Chimerism and allorecognition in the broadcast spawning coral *Acropora millepora* on the Great Barrier Reef

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ABSTRACT

The ability to differentiate between self and non-self is a key feature of all living organisms and provides the first line of defense against invading pathogens, however, the allorecognition systems of many marine invertebrates allow fusion of two or more genetically distinct individuals, resulting in the formation of genetic chimeras. In scleractinian corals, fusion among allogeneic juveniles is known to occur following aggregated larval settlement in some brooding species, but no studies have investigated chimerism in juveniles of broadcast spawning corals or in adult populations of any coral species.

Accordingly, the potential of the broadcast spawning coral species, *Acropora millepora*, to form chimeras during early life stages was explored under experimental conditions, and the persistence of chimeras deployed in the field monitored for 23 months post-settlement. Larvae settled in aggregations in high numbers with 47% of juveniles originating from aggregated settlement. Genotyping at 9 microsatellite loci revealed that 50% of juveniles tested were chimeras. Therefore, *Acropora millepora* shows high potential for chimera formation following gregarious larval settlement. Relatedness analysis highlighted that the majority of chimeric colonies were either full or half sibling associations. Fusion at settlement confered greater size for chimeras (~three-fold greater) compared to solitary juveniles through to at least three months. Consequently, chimerism is likely to be an important strategy for maximizing survival of vulnerable early life history stages of corals.

To determine if the lack of a mature allorecognition system might facilitate chimera formation, I compared the development of allorecognition in full sibling, half sibling and non-sibling contact reactions between newly settled juveniles of *Acropora millepora*. In the first two months post-settlement, fusions among juveniles of all kinship levels indicate that *A. millepora* juveniles lack a mature allorecognition system in early life history stages. Relatedness governed the rate of allorecognition maturation, with all contact reactions between non-siblings rejecting by 3 months, while it took at least 5 months for all contact reactions between half siblings to reject and longer than 13 months for full siblings. The comparatively slow maturation of allorecognition in spawning corals (more than 13 months) compared to brooders (4 months) constitutes a significant difference in their life history strategies, and may contribute to flexibility in *Symbiodinium* uptake in the early ontogeny of broadcast spawning corals.

Differential expression of putative immune response genes (apextrin, complement C3, and two CELIII type lectin genes) was monitored in *Acropora millepora* juveniles for six months post-settlement, to explore the molecular basis of allorecognition maturation. Using quantitative real-time PCR (qPCR), I found that expression of the lectin gene A036-E7 peaked and was significantly greater in the fourth month than at any other time for the majority of coral juveniles sampled. Complement C3 and apextrin were also highly expressed concurrently with A036-E7, suggesting that there may be a number of genes co-expressed and influencing the immune system of corals during development. Increased expression levels of one lectin gene may be linked to allorecognition maturation, or alternatively may represent a response to non-self recognition challenges. Although my data are preliminary, they confirm, as highlighted by recent studies of *A. millepora*, the crucial role lectins may play in the allorecognition and innate immunity of corals.

The extent of chimerism was explored within two wild populations of a common coral, *Acropora millepora*, on the Great Barrier Reef, Australia, by using up to 12 polymorphic DNA microsatellite loci. At least 2% and 5% of Magnetic Island and Pelorus Island populations of *A. millepora*, respectively, were found to be chimeras (3% overall), based on conservative estimates. These values are likely to be vast underestimates of the true extent of chimerism in wild populations, as the sampling protocol was restricted to a maximum of eight branches per colony, whereas most colonies consist of hundreds of branches. Genotypes within chimeric corals showed high relatedness, indicating that genetic similarity is a prerequisite for long-term acceptance of non-self genotypes within coral colonies. Detection of chimeras in wild populations of *Acorpora millepora* validates results of experimental studies showing fusion and chimera persistence in juveniles.

Taken together, the high potential for chimera formation following aggregated larval settlement found in experimental studies and the occurrence of chimerism in wild populations of *A. millepora* suggest that chimerism is likely to be an important strategy in the early life cycle of broadcast spawning corals and may be more widespread in corals than previously thought. Chimerism and associated increased genetic diversity within colonies are likely to have important implications for the resilience of reef corals, potentially enhancing their capacity to compete for space and respond to environmental stressors and pathogen infection.

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Chapter 1.0 Background and General Introduction

1.1 Background

The ability to differentiate between self and non-self is a key feature of all living organisms. Allorecognition and histocompatibility are ancient mechanisms governing self-recognition and underlie the ability of organisms to discriminate foreign genetic material (Dishaw & Litman, 2009), which in combination with innate immunity, provides the first line of defense against pathogen invasion in both plant and animal species (Nürnberger *et al.*, 2004). The capacity of some organisms, particularly colonial marine invertebrates, to form chimeras (from the Latin "chimaera" meaning monster), which are entities containing tissues or cells of two or more genetically distinct individuals (Rinkevich & Weissman, 1987), indicates that some allorecognition systems are not precise in recognising self, or alternatively, allow or even potentially enable fusion between genetically different entities. The occurrence of chimerism in colonial marine animals challenges notions of genetic uniqueness within clonal organisms (Santelices, 1999) and raises questions about the potential roles that chimerism might play in the ecology and evolution of clonal organisms. A review of studies of allorecognition and chimerism in such organisms represents the first step in revealing potential benefits and costs associated with lack of precision in allorecognition and in assessing the ecological and evolutionary significance of such a strategy.

In this chapter, I review what is known about allorecognition and chimerism in colonial marine invertebrates, focussing particularly on the phyla Porifera, Cnidaria, Bryozoa and Tunicata. Species in these phyla are typically both sessile and modular and can be defined as organisms in which members of a colony are physically connected and have common ancestry through asexual replication of modules (Jackson, 1977). Colonial marine invertebrates represent a major component of global marine biodiversity (Jackson, 1977) and their sessile, modular nature (Sommerfeldt *et al.*, 2003) enables

them to dominate hard substratum communities of the shallow seas. The sessile habit of most modular marine invertebrates also promotes aggressive competition for space among both conspecifics and heterospecifics. Thus, widespread asexual reproduction within these groups, in combination with aggregated settlement of sexually produced larvae, provide many opportunities for contact reactions among kin (Hart & Grosberg, 1999). In general, traits such as indeterminate growth, asexual reproduction, long lifespan and dense larval settlement are expected to favour the evolution of allorecognition systems within colonial marine invertebrates to enable individuals to discriminate self from nonself during contact reactions with neighbouring colonies (Grosberg, 1988; Hart & Grosberg, 1999).

In this chapter, I first review studies of self-recognition in adult and juvenile colonial marine invertebrates to highlight and compare characteristics of their allorecognition systems and evaluate how such systems change through ontogeny. Allorecognition comprises a series of events triggered by contact between genetically different tissues, followed by a specific answer or reaction to nonself. Contact between adult or juvenile colonial marine invertebrates may thus express a wide variety of responses to the same aim: maintaining the integrity of self (Grosberg, 1988). Secondly, I review to what extent chimeras occur within natural populations of colonial marine invertebrates and the potential ecological and evolutionary costs and benefits of chimeras. This chapter ends with an overview of the aims for this thesis and an outline of chapters addressing each of my research objectives.

1.2 Allorecognition in colonial marine invertebrates

The widespread occurrence of asexual reproduction in the life histories of modular marine invertebrates highlights the need for allorecognition systems to control fusion and

rejection reactions among isogeneic (same species, same genotype), allogeneic (same species, different genotype) and xenogeneic (different species) entities when they come into contact (Grosberg, 1988). A variety of techniques have been used to evaluate allorecognition in marine invertebrates, and based on the outcomes of contact reactions within both adult and juvenile life history stages, these studies highlight a number of exceptions to the dogma that allorecognition functions mainly in maintaining the integrity of self.

1.2.1 Contact reactions between adult colonies of marine invertebrates

To investigate the capacity of colonial marine invertebrates to discriminate self from nonself, contact reaction experiments are commonly conducted using adult colonies (Table 1.1). The outcomes of contact reactions are typically diverse, with fusion and rejection of tissues representing the extremes of the range. However, incompatible contact responses can occur at a variety of different levels, including behavioural, morphological, energetic and physiological, and chemical (Rinkevich & Loya, 1985). In the following sections, I focus on tissue reactions, as these are the most readily interpretable indicators of the outcomes of contact reactions.

The self-recognition bioassay as an indicator of clonal identity

Early studies assumed that allorecognition systems were precise in marine invertebrates and used histocompatibility bioassays to identify clonemates and clonal population structures. Bioassays involved placing branches from two colonies in tissue contact and recording fusions, which were assumed to identify clones (i.e., identical genotypes), and rejection (or non-fusion) reactions, which were presumed to identify non-clonemates. For example, self-recognition bioassays between adult colonies of the branching coral *Acropora cervicornis* were used to investigate the genotypic structure and extent of asexual reproduction within populations of this species (Neigel & Avise, 1983). Among

62 known isogeneic or inferred allogeneic contacts between branches of *A. cervicornis*, no exception to "precise clonal discrimination" was observed. However, clonal identities were inferred from observations of fusion, without verification by an independent genetic technique, thus such data need to be interpreted with caution.

In a study of the scleractinian coral *Pavona cactus*, two techniques, tissue grafting and allozyme electrophoresis, were applied to examine the genetic structure of a population and also to investigate the reliability of the self-recognition bioassay in comparison to a molecular technique (Willis & Ayre, 1985). Ten months following the initiation of contact reactions, electrophoresis revealed fusions between both genetically different and identical colonies (based on allozymes), indicating that tissue grafting is not always a reliable technique to identify clonemates. Fusion between electrophoretically distinct tissues was also found for the corals *Montipora dilatata* and *Montipora verrucosa* (Heyward & Stoddart, 1985), with nearly 50% of allogeneic tissues fusing. However, contact reactions were assessed after only 5 weeks and so the long-term stability of such fusions is unknown. A study comparing tissue grafting and electrophoresis in the corals *Porites cylindrica*, *Seriatopora hystrix*, and *Porites nigrescens* also revealed that tissue grafting was an inadequate indicator of clonal identity, as fusions between electrophoretically distinct colonies (i.e. different genotypes) occurred in 20-40% of allografts after 6-17 weeks. However, no fusions were detected between allografts of the brooding coral *Stylophora pistillata* (Resing & Ayre, 1985). Thus, comparative histocompatibility and genetic studies have revealed that precision of the self-recognition response can vary considerably in adult populations of corals.

Table 1.1. Overview of contact reaction experiments and observations conducted on colonial marine invertebrates.

Genus species		Type of contact and responses			References
		Xenogeneic	Allogeneic	Isogeneic	
Hydractinia	juvenile	-	Fusion	fusion	(Fuchs *et al.*, 2002)
	adult	-	non-fusion	fusion	
Acropora formosa	juvenile	-	-	-	(Hildemann *et al.*, 1975)
	adult	non-fusion	non-fusion	fusion	
Acropora nasuta	juvenile	-	-	-	(Hildemann *et al.*, 1975)
	adult	non-fusion	non-fusion	fusion	
Fungia fungites	juvenile	-	-	-	(Hildemann *et al.*, 1975)
	adult	acute rejection	-	fusion	
Agaracia tenufolia	juvenile	-	-		(Chornesky, 1991)
	adult	-	fusion (2%)	fusion	
Stylophora pistillata	juvenile	-	-	-	(Chadwick-Furman & Rinkevich, 1994)
	adult	-	non-fusion or unilateral rejection	fusion	
Millepora dichotoma	juvenile	-		-	(Frank & Rinkevich, 1994)
	adult	-	overgrowth (n=42) and fusion (n=3)	fusion	
Stylophora pistillata	juvenile	-	Fusion	fusion	(Frank *et al.*, 1997)
	adult	-	-	-	
Pocillopora damicornis	juvenile	-	fusion, non-fusion or incompatible fusion	fusion	(Hidaka, 1985b; Hidaka *et al.*, 1997; Raymundo & Maypa, 2004)
	adult	-	non-fusion	fusion	
Nephtea sp.	juvenile		Fusion	-	(Barki *et al.*, 2002)
	adult	-	-	-	
Heteroxenia fuscescens	juvenile		Fusion	-	
	adult	-	-	-	
Parerythropodium fulvum fulvum	juvenile		Fusion	-	
	adult	-	-	-	
Clavularia hamra	juvenile		Fusion	-	
	adult	-	-	-	
Hydractinia symbiolongicarpus	juvenile	-	fusion (68%) and rejection (32%)		(Wilson & Grosberg, 2004)
	adult	-	-	-	
Seriatopora caliendrum	juvenile	non-fusion or death	fusion, non-fusion or incompatible fusion	-	(Nozawa & Loya, 2005)
	adult	non-fusion	-	-	
Seriatopora hystrix	juvenile	non-fusion or death	fusion, non-fusion or incompatible fusion	-	
	adult	non-fusion	-	-	
Botryllus schlosseri	juvenile	-	fusion or rejection	fusion	(Rinkevich & Weissman, 1989; Rinkevich *et al.*, 1993; Chadwick-Furman & Weissman, 1995 & 2003; Rinkevich, 2004b)
	adult	-	fusion or rejection	fusion	
Galaxea fascicularis	juvenile				(Hidaka, 1985a)
	adult		fusion or rejection	fusion	
Acropora hemprichi	juvenile	-	-	-	(Rinkevich *et al.*, 1994)
	adult	-	non-fusion or rejection	fusion	
Tedania ignis	juvenile	-	fusion (but no natural fusion)	-	(Maldonado, 1998)
	adult	-	-	-	

