokranos*, respectively, using Type-it Microsatellite PCR kit (QIAGEN), with genomic DNA isolated using a standard salting out protocol. Individual amplifications (10µl reactions) contained 20-50ng DNA template, 2X Type-it Multiplex PCR Master Mix (QIAGEN), and 2µM of each primer (forward and reverse). Tailed forward primer and reporter primer (5' labelled with fluorescent dye modification FAM) were included in indirectly labelled reactions (1:4; total = 0.2µM). Reaction conditions were as follows: initial 3 min denaturation at 94°C, 28 cycles of 95°C for 30 s (denaturation), 60°C for 1 min 30 s (annealing) and 72°C for 30 s (extension), and final extension at 60°C for 30 min using a Bio-Rad C1000 Thermal Cycler (Bio-Rad, Australia). PCR products were visualised by gel electrophoresis using 2.0% agarose, and column purified using GE Illustra Sephadex G-50. Genotypes were run on an ABI 3730XL Genetic Analyser (Applied Biosystems) with a GeneScan LIZ-600bp size standard and scored using GeneMarker (SoftGenetics, USA). The details of 8 loci with interpretable peak profiles, chosen for further use, are presented here (Table 2.1), although amplification success and polymorphism varied across species and sampling location (Table 2.2).

Table 2.1 Details of 8 novel microsatellite markers developed from *Amphiprion sandaracinos* 454 sequencing data.

Locus		Primer sequence (5'-3')	Repeat motif	T_A (°C)	Accession no.
As9	F:	[FAM]GGCCCTACAGAGGATTAAGCA	$(ATGG)_{10}$	60	KJ434608
	R:	CAGGATTGCTTGTCATCATTG			
As16	F:	[FAM] TGGAGACGGTGATGGACATA	$(GAT)_{13}$	60	KJ434615
	R:	ATCATCCTCACCAACCAAGC			
As17	F:	[FAM]GCTGGAGAACTGAGGCTGAC	$(ATA)_{13}$	60	KJ434616
	R:	GAAACCCACTAAAGGCGACA			
As19	F:	[FAM]TCCAGTAGTGAGTTTATTCTCCTGG	$(TTA)_{13}$	60	KJ434618
	R:	TTGGACCAGTTGATTGCTGA			
As21	F:	[FAM]GCAACAATACAAACACCGCA	(ACT)10	60	KJ434620
	R:	TCATATTACATCTGTGCTCATTTCA			
As22	F:	[FAM]CTGGTGCTGTGTTGGCTAAA	$(CAG)_9$	60	KJ434621
	R:	CGAGGCTGAAGGGATTAGAG			
As23	F:	[FAM]TGTTGGAGTGTCACCTGGAG	$(TGA)_9$	60	KJ434622
	R:	TGCAGCTGTGGAGACAAAGT			
As24	F:	[FAM]TGTAGGTCAGAGCCGCAGTA	$(AGC)_9$	60	KJ434623
	R:	AGCTGTCGATCAGAACTCGG			

T_A annealing temperature

Table 2.2 Amplification success and characterisation of 12 *A. sandaracinos*, 20 *A. latezonatus*, 5 *A. mccullochi* and 5 *A. chrysopterus* microsatellite markers, developed from 454 sequencing data, in 8 individuals of each of *Amphiprion chrysopterus, Amphiprion sandaracinos* and their natural hybrid, *Amphiprion leucokranos,* collected from Melanesia; where N_A = number of alleles, H_O = observed heterozygosity, H_E = expected heterozygosity (* indicates significant departure from HWE at $P < 0.016$ after FDR correction), *PIC* = polymorphic information content (PIC > 0.500 indicated in bold), and [A, B, C, D, E, F, G] multiplex assignment are indicated.

Locus	Success	N_A	Allelic size (bp)	H_O	H_E	*PIC*	Success	N_A	Allelic size (bp)	H_O	H_E	*PIC*	Success	N_A	Allelic size (bp)	H_O	H_E	*PIC*
	A. sandaracinos						*A. chrysopterus*						*A. leucokranos* (hybrid)					
A. sandaracinos (Christmas Is., Indian Ocean); source: present study & Gainsford et al. 2015																		
As9	0/8	-	-	-	-	-	7/8	5	129-145	0.571	0.593	**0.521**	0/8	-	-	-	-	-
As16	0/8	-	-	-	-	-	0/8	-	-	-	-	-	8/8	2	225-231	0.000	0.533	0.375
As17	0/8	-	-	-	-	-	0/8	-	-	-	-	-	8/8	8	108-228	0.875	0.825	**0.746**
As19	1/8	1	160	0.000	0.000	-	8/8	4	144-230	0.625	0.658	**0.572**	7/8	5	144-160	0.429	0.505	0.448
As21	0/8	-	-	-	-	-	8/8	5	115-124	0.625	0.683	**0.584**	8/8	5	124-142	0.625	0.783	**0.685**
As22	2/8	1	115	0.000	0.000	-	8/8	4	121-133	0.500	0.675	**0.570**	8/8	5	115-133	0.375	0.650	**0.561**
As23	8/8	4	103-136	0.625	0.517	0.443	8/8	5	124-136	0.625	0.767	**0.679**	8/8	7	97-136	1.000	0.750	**0.678**
As24	3/7	1	115	0.000	0.000	-	5/7	4	128-171	0.400	0.644	**0.535**	3/7	4	131-171	0.667	0.867	**0.671**
As6[C]	8/8	6	144-178	0.625	0.817	**0.733**	8/8	4	144-156	0.500	0.442	0.387	8/8	1	144	0.000	0.000	-
As8[G]	8/8	4	122-154	0.500	0.650	**0.530**	8/8	5	122-158	0.625	0.825	**0.737**	8/8	4	122-158	0.250	0.650	**0.530**
As18[D]	8/8	2	81-93	0.000	0.233*	0.195	1/8	1	208	0.000	0.000	-	8/8	4	81-96	0.625	0.692	**0.592**
As20[A]	8/8	2	213-239	0.125	0.325	0.258	8/8	1	213	0.000	0.000	-	7/8	2	213-239	0.429	0.495	0.354
A. latezonatus (Lord Howe Is.); source: Steinberg et al. 2015																		
Alat1[B]	0/8	-	-	-	-	-	2/8	2	107-140	0.000	0.667	0.375	1/8	1	107	0.000	0.000	-
Alat3[C]	8/8	1	91	0.000	0.000	-	8/8	1	91	0.000	0.000	-	8/8	1	91	0.000	0.000	-
Alat4[A]	8/8	3	117-134	0.125	0.242*	0.215	8/8	7	119-142	0.875	0.858	**0.779**	7/8	4	119-127	1.000	0.714	**0.615**
Alat5[D]	8/8	2	98-100	0.000	0.233*	0.195	8/8	6	92-120	0.875	0.867	**0.785**	8/8	5	92-102	0.875	0.750	**0.657**
Alat7[E]	8/8	2	91-93	0.000	0.233*	0.195	8/8	5	91-112	0.625	0.650	**0.561**	8/8	6	91-121	1.000	0.717	**0.631**
Alat8[E]	8/8	5	102-148	0.625	0.608	**0.539**	8/8	6	102-140	1.000	0.817	**0.728**	8/8	8	102-144	1.000	0.875	**0.799**
Alat9[E]	8/8	2	89-98	0.125	0.325	0.258	7/8	2	98-101	0.143	0.143	0.124	8/8	2	89-98	0.500	0.500	0.359
Alat10[A]	8/8	5	100-124	1.000	0.750*	**0.657**	8/8	3	100-121	0.500	0.567	0.468	8/8	5	106-130	0.625	0.825	**0.737**
Alat11[B]	7/8	2	232-238	0.286	0.527	0.370	7/8	5	232-287	0.000	0.848	**0.744**	7/8	3	232-246	0.143	0.626	**0.517**
Alat12[C]	8/8	3	245-251	0.625	0.575	0.447	8/8	3	248-257	0.625	0.592	0.456	8/8	4	245-257	0.750	0.650	**0.559**
Alat13[D]	8/8	4	301-316	0.250	0.692	**0.592**	6/8	5	301-334	0.667	0.848	**0.741**	6/8	4	298-313	0.000	0.727	**0.620**
Alat14[A]	8/8	2	293-299	0.000	0.233*	0.195	8/8	1	299	0.000	0.000	-	7/8	2	293-299	0.571	0.527	0.370
Alat16[B]	7/8	6	162-187	0.571	0.791	**0.701**	7/8	8	106-192	0.857	0.923	**0.842**	8/8	13	107-187	0.875	0.975	**0.908**
Alat17[D]	7/8	7	191-277	0.429	0.846	**0.759**	5/8	2	187-191	0.000	0.356	0.269	8/8	2	191-221	0.125	0.125	0.110

Alat18[E]	8/8	1	222	0.000	0.000	-	8/8	2	222-274	0.125	0.125	0.110	8/8	1	222	0.000	0.000	-
Alat19[E]	7/8	5	324-352	0.857	0.758	**0.657**	0/8	-	-	-	-	-	5/8	5	328-352	0.200	0.867	**0.745**
Alat20[A]	7/8	3	270-278	0.571	0.582	0.453	0/8	-	-	-	-	-	6/8	3	270-278	0.167	0.439	0.363
Alat21[D]	8/8	6	221-258	0.750	0.817	**0.733**	8/8	6	195-292	0.125	0.875	**0.795**	8/8	6	226-262	0.375	0.858	**0.778**
Alat22[F]	8/8	8	166-296	0.500	0.908	**0.835**	8/8	1	166	0.000	0.000	-	8/8	6	166-296	0.750	0.742	**0.666**
Alat23[A]	8/8	2	204-209	0.000	0.233*	0.195	8/8	4	204-229	0.375	0.350	0.313	7/8	3	204-229	0.714	0.538	0.427
A. mccullochi (Lord Howe Is.); source: Van Der Meer et al. 2011.																		
Am5[G]	8/8	5	72-98	0.375	0.708*	**0.618**	8/8	4	78-94	0.500	0.517	0.443	8/8	6	72-92	0.625	0.675	**0.599**
Am9[E]	8/8	8	142-232	1.000	0.875	**0.799**	8/8	11	176-234	1.000	0.950	**0.881**	8/8	11	145-232	1.000	0.950	**0.881**
Am18[A]	7/8	1	124	0.000	0.000	-	0/8	-	-	-	-	-	2/8	1	124	0.000	0.000	-
Am21[F]	8/8	1	91	0.000	0.000	-	8/8	1	91	0.000	0.000	-	8/8	1	91	0.000	0.000	-
Am24[C]	0/8	-	-	-	-	-	1/8	2	246-252	1.000	1.000	0.375	0/8	-	-	-	-	-
A. chrysopterus (Moorea); source: Beldade et al. 2009.																		
A115[B]	7/8	1	174	0.000	0.000	-	7/8	8	107-231	0.429	0.901	**0.818**	8/8	4	123-215	0.250	0.350	0.313
A130[C]	8/8	10	268-300	0.875	0.933	**0.862**	8/8	8	254-300	1.000	0.892	**0.816**	8/8	10	250-294	1.000	0.933	**0.864**
A131[A]	3/8	2	200-210	0.000	0.533	0.346	8/8	1	204	0.000	0.000	-	7/8	2	204-210	0.571	0.440	0.325
D1[A]	8/8	10	259-344	1.000	0.917	**0.845**	8/8	7	316-340	0.625	0.850	**0.770**	7/8	7	267-344	0.429	0.879	**0.792**
D114[B]	7/8	7	219-263	0.714	0.813	**0.730**	7/8	12	223-363	1.000	0.978	**0.901**	8/8	8	231-322	0.500	0.858	**0.780**

Thirty-four microsatellite loci, in addition to the 8 loci developed here, were tested in seven to eight individuals representing *A. sandaracinos, A. chrysopterus* and *A. leucokranos* (hybrid) to identify the utility of published *Amphiprion* markers for population genetic and parentage analyses among study taxa. Directly labelled markers were amplified in optimised multiplex reactions, based on locus sizes using Multiplex Manager 1.0 software (Holleley and Geerts 2009), for inclusion of multiple loci of the same size (Table 2.2). Number of alleles (N_A), observed (H_O) and expected (H_E) heterozygosity and probabilities of departure from Hardy-Weinberg Equilibrium (*P* HWE) were calculated using GENALEX 6.41. Polymorphic information content (PIC) was calculated for each locus using CERVUS 3.0.7. Linkage disequilibrium among loci was tested using GENEPOP 4.2 default settings.

Among the 8 markers developed here, one locus was found to be out of HWE (As18) and no significant linkage disequilibrium was detected following FDR correction in locus pairs (Benjamini and Hochberg 1995). Cross-amplification success of markers and levels of polymorphism were variable among taxa. Four markers consistently failed to amplify in multiplex reactions (Alat2^{C}, Alat6^{E}, Alat15^{D} and Alat24^{C}). Eight of 42 loci showed significant departure from HWE before and after False Discovery Rate (FDR) correction (Steinberg et al. 2015) (Table 2.2) in *A. sandaracinos* individuals only, possibly due to relatedness among samples or null alleles. Low allelic richness (mean $N_A = 2.88 \pm 0.479$, range 2-5), expected heterozygosity (mean $H_E = 0.358 \pm 0.081$, range 0.233-0.750), and PIC (mean $PIC = 0.308 \pm 0.072$, range 0.195-0.657) were evident in loci departing from HWE (As18, Alat4, Alat5, Alat7, Alat10, Alat14, Alat23, Am5). Mean PIC was 0.513 ± 0.045, 0.589 ± 0.040, and 0.590 ± 0.033, for *A. sandaracinos, A. chrysopterus* and *A. leucokranos* hybrids, respectively, indicating moderate discrimination between individuals. Nine highly polymorphic loci (PIC > 0.5) consistently amplified across all three taxa, with 15, 20 and 24 highly polymorphic loci amplifying specifically within *A. sandaracinos*, *A. chrysopterus,* and *A. leucokranos* individuals, respectively. *A. sandaracinos* markers amplified poorly overall in *A. sandaracinos* individuals, possibly reflecting the sampling location of samples used for marker

development, where Christmas Island, Indian Ocean, represents an isolated and discrete population of the species.

2.4 Conclusions

Microsatellites provide a useful resource for resolving population structure within and among taxa, offering an informative tool for investigating ecological and evolutionary processes such as hybridization. Markers described herein, including 8 novel microsatellite loci, show versatility for use in studies of *A. sandaracinos, A. chrysopterus* and the hybrid *A. leucokranos*. The twenty polymorphic loci that consistently amplified across all three taxa highlight the utility of these markers to inform conservation and management through parentage and population genetic studies; particularly in the case of commercially targeted taxa with rare phenotypes, prized in the ornamental aquaria trade.

CHAPTER 3: Hierarchical behaviour, habitat use and species size differences shape evolutionary outcomes of hybridization in a coral reef fish.

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3.1 Abstract

Hybridization is an important evolutionary process, with ecological and behavioural factors influencing gene exchange between hybrids and parent species. Patterns of hybridization in anemonefishes may result from living in highly specialized habitats and breeding status regulated by size-based hierarchal social groups. Here morphological, ecological and genetic analyses in Kimbe Bay, Papua New Guinea examine the hybrid status of *Amphiprion leucokranos,* a nominal species and presumed hybrid between *Amphiprion sandaracinos* and *Amphiprion chrysopterus*. I test the hypothesis that habitat use and relative size differences of the parent species and hybrids determine patterns of gene exchange. There is strong evidence that *A. leucokranos* is a hybrid of smaller *A. sandaracinos* and larger *A. chrysopterus*, where *A. chrysopterus* is exclusively the mother to each hybrid, based on mtDNA cytochrome *b* and multiple nDNA microsatellite loci. Overlap in habitat, depth and host anemone use was found, with hybrids intermediate to parents and co-habitation in over 25% of anemones sampled. Hybrids, intermediate in body size, colour and pattern, were classified 55% of the time as morphologically first generation hybrids relative to parents, whereas 45% of hybrids were more like *A. sandaracinos*, suggesting back-crossing. Unidirectional introgression of *A. chrysopterus* mtDNA into *A. sandaracinos* via hybrid back-crosses was found, with larger female hybrids and small male *A. sandaracinos* mating. Potential nDNA introgression was also evident through distinct intermediate hybrid genotypes penetrating both parent species. Findings support the hypothesis that anemonefish hierarchical behaviour, habitat use and species-specific size differences determine how hybrids form and the evolutionary consequences of hybridization.

3.2 Introduction

Hybridization may produce viable, genetically distinct offspring based on one or multiple heritable characters when heterospecific breeding occurs (Mayr, 1963, Arnold, 1997). Once considered uncommon and insignificant to evolution (Mayr, 1942, Dowling and Secor, 1997, Coyne and Orr, 2004), hybridization is now recognised as widespread in plants and animals (Mallet, 2005). Hybridization most often occurs between closely related species (Seehausen, 2004) on secondary contact due to range expansion or disturbance (Abbott et al., 2003, Johannesen et al., 2006, Zinner et al., 2009), where typically one species is rare (Currat et al., 2008). Evolutionary outcomes of hybridization include introducing a significant source of genetic variation within a species through introgression, at a greater rate than solely through mutation (Grant and Grant, 1994, Dowling and Secor, 1997, Baack and Rieseberg, 2007). Hybridization may also lead to a balance between selection and hybridization where species remain differentiated, such as in tension zones (Barton and Hewitt, 1985) or in species adapted to discrete habitats (Nosil et al., 2009). It can either result in the breakdown of gene exchange barriers resulting in merging of species (Taylor et al., 2006, Kleindorfer et al., 2014) or strengthen gene exchange barriers, where species integrity is protected (Wu, 2001, Via, 2009, Servedio and Noor, 2003). The contribution of hybridization to individual speciation events has also been recognised (Bullini, 1994, Mallet, 2007, Abbott et al., 2010). However, among nominal hybrids identified, parent species are often unknown and the mechanisms that promote hybridization and explain patterns of introgression are poorly understood (Abbott et al., 2013).

Species behaviour and ecology are significant factors which shape hybridization events, and are fundamental to understanding the long-term evolutionary consequences. Patterns of overlap in distribution and habitat use may determine how often the two parental species come into potential reproductive contact and their relative abundances. Whether or not individuals from different species form pairs or groups and interbreed may depend on resource use and social structure. For many taxa, reproductive success is highly skewed towards large, socially dominant, high ranked individuals within groups (Buston and Cant, 2006, Wong, 2011). In this way, reproductive traits

and mating systems may shape hybridization outcomes and the specific gene exchange dynamics among species. Where there is a strong size disparity between parent taxa, larger species are typically dominant. Some individuals may be more or less predisposed to mating with other species or hybrids, and similarly, intermediate hybrids may be more or less fit than one or the other parent species, depending on mating traits under selection (Kingsolver et al., 2012, Kingsolver and Pfennig, 2007, Hoskin and Higgie, 2010).

Hybridization appears to be an important evolutionary process in highly diverse ecosystems such as coral reefs (Gardner, 1997, Marie et al., 2007). It is facilitated by the presence of co-existing, closely related species assemblages of relatively recent origins (Choat and Bellwood, 1991, Bellwood and Hughes, 2001) and rapid environmental changes associated with glacial-inter-glacial cycles (Palumbi, 1994, McMillan and Palumbi, 1995, Timm and Kochzius, 2008). Hybridization is potentially an important mechanism for speciation in a range of coral reef taxa (Rocha et al., 2005, Mallet, 2007). Hybridization has been recognised in several coral reef fish families, primarily Pomacanthidae (Pyle and Randall, 1994) and Chaetodontidae (McMillan et al., 1999, Montanari et al., 2012, Hobbs et al., 2013), where over a quarter of species have been found to hybridize. Hybrids are also reported for a number of other coral reef fish taxa, with many having nominal species status. In these cases it is not known who parent species are, how often they come into contact in terms of distribution or resource use, or whether hybrids interbreed with one or both parent species. Advanced genetic techniques are now available to confirm hybrid status and patterns of gene exchange among hybridizing taxa.

Anemonefishes provide a unique opportunity to understand how patterns of hybridization and introgression can be controlled by resource use and reproductive behaviour. Parent species have specific habitat requirements and may only interbreed where they overlap and co-occur. Anemonefish are well studied for size-based hierarchical social structure (Buston and Cant, 2006, Buston, 2004), protandrous hermaphroditism (Fricke and Fricke, 1977, Moyer and Nakazono, 1978) and an obligate association with anemones (Fautin, 1986). Within groups there is a strong size-based dominance hierarchy that determines sexual rank and breeding status. Within groups the

female dominates and is largest, followed in size and dominance by the male and non-breeding subordinates that are progressively smaller in size (Fricke, 1979, Hattori, 1991). Subordinate individuals 'queue' for reproductive rights, usually acquiring social dominance passively through outliving higher ranked individuals within the anemonefish group (Mitchell, 2003, Buston, 2004). In these groups the dominant females, in social rank and size, suppress growth and sexual maturation of subordinates, including the sub-dominant male; termed 'female control protandrous hermaphroditism' (Ross, 1990).

The nominal anemonefish species, *Amphiprion leucokranos,* described by G.R. Allen in 1973, is now hypothesised to be of hybrid origin based on intermediate colouration and patterns, compared to species found within its range (Fautin and Allen, 1997). The putative parent species, *A. sandaracinos* (Allen, 1972) and *A. chrysopterus* (Cuvier, 1830) occur throughout the west and central Pacific Ocean, respectively. The region where these allopatric ranges overlap marks the restricted distribution of the putative hybrid *A. leucokranos*, from the north-western regions of Papua New Guinea (PNG) to the Solomon Islands (Fautin and Allen, 1997), along the line of convergence between Indo-Australian and Pacific plates. At local scales within this narrow zone, the extent of ecological contact and co-occurrence, as well as how long species ranges have overlapped is unknown. There has been no published study on genetic relatedness between *A. leucokranos* and the putative parent species, the level and direction of genetic introgression between them, or hybrid viability and fertility. Due to the size-based hierarchal behaviour of anemonefish, relative sizes of parent species and hybrids will be critical in shaping the evolutionary outcome of hybridization.

The overall aim of this study was to assess how ecological and behavioural traits shape evolutionary outcomes of hybridization in anemonefishes. I combined genetic, ecological and behavioural approaches to examine the status of the putative hybrid anemonefish *A. leucokranos* and determine patterns of interbreeding between hybrids and their parent species. Ecological and social contact among parent species and hybrids was examined by quantifying patterns of anemone use, depth distributions and co-occurrence within Kimbe Bay, PNG. Patterns of morphological

intermediacy between putative hybrids and parent species were examined and genetic analyses were used to test the hypothesis that the novel species *A. leucokranos* is a hybrid of *A. sandaracinos* and *A. chrysopterus*. Genetic analyses were applied to quantify the degree to which parent species and putative hybrids are genetically distinct, whether gene exchange is ongoing and in which direction contemporary gene exchange occurs. Finally, considering the strong social constraints on anemonefish reproductive behaviour based on size, it is hypothesised that the relative size differences of parent species will impact patterns of gene exchange among hybridizing species.

3.3 Methods

Study location

This study was conducted during April-May 2011 in Kimbe Bay, PNG (5°30’S, 150°05’E), centrally located in the putative hybrid zone (Fig. 3.1). Sampling was carried out opportunistically at 20 reef sites on the eastern side of the bay, from nearshore to offshore reefs 20km from the coast, with equal effort applied to searching for each species. Opportunistic sampling was essential for adequate sample sizes, with many reefs (not included in the 20 sampled reefs) surveyed not found to have the taxa of interest, as species are relatively rare and patchily distributed. All fish were captured using hand nets and clove oil, and subsequently released back onto their respective anemones post sampling.

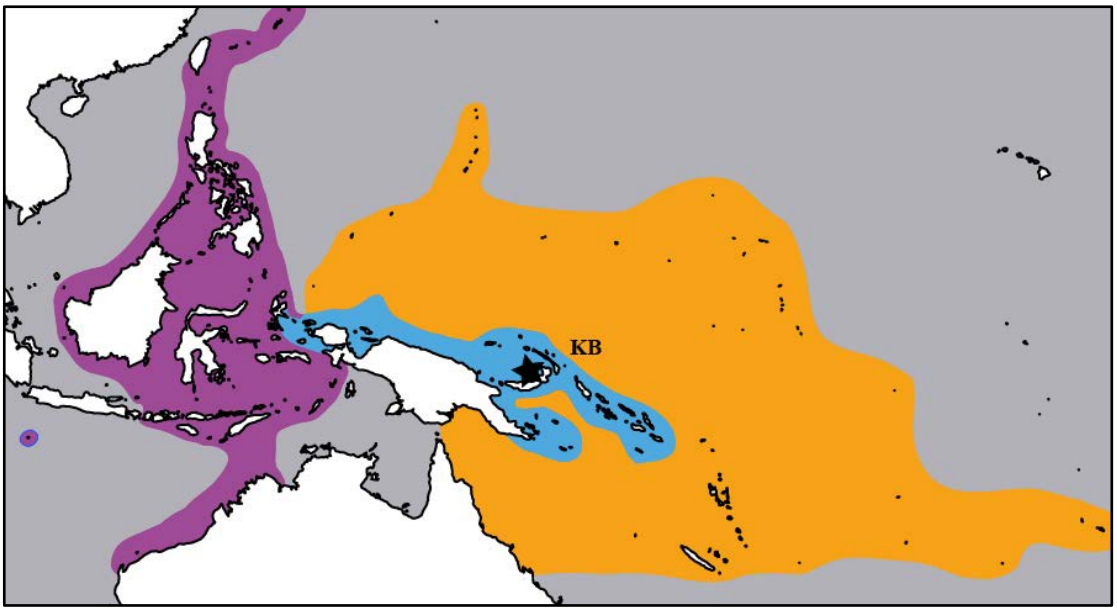

Figure 3.1 Distribution ranges: *A. sandaracinos* (purple), *A. chrysopterus* (orange), and the hybrid zone where *A. leucokranos* is found (blue). The narrow putative hybrid zone exists where putative parental distributions overlap, along the northern coastal region of Papua New Guinea to the Solomon Islands. The sampling region, Kimbe Bay (KB), within the hybrid zone is indicated by a black star.

Ecological and morphological perspectives

Distribution, habitat use and co-occurrence

Field data on distribution, depth, habitat use, frequency of co-habitation, body size and colouration of the proposed hybrid *A. leucokranos* and two putative parent species (*A. sandaracinos* and *A. chrysopterus*) were collected on reefs close to, mid-way and offshore from the coastline in order to establish broad patterns of distribution. Each anemone was identified to species level and the number and type of *Amphiprion* species were recorded for each. The total length of individuals was measured, and depth (to 20m), reef aspect (flat, crest, slope), and GPS position were recorded.

Species identification: morphological and colour variation

Consistent identification of individuals to species was vital for accurately informing analyses and conclusions within the study. Due to the nature of hybridization, where a variety of phenotypes may exist based on the respective individual's genotype, photographs documented each

anemonefish sampled, as well as total length (to the nearest millimetre). This record of variability in morphological features of species and hybrids could then be cross-referenced with ecological and genotypic data for each individual. Fish were identified as either *A. chrysopterus* or *A. sandaracinos* only if their phenotype conformed to relevant species identifications (Fautin and Allen, 1997). Conversely, all intermediate morphologies were categorised as *A. leucokranos,* ranging from directly intermediate between apparently pure-bred parents, to intermediate between hybrids and pure putative parental phenotypes; based on the assumption that *A. leucokranos* represents all hybrids within this phenotypically intermediate complex, including back-crosses and first generation (F1) variants. The frequency of hybrid morphologies was quantified as the total number of hybrids sampled.

Genetic evaluation of hybrid status and gene exchange

Phylogenetic analyses were applied to infer the evolutionary relationship between putative parent species, and assess whether hybrids are genetically distinct. Population genetic analyses, informed by phylogenetics, were applied to determine contemporary level of gene exchange among and within species, and infer whether hybridization is ongoing. Two molecular marker types were selected. The mtDNA cytochrome *b* marker allowed for analysis of female mediated gene flow, with high resolution inference of recent evolutionary events. Conversely, nDNA microsatellite markers were used to assess overall gene exchange (both maternal and paternal) occurring among study species.

Sampling

Genetic analyses were based on 174 *A. sandaracinos,* 26 *A. leucokranos* (hybrid), and 50 *A. chrysopterus* individuals collected predominantly from Kimbe Bay, PNG (n = 165, 26, and 50, respectively); where additional *A. sandaracinos* were sampled from Eastern New Britain (n = 2), PNG and Christmas Island (n = 7), Indian Ocean. Christmas Island samples allowed identification of pure-bred *A. sandaracinos* genotypes in the absence of contributions from other species, due to possible hidden hybridization and introgression as documented for other hybridizing reef fishes

including coral trout (van Herwerden et al., 2006) and surgeonfish (Marie et al., 2007). Caudal fin clippings were taken from each individual *in situ* and preserved in 80% ethanol following each dive.

Laboratory procedures

DNA was extracted from fin clips following a modified 5% chelex extraction protocol (Walsh et al., 1991). Universal primers (CB3H; 5'- GGCAAATAGGAARTATCATTC-3' and L15162; 5'- GCAAGCTTCTACCATGAGGACAAATATC-3') were used to amplify 387 bp of the cytochrome *b* region of the mitochondrial genome (Irwin et al., 1991, Palumbi, 1996). Polymerase chain reactions (PCR) of cytochrome *b* primers were prepared using a BIOTAQ DNA Polymerase kit (Bioline^{TM}) as follows: 20µL reactions containing 10x NH_4 Reaction Buffer, 50µM $MgCl_2$, 2mM dNTP mix, 5U/µL TAq enzyme, 10µM of each primer and 1µL gDNA. Thermocycling followed a touchdown PCR protocol (Korbie and Mattick, 2008) with initial denaturation of 94°C for 3 min, three touchdown denaturation steps, annealing and extension (5 cycles of 94°C for 30 s, 51°C for 30 s, 72°C for 60 s; 5 cycles of 94°C for 30 s, 49°C for 30 s, 72°C for 60 s; and 25 cycles of 94°C for 30 s, 48°C for 30 s, 72°C for 60 s), and a final extension of 72°C for 10 min for *A. sandaracinos*. Alternatively an initial denaturation of 94°C for 3 min, two touchdown denaturation steps, annealing and extension (5 cycles of 94°C for 30 s, 53°C for 30 s, 72°C for 60 s; and 30 cycles of 94°C for 30 s, 51°C for 30 s, 72°C for 60 s), and final extension of 72°C for 10 min for *A. chrysopterus* and *A. leucokranos* samples was applied. PCR products were column purified using a modified Sephadex protocol, with GE-Illustra Sephadex slurry, and confirmed visually by gel electrophoresis. Purified PCR products were then sequenced with each primer using ABI technologies (Macrogen, South Korea). Genbank accession numbers for sequences are KJ434624 - KJ434778.

Next generation 454 sequencing was used to develop a library of microsatellite markers for population genetic analyses among study species, based on *A. sandaracinos* sampled from Christmas Island, as the location is known to be representative of this species alone. Shotgun pyrosequencing was performed on Roche GS FLX instrumentation (AGRF, Adelaide) (Gardner et

al., 2011). *A. sandaracinos* microsatellite loci which require further optimisation were eliminated during screening and will be revisited subsequently. The 4 best performing *A. sandaracinos* markers were selected for genotyping, in addition to 6 microsatellite loci isolated from *A. chrysopterus* (Beldade et al., 2009) (Table 3.1). Primer design followed a fluorescent labelling protocol (Shimizu et al., 2002) to allow for multiplex PCR of microsatellite loci. Following preliminary marker testing, multiplex allocation was optimised based on locus sizes. Two multiplex mixes were designed, with primers synthesised to include complementary directly-labelled forward primers (NED, PET, FAM or VIC), allowing for downstream PCR of 5 loci per multiplex reaction. Amplification was carried out using Type-it Microsatellite PCR Kits including 5µL of 2X Type-it Multiplex PCR Master Mix (QIAGEN), prepared as follows: reactions containing 2µL of a 2µM per primer mix (10X), 2µL gDNA and molecular grade, RNase-free water to final reaction volume of 10µL. Thermocycling was carried out with an initial denaturation (95°C for 5 m), 28 cycles of denaturation, annealing and extension (95°C for 30 s, 60°C for 90 s, 72°C for 30 s) and a final extension (60°C for 30 m). Multiplex PCR products were purified and checked for quality as above. Preliminary analysis of a sample subset confirmed success of multiplex reactions across species; however large variation in amplification success was observed within species between individuals. Problematic samples were optimised for primer concentration. Final genotyping was performed by capillary separation of fluorescently labelled DNA fragments using an AB3730 DNA analyser (AGRF, Melbourne), including LIZ 500 bp internal size standard.

Table 3.1 Details of primers and microsatellite markers used, as described in the Methods; where T_A = annealing temperature. Loci in multiplex 1 and 2 are represented by (locus) $^{1A\text{-}E}$ and (locus) $^{2A\text{-}E}$, respectively. Microsatellite loci used in nDNA analyses are highlighted in bold.

Locus	Primer sequence 5' to 3'	Motif repeat	T_A (°C)	Size range (bp)	Source
Cytochrome *b*					
CB3H L15162	F: GGCAAATAGGAARTATCATTC R: GCAAGCTTCTACCATGAGGACAAATATC	n/a	53 to 48	380 380	(Palumbi et al., 1991) (Irwin et al., 1991)
Amphiprion chrysopterus microsatellite loci					
ACRY_A115^{1A}	F: [TET] GACTCGTGTTCGGAGGAC R: CGGGATAATAACGGAGAGC	$(CA)_{27}$	60	173-280	(Beldade et al., 2009)
ACRY_A130^{2A}	F: [HEX] GCACTCAACACAAAGACCTTA R: ACCCAAACAACATCCAGTC	$(CA)_{24}$	60	120-194	
ACRY_A131^{1B}	F: [FAM] CCTCAGCAGTGTGAAATGA R: CTCCACCTCTCTCTTCTTGAC	$(CA)_{39}$	60	200-250	
ACRY_D1^{1C}	F: [TET] CCAAAAGTTTAGGAAGCTACC R: AACCAGACTGCCCTGATAC	$(GATA)_{25}$	60	220-355	
ACRY_D108^{2B}	F: [FAM] GAAGGATGTGCTTTGTTGTTC R: GCTTTACGATTTTACAATGCAC	$(GATA)_{30}$	60	210-350	
ACRY_D114^{2C}	F: [HEX] TGTTCCAGCTCTGATATTTGAC R: TTGGCAGTGTTTTATACCTGTC	$(GATA)_{19}$	60	205-285	
Amphiprion sandaracinos microsatellite loci					
As6^{2D}	F: [NED] TATGAAGCCACTTCACAGCG R: GGGCGGATGTTTACAAGTCA	$(TCAA)_{12}$	60	140-177	(Present Study) *Accession no. KJ434605*
As8^{2E}	F: [FAM] AAACAAATATGCCCACAGCC R: AGGAGACACCTCACAGCCAG	$(TGTC)_{11}$	60	140-165	*Accession no. KJ434607*
As18^{1D}	F: [NED] GAGACAGAGACATCGGCAGT R: CTCGGTGCTCTTTCATGGAT	$(CAG)_{13}$	60	90-115	*Accession no. KJ434617*
As20^{1E}	F: [NED] CTGAAGGACACCTCTGGCTC R: CTCCTGCTGTCCTGTGATGA	$(GAG)_{10}$	60	190-275	*Accession no. KJ434619*

Data compilation

mtDNA cytochrome *b* sequences were automatically aligned using ClustalW (Thompson et al., 1994), and manually edited using BioEdit v7.0.9.0 (Hall, 1999). nDNA microsatellite loci were scored using PeakScanner v1.0 and edited using Genalex 6.1 (Peakall and Smouse, 2006).