Grafting studies reveal a range of allogeneic reactions among adult colonies

Grafting experiments involving different colour morphs of adult colonies of *Pocillopora damicornis* revealed complexity beyond a simple fusion-rejection system in allogeneic contact reactions of scleractinian corals (Hidaka 1985). While isografts always fused and most allografts between colour morphs showed clear signs of rejection, some were classified as non-fusions because, although tissues were confluent across some parts of the contact zone, stimuli were not propagated across the interface and a white line, potentially representing reduced numbers zooxanthellae and/or bare skeleton, was visible at the interface. In addition, a category named "apparent fusions" was identified, although closer inspection revealed a thin skeletal ridge at the interface, indicating that this category also represented a rejection response (Hidaka, 1985b). Thus, when colour was used to distinguish allogeneic tissues, *Pocillopora damicornis* appears to possess a functional histocompatibility system that is capable of exhibiting variation in the intensity of rejection responses (Hidaka, 1985b, Table 1.1).

Complexity in allogeneic reactions was also found in adult contact reactions for another pocilloporid coral, *Stylophora pistillata* (Chadwick-Furman & Rinkevich, 1994). While isografts always fused, no complete fusions were observed for allografts. The authors described complete fusion as the continuity of tissue and skeleton across the contact zone, but rejection reactions expressed several different levels of incompatibility. Rejection was identified as unilateral tissue destruction extending from the contact zone, with tissue being replaced by bacterial communities or algal turf (Chadwick-Furman & Rinkevich, 1994). Non-fusions resembled fusions, but a microscopic gap was observable between tissues at the contact zone, which later developed into a suture line extending along the contact interface, and was usually followed by overgrowth of one colony by the other. The consistency with which *Stylophora pistillata* responded

selectively and reproducibly to isogeneic or allogeneic grafts suggested that these corals were able to efficiently distinguish self from nonself in mature life history stages (Chadwick-Furman & Rinkevich, 1994).

Incompatible responses between allogeneic colonies have also been found to vary in a range of marine invertebrates, from mild rejection reactions, where barriers or suture lines form along the contact interface, to clear rejection reactions involving cytotoxicity leading to resorption and sometimes overgrowth. Resorption of tissues by the dominant genotype is a common outcome (at least in laboratory experiments) of incompatible contact reactions between colonies of the ascidian *Botryllus schlosseri* (Rinkevich *et al.*, 1993). In contrast, fusion may lead to stable long-term chimeras under field conditions (Chadwick-Furman & Weissman, 1995, 2003). Overgrowth was a common outcome of incompatible contact reactions between adult colonies of the hydrocoral, *Millepora dichotoma.* Following contact, the majority of allogeneic combinations resulted in reproducible and unilateral tissue and skeleton overgrowth during the 10 weeks following first contact (Frank & Rinkevich, 1994). However, after this first contact reaction, four types of secondary responses were observed: reversals in overgrowth directionality, tissue necroses, stand-offs and abnormal growth patterns (Frank & Rinkevich, 1994).

Outcomes of xenogeneic contact reactions range from mild to severe

Two levels of immunoreactivity have been described for xenogeneic contact reactions between adult colonies of sessile marine invertebrates (Hildemann *et al.*, 1975; Nozawa & Loya, 2005). In corals, many incompatible xenografts appeared to reflect “normal transplantation immunity”, where neither fusion nor acute aggression was observed; these reactions were designated as chronic xenogeneic incompatibility. For example, a thin wall often separated xenogeneic tissues (Hildemann *et al.*, 1975). However, in

other cases, severe incompatibility reactions destroyed polyps and zooxanthellae unidirectionally, indicating acute xenogeneic incompatibility (Hildemann *et al.*, 1975). Indeed, xenogeneic contact interactions between *Fungia fungites* and a range of species, including *Acropora rotumana*, *Pocillopora elegans*, and *Pocillopora eydouxi,* resulted in cytotoxicity over 5 to 6 mm from the contact zone. Xenogeneic contacts between five species of *Porites* resulted in incompatibility and overgrowth of one species according to a linear hierarchy of aggressiveness; *P. rus* was the dominant species, whereas *P. lutea* was overgrown by other species in most interactions (Rinkevich & Sakai, 2001). Thus, xenogeneic contact experiments clearly demonstrate that corals are able to differentiate species and reject non-self at this level with differing degrees of aggressiveness.

Contact reactions and immune memory

Early allogeneic contact studies with *Montipora verrucosa* suggested that corals had an immune memory that persisted for 4 to 8 weeks, based on shorter rejection times (by almost half) at second contact (Hildemann *et al.*, 1977). The hypothesis that the immune system of a basal marine invertebrate possesses a memory similar to that observed in vertebrate immune systems was further tested using the hydrocoral *Millepora dichotoma* (Frank & Rinkevich, 2001). Allografts involving the same genotype combination took the same length of time to mount a rejection response at first contact as they did at second and third contact. Thus, *Millepora dichotoma* lacks an alloimmune memory in contact interactions with non-self (Frank & Rinkevich, 2001). Such contrasting results highlight the complexity of the immune systems of basal marine invertebrates, potentially because immune memory might not always be essential for such organisms given the very wide scope of possible interactions and contacts with self and nonself.

Mechanisms and pathways underlying the self-recognition response

There have been few studies of the genetic basis of allorecognition in marine invertebrates, with the exception of the ascidian *Botryllus schlosseri*. Genetic studies have shown that allorecognition in this species is governed by multiple, co-dominantly expressed alleles at a single, highly polymorphic locus called the fusibility/histocompatibility locus (Fu/HC locus) (Rinkevich *et al.*, 1993). Thus two colonies sharing one or both alleles at this locus can fuse (Rinkevich *et al.*, 1993). Interestingly, allorecognition in *B. schlosseri* involves multilevel organization of histocompatibility alleles (Rinkevich *et al.*, 1993), leading to the hierarchical resorption of subordinate partners by dominant partners within chimeras under laboratory but not field conditions (Chadwick-Furman & Weissman, 1995, 2003).

Recent advances in molecular techniques have stimulated studies of innate immunity in marine invertebrates and are beginning to unravel mechanisms involved in activation of the innate immune responses that underlie allorecognition systems. Innate immune responses are activated by the detection of PAMPs (Pathogen-Associated-Molecule-Patterns). Such detection is achieved by Pattern Recognition Receptors (PRRs) that have the ability to detect and recognise PAMPs, and initiate an immune response (Janeway Jr. & Medzhitov, 2002). These receptors (PRRs) include C-type lectins (Robinson *et al.*, 2006). Indeed, C-type lectin receptors (CLRs) can be expressed as transmembrane proteins on myeloid cells of vertebrates and invertebrates, and are able to bind PAMPs. However, in addition to their PRR role, it has been hypothesized that some C-type lectins might also be able to recognize self and maintain an immune homeostasis (Robinson *et al.*, 2006). Consequently, lectin-type genes have been explored in recent studies of marine invertebrates for their potential involvement in allorecognition.

Lectin-type genes show high levels of expression in alloimmune-challenged ascidians (Oren *et al.*, 2007). Indeed, lectins were highly expressed at the point of rejection between incompatibly paired ascidians. Therefore, it is possible that ascidians possess a lectin-based opsonisation system, where lectins play a role in opsonisation of dead cells at the point of rejection (Oren *et al.*, 2007). Furthermore, an immunity study in a solitary ascidian linked Complement C3 to lectins (Sekine *et al.*, 2001). The presence of complement C3 in corals (Dishaw *et al.*, 2005; Miller *et al.*, 2007) and the involvement of lectins in immunity (Kvennefors *et al.*, 2008) might indicate a strong link between lectin and complement genes in coral immunity. This hypothesis is supported by the finding that Millectin, a mannose-binding lectin gene recently identified in *A.millepora,* is able to recognize various types of PAMPs (Kvennefors *et al.*, 2008). The binding region of this functional mannose-binding lectin showed high sequence diversity, suggesting that Millectin could recognize and bind to various groups of bacteria and genetic types of the algal endosymbiont, *Symbiodinium* (Kvennefors *et al.*, 2008). Consequently, Millectin may play a key role in the innate immune system of corals.

Following larval settlement, CELIII type lectins (A036-E7 & A049-E7) have been shown to be expressed exclusively on the oral surface of primary polyps of *A.millepora* (Grasso *et al.*, 2008), suggesting that they are potentially involved in cell recognition and self-defence. Similarly, a hemolytic lectin CEL III in the sea cucumber *Cucumaria echinata*, was able to bind to cell surface carbohydrate chains (i.e., PAMPs), leading to the formation of ion permeable pores in target cell membranes (Kouzuma *et al.*, 2003). Therefore, lectins expressed on the oral surface of corals could have a similar function and be involved in self-nonself recognition mechanisms.

In summary, contact reactions between adult colonies of modular marine invertebrates involve complex molecular pathways, the majority of which are still to be described. It

is clear that these organisms have allorecognition systems that are able to distinguish a range of genetic relatedness in tissue contact interactions, as evidenced by the range of outcomes described above. Generally it has been assumed that fusions identify genetically identical tissues and rejections identify non-self. However, studies that have tested the precision of the self-recognition response of adult colonies have revealed that fusion of allogeneic tissues can occur in a range of coral species, most notably for broadcasting species, highlighting the potential for formation of genetic chimeras.

1.2.2 Contact reactions between juveniles and alloimmune maturation

Aggregated settlement of larvae occurs commonly in a number of modular, sessile marine invertebrates (Lewis, 1974; Sammarco, 1982; Smith, 1997; Zilberberg & Edmunds, 2001; Barki *et al.*, 2002), enhancing the likelihood of contact reactions between newly settled larvae of varying genetic relatedness. To investigate allorecognition in these early life history stages, pairs of newly established colonies of the brooding coral, *Pocillopora damicornis,* which had originated from either the same or different parent colony, were brought into contact from 7 days to 3 months after planulation (Hidaka *et al.*, 1997). Fusion always occurred between juvenile colonies originating from larvae released from the same source colony (i.e. allografts, assuming that larvae were sexually produced). Although a small proportion of juveniles derived from different colonies also fused, contact reactions between allogeneic tissues of these more distantly related juveniles resulted primarily in non-fusion or incompatible fusion, outcomes that were similar in appearance to those described above for adult contact reactions (see section 1.2.1). In some cases, incompatible fusions changed to non-fusion or to disconnection when tissues at the interface died (Hidaka *et al.*, 1997). However, overall, much higher rates of allogeneic fusions were found among juveniles (Table 1.1) compared to the very rare fusion events detected among allogeneic tissues of adult

colonies of *P. damicornis*. Thus, this study revealed that allorecognition systems may differ between juvenile and adult colonies of the same species, providing the first evidence that newly settled larvae of corals lack a functional allorecognition system, as expressed by adult colonies (Hidaka, 1985b).

While post-settlement fusion between closely related (isogeneic or allogeneic) colonies may be comparatively common in early juvenile stages of colonial marine invertebrates, this initial fused state is not always stable. Indeed, young fused zooids of *Botryllus schlosseri* responded with a variety of outcomes after initial fusion (Rinkevich & Weissman, 1989). Resorption of the subordinate colony in the chimera, disconnection of allogeneic tissues, or even death of the chimera was observed. Nevertheless, stable chimeras were also formed, although it was suggested that stable chimeras could only be formed by homozygotic partners or heterozygotic partners sharing all alleles at gene loci governing fusibility and resorption (Rinkevich & Weissman, 1989). The development from a fused state to different compatible or incompatible states could be linked to the onset of a functional and mature allorecognition system.