Phylogenetic analyses

An alignment of all sequences obtained from all sampling locations, including two *A. sandaracinos* cytochrome *b* sequences sourced from Genbank (DQ343962, AF097929), were used in phylogenetic analyses to assess whether species are genetically distinct, infer evolutionary history, and examine the relationships among haplotypes. The best substitution model for alignment data was selected using a likelihood approach implemented in jModelTest v0.1 under default settings (Posada, 2008). The preferred model was GTR+I+G.

Phylogenetic analyses including maximum parsimony (MP), maximum likelihood (ML), and Bayesian inference (BI) performed with the GTR+I+G model (ML and BI), inferred evolutionary relationships between species, assuming a Strict Evolutionary Clock, based on a Bayes Factor (BF) value <10 calculated in BEAST v1.6.2 and Tracer v1.5 (Drummond and Rambaut, 2007, Rambaut and Drummond, 2007). All trees were outgroup rooted with *Amphiprion clarkii* sequences from Genbank (AY208513, AF097931, DQ343949-51) and 4 *A. clarkii* sequences from Kimbe Bay. MP analyses were undertaken in MEGA 5.0 (Tamura et al., 2011) with 10 independent runs, with trees showing variation in length and topology. The MP analysis with the shortest set of trees overall was selected for comparison to those obtained by previously described phylogenetic analyses. ML analyses, performed in GARLI v2.0 using an extended Majority Rule consensus (Zwickl, 2008), included 10 independent runs, all displaying identical tree length and topology. For both MP and ML analyses, ≥50% majority rule support values derived from consensus trees generated with 1000 bootstrap replicates were accepted. Finally, BI analyses were conducted in Geneious v5.4.6 using MrBayes Plug-in (Huelsenbeck and Ronquist, 2001), with 1,100,000

iterations and 100,000 tree burn-in under the GTR+G+I substitution model, and otherwise default settings. Consensus support values of ≥50% are displayed on the best ML tree.

Population genetic analyses

Population genetic analyses estimate the level of gene exchange among and within study species within Kimbe Bay. Samples from other regions of PNG were excluded if sample sizes were <10.

All population genetic analyses of cytochrome *b* haplotypes were performed in Arlequin v3.5.1.2 (Excoffier and Schneider, 2005), with species sequence sets defined within the alignment using DnaSP v5.10.1 (Librado and Rozas, 2009). In order to illustrate spatial patterns of cytochrome *b* haplotype distributions within and among species, a minimum spanning tree was constructed manually and edited using Adobe Illustrator CS5.1 (Adobe Systems Inc.). Pairwise F_{st} values and Analysis of Molecular Variance (AMOVA) were calculated with 10,000 permutations at a significance level of 0.05 to assess spatial heterogeneity of cytochrome *b* haplotypes. AMOVA was conducted using the Pairwise Difference model, analysing variance among species within the region (F_{sc}) and within species variance relative to overall genetic variance (F_{st}) only, as species within Kimbe Bay are thought to be representative of the region. Genetic diversity indices of cytochrome *b* haplotypes, including haplotype diversity (*h*) and nucleotide diversity (π) were estimated. Based on phylogenetic analyses, where a number of *A. sandaracinos* individuals appear outside their clade, repeated analysis of haplotype and nucleotide diversity estimates, and AMOVA were conducted to assess variation in outcomes when back-crosses are either included or excluded.

Of the 10 microsatellite loci genotyped, 8 were successfully scored and 2 were excluded (As6 and As8) from further analyses due to inconsistent amplification. Microsatellite summary statistics including observed number of alleles (N_A), private alleles (P_A), observed (H_O) and expected (H_E) heterozygosities, and average inbreeding coefficient (F_{IS}) were estimated using Genalex (Peakall and Smouse, 2006) and Genepop 4.0.11 (Rousset, 2008). Genepop was also used to calculate probabilities of departure from Hardy-Weinberg equilibrium (HWE) via Markov chains

with dememorization of 10,000, across 20 batches, with 5,000 iterations per batch. Micro-checker v2.2.3 (van Oosterhout et al., 2004) was used when departure was observed to detect the presence of null alleles, large allelic dropout and other scoring biases. Locus by locus raw estimates of species differentiation, were calculated as the average over 7 loci using an AMOVA with 10,000 permutations in Arlequin (Excoffier and Schneider, 2005). Species specific genotypic diversity (*gd*) was also estimated in Arlequin. Individuals were excluded from AMOVA and *gd* calculations if data was missing for >1 loci, or loci were monomorphic (As 20). Therefore, 181 individuals with a minimum of 5 loci with <20% missing data, were retained; where null homozygous loci contributed to increased allowed level of missing data. An "excluding null alleles" (ENA) correction was used with 1000 bootstrap replicates in FreeNA (Chapuis and Estoup, 2007) to estimate species differentiation corrected for null allele frequencies.

Estimates of actual differentiation (D_{est}) were calculated online via SMOGD v1.2.5 (Crawford, 2010). The number of differentiated genetic populations (K) represented by the samples analysed was estimated using Structure v2.3.3 (Pritchard et al., 2000); where K ranges from 1 (unstructured populations) to the maximum number of sampled locations (highly differentiated populations). Structure was run using the admixture ancestry model informed by location and with correlated allele frequencies, for each K value for 10 independent repetitions each, at 1,000,000 MCMC iterations following a 100,000 burn-in. The best K was assessed using Evanno's method (Evanno et al., 2005) via StructureHarvester (Earl and von Holdt, 2012), and Distruct v1.1 (Rosenberg, 2004) was used to visualize the Structure analysis. New Hybrid (Anderson and Thompson, 2002), a model-based method used to identify hybrids within a multilocus genetic data set, was used with a 150,000 burn-in and 1,500,000 steps. Finally, a discriminant analysis of principal components (DAPC) was executed using microsatellite data, to describe relationships between predefined species clusters, with 95% genotypic inertia ellipses (IE) identified (Jombart et al., 2010, Jombart et al., 2009). DAPC retained 68 principal components, accounting for 95% of the genotypic variability present. DAPC was implemented using the dudi.pca function within the R

package ade4 (Dray and Dufour, 2007) and adegenet (Jombart, 2008) in R v2.13.2 (R Development Core Team, 2016).

Analysis of hybrid frequency

To compare putative parental species and hybrid frequency within Kimbe Bay, to that of expected frequencies within a theoretical hybrid zone, the HWE hypothesis was tested based on the assumption of random assortative mating; where hybrid frequency acts as a proxy for random assortative mating between putative parental contributors. Observed frequencies are representative of observed species frequency, based on identification during sampling, where equal effort was applied to searching for all species. Expected frequencies were derived from theoretical parameters of HWE following the function: $p^2 + 2pq + q^2 = 1$. Observed and expected frequencies were then evaluated to test the null hypothesis of random mating, using a chi-square test.

3.4 Results

Ecological and morphological perspectives

Ecological contact

Patterns of distribution, co-occurrence across reefs in Kimbe Bay, at depth, and in anemone usage indicate consistent ecological contact between parent species and putative hybrids. Pairwise overlap was generally higher on midshore reefs, where *Amphiprion sandaracinos, A. leucokranos* and *A. chrysopterus* relative abundances were greatest (>70%, 90% and >30%, respectively). The relative abundance of *A. chrysopterus* was more than two-fold greater on offshore than midshore reefs (Fig. 3.2A). Depth ranges of *A. sandaracinos, A. leucokranos* and *A. chrysopterus* overlapped substantially (1-9m, 1-10m, and 1-14m, respectively). *A. chrysopterus,* however, was recorded at depths greater than 5m in over 65% of individuals surveyed. There were no differences among species associated with reef aspect. Preferential anemone usage by species was fairly consistent,

with *A. sandaracinos, A. leucokranos* and *A. chrysopterus* (n = 45, 20, and 55 individuals, respectively) recorded primarily in *Stichodactyla mertensii* (100%, 90%, 69.1% individuals, respectively). *Heteractis crispa* was the alternative host at all other times.

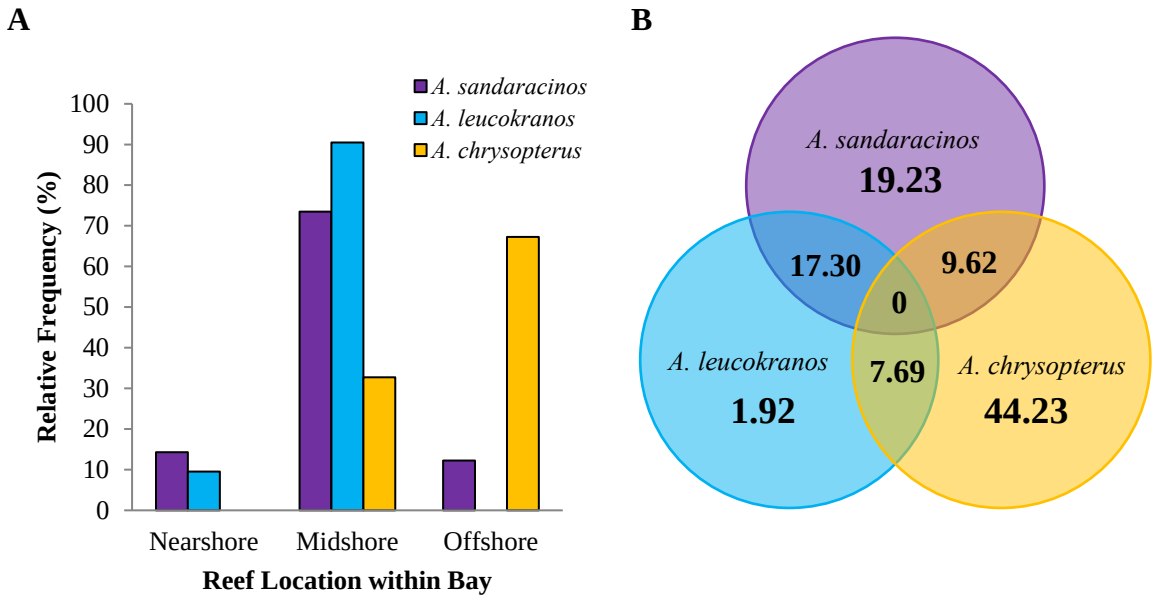

Figure 3.2 (A) Relative frequency of *A. sandaracinos, A. leucokranos,* and *A. chrysopterus* individuals observed across coral reefs at varied locations within Kimbe Bay; where n = 49, 21, and 55 individuals, respectively. (B) Comparison of conspecific and heterospecific associations (%) between *A. sandaracinos, A. leucokranos,* and *A. chrysopterus* across 52 anemones surveyed within the study region (n = 49, 20, and 48 individuals, respectively). Data in both panels represents a complete census, with all individuals from adults to recruits included.

Co-occurrence: conspecific and heterospecific group assemblages

Overall, 65% of anemones surveyed hosted conspecific groups, while 35% of anemone assemblages were heterospecific combinations of the study species (Fig. 3.2B). *A. sandaracinos* and *A. chrysopterus* were predominantly in conspecific group assemblages (>19% and >44%, respectively), compared to *A. leucokranos* which was rarely found with conspecifics (<2%; Fig. 3.2B). Putative parental species *A. sandaracinos* and *A. chrysopterus* lived together in over 9% of anemones surveyed, and almost twice as many *A. sandaracinos* co-occurred with *A. leucokranos*

(>17%; Fig. 3.2B). Hybrids were found with *A. sandaracinos* (25%) over three times more often than with *A. chrysopterus* and over an order of magnitude more often than with other hybrids.

No observations were made of all three species co-inhabiting a single anemone, nor were eggs observed at any sampled anemones. Interestingly, *A. sandaracinos* adults were living with subordinates of another anemonefish species (*A. clarkii*) on three occasions.

Morphological intermediacy

Individuals identified as putative hybrids were intermediate in size (73.93mm +/- 5.34mm) to *A. sandaracinos* and *A. chrysopterus* individuals, with over 40% of *A. leucokranos* sampled within the 91 to 110mm size class (Fig. 3.3), encompassing the range of size overlap between parent species. Putative parental species dominated size classes at opposite ends of the size distribution, where *A. sandaracinos* is the smaller (54.13mm +/- 1.45mm) and *A. chrysopterus* the larger (97.06mm +/- 3.84mm) species (Fig. 3.3).

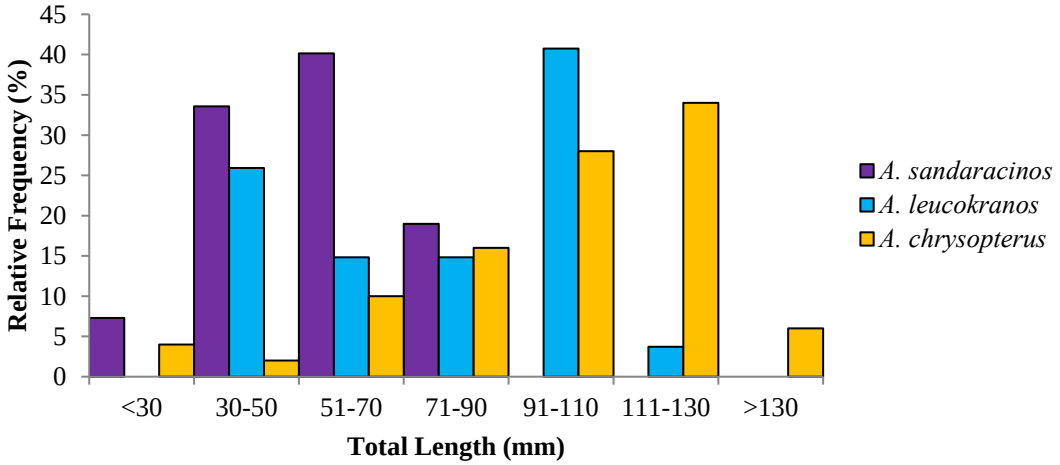

Figure 3.3 Relative frequency distribution comparing size structure in *A. sandaracinos, A. leucokranos* and *A. chrysopterus* in Kimbe Bay (n = 137, 27, and 50, respectively). Data is inclusive of all age classes from adults to recruits.

No individuals identified as putative hybrids based on morphology appeared intermediate between the *A. leucokranos* and *A. chrysopterus* colouration (Fig. 3.4A). However, hybrids

displayed a variety of colour patterns intermediate to putative parental contributors ranging from the characteristic *A. leucokranos* morphology, with 'white-bonnet' pattern, orange to brown broad body, and rounded tail (Fig. 3.4B). Of the *A. leucokranos* individuals sampled, 55% displayed the intermediate condition for which the nominal species is described (n = 20), compared to those displaying morphology, colour and pattern more similar to *A. sandaracinos* (45%; Fig. 3.4C-K). Hybrid variant characteristics included an orange, slender body shape, with varying degrees of dorsal white stripe, and remnants of white side-bars on the head, almost resembling *A. sandaracinos* (Fig. 3.4L).

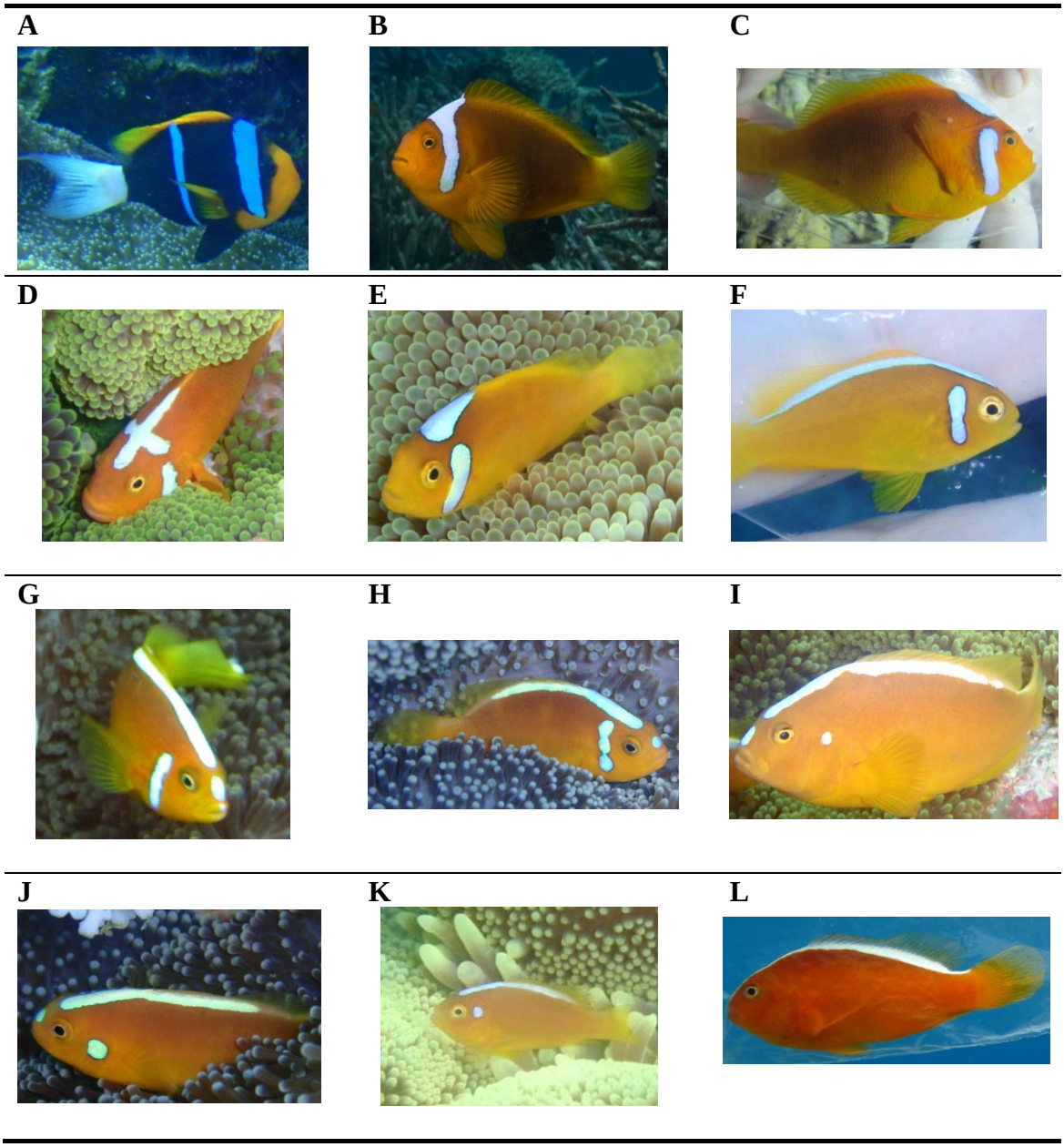

Figure 3.4 Comparison of colours and patterns observed during the study of individuals identified as *A. leucokranos* hybrids, ranging from the typical F1 hybrid with intermediate morphology and characteristics (B), to a variety of back-crossed individuals also identified here as *A. leucokranos* (C-K). For visual reference pure looking *A. chrysopterus* (A) and *A. sandaracinos* (L) individuals are also shown. Photo credits: A. Gainsford.

Genetic evaluation

Three hundred and eighty-seven alignment positions were resolved for 153 individuals in the mtDNA cytochrome *b* region (summary statistics in Table 3.2), including 41 polymorphic sites, of which 22 were parsimony informative. Twenty-eight haplotypes were detected in the data set, where 13 were shared and 15 were unique, suggesting recent gene exchange between parent species in Kimbe Bay via female mediated gene flow.

Summary statistics for microsatellite loci revealed *A. chrysopterus* and *A. sandaracinos* species had the highest number of private alleles (45 and 16, respectively), compared to *A. leucokranos* (6; Table 3.2). Significant single-locus departures from HWE were detected in 11 of 24 tests at species level before sequential Bonferroni correction and 10 afterwards (α=0.00208; Table 3.3). Null alleles might contribute to departures from HWE in loci A131 and A130 as indicated in Micro-checker; however, departures from HWE in loci As18, As20 and As115 are more likely due to apparent fixed differences between species, where A115 is homozygous for a null allele in *A. chrysopterus*. Fixed differences of these loci are in line with expectations of hybrid genotypes, where hybrids are heterozygous for alleles of either parent species, albeit sometimes a null allele.

Evolutionary relatedness

Phylogenetic analyses delineated study species into two distinct clades, depicting likely evolutionary history (Fig. 3.5A). Clade 1 included representatives of all species, where 100% of *A. chrysopterus* and *A. leucokranos* samples, but only 21.2% of *A. sandaracinos,* were clustered. In contrast, Clade 2 included only 76.5% of *A. sandaracinos* representatives. Remaining *A. sandaracinos* individuals (2.4%) grouped with the outgroup species *A. clarkii,* which was supported by a >50% majority rule consensus value. Despite reasonable Bayesian posterior probability support for the majority of clades, there is no such support for the majority of within clade nodes, evident from numerous polytomies and relatively few supported sub-clades (Fig. 3.5A). Evolutionary relatedness was revealed using three phylogenetic methods to identify

genetic differentiation based on mtDNA between species. MP trees recovered different topologies from ML and BI analyses, with the best ML tree used out of ten independent analyses (Fig. 3.5A).

Table 3.2 Marker credentials for Kimbe Bay species only, derived from cytochrome *b*: Number of individuals (n), haplotypes (n_h), haplotype diversity (h), and nucleotide diversity (π); derived from microsatellites: Number of individuals (n), alleles per locus (Na), observed number of private alleles (Pa), observed heterozygosity (H_O), and expected heterozygosity (H_E) averaged over eight loci.

	Cytochrome *b*				Microsatellite loci				
	n	n_h	h	π	n	N_a	P_a	H_O	H_E
A. chrysopterus	46	7	0.492	0.251	46	10.8	45	0.601	0.603
A. leucokranos	22	3	0.385	0.210	20	6.4	6	0.735	0.603
A. sandaracinos	76	18	0.571	2.094	160	7.4	16	0.264	0.304

Table 3.3 Summary statistics for eight microsatellite loci: Sample size (n), observed number of alleles (N_a) and private alleles (P_a), observed heterozygosity (H_O) expected heterozygosity (H_E), and average inbreeding coefficient (F_{IS}). Probability of departure from HWE for each locus at each species (P); where the significance of departure $P < 0.05$* and significance of departure following sequential Bonferroni adjustment $P < 0.00208$** (bold) are indicated.

Species		As18	As20	A131	A115	D1	A130	D108	D114
A. chrysopterus (total n = 46)	n	46	42	42	1	34	32	30	44
	N_a	2	1	13	2	15	14	23	16
	P_a	1	0	6	2	10	6	16	4
	H_O	0.022	0.000	0.619	1.000	0.853	0.469	0.933	0.909
	H_E	0.022	0.000	0.789	0.500	0.894	0.848	0.922	0.848
	F_{IS}	0.000	-	0.227	-	0.060	0.460	0.005	-0.060
	P	0.940	-	**0.000****	0.317	**0.002****	**0.000****	0.445	**0.002****
A. leucokranos (total n = 20)	n	18	18	18	0	15	17	16	19
	N_a	2	2	9	0	10	9	8	11
	P_a	0	0	1	0	2	1	1	1
	H_O	0.944	0.889	0.833	0.000	0.800	0.706	0.813	0.895
	H_E	0.498	0.494	0.711	0.000	0.862	0.784	0.611	0.861
	F_{IS}	-0.889	-0.789	-0.144	-	0.106	0.129	-0.300	-0.012
	P	**0.000****	**0.001****	0.732	-	0.570	0.121	1.000	0.420
A. sandaracinos (total n = 160)	N	2	3	8	1	14	8	2	21
	N_a	1.045	1.031	1.170	1.000	4.870	2.123	1.007	8.760
	P_a	0	0	1	0	3	2	10	0
	H_O	0.015	0.031	0.098	0.000	0.766	0.280	0.007	0.919
	H_E	0.043	0.030	0.145	0.000	0.795	0.529	0.007	0.886
	F_{IS}	0.661	-0.009	0.330	-	0.041	0.473	0.000	-0.035
	P	**0.000****	0.999	**0.000****	-	0.005*	**0.000****	0.967	**0.000****

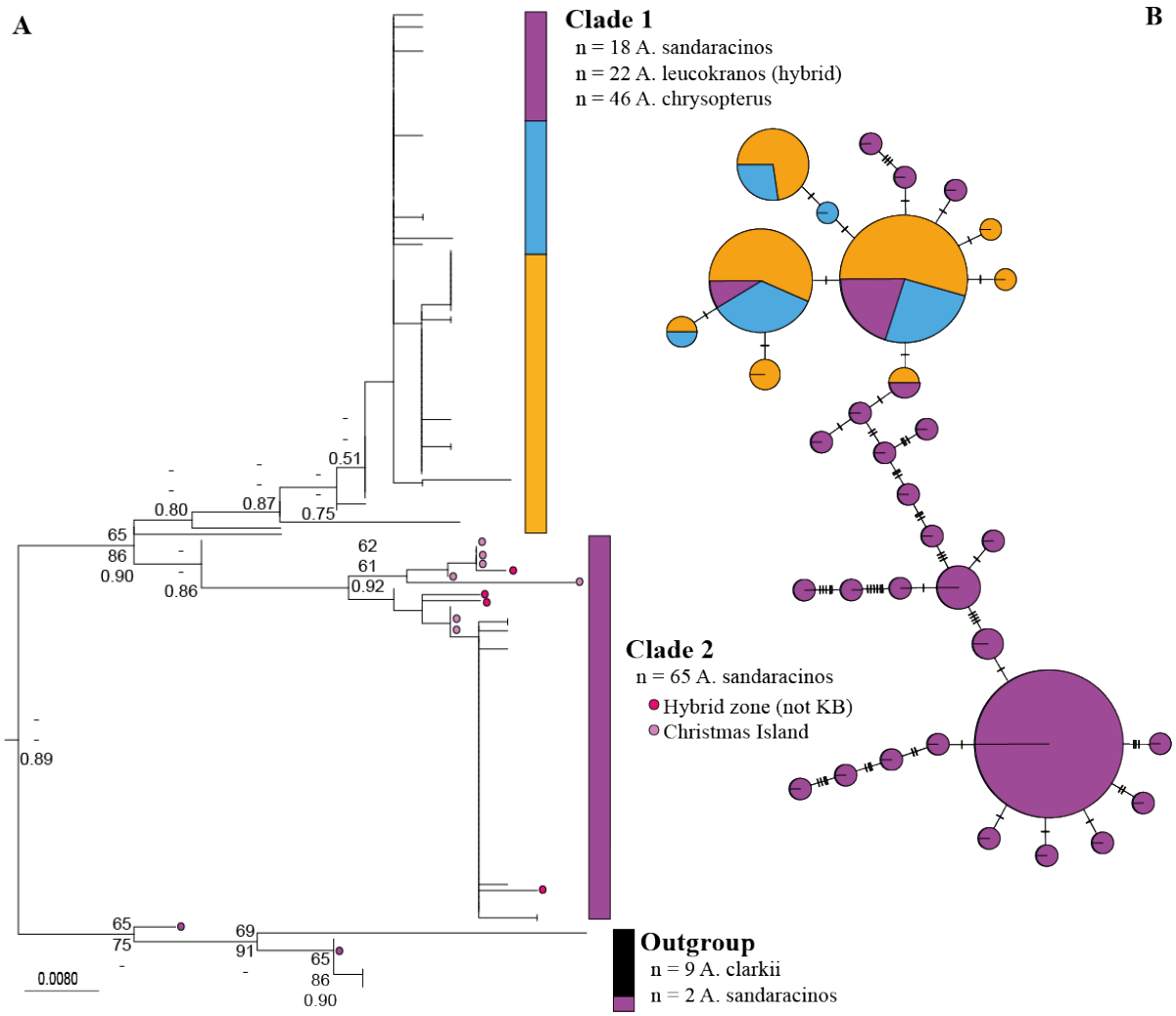

Figure 3.5 Outgroup rooted phylogenetic tree of cytochrome *b* for the *A. sandaracinos, A. leucokranos* and *A. chrysopterus* complex (A). Stacked numbers adjacent to nodes indicate support from Maximum Parsimony (MP, upper), Maximum Likelihood (ML, middle), and Bayesian Inference (BI, lower) analyses; where **–** indicates that support was lacking (<50) for that particular analysis. Species are represented as follows: *A. sandaracinos* (dark purple), *A. leucokranos* (light blue), *A. chrysopterus* (orange), and *A. clarkii* outgroup (black), and the number of species represented within each clade are given. Dark purple dots falling within the outgroup represent two *A. sandaracinos* individuals; and alternatively coloured *A. sandaracinos* within Clade 2 represent individuals from within the hybrid zone but outside Kimbe Bay (dark pink), and those from Christmas Island (light pink). Relationships between *A. sandaracinos, A. chrysopterus* and *A. leucokranos* mtDNA haplotypes are displayed in a minimum spanning network (B). Species are represented by colour as identified above, where all *A. sandaracinos* samples including those outside Kimbe Bay are blue. Circle size is proportional to the number of individuals which share a unique haplotype, where the fraction of individuals of a particular species is indicated. The number of substitutions separating haplotypes is represented by crossbars, either for one (thin) or five (thick) substitutions, respectively.

As was found in phylogenetic analyses (Fig. 3.5A), the minimum spanning network clearly separated an *A. sandaracinos* group from a mixed assemblage of haplotypes representing all *A. leucokranos* and *A. chrysopterus* individuals, and a minority of *A. sandaracinos* individuals, based on haplotype distributions among species (Fig. 3.5B). These results suggest some level of introgression of mtDNA haplotypes from *A. chrysopterus* into *A. sandaracinos*, with all hybrids appearing maternally related to *A. chrysopterus.* Common haplotypes exist within both groups. Specifically within the *A. sandaracinos* assemblage, a single common haplotype is shared by many individuals, with the majority of haplotypes unique to single individuals. Consequently, the *A. sandaracinos* assemblage has the greatest number of haplotypes. Comparatively within the mixed group, three common haplotypes exist, where two of these are shared by individuals from all species. When all back-crossed *A. sandaracinos* individuals are excluded, the number of mtDNA haplotypes is reduced within *A. sandaracinos* by over 61%.

Contemporary gene exchange

Genetic diversity indices

Species populations associated with hybridization events and ongoing gene exchange via back-crossing are expected to show an increase in estimated diversity indices. For mtDNA cytochrome *b, A. leucokranos* and *A. chrysopterus* showed similarly low haplotype (0.385-0.492) and nucleotide (0.210-0.251) diversities (Table 3.2). In comparison, *A. sandaracinos* nucleotide diversity was approximately 10-fold greater than other species when all sequences were included from phenotypically identified *A. sandaracinos* (2.094; Table 3.2). When back-crossed individuals identified in phylogenetic analyses were excluded from the *A. sandaracinos* group, nucleotide diversity was comparable to *A. chrysopterus* and hybrids (0.274). Similarly, haplotype diversity of *A. sandaracinos,* which was most diverse when phenotypically identified *A. sandaracinos* were considered, was lowest compared to other species when back-crosses were excluded.

Comparatively, genotypic diversity based on microsatellite data of *A. sandaracinos* (0.186 +/- 0.129) in Kimbe Bay was approximately half that of *A. chrysopterus* (0.368 +/- 0.249) and almost a third that of *A. leucokranos* (hybrid, 0.532 +/- 0.312; n = 160, 46, and 20, respectively).

Population structure

Population genetic analyses, which determined the level of mtDNA gene exchange, revealed high differentiation between hybrids and parent species (Tables 3.4-6). Pairwise species comparisons for cytochrome *b* revealed *A. sandaracinos* to be most differentiated relative to other species (Table 3.4), reaffirming the delineation observed previously (Fig. 3.5A-B). This finding was upheld by microsatellite loci with respect to connectivity between *A. sandaracinos* and *A. chrysopterus* in Kimbe Bay, indicating limited gene flow. Raw values of species differentiation were comparable to values corrected for null alleles (Table 3.5). Strong genetic differentiation was upheld regardless of inclusion or exclusion of *A. sandaracinos* back-crossed individuals, whereas AMOVA fixation indices for cytochrome *b* were 0.669 ($P<0.0001$) and 0.952 ($P<0.001$) when back-crosses were included or excluded, respectively (Table 3.6). However, when all back-crosses were removed, pairwise F_{st} values and among species variation was considerably greater, and within species variation an order of magnitude lower than other comparisons. Overall, the highest levels of genetic variation detected by AMOVA were among species (67-95%; Table 3.6). These results are consistent with reduced gene flow between species when introgressed individuals which look like one parent (*A. sandaracinos*), but carry the mtDNA of the other parent (*A. chrysopterus*), are removed.

Table 3.4 Pairwise species comparisons: F_{st} calculated from 387 bp of mitochondrial cytochrome *b* with corresponding *P* values in parenthesis (above diagonal), where significant values are given in bold and * indicates $P < 0.001$; and the harmonic mean of the estimator of actual differentiation (D_{est}) across 8 microsatellite loci (below diagonal).

	CH	LU	SA
CH		-0.030 (0.98+/-0.01)	**0.664** (*)
LU	0.458		**0.623** (*)
SA	0.792	0.144	

Table 3.5 Raw species differentiation from microsatellite allele frequencies**,** species differentiation corrected for null allele frequencies using the ENA correction and estimator of actual differentiation (D_{est}); results are presented locus-by-locus and as an average over 8 loci, where * indicates all values are significant to the 95% confidence interval.

Locus	Raw	ENA corrected	D_{est}
As18	0.897	0.846	0.582
As20	0.912	0.911	0.592
A131	0.534*	0.486*	0.598
A115	0.933	0.915	1.000
D1	0.122	0.121	0.748
A130	0.180	0.145	0.388
D108	0.644*	0.644*	0.573
D114	0.028	0.028	0.128
Mean	0.458	0.442	0.483

Table 3.6 Hierarchical AMOVA for mitochondrial cytochrome *b* when all sequences included (A), and all back-crosses are removed (B); where each species represents a population within Kimbe Bay, only allowing for calculation for the Φ_{st} statistic. Results of AMOVA for microsatellite loci (C) are also included. Asterisks *, and ** are indicative of $P < 0.001$ and $P < 0.0001$, respectively.

Source of variation	*df*	Sum of squares	Variance components	% variation	Φ statistic (*P*)
(A) Cytochrome *b* – back-crosses included					
Among populations	2	406.16	4.678 Va	66.97	**Φ_{st} 0.669 (**)**
Within populations	141	325.33	2.307 Vb	33.03	
Total	143	731.49	6.985		
(B) Cytochrome *b* – back-crosses removed					
Among populations	2	609.66	7.832 Va	95.17	**Φ_{st} 0.952 (*)**
Within populations	121	48.15	0.398 Vb	4.83	
Total	123	657.81	6.924		
(C) Microsatellite loci					
Among populations	2	64.38	0.397 Va	30.22	**Φ_{st} 0.260 (**)**
Among individuals within populations	178	175.45	0.069 Vb	5.24	**Φ_{is} 0.108 (**)**
Within individuals	181	153.50	0.848 Vc	64.54	**Φ_{it} 0.340 (**)**
Total	361	393.32	1.314		

Clear genetic structuring derived from microsatellite loci separated the hybrid group from putative parental species, *A. sandaracinos* and *A. chrysopterus* (Fig. 3.6A). Additionally, extensive gene flow between *A. leucokranos* and parental species, *A. sandaracinos*, of Kimbe Bay was

apparent from similar Structure (Fig. 3.6B) and New Hybrid analyses. As expected, *the A. leucokranos* segment of the Structure plot shows equal proportions of microsatellite assignments to both putative parental species. Furthermore, as back-crosses of F1 hybrids to *A. sandaracinos* carry increasingly more *A. sandaracinos* alleles (assuming selective neutrality), the *A. sandaracinos* segment shows predominance of *A. sandaracinos* assignments. Bars representing *A. chrysopterus* assignments in the *A. sandaracinos* segment may suggest extensive back-crossing of hybrids with *A. chrysopterus*; however, this is not evident in the DAPC. Delta K was greatest when K=2, identifying two differentiated genetic clusters within Kimbe Bay (*A. sandaracinos* and *A. chrysopterus*). The DAPC showed separation of parental species along the X-axis (Fig. 3.6A). Conversely, *A. leucokranos* and *A. sandaracinos* were similarly positioned along the X-axis; however, they appeared distinct and clearly separated along the Y-axis (Fig. 3.6A). Despite separation of genotypic clusters, some *A. leucokranos* and *A. sandaracinos* individuals appeared outside the 95% inertia ellipse (IE). These individuals were identified as hybrid back-crosses to *A. sandaracinos* based on mtDNA. Also a number of *A. chrysopterus* individuals appeared outside the 95% IE, which may suggest penetration of *A. leucokranos* genotypes into *A. chrysopterus* at Kimbe Bay, which was not reflected in mtDNA analyses.

Hybrid frequency

The observed frequency of *A. leucokranos* (F1 and back-cross hybrids combined) within the study region conforms to an expected hybrid frequency, given species abundance and assuming random mating ($\chi 2 = 0.118$, $df = 2$, $p>0.05$).

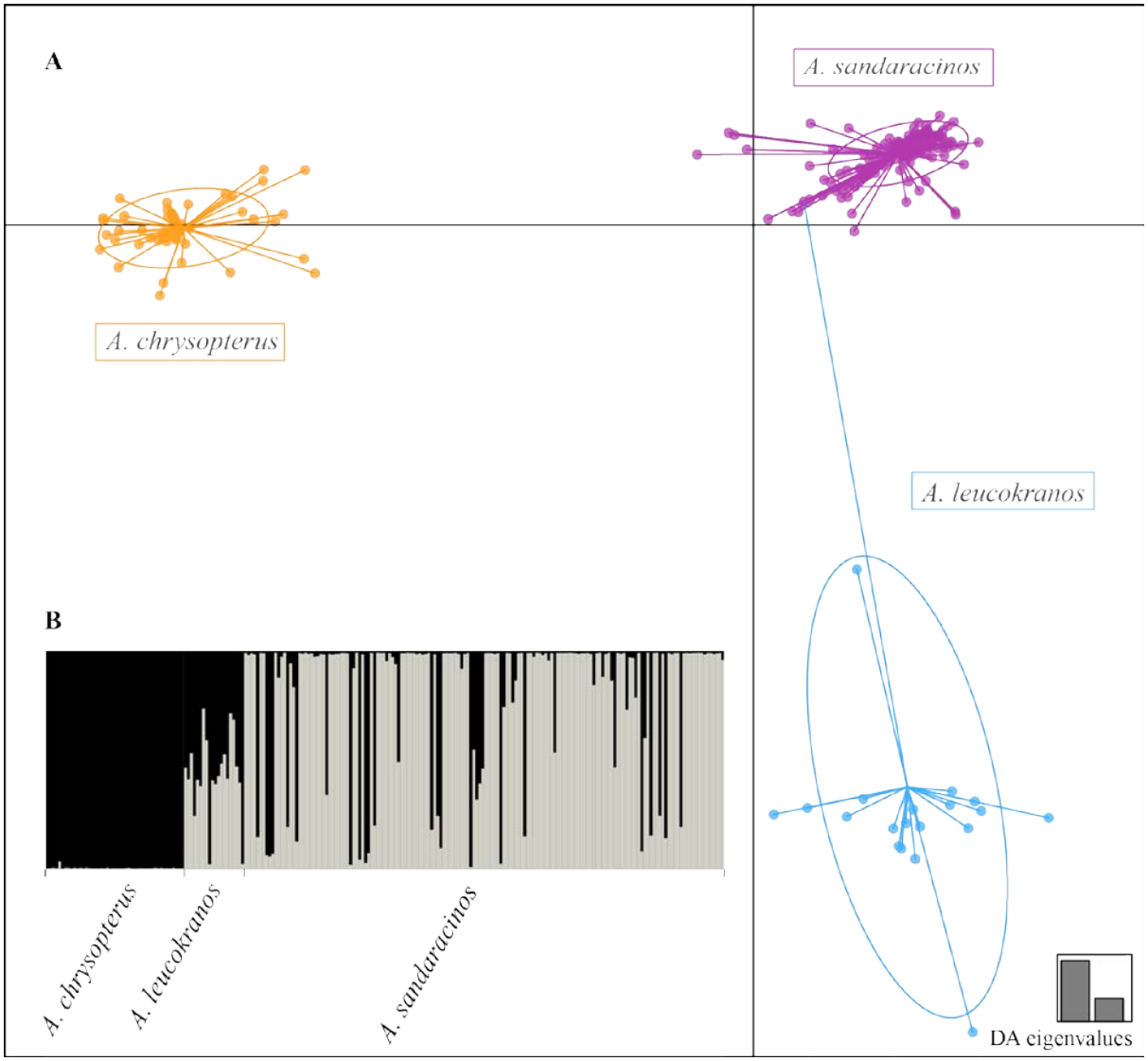

Figure 3.6 Differentiation between *A. sandaracinos, A. leucokranos,* and *A. chrysopterus* within Kimbe Bay based on eight microsatellite loci. Species were used as priors for genetic clusters for DAPC (A) and Structure analysis (B). Individual genotypes are expressed in DAPC as dots and species represented by colours as shown; 95% IE are indicated for each defined cluster by ovals; DA (discriminant analyses) eigenvalues shown depict the amount of genetic information contained in each successive principal component; X and Y axis represent the first two principal components, respectively.

3.5 Discussion

This study provides consistent ecological, morphological and genetic evidence to support the hybrid status of *Amphiprion leucokranos*, which is a product of interbreeding between *A. sandaracinos* (small species) and *A. chrysopterus* (large species). Parent species overlap in

geographic range, anemone host use, local distribution and depth range, and frequently co-occur in the same anemone. The hybrid 'species' is intermediate in all respects including size, colour and ecological traits, and often co-occurs with either parent species. The frequency of *A. leucokranos* (hybrids) conforms to expectations for a theoretical hybrid zone, given the relative abundance of parent species and random mating.

Genetic analyses not only confirmed the hybrid status of *A. leucokranos*, but also revealed informative patterns of ongoing genetic exchange occurring. MtDNA indicated unidirectional hybridization and introgression of *A. chrysopterus* mtDNA into *A. sandaracinos* via hybrid back-crossing, always with larger hybrids as mothers during back-crosses with smaller male *A. sandaracinos*. Furthermore, predominantly, but not exclusively, unidirectional nDNA introgression was detected, with distinct intermediate *A. leucokranos* genotypes. Intermediately-sized hybrids were always female in crosses with smaller male *A. sandaracinos.* These results strongly suggest that hybridization in the case of *A. leucokranos* has been shaped through strong sexual selection for size within the social dominance hierarchy that determines anemonefish mating patterns.

Factors facilitating hybridization

Hybridization between *A. chrysopterus* and *A. sandaracinos* appears to be facilitated by co-occurrence of hybrids and parent species at midshore reefs within Kimbe Bay. The reduced abundance of *A. chrysopterus* relative to *A. sandaracinos* on these reefs may contribute to the extent of hybridization, as proposed for other reef fishes (Frisch and van Herwerden, 2006, Marie et al., 2007, Montanari et al., 2012). Furthermore, shared host specificity by taxa, an established obligate association (Fautin, 1986), facilitates co-inhabitation of anemones on reefs where their respective ranges overlap. Direct observations of *A. sandaracinos* and *A. chrysopterus* sharing hosts across reefs within Kimbe Bay were greater than expected. However, relative to the frequency of each species living with conspecifics, interspecific associations between parents were two-fold to five-fold lower.

The extent of ecological overlap and shared resource use in the hybrid zone appears to promote and maintain hybridization in other taxa, including wrasses (Labridae) (Yaakub et al., 2006), butterflyfishes (Chaetodontidae) and surgeonfishes (Acanthuridae) in the suture zone at Christmas Island, Indian Ocean (Montanari et al., 2012, Marie et al., 2007, Hobbs et al., 2009) and elsewhere (van Herwerden et al., 2006). This study has found hybrids co-inhabiting with *A. sandaracinos* more than twice as often as co-inhabiting with *A. chrysopterus* and almost an order of magnitude more often than living with other hybrids; a preference that also holds true when *A. sandaracinos* individuals identified as hybrid back-crosses were included in analyses as hybrids, based on genetic data.

Direction of back-crossing

Given the apparent preference for interspecific co-habitation among hybrids and *A. sandaracinos,* the observed diversity in hybrid morphological traits (appearing more like *A. sandaracinos*) likely results from back-crosses, where F1 hybrids (from original interspecific hybridization) have interbred with *A. sandaracinos.* Conversely, despite the frequency of co-occurrence with hybrids in Kimbe Bay, there is paucity in individuals representing back-crosses with *A. chrysopterus*, based on morphological intermediacy of hybrids and parent species. Taken together, ecological and morphological observations suggest several interacting factors, including availability of conspecific versus interspecific partners, size-hierarchical behaviour and selection (sexual and ecological) are likely implicated, as illustrated theoretically for evolution under asymmetric competition (Law et al., 1997).

Genetic structure of species and hybrids in Kimbe Bay confirmed unidirectional mating asymmetry apparent from ecological and morphological findings. Similar patterns of asymmetric gene-flow between different colour morphs or sister species of reef fishes were found for another pomacentrid (van Herwerden and Doherty, 2006); where asymmetry was attributed to mating opportunities provided in contact zones when abundance disparities were evident, not due to behaviour and size dissimilarities. However, in several fish species differences in body size are

associated with reproductive isolation, including sympatric cichlids (Schliewen et al., 2001), lake whitefish ecotypes (Lu and Bernatchez, 1999), and sockeye salmon forms (Foote and Larkin, 1988, Wood and Foote, 1996).

Here, findings suggest that *A. sandaracinos* is genetically divergent from *A. chrysopterus* and *A. leucokranos* hybrids, which consistently appear undifferentiated based on mtDNA. Given the significant molecular variation among species, dominated by high diversity within *A. sandaracinos*, results suggest secondary hybridization or back-crossing of hybrids with parental species *A. sandaracinos* may prevent erosion of genetic diversity and differentiation within the species through introgression; where back-crosses identified by phylogenetic analyses suggest that pure *A. sandaracinos* individuals within Kimbe Bay are rare, compared to other species. Analogous to these findings, Marie *et al* (2007) reported similarly rare *Acanthurus leucosternon*, relative to *A. nigricans*, the more abundant sister species that it hybridizes with.

Ongoing hybridization and introgression

Introgression has two implications. Firstly, F1 hybrids represent fertile, viable offspring, which successfully reproduce with *A. sandaracinos*. Secondly, back-crossed hybrids, which are not always positively distinguishable as hybrids based on morphology, also produce fertile, viable offspring through further back-crossing. Findings of back-crossing and introgression support hypotheses of unidirectional hybridization with respect to the female lineage, consistent with size-based dominance of female anemonefish, rather than a result of Darwin's corollary, where asymmetric post-mating isolation is achieved from differential viability in reciprocal crosses between hybridizing species (Turelli and Moyle, 2007).

The frequency of individuals classified as *A. sandaracinos* based on phenotype, but identified as hybrids by sharing *A. chrysopterus* mtDNA, reveal similar patterns to those observed ecologically. Animals displaying a combination of parental traits more similar in colour and form to *A. sandaracinos,* but bearing traces of *A. chrysopterus* patterns, were relatively common and proportionally as abundant as expected under random mating. These phenotypic patterns are

explained by an increasing accumulation of *A. sandaracinos* nDNA and corresponding decrease of *A. chrysopterus* nDNA in back-crossed individuals as a result of consistently back-crossing with the smaller parental species (*A. sandaracinos*).

Finally, the presence of nuclear genotypes similar to *A. leucokranos* in both parent species is consistent with inter-specific hybridization, which produced *A. leucokranos*. Here, findings suggest back-crossing may occur with both parents, but size-dominant behaviour has limited gene flow to one direction. Analyses identified genetically intermediate hybrids, suggesting that through time, as a consequence of consistent back-crosses with *A. sandaracinos*, multi-generational back-crossed hybrids (morphologically indistinguishable from *A. sandaracinos*) are reproducing with *A. leucokranos* as subdominant '*A. sandaracinos*' males. This validates findings that size-dominant behaviour appears to influence hybridization dynamics in these taxa, with hybrids distinct from both parental species, further suggesting that hybridization is ongoing in demographic time and may eventually produce a hybrid swarm on midshore reefs of Kimbe Bay. Such results (termed reverse speciation) were shown for benthic and limnetic sticklebacks, traced over 25 years, following introduction of a benthic predator (Taylor et al., 2006). Hybrid swarms often develop under secondary contact of closely related allopatric species, where isolating mechanisms have not completely evolved (Forbes and Allendorf, 1991, Leary et al., 1995).

Mechanisms shaping hybridization outcomes

Several ecological mechanisms specifically related to interbreeding between fishes are recognised as potential reasons for a variety of naturally occurring fish hybridization examples (Wood and Foote, 1996). These include external fertilisation (Hubbs, 1955), non-assortative mating (McMillan et al., 1999, Montanari et al., 2012), resource overlap (van Herwerden et al., 2006, Yaakub et al., 2007), and sneak mating (Wirtz, 1999, Taylor, 2004, Frisch and van Herwerden, 2006, van Herwerden et al., 2006). These processes represent valid means of explaining hybridization, relative to ecology and mating systems of species involved. For example, sneak mating was found to promote asymmetric hybridization between species that differ in size at sexual

maturity (McGowan and Davidson, 1992, Ostberg et al., 2004). Conversely, asymmetry in fertilization success of freshwater darters resulted from behavioural ecology, where one species spawns in open-water and the other buries eggs, with selection favouring increased fertilization specificity in one species only (Mendelson et al., 2006). Accordingly, in the anemonefish *A. leucokranos*, the mechanism proposed to explain hybridization outcomes is specifically linked to ecology and behaviour.

The characteristic size dominant behaviour of anemonefish has important implications for hybridization. Larger species remain dominant through size advantage, as is true for other coral reef fishes, such as in coral gobies (Munday et al., 2004). Considering the findings of the present study, confirming size disparities between parent species within the broader context of anemonefish behaviour, it is suggested that larger *A. chrysopterus* will always be the female contributor during hybridization, due to size dominance. This behaviour appears to generate a barrier to hybridization in the other direction (*A. sandaracinos* as female contributor). It follows that in back-crossing between intermediate-sized hybrids and parent species, as takes place exclusively with *A. sandaracinos*, the larger *A. leucokranos* (hybrid) would dominate and contribute to the back-cross as female. Similarly, asymmetrical mtDNA introgression between hybridizing freshwater fishes (Centrarchidae) has been found to correlate with adult body size disparities; however, the study concluded this as a likely effect of 'Darwin's corollary' (Bolnick et al., 2008).

Comparatively, passive acquisition of social dominance may explain why back-crossing does not (or rarely) occur between *A. leucokranos* and *A. chrysopterus*. When they do back-cross, it appears to be the subordinate (smaller) male *A. leucokranos* crossing with a dominant (larger) female *A. chrysopterus,* based on the combined patterns of mtDNA and nDNA transmission. In cases where individuals choose to passively increase in rank, the dominant female may choose not to engage in reproduction, but rather wait for a fitter sub-ordinate to increase in rank; where in the case of *A. chrysopterus* and hybrids associating, the larger parent species *A. chrysopterus* is dominant. Similarly, putative hybrid individuals may choose to wait for the dominant female to disappear, for ensured inheritance of territory and reproductive opportunities as the dominant sex

when associating with *A. sandaracinos*. Passive coexistence is plausible when considering improbabilities of success of group members attempting to increase rank by methods other than queuing; such as dispersal or contest. Both active methods of increasing dominance come with increased risk, where dispersal and contest render animals vulnerable to predation, rejection, and eviction (Buston, 2004, Wong et al., 2008). Here, behavioural isolation appears to be the most parsimonious justification for the ecological outcomes of hybridization, although a number of other plausible mechanisms may be invoked to explain the ecological findings.

Behavioural isolation constitutes a pre-zygotic reproductive barrier. Pre-zygotic barriers obstruct mating or impede fertilisation when mating occurs through various means of isolation, including spatial, temporal, behavioural and mechanical isolation. In comparison, post-zygotic barriers prevent hybrid development into viable, fertile adults through gametic isolation, reduced hybrid viability and/or fertility and hybrid breakdown (Littlejohn, 1981, Howard, 1993). As such, an alternative explanation for the observed general deficiency of back-crossing of hybrids with *A. chrysopterus* in Kimbe Bay is hybrid breakdown and reduced hybrid viability and/or fertility; where F1 hybrids would either be infertile when reproducing as the female or alternatively F2 offspring would be unviable or infertile. Although plausible, these explanations do not account for known anemonefish behavioural ecology, nor do they reflect genetic findings presented here, and are thought to be less likely given the data.

Implications for genetic diversity

Based on mtDNA and nDNA, *A. sandaracinos* has substantially reduced genotypic diversity compared to hybrids and *A. chrysopterus* in Kimbe Bay. These results raise concerns over possible local threats of low genetic diversity for this species, including increased risk of inbreeding depression, lowered adaptive resilience and reduced viability, which likely interact with demographic and ecological processes (Lacy, 1997). Hybridization appears to reduce potential genetic threats to viability by increasing genetic diversity within this species in the short term. Similar findings have been reported for rare Acroporid coral species, which appear to overcome

extinction risk associated with lowered genetic diversity due to a propensity for hybridization (Richards et al., 2008). However, I note that although introgression may increase diversity initially, it can act to reduce diversity through time (Seehausen, 2006).

Conclusions

This study strongly suggests that hierarchical behaviour, habitat use and species size differentiation have shaped the evolutionary outcomes of hybridization for the anemonefish *A. leucokranos*. As hybrids persist as distinct forms, intermediate to parental species, I predict that hybrid resilience, behaviour and adaptive capacity likely increase hybrid fitness and reproductive viability. I hypothesize that hybridization may enable locally rare *A. sandaracinos* to persist within Kimbe Bay by increasing genetic diversity through substantial hybrid back-crossing, although this may lead to reduced differentiation of the species in the long term, if a hybrid swarm emerges. More broadly, this study provides an example of enhanced diversity and resilience of a locally rare coral reef fish due to hybridization, adding support to the evolutionary importance of hybridization in nature. Novel insights into the relevance of "asymmetric behavioural isolation" to hybridization on coral reefs are also provided. Finally, although findings suggest that the species status of *A. leucokranos* is questionable, taxonomic changes to the group in the absence of additional ecological and genetic data from elsewhere within the hybrid zone would be premature, and future changes may need to consider the impacts on the conservation and management of closely related taxa.

CHAPTER 4: Balancing introgression and species integrity across a coral reef fish hybrid zone.

To be submitted as: Gainsford, A., Jones G.P., Hobbs J-P.A., and van Herwerden, L. Balancing introgression and species integrity across a coral reef fish hybrid zone.

4.1 Abstract

Hybridization is an evolutionarily significant phenomenon common in nature. However, the understanding of the mechanisms driving and maintaining hybridization are limited. Hybrid zones, where hybridization takes place, are ideal environments to address questions on hybridization and speciation. Here, the anemonefish *A. leucokranos* hybrid zone was evaluated to determine if the social and ecological processes that facilitate hybridization vary across the hybrid zone, and whether spatial variation in social and ecological factors affect patterns of hybridization and introgression among species. Parent species frequencies and size disparities appear to drive regional ecological patterns and gene flow among taxa. Conspecific groups were most common in Kimbe Bay (65%) where parent species relative frequency was similar. Mixed species groups dominated the Solomon Islands (82%), with larger *A. chrysopterus* found over 1.5 times more often than smaller *A. sandaracinos*. Hybrid phenotypes were highly variable across the hybrid zone, reflecting extensive back-crossing among hybrids and parent species relative to region. nDNA microsatellites defined two genetic clusters in the hybrid zone that represent parent species, despite ongoing back-crossing. Pure parent species size and relative frequency explained the existing genetic structure throughout the hybrid zone, reflecting the characteristic size-based dominance behaviour of anemonefish. Findings suggest the hybrid *A. leucokranos* may differentiate from pure parent taxa in time, emphasizing the importance of protection for hybrids that may contribute to the biodiversity of coral reef systems.

4.2 Introduction

Hybridization can play an evolutionarily significant role in speciation, however the mechanisms driving and maintaining hybridization in nature remain poorly understood. Originally thought to be an evolutionary dead end (Mayr, 1942, Dowling and Secor, 1997, Coyne and Orr, 2004), it is now clear that hybridization among species is common (Mallet, 2005) and can contribute to speciation events in a variety of ways, including through introgression (Abbott et al., 2013, Meier et al., 2017). Hybridization can facilitate genetic exchange between distinct groups of taxa and may promote evolutionary novelty within a system faster than through mutation alone (Grant and Grant, 1994, Kunte et al., 2011). The outcomes of hybridization events are diverse and include fusion of species, reinforcement of reproductive barriers and generation of new distinct populations of mixed ancestry, and may provide the foundation for speciation and diversification to take place (Wu, 2001, Via, 2009, Servedio and Noor, 2003, Mallet, 2007, Abbott et al., 2010, Taylor et al., 2006, Meier et al., 2017). The study of young hybrid taxa therefore allows contemporary insights into potential speciation events in motion, which can take place at secondary contact zones between closely related taxa that may be undergoing rapid adaptive radiations (Seehausen, 2004, Gourbiere and Mallet, 2010, Price and Bouvier, 2002, Meier et al., 2017).

Hybrid zones provide natural laboratories for studying hybridization and investigating the patterns of variation among hybridizing species. Hybrid zones may vary spatially and over time, with taxa subjected to demographic processes in which novel ecological opportunities may arise (Abbott et al., 2013). Most commonly, hybrid zones have been recorded at the parapatric boundaries of species, with spatial overlap ranging from dispersal distance between locally adapted populations, to secondary contact between independently evolved sister taxa on a biogeographical scale. Ecological factors often associated with hybridization include abundance disparities between closely related taxa and the shared use of a limited resource (i.e. host, food source, and habitat). As such, the causes and consequences of hybrid zones are complex and varied, and patterns of gene flow represent single observations in time of a dynamic interaction between species (Abbott et al., 2013).

Hybridization was once considered rare in the marine environment (Arnold, 1997), however a surge of recent studies have challenged these traditional perceptions of hybrid scarcity (Gardner, 1997; Willis et al., 2006; Montanari et al., 2016). In the marine environment, hybrid zones have mainly been found along coastal areas (Gardner, 1997), including coral reef systems (Marie et al., 2007, Vollmer and Palumbi, 2002, Yaakub et al., 2006), sub-tidal zones (Koehn, 1991, Rawson and Hilbish, 1995) and in open ocean habitats such as along hydrothermal vents (Johnson et al., 2013, Van Dover et al., 2002, O'Mullan et al., 2001). Biogeographic borders often have many, largely allopatric sister species hybridizing on secondary contact. Hybrid hotspots along biogeographic borders are termed suture zones (Remington, 1968, Hewitt, 2000). Four key suture zones have been identified in the marine environment, including in temperate regions along the southern Atlantic coast of Florida (Bowen and Avise, 1990, Karl and Avise, 1992) and the Baltic Sea (Riginos and Cunningham, 2005, Johannesen et al., 2006), as well as two examples on tropical coral reefs (Hobbs and Allen, 2014, DiBattista et al., 2015).

For coral reef species, hybridization appears concentrated at two recognised suture zones. The biogeographic border between the Indian and Pacific Oceans, near Christmas and Cocos (Keeling) Islands, marks one region where regionally distinct sister taxa come into contact and hybridize frequently (Hobbs et al., 2009, Marie et al., 2007, Hobbs and Allen, 2014). At the Christmas Island hybrid hotspot, fifteen hybrid fishes involving 27 species across eight families have been confirmed (Hobbs and Allen, 2014). The other recognised marine suture zone, the Socotra Archipelago, where fourteen putative hybrid coral reef fishes from four families have been recorded (DiBattista et al., 2015), is the junction of four marine biogeographic provinces (Red Sea - Gulf of Aden, Arabian Sea, western Indian Ocean and greater Indo-Polynesian). Suture zones where hybridization occurs provide ideal environments to address evolutionarily important questions regarding hybridization.

The line of convergence between Indo-Australian and Pacific plates from north-western Papua New Guinea (PNG) to the Solomon Islands (SO), is a unique region and potential emerging suture zone, where the ranges of many sister species ranges also overlap, and taxa hybridize

(McMillan et al., 1999, Gainsford et al., 2015, Hobbs et al., 2013). The 'PNG-Solomon suture zone' has a dynamic history of disturbance associated with climatic changes and sea level fluctuations, and occurs within the Coral Triangle – the global centre of marine biodiversity (Hughes et al., 2002). In this centre of diversity, many species share habitats, increasing the likelihood of hybridization (Camp et al., 2016). Thus, the 'PNG-Solomon suture zone' can provide unique insights into what processes promote cohabiting species to hybridize and how this hybridization affects biodiversity on coral reefs.

The hybridization between *Chaetodon punctatofasciatus* and *C. pelewensis* provides one example of coral reef fish hybridization within the 'PNG-Solomon suture zone'. McMillan et al. (2015) found a greater frequency of hybrid phenotypes in comparison to parental phenotypes, suggesting greater fitness of hybrids to parental species within the hybrid zone. Another group known to hybridize in the PNG-Solomon suture zone is the anemonefishes. Anemonefish have attributes that make them ideal for studying hybridization including site attachment, relatively small size, ease of capture, known hierarchical group structure and monogamous breeding pairs that lay an egg clutch (Buston, 2004, Buston and Cant, 2006, Fautin, 1986, Moyer and Nakazono, 1978, Fricke and Fricke, 1977). Moreover, being an evolutionarily young and rapidly diversifying group, anemonefishes are prone to hybridization (Santini and Polacco, 2006, Timm et al., 2008), providing an ideal system to test evolutionary questions on hybridization (Abbott et al., 2013). Hybridization in the 'PNG-Solomon suture zone' occurs between anemonefish species *Amphiprion chrysopterus* and *Amphiprion sandaracinos* (Gainsford et al., 2015). These species have predominantly allopatric distributions; however, they cohabit and hybridize within a narrow area of overlap, termed the *A. leucokranos* hybrid zone. Due to its distinct colouration, the hybrid of the two species was initially described as a nominal species, *A. leucokranos,* but later confirmed to be a hybrid based on intermediate morphology, ecological relatedness and genetic relatedness (Gainsford et al., 2015). Size differences between hybridizing species in the context of anemonefish hierarchical behaviour were most significant in driving ecological and evolutionary patterns observed in this hybridization. Anemonefish groups are structured based on size, where individuals queue to breed (Buston, 2004,

Buston and Cant, 2006). Females are largest and dominant, followed in size by sub-dominant males, and progressively smaller non-breeding subordinates (Fricke, 1979, Hattori, 1991). Groups are site attached to host anemones (Fautin, 1986) and sex change to higher social status is passively acquired when dominant individuals are removed (Fricke and Fricke, 1977, Moyer and Nakazono, 1978, Mitchell, 2003, Buston, 2004). In this way dominance is ascertained through size, with dominant females suppressing growth and sexual maturation of the male and subordinates (Ross, 1990). The legacy of this size-based reproductive behaviour was highlighted in previous research on *A. leucokranos* in Kimbe Bay, PNG, which found the significantly larger parent species, *A. chrysopterus*, to always mate as the female when reproducing with the smaller parent species, *A. sandaracinos* (Gainsford et al., 2015). Furthermore, the intermediately sized hybrid was always the female when back-crossing with the significantly smaller parent species, *A. sandaracinos,* which was consistent with the strong size dominant behaviour observed.

Body size within animal societies is tightly linked with advantageous physiological and fitness characteristics, including social rank, particularly for fishes (Blanckenhorn, 2000). Larger individuals within groups are typically better competitors, higher ranked and more dominant (Forrester, 1991, Balshine-Earn et al., 1998, Buston, 2004, Mitchell, 2005, Hamilton et al., 2005), stabilising conflict within breeding queues through regulating subordinate growth rates (Wong et al., 2007, Buston, 2004, Heg et al., 2004, Buston and Cant, 2006). In addition to anemonefish involved in the *A. leucokranos* hybridization, differences in body size between other hybridizing taxa have resulted in reproductive isolation for a number of fish species including cichlids, lake whitefish and sockeye salmon (Schliewen et al., 2001, Lu and Bernatchez, 1999, Foote and Larkin, 1988, Wood and Foote, 1996). As such, mating behaviour associated with size has significant implications for the evolutionary outcomes of taxa involved in hybridization, directing gene flow and the degree of hybridization between species.

Factors such as abundance disparities, overlapping patterns of resource use and breakdown in assortative mating are known to promote hybridization in marine fishes (Montanari et al., 2016); however, these factors may vary across the hybrid zone and, as a result, the causes and consequences

of hybridization could also vary. For the *A. leucokranos* hybrid zone, it is not known how these factors vary regionally, or if the dynamics and outcomes of hybridization throughout the hybrid zone are comparable to Kimbe Bay, PNG. If relative abundance between hybridizing species differs, the prevalence of hybrids and level of introgression between species is expected to be strikingly different. Furthermore, although body size is an important driver of ecological and evolutionary patterns in one region of the hybrid zone, other factors such as hybrid fitness, vigour and a potential increase in deleterious mutations may also be important. Throughout the Coral Triangle, it is becoming apparent that various evolutionary processes are important for marine species to diversify and persist (Carpenter et al., 2010, Bowen et al., 2013, Bellwood and Meyer, 2009). In order to detect differences between regions and mechanisms important to the maintenance of the hybrid zone, a multidisciplinary approach is key, and can include investigating multiple genetic markers as well as ecological and behavioural data (Montanari et al., 2016).

This study investigates the *A. leucokranos* hybrid zone, evaluating the mechanism driving ecological patterns and gene flow among hybridizing taxa. The aims of the study are twofold. Firstly, I determine if the social and ecological processes that facilitate hybridization vary across the hybrid zone. Secondly, I examine whether spatial variation in social and ecological factors affect patterns of hybridization and introgression among species. A combination of ecological observations, phenotypic measurements and genetic analyses (using mitochondrial and nuclear DNA markers) were applied to address the aims; where comparison of each species and their hybrids across three regions of the hybrid zone was expected to reveal patterns of genetic regional differentiation reflective of underlying ecological differences. Overall, this study determines the processes that underpin the initiation and persistence of the *A. leucokranos* hybrid zone, in an effort to understand the evolutionary resilience of hybridizing taxa and how hybridization affects biodiversity on coral reefs.

4.3 Methods

Study taxa and locations

This study was conducted at sites within and outside the hybrid zone relative to parent species distributions between 2011 and 2014 (Figure 4.1). The yellow anemonefish, *Amphiprion sandaracinos* (Figure 4.2A), occurs from Japan south to the Solomon Islands and west to north-western Australia and Christmas Island (Indian Ocean). The orange-fin anemonefish, *Amphiprion chrysopterus* (Figure 4.2C), occurs throughout the Pacific from Palau, Papua New Guinea (PNG) and northern Great Barrier Reef in Australia, eastward to French Polynesia (Fautin and Allen, 1997). The *A. leucokranos* hybrid zone is found where these parent species distributions overlap, along the northern PNG coastline to the Solomon Islands (104'N, 12842'E to 1050'S, 16228'E). Within the hybrid zone, the two parent species form heterospecific groups and various novel hybrid phenotypes are present. In Kimbe Bay (PNG), hybrid phenotypes range from directly intermediate to parent species phenotypes (Figure 4.2B) to those that resemble *A. sandaracinos* (Gainsford et al., 2015).

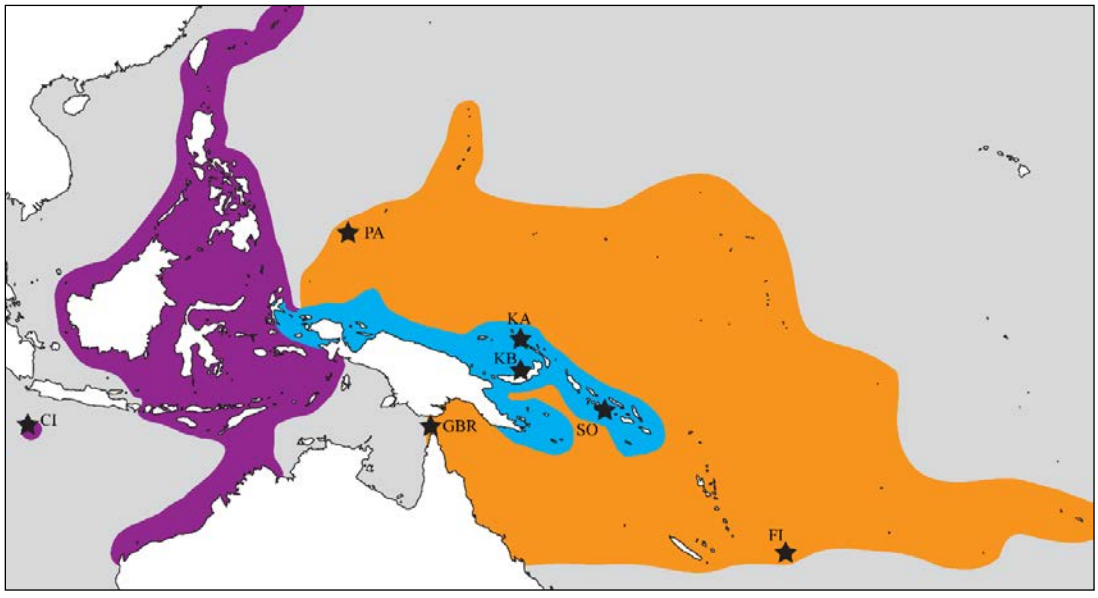

Figure 4.1 Distribution map indicating sampling sites (black stars) within and outside the *Amphiprion leucokranos* hybrid zone (blue), where species *A. sandaracinos* (purple) and *A. chrysopterus* (orange) known biogeographical distributions overlap. Sites abbreviated as follows: Christmas Island (CI), Palau (PA), Great Barrier Reef (GBR), Kimbe Bay (KB), Kavieng (KA), Solomon Island (SO), and Fiji (FI).