Fusions facilitated by aggregated settlement of juveniles

Fusions during the early life history stages of colonial marine invertebrates are enhanced by gregarious settlement of larvae, leading to allogeneic contact as colonies grow in size. If neighbouring colonies are genetically distinct, their fusion would therefore generate a chimera (Hughes, 2005). Short distance dispersal, which is common in brooding corals, increases contact reactions among closely-related individuals, increasing the probability of fusion between siblings. The greater probability of fusions between siblings than between genetically distant individuals in species with short distance larval dispersal could thus decrease the potential costs of fusion associated with inter-genotype competition (Jackson, 1986). While fusion of

allogeneic larvae post settlement in laboratory experiments has been observed in sponges, hydroids, corals and ascidians, Jackson (1986) considers fusion between aclonal marine invertebrates in the field a rare event.

Maturation of the allorecognition system

The common occurrence of fusion between allogeneic individuals or colonies is likely to be linked to a lack of maturity in allorecognition systems in early stages of life of colonial marine invertebrates. For example, fusion of genetically distinct juvenile colonies of the hydrozoan *Hydractinia symbiolongicarpus* is commonly observed, however, the rate of fusions between pairs of sibling colonies decreases through time during the post-settlement period (Wilson & Grosberg, 2004). The shift to a more "restrictive conspecific acceptance threshold" indicates that the allorecognition system matures within this early period of sessile life and becomes increasingly able to distinguish self from nonself. Studies on juveniles of the brooding corals, *Seriatopora caliendrum* and *S. hystrix,* revealed that fusions between grafted allogeneic colonies were only observed during the first four months post-settlement, suggesting that complete maturation of the allorecognition system requires at least four months (Nozawa & Loya, 2005). Nevertheless, the self-nonself recognition system of *Seriatopora* functions to some extent during the first four months post-settlement, given that juveniles rejected genetically distant tissues more consistently than closely related tissues (Nozawa & Loya, 2005). Lack of an allorecognition system in the early stages of post larval settlement was also documented in four species of soft corals: *Nephthea sp.*, *Heteroxenia fuscescens*, *Parerythropodium fulvum* and *Clavularia hamra,* as evidenced by high frequencies of allogeneic fusions following co-settlement of larvae (Barki *et al.*, 2002). Consequently, the lack of a mature allorecognition system appears to be widespread in the early life stages of marine invertebrates.

A study of contact reactions between allogeneic juveniles revealed step-wise progression in the maturation of the juvenile allorecognition system in the brooding coral *Stylophora pistillata,* and identified three distinctive stages in the maturation process (Frank *et al.*, 1997). When juveniles were younger than 2 months, almost all allogeneic colonies fused to form morphologically stable chimeras. Then, for contacts that occurred between 2 and 4 months post-settlement, fusion was transitory and ended by tissue separation or death of a partner at the age of 4 months. After 4 months, no fusions between allogeneic tissues were recorded, indicating maturation of the allorecognition system. Thus, maturation of the allorecognition system in this brooding coral took approximately four months, but similar studies have not been done for broadcast spawning species.

In summary, within the first part of this chapter, I have shown that colonial marine invertebrates are able to discriminate self from nonself with a higher degree of precision in adults than in juveniles. Although the juvenile allorecognition system distinguishes distantly related tissues in early stages of life, it is not fully functional for up to four months post settlement in brooding corals. Thus, the creation of chimeric colonies in colonial marine invertebrates may primarily arise from fusion of allogeneic tissues in early life history stages. However, demonstrations of allogeneic tissue fusion among adult corals (Heyward & Stoddart, 1985; Resing & Ayre, 1985; Willis & Ayre, 1985) indicates that even mature allorecognition systems at least occasionally allow the fusion of genetically non-identical entities and the formation of chimeras. The greatest precision in self-recognition in adult contact reactions has been found for brooding species of corals, but this pattern cannot be assessed for juveniles because contact reactions studies have not yet been attempted for broadcast spawning species.

1.3 Chimerism in colonial marine invertebrates

Chimeras can originate from the fusion of closely settling larvae or from the fusion of colonies that come into contact, either while growing or after movement (Sommerfeldt *et al.*, 2003). Depending on the organisms under study, colonial marine invertebrates can form sectorial or cytomictical chimeras. Cytomictical chimeras are created when fusion establishes a common blood circulation system that mixes blood cells from each partner of the chimera, for example within chimeras of the colonial tunicate *Botryllus schlosseri*. Such fusions are termed "cytomictical" because some cells of the two parent organisms have become so mixed that they can no longer be separated into individuals (Rinkevich & Weissman, 1987). In contrast, natural coral chimeras (e.g. *Stylophora pistillata*) show no evidence of mixed cellular elements and each partner retains its original colour phenotype (Rinkevich & Weissman, 1987). Such fusions are termed "sectorial chimeras" because each partner maintains its individuality (Rinkevich & Weissman, 1987).

1.3.1 The origins of chimerism

Naturally occurring chimeras are known to exist within at least nine phyla of protists, plants and animals (Buss, 1982). Natural chimeras usually originate from allogeneic fusions (i.e., fusions between different individuals of the same species). These natural allogeneic fusions may be restricted to species in which fragmentation and fusion are normal features of the life cycle (Hughes, 1989). Although chimerism occurs in a wide range of organisms (Buss, 1982) and has even been recorded in mammals (Rinkevich, 1998; Rinkevich, 2001), including humans (a variety of autoimmune diseases, such as the persistence and proliferation of foetal cells in maternal blood circulation for several decades postpartum, were found to be linked to human microchimerism (Rinkevich, 2004b)), it has been reported much more frequently from the marine environment,

primarily from benthic organisms with planktonic larvae or propagules, such as red algae (Santelices *et al.*, 1999) or colonial marine animals including corals, bryozoans and ascidians (Santelices, 2004). Therefore, the occurrence of chimerism in colonial marine animals further challenges notions of genetic uniqueness within clonal organisms (Santelices, 1999) and the commonly held view that clonality is a mechanism for maintaining well-adapted lineages (Strassmann & Queller, 2004).

1.3.2 Extent of chimerism in natural populations of marine invertebrates

The application of molecular tools to the detection of chimerism in non-cnidarian colonial marine invertebrates has revealed relatively high levels within wild populations. Random Amplified Polymorphism DNA (RAPD) analysis assessed the presence and extent of chimerism in the colonial ascidian, *Diplosoma listerianum* (Sommerfeldt & Bishop, 1999), and revealed that 34% of *Diplosoma listerianum* colonies in a wild population on the Langness peninsula, Isle of Man (British Isles) possessed multiple genotypes (i.e., were chimeras). A similar study on one population from artificial settlement plates and seven natural populations of *Diplosoma listerianum* at sites adjacent to the Isle of Man, North Wales, Cornwall and Devon (UK) also revealed high levels of chimerism (Sommerfeldt *et al.*, 2003). In this latter study, RAPD analyses revealed that chimeric colonies were present in all populations studied, at frequencies ranging from 3% to 61%, with up to six different genotypes present in some colonies. Investigations of the population structure of the colonial hydroid *Hydractinia symbiolongicarpus,* which lives on gastropod shells inhabited by hermit crabs (*Pagurus longicarpus*) in Barnstable Harbor, Massachussetts (USA), estimated that the frequency of chimeras was around 6 to 7%, also based on RAPD analyses (Hart & Grosberg, 1999). Consequently, RAPD analyses has detected chimeras and demonstrated that chimerism is not rare in a range of colonial marine invertebrates.

Polymorphic microsatellites have also proven to be a useful molecular tool for assessing chimerism within natural populations (Pancer *et al.*, 1994). Microsatellites revealed "surprisingly high" levels of chimerism in natural populations of a colonial ascidian (*Botryllus schlosseri*) from New Zealand (Ben-Shlomo *et al.*, 2001). Patterns of genetic diversity analyzed among six populations based on 5 microsatellites loci, revealed an average chimerism level of 8%, reaching 14% within one population (Ben-Shlomo *et al.*, 2001). However, as natural chimeras in this study were defined as colonies expressing more than two alleles per individual at a locus and colonies that showed visual evidence of chimerism were purposely not collected, these levels of chimerism are likely to be underestimates. The use of highly polymorphic microsatellite loci in two different populations of the ascidian, *Botryllus schlosseri* (one native population from Caesarea (Israel) and one recently introduced population at Woods Hole marina (MA, USA)) revealed that ~9% of colonies were chimeras in these two distinct populations (Ben-Shlomo *et al.*, 2008). Consequently, the use of molecular tools to investigate the presence of chimeras in natural populations of colonial marine invertebrates demonstrates that levels of chimerism can be very high.

Examples described above illustrate that natural chimerism among allogeneic individuals is fairly common in colonial marine invertebrates, however, chimeras between xenogeneic individuals or colonies appear to be extremely rare. Nozawa and Loya (2005) reported a potential "chimera" of *Seriatopora caliendrum* and *Seriatopora hystrix* in the field, but analysis of the contact zone revealed that colonies were not fused, thus they did not constitute a real chimera. Thus, chimeras are more likely to be entities arising from fusions of colonies within the same species. Even in taxa and populations where allorecognition does not perfectly distinguish between self and

nonself, recognition "errors" leading to the formation of chimeras are likely to be confined to encounters between closely related kin (Grosberg, 1988).

1.3.3 Ecological implications of chimerism

The costs and benefits associated with the chimeric state have provoked ongoing debate concerning the ecological and evolutionary merits of chimerism. As chimerism can be costly, why are there so many cases of chimerism in natural populations of colonial marine invertebrates? Does chimerism simply constitute recognition errors as suggested by Grosberg (1988) or could it be linked to benefits that overcome costs that are often associated with the chimeric state?

Costs associated with chimerism

Both fusion and rejection outcomes of allogeneic contacts have been shown to significantly reduce the fitness of interacting colonies through reductions in growth and reproduction in the colonial ascidian *Botryllus schlosseri* under field conditions (Chadwick-Furman & Weissman, 2003). Reduced fitness of genetically distinct colonies in contact is likely to result from energetic costs associated with recognizing and reacting to nonself (Chadwick-Furman & Weissman, 2003). Previous evaluations of the potential fitness costs and benefits of chimerism in the same ascidians under laboratory conditions showed that the formation of chimeras did not improve survivorship, growth rate or the timing of reproductive maturity compared to non-chimeric colonies (i.e. no improved fitness) (Rinkevich & Weissman, 1992).

Fusion between compatible colonies of the coral *Stylophora pistillata* also decreased the overall growth rate and the reproductive output of fused colonies (Rinkevich & Loya, 1985). Similarly, detrimental effects from the chimeric state were noted during follow up observations on chimeras in 4 soft coral species: *Nephthea sp.*, *Heteroxenia*

fuscescens, *Parerythropodium fulvum* and *Clavularia hamra* (Barki *et al.*, 2002). Three main detrimental outcomes were seen: morphological separations of the partners of a chimera, sudden death of one or more partners, and the unilateral or reciprocal resorption of polyps leading to the morphological disappearance of some partners from the chimeras. Such negative outcomes of chimerism suggest that soft coral chimeras do not possess long term advantages over non-chimeric colonies and were likely to be less fit than non chimeras in natural field populations.

Evaluation of the selective advantages of forced chimerism in the sponge *Tedania ignis* indicated that although forced chimeric sponges were bigger, they did not have increased rates of survival (Maldonado, 1998). In this species of sponge, larvae did not show any significant tendency to settle close to siblings. Moreover, no natural fusions were observed between siblings (Maldonado, 1998). In summary, for both natural and forced chimerism, allogeneic fusion does not appear to provide fitness advantages through the chimeric state; on the contrary, this state appears to be costly.

One of the main costs to individuals in chimeric colonies is germ and somatic cell parasitism (Chadwick-Furman & Weissman, 2003). Germ cell parasitism occurs when a partner in a chimera uses the somatic resources of the other(s) in order to produce its own germ cells (Chadwick-Furman & Weissman, 2003). In such cases, fusion is associated with competition at the cellular level (Buss, 1990). Both germ and somatic cell parasitism (G/SCP) were found to be common in natural and laboratory chimeras of the colonial ascidian *Botryllus schlosseri,* reaching 40% within natural chimeras (Stoner & Weissman, 1996). As a single highly polymorphic histocompatibility locus (called Fu/HC) is responsible for rejection versus fusion reactions in these ascidians, high rates of G/SCP in fused colonies may explain why high levels of polymorphism for Fu/HC is necessary to limit G/SCP in sibling offspring (Stoner & Weissman, 1996). Thus, the

occurrence of two genotypes (or more) within the same individual or colony could eventually lead to cell linage competition for position in the germ line (Buss, 1982). This cell lineage competition represents a potentially severe cost to chimeric colonies (Buss, 1982).

Another potential cost of fusion between genetically different colonies is possible facilitation of pathogen transmission, as contact between different individuals represents a direct path for transmission (Grosberg, 1988). Overall, although chimeras express higher genetic diversity and likely possess more physiological attributes than non-chimeric colonies and thus may be more tolerant in heterogeneous environments (Rinkevich & Weissman, 1992), the chimeric state appears to be costly. If costs are too high, there will be selection against chimerism. This apparent paradox, highlighted by (Rinkevich & Weissman, 1992), may largely reflect outcomes from laboratory studies. Indeed, resorption of individuals following fusion in allogeneic contacts involving the tunicate *Botryllus schlosseri* was observed in laboratory studies, whereas field studies revealed that such resorptions are rare events in the wild (Rinkevich & Weissman, 1992; Chadwick-Furman & Weissman, 1995, 2003). According to Buss (1982), chimeras formed between compatible individuals are often stable, suggesting that naturally formed chimeras could be more stable than laboratory "forced" chimeras, because the latter are more likely to involve incompatible individuals (Buss, 1982).

Benefits of chimeric formation

Although natural fusion between allogeneic colonies may carry substantial fitness costs over the long term for species like *Botryllus schlosseri* and *Stylophora pistillata* (Rinkevich & Weissman, 1987), it has been speculated that such costs may be outweighed by benefits associated with the chimeric state, thus selective pressures maintain chimerism within natural populations (Rinkevich & Weissman, 1992).

In fossil reef complexes in Europe, many cases of intraspecific fusion have been observed between branches of the coral *Thamnasteria dendroida* (Helm & Schülke, 2000), revealing the existence of self-recognition and histocompatibility reactions in corals as early as the late Jurassic. Fusion of *Thamnasteria dendroida* branches strengthened the capacity of this species to face physical damage for these normally fragile ramose corals (Helm & Schülke, 2000). Thus intraspecific and interclonal fusion and cooperation have played and continue to play an important ecological role on coral reefs. These observations also suggest that chimeras were probably common, for corals at least, in the late Jurassic. Similar structural benefits are observable today in intraspecific contacts between different clones of *Agaracia tenuifolia* on Belizean shallow fore-reef buttresses (Chornesky, 1991). Fusion occurred in 2% of contacts between different clones, although use of colour to assign genotypes probably underestimated the real genetic diversity. Inter-clonal contact did not appear to result in competitive interactions, tissue bleaching, altered colony growth, or death. Instead, inter-clonal contact tended to anchor corals against each other, making them more resistant to physical disturbances on these shallow high-energy reefs. Comparisons of positive and negative aspects of inter-clonal contacts in *Agaracia tenuifolia* suggested that benefits exceeded the potential costs of a small localized loss of polyps in contact zones. Thus, chimerism has probably contributed to the ecological success of *Agaracia tenuifolia* in shallow high-energy reef habitats.

Another obvious advantage of chimera formation relates to rapid increases in size of early life history stages enabled by fusion, which can be more rapid than through growth alone (Raymundo & Maypa, 2004). For species with size-dependent survivorship (Sammarco, 1982; Jackson, 1986), such rapid increases in size following fusion provide clear advantages (Rinkevich & Weissman, 1987). Accordingly, young

recruits might find benefits within multipartner-chimeras which outweigh costs associated with interacting genotypes (Chadwick-Furman & Weissman, 2003), Indeed, one of the main advantages of multipartner kin aggregations at settlement is associated with an immediate and long term size increase (Amar *et al.*, 2008).

Studies of juveniles of *Pocillopora damicornis* demonstrated that fusion within 8 months post-settlement enhanced survival and growth of colonies (Raymundo & Maypa, 2004). Mortality was much lower in fused colonies compared to colonies comprised of one genotype, and lowest among multiple chimeras. The greatest benefits occurred for groups of multiple fusions because of a greater stability of the colony (chimeras formed prior to 8 months remained stable to 1 year of age) and fewer polyp resorptions were found in multi-colony chimeras than in bi-chimeras. Thus multi-chimeras have been suggested to have potential applications for reef rehabilitation (Raymundo & Maypa, 2004) and may play an important role in coral reef replenishment following major disturbances. Nevertheless, although this study established short term benefits of chimeras, the potential for long term costs remains.

Fusion between allogeneic colonies can also lead to oriented translocation of organic products of algal photosynthesis and was observed during grafting experiments involving *Stylophora pistillata* (Rinkevich & Loya, 1983). The “recipient” coral metabolised the photosynthetic products derived from the “donor” coral. Even if this energy translocation could be considered a classical case of parasitism, such energy translocation could provide benefits to the chimera and could be ecologically important in the recovery of coral reefs after storm or hurricane damage (Rinkevich & Loya, 1983). For example, fusions between allogeneic branches and their fast recovery following destruction might be explained by the oriented translocation of energy

observed in grafting experiments of the coral *Stylophora pistillata* (Rinkevich & Loya, 1983).

Finally, chimeras with mixed cells from two different genotypes may reduce the risk of genotype extinction by spreading a genotype (Grosberg, 1988). This benefit may be especially important in clonal organisms, such as corals, that can suffer partial mortality (Grosberg, 1988). Overall, six potential benefits of the chimeric state have been proposed (Rinkevich and Weissman 1987):

-1- Theoretically, chimeric colonies may have a greater store of genetic variability and hence a wider range of physiological qualities and characteristics compared to non-chimeric colonies (Ben-Shlomo *et al.*, 2001). A greater store of genetic variability within one colony could be of particular importance in clonal organisms, and could prevent favourable genetic combination from being lost in the sexual process (Buss, 1982).

-2- A chimera formed from two aberrant genotypes could form a synergistic complement with normal structures.

-3- Fusion provides a mechanism for more rapid size increase than through growth alone, and could thus increase chances of survival (Sammarco, 1982; Jackson, 1986). This could be beneficial for species where survivorship is size dependent (Rinkevich & Weissman, 1987).

-4- The onset of reproduction can also be size dependent. The formation of a chimera may thus lower the age of reproductive maturity due to the increased size after fusion.

-5- The increased body size of chimeric colonies may also provide some benefits to sedentary species competing for available substratum. Moreover, other ecological benefits such as more environment tolerance can come from this increased size.

-6- Chimerism may also be beneficial for mate location in sedentary organisms (Buss, 1982).

The selective forces behind chimerism

As outlined above, fusion between closely related colonies or individuals may confer benefits, thus it is probable that the self-nonself recognition systems of colonial marine invertebrates limit fusions to those between close relatives to maximise benefits and minimise costs associated with fusion (Feldgarden & Yund, 1992).

Heterosis, or chimeric vigour, is a potential selective advantage of chimerism. In fact, colonies that are heterozygous at allorecognition loci would tend to be selected for after colony resorption, which sometimes follows chimera formation, rather than homozygote colonies that might be less adaptive under unstable environmental conditions (Rinkevich, 2004b). Moreover, Rinkevich also highlighted the fact that many previous studies have examined chimerism in bi-chimeras, whereas gregarious settlement of larvae would rather favour formation of multi-chimeras. Multi-partner chimeras form more stable and vigorous entities (Raymundo & Maypa, 2004) and their formation sets the "group level" as the key level at which natural selection may act (Rinkevich, 2004b).

1.4 Aims of thesis

Chimerism appears to be common in plants, vertebrates and invertebrates but remains a "mysterious" phenomenon, because efficient self-nonself recognition systems would not be expected to accept different genotypes within the same colony.While earlier studies of juvenile brooding corals have shown a high potential for chimerism under experimental conditions, no genetic study has demonstrated the occurrence of mixed genotypes within these coral chimeras. Furthermore, chimera formation in broadcast

spawning corals, the dominant type of coral on Indo-Pacific reefs, has never been examined.

Accordingly, in Chapter 2, I investigated whether a representative of the most widespread type of coral (i.e. broadcast spawning corals) was able to form chimeras under experimental laboratory conditions, and the fate of different genotypes following fusion in the field. The model organism used was *Acropora millepora*, a common broadcast-spawning coral that is both abundant and widespread on the Great Barrier Reef. *A. millepora* is also the best characterized coral at the molecular level and appropriate molecular markers to explore chimerism were readily available (van Oppen *et al.*, 2007).

Evidence that allogeneic fusion among juveniles of brooding coral species is facilitated by immature allorecognition systems in the first four months following settlement suggests that allorecognition in broadcast spawning corals might also require time to mature. In Chapter 3, I followed allorecognition maturation and investigated if broadcast spawning corals require a period of time during their early life to reach a mature state of allorecognition. Contact reactions between juvenile *A. millepora* up to 12 months following settlement were regularly monitored in the field in order to assess the maturation of allorecognition in spawning corals.

In Chapter 4, I explore whether immune related genes were differentially expressed in *Acropora millepora* juveniles in the field during the period of allorecognition maturation following settlement and during the establishment of symbiosis with *Symbiodinium*. Using real time PCR, the expression of apextrin, complement C3, and 2 CELIII type lectin genes was followed in early post-settlement life (up to 6 months) of *A. millepora,* in order to assess the potential involvement of these genes in allorecognition.

Finally, as the extent to which natural chimeras occur in populations of reef corals is currently unknown, in Chapter 5 I explore the extent of genetic chimera occurrence (i.e., the cohabitation of different genotypes within a single coral colony) within two populations of *Acropora millepora* on the Great Barrier Reef (Australia), using genetic characterization of coral tissues at 12 polymorphic DNA microsatellite loci.

Chapter 2.0 High potential for formation and persistence of chimeras following aggregated larval settlement in the broadcast spawning coral, *Acropora millepora*

2.1 Introduction

Chimerism is defined as the co-habitation of more than one genetically distinct cell line originating from more than one zygote within the same individual (Rinkevich & Weissman, 1987) and is known to occur naturally in at least nine phyla of protists, plants and animals (Buss, 1982). Chimerism is also known from humans (Rinkevich, 1998; Rinkevich, 2001) but it has been reported more frequently in marine environments for benthic organisms with planktonic larvae or propagules, including seaweeds or colonial marine animals, such as sponges, hydroids, corals, bryozoans and ascidians (Santelices, 2004). Chimerism typically follows allogeneic fusions (i.e. fusions between genetically different individuals of the same species) and may be most common in species for which fragmentation and fusion are normal features of the life cycle (Hughes, 1989). However, the formation of chimeras in broadcast spawning corals, the spatially dominant and numerically most abundant group of reef corals, has not been investigated.

Sessile, modular marine animals have a number of life history traits that increase the probability of prolonged contact among neighboring colonies, which enhances opportunities for fusion and thus the potential for chimera formation (Sommerfeldt *et al.*, 2003). In particular, their larvae tend to settle in proximity to one another (Keough, 1984) and adult colonies often come into physical contact when colonies increase in size through growth or after movement of fragments produced through asexual reproduction (e.g. sponges, cnidarians, bryozoans, and ascidians). The occurrence of chimeras in natural populations (Sommerfeldt & Bishop, 1999; Ben-Shlomo *et al.*, 2001; Sommerfeldt *et al.*, 2003; Rinkevich, 2005; Ben-Shlomo *et al.*, 2008) suggests that fusion of non-identical, conspecific genotypes is sometimes permitted, despite the fact that colonial marine invertebrates generally discriminate between clone mates and

non-clone mates (Heyward & Stoddart, 1985; Stoddart *et al.*, 1985; Willis & Ayre, 1985; Grosberg, 1988). Rather than representing allorecognition failure, it is possible that by potentially increasing survival of early life history stages, chimerism is adaptive in sessile, colonial marine animals (Amar *et al.*, 2008). However, this hypothesis has not been explicitly tested.

In cnidarians, chimerism has been well-studied in brooding corals (Neigel & Avise, 1983; Hidaka, 1985a; Hidaka, 1985b; Stoddart *et al.*, 1985; Chadwick-Furman & Rinkevich, 1994; Frank & Rinkevich, 1994; Frank *et al.*, 1997; Hidaka *et al.*, 1997; Frank & Rinkevich, 2001; Barki *et al.*, 2002; Rinkevich, 2004a; Nozawa & Loya, 2005; Amar *et al.*, 2008), however, there has been only one study of chimerism in a broadcast spawning coral, and it focused on adult patterns of chimerism (Puill-Stephan *et al.*, 2009). In studies of brooding corals, kin aggregations of larvae at settlement and subsequent fusion have been shown to promote the occurrence of chimerism in coral juveniles (Rinkevich, 2004a; Amar *et al.*, 2008). In young colonies of the coral *Pocillopora damicornis*, fusion always occurred between colonies originating from larvae released from the same source colony (i.e. between full or half siblings). Larvae derived from different colonies (non siblings or half siblings) rarely fused, instead contact reactions between these allogeneic larvae mainly resulted in non-fusion or incompatible fusion (Hidaka *et al.*, 1997), indicating that fusion is strongly influenced by relatedness among the different entities in contact. In addition, a delay in maturation of the coral allorecognition system (Frank *et al.*, 1997) has been hypothesized to give rise to a "window in ontogeny" during which fusion of genetically distinct conspecifics is facilitated (Rinkevich, 2004c). It has been suggested, however, that chimerism in corals is unimportant or non-beneficial because chimeric partners may separate or because death or resorption of one partner may occur (Rinkevich 2004b). Moreover,

chimerism challenges evolutionary theory developed for genetically homogeneous individuals (Santelices, 1999) and the commonly held view that clonality is a mechanism for maintaining genotypes intact, particularly for well-adapted lineages (Strassmann & Queller 2004).