Three regions within the hybrid zone were explored for parent species and hybrid taxa, including Kimbe Bay (530'S, 15005'E) and Kavieng (236'S, 15041'E) in PNG, and southern New Georgia islands (845'S, 15815'E) in the Solomon Islands (Figure 4.1). Anemonefish groups were opportunistically sampled due to their patchy distribution and relative rarity of anemones, resulting in samples from 43 reef sites. Outside the hybrid zone, representative samples from 'pure' populations of parent taxa were collected. *A. sandaracinos* were collected from Christmas Island (1030'S, 10540'E) and *A. chrysopterus* from Palau (705'N, 13415'E), Fiji (1829'S, 17808'E) and north eastern Australia (1628'S, 14801'E). Fish were captured using hand nets and anaesthetized with clove oil *in situ* and released post-sampling, following recovery.

Ecology and demographics

To determine how social and ecological factors vary across the hybrid zone, habitat use and social group composition of the hybridizing species were characterised among regions within the hybrid zone. The majority of individuals were encountered between 1 m and 20 m depth, where depth, host anemone species, immediate surrounding habitat and reef zones (reef flat, crest, and slope) were recorded for all groups found in this depth zone. For each individual captured, the following data were recorded: phenotype (photographed), total length (measured to the nearest mm), sex (assigned based on relative social position), the number of individuals of each species in the group and the rank of each individual within the social hierarchy. Presence of egg clutches was recorded when observed, and putative parent species identified. Relative frequencies of species, hybrid frequency and cohabitation among taxa were quantified and tested as per Gainsford *et al.* (2015). The relative frequency of hybrid phenotypes was calculated from the photographs using seven qualitative traits including tail shape and colour, dominant body colour, presence and completeness of dorsal stripe and side bars, as well as latitudinal body shape (see Table S4.1 for phenotypic categories ranging from pure *A. chrysopterus* to hybrids, and pure *A. sandaracinos*).

Regional variation in genetic structure

The population genetic and phylogenetic structure within and outside the hybrid zone was compared to assess regional variation in hybridization propensity among regions. Whilst anaesthetized, each individual was fin-clipped for genetic analyses. Small ($4mm^2$) caudal fin clips were taken from all captured fish and preserved in 80% ethanol. Samples of 'pure' parental species from outside the hybrid zone were included to allow for comparison of species-specific genetic signals. Both mitochondrial cytochrome *b* and nuclear microsatellite markers were employed to estimate historical and contemporary gene flow.

Laboratory protocols

For all laboratory methods described herein, genomic DNA was isolated from fin clips using a standard salting out protocol (after Sunnucks and Hales, 1996), and Polymerase Chain Reaction (PCR) products were column purified with GE Illustra Sephadex G-50 for sequencing.

Approximately 430 bp of mitochondrial cytochrome *b* gene was amplified in parent species and hybrids using universal primers (CB3H; 5'- GGCAAATAGGAARTATCATTC-3'and L15162; 5'-GCAAGCTTCTACCATGAGGACAAATATC-3') following amplification procedures in Gainsford *et al.* (2015). Genbank accession numbers for sequences to be attained.

Forty-two *Amphiprion* spp. microsatellite markers, including 8 novel loci, were tested on seven to eight individuals of *A. sandaracinos, A. chrysopterus* and hybrid taxa. Novel primer development and cross-amplification success of markers is detailed in Chapter 2. Of those markers tested, 23 highly polymorphic loci that consistently cross-amplified in the study taxa across regions were used in optimised multiplex reactions, based on locus sizes using Multiplex Manager 1.0 software (Holleley and Geerts, 2009). PCRs of seven multiplex sets of two to six markers, were carried out in 10µl reactions with 50ng template, 2X Type-it Multiplex PCR Master Mix (QIAGEN), and 2µM each primer (forward and reverse). Reaction conditions were as follows: initial 3 min denaturation at 94°C, 28 cycles of 95°C for 30 s (denaturation), 60°C for 1 min 30 s

(annealing) and 72°C for 30 s (extension), and final extension at 60°C for 30 min using a Bio-Rad C1000 Thermal Cycler (Bio-Rad, Australia). PCR products were visualised by gel electrophoresis using 2.0% agarose, purified as above, and genotyped on an ABI 3730XL Genetic Analyser (Applied Biosystems) with GeneScan LIZ-600bp size standard. All genotypic data will be available on a Dryad repository at a later time.

Data compilation and analyses

Cytochrome *b* sequences were MUSCLE aligned (Edgar, 2004b, Edgar, 2004a), and manually edited in Geneious v9.0.4. An alignment including sequences from all regions sampled was used to estimate phylogenetic evolutionary history of taxa and relationships among haplotypes. The best substitution model for the alignment was the HKY + G model chosen from 21 models using a likelihood approach under default settings in MEGA6 (Tamura et al., 2013). Phylogenetic relationships were inferred using standard approaches including maximum parsimony (MP) and maximum likelihood methods in MEGA6 (Tamura et al., 2013), and Bayesian inference (BI) using the MrBayes 3.2 plug-in (Ronquist et al., 2012) through Geneious v9.0.4 (Fig. S2-4). For all analyses, the HKY + G substitution model was implemented and trees were outgroup rooted using individuals from *Amphiprion ocellaris* (DQ343956-7, KF264293-4). MP analyses included 10 independent runs using 1000 bootstrap replicates, with all ten best MP trees showing identical length and topology. ML analyses were performed using 1000 bootstrap replicates under a likelihood approach, and BI analyses were conducted with 1,100,000 iterations and 100,000 tree burn-in.

All population genetic analyses were performed in Arlequin v3.5.1.2 (Excoffier and Lischer, 2010) to estimate levels of gene exchange between and within populations, where populations outside the hybrid zone with sample sizes < 10 were excluded. Species sequence sets were defined *a priori* in DnaSP v5.10.1 (Librado and Rozas, 2009). A minimum spanning tree (MST) was constructed manually and edited in Illustrator (Adobe Systems Inc.) to elucidate patterns of haplotype distribution among and within populations. Genetic diversity indices including

haplotype diversity (*h)* and nucleotide diversity (π) were calculated for all populations. Spatial heterogeneity for cytochrome *b* was assessed through population pairwise F_{ST} and analysis of molecular variance (AMOVA) following 1000 permutations, where the proportion of variance among species groups (F_{CT}), the proportion of variation among populations within species groups (F_{SC}), and the proportion of variation within populations (F_{ST}) were estimated using a pairwise difference model.

Microsatellite genotypes were scored and manually edited using GeneMarker (SoftGenetics, USA). Of the 23 markers tested, 21 could be confidently scored. Population genetic analyses of microsatellite markers were based on a total of 124 *A. chrysopterus,* 113 hybrid (*A. leucokranos*), and 122 *A. sandaracinos* individuals collected from Kavieng (n = 26, 25, 28, respectively), Kimbe Bay (n = 31, 35, 66, respectively), and the Solomon Islands (n = 65, 53, 30, respectively; Table S4.3). Sample sizes of populations outside the hybrid zone were too small (n < 5) and therefore excluded from further population genetic analysis. Number of alleles (N_A), private alleles (P_A), observed (H_O), and expected (H_E) heterozygosities were calculated in Genalex (Peakall and Smouse, 2006, Peakall and Smouse, 2012), and the average inbreeding coefficient (F_{IS}) was estimated in Arlequin v3.5.1.2 (Excoffier and Lischer, 2010). Probabilities of departure from Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium (LD) were calculated in Genepop (Rousset, 2008) using Markov chains with dememorisation of 10000, 20 batches, and 5000 iterations per batch. The presence of null alleles, large allelic dropout and scoring bias was estimated using Micro-checker (van Oosterhout et al., 2004). Raw estimates of population structure were calculated locus-by-locus and as an average over 21 loci using Analysis of Molecular Variance (AMOVA) with 10000 permutations, in Arlequin v3.5.1.2 (Excoffier and Lischer, 2010), as well as genotypic diversity (*gd*) estimates. An excluding null allele correction (ENA) was carried out in FreeNA with 1000 bootstrap replicates (Chapuis and Estoup, 2007) to estimate species differentiation corrected for null allele frequencies. SMOGD v1.2.5 (Crawford, 2010) was used to calculate estimates of actual differentiation (D_{est}), and Structure v2.3.4 (Pritchard et al., 2000) was used to estimate the number of differentiated genetic populations (K) represented by samples.

Structure was run using the admixture ancestry model informed by location, with correlated allele frequencies for each K value for 10 individual repetitions, at 1,000,000 MCMC iterations following a 100,000 burn-in. Structure Harvester (Earl and von Holdt, 2012) was used to assess the best K following Evanno's method (Evanno et al., 2005). To visually assess relationships between predefined population clusters, a discriminant analysis of principle components (DAPC) was executed using the adegenet package (Jombart, 2008) in R v2.13.2 (R Development Core Team, 2016). DAPC retained 198 principle components, accounting for 95% of the variability present, and is visually represented in a scatterplot of the first two principle components with 95% genotypic inertia ellipses (IE) for each population.

4.4 Results

Regional ecological and phenotypic effects of hybridization

Cohabitation prevalence and relative frequency

All combinations of con- and hetero-specific groups were observed across all hybrid zone survey locations, including hybrid only groups (Figure 4.2). However, the proportion of conspecific versus heterospecifics varied across the hybrid zone. The proportion of conspecific groups was highest in Kimbe Bay (65%), compared to 18% in the Solomon Island region (Figure 4.3). Thus, 82% of groups in Solomon Islands contained heterospecifics. Across all three locations, there was a much greater proportion of conspecific groups of *A. chrysopterus* than *A. sandaracinos* (12-44% and 5-19%, respectively), however both parent species showed a similar pattern of group composition across the hybrid zone. The proportion of conspecific groups for both *A. chrysopterus* and *A. sandaracinos* was greatest at Kimbe Bay (44% and 19%, respectively) and least at Solomon Islands (12% and 5%, respectively). Therefore, the proportion of heterospecific groups, and thus the incidence of hybridization, varied across the hybrid zone.

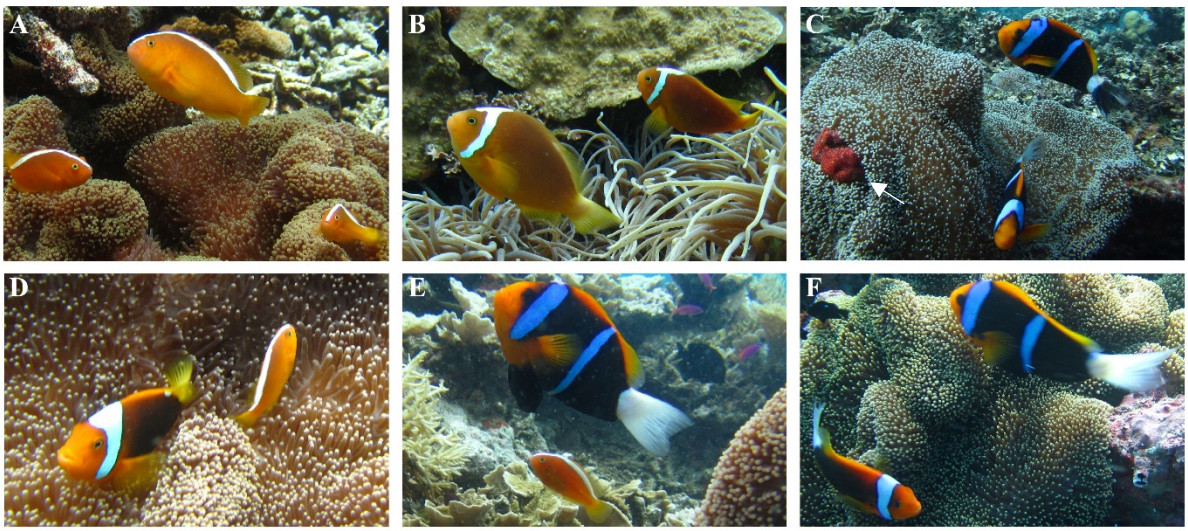

Figure 4.2 Study species group combinations found within the hybrid zone including: (A), 'pure' *A. sandaracinos;* (B) hybrid *A. leucokranos* only (observed with egg clutch); (C) 'pure' *A. chrysopterus* (egg clutch indicated with white arrow; note pigmentation of top individual), (D) hybrid with *A. sandaracinos*, (E) *A. chrysopterus* with *A. sandaracinos*, and (F) a putative *A. chrysopterus* & *A. leucokranos*' hybrid with *A. chrysopterus.* Note host anemone species are *Stichodactyla mertensii* (A, C-F) and *Heteractis crispa* (B). Photo credits: A. Gainsford.

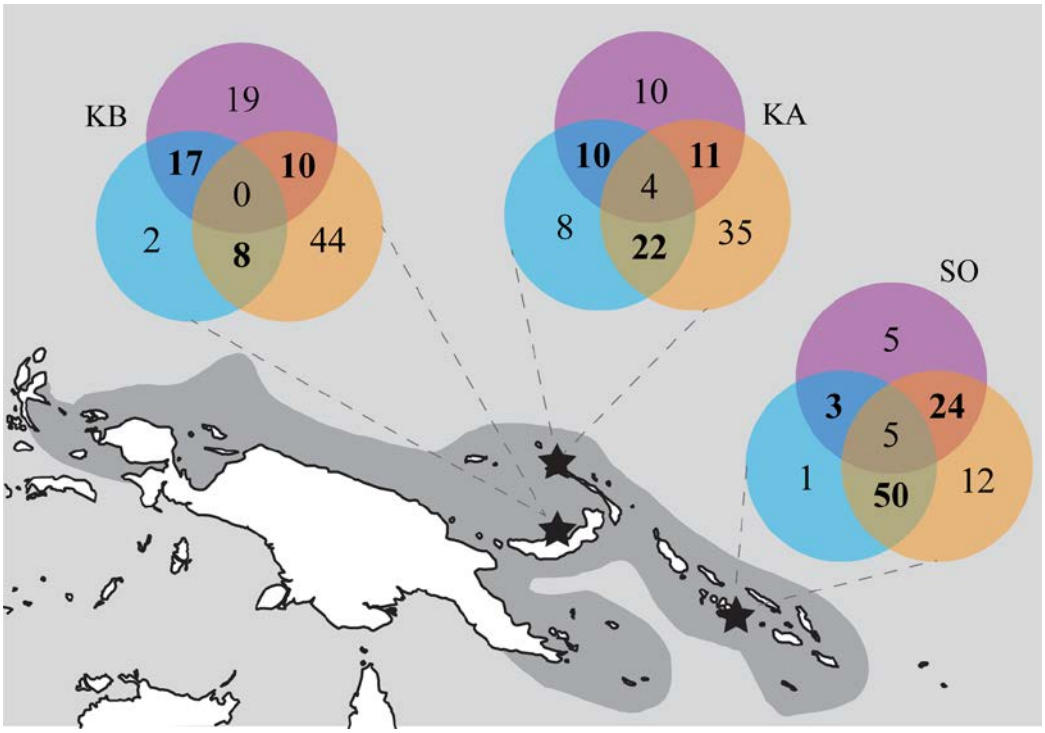

Figure 4.3 Relative abundance of hybrid zone group assemblages (%) for 'pure' *A. chrysopterus* (orange), hybrid (blue) and *A sandaracinos* (purple) groups, as well as mixed taxonomic groups (circle intersects) within the hybrid zone. Total sample sizes were: KB (n = 101), KA (n = 72), and SO (n = 77).

The formation of hybrid only groups was generally low and varied across the hybrid zone between 8% (Kavieng) to 1% (SO). Interestingly, the proportion of groups containing hybrids and *A. chrysopterus* varied six-fold across the hybrid zone from 8% at Kimbe Bay to 50% at Solomon Islands, whereas the proportion of hybrid - *A. sandaracinos* groups increased at similar magnitude in the opposite direction from 3% at Solomon Islands to 17% at Kimbe Bay. Groups containing both parental species and the hybrid were present at Kavieng (4%) and Solomon Islands (5%). This geographic variation in the composition of social groups containing hybrids is important to document because these patterns could lead to differences in the level and direction of introgression across the hybrid zone.

Across the hybrid zone, the relative frequency of hybrid individuals was comparable among the three surveyed locations (22-30%; Figure 4.4). However, when considering parent species, the relative frequency of *A. sandaracinos* was over two-fold greater than *A. chrysopterus* in Kavieng (56% and 23%, respectively), in contrast to the Solomon Islands where *A. chrysopterus* was more prevalent than *A. sandaracinos* (50% and 19%, respectively; Figure 4.4). Comparatively, parent species, *A. chrysopterus* and *A. sandaracinos*, were observed at relatively equal frequency in Kimbe Bay.

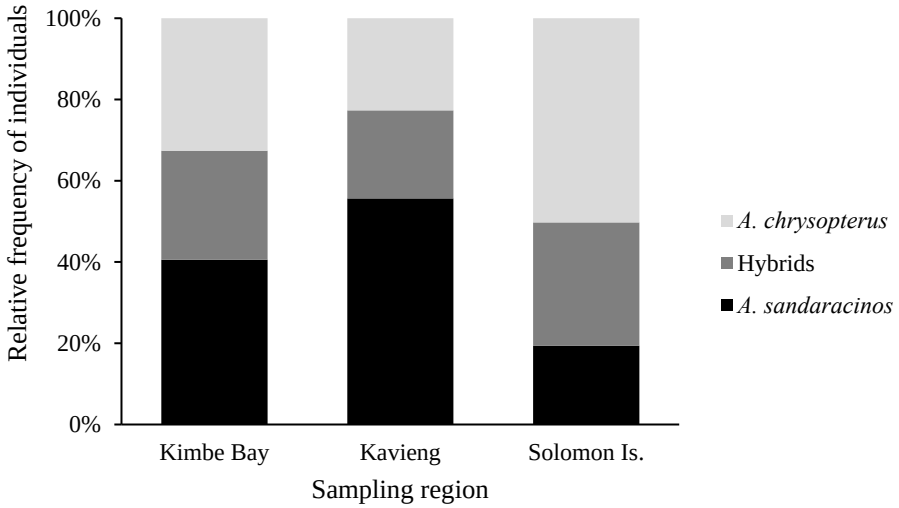

Figure 4.4 Relative frequency of 'pure' *A. chrysopterus* and *A. sandaracinos* individuals, and hybrids across three sampling regions within the hybrid zone; including all ranks from recruits to adults.

Phenotype variation and abundance

Relative frequency of phenotypic traits revealed regional variation in *A. chrysopterus* and hybrids (Table S4.2). Hybrids at Solomon Islands predominantly had an elongated tail shape similar to *A. chrysopterus* (96%), in comparison to most hybrid individuals at Kimbe Bay and Kavieng that had the *A. sandaracinos*-like round tail phenotype (96%, respectively; Table S4.2). Body colour was highly variable for *A. chrysopterus*, with black body colour most common in Kimbe Bay and Solomon Islands (94% and 79%, respectively), compared to equal black and brown coloured individuals in Kavieng (52% and 48%, respectively).

The highly variable hybrid phenotypes previously reported at Kimbe Bay (Gainsford et al., 2015) were also found at the other survey locations in the hybrid zone. During extensive surveys, an additional hybrid phenotype thought to represent a hybrid - *A. chrysopterus* back-cross was also observed. These individuals were found to have characteristics of body shape, pectoral and anal fin colouration, and singular 'white bonnet' side bar pattern consistent with known *A. leucokranos* hybrid phenotypes (Figure S4.1D), as well as caudal fin shape and colour, blue tinge to white side bar, and facial features consistent with the most common *A. chrysopterus* phenotype in the sampling region. Body colouration faded from dark orange/brown to black. These hybrid – *A. chrysopterus* back-cross individuals were always found as a male mated with *A. chrysopterus* female, and displayed particularly bold behaviour (n = 3). Additionally, a range of *A. chrysopterus* phenotypes were found across the hybrid zone, including brightly pigmented Solomon Island morphs (approximately 9% of population), individuals with half side bars, and significantly smaller, light morphs (approximately 2% and 19% of Kimbe Bay and Solomon Island populations, respectively; Figure S4.1).

Population demographics and host use

Among the egg clutches sampled throughout the hybrid zone, over half were from mixed species and hybrid groups (53%, total n = 17; specifically, *A. chrysopterus*-hybrid n = 2, hybrid-*A. sandaracinos* n = 4, and hybrid-hybrid n = 3). Seven *A. chrysopterus* mated pairs and one

A. sandaracinos mated pair were found with eggs. Recording of egg clutches was opportunistic due to natural variation in the timing of reproduction in anemonefish. Egg clutches were most often recorded in Kimbe Bay and Solomon Island sampling sites, but rarely in Kavieng.

Relative frequency of size classes for parent species and hybrids were consistent across the hybrid zone and mirrored previous results reported from Kimbe Bay (Gainsford *et al.* 2015).

Three species of anemone were used by study taxa, including *Heteractis crispa, Stichodactyla mertensii* and rarely *Heteractis aurora*. *A. sandaracinos* was almost exclusively found in *S. mertensii* (99%, n = 226), whereas hybrids (n = 181) and *A. chrysopterus* (n = 230) consistently used both *S. mertensii* (65% and 61%, respectively) and *H. crispa* (34% and 39%, respectively).

Gene flow and population variation across the hybrid zone

Four hundred and thirty alignment positions were resolved for 388 individuals in mtDNA cytochrome *b* region (Table 4.1), including 363 individuals from the hybrid zone, which had 184 polymorphic sites. Seventy-two haplotypes were detected in the hybrid zone data set, where 15 haplotypes were shared and 57 were unique, suggesting an accumulation of mutations over time via female mediated gene flow throughout the hybrid zone.

Table 4.1 Marker credentials for Hybrid Zone populations, derived from mtDNA cytochrome *b*: number of individuals (n), haplotypes (n_h), haplotype diversity (*h* +/- SE), and nucleotide diversity (π +/- SE); and nDNA microsatellites: number of individuals (n), alleles per locus (n_a), observed number of private alleles (P_a), genotypic diversity (*gd* +/- SE), observed heterozygosity (H_O), and expected heterozygosity (H_E) averaged over 21 loci. Populations include Kimbe Bay (KB), Kavieng (KAV), and Solomon Islands (SOLO).

	mtDNA cytochrome *b*				nDNA microsatellite loci					
Population	n	n_h	h	Π	n	n_a	P_a	gd	H_O	H_E
CHKB	76	17	0.819 +/- 0.03	0.017 +/- 0.01	31	198	8	0.573 +/- 0.30	0.556	0.675
CHKA	31	7	0.656 +/- 0.06	0.010 +/- 0.01	26	179	9	0.624 +/- 0.32	0.677	0.715
CHSO	56	9	0.651 +/- 0.04	0.010 +/- 0.01	65	266	27	0.634 +/- 0.33	0.634	0.717
CH overall	163	26	0.739 +/- 0.02	0.013 +/- 0.01	122					
LUKB	45	13	0.813 +/- 0.04	0.028 +/- 0.02	35	155	1	0.623 +/- 0.32	0.636	0.634
LUKA	23	5	0.640 +/- 0.07	0.003 +/- 0.00	25	194	3	0.742 +/- 0.37	0.765	0.750
LUSO	55	6	0.575 +/- 0.03	0.008 +/- 0.01	53	258	16	0.735 +/- 0.37	0.701	0.757
LU overall	123	18	0.685 +/- 0.03	0.015 +/- 0.01	113					
SAKB	30	14	0.798 +/- 0.07	0.063 +/- 0.03	66	160	10	0.478 +/- 0.25	0.460	0.503
SAKA	23	12	0.881 +/- 0.05	0.073 +/- 0.04	28	151	1	0.569 +/- 0.29	0.592	0.584
SASO	24	15	0.909 +/- 0.05	0.136 +/- 0.07	30	122	3	0.507 +/- 0.26	0.528	0.556
SA overall	77	37	0.892 +/- 0.03	0.102 +/- 0.05	124					

Mitochondrial DNA: phylogenetic analyses

Evolutionary history was inferred using three phylogenetic methods, all producing similar tree topologies with comparable branch lengths (Figure S4.2-4). Limited phylogenetic structure was evident, with only a group of Kimbe Bay *A. chrysopterus* (n = 5) and Solomon Island *A. sandaracinos* (n = 11) delineated from other sequences in all analyses. *A. chrysopterus* and hybrid populations shared six common haplotypes, with a minority of *A. sandaracinos* representatives, indicating a high level of maternal relatedness of hybrids to the parent species, *A. chrysopterus,* throughout the hybrid zone (Figure 4.5A). Two common haplotypes connect *A. sandaracinos* populations, with some evidence for maternal relatedness of *A. sandaracinos* to hybrids in Kimbe Bay only. Rare haplotypes were mostly evident in the Kimbe Bay *A. sandaracinos* population, with *A. sandaracinos* contributing to 43% of rare alleles in hybrid zone overall. Results suggest variation in the degree of mtDNA introgression across regions. Kavieng shows exclusively *A. chrysopterus* female mediated gene flow into *A. sandaracinos* via hybrids. Kimbe Bay shows a similar pattern, reflecting the importance of the size based mating hierarchy of anemonefish in mediating gene flow. However, evidence of common *A. sandaracinos* haplotypes shared with

limited hybrid individuals in Kimbe Bay suggests that extensive back-crossing has led to feedback of *A. sandaracinos* mtDNA haplotypes into the hybrid population (Figure 4.5A). There is no evidence for mtDNA introgression of *A. chrysopterus* haplotypes into the *A. sandaracinos* population for the Solomon Islands.

Mitochondrial DNA: genetic diversity and population structure

The level of population differentiation was high for all comparisons (Table 4.2, Table 4.3). Pairwise population comparisons of cytochrome *b* revealed *A. sandaracinos* to be most differentiated from other taxa overall. All 'pure' *A. sandaracinos* populations appeared highly differentiated from all other populations, with the exception of Kavieng and Kimbe Bay, which were not significantly different (Table 4.2). 'Pure' *A. chrysopterus* populations from Solomon Islands and Kimbe Bay were also highly differentiated from each other before and following Bonferroni correction. Similarly, species level differences between *A. sandaracinos* compared to *A. chrysopterus* and hybrids were significant (F_{ST} = 0.528 p < 0.000 and F_{ST} = 0.486 p < 0.000, respectively), reaffirming previous findings (Figure 4.5A).

Table 4.2 Pairwise population comparisons: F_{ST} (p-value) calculated from 430bp mitochondrial cytochrome *b* (above diagonal), where significance levels of $p < 0.05$* before sequential Bonferroni correction, and $p < 0.00138$ (bold) following the correction are indicated; and the harmonic mean of the estimator of actual differentiation (D_{est}) across 21 microsatellite loci (below diagonal).

	CHKB	CHKA	CHSO	LUKB	LUKA	LUSO	SAKB	SAKA	SASO
CHKB		0.008	**0.034***	0.006	0.007	0.024*	**0.659***	**0.578***	**0.574***
CHKA	0.006		0.013	0.000	-0.000	-0.004	**0.619***	**0.530***	**0.487***
CHSO	0.013	0.000		0.029*	-0.001	0.010	**0.669***	**0.597***	**0.573***
LUKB	0.591	0.636	0.609		0.011	0.022	**0.540***	**0.436***	**0.452***
LUKA	0.358	0.346	0.33	0.140		-0.017	**0.619***	**0.534***	**0.472***
LUSO	0.292	0.293	0.266	0.176	0.040		**0.688***	**0.617***	**0.580***
SAKB	0.774	0.835	0.809	0.017	0.275	0.309		0.014	**0.260***
SAKA	0.788	0.820	0.786	0.107	0.136	0.237	0.082		**0.193***
SASO	0.795	0.819	0.791	0.159	0.229	0.194	0.129	0.062	

All AMOVA fixation indices were significant for cytochrome *b* (Table 4.3). Variance within populations was greatest (Φ_{ST} = 0.481, 51.87%). Variation among populations (Φ_{CT} = 0.396) explained 39.63% of the variation (Table 4.3), and variance among populations within species groups was smallest (Φ_{SC} = 0.141, 8.5%), highlighting species-specific signal. Neutrality tests of Tajima's D and Fu's F_S revealed Kimbe Bay *A. chrysopterus* and *A. sandaracinos* population size may be increasing or showing evidence of purifying selection at cytochrome *b,* indicated from significant ($p < 0.05$), negative Tajima's D (Table S4.4). Negative Fu's F_S for Kavieng hybrids indicates an excess number of alleles, as would be expected from recent population expansion. All other populations displayed positive Fu's F_S, suggesting a deficiency in alleles as expected following a recent population bottleneck or over dominant selection (Fu, 1997). However, no populations showed significant Fu's F_S (where $p < 0.02$), providing no evidence that population expansion has taken place. High levels of haplotype diversity ($h > 0.5$) and low nucleotide diversity ($\pi < 0.5$) were recorded among parental and hybrid populations across the hybrid zone (Table 4.1), consistent with recent population expansions. These results provide evidence for a historical bottleneck followed by population expansion in the hybrid zone.

Table 4.3 Analysis of Molecular Variance (AMOVA) for mitochondrial cytochrome *b* and 21 microsatellite loci, respectively, for species-level groups. Significant *p*-values indicated in bold.

Source of variation	*df*	Sum of squares	Variance components	% variation	Φ-statistic (*p*-value)
mtDNA cytochrome *b*					
Among populations	2	1055.654	4.183 Va	39.63	Φ_{CT} **0.396** (0.021 +/- 0.01)
Among pop within groups	6	240.024	0.897 Vb	8.50	Φ_{SC} **0.141** (0.000 +/- 0.00)
Within populations	354	1937.945	5.474 Vc	51.87	Φ_{ST} **0.481** (0.000 +/- 0.00)
Total	362	3233.623	10.554		
nDNA microsatellite loci					
Among populations	2	722.758	1.380 Va	23.58	Φ_{CT} **0.236** (0.006 +/-0.00)
Among pop within groups	6	155.571	0.294 Vb	5.01	Φ_{SC} **0.066** (0.000 +/- 0.00)
Among individuals within populations	350	1507.118	0.126 Vc	2.15	Φ_{IS} **0.030** (0.002 +/- 0.00)
Within individuals	359	1455.500	4.054 Vd	69.26	Φ_{IT} **0.307** (0.000 +/- 0.00)
Total	717	3840.947	5.854		

Nuclear DNA: contemporary population structure

Summary statistics for 21 microsatellite loci are presented in Table S4.3. Significant single-locus departures from HWE were detected in 100 of 189 tests at population level before and 71 of 189 after sequential Bonferroni correction ($a < 0.001$; Table S4.3). Departure from HWE at locus As20, with homozygous excess revealed during analysis may be influenced by a null allele.

Genotypic diversity, based on microsatellite data, was moderate to high (0.478 +/- 0.25 to 0.742 +/- 0.37), with greater genotypic diversity estimates for hybrid populations compared to parent taxa across all three regions (Table 4.1). *A. chrysopterus* from Solomon Islands had the highest number of private alleles ($P_a = 26$), more than double that of Solomon Island hybrids and Kimbe Bay *A. sandaracinos* (11 and 10, respectively), which were next highest.

For all comparisons, population genetic differentiation was high (Table 4.2, Table S4.5, and Table 4.3). Low estimates of actual differentiation (D_{est}), between populations within species, indicate that region may not be important in structuring populations of parental and hybrid taxa (Table 4.2). There is a cascade of structure among taxa, where *A. sandaracinos* and *A. chrysopterus* were highly differentiated, *A. chrysopterus* and hybrids moderately differentiated and *A. sandaracinos* and hybrids least differentiated. This indicates species level is the most important factor structuring the various populations, despite ongoing hybridization and back-crossing (Table 4.2). Variation within individuals relative to the total was greatest ($\Phi_{IT} = 0.307$, 69.25%), followed by variation among populations ($\Phi_{CT} = 0.236$, 23.58%), based on AMOVA estimates (Table 4.3). Although significant, variation among populations within species groups and among individuals within populations contributed only 5.01% and 2.15%, respectively, to overall variation (Table 4.3).

DAPC visually defined clustering of populations in the hybrid zone (Figure 4.5B). *A. chrysopterus* populations grouped together and separated from all other populations along the x-axis. Comparatively, *A. sandaracinos* and hybrid populations are differentiated along the y-axis, where Kimbe Bay populations group loosely together and appear most different from the *A. chrysopterus* cluster. The Solomon Islands *A. sandaracinos* population was distinct from other

populations, where Kavieng and Solomon Islands hybrid populations group together with Kavieng *A. sandaracinos* in the plot centre (Figure 4.5B). Evidence of back-crossing and individual unique genotypes are indicated by dots falling outside 95% ellipses for all populations. The structure analysis used to inform DAPC supported two differentiated genetic clusters representing each parent species (Figure S4.5-6). When K = 2 clusters, an approximate 50% contribution of both parent species to hybrid populations is clearly defined in Kavieng and Solomon Islands regions (Figure 4.5C). In Kimbe Bay, closer to a 75% contribution to hybrids is evident from *A. sandaracinos.* Some individuals identified as *A. sandaracinos* were more similar to hybrids, providing evidence of ongoing back-crossing of hybrids with the smaller parent species in this region (Figure 4.5C). For comparison, when K=3, a third cluster appears revealing Kimbe Bay hybrid and *A. sandaracinos* populations to be similar, but differentiated from all other populations. This distinct cluster may result from ongoing back-crossing between these Kimbe Bay populations, where they are more genotypically similar to each other than to their conspecific populations (Figure 4.5C).