Recent evidence that chimerism occurs in wild populations of the broadcast spawning coral *Acropora millepora* (Puill-Stephan *et al.*, 2009) indicates that chimerism is not limited to brooding species with limited larval dispersal and raises questions about mechanisms promoting chimera formation in species with dispersive larvae. Philopatric settlement of brooded larvae increases the probability that closely related juveniles come into contact, thereby enhancing the likelihood of chimera formation, while encounters between closely related larvae of broadcast spawning corals appear to be less likely. However, for broadcast spawning corals that breed annually and release thousands to millions of gametes with high synchrony (Babcock *et al.*, 1986), localised mixing of gametes would enhance relatedness of the larvae produced. Furthermore, closely related larvae may remain aggregated in dense spawning slicks that form during low to moderate wind conditions typical of coral spawning seasons (Oliver & Willis, 1987). Larvae within spawning slicks tend to reach settlement competency at the same time (Graham *et al.*, 2008; Siegel *et al.*, 2008; Heyward & Negri, 2010), further increasing the probability that closely related larvae might settle in the same area, fuse and form chimeras. Hence, broadcast spawning corals may also have life history traits that promote chimera formation, but the potential for chimerism in such species has not been directly tested.

Here I investigate the potential for a broadcast spawning coral to form chimeras during early life history stages and the fate of different genotypes following fusion when chimerism occurs. *Acropora millepora*, a common broadcast-spawning coral, was

selected as the model organism because, in addition to being abundant and widespread on the Great Barrier Reef, it is also the best characterized coral at the molecular level, and molecular markers appropriate for exploring chimerism are readily available (van Oppen *et al.*, 2007). Using 9 polymorphic microsatellites, I also investigate whether multiple genotypes co-occurring within the same colony persist over time, and whether persistence correlates to level of genetic relatedness.

2.2 Materials and methods

2.2.1 Rearing of coral larvae

Eight mature colonies of the hermaphroditic, broadcast spawning coral *Acropora millepora* were collected from each of two field sites (Nelly Bay, Magnetic Island, and South West Pelorus Island; both located in the central Great Barrier Reef and separated by 70 km) prior to the predicted spawning dates in November (Magnetic Island) and December 2006 (Pelorus Island). Colonies were transferred to 1000 litre aquaria with running, temperature-controlled (28.5°C), 1 µm filtered sea water (FSW) at the Australian Institute of Marine Science for Magnetic Island corals and at Orpheus Island Research Station for Pelorus Island corals. The genotype of each coral colony collected was determined prior to spawning based on analyses of 3 microsatellite loci (van Oppen *et al.*, 2007) to ensure colonies were genetically distinct and to avoid crosses between clone mates.

Colonies were maintained in 1000 litre aquaria for up to 10 days after collection, and were isolated just prior to spawning in individual 70 L aquaria filled with FSW, and kept isolated for at least 45 minutes after spawning had commenced. Gametes from the 5 most prolific colonies were collected from the water surface of respective isolated aquaria and mixed with gametes from a second colony in a new 70 L fertilization

aquarium filled with FSW, according to the experimental design shown in Figure 2.1A. In summary, gametes from each pair-wise combination of the five selected parent colonies at each site were mixed to create one of three gamete crosses (Figure 2.1A), which resulted in the production of three larval batches or broodstocks that represented three full sibling cultures (A, B, and C). Gametes were allowed to fertilise for at least 1.5-2 hours, after which time a small subset of eggs was sampled for microscopic confirmation of fertilisation and development. Embryos were cleaned in three consecutive water changes by draining ~90% of water from the bottom of the fertilization aquaria and slowly filling from the top. Embryos were then transferred into 500 L larval culture aquaria supplied with running, temperature-controlled (28.5°C) FSW in a controlled environment facility, at a density of approximately one larva per mL. Embryos were checked microscopically in order to assess their development until ~48 hours following fertilisation, when the fully developed, ciliated planula stage was reached. Once swimming larvae were elongated and demonstrated searching behaviour for settlement, larvae were transferred, at stocking densities of ~1 larva/mL, into smaller aquaria (40 L), the bottoms of which were covered with settlement plates.

To establish mixtures of larvae with differing kinship levels for settlement purposes, two samples of approximately 20 000 larvae from the broodstock cultures (i.e., from the 500L larval aquaria) were mixed into 40L settlement aquaria. Larvae were distributed among settlement aquaria to create three kinship levels: full sibling mixes (broodstock A with A, B with B, C with C); half sibling mixes (broodstock A with B); and non-sibling mixes (broodstock A with C, and B with C) (illustrated in Figure 2.1). Note that both half sibling and non-sibling mixtures also contained full sibling larvae, such that any given larva had a similar probability (~50%) of contacting full sibling and either half or non-sibling larvae, respectively. Each kinship level was established in 4 replicate

settlement aquaria (Figure 2.1B), each of which contained 9 autoclaved terracotta tiles (8 cm x 8 cm = 64cm^2). Larval mixtures provided opportunities for larvae of varying genetic relatedness to settle on the same piece of substratum. Juveniles that settled in groups of two or more recruits that were in physical contact with one another were defined as aggregations.

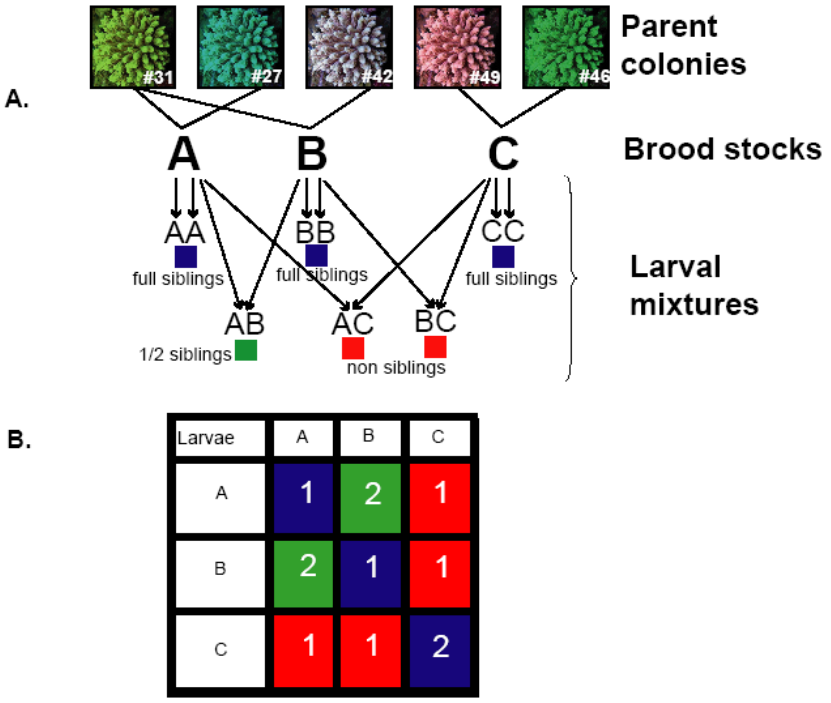

Fig. 2.1. Schematic diagrams showing: **A)** experimental design for mixing gametes from different colonies (identified by numbers) to produce larvae that differed in kinship level (full siblings, half siblings, or non siblings); and **B)** the number of replicates (indicated by numbers within each square) for combinations of broodstock larvae (A, B, and C) that were mixed together and added to settlement aquaria. Overall, there were 4 replicate aquaria established for settlement purposes per kinship level. Note that both half sibling and non-sibling mixtures also contained full sibling larvae, such that any given larva had a similar probability (~50%) of contacting full sibling and either half or non-sibling larvae, respectively.

Twenty days after spawning, the laboratory-reared juvenile corals were placed in the field. The terracotta tiles were skewered on rods through a hole in the centre of the tiles and spacers (2-3 cm long) placed between each tile. The rods were suspended horizontally between two star pickets that had been driven into dead substratum at a depth of 5m on an inshore reef at Nelly Bay, Magnetic Island. Tiles containing corals were oriented vertically. Tiles were labelled, tagged and photographed prior to deployment in the field. In order to investigate the persistence of chimeras, tiles from Magnetic Island were monitored and photographed every 4-8 weeks during the first year and less frequently in the second year until 23 months post-settlement. Photographs enabled the fusion history of colonies that were sampled for genetic determination of chimeric state, to be traced from settlement.

Sampling for genotyping was performed 6 times (4 to 10 samples collected at each time point) from about 6 months through to 2 years after settlement. Juvenile colonies were sampled haphazardly at each sampling time without knowing their origin and without regard to size (i.e. whether colonies were large and potentially represented aggregations or small and more likely to have originated from a solitary polyp), however, healthy looking colonies were preferentially sampled. At each sampling time, 1 recruit was haphazardly selected from each of 4-10 tiles that were also haphazardly selected. Because of the small size of coral juveniles ($<1cm^2$), sampling resulted in sacrificing the whole colony. Colonies were sub-sampled and divided into either 4 fragments (named 1-4) or 8 fragments (named 1-8) from 15 months post-settlement because older corals were large enough to allow higher levels of sub-sampling. DNA was extracted from each colony or sub-sample and was genotyped using nine microsatellite loci (Table 2.1). Samples were named according to: broodstock origin of larvae in the mixture, thus AB represented a mixture of full sibling and half sibling larvae from broodstocks A and B;

tile number (up to 36 depending on broodstock mixture); recruit ID letter (a-h, used to locate recruits on photographs); and the recruit subsample number (up to 8, depending on the size of the recruit, which governed the number of subsamples). For example, the sample AC4e1 is subsample #1 (out of 8) of the recruit named "e" on tile # 4 of the sibling/non-sibling larval mixture A x C. The precise location of each colony sampled (as identified by letters a-h) on each tile was noted so that colonies could be identified in monitoring photographs and the origin of each colony, from either a single larval recruit or an aggregation of larvae, determined.

2.2.2 DNA extraction and genotyping

DNA was extracted using "Wayne's method" (Wilson *et al.*, 2002). DNA pellets were re-suspended in 200 mL of 0.1 M Tris (pH = 9.0) and stored at 4°C. Prior to amplification, DNA was diluted at 1:10 in milliQwater. Microsatellite loci were amplified in 10 µL multiplex PCR reactions, in PTC-100® Peltier Thermal Cyclers. Three different primer mixes (MP2, MP3, and MP5, see Table 1), each amplifying three microsatellite loci developed for *A. millepora* (van Oppen *et al.*, 2007), were used. Reactions contained 1 µL DNA template, 1 µL 10x primer mix, 5 µL 2x Qiagen multiplex PCR kit, and 3 µL milliQwater. The cycling protocol was: 1 x 95°C (15 min), 35 x (30 sec at 94°C, 90 sec at 50°C, and 60 sec at 72°C), 1 x 60°C (30 min), and 4°C. PCR products were diluted in Sample Loading Solution (SLS from Beckman Coulter) at 1:10. Then, 2.5 µL of the diluted PCR products were loaded, together with 37.25 µL of SLS and 0.25 µL of 400 bp size standard, into a Genetic Analysis System CEQ 8800 from Beckman Coulter, for separation and subsequent PCR product size determination.

Table 2.1. Primer mixes, associated microsatellites and dyes, and concentrations

Primer mix name	Microsatellite loci	Repeat motif	Associated WellRED dye	Concentration in 10x primer mix
MP2	Amil2_006	(CA)4TA(CA)4	D2	0.8 µM
MP2	Amil5_028	(TCACA)7TCAC (TCACA)4 TCACTCACTCACA	D3	0.8 µM
MP2	Amil2_002	(TG)10	D4	0.28 µM
MP3	Apam3_166	(AAT)28	D2	1.5 µM
MP3	Amil2_22	(AC)10	D3	1.0 µM
MP3	Amil2_23	(AG)7	D4	0.6 µM
MP5	Amil2_010	TA(TG)11	D2	0.5 µM
MP5	Amil2_012	GA(CA)6GA(CA)2	D3	0.3 µM
MP5	Amil2_007	(TG)7AG	D4	0.5 µM

2.2.3 Scoring

Once samples were run through the CEQ 8800, data were analyzed with Fragment Analysis software from the Genetic Analysis System CEQ 8800 from Beckman Coulter (400FragmentAnalysisParameter). All results were scored manually. Based on peak values for Negative Controls, peaks under 5 000 RFU were not scored. Fragment sizes were then entered into Microsoft Excel for further analysis.

Estimates of the rate of somatic mutations per locus per cell generation (10^{-7}) for multicellular clonal organisms (e.g., *Goniastrea aspera, G. favulus,* and *Platygyrus sinensis*; Orive, 2001) suggest that it is highly unlikely that two independent somatic mutations occur in the same tissue. We are therefore confident that if genotypes of two or more subsamples within a single recruit differed at two or more alleles, the colony was chimeric. Consequently, when genotypes of subsamples from a single colony displayed at

least two non-shared alleles and no obvious sign of tissue rejection was detected in photographic series (see figure 2.4) the colony was considered a chimera.

2.2.4 Surface area and Survival

To compare the size of colonies originating from solitary versus aggregated larval settlement, the surface area of coral juveniles was measured from photographs using the software package Canvas (ACD systems). Such measurements adequately capture growth up to 4 months post-settlement because of the primarily 2-dimensional structure of colonies at this early life history stage. Accordingly, the surface areas of 30 randomly selected colonies, which did not show signs of rejection among interacting genotypes, from each of the solitary and aggregated categories of juveniles raised at Magnetic Island were measured at settlement and at monthly intervals until 4 months post-settlement. To compare survival of Magnetic Island juveniles in each category, the number of colonies originating from solitary versus aggregated larval settlement on each tile was counted at settlement and at monthly intervals until 4 months post-settlement.

2.2.5 Analysis

Statistical tests were performed with Statistica 6.0. Normality of settlement, surface area, and survival data were investigated with the Shapiro-Wilk test (if $N<50$) or with the Kolmogorov-Smirnov test (with Lillefors significance correction). As the normality assumption was not met, a non-parametric Mann-Whitney U test was performed for comparisons of the mean number and mean surface area of juveniles per tile originating from solitary versus aggregated larval settlement.

Relatedness between genotypes within chimeras and among genotypes of rejecting colonies was calculated with the Queller and Goodnight estimator in GenAlEx 6.1

(Peakall & Smouse, 2006). Queller and Goodnight's pairwise relatedness estimator (QG) values are expected to be equal or higher than 0.5 (i.e. QG≥0.5) for full siblings. Half siblings are expected to have values around 0.25, and QGs of unrelated individuals (i.e., non-siblings) are expected to be close to zero (Queller & Goodnight, 1989).

2.3 Results

2.3.1 Gregarious versus solitary larval settlement

Under the laboratory experimental conditions of this study, more than 47% of *Acropora millepora* juveniles monitored originated from gregarious larval settlement (n = 2168 juveniles examined; Figure 2.2A). In the Magnetic Island study, the mean number of colonies per tile originating from gregarious settlement (14.3±1.7) was similar to the mean number originating from solitary larval settlement (14.5±1.5) (U=1473, n1=n2=56, P=0.580; Figure 2.2A). However, significantly more (~1.5 fold more) colonies originated from solitary polyps (10.2±2.0) than from aggregations (7.0±2.8) in the Pelorus Island study (U=326.5, n1=n2=32, P=0.012; Figure 2.2A). Nevertheless, because each aggregation represented a minimum of two fused recruits, overall, the majority of larvae settled in aggregations. Indeed, at least a two-fold greater number of larvae settled gregariously in the Magnetic Island study (i.e. a minimum of 1600 larvae assuming that aggregations originated from only 2 larvae) and at least a 1.3-fold greater number settled gregariously in the Pelorus Island study (i.e. a minimum of 456 larvae).

To determine whether the high numbers of aggregated larvae found were likely to have arisen as a consequence of random settlement patterns rather than gregarious settlement, the mean size and number of new recruits were compared to the surface area available for recruitment. Combining the solitary and aggregated categories of larval settlement, the approximately 30 juveniles that recruited on average to each of the settlement tiles

represented 1 recruit per 2 cm^2 of available surface area (tile dimensions were 8cm x 8cm). Considering that the mean surface area of a recruit originating from aggregated settlement was 0.018 cm^2 ±0.001 (n=30 recruits derived from aggregated settlement), thirty recruits would have occupied a maximum surface area of 0.55cm^2 of the 64cm^2 available for settlement. Thus, on average, larvae recruited to less than 1% of the available surface area, suggesting non-random patterns of settlement.

To evaluate whether genetic relatedness was a driving factor behind aggregated settlement, the numbers of juveniles with aggregated versus solitary origins were compared between larval mixtures representing full siblings only (i.e. AA, BB, and CC larval mixtures in Figure 2.1B) and larval mixtures including all relatedness groups (full siblings, half siblings and non-siblings, i.e. AB, AC, BC larval mixtures in Figure 2.1B). No significant difference was found between the mean number (11.8±2.9) of juveniles originating from aggregated settlement in full sib larval mixtures versus the mean number (11.6±1.6) of juveniles originating from aggregated settlement in larval mixtures with the full range of kinship types (U=860.5, n1=54, n2=34, P=0.622; Fig. 2.2B).

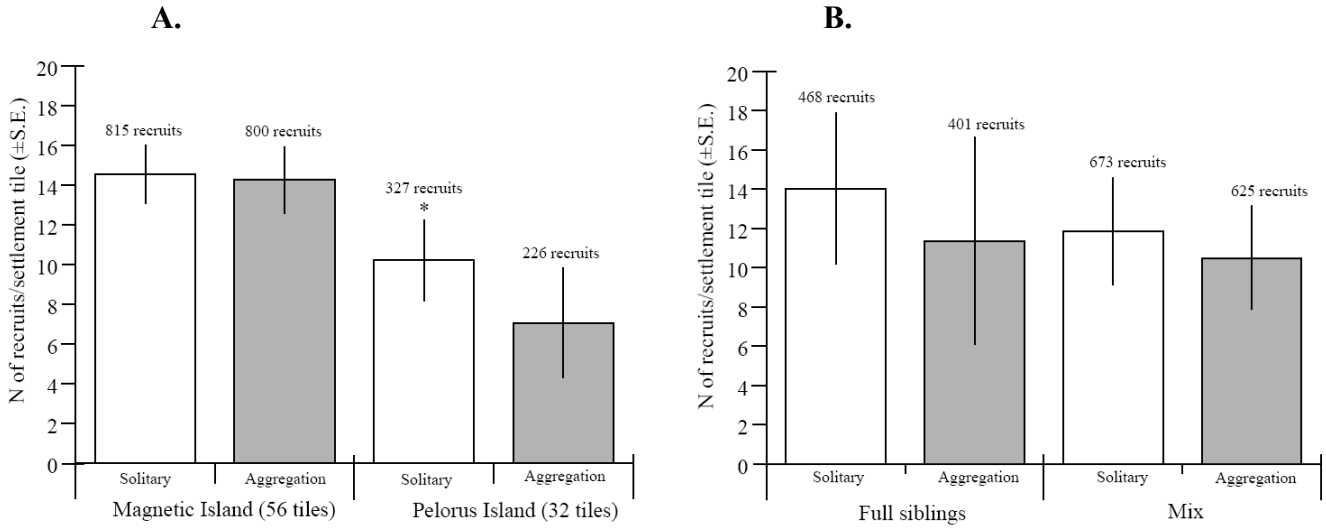

Fig. 2.2. Mean (±S.E.) number of *Acropora millepora* juveniles per tile that settled in aggregations (■) or solitarily (□) for: **A)** juveniles originating from spawning at Magnetic Island (n=56 tiles) or Pelorus Island (n=32 tiles). *: denotes a significant ($P<0.05$) difference in the number of recruits originating from aggregations versus solitary juveniles in the Pelorus Island study; and **B)** juveniles from the combined Magnetic and Pelorus Island studies originating from full sibling cultures or from a mix of full, half and non-sibling larvae. Numbers above each histogram represent the total number of juveniles counted in each category (i.e. numbers of recruits originating from aggregated versus solitary larval settlement).

Significant differences in the mean size of recruits derived from solitary versus gregarious larval settlement were detected both at settlement (U=18, n1=n2=30, P<0.001; Figure 2.3A) and at 3 months post settlement (U=185.5, n1=n2=30, P<0.001; Figure 2.3A). Mean surface area of recruits originating from gregarious settlement was approximately three-fold greater than mean surface area of recruits originating from solitary larvae at settlement and 2.5-fold greater at three months post-settlement.

No significant difference was observed in the mean number of recruits per tile originating from solitary versus aggregated settlement at any time in the first four months post settlement (e.g., at 3 months: 4.6±0.7 solitary recruits/tile versus 4.3±0.7 aggregated recruits/tile, N.S., U=1496, n1=n2=56, P=0.675; Figure 2.3B). Consequently, survival of both aggregated and solitary recruits was similar.

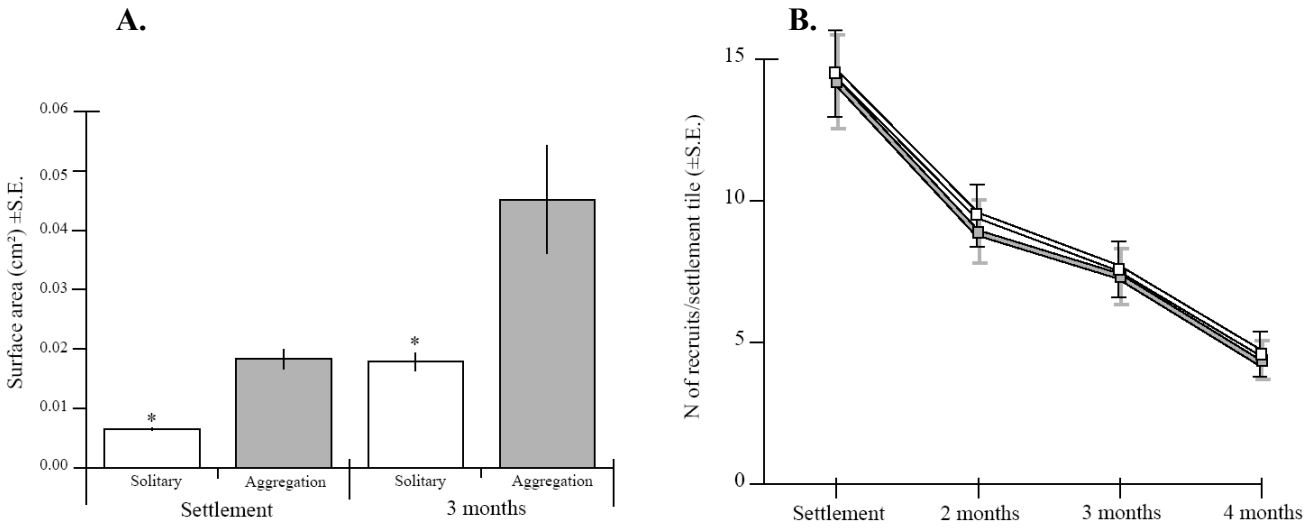

Fig. 2.3. Comparisons among means (±S.E.) from the Magnetic Island study for: **A)** surface area of juvenile colonies of *Acropora millepora* that had originated from solitary (□, n=30) versus aggregated (■, n=30) larval settlement, at both settlement and 3 months post-settlement; and **B)** survival of *Acropora millepora* juveniles originating from aggregated (■) versus solitary (□) settlement in the 4 months post-settlement. *: denotes significant (P<0.05) differences in surface areas between colonies originating from solitary versus aggregated larvae.

2.3.2 Persistence of chimerism in coral juveniles

A total of 42 of the experimental juvenile colonies of *A. millepora* that were settled in the laboratory and then raised at Magnetic Island were sampled for molecular analyses over a period of 2 years (between settlement in November 2006 and termination of the study in October 2008). One-half of the corals sampled were chimeras (i.e. 21 of the 42 colonies; Table 2.2). Chimeras originated either from fusion of recruits at settlement (i.e. 10 of the chimeric juveniles detected; e.g. Fig. 2.4C), from fusion of colonies coming into contact through growth (6 cases; e.g. Fig. 2.4B), or potentially from both types of fusions (5 cases, including AB4g). The origin of colony AB4g could not be determined as it was not observed at settlement because of its location on the underside of the tile.