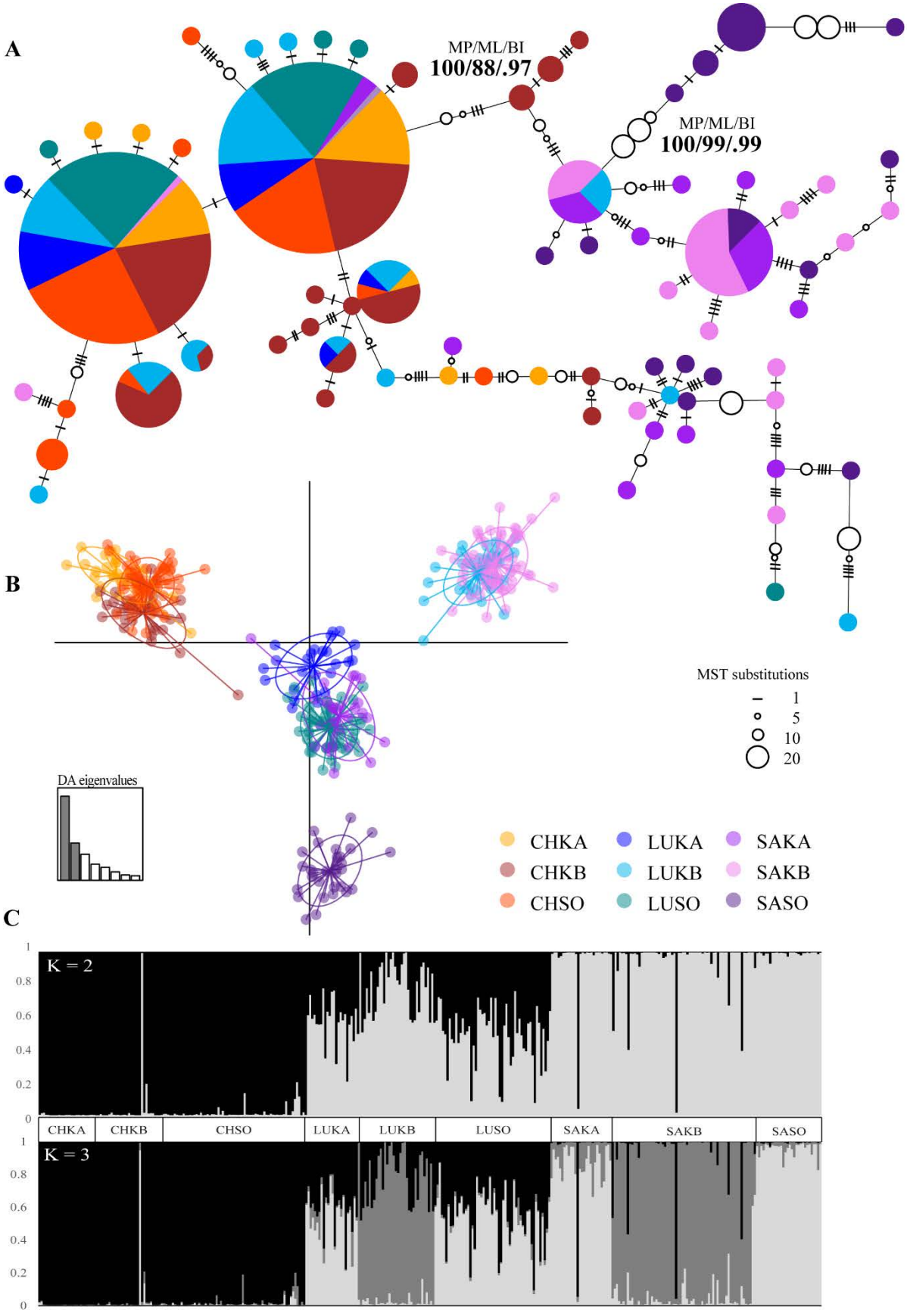
A
MP/ML/BI
100/88/.97
MP/ML/BI
100/99/.99
B
MST substitutions
1
5
10
20
DA eigenvalues
CHKA
CHKB
CHSO
LUKA
LUKB
LUSO
SAKA
SAKB
SASO
C
K = 2
K = 3
1
0.8
0.6
0.4
0.2
0
CHKA
CHKB
CHSO
LUKA
LUKB
LUSO
SAKA
SAKB
SASO

Figure 4.5 (A) Minimum spanning tree of mtDNA cytochrome *b* haplotype relationships for hybrid zone *A. sandaracinos*, hybrid *A. leucokranos,* and *A. chrysopterus* estimated under a median-joining algorithm. Each 'pie' represents an individual haplotype, the size of which is proportional to the total number of individuals that share each haplotype, where individual population identity is indicated by colour. Substitutions separating haplotypes are indicated in the legend for one, five, ten and twenty substitutions, respectively. Phylogenetic relationship structure is inferred through MP and ML bootstrap support values, and BI posterior probabilities. See Table 4.1 for number of individuals per population. (B) Inferred ancestry of individuals using Bayesian population assignment to K=2 and K=3 clusters, as indicated, using 21 microsatellite loci. Each vertical line represents an individual, with proportional genotype assignment to K clusters indicated by different colours. For panels A and B, colours relate to populations with first two letters denoting species, *A. chrysopterus* (CH), *A. leucokranos* (LU), *A. sandaracinos* (SA), and last two letters indicating region, Kavieng (KA), Kimbe Bay (KB), and Solomon Islands (SO). (C) Scatterplot of DAPC performed on 21 microsatellite loci for 9 populations within the hybrid zone as indicated in the legend. Individual genotypes are represented by dots and population clusters are defined by 95% inertia ellipses. The screeplot (bottom left of panel B) of discriminant analysis (DA) eigenvalues provides a graphical representation of variance of each discriminant function; where shaded bars highlight those retained in analysis. Axes represent the first two discriminant analysis functions.

nDNA contribution relative to parent species and hybrid abundance

The degree to which parent species nDNA contributed to hybrid populations varied regionally regardless of the relative abundance of species. In Kimbe Bay, where the abundance of each pure parent species and hybrids were near equal, there is an asymmetric 25:75 contribution by *A. chrysopterus* and *A. sandaracinos* to hybrid populations. In Kavieng and Solomon Islands, there is a near 50:50 input by *A. chrysopterus* and *A. sandaracinos,* despite relatively high and low abundance, respectively, of *A. sandaracinos* compared to *A. chrysopterus* at these two locations.

4.5 Discussion

Studying anemonefish hybridization in the 'PNG-Solomon suture zone' revealed that size differences and regional disparities in parent species frequency drive variation in gene flow among taxa across the hybrid zone. The relative abundance of parent species and hybrids varied across the hybrid zone and observed levels of cohabitation did not reflect a scenario whereby rare species 'seek out' heterospecific mates in absence of conspecifics, as is often associated with

hybridization events. Subsequently, hybrid phenotypes were highly variable across the hybrid zone, reflecting the degree of back-crossing among hybrids and parent species relative to region. Species level was most significant in structuring populations based on nDNA microsatellites, despite ongoing hybridization and persistent back-crossing throughout the hybrid zone, where two genetic clusters representing the parent species were defined. I propose that the maximum size of parent species, rather than species identity *per se*, explains the revealed genetic structure, reflecting strong size-based dominance behaviour characteristic of anemonefish, where size is associated with taxonomic status (Gainsford et al., 2015). In this way, mtDNA revealed unidirectional hybridization among species, where the larger species was consistently female and the smaller species was consistently male when interbreeding.

In contrast, the degree to which parent species nDNA contributed to hybrid populations varied regionally regardless of species relative abundances, with an asymmetric 25:75 contribution in Kimbe Bay, and 50:50 input elsewhere by *A. chrysopterus* and *A. sandaracinos, respectively*. This may reflect the extent of back-crossing in each region. High haplotypic diversity and low nucleotide diversity in all populations indicate that, historically, a bottleneck followed by a population expansion contributed to the generation and subsequent expansion of the hybrid zone. Collectively, results suggest the hybrid (originally described as *A. leucokranos*) may persist in the hybrid zone and differentiate from parent species over time. This study shows, for the first time in the marine environment, that the outcome of hybridization is dependent on the social and ecological context in which taxa hybridize.

Regionally disparate species prevalence and cohabitation

In Kavieng and Solomon Islands regions, where abundance disparities between parent species are evident, significantly more mixed species group assemblages occur than in Kimbe Bay, where conspecific groups are twice as common. In contrast, the frequency of each parent species in Kimbe Bay is relatively equal and overall conspecific assemblages predominate. Abundance disparities between species are considered a key factor facilitating hybridization between sister taxa

in regions of range overlap (Hubbs, 1955). In a recent review of fish hybridization, rarity of one or both parent species was reported as the primary ecological factor implicated in promoting hybridization among marine fishes (Montanari et al., 2016). This was followed by shared resource use, specifically the degree of habitat and dietary overlap. Mate choice experiments on hybridizing marine fishes are not currently available, however experimentally altering the relative abundance of two largely sympatric grasshopper species increased hybridization propensity when relative frequencies of sister taxa were increasingly disparate, due to additional inter-species encounters (Rohde et al., 2015). Authors concluded that abundance disparities are a major driver of hybridization, and experimentally found for the first time that hybridization probability increased with decreasing relative frequency of conspecific taxa (Rohde et al., 2015). Hybrid systems in which one species is rare and the other abundant are widely reported, where rare species are generally purported to choose mates from an abundant sister species in the absence of conspecifics (Frisch and van Herwerden, 2006, Marie et al., 2007, Montanari et al., 2014, Allen, 1979, Moyer, 1981, Randall et al., 1977a, van Herwerden et al., 2006, Hobbs and Allen, 2014). Within the *A. leucokranos* hybrid zone, the less abundant species was not consistently found to have a greater propensity for cohabitation with the more abundant species. For example, *A. chrysopterus* was more abundant than *A. sandaracinos* in Kavieng, and less abundant than *A. sandaracinos* in the Solomon Islands, yet showed a relatively greater propensity for cohabitation and hybridization with other taxa at both locations. This resulted regardless of who the most abundant species was, considering both mtDNA and nDNA exchange.

The data show that common species mate with less common species. Pyle and Randall (1994) asserted that the general assumption of rare species seeking out heterospecific mates, does not consider why individuals from a common species might choose to mate with individuals from a rare species when conspecifics are abundant. It was suggested that particular social systems may provide alternative opportunities for reproduction at more favourable times for dominant individuals of a particular sex (Pyle and Randall, 1994), such as in the harem forming *Centropyge* species that hybridize (Moyer, 1981, Moyer, 1990, Kosaki et al., 1991, Lutnesky, 1992a, Lutnesky,

1992b). However, in *Centropyge* spp., gender frequency disparities appear to be more important drivers than species abundance disparities.

I propose that in the *A. leucokranos* hybrid zone, although abundance disparities clearly appear to be associated with hybridization propensity, the underlying reason that abundant *A. chrysopterus* may choose to engage in hybridization is more closely associated with its need for a limited resource, the host anemone on which groups live and reproduce. In the *A. leucokranos* hybrid zone, intraspecific competition for limited host anemones is great (Hattori, 2005), and the larger species in a given scenario holds a significant size advantage when joining and living in mixed groups.

Drivers of population structure across hybrid zone

What is driving the structure found across the *A. leucokranos* hybrid zone, where abundance disparities appear to promote hybridization? In considering preferences for conspecific or interspecific group formation, it is widely assumed that all individuals have equal choice in determining breeding partners. However, the assumption that mate choice is a level playing field in hybridization between species in hierarchical groups is fundamentally false, as the factor on which dominance depends may not be equally distributed among taxa (Bronson et al., 2003, Reudink et al., 2006). In the case of anemonefish, dominance is dependent on size, and anemonefish are well known for living in hierarchical groups in which size dominance determines an individual's right to reproduce as either a female or male (Buston, 2004, Buston and Cant, 2006, Fricke and Fricke, 1977, Moyer and Nakazono, 1978, Buston, 2003). In the case of hybridizing anemonefish, Gainsford *et al.* (2015) found that the maximum size of hybridizing taxa drives which species reproduces as the dominant female or sub-dominant male in mixed species group assemblages. Based on previous research across the *A. leucokranos* hybrid zone, it is likely that the bigger (i.e. dominant) fish always gets first choice of a mate. At all locations sampled, *A. chrysopterus* was always the larger species and apparently prefers conspecifics followed in choice by intermediately sized hybrids, and last - smaller *A. sandaracinos*. As in many group forming fish species, size of

individuals can be very important in shaping ecological interactions. Reproductive success is highly skewed towards individuals that are socially dominant due to greater size, aggression and fitness, thus attaining greater access to mates and limited resources (Vehrencamp, 1983, Keller and Reeve, 1994, Reeve and Keller, 2001, Wong, 2011). In this way, moderately sized hybrids and small *A. sandaracinos* are disadvantaged against the more dominant species, and must continue to queue in the hope of reproducing, rather than facing eviction and becoming vulnerable to mortality outside the group (Buston, 2004, Wong et al., 2008). However, individuals may benefit from remaining in queues for reproductive positions, rather than recruiting to another group (Wong et al., 2007, Mitchell, 2005), and that experience of individuals can compensate for size disadvantages in fish social hierarchies and potentially overcome them (Alcazar et al., 2014). Smaller hybrid and *A. sandaracinos* mate preferences are not evident in the ecological data, as results retain the signature of larger species mate choice throughout the hybrid zone. I hypothesise that hybrids may prefer to mate with other hybrids, if available, but due to hybrid rarity would preferentially cohabit with the relatively smaller species, leaving hybrids with an inherent advantage over the smaller, sub-dominant species.

The influence of size dominance on gene flow is evident in genetic structure found among populations. Species level was most important in structuring genetic populations; however, as size is not independent of taxonomic status in this hybridization scenario, I propose that the size of the parent species, rather than the species itself, is structuring populations and driving the direction of gene flow among species. mtDNA reflects a pattern of haplotype introgression from larger *A. chrysopterus* to *A. sandaracinos* via the intermediately sized hybrid conduit. When mixed species mated, hybrids appeared more genetically similar to the larger, dominant parent species. This larger, dominant species would exclusively be the mother based on maternally inherited DNA. Therefore, hybrid phenotypic diversity was ultimately influenced by the proportion of mixed species groups within each region, in addition to the taxonomic assemblage of groups. In this way, parent species size was important in shaping observed hybrid phenotypes due to the influence of hierarchical size-dominance behaviour involved anemonefish reproduction.

Genetic data also revealed two defined parental clusters, one representing the larger *A. chrysopterus* and the other representing the smaller *A. sandaracinos.* As would be expected when examining nuclear loci, hybrid populations have an intermediate 50:50 contribution of each parent species, except in Kimbe Bay where more of the hybrid genotypes are similar to the *A. sandaracinos* parent based on structure assignment. This apparently highlights extensive back-crossing amongst hybrid and *A. sandaracinos* populations in Kimbe Bay, where hybridization has most likely been occurring for longer than other regions sampled as suggested by the level of introgression at Kimbe Bay. This is not to say that back-crossing is not extensive in other regions. DAPC analysis showed, based on 21 highly polymorphic nDNA loci, that hybrid phenotypes are more *A. sandaracinos*-like in each region, reflecting the hybrid choice, in the absence of other hybrids, to mate as the larger dominant female with the sub-dominant parent species males. An exception to this generalisation is that in the Solomon Islands *A. sandaracinos* appears isolated from other taxa and is particularly rare.

Persistence of hybrid *A. leucokranos*

High haplotype and low nucleotide diversities throughout the hybrid zone suggest that hybridizing species have historically experienced a population bottleneck followed by rapid population growth, which has led to an accumulation of mutations (Grant and Bowen, 1998, Avise et al., 1984, Rogers and Harpending, 1992). Grant and Bowen (1998) categorised such scenarios as examples of species which contain dominant haplotypes connected to clusters of unique haplotypes by only a few mutations, and are mostly evolutionarily 'young' species. Recently diverged sister species, reveal the association between biogeographical barriers and evolutionary patterns. For example, the evolutionary trajectories of many Indo-Pacific marine fauna are directly related to glacial sea level fluctuations during the Pleistocene, which divided the Red Sea, Indian and Pacific Oceans (Palumbi, 1994, McMillan and Palumbi, 1995, Timm and Kochzius, 2008, DiBattista et al., 2016). Anemonefish species studied in the Indo-Pacific are highly related based on morphometrics, phylogenetic and population genetic data (Gainsford et al., 2015, Timm et al., 2008). Specifically *A. leucokranos* hybrids regardless of region have greater genotypic diversity than parent species as

is expected to be required for the persistence of hybrid populations. Hybrid zones, where the process of divergence is underway, offer insights into the importance of biogeography and ecology in shaping population histories and future evolutionary patterns. Despite drawing the conclusion that *A. leucokranos* may be a true species, Santini and Polacco (2006) concluded the probable area of origin for Amphiprionidae is the Coral Triangle, beginning somewhere between the Philippines and the Great Barrier Reef on east coast Australia, and Sumatra and Melanasia. This finding is in agreement with the Coral Triangle being the most significant hot spot for biodiversity and evolution of endemism (Roberts et al., 2002). Thus, it is not surprising that the hybrid zone examined here is located within the Coral Triangle, where Amphiprionidae first appeared and diversified (Litsios and Salamin, 2014), and where adaptive radiation of species through hybridization continues. Size-based behaviour, limiting bidirectional gene flow, and hybrid zone location along species distribution boundaries may contribute to *A. chrysopterus* and *A. sandaracinos* not merging, whilst promoting hybridization when abundance disparities exist.

The persistence of the hybrid *A. leucokranos* is associated with six key factors, which may contribute to speciation through time. Firstly, hybrid - hybrid pairs with egg clutches are consistently found, where offspring are viable based on both phenotypic and genetic evidence. Throughout the hybrid zone, hybrids regularly share resources with parent species, facilitating back-crossing with parent taxa, particularly with the smaller, sub-dominant *A. sandaracinos*. Back-crosses in the other direction (with dominant *A. chrysopterus*) are also evident, albeit rare, due to the size dominant behaviour structuring anemonefish groups.

There is a strong case for recognising the status of this hybrid as more than an evolutionary dead end in light of overwhelming evidence for the importance of hybridization to the two parent taxa, as well as the indication that *A. leucokranos* may be in an evolutionarily early stage of speciation. Recently, authors have highlighted the importance of acknowledging hybrid species (Allendorf et al., 2001, Richards and Hobbs, 2015). Legislation regarding hybrids is inherently vague and generally does not consider protection or conservation policy measures. Losses of taxonomic evolutionary novelty and phylogenetic diversity, as well as increased species extinctions

are predicted from inadequate management of hybridization and hybridizing lineages (Dowling and Secor, 1997, Forest et al., 2007, Van Dyke, 2008). Pertinently, *A. leucokranos,* a highly prized aquarium trade species that is iconic, rare and easily caught due to reliance on sessile anemone hosts, is likely to be detrimentally impacted by removal of its current species status. Taxonomic delisting of this species, already prized by aquarium traders, may lead to increased harvest and thus increased rarity of this already locally rare and endemic taxon, simultaneously driving an increase in market value of individual fish and hence greater motivation for trade. Richards and Hobbs (2015) concluded that in order to conserve coral reef biodiversity, and the processes that are implicit in initiating and maintaining biodiversity, such as hybridization, policies regarding conservation and management must be addressed on an individual case basis, as removal of species status or lack of protection may indirectly impact evolution and biodiversity of species overall.

Future study of this system and evolutionarily young taxa should aim to address questions of hybrid vigour and differential fitness specifically which could not be directly addressed here, however with the use of genomic data may be resolved. Additionally, given the logistics of doing so, manipulation of the natural mating system in a closed laboratory environment whereby *A. sandaracinos* eggs are cross-fertilised with *A. chrysopterus* sperm to test whether post-zygotic barriers limit fertilization in addition to pre-zygotic ecological and behavioural barriers would further confirm conclusions presented here. Differentiation in egg development would then provide a measure of fitness in comparison to naturally occurring hybrids.

Conclusions

Extensive investigation of the *Amphiprion leucokranos* hybrid zone revealed that parent species frequencies and size disparities drive regional ecological patterns and gene flow among taxa. The size of parent species, rather than the species itself, better explains the existing genetic structure, reflecting the characteristic size-based dominance behaviour of anemonefish. This study demonstrated that rare species may not always choose to hybridize with abundant species when frequency disparities arise, such as along the edges of their biogeographical distributions. High

haplotypic diversity and low nucleotide diversity in all populations examined suggest a bottleneck followed by recent population expansion that has led to initiation and persistence of this hybrid zone, where the hybrid *A. leucokranos* appears to be differentiating from the parent taxa. This study emphasizes the need and importance of protection for hybrid species. Not only are *A. leucokranos* vulnerable to over-harvesting by aquarium traders, but they are also important contributors to both the evolutionary resilience of hybridizing parent species and the biodiversity of coral reef systems.

CHAPTER 5: *Amphiprion leucokranos* joins the queue: are hybrids on an equal playing field with parent species in a size-based hierarchical queue?

To be submitted as: Gainsford, A., Bonin, M.C., van Herwerden, L., and Jones G.P. *Amphiprion leucokranos* joins the queue: are hybrids on an equal playing field with parent species in a size-based hierarchical queue? Balancing introgression and species integrity across a coral reef fish hybrid zone.

5.1 Abstract

Hybrids are often considered inferior based on classical hybrid theory, a generalised assumption driven by limited empirical studies involving direct comparisons to parent species, particularly in the marine environment. This Chapter aims to address this significant gap in understanding hybrid inferiority in an ecological and behavioural context, directly comparing hybrids to pure parent species within an anemonefish dominance hierarchy. Assumptions of hybrid inferiority were tested within the *Amphiprion leucokranos* hybrid system. Findings highlight that hybrids may not necessarily be inferior to parent species; where behaviour generates important ecological, and evolutionary implications for hybridization outcomes in such species. Hybrid intermediate size to markedly larger and smaller parent species, respectively, drove advancement in rank within mixed species groups; where hybrids within mixed groups positively changed rank faster and occupied dominant ranking positions more often than did the smaller parent species, *Amphiprion sandaracinos*. With hybrids present in mixed groups, the growth rate of all individuals was significantly greater, but no impact on survivorship, eviction and recruitment was evident in such groups. Additionally, groups, post male removal, displayed courting behaviours such as swimming together as frequently as did pure species after the same disturbance. Nevertheless, mixed groups took longer to display courting associated behaviours, including nest preparation. Results reveal that when dominance is associated with a predetermined factor such as size, hybrids may have an innate advantage. Larger parent species, *Amphiprion chrysopterus,* evidently also benefitted by 'jumping the queue' altogether, indicating a benefit to joining mixed groups at a dominant size, thereby avoiding competition for higher ranks in conspecific groups, where higher ranked individuals are already reproductively active.

5.2 Introduction

Hybrids, derived from interspecific breeding of closely related taxa, are generally expected to have reduced fitness compared to offspring of same species pairs. Hybrid inferiority is often assumed based on classical hybrid theory, but limited empirical studies with direct comparisons to parent species exist, particularly in the marine environment. Studies of hybrid fitness are commonly done in controlled laboratory settings, isolated from the natural environment and associated selection pressures which may impact on hybridization outcomes and ultimately on evolution of species (Hatfield and Schluter, 1999). This reveals a significant gap in understanding natural hybrid fitness and highlights the challenge of forecasting outcomes of hybridization in an ecological context

Group living species provide a unique framework in which hybrids and parent species can be directly compared in a natural ecological and behavioural context. Mating systems are tightly aligned with evolutionary diversification of many species in nature, where group-living taxa often form strict dominance hierarchies. Within many group-living species, dominant breeding positions are acquired by subordinates through queuing in a rank based hierarchy. Favourable ranks, which place individuals in contention to breed within queues, may be ascertained either passively by outlasting competitors or actively through asserting dominance. The basis of dominance within animal mating systems is highly variable and has been associated with intelligence, aggression, division of labour and ability to suppress subordinate growth (Wittig and Boesch, 2003, Mech, 1999, Wong et al., 2008). However, across different taxa and types of mating systems, a common factor in dominance is body size, which has been linked with advantageous physiological and fitness characteristics, particularly in fishes (Blanckenhorn, 2000). In this way, larger individuals within groups are typically better competitors, higher ranked and more dominant (Forrester, 1991, Balshine-Earn et al., 1998, Buston, 2004, Mitchell, 2005, Hamilton et al., 2005).

Anemonefish groups provide an acute example of a dominance hierarchy in which subordinates queue to inherit breeding rights, and relative size provides a strict dominance

framework (Buston, 2004, Buston and Cant, 2006). Females are largest and most dominant, followed in size by sub-dominant males, and progressively smaller non-breeding subordinates (Fricke, 1979, Hattori, 1991). Groups are site attached to host anemones (Fautin, 1986) and sex change to higher social status is passively acquired when dominant individuals are removed through mortality or eviction (Fricke and Fricke, 1977, Moyer and Nakazono, 1978, Mitchell, 2003, Buston, 2004). Furthermore, dominant females suppress growth and sexual maturation of the male and subordinates, controlling the queue from the top down (Ross, 1990).

The *Amphiprion leucokranos* hybrid system is well studied (Chapter 3, Chapter 4) and provides an ideal framework and case study to compare hybrid and pure species in a naturally relevant ecological and behavioural context. The aim of this study is firstly to elucidate in a natural setting, within mixed species groups**,** how hybrids might perform compared to within pure parent species groups. Specifically this study monitors mixed and pure species groups to test (1) how the presence of hybrids within mixed groups affects survivorship, eviction and recruitment, (2) how the presence of hybrids affects growth rates within mixed groups, (3) if hybrids have a growth advantage or disadvantage within mixed species groups, (4) whether the proportion of each species at each rank differs within mixed groups, and (5) if hybrids progress up the queue in a similar way to pure species. Secondly, this study then manipulates and investigates - in a natural setting, whether: (6) courting behaviours associated with reproduction are displayed as early and as often in mixed species groups, and (7) the onset and frequency of courting in hybrid only groups differs from that in pure conspecific species groups.

Overall, it is expected that pure groups will court, display nest preparation, and reproduce sooner than mixed groups, particularly groups with hybrids, as hybridization should be selected against through reinforcement of reproductive barriers to maintain distinct species. Following the classic assumption of hybrid inferiority, hybrids are likely to be disadvantaged when queuing for reproductive positions within a mixed species group. Reduced fitness of hybrids may lead to limited survivorship within mixed groups, where hybrid individuals grow slower and may be evicted at a greater rate than pure species. Mixed groups may have limited recruitment, and hybrids might be

less likely to progress to breeding positions and take longer than pure species to engage in courting behaviour associated with reproduction, rendering hybrids less likely to reproduce and less fit than pure parent species. However, hybrids are not always inferior to pure species (Arnold, 1997, Arnold et al., 2001, Burke and Arnold, 2001), and in the case of *A. leucokranos,* hybrids develop into viable, fertile adults and go on to reproduce, growing to a larger maximum size than the adults of the smaller of the two parent species, *A. sandaracinos*, which may favour hybrids when queuing in the size based hierarchical setting of anemonefishes.

Understanding mixed species group dynamics will help elucidate why some hybrid populations persist and others do not, and how these hybridization events can contribute to the evolution or devolution of species. This study will provide insight into the persistence of a young hybrid taxon (*A. leucokranos*), by testing classical assumptions of hybrid inferiority compared to 'true' species.

5.3 Methods

Study species

High site fidelity, strong hierarchical group behaviour and ease of manipulation make the hybrid coral reef fish *Amphiprion leucokranos*, and parent taxa *Amphiprion chrysopterus* and *Amphiprion sandaracinos*, ideal focal species for this study. The *A. leucokranos* hybrid zone is located where parent species distributions overlap, along the northern coast and islands of Papua New Guinea and the Solomon Islands. Here, hybrid *A. leucokranos* is intermediately sized compared to large and small parent species, *A. chrysopterus* and *A. sandaracinos*, respectively. These three taxa share host anemone species choice and regularly co-inhabit anemones, naturally forming mixed species mated pairs, with ongoing back-crossing of hybrids and parent taxa within the hybrid zone (dependent on relative local species abundance; Gainsford et al., 2015; Chapter 4). As is characteristic of anemonefish, individuals queue within size-based hierarchical groups for dominant female and male breeding positions.

Observational study

Annual monitoring of a total 25 groups of *A. chrysopterus*, the hybrid *A. leucokranos*, and *A. sandaracinos* (n = 17, n = 39, and n = 58 individuals, respectively) was carried out in Kimbe Bay, Papua New Guinea (5.30'S, 150.05'E; Figure 1) from 2011 to 2013. Over a month long search period during April 2011, 25 focal anemone groups were selected from eleven reefs to examine naturally occurring species assemblages, including *A. chrysopterus* only (n = 2), hybrid only (n = 1), *A. sandaracinos* only (n = 1), *A. chrysopterus* and *A. sandaracinos* (n = 2), *A. chrysopterus* and hybrid (n = 3), hybrid and *A. sandaracinos* (n = 14), all three species together: *A. chrysopterus,* hybrid and *A. sandaracinos* (n = 2). No *A. chrysopterus* only groups were included in subsequent analysis due to inconsistent annual monitoring as a result of logistical and weather related challenges. An initial census of each group included photographic records, species identification (and subsequent unique elastomere tagging of each individual), recording total length (mm) and size based putative relative rank of individuals within group, as well as host anemone species inhabited, GPS location, reef aspect, and immediate habitat surrounding anemone groups. Group sizes ranged from 1 to 6 individuals at the start of monitoring.

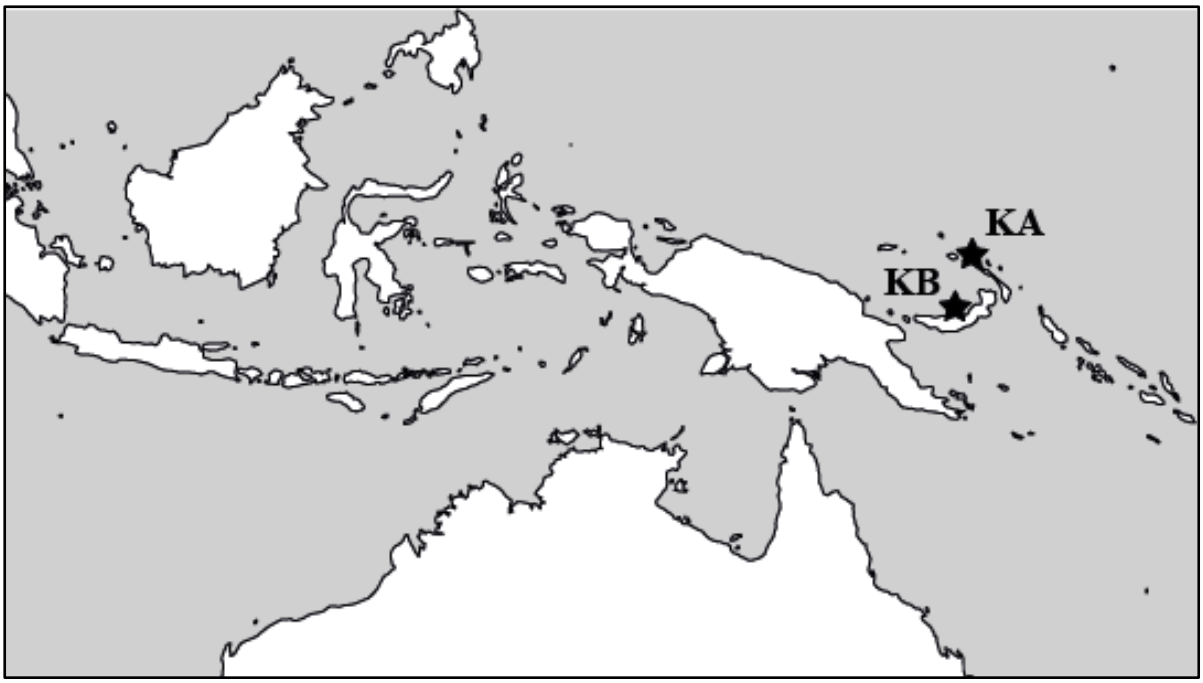

Figure 5.1. Map indicating sampling sites (black stars). Two separate sampling components took place; including (1) a monitoring study in Kimbe Bay (KB; n = 17, n = 39 and n = 58, individuals respectively) and (2) an experimental manipulation of groups in Kavieng (KA; n = 19, n = 14 and n = 28, individuals respectively), Papua New Guinea, of *A. chrysopterus,* hybrid *A. leucokranos* and *A. sandaracinos.*

The presence or absence of hybrids (n = 22 and n = 3, respectively) and group status as mixed or pure (n = 23 and n = 2, respectively), were compared to test for an effect of hybrids on survivorship, eviction and recruitment in Kimbe Bay, PNG, over time. For the purposes of this study, survivorship was recorded as the proportion of tagged individuals remaining from previous sampling year; recruitment is the proportion of new individuals in group since previous sampling year; eviction is the proportion of individuals which no longer remain within the group since previous sampling year. For the purposes of this study, the term eviction represents the disappearance of any individuals either due to eviction or natural mortality, as they cannot be distinguished. The effects of these explanatory variables were statistically compared in S-Plus 8.0 (TIBCO Software Inc, 2010). Observational study data was visualized using diagnostic histograms, boxplots, and QQ plots. To test for normality, a Shapiro-Wilks test was also applied. Data for survivorship, eviction, and recruitment were highly skewed and not normally distributed, and the variances between explanatory variable groups were not equal. Therefore, it was not appropriate to

use a two-sample t-test, which assumes a normal distribution of the data and homogeneity of variances. Data transformations including arcsine (as data are proportions), reciprocal (for eviction and recruitment data which were right skewed), square and antilog (for survivorship data which was left skewed) were unsuccessful, where data normality and homoscedasticity did not improve.

Two non-parametric alternatives, the Kolmogorov-Smirnov (KS) and Kruskal-Wallis rank sum (KW) tests, were ultimately applied to compare group demographics of survivorship, eviction and recruitment when hybrids were either present or absent, and when groups were either mixed or pure. The Kolmogorov-Smirnov test was used to test for more general differences in the overall shape and position of the distribution (i.e. median, skewness, dispersion), while allowing for non-normality and unequal variance. The Kruskal-Wallis rank sum test was employed to compare means between explanatory variable groups, assuming measurements in each group are a random sample and the distribution shapes are similar. Mean survivorship, eviction and recruitment were also graphically compared based on hybrid presence or absence and group status (+/- SEM) using GraphPad Prism 7.01 (GraphPad Software).

To assess whether hybrids may be disadvantaged or advantaged within mixed anemonefish group queues, growth rates of *A. chrysopterus*, hybrid, and *A. sandaracinos* individuals were compared over a 2-year period (in 2011, 2012, and 2013). Growth rate was estimated using the final total length divided by the initial total length. Change in rank was inferred from start rank and final rank of individuals within mixed groups. A one-way analysis of variance (ANOVA) was employed in S-Plus 8.0 (TIBCO Software Inc, 2010) to determine whether growth rates significantly differ between species at each starting rank within mixed groups. It was assumed that rank within group predicts the order in which individuals inherit breeding positions; relative size infers rank within group; lower ranks have higher growth rates and higher mortality; group size is dependent on host anemone size; and growth rates within groups are not independent as they are relative to the size of other individuals in the hierarchy. Additionally, to assess whether hybrids influence overall growth rate of mixed groups, a comparison of groups where hybrids were either present or absent was carried out using a non-parametric Wilcoxon rank-sum test, as data were not normally distributed,

but were skewed to the right and had similar ranges. The Wilcoxon rank-sum test assumes the shape of the distribution of data for both groups within the explanatory variable are similar (i.e. skewness and variances), and tests for differences between the medians of these explanatory variable groups. Mean growth rate (+/- SEM) of groups either having hybrids present or absent was visualised using GraphPad Prism 7.01 (GraphPad Software).

Total length of *A. chrysopterus* individuals (disregarding the variant *A. chrysopterus*) in adult female and male pairs were also compared among mixed and conspecific groups in Kimbe Bay, PNG, using an ANOVA in RStudio v0.99.902 (RStudio Team, 2015, R Development Core Team, 2016) following the aov function; where data met all assumptions of test. The interaction term was omitted from the model due to no significant interaction between the explanatory variables found. Relative total length was visually compared using GraphPad Prism 7.01 (GraphPad Software).

aov (response variable ~ explanatory variable + explanatory variable, data = matrix)

Removal experiment

The experimental component of this study was conducted near Kavieng, Papua New Guinea (02.38'S, 150.38'E; Figure 5.1) during November and December 2014. For a period of 3 days, five island reef sites were searched for groups of pure and mixed study species. Of 66 candidate groups located, 24 groups from four island reef sites, including Nago, Raal, Lemus and Nasaum, were selected for inclusion in the experiment. Criteria for group selection included group size (>4 individuals per group), species identity of individuals within groups, size of individuals within groups, anemone depth (1-15m) and relative location of groups. Relative location of groups was an important consideration logistically in order to sample 24 groups every second day for two weeks on SCUBA; preference was given to groups on similar islands for efficient use of resources and time, as well as safety of SCUBA divers.

To determine whether there is a reduced potential to mate when mixed species breeding pairs form, courting behaviours of females and sub-adult individuals advancing to male rank within groups were quantified following the removal of the original male in selected groups. Firstly, total length (to the nearest mm) of all individuals within each group was recorded. Male individuals were then removed from group queues to initiate sub-adult to male transition and rank advancement. All remaining individuals were elastomere tagged with unique colour and tag placement identifiers near the dorsal fin. Groups were given an acclimation period of 5 days post manipulation, where fish were tagged and males removed, before behavioural monitoring commenced as initial observations are thought to be tainted by the disturbance of manipulation. Groups were monitored from day 6 to 12 post manipulation, every second day for 10-minute observation periods. Group status varied, including pure groups of *A. chrysopterus*, *A. sandaracinos*, and hybrids (n = 4, n = 7 and n = 2 respectively), as well as mixed groups that naturally occur. These naturally occurring mixed groups included *A. chrysopterus* & *A. sandaracinos* (n = 3), *A. chrysopterus* & hybrid (n = 4), and hybrid & *A. sandaracinos* pairs (n = 4; species in female rank first, followed by species in sub-adult rank). Courting behaviours included (1) aggression, whereby female or sub-adult chases or charges at other individuals within group, displacing them from previous position on anemone and asserting dominance over the individual, (2) female and largest sub-adult swimming towards each other, turning to pause and touch sides, (3) shivering by female or sub-adult, whereby one approaches the other and shakes whole body in display of submission or affiliation and (4) nest preparation by sub-adult, which is defined as biting at substrate near anemone (total bites and total bouts of biting behaviour over a 10 minute period).

To assess how differences in group type and mixed or pure species status of groups might affect the frequency of courtship behaviours, mean frequency of courtship behaviours were plotted against explanatory variables. Log-linear models were applied to compare the response variables (behavioural counts) with explanatory variables (group type or status) over observation time; see Figure S5.1 for R-script). Standard Generalized Linear Models (i.e. regression and ANOVA) were not used here because they assume data to be linear and residuals to be normally distributed. Count

data rarely meet these assumptions due to counts not being continuous. Additionally, residual variances of count data generally increase with the mean, and are consequently not homogenous. Assumptions of all models were assessed using a residual diagnostic plot of residuals against predicted values, and model selection was based on residual deviance in relation to residual degrees of freedom, as well as AIC values (Table 5.1), where the model with the lower AIC value is a better fit to the data. Models were run using the MASS library in RStudio v0.99.902 (RStudio Team, 2015, R Development Core Team, 2016) rather than S-Plus which does not have the capability to execute the models required. Initially, a Poisson Regression (PR) model was fitted to data using the glm function (Chambers & Hastie, 1992). PR is appropriate for modelling count data as it uses a link function to apply a logarithmic transform to the response variable and specifies the Poisson distribution of residuals, where the variance is equal to the mean.

Table 5.1. Model selection for modelling count data based on AIC values compared between Poisson Regression (PR) and Negative Binomial Distribution Regression (NBR). Model chosen is in bold font. * indicates interaction term included in model, and + when non-significant interaction term omitted from model.

Response Variable	Explanatory Variables	PR	NBR
Female aggression	group.type + observation	135.42	**130.16**
	status + observation	134.16	**129.51**
Sub-adult aggression	group.type * observation	111.58	**105.01**
	status * observation	102.38	**102.11**
Female shivering	group.type + observation	180.09	**170.60**
	status + observation	180.42	**171.77**
Sub-adult shivering	group.type + observation	182.92	**165.92**
	status + observation	173.05	**160.10**
Swimming together	group.type + observation	**227.02**	-
	status + observation	**226.45**	-
Total bites	group.type + observation	614.12	**561.27**
	status + observation	824.42	**239.31**
Total bouts of biting	group.type + observation	234.69	**171.38**
	status + observation	288.71	**178.60**

*glm (response variable ~ explanatory variable * explanatory variable, data = matrix, family = Poisson (link="log"))*

In cases where the residual distribution increased more rapidly than the mean (i.e. residuals over-dispersed), a Negative Binomial Regression (NBR) was subsequently fitted to data using the glm.nb function (Venables and Ripley, 2002). The NBR allows the residual variance to increase more rapidly than the mean through including an additional parameter, theta, estimated by the model.

*glm.nb (response variable ~ explanatory variable * explanatory variable, data = matrix)*

Models were refitted to the data matrix with the interaction term omitted when no significant interaction was detected. In cases where the response variable could be predicted from an explanatory variable, the coefficient estimate was exponentiated to investigate the multiplicative effect of the explanatory variable on the response variable.

5.4 Results

Queue advancement

Of a total 84 fish from 25 groups at the onset of monitoring in 2011, 79 fish from 25 groups remained at the end of the monitoring study. Average group size remained similar throughout the monitoring period, with an average of 3.36 in the beginning compared to 3.16 at the end of the study. A total of 48 original fish (57.14%) survived the three-year period, 36 individual fish (42.86%) were evicted and 31 individual fish (39.24%) were recruited to the study groups.

Means for survivorship, eviction and recruitment to study anemonefish groups were visually compared when hybrids were either present in or absent from the group (Figure 5.2), and between mixed and pure groups (Figure 5.3). Survivorship and recruitment did not appear to differ between groups, regardless of hybrids being present or absent or groups being mixed or pure. Groups with hybrids and those of mixed species status did appear to have more evictions over the

three-year monitoring period (Figure 5.2C-D, Figure 5.3C-D). However, these results must be taken with caution, due to relatively small sample sizes for pure groups and for groups lacking hybrids.

Non-parametric tests revealed that median survivorship and overall shape of the survivorship distribution (KS: ks = 0.288, p = 0.687), and individuals surviving (KW: $\chi^2 = 1.176$, *df* = 1, p = 0.278) did not differ significantly between groups, regardless of whether hybrids were present or absent. Similarly, median survivorship and overall shape of data distribution (KS: ks = 0.457, p = 0.333), and individuals surviving (KW: $\chi^2 = 2.819$, *df* = 1, p = 0.093) did not differ significantly regardless of whether groups were mixed or pure.

Median eviction and the overall shape of eviction distributions (KS: ks = 0.288, p = 0.687), and incidence of eviction (KW: $\chi^2 = 1.176$, *df* = 1, p = 0.278) did not differ significantly between groups with respect to hybrid presence or absence. Mixed and pure groups did not differ significantly in median eviction, overall distribution shape (KS: ks = 0.457, p = 0.333) or incidence of eviction (KW: $\chi^2 = 2.819$, *df* = 1, p = 0.333).

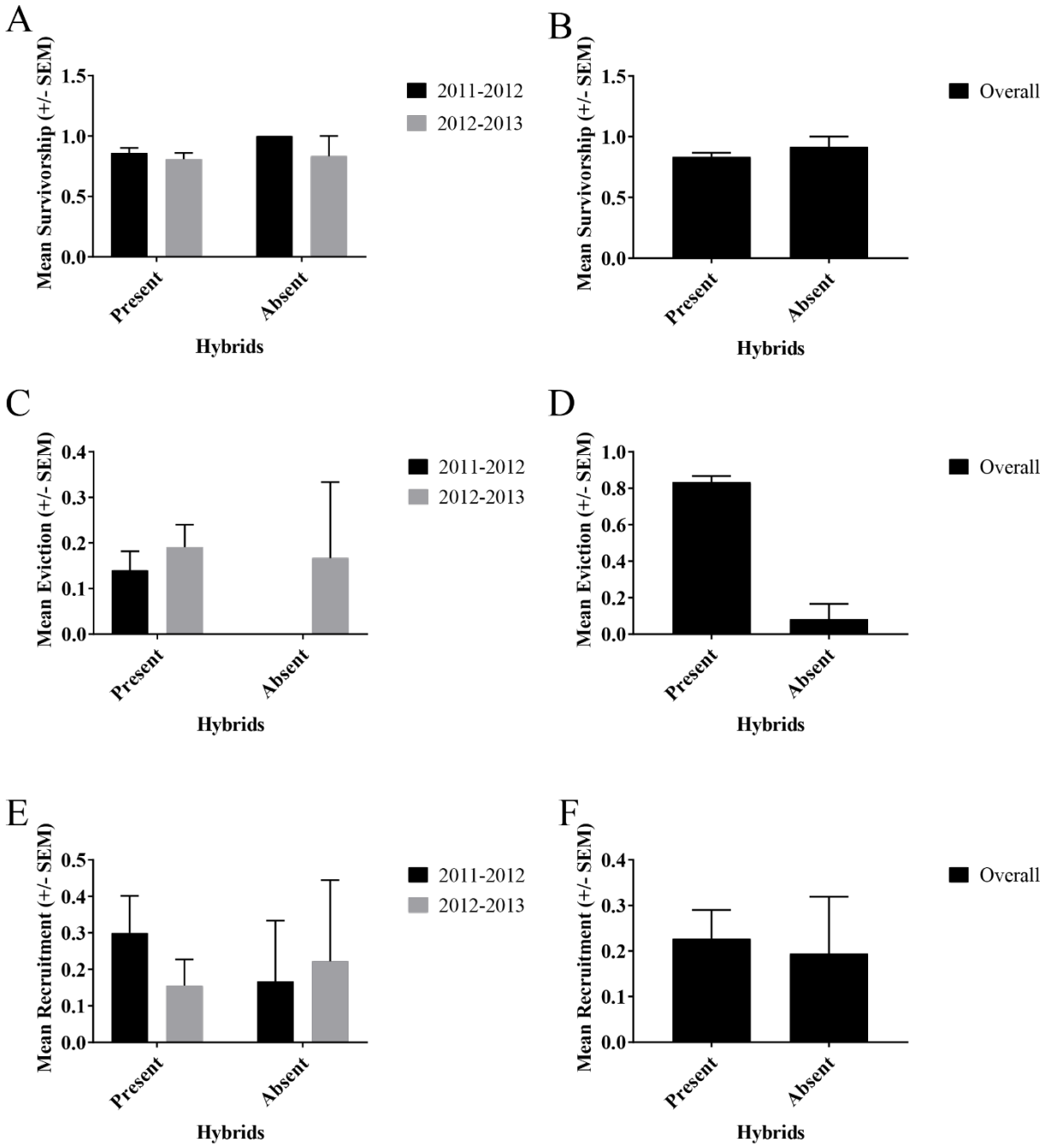

Figure 5.2 Mean (+/- SEM) survivorship (A-B), eviction (C-D) and recruitment (E-F) within focal anemonefish groups in Kimbe Bay, PNG when hybrids are either present or absent (n = 22 and n = 3, each year respectively) during 2011 to 2012, 2012 to 2013, and overall (n = 44 and n = 6).

Median recruitment, overall recruitment distribution (KS: ks = 0.129, p = 0.999) and recruitment to groups (KW: χ^2 = 0.003, *df* = 1, p = 0.986) did not differ significantly based on whether hybrids were present or absent in groups monitored. Similarly, median recruitment and

overall shape of recruitment distribution (KS: ks = 0.163, p = 0.999) or number of individuals recruiting (KW: χ^2 = 1.0.108, *df* = 1, p = 0.743) did not differ significantly between mixed and pure groups.

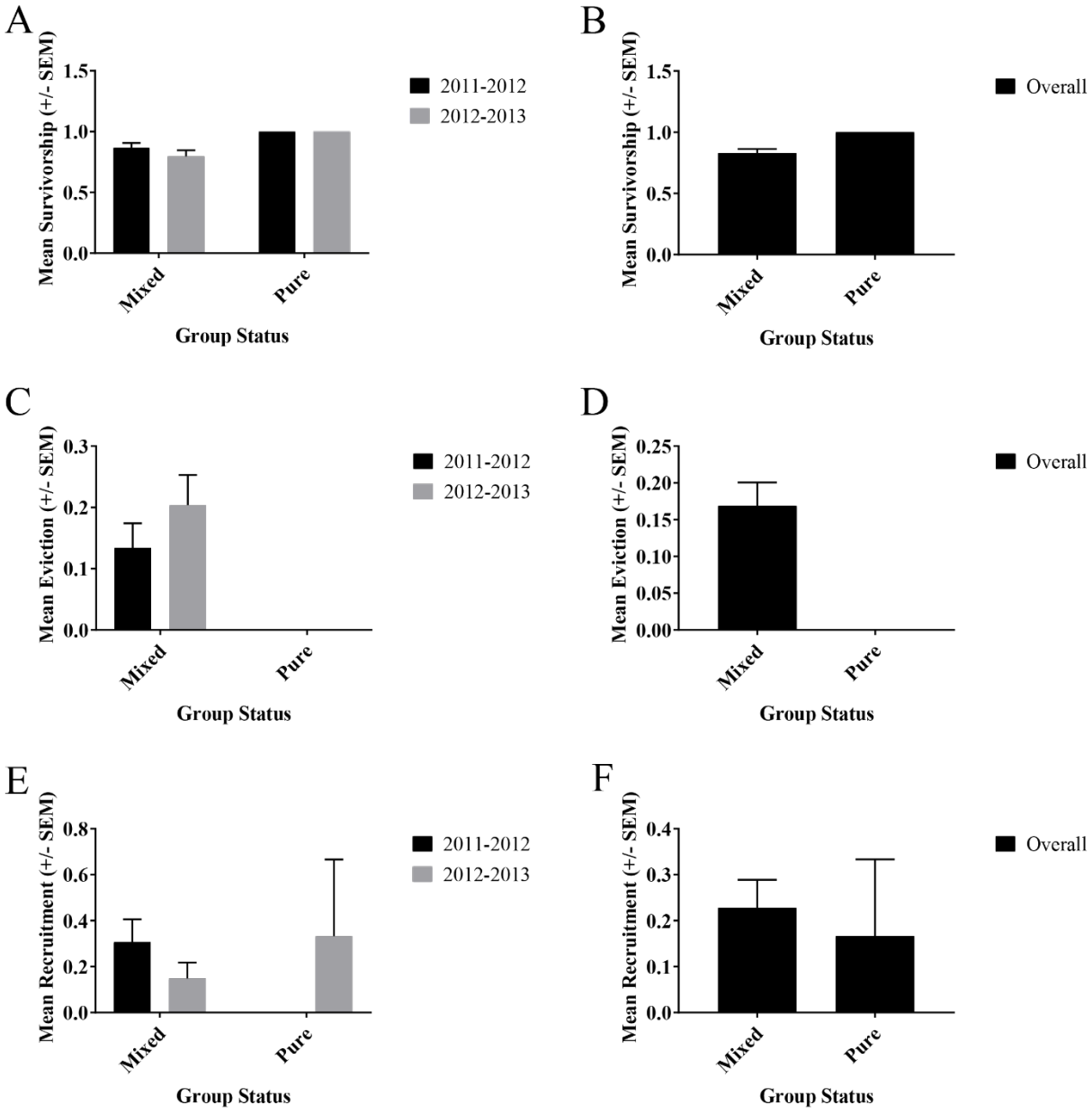

Figure 5.3 Mean (+/- SEM) survivorship (A-B), eviction (C-D), and recruitment (E-F) within focal anemonefish groups in Kimbe Bay, PNG, which are mixed and pure (n = 23 and n = 2, each year respectively) during 2011 to 2012, 2012 to 2013, and overall (n = 46 and n = 4).

Within mixed groups, only a positive change in rank was detected over a period of two years (2011-2013). Growth rate of pure and hybrid taxa within mixed groups differed significantly at each rank (1-way ANOVA: $F_{3, 72} = 7.168$, $p = 0.0003$; Figure 5.4), indicating a significant difference in change of rank between species. Growth rate in mixed groups significantly depended on starting rank; however, species, status as hybrid or pure, and mixed or pure group type did not affect growth rate significantly. Overall, the smaller, lower ranked individuals grew at an overall faster rate than the larger and higher ranked individuals. Hybrids appear to positively change rank faster than the pure parent species *A. sandaracinos,* although this did not prove statistically significant. Furthermore, hybrids are more variable in growth rate at any one rank than other species (Figure 5.4). No linear fit applied to *A. chrysopterus* individuals as they always occupied the most dominant ranking position (female) within all groups that they were members of. In the presence of hybrids, growth rate (+/- SEM) for all individuals in a mixed group was significantly higher than when hybrids were absent from mixed and species-specific groups (WRS: $Z = -2.598$, $p = 0.009$; Figure 5.5).

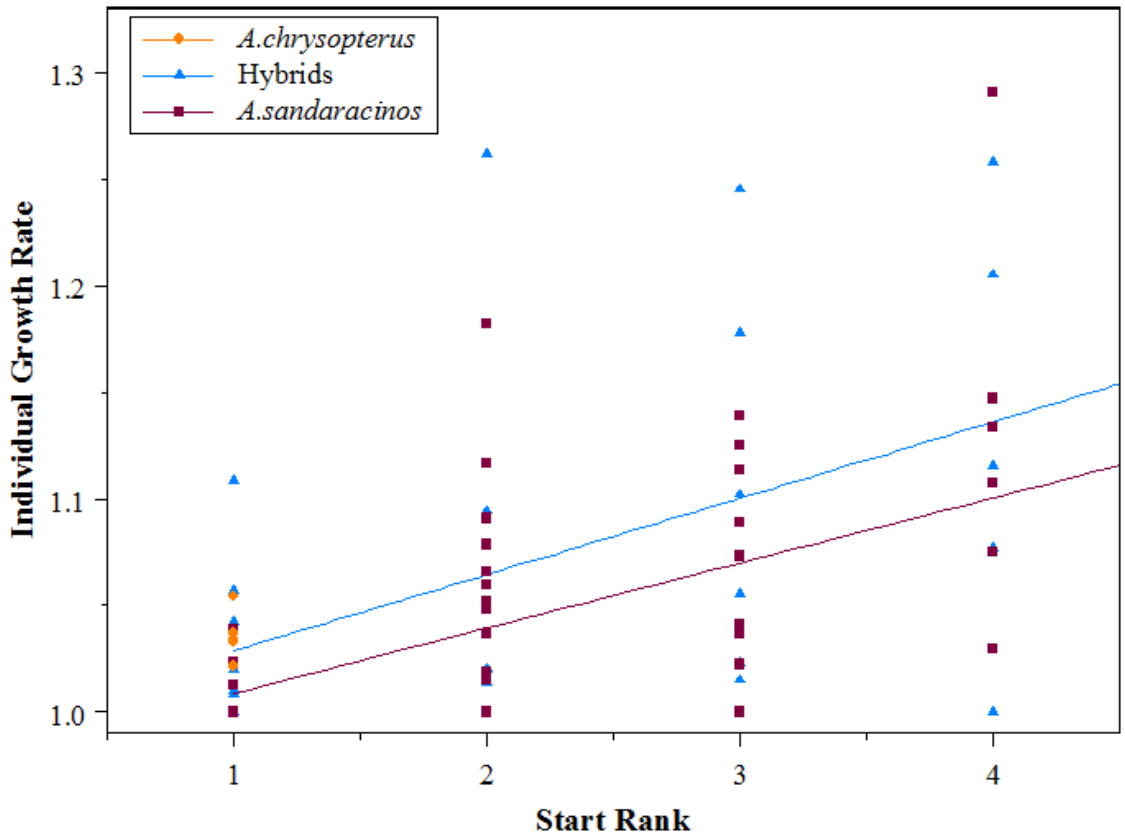

Figure 5.4. Growth rate of *A. chrysopterus,* hybrids, and *A. sandaracinos* compared across starting rank within mixed anemonefish groups in Kimbe Bay, PNG (n = 25). Starting ranks are as follows: 1 = Female, 2 = Male, 3 = Sub-adult, and 4 = Recruit. Linear fit applied where appropriate to taxa data points, including hybrids and *A. sandaracinos*. Note: *A. chrysopterus* was only recorded in most dominant female rank within groups, and as such no linear fit is applied.

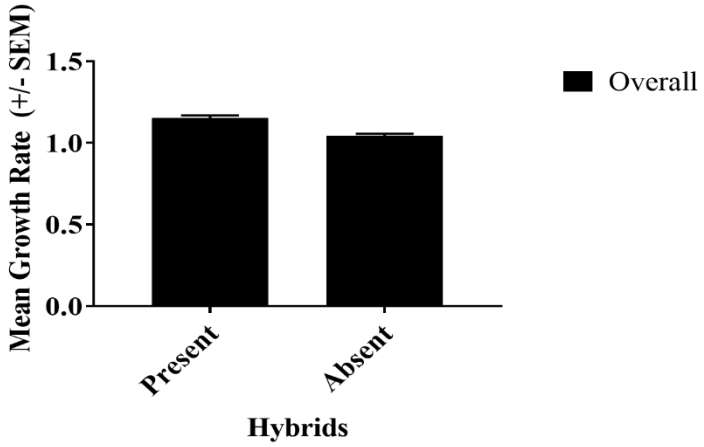

Figure 5.5 Overall mean growth rate (+/- SEM) for individuals within mixed anemonefish groups in Kimbe Bay, PNG; when hybrids were present in or absent from the group (n = 68 and n = 8).

Female *A. chrysopterus* were found to be significantly larger than male *A. chrysopterus* (2-way ANOVA: $F_{1, 26} = 6.467$, p = 0.017) as expected based on known anemonefish hierarchical size structure. *A. chrysopterus* individuals in groups of mixed and pure species status showed no significant difference in mean total length (2-way ANOVA: $F_{1, 26} = 0.064$, p = 0.802) indicating that female and male *A. chrysopterus* in mixed groups (with smaller heterospecific individuals) maintain a similar size to those queuing with conspecifics.

Courting behaviour

Observations of courtship behaviour were restricted to the two largest individuals following removal of the male (second rank holder) from the group; the mature female held the most dominant rank within all groups examined (n = 24). All groups displayed courting behaviours following male removal, however differences in both frequency and timing of behaviours were observed. Time following male removal and mixed or pure group status were the most important predictors of courting behaviours in this study.

The change in sub-adult aggression over time differed between group type ($NBR_{35, 36}$; $df = 5$, p=0.004; Figure 5.6); where in some groups, aggression tended to decline, while in others it increased. Pure or mixed species group status was also predictive of change in sub-adult aggression over time; where aggression of sub-adults declined significantly in pure groups, but aggression tended to increase in mixed groups, following male removal ($NBR_{41, 44}$; $z = -2.545$, $df = 1$, $p = 0.011$; Figure 5.6). When a group included pure species only, sub-adults displayed aggressive behaviour 40.55% less over time. The inverse of this interaction was found in mixed species groups. Aggression of female individuals increased significantly over time following male removal, regardless of group type ($p = 0.003$), where females were observed to be 46.6% more aggressive at 12 days post removal, compared to day 6 post removal ($NBR_{44, 41}$; $z = 2.841$, $df = 1$, $p = 0.005$; Figure 5.7). This increase in aggression was observed regardless of mixed group status ($p = 0.005$),

where females were 46.22% more aggressive at 12 days post removal, compared to 6 days post removal ($NBR_{46, 45}$; $z = 2.719$, $df = 1$, $p = 0.007$; Figure 5.7).

Frequency of female shivering behaviour was not significantly different depending on group status ($p = 0.36$); however, across all groups, female shivering behaviour significantly increased approximately 29.2% between 6 and 12 days post male removal ($NBR_{52, 45}$; $z = 2.463$, $df = 1$, $p = 0.014$; Figure 5.6). Shivering behaviour of sub-adults was not significantly different between mixed or pure status groups, nor was it different over time post male removal ($NBR_{51, 45}$; $z = 1.19$, $df = 1$, $p = 0.24$ and $z = 0.80$, $df = 1$, $p = 0.42$ respectively; Figure 5.6). There was no evidence for a relationship between sub-adult shivering behaviour and group status.

Swimming together courting behaviour of females and the dominant sub-adult was displayed 26.9% more often in pure groups compared to mixed groups following male removal ($PR_{41, 45}$; $z = 2.154$, $df = 1$, $p = 0.031$), regardless of the time since male removal ($z = 1.192$, $df = 1$, $p = 0.233$; Figure 5.6). Frequency of males biting the substrate surrounding anemone groups, which displays nest preparation behaviour, decreased by 61.94% over time for both pure and mixed species groups ($NBR_{40, 45}$; $z = -2.101$, $df = 1$, $p = 0.036$; Figure 5.6; Figure 5.7). Likewise, total bouts of biting behaviour during nest preparation behaviour were displayed by males 68.88% less often 12 days post male removal from groups compared to 6 days post removal ($NBR_{41, 45}$; $z = 1.989$, $df = 1$, $p = 0.047$), regardless of group status. Eggs were not found at any groups during this experiment.

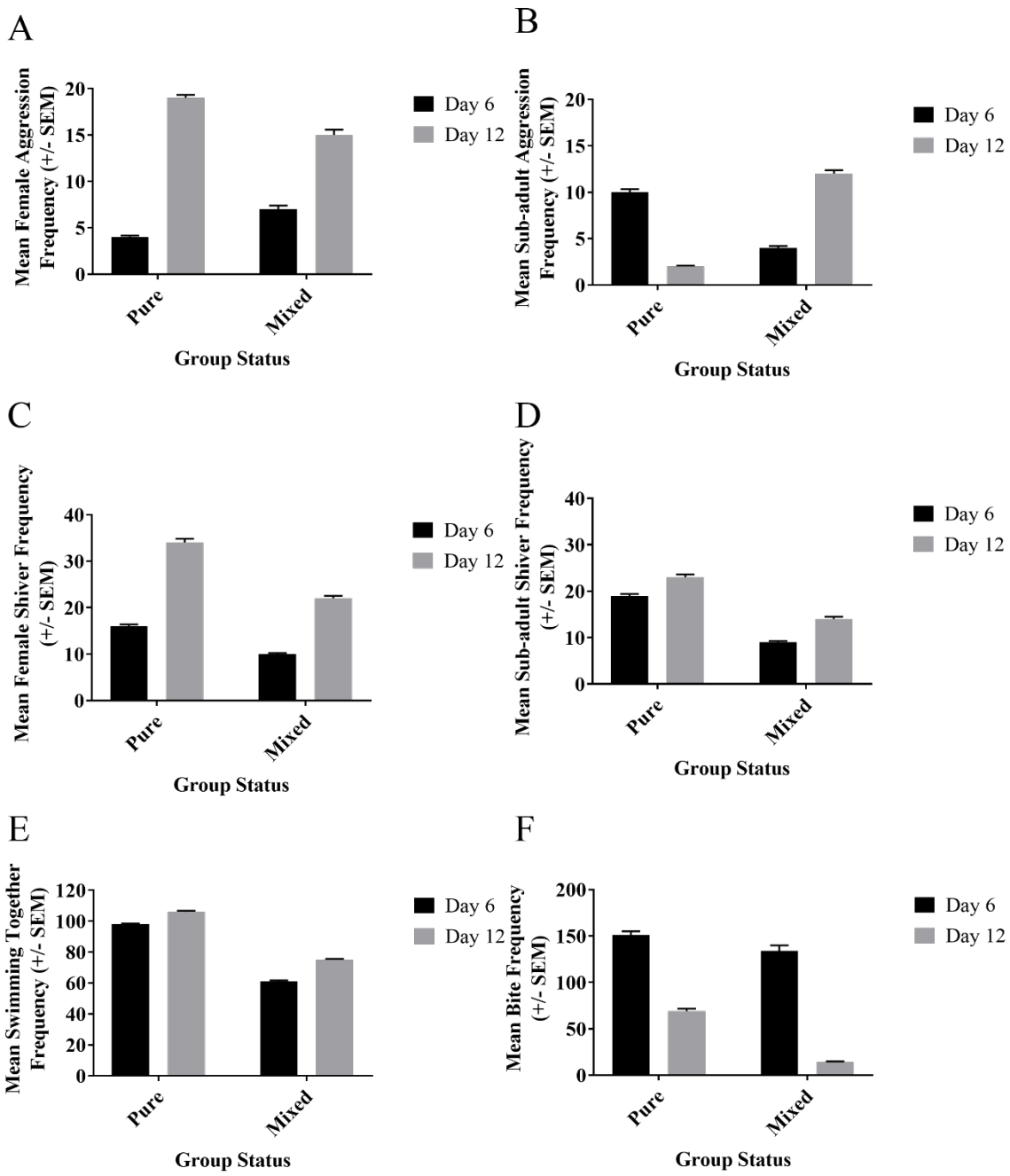

Figure 5.6 Comparison of mean courting behaviour frequency (+/- SEM) at day 6 and 12 following male removal from pure and mixed species groups (n = 13 and n = 11, respectively); including female and sub-adult aggression (A-B), female and sub-adult shivering (C-D), swimming together (E), and nest preparation behaviour (F).

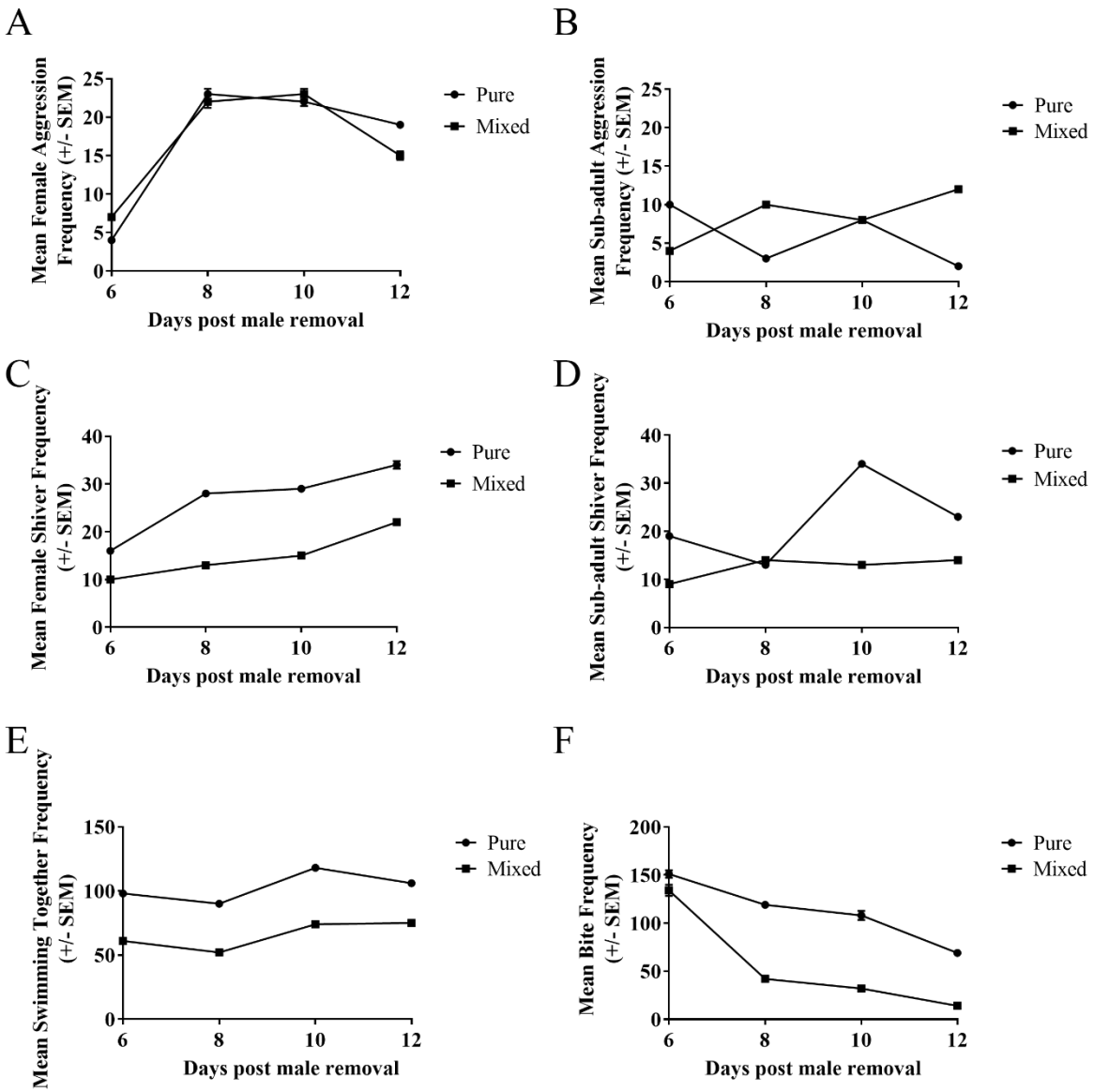

Figure 5.7 Mean courting behaviour frequency (+/- SEM) over time, at days 6 through 12, post male removal from pure and mixed species groups (n = 13 and n = 11, respectively); including female and sub-adult aggression (A-B), female and sub-adult shivering (C-D), swimming together (E), and nest preparation behaviour (F). Note: SEMs are limited and therefore may not be obvious in graphic.

5.5 Discussion

Hybrids are often assumed as inferior to pure species, based on classical hybrid theory and due to a paucity of empirical studies. This study provides a direct comparison of anemonefish hybrids with pure parent species within a size based dominance hierarchy and has tested assumptions of hybrid inferiority when compared with pure species. The strict dominance hierarchy of anemonefish provides a framework in which to test how hybrids fare compared to parent species within a queue for breeding positions. Results of this investigation into the *A. leucokranos* hybrid system, in which hybrid maximum size is intermediate to parent species size, have revealed a more complicated story suggesting hybrids are not always inferior to their pure species counterparts.