Fusion events were observed from the time of settlement (November 2006) until about 10-11 months post-settlement (September 2007, i.e. sampling period 3 in Table 2.2). Fusion was assumed when no discontinuity in tissues could be observed along the zone of contact, and newly formed polyps appeared in the contact area (Fig. 2.4B in July 2007). Histological confirmation of fusion (and rejection reactions) could not be performed because juveniles were sacrificed for DNA extraction. Chimeras were found at every sampling time, even 23 months post-settlement (Table 2.2).

A slight majority of chimeras (12 out of 21) were multi-partner chimeras, resulting from the association of three or more initial recruits (Table 2.2). Eight of the remaining chimeras resulted from the fusion of two initial recruits (Table 2.2), and were defined as bi-partner chimeras. As colony AB4g was not observed at settlement, it could not be defined as either a bi or multi-partner chimera.

Colonies BC4c, AC3d, BC7k and the contact interaction between colonies BB4g and BB4h showed clear rejection reactions, characterized by a white line along tissue

margins where the two colonies met in the contact zone, and clear tissue discontinuity (Fig. 2.4A). All rejections detected (4 in total) originated from the rejection of juveniles coming into contact during growth. The first observation of rejection reaction were noted ~6 months post-settlement (May 2007, sampling period 1 in Table 2.2).

Table 2.2. Summary of fused versus isolated status of *Acropora millepora* juveniles raised at Magnetic Island and collected at 6 different sampling times, denoted as sampling periods: 1 (May 07), 2 (July 07), 3 (September 07), 4 (February 08), 5 (March 08), and 6 (October 08). Chimeras were identified based on 9 microsatellites and are indicated in **bold**. *: denotes cultures comprising only full-sibling larvae.

Sample	State at settlement (16/11/06)	genotype	State at sampling (sampling period)	Comments during growth (date of observation: month/year)
* CC8a	1 juvenile	1 genotype	1 colony (1)	
AC6a	1 juvenile	1 genotype	1 colony (1)	
* CC3c	1 juvenile	1 genotype	1 colony (2)	
AB14c	1 juvenile	1 genotype	1 colony (2)	
* AA1d	1 juvenile	1 genotype	1 colony (3)	
AC7e	1 juvenile	1 genotype	1 colony (4)	
AC4g	1 juvenile	1 genotype	1 colony (5)	
BC9k	1 juvenile	1 genotype	1 colony (6)	
AC7k	1 juvenile	1 genotype	1 colony (6)	
AC4d	2 isolated juveniles	2 genotypes	2 colonies fusing (3)	
*** BB3a**	**2 isolated juveniles**	**Chimera**	**1 colony (1)**	**contact & fusion (04/07)**
AC8c	**2 isolated juveniles**	**Chimera**	**1 colony (2)**	**contact & fusion (05/07)**
*** BB4c**	**2 isolated juveniles**	**Chimera**	**1 colony (2)**	**contact & fusion (06/07)**
BB4g	**2 isolated juveniles**	**Chimera**	**1 colony rejecting BB4h (5)**	**Contact & fusion (08/07), Contact with BB4h & rejection (01/08)**
*** BB4h**	**2 isolated juveniles**	**chimera**	**1 colony rejecting BB4g (5)**	**Contact & fusion (08/07), Contact with BB4g & rejection (01/08)**
AC3d	3 isolated juveniles	2 genotypes	2 rejecting colonies (3)	contact & fusion between 2 juveniles (08/07) contact & rejection with 3rd juvenile in (08/07)
BC8a	2 fused juveniles	1 genotype	1 colony (2)	
*BB4k=BB4g &h	4 isolated juveniles	2 genotypes	2 colonies no obvious rejection (6)	Contact & fusion by pair (08/07), contact & rejection of pairs (01/08)
* AA5d	2 fused juveniles	1 genotype	1 colony (3)	
AB16a	**2 fused juveniles**	**Chimera**	**1 colony (1)**	
BC9a	**2 fused juveniles**	**Chimera**	**1 colony (1)**	
AC3c	3 fused juveniles	1 genotype	1 colony (2)	
BC9b	**3 fused juveniles**	**Chimera**	**1 colony (1)**	
*** AA5e**	**3 fused juveniles**	**Chimera**	**1 colony (4)**	
AB4e	**3 fused juveniles**	**Chimera**	**1 colony (4)**	
AC3e	**3 fused juveniles**	**Chimera**	**1 colony (4)**	
AB1e	4 fused juveniles	1 genotype	1 colony (4)	
AC1a	**4 fused juveniles**	**Chimera**	**1 colony (1)**	**contact & fusion with another small colony (04/07)**
*** AA4c**	**4 fused juveniles**	**Chimera**	**1 colony (2)**	
AB7c	**6 fused juveniles**	**Chimera**	**1 colony (3)**	
* BB6c	Juvenile aggregation	1 genotype	1 colony (3)	
AB1f	Juvenile aggregation	1 genotype	1 colony (4)	
AB2a	**Juvenile aggregation**	**Chimera**	**1 colony (1)**	
AB2b	**Juvenile aggregation**	**Chimera**	**1 colony (1)**	
*** BB3c**	**Juvenile aggregation**	**Chimera**	**1 colony (2)**	**2 separated colonies surviving, contact & fusion (06/07)**
AB2k	**Juvenile aggregation + 2 juveniles**	**Chimera**	**1 colony no sign of rejection (6)**	
BC7k	1 juvenile + aggregation	1 genotype	1 colony (6)	contact and rejection with solitary juvenile, death of solitary (05/07)
BC4c	1 juvenile & 4 fused juvenile	2 genotypes	2 rejecting colonies (2)	contact & rejection (05/07)
* AA3e	3 fused juveniles + 1 isolated juvenile	1 genotype	1 colony (4)	contact & fusion (09/07)
AC4e	**2 fused juveniles + 2 isolated juveniles**	**Chimera**	**1 colony (4)**	**contact & fusion (09/07)**
AC8e	**3 fused juveniles + 2 fused juveniles**	**Chimera**	**1 colony (4)**	**contact & fusion (08/07)**
AB4g	**unknown…**	**Chimera**	**1 colony (5)**	

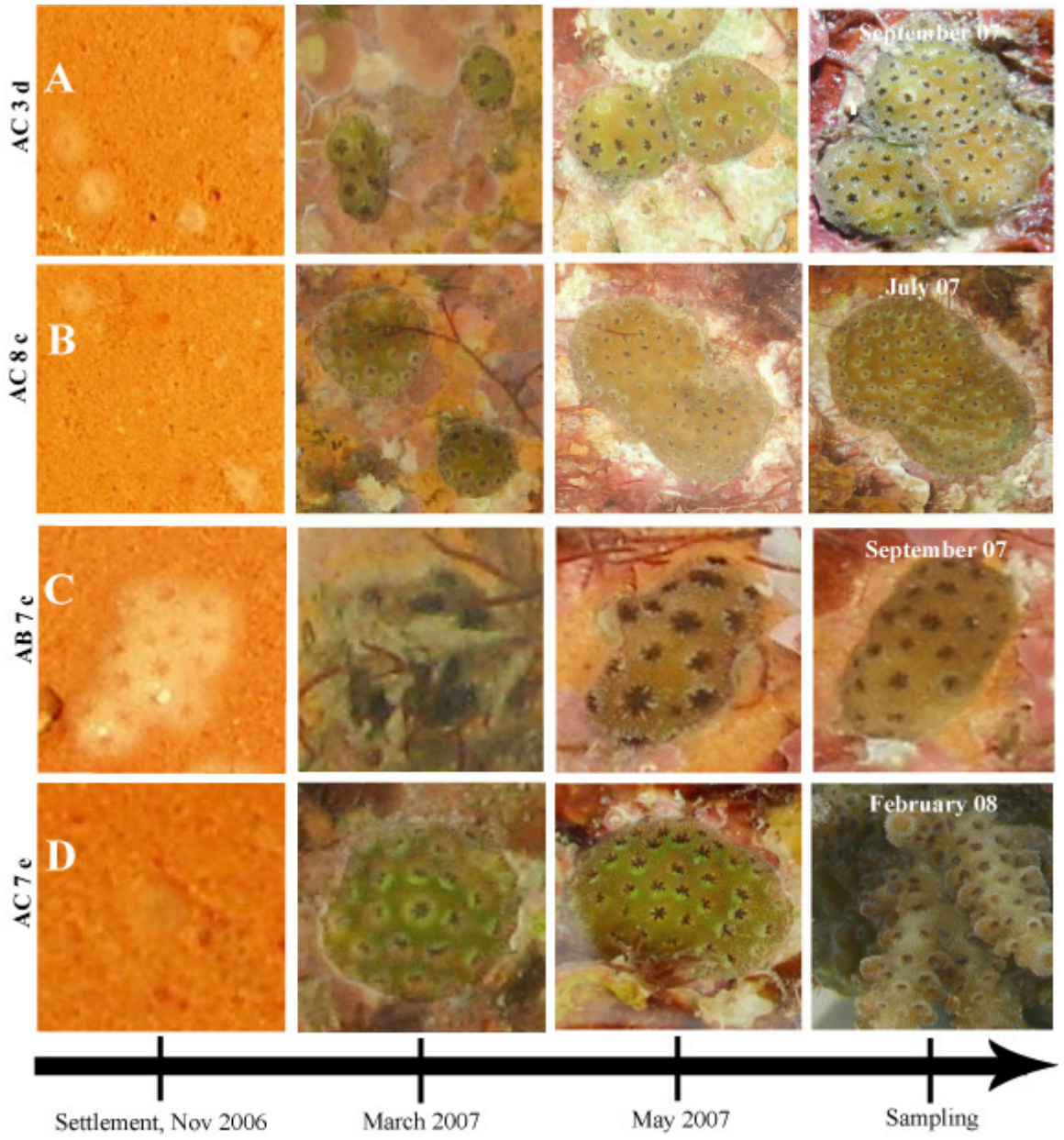

Fig. 2.4 A-D. Summary of four different outcomes following settlement and growth of juveniles of *Acropora millepora* showing: **A)** rejection reaction between 3 distinct genotypes (colony AC3d); **B)** chimera originating from the fusion of two isolated juveniles that came into contact through growth (colony AC8e); **C)** chimera originating from gregarious larval settlement (colony AB7c); and **D)** a single isolated recruit, remaining as a single genotype from settlement to collection at 15 months (colony AC7e). Genotypes were determined based on analyses of 9 microsatellite loci.

2.3.3 Relatedness

Relatedness within chimeric colonies was high, with an average Queller and Goodnight's pairwise relatedness estimator (QG) of 0.54 ± 0.01 (Table 2.3). All genotypes within chimeric colonies were compared with each other (n=58 paired genotype combinations). A large majority of genotypes within chimeric colonies were either full siblings (n=36 genotype comparisons with QG>0.5) or half siblings (n=11 genotype comparisons with 0.25<QG<0.5). Hence, more than 62% of the chimeras detected were full sibling associations, rising to more than 81% when half siblings are included. Rejecting colonies (n=3 paired genotypes) displayed variable levels of relatedness (QG = 0.20 ± 0.29, Table 2.3). Through time, the percentage of fused colonies originating from full sibling larval cultures and persisting as chimeras increased (Figure 2.5). In the second year of the study, the percentage of juvenile colonies tested that were identified to be chimeras was nearly two-fold greater for those originating from full sibling cultures compared to those originating from mixes of sibling and non-sibling larvae (Figure 2.5).

Table 2.3. Average (± SE) pairwise relatedness for chimeras versus all other paired genotypes, calculated according to the Queller and Goodnight (1989) pairwise relatedness estimator.