Hybrid inferiority complex

Based on the classic view of hybrids, it might be expected that hybrids would be disadvantaged when queuing with pure parent species. In the *A. leucokranos* system, however, hybrids are both capable of reproducing with pure parent species and other hybrids, as well as viable, where secondary and subsequent generations produce gametes and reproduce with pure parent species and other hybrids (Chapter 3, Chapter 4). Furthermore, the maximum size of hybrids is larger than the smaller of the two parent species, which suggests a potential size advantage among taxa in a mating system based on size dominance (Chapter 3).

In this study, hybrids were found to positively change rank faster and occupy dominant ranking positions more often than the smaller parent species, *A. sandaracinos,* within mixed group queues. Growth rate in mixed groups was dependent on the starting rank of individuals and was not affected by species identity, status as pure or hybrid, or mixed group type; where overall, the smaller, lower ranked individuals grew at a faster rate. However, when hybrids were present in mixed groups the growth rate of all individuals was significantly greater while hybrid growth rates appeared to be most variable at any one rank compared to pure species. Based on previous research across the *A. leucokranos* hybrid zone, it is not surprising that the bigger (i.e. dominant) fish always

gets first choice of a mate, and as such, larger individuals, including hybrids, can hold a size advantage in mixed species queues, not afforded in conspecific queues. Ultimately, it appears that pairing with conspecifics facilitates a smoother transition for sub-adults progressing up the queue, including for hybrids. Results also indicated that when dominance is associated with a predetermined factor such as size, hybrids apparently have an innate advantage. Intermediately sized hybrids appear to change rank faster within mixed groups and may have a fitness advantage when present in mixed groups comprising hybrids and the smaller parent species, *A. sandaracinos,* by essentially jumping the queue.

In this way, moderately sized hybrids and small *A. sandaracinos* are disadvantaged against the more dominant (largest) species, and must continue to queue in the hope of reproducing, rather than facing eviction and becoming vulnerable to mortality outside the group (Buston, 2004, Wong et al., 2008). However, individuals may benefit from remaining in queues for reproductive positions, rather than recruiting to another group (Wong et al., 2007, Mitchell, 2005), and the experience of individuals in a group may compensate for size disadvantages in fish social hierarchies and potentially overcome them (Alcazar et al., 2014). It appears that hybrids may prefer to mate with other hybrids, if available, but due to hybrid rarity, they often cohabit with the relatively smaller species, *A. sandaracinos,* providing hybrids with an inherent fitness advantage over the smaller, sub-dominant parent species in mixed group queues. Accordingly, hybrids may increase in abundance over time, at least within the hybrid zone and possibly beyond, as was documented in other hybridizing marine fishes, including pair forming butterflyfishes from the same region as the presently studied hybrid zone (McMillan et al., 1999) and another pair of anemonefish species from elsewhere, based on genetic data alone (van der Meer et al., 2012).

Jumping the queue?

Unexpected benefits of hybridization to the larger parent species, *A. chrysopterus,* were observed in mixed groups. The queue system was skipped altogether by adult *A. chrysopterus,* through movement into established mixed groups, evident through an absence of *A. chrysopterus*

recruits which appear to avoid recruitment to mixed groups, and adults consistently found in the highest ranked female position in mixed groups of *A. chrysopterus* and *A. leucokranos*. Although there was no significant difference in size (based on total length) between adult ranking individuals found in mixed and pure groups, findings suggest that *A. chrysopterus* individuals in mixed groups benefit compared to those queuing with larger conspecifics, where they do not hold an inherent size advantage over the conspecific individuals they are competing with for the highest ranks.

These findings are not consistent with known anemonefish behaviour (Buston, 2004, Fautin, 1992, Fautin, 1991, Hattori, 1991, Hirose, 1995) but provide an example of an ecological and behavioural consequence of hybridization directly impacting hybridizing species, at the scale of the shared anemone resource, where abundance disparities and relative size differences between parent taxa provide an opportunity to the larger species in mixed groups. I hypothesise that subdominant individuals of the larger species, *A. chrysopterus,* may 'jump the queue' and become the dominant female in mixed groups more effectively due to an inherent size advantage when in mixed groups facilitated by the size-based dominance hierarchy. Could it be that the mechanism underlying original hybridization between these taxa is derived from this 'queue jumping' opportunistic behaviour by the larger *A. chrysopterus* in the context of a highly abundant species with a relative size advantage to reproductive fitness? To further understand this opportunistic behaviour with more certainty, future manipulative studies should include a more balanced design, with increased numbers of all group types, but particularly pure parent species groups for comparison.

Conclusions

Hybrid theory suggests that hybridization should be selected against through reinforcement of reproductive barriers to maintain species delineation. Here, assumptions of hybrid inferiority within the *A. leucokranos* hybrid system, based on mixed groups of hybridizing taxa, were tested. It was expected that mixed groups, particularly those including hybrids, would court, display nest preparation, and reproduce later than pure groups. Instead, hybrid inferiority was not found. Hybrids

were more fit within the ecological context of group queues than the smaller parent species, *A. sandaracinos,* due to their inherent dominant size advantage. Hybrids positively changed rank faster and occupied dominant ranking positions more often than the smaller parent species, and hybrid only pairs displayed courting behaviours as soon and frequently as pure groups studied. Furthermore, unusual (and unexpected) 'queue jumping' by the larger *A. chrysopterus* indicates that abundance disparities and relative size differences between species have provided a fitness opportunity for one hybridizing taxon, and may identify the mechanism – queue jumping - underlying original hybridization between these taxa. This novel *in situ* study provides a direct comparison of hybrids with pure parent species and highlights the importance of empirically investigating evolutionary questions in the context of ecology and behaviour in natural systems.

CHAPTER 6: General discussion

Hybridization drives major and rapid evolution of species in nature, challenging established phylogenies, theoretical concepts of what constitutes a species and questions how to best implement conservation of biodiversity and evolutionary novelty. The study of natural hybrid zones is key to understanding patterns of variation among species and the consequences of hybridization to evolution. In this thesis, I addressed how ecology and behaviour contribute to maintenance and persistence of the *Amphiprion leucokranos* hybrid zone. This research employed a combination of ecological, phylogenetic and population genetic assays, as well as an observational study and behavioural experiment to test key concepts of the importance of hybridization to evolution of species.

In Chapter 2, a suite of microsatellite markers were developed and tested to facilitate investigation into the relatedness of taxa within the hybrid zone. This genetic toolkit, comprised of 42 novel and published markers, was used extensively to inform subsequent chapters of this thesis. Chapter 3 resolved the influence of ecology and behaviour on the outcomes of hybridization, specifically testing how habitat use and relative size differences of parent species and hybrids may drive patterns of gene exchange between hybridizing taxa. Results confirmed *A. leucokranos* is a hybrid of closely related *A. chrysopterus* and *A. sandaracinos,* and verified that behavioural isolation (i.e. anemonefish hierarchical behaviour), habitat use and species-specific size differences dictate the direction and degree of back-crossing and subsequent introgression. Chapter 4 elucidated the mechanisms driving and maintaining hybridization through investigating whether social and ecological factors facilitating hybridization varied across the hybrid zone, and if this influenced gene flow and introgression regionally. Findings revealed that the relative frequency and size disparities of parent species drive regional ecological patterns and gene flow among taxa, where species integrity is maintained despite extensive mixed species group cohabitation and back-crossing. Chapter 5 experimentally tested how hybrids directly compare to pure species when queuing within mixed groups for reproductive breeding positions. This chapter focused on understanding why species barriers do not break down in the face of persistent hybridization and

back-crossing. Chapter 5 demonstrated that hybrids are not always inferior to pure species, particularly when a predetermined factor such as maximum size influences dominance within a group. Hybrids positively changed rank faster and held dominant ranks more often than the smaller parent species, *A. sandaracinos,* indicating a fitness advantage to hybrids in the context of size-based hierarchical anemonefish breeding queues. The presence of hybrids lead to an increased growth rate overall, but no significant effects were identified pertaining to survivorship, eviction or recruitment in mixed groups. Ultimately, hybrid only groups displayed courting behaviours associated with reproduction just as early and frequently as pure parent species. This thesis represents an important contribution to our understanding of how this hybridization is persisting through time and what the evolutionary outcomes might be for the taxa involved. Overall, the thesis outcomes highlight the importance of ecology and behaviour to the consequences of hybridization, by driving patterns of gene flow and introgression across the hybrid zone.

Importance of behaviour and social system to outcomes of hybridization

Fundamental to elucidating hybridization events is understanding the way that ecological and behavioural factors contribute to hybridization outcomes for species and ecosystems (Montanari et al., 2016). Hybridization studies of marine fishes tend to be molecular-centric, with as few as 24% of studies incorporating quantitative information on ecology or behaviour in order to contextualise the hybridization event and forecast potential consequences (Montanari et al., 2016). This is in contrast to the study of freshwater fishes, where the importance of ecology is well documented, and adds habitat loss (or change), range expansion and limited spawning habitat as major factors facilitating hybridization (Scribner et al., 2001). In order to truly advance the understanding of adaptive evolutionary significance of hybridization and its importance in maintenance of reproductive isolation and speciation processes (Meier et al., 2017), the study of natural hybridization in marine fishes must incorporate ecological and behavioural data within hybrid zones. Most commonly, three factors are associated with readily hybridizing species; including (1) rarity of one or both parent species (Randall et al., 1977b, Frisch and van Herwerden, 2006, van Herwerden et al., 2006, Marie et al., 2007, Hobbs and Allen, 2014, Montanari et al., 2014), which

have (2) significant overlap in habitat or resource use (Marie et al., 2007, Montanari et al., 2012, Montanari et al., 2014, van Herwerden and Doherty, 2006, Yaakub et al., 2006), leading to (3) a breakdown in assortative mating between closely related species (McMillan and Palumbi, 1995, Mallet, 2005, Hubbs, 1955, Frisch and van Herwerden, 2006). Furthermore, the lack of conspecific partners has been reported to be a common factor contributing to hybridization, despite hybridizing coral reef fish having mating systems which are pair forming, haremic, and aggregate spawning.

Applying a multidisciplinary approach to address the overarching theme of this thesis was key to identifying the importance of hierarchical behaviour, species size differences and habitat use in driving the formation of the hybrid, *A. leucokranos,* and subsequent backcrossing with parent species. Here, important ecological and behavioural factors were identified, including regional differences in the relative frequency of parent species (Chapter 4), shared host use and ecological niche space (Chapter 3), and assortative mating breakdown among closely related species through high incidence of cohabitation (Chapter 3, Chapter 4), all of which were potentially driven by limited anemone host abundance. Hybridization appears to be facilitated by co-occurrence on mid-shore reefs within one region of hybrid zone (Chapter 3), and the characteristic size dominant behaviour of anemonefish where larger species remain dominant through inherent size advantage, dictates hybridization outcomes on both an evolutionary scale (Chapter 4) and within queues for breeding positions directly (Chapter 5). Furthermore, on an ecological scale at the level of the shared anemone resource, abundance disparities between parent taxa again appear to drive outcomes of hybridization for species, where it is hypothesised that subdominant individuals of the larger *A. chrysopterus* parent species 'jump the queue' to assume the dominant breeding position in mixed species groups due to its inherent size advantage within the size-based dominance hierarchy (Chapter 5).

Implications of hybridization for the loss of taxonomic status

Confirmation of hybrid status for the previously described *A. leucokranos* dictates subsequent loss of taxonomic status and associated protections throughout its range. Hybrids are currently not afforded substantiative protection and do not qualify for consideration for threatened

species listing. As legislation regarding hybrids remains unclear globally, protection of hybrid taxa poses complicated challenges for conservation (Haig and Allendorf, 2006, Garnett et al., 2011). The threat of hybridization negatively impacting on the integrity of distinct species lineages has driven the cautious approach to protection of hybrid taxa by policy makers (Mayr, 1963, Richards and Hobbs, 2015). In this way, a potential conservation opportunity is missed, where hybridization may introduce potentially novel genotypes with adaptive traits, promote speciation through introgression and increase genetic diversity of hybridizing taxa (Seehausen, 2004, Arnold, 1997, Montanari et al., 2014, van der Meer et al., 2012), as was reported for *A. sandaracinos* (Chapter 3; Gainsford et al., 2015). More broadly, with climate models predicting the collapse of coral reef ecosystems over the next 200 years (Hoegh-Guldberg et al., 2007), the protection of hybrids may act to safeguard against some loss of evolutionary novelty within these natural systems under environmental change through increased adaptive potential and advantageous fitness outcomes for species (Stebbins, 1959, Van Oppen and Gates, 2006, Willis et al., 2006, Lewontin and Birch, 1966).

The well documented example provided by the Caribbean *Acropora* system, where a once rare hybrid coral has proliferated, increasing in abundance and extending its known range (Willis et al., 2006, Fogarty, 2010, Fogarty, 2012), draws attention to the need for hybrid protection. Stony corals such as *Acropora* species are functionally important, as both a food source for corallivores and habitat structure provider for coral reef organisms. Protection of reef building corals, particularly in the face of coral reef degradation in regions like the Caribbean, is considered advantageous to biodiversity outcomes. Despite this, the hybrid, *Acropora prolifera,* has been explicitly excluded from IUCN Red List assessment for protection, not warranting threatened species listing under the US Endangered Species Act by NOAA, as a hybrid (Carpenter et al., 2008).

Conservation managers must consider evolutionary theory in order to effectively manage biodiversity challenges in a changing climate (Eizaguirre and Baltazar-Soares, 2014, Wayne and Shaffer, 2016). Policy guidelines (Haig and Allendorf, 2006). Decision and decision-free frameworks (Richards and Hobbs, 2015, Wayne and Shaffer, 2016) have been advocated for implementation prior to management of hybrid taxa; however, to date no changes have been

adopted. Overall, authors agree that approaching legislative solutions to adequately address hybridization scenarios must be done on a case-by-case basis, taking into account how hybridization was facilitated (via natural or anthropogenic means) and the potential consequences for the ecosystem (Allendorf et al., 2001, Wayne and Shaffer, 2016, Shafer et al., 2015, Richards and Hobbs, 2015), as is common practice in plant conservation science (Van Dyke, 2008).

Richards & Hobbs (2015) argue that disregarding hybrids in conservation efforts of coral reef systems may act to perpetuate policy status quo while ignoring real-world conservation needs. In this way, policy may miss an opportunity to enhance coral reef biodiversity through protecting important structure forming hybrid stony corals in the face of coral reef decline. Increased shifts in species distributions due to climate change are predicted, and may lead to greater incidence of natural hybridization and a greater need for legislative consideration of hybridization (Pauls et al., 2013, Moritz and Agudo, 2013). Introgressive hybridization has theoretically been found to provide a mechanism for species recovery following disturbance or due to environmental change, with models suggesting improved likelihood of successful introgression rather than extinction when species showed intermediate assortative mating and limited mating system promiscuity (Baskett and Gomulkiewicz, 2011). Similarly, other authors argue that interspecific gene flow may mediate extinction risk and the consequences of limited adaptive potential to change through enabling enhanced demographic recovery, thereby conserving evolutionary potential within a system (Hamilton and Miller, 2016, Carlson et al., 2014, Kremer et al., 2012).

For parent species, which appear to remain distinct in the face of ongoing back-crossing, benefits derived from the hybridization event studied here include enhanced genetic diversity of the smaller, *A. sandaracinos*, through introgression; where in Kimbe Bay genotypic diversity was 10-fold greater in *A. sandaracinos* (based on phenotype identification) than hybrids or larger *A. chrysopterus* (Chapter 3). When back-crossed hybrid individuals (based on genotypic identification) were removed from analysis, *A. sandaracinos* genotypic diversity was greatly reduced in comparison to pure *A. sandaracinos* populations found elsewhere. Finally, if reproductive isolation through assortative mating is achieved, or hybrids move to occupy a separate

niche, *A. leucokranos,* the hybrid taxon may ultimately differentiate from its parent species over time, and provide an example of hybridization adding to coral reef biodiversity. However, given the legislative uncertainty regarding hybrids, is the hybrid *A. leucokranos* at risk of overexploitation now following the loss of taxonomic protection as a 'true species'?

Vulnerability to overexploitation in the face of aquarium trade demand

Coral reef fish most at risk of species loss and decline share key biological characteristics including being small-bodied, tightly habitat associated and highly specialised (Munday, 2004, Munday and Jones, 1998); characteristics which also tend to be targeted by the aquarium trade. Globally, the aquarium trade industry has previously been valued at US$15 billion per year, and is known to trade in over 1400 species (Whittington and Chong, 2007, Donnelly, 2011), where the United States of America (US) represents the majority of the marine aquarium trade market (Wabnitz et al., 2003). The Banggai Cardinalfish, *Pterapogon kauderni,* provides an example of negative effects observed following exploitation by the aquarium trade (Kolm and Berglund, 2003), which have led to its listing as Endangered on the IUCN Red List of Threatened Species in 2007 (Allen and Donaldson, 2007). Due to its popularity and unfavourable biological traits including limited dispersal capability, endemicity and reduced reproductive rate, fishing pressure negatively affected both density and group size of this species; where in one region alone, upwards of 118,000 fish per month were sold during one study period (Lunn and Moreau, 2004).

Anemonefish are also popular among aquarium enthusiasts due to their small size, relative longevity, conspicuous colouration and patterns, limited dispersal and obligate association with sedentary anemone hosts (Roelofs, 2008, Shuman et al., 2005, Fautin and Allen, 1997); qualities that also increase their risk to overexploitation. Anemonefish have previously been suggested as indicator species for overexploitation on the Great Barrier Reef due to these traits (Ryan and Clarke, 2005), however one investigation into fished and unfished northern populations found little difference (Butler, 1991). The hybrid, *A. leucokranos*, has biological traits which make it inherently more susceptible to overexploitation; including endemicity, being small-bodied, highly specialised,

site attached, with limited post-recruitment ability and having striking variation in phenotypes throughout the hybrid zone (Chapters 3 and 4). In Papua New Guinea (PNG), the National Fisheries Authority (NFA) reports very limited export of *A. leucokranos* between 2008 (when commercial aquarium export began) and 2012; however unreported trade between local fisherman with tourists and collectors sometimes occurs opportunistically. Prior to 2008, dating back to PNG's Independence in 1975, NFA reports show no record of export, and similarly no record after 2012 due to fishery inactivity. Market price for *A. leucokranos* is approximately US$18.00 per fish; significantly greater than other anemonefish species from Melanasia, which range from US$1.00-$4.50 on average. At the retail end, *A. leucokranos* are sold at approximately US$150.00 to US$250.00 each. Comparatively, rare, naturally occurring phenotypes of *Amphiprion percula* and *Premnas biaculeatus* are known to trade at up to US$150.00 each. Similarly, in the Solomon Islands (SI) data is limited. In 2007, the trade of 69 *A. leucokranos* (at US$13.50 each on average) was reported, making up approximately 0.06% of the overall marine aquarium market reported that year (Kinch, 2008). Overall, global trade of *A. leucokranos* from the Solomon Islands has been estimated to have fallen from 106 individuals in 2004 to 7 individuals in 2011 (Source: Marine Aquarium Biodiversity Trade Flow). Of concern is the presumed unreported level of trade, particularly when considering the financial value of such trade in communities that may otherwise have limited opportunities for equally lucrative earnings.

Despite having characteristics that place higher risk of vulnerability on the hybrid taxa, *A. leucokranos* appears to have limited reported trade globally in comparison to other anemonefish species. Limitations on their trade are thought to include high retail cost for average aquarium trade consumers and the remoteness of their distribution throughout PNG and SI; where targeting export of the hybrid is not realistic given international and domestic flight paths and airports (Kinch, 2008). This has led to increased interest in closing the breeding cycle for parent species and producing hybrids in captivity, where success has been very limited and not yet economically viable (T. Millitz, pers. comm). Therefore, given current limitations to trade it is not likely that this hybrid will be overexploited by the aquarium trade. However, if accessibility to reefs is aided through

increased development and infrastructure connecting more remote locations to ports of trade like Port Moresby, PNG, the risk of vulnerability may increase.

Concluding remarks

Hybridization plays an evolutionarily significant role in driving rapid evolution of species in nature. Despite challenging established phylogenies and fundamental ideas of the purity of 'true species', hybridization may hold value in conserving biodiversity, particularly on coral reefs in global decline (Hoegh-Guldberg et al., 2007, Eizaguirre and Baltazar-Soares, 2014, Wayne and Shaffer, 2016). As such, conservation management must consider evolutionary theory and legislate for the protection of hybrid taxa on a case-by-case basis when appropriate in order to effectively manage biodiversity challenges in a changing climate. In this thesis, behaviour and ecology have been identified as key drivers in the evolutionary outcomes of the *A. leucokranos* hybridization event. This research employed a combination of ecological, phylogenetic and population genetic assays, as well as an observational study and a behavioural experiment to test key theories and assumptions of the importance of hybridization to the evolution of species. Gene flow between parent species was strongly influenced by differences in maximum size and regional relative abundance of parent taxa within this size-based dominance hierarchy, which defines anemonefish mating behaviour. Findings suggest the hybrid *A. leucokranos* may differentiate from pure parent taxa in time, emphasizing the importance of protection for hybrids that may contribute to the biodiversity of coral reef systems, particularly in the face of current coral reef declines and projected demise.

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APPENDIX A: Supplementary materials for Chapter 3

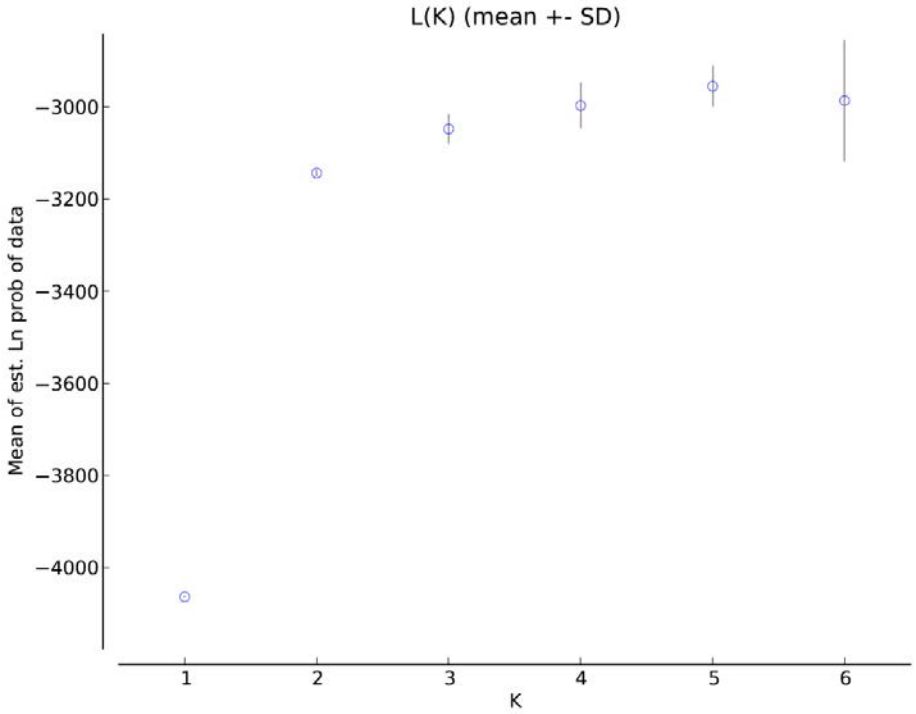

Figure S3.1 Mean log likelihood and standard deviation based on 8 microsatellite loci across Kimbe Bay populations

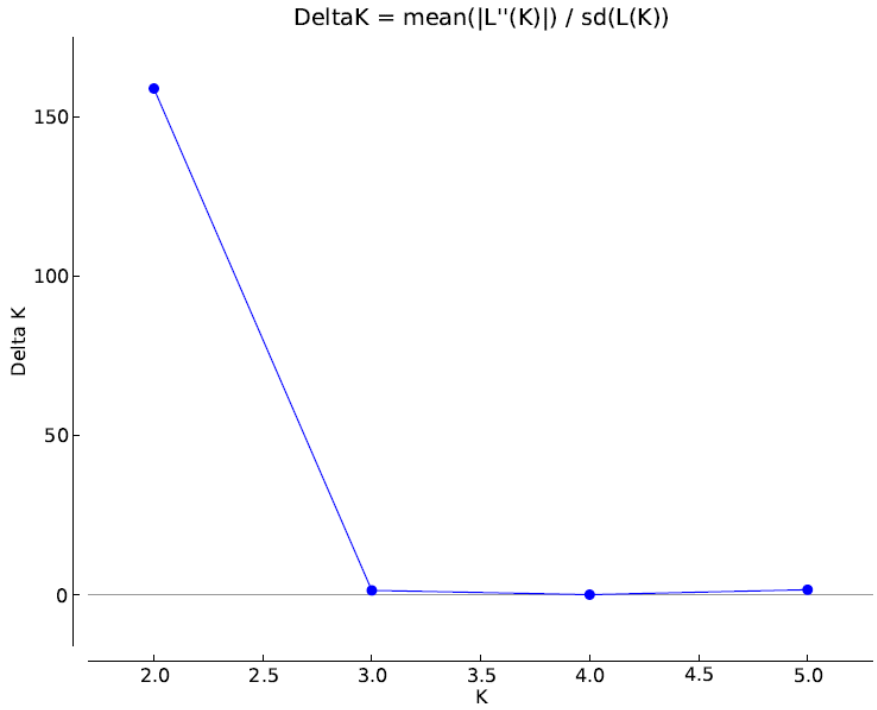

Figure S3.2 Delta K based on 8 microsatellite loci across Kimbe Bay populations

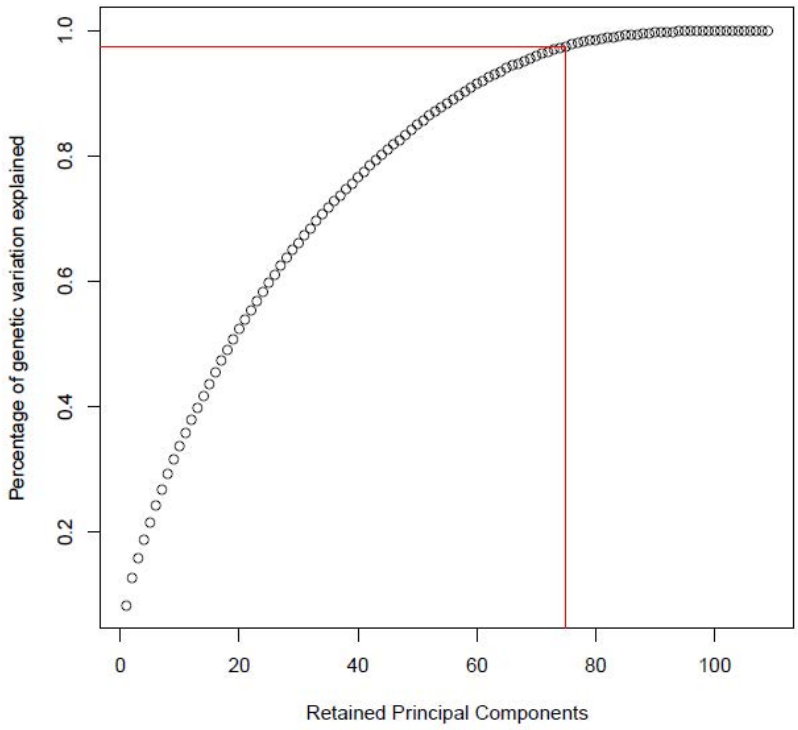

Figure S3.3 Retained principle components for DAPC which explain 95% of genetic variability

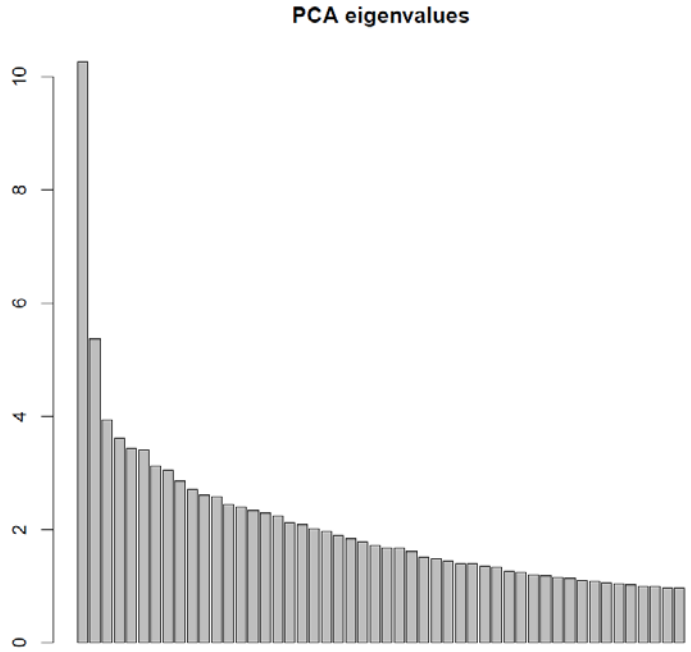

Figure S3.4 Principle component analysis eigenvalues for DAPC

APPENDIX B: Supplementary materials for Chapter 4

Table S4.1 Phenotype expectations for pure *A. chrysopterus* (CH), *A. chrysopterus* back-crosses (CH b/c), first generation hybrids (F1 hybrid), *A. sandaracinos* back-crosses (SA b/c), and pure *A. sandaracinos* (SA), throughout the hybrid zone. Pigmentation within the Solomon Islands region is also expected for *A. chrysopterus* individuals, additional to this table.

Trait	Pure CH	CH b/c	F1 hybrid	SA b/c	Pure SA
Tail shape	Elongated	Elongated	Round	Round	Round
Tail colour	White	White	Orange	Orange	Orange
Body colour	Black	Black/brown	Orange	Orange	Orange
Dorsal stripe	Absent	Absent	White cap	Partial white cap and/or stripe	Full white stripe
1st side bars	Complete	Complete	Partial	Partial	Absent
2nd side bars	Complete	Partial/ complete	Absent	Absent	Absent
Lateral body shape	Deep	Deep	Deep	Narrow	Narrow

Table S4.2 Relative frequency of qualitative phenotypic traits observed in hybrid zone; where in CHKB and CHSO populations, 2% and 19%, respectively were light variants (see Fig S4.1C), and 9% showed pigmentation (see Fig S4.1A).

	CHKB	CHKA	CHSO	LUKB	LUKA	LUSO	SAKB	SAKA	SASO
Total *n*	54	29	90	26	23	52	62	29	35
Tail Shape									
1. Round	0.00	0.00	0.00	0.96	0.96	0.04	1.00	1.00	1.00
2. Elongated	1.00	1.00	1.00	0.04	0.04	0.96	0.00	0.00	0.00
Tail Colour									
1. White	1.00	1.00	0.95	0.04	0.09	0.08	0.00	0.00	0.00
2. Orange	0.00	0.00	0.00	0.96	0.91	0.92	1.00	1.00	1.00
3. Brown	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.00
4. Black	0.00	0.00	0.03	0.00	0.00	0.00	0.00	0.00	0.00
Body Colour									
1. Black	0.94	0.52	0.79	0.00	0.00	0.00	0.00	0.00	0.00
2. Brown	0.06	0.48	0.21	0.12	0.13	0.17	0.00	0.00	0.00
3. Orange	0.00	0.00	0.00	0.88	0.87	0.83	1.00	1.00	1.00
Dorsal Stripe									
1. Cap	0.00	0.00	0.00	0.42	0.70	0.58	0.00	0.00	0.00
2. Full	0.00	0.00	0.00	0.12	0.04	0.08	1.00	1.00	1.00
3. Partial	0.00	0.00	0.00	0.04	0.22	0.15	0.00	0.00	0.00
4. Missing	1.00	1.00	1.00	0.42	0.04	0.19	0.00	0.00	0.00
1st Side Bars									
1. Complete pair	0.98	1.00	1.00	0.31	0.65	0.58	0.00	0.00	0.00
2. Partial pair	0.02	0.00	0.00	0.54	0.30	0.38	0.02	0.00	0.00
3. Complete single	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
4. Partial single	0.00	0.00	0.00	0.04	0.00	0.04	0.00	0.00	0.00
5. Both missing	0.00	0.00	0.00	0.12	0.04	0.00	0.98	1.00	1.00
2nd Side Bars									
1. Complete pair	0.98	0.90	0.94	0.00	0.00	0.02	0.00	0.00	0.00
2. Partial pair	0.02	0.10	0.06	0.00	0.04	0.00	0.00	0.00	0.00
3. Complete single	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
4. Partial single	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00
5. Both missing	0.00	0.00	0.00	1.00	0.96	0.96	1.00	1.00	1.00
Lateral Body Shape									
1. Narrow	0.00	0.00	0.00	0.42	0.13	0.37	1.00	0.93	1.00
2. Deep	1.00	1.00	1.00	0.58	0.87	0.63	0.00	0.07	0.00

Table S4.3 Summary statistics for 21 microsatellite loci across nine populations: Sample size (n), observed number of alleles (N_a) and private alleles (P_a), observed heterozygosity (H_O) expected heterozygosity (H_E), and average inbreeding coefficient (F_{IS}). Probability of departure from HWE for each locus at each species (P); where significance of departure before $P < 0.05$* and following sequential Bonferroni adjustment $P < 0.001$ (bold) are indicated. Loci listed are as follows: Alat10, Alat23, D1, As20, Alat14, Alat16, D114, Alat11, Alat12, A130, Alat5, Alat21, Alat13, Alat7, Am9, Alat19, Alat8, Am21, Alat22, Am5, and As8.

		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**
CHKB	n	30	30	29	31	30	31	31	22	31	29	31	31	29	31	30	28	29	29	31	30	30
	N_a	7	4	15	3	2	13	17	11	7	15	6	9	6	12	17	16	12	8	5	7	6
	P_a	0	0	0	1	0	0	0	1	2	1	1	1	0	3	0	0	1	0	0	0	1
	H_O	0.567	0.133	0.586	0.032	0.000	0.839	0.871	0.364	0.581	0.897	0.613	0.194	0.552	0.677	0.900	0.857	0.862	0.586	0.774	0.500	0.300
	H_E	0.658	0.243	0.908	0.122	0.064	0.888	0.906	0.849	0.589	0.857	0.669	0.745	0.680	0.682	0.873	0.907	0.837	0.807	0.535	0.636	0.472
	F_{IS}	0.155	0.464	0.370	0.744	1.000	0.072	0.055	0.587	0.031	-0.029	0.100	0.747	0.205	0.023	-0.014	0.073	-0.012	0.290	-0.433	0.230	0.379
	P	0.029*	**0.000***	**0.000***	**0.000***	**0.000***	0.363	0.209	**0.000***	**0.000***	0.063	**0.000***	**0.000***	**0.000***	0.762	**0.000***	0.016*	0.572	0.132	**0.000***	0.005*	**0.000***
CHKA	n	26	26	26	26	26	26	26	21	26	26	26	26	24	26	26	24	26	26	26	26	25
	N_a	5	3	13	1	1	14	25	9	3	11	7	7	4	8	17	14	10	11	2	7	7
	P_a	0	0	0	0	0	0	4	2	0	0	1	2	0	0	0	0	1	0	0	0	0
	H_O	0.577	0.231	0.654	0.000	0.000	0.923	1.000	0.381	0.538	0.923	0.923	0.346	0.583	0.692	0.846	0.917	0.885	0.769	0.885	0.423	0.360
	H_E	0.490	0.211	0.891	0.000	0.000	0.865	0.940	0.783	0.533	0.851	0.667	0.753	0.608	0.749	0.902	0.890	0.848	0.839	0.493	0.547	0.466
	F_{IS}	-0.157	-0.075	0.284	-	-	-0.048	-0.044	0.531	0.010	-0.065	-0.367	0.554	0.061	0.095	0.081	-0.009	-0.024	0.102	-0.786	0.246	0.246
	P	0.982	0.931	**0.000***	-	-	0.151	0.600	**0.000***	0.996	0.431	0.456	**0.000***	0.525	0.653	0.171	0.314	0.490	0.402	**0.000***	0.101	0.006*
CHSO	n	65	63	62	61	63	64	52	58	64	63	65	65	55	61	63	60	61	56	65	63	64
	N_a	12	7	17	1	2	17	35	14	5	16	10	11	9	12	24	19	16	10	8	13	8
	P_a	3	2	1	0	0	1	5	2	0	1	2	3	1	2	6	0	3	0	3	0	1
	H_O	0.708	0.270	0.613	0.000	0.000	0.938	0.923	0.207	0.438	0.905	0.785	0.338	0.709	0.623	0.889	0.917	0.852	0.732	0.708	0.762	0.359
	H_E	0.754	0.322	0.902	0.000	0.031	0.875	0.947	0.845	0.559	0.844	0.703	0.790	0.714	0.620	0.913	0.924	0.866	0.816	0.497	0.722	0.576
	F_{IS}	0.069	0.171	0.328	-	1.000	-0.064	0.035	0.759	0.225	-0.064	-0.109	0.577	0.016	0.004	0.035	0.017	0.024	0.112	-0.416	-0.047	0.383
	P	**0.000***	**0.000***	0.002*	-	**0.000***	0.047*	0.303	**0.000***	**0.000***	0.517	**0.000***	**0.000***	**0.000***	0.005*	0.123	**0.000***	0.007*	**0.000***	**0.000***	**0.000***	**0.000***
LUKB	n	34	35	34	34	35	35	35	34	35	34	35	35	35	33	35	33	35	34	35	35	35
	N_a	6	4	13	4	2	14	11	5	3	7	6	7	3	6	12	15	9	9	9	7	3
	P_a	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	H_O	0.971	0.571	0.824	0.559	0.486	1.000	0.829	0.088	0.629	0.647	0.657	0.514	0.114	0.455	0.943	0.758	0.686	0.794	0.914	0.657	0.257
	H_E	0.771	0.446	0.863	0.431	0.396	0.818	0.871	0.333	0.589	0.667	0.661	0.760	0.160	0.385	0.749	0.862	0.706	0.723	0.790	0.580	0.564
	F_{IS}	-0.245	-0.269	0.061	-0.282	-0.214	-0.209	0.063	0.742	-0.053	0.045	0.020	0.336	0.299	-0.166	-0.246	0.136	0.043	-0.084	-0.143	-0.119	0.554
	P	**0.000***	0.469	0.314	**0.000***	0.177	0.080	0.077	**0.000***	0.094	0.999	0.027*	0.001*	**0.000***	1.000	0.948	0.740	0.912	0.997	0.656	0.123	**0.000***
LUKA	n	25	25	25	25	25	25	25	24	24	25	25	25	25	25	25	25	25	24	25	25	25
	N_a	7	4	19	2	2	16	19	6	4	14	5	9	4	9	14	16	11	8	12	9	4
	P_a	0	0	0	0	0	0	1	1	0	0	0	0	0	1	0	0	0	0	0	0	0
	H_O	0.800	0.840	0.880	0.920	0.840	1.000	0.880	0.167	0.833	1.000	0.800	0.320	0.160	0.880	0.960	1.000	0.880	0.833	0.880	0.880	0.320
	H_E	0.714	0.551	0.911	0.497	0.500	0.888	0.910	0.661	0.672	0.839	0.656	0.846	0.545	0.722	0.863	0.924	0.866	0.828	0.665	0.758	0.618
	F_{IS}	-0.100	-0.509	0.055	-0.846	-0.669	-0.106	0.053	0.757	-0.220	-0.172	-0.200	0.634	0.716	-0.199	-0.092	-0.062	0.004	0.015	-0.305	-0.142	0.498
	P	**0.000***	0.053	0.012*	**0.000***	0.001*	0.887	0.978	**0.000***	0.608	0.074	0.111	**0.000***	**0.000***	0.681	0.913	0.100	0.385	0.564	1.000	0.594	**0.000***

Table S4.3 Continued.

		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**
LUSO	n	52	53	47	53	53	53	52	48	53	53	53	53	53	53	53	52	53	22	23	53	53
	N_a	10	6	22	5	2	17	24	9	6	21	10	15	7	11	18	25	18	9	11	8	4
	P_a	0	0	0	0	0	0	1	1	0	1	3	3	2	1	0	0	3	0	1	0	0
	H_O	0.269	0.887	0.851	0.849	0.698	0.943	0.731	0.229	0.585	0.981	0.755	0.415	0.113	0.811	0.962	1.000	0.962	0.818	0.826	0.962	0.075
	H_E	0.727	0.602	0.941	0.530	0.497	0.896	0.878	0.810	0.593	0.912	0.686	0.848	0.718	0.653	0.818	0.943	0.867	0.830	0.680	0.766	0.537
	F_{IS}	0.636	-0.465	0.106	-0.596	-0.396	-0.044	0.177	0.722	0.024	-0.066	-0.090	0.517	0.845	-0.234	-0.167	-0.051	-0.100	0.037	-0.194	-0.248	0.862
	P	**0.000***	0.003*	0.001*	**0.000***	0.003*	0.830	0.943	**0.000***	0.049*	0.301	**0.000***	**0.000***	**0.000***	0.989	0.011*	0.042*	0.094	0.126	0.370	**0.000***	**0.000***
SAKB	n	65	64	49	60	62	63	60	56	66	62	65	64	64	65	65	59	63	60	66	66	65
	N_a	7	4	11	2	3	12	13	8	7	6	5	9	4	6	8	13	6	7	16	8	5
	P_a	1	0	1	0	1	0	0	0	1	1	1	1	0	0	0	2	0	0	1	0	0
	H_O	0.815	0.063	0.735	0.050	0.048	0.810	0.933	0.161	0.515	0.532	0.292	0.656	0.047	0.185	0.585	0.797	0.508	0.400	0.712	0.212	0.600
	H_E	0.735	0.061	0.847	0.049	0.047	0.738	0.846	0.359	0.577	0.515	0.370	0.734	0.159	0.213	0.632	0.842	0.566	0.564	0.808	0.273	0.543
	F_{IS}	-0.101	-0.014	0.142	-0.017	-0.011	-0.088	-0.095	0.559	0.115	-0.025	0.218	0.114	0.710	0.143	0.082	0.063	0.111	0.298	0.126	0.231	-0.098
	P	0.029*	1.000	**0.000***	0.843	0.998	**0.000***	0.954	**0.000***	**0.000***	**0.000***	**0.000***	**0.000***	**0.000***	**0.000***	0.175	**0.000***	0.270	0.001*	**0.000***	**0.000***	**0.000***
SAKA	n	28	28	26	27	28	28	28	28	27	28	28	28	28	28	28	28	28	28	28	28	28
	N_a	7	3	11	2	3	10	12	7	3	11	4	7	5	3	8	11	9	5	19	6	5
	P_a	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
	H_O	0.536	0.071	0.885	0.000	0.036	0.893	0.893	0.536	0.556	0.786	0.750	0.786	0.571	0.143	0.643	0.893	0.750	0.571	0.964	0.500	0.679
	H_E	0.554	0.135	0.825	0.071	0.103	0.786	0.827	0.589	0.516	0.769	0.531	0.781	0.570	0.135	0.638	0.874	0.705	0.615	0.893	0.528	0.595
	F_{IS}	0.052	0.486	-0.052	1.000	0.663	-0.118	-0.062	0.108	-0.057	-0.003	-0.397	0.012	0.015	-0.043	0.011	-0.003	-0.045	0.089	-0.061	0.071	-0.123
	P	**0.000***	**0.000***	0.324	**0.000***	**0.000***	0.002*	0.002*	0.047*	0.717	0.006*	0.121	0.001*	0.920	0.983	**0.000***	**0.000***	0.111	**0.000***	**0.000***	**0.000***	0.650
SASO	n	30	30	27	30	30	30	30	30	30	30	30	30	30	30	30	30	30	28	30	30	30
	N_a	7	2	10	3	1	9	8	5	2	11	2	8	5	2	4	11	7	6	12	4	3
	P_a	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	2	0	0
	H_O	0.900	0.033	0.815	0.033	0.000	0.767	0.700	0.333	0.300	0.700	0.033	0.800	0.600	0.067	0.567	0.867	0.667	0.536	0.967	0.467	0.400
	H_E	0.704	0.033	0.834	0.156	0.000	0.741	0.679	0.474	0.473	0.753	0.033	0.740	0.624	0.064	0.552	0.885	0.669	0.621	0.849	0.534	0.504
	F_{IS}	-0.262	0.000	0.042	0.793	-	-0.018	-0.013	0.313	0.380	0.088	0.000	-0.064	0.056	-0.018	-0.010	0.038	0.020	0.154	-0.121	0.143	0.223
	P	0.844	0.926	0.910	**0.000***	-	0.960	0.657	0.809	0.045*	0.997	0.926	0.102	0.777	0.850	0.684	0.024*	0.027*	0.207*	0.895	0.695	0.181

Table S4.4 Neutrality tests using 1000 simulations of the infinite site model in Arlequin on mtDNA sequences from nine hybrid zone populations. Significant values in bold.

	CHKB	CHKA	CHSO	LUKB	LUKA	LUSO	SAKB	SAKA	SASO	Mean	s.d.
Tajima's D test											
Sample size	76	31	56	45	23	55	30	23	24	40.333	18.762
S	33	3	4	25	2	6	35	27	74	23.222	23.333
Pi	6.022	3.708	3.855	10.620	0.933	2.981	23.720	27.316	50.583	14.415	16.525
Tajima's D	**-1.533**	-0.155	-0.669	-1.102	0.243	-1.185	**-1.875**	-0.554	1.593	-0.582	1.049
Tajima's D p-value	**0.038**	0.468	0.290	0.128	0.698	0.100	**0.012**	0.300	0.966	0.333	0.324
Fu's FS test											
Real no. of alleles	16	7	9	13	5	6	14	12	15	10.778	4.116
Orig. no of alleles	17	7	9	13	5	6	14	12	15	10.889	4.285
Theta pi	6.023	3.708	3.855	10.620	0.933	2.981	23.720	27.316	50.583	14.415	16.525
Exp. no. of alleles	16.205	8.761	11.062	17.997	3.589	9.348	19.672	16.917	19.803	13.706	5.707
FS	0.315	1.716	1.625	3.486	**-1.117**	2.969	5.020	5.292	5.658	2.774	2.342
Significance at p<0.02	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

Table S4.5 Raw species differentiation from microsatellite allele frequencies**:** species differentiation corrected for null allele frequencies using the ENA correction, where values significant to the 95% confidence interval are in bold; and estimator of actual differentiation (D_{est}). Results are presented locus-by-locus and as an average over 21 loci, and reveal comparable raw values to values corrected for null alleles.

Locus	Raw	ENA corrected	D_{est}
Alat10_tri	0.164	0.163	0.482
Alat23_penta	**0.523**	**0.498**	0.442
D1_tetra	**0.061**	**0.058**	0.565
As20_tri	**0.644**	**0.610**	0.435
Alat14_tri	**0.675**	**0.630**	0.440
Alat16_tetra	**0.103**	**0.103**	0.597
D114_tetra	**0.050**	**0.051**	0.422
Alat11_tri	0.207	0.186	0.476
Alat12_tri	**0.119**	**0.111**	0.173
A130_di	**0.135**	**0.132**	0.509
Alat5_di	0.232	0.225	0.393
Alat21_penta	**0.114**	**0.100**	0.493
Alat13_tri	0.200	0.178	0.249
Alat7_di	**0.315**	**0.307**	0.402
Am9_tri	**0.116**	**0.114**	0.459
Alat19_tetra	**0.049**	**0.048**	0.501
Alat8_di	**0.128**	**0.124**	0.484
Am21_penta	**0.130**	**0.117**	0.402
Alat22_penta	0.195	0.195	0.504
Am5_di	**0.313**	**0.295**	0.632
As8_tetra	0.271	0.239	0.460
Mean	0.204	0.194	0.450

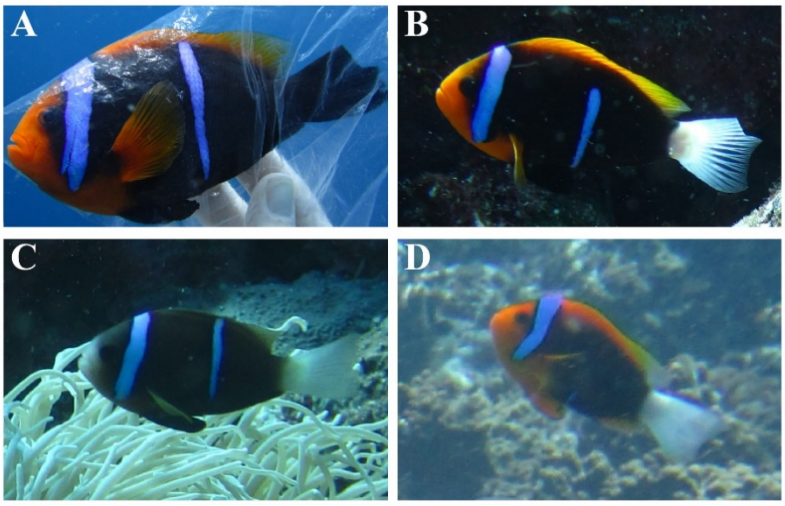

Figure S4.1 *A. chrysopterus* hybrid zone phenotypes including: (A) black pigmented morph, (B) half second sidebar, and (C) light morph, New Georgia Province, Solomon Islands, and (D) putative *A. chrysopterus* & '*A. leucokranos*' hybrid morph, Kimbe Bay, Papua New Guinea. Segments of caudal fin missing due to fin-clip sampling (A, B). Photo credits: A. Gainsford.

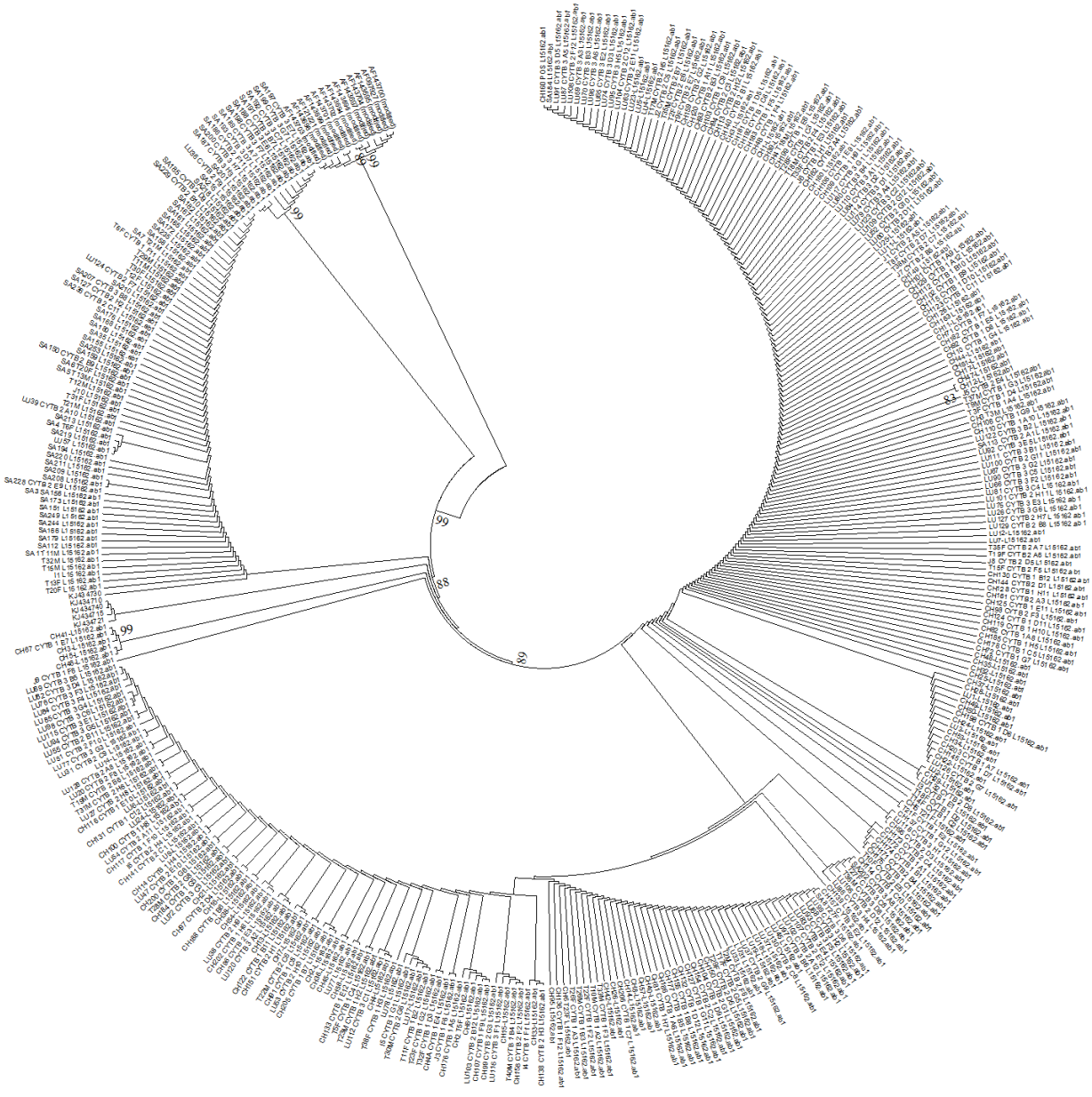

Figure S4.2 Maximum Parsimony tree (tree 1 out of 7 parsimonious trees, length 285) generated in MEGA6 is shown. Analysis involved 388 mtDNA cytochrome *b* sequences and the tree is outgroup rooted with *Amphiprion ocellaris* sequences obtained from Genbank. Support values were inferred from 1000 replicate bootstrap consensus MP tree.

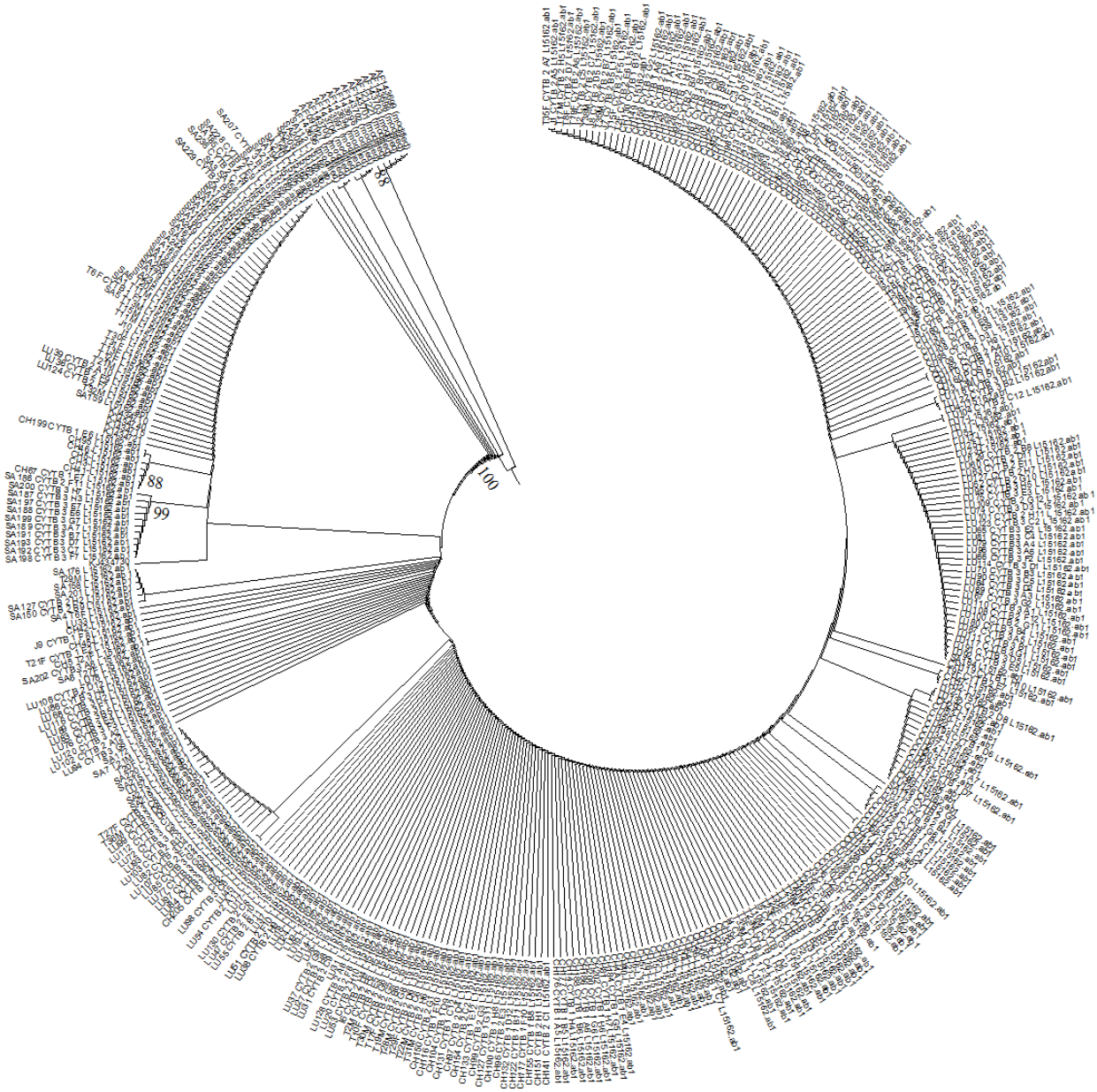

Figure S4.3 Maximum Likelihood tree with highest log likelihood (-1822.1001) based on HKY+G model in MEGA6. Analysis involved 388 mtDNA cytochrome *b* sequences and the tree is outgroup rooted with *Amphiprion ocellaris* sequences obtained from Genbank. Support values included are inferred from 1000 replicate bootstrap consensus ML tree.

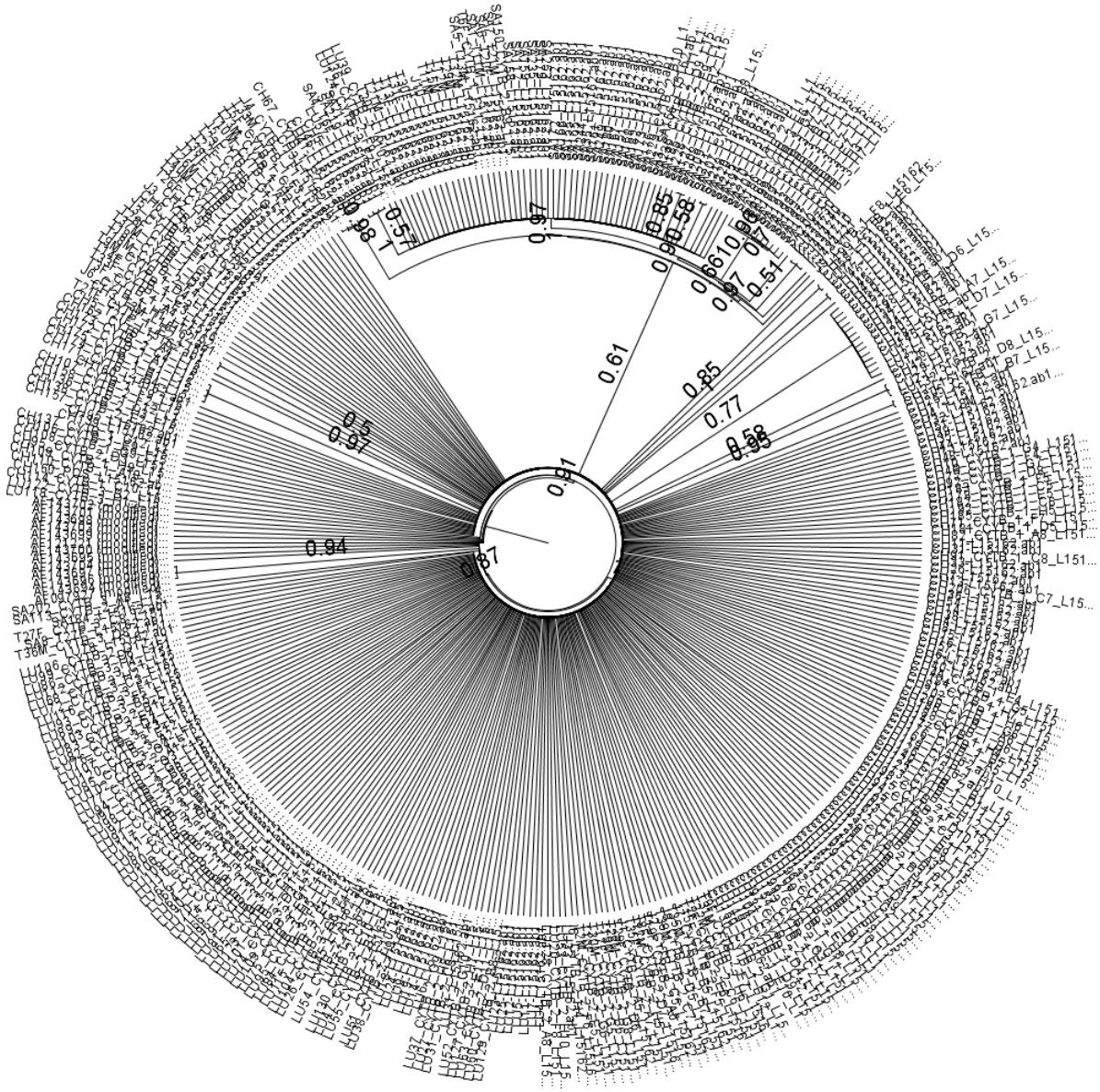

Figure S4.4 Bayesian Inference tree with highest log likelihood (-2880.726) based on HKY+G model in MrBayes plug-in for Geneious v9.0.4. Analysis involved 388 mtDNA cytochrome *b* sequences and the tree is outgroup rooted with *Amphiprion ocellaris* sequences obtained from Genbank. Posterior probabilities indicate phylogenetic support on proportionally transformed branches.

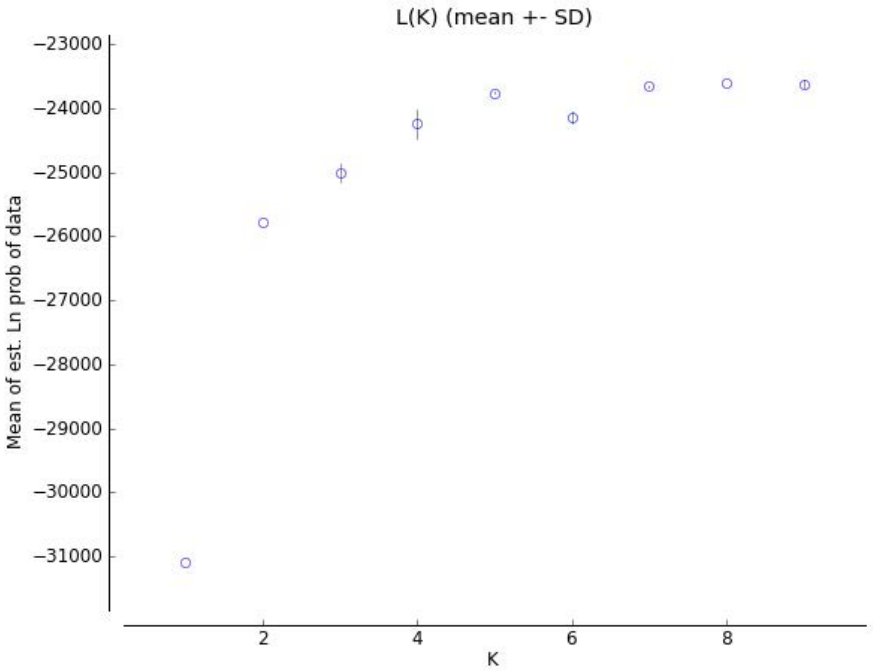

Figure S4.5 STRUCTURE Harvester output of mean log likelihood and standard deviation based on STRUCTURE analyses defines K = 2 clusters.

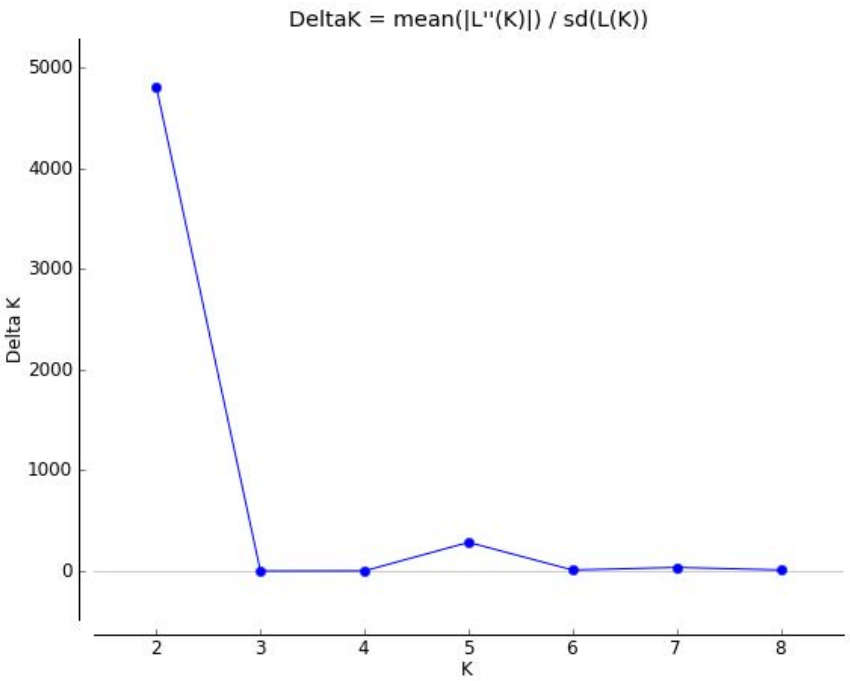

Figure S4.6 STRUCTURE Harvester output showing Delta K for each number of potential clusters (K) based on STRUCTURE analysis which clearly defines K = 2 clusters for dataset.

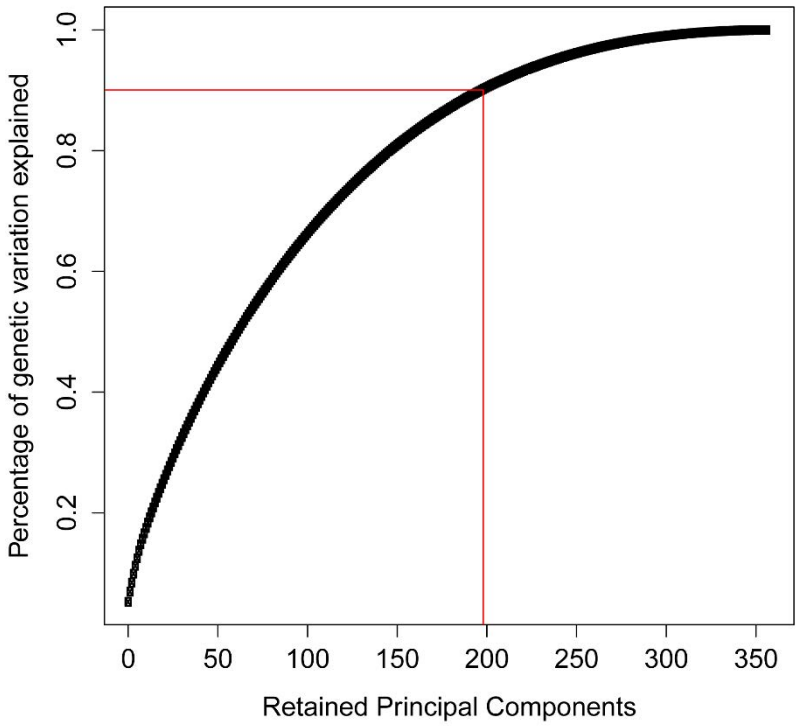

Figure S4.7 DAPC output revealing 198 principle components explain 90% of genetic variability (indicated by red intercept lines).

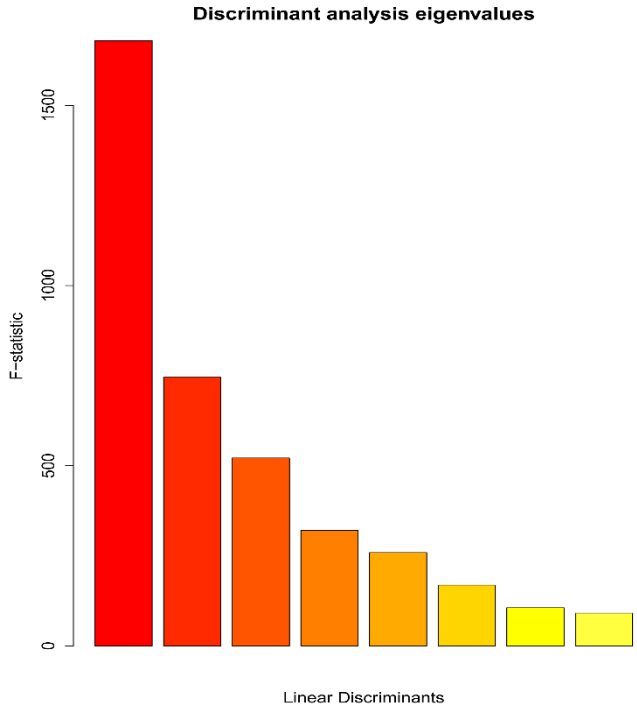

Figure S4.8 DAPC output of discriminant analysis eigenvalues which selected the first two discriminant functions to retain.