Samples	Average relatedness	S.E.
Chimeras (n=58 paired genotypes)	0.54	0.04
Rejections (n=3 paired genotypes)	0.20	0.29

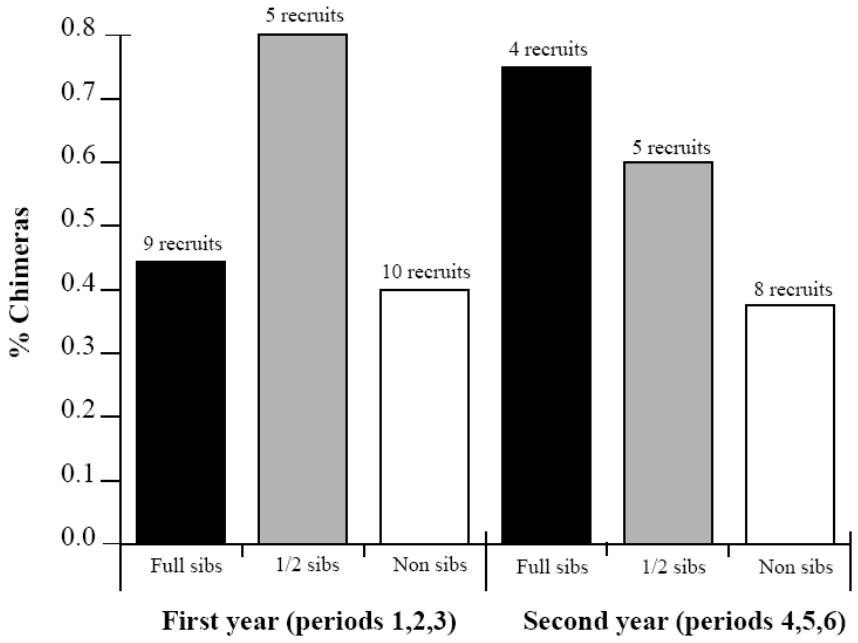

Fig. 2.5. Percent of chimeras detected from 6 months post-settlement (sampling period 1) up to two years post-settlement (sampling period 6), according to the kinship of the juveniles mixed together. Recruit numbers correspond to the total number of recruits genotyped (chimeras and non-chimeras) in each category. Both half- and non-sibling mixtures contained full sibling larvae (see methods)

2.4 Discussion

The study demonstrates that under experimental laboratory conditions, larvae of the broadcast spawning coral *Acropora millepora* tend to settle in aggregations that enhance the likelihood of chimera formation. At least 1.5-fold more larvae settled gregariously than solitarily in the combined Magnetic and Pelorus Island studies, and overall, 47% of juveniles originated from gregarious larval settlement. Considering that, on average, new larval recruits occupied less than 1% of the surface area available for settlement, it is unlikely that the aggregated settlement patterns detected occurred solely by chance. Aggregated larval settlement is commonly observed in coral recruitment studies (Lewis, 1974; Smith, 1997; Zilberberg & Edmunds, 2001; Rinkevich, 2004a; Amar *et al.*, 2008)

and is considered to be a characteristic settlement pattern for a number of coral species (Sammarco, 1982; Barki *et al.*, 2002). For example, Amar *et al.* (2008) recorded that 67% of all newly settled juvenile colonies of the brooding coral, *Stylophora pistillata,* arose from aggregated larval settlement in experimental conditions. Although densities of larvae used in my experimental studies (~1 larvae/mL) were likely to be substantially higher than densities typically found in the field, based on the high numbers of larvae I found settling gregariously in aquaria and multiple reports of aggregated coral settlement in field studies, at least modest numbers are likely to settle in aggregations in wild populations of *A. millepora*.

Significantly, fifty percent of experimental juvenile colonies tested (n=42 colonies) were chimeras and all chimeric colonies formed as a consequence of aggregated settlement, further suggesting that chimerism is likely to occur in the wild and that aggregated larval settlement of broadcast spawning corals contributes to this likelihood. However, the high proportion of chimeras was likely enhanced by the low genetic diversity of the larvae used here. Chimeric juveniles persisted for up to two years, indicating that allogeneic fusions can be stable for extended periods of time. The recent discovery that a minimum of 2-5% of adult colonies are chimeric in populations of *A. millepora* on these same reefs (Puill-Stephan *et al.*, 2009) provides corroborative evidence that aggregated larval settlement gives rise to persistent allogeneic fusions. The alternative, that naturally-occurring adult chimeras represent fusions between allogeneic fragments, is less likely, given the low likelihood of successful survival of fragments in this species because its morphology (*sensu* Jackson, 1979) is dependent on an intact stalk. The persistence of chimeric colonies of *A. millepora* in the field for almost two years post-settlement, combined with evidence of chimerism in adult field populations, suggest that two or more genotypes are able to cohabit within the same

colony indefinitely, with compatibility potentially arising from allelic similarities at currently unknown allorecognition loci.

Evidence that over 60% of persistent chimeras detected formed as a consequence of full sibling associations indicates that relatedness of larvae also plays an important role in chimera formation in this species. Although it is possible that non-related conspecific larvae form chimeras but only closely related individuals survive and maintain a chimeric state, the high relatedness I found between genotypes within chimeric colonies from the earliest sampling at six months suggests that the probability of allogeneic fusion and chimera formation is increased if coral planulae settle in kin aggregations. The limited pool of parents (n=5 colonies per site, Fig 2.1A) and subsequent relatedness of a large number of larvae in my study are likely to have contributed to the gregarious larval behaviour observed at settlement. Such an interpretation is consistent with studies that have shown that larvae of other colonial marine invertebrates tend to aggregate with closely related larvae (Keough, 1984; Grosberg & Quinn, 1986; Barki *et al.*, 2002; Amar *et al.*, 2008), the net result being to minimise the frequency of rejections attributed to recognition of "non-self". For example, in distribution studies of the ascidian *Botryllus schlosseri,* sibling colonies aggregated strongly, whereas unrelated colonies were significantly overdispersed (Grosberg & Quinn, 1986). Moreover, ascidian larvae which shared a histocompatibility allele settled in aggregations and formed temporally stable chimeric colonies *in situ*. Taken together, my results suggest that the frequency of chimera formation in early life history stages of corals is related to intrinsic gregarious settling behaviour of larvae and their level of kin relatedness. For broadcast spawning corals on the Great Barrier Reef, highly synchronized breeding (Willis *et al.*, 1985; Babcock *et al.*, 1986), combined with larval development in dense spawning slicks (Oliver & Willis, 1987) and comparatively short pre-competent periods

(Babcock & Heyward, 1986; Miller & Mundy, 2003) that promote synchronous settlement would all favour aggregated settlement of closely related larvae.

A third factor likely contributing to the high potential for chimerism in *A. millepora* is delayed maturation of allorecognition mechanisms required to discriminate genetically distinct conspecifics during early life history stages. The first rejection reactions were observed ~6 months post-settlement, but fusions between some allogeneic juveniles continued to occur for up to 10-11 months post-settlement (Table 2.2). Thus maturation of allorecognition mechanisms appears to take at least six months in *A. millepora* and may not be fully established even by 11 months post-settlement. Fusions among both aggregated conspecific larvae at settlement and conspecific juveniles coming into contact during their early growth may be facilitated by a "window in ontogeny", during which self-recognition responses have not matured, as proposed by Rinkevich (2004). Similar to cases of natural chimerism in humans that are initiated during pregnancy (blood chimeras, whole body, foetal-maternal, germ cell, and tumor chimeras) (Rinkevich, 2001), immaturity of allorecognition systems in colonial marine invertebrates facilitates the formation of chimeric entities (Rinkevich, 2004c). It is known that the period of time required for many marine invertebrates to acquire a mature state of allorecognition varies from less than two weeks after metamorphosis in the hydrozoan *Hydractinia symbiolongicarpus* (Wilson & Grosberg, 2004), to more than two weeks in the bryozoan *Celleporella hyalina* (Hughes *et al.*, 2004), and up to four months post-settlement for the corals *Stylophora pistillata* (Frank *et al.*, 1997) and *Seriatopora* spp. (Nozawa & Loya, 2005). Interestingly, maturation of allorecognition in the brooding coral *S. pistillata* occurs in a step-wise manner, culminating in full allogeneic incompatibility at four months post-settlement (Frank *et al.*, 1997). In general, lack of an efficient allorecognition system in the early stages of ontogeny in

scleractinian and soft corals is probably universal. However, rather than chimera formation representing allorecognition "failure", such a universal pattern suggests that lack of precision in self-recognition might be adaptive. Consistent with the possibility that delayed allorecognition is adaptive, it is noteworthy that during the first few months post-settlement, juveniles of *Acropora tenuis* and *A. millepora* are able to take up a variety of genetic types of the dinoflagellate endosymbiont, *Symbiodinium*, after which they become dominated by one *Symbiodinium* type (Abrego *et al.*, 2009). The non-selectivity of *Symbiodinium* uptake in the first few months post-settlement provides corroborative evidence that acroporid corals lack a mature allorecognition system early in life.

A major advantage of multi-partner kin aggregations at settlement is thought to reside in ensuing immediate and long term increases in colony size (Chadwick-Furman & Weissman, 2003). Size-related benefits may overcome costs associated with interacting genotypes, for example intra-colony competition resulting in colony fission if incompatible genotypes reject each other, and potentially absorption or mortality of one or more genotypes within chimeric colonies (Amar *et al.*, 2008). In my study, 12 out of a total of 21 chimeras were formed from the fusion of three or more juveniles (Table 2.2), thus chimeras were significantly larger in size (by almost 3-fold) than juveniles settling solitarily, even at 3 months post-settlement. My results support hypotheses suggesting that chimera formation represents an important opportunity for substantial increases in the size of recruits, as well as in surface area occupied at settlement, both of which would be much greater than those possible through growth alone. Although escape in size is widely accepted as an important survival strategy for early life history stages of sessile marine invertebrates (Jackson & Hughes, 1985), the lack of difference found for survival rates between chimeras and single-genotype juveniles in the first 4

months post-settlement raises questions as to whether the benefits of allogeneic fusion relate primarily to rapid increase in colony size. Longer-term studies of comparative mortality rates of chimeras and single-genotype colonies are needed to more fully evaluate potential adaptive benefits of chimerism, and may reveal differential size-related survival after 4 months post-settlement. The survival rate of chimeric versus single genotype colonies did not differ over time periods studied in the field, but earlier attainment of sexual maturity could eventually benefit the fused corals. Alternatively, benefits of chimerism may relate to the presence of increased genetic variation within colonies, which might have implications for the survival of later life history stages. Higher stress tolerance of one genotype within a chimera could play an important role in ensuring at least partial colony survival following stress, and could explain observations of partially bleached corals following exposure to thermal stress.

In summary, juveniles of the broadcast spawning coral *A. millepora* show a high potential to form bi- and multi-partner chimeras following gregarious larval settlement. High potential for co-settlement of closely-related larvae and delayed maturation of the allorecognition system also contribute to the propensity for fusion between genetically different individuals in early life history stages of this species. While there may be costs associated with chimerism, its occurrence and persistence for up to two years in the present study and also in wild adult populations of this species (Puill-Stephan *et al.*, 2009) indicate that, at least in some cases, there may be net benefits associated with chimerism. Further investigations of the benefits and costs of chimerism in modular marine invertebrates should provide important evolutionary insights into this little studied aspect of coral life histories.

Chapter 3.0 Allorecognition maturation in the broadcast spawning coral *Acropora millepora*

3.1 Introduction

The ability to differentiate between self and non-self is a key feature of all living organisms and is integral to activating mechanisms of innate immunity essential for resisting pathogen invasion (Nürnberger *et al.*, 2004). Precision in non-self recognition mechanisms enables an organism to discriminate foreign genetic material, thereby providing the first line of defence against invading pathogens in both plants and animals. Allorecognition comprises a series of events triggered by contact between genetically different tissues, followed by a rejection reaction in order to maintain the integrity of self (Grosberg, 1988). However, sessile marine invertebrates like corals and ascidians are able to form entities containing tissues or cells of two or more genetically distinct individuals, i.e. chimeras (Rinkevich & Weissman, 1987), indicating either lack of precision in the self-recognition response or potentially delayed onset of precision early in ontogeny. Chimerism challenges many aspects of the purportedly accurate discrimination between self and non-self required for immunocompetence, and also challenges the notion of genetic uniqueness within individuals and colonial organisms (Santelices, 1999). Studies of allorecognition early in ontogeny may provide important insights into processes leading to the establishment of chimeras in sessile marine invertebrates.

As adults, many sessile, modular marine invertebrates, such as sponges, cnidarians, bryozoans, and ascidians, are able to discriminate self from non-self with great precision (Grosberg, 1988). Because these marine invertebrates typically include asexual reproduction in their life histories, colonies originating from fragmentation or other asexual processes may come into contact with clone mates as they grow in size. Thus allorecognition systems are essential for identifying colonies that are isogenic (same species, same genotype), allogeneic (same species, different genotype), or xenogeneic

(different species), and represent the first step leading to fusion or rejection reactions following contact. Contacts between xenogeneic individuals invariably result in a rejection (or non-fusion) reaction, but contact between allogeneic or isogeneic individuals can lead to fusion, with allogeneic fusions resulting in the establishment of two or more genotypes within the same colony (Hart & Grosberg, 1999). Because the allorecognition systems of adult colonial marine invertebrates generally discriminate between clone mates and non-clone mates effectively (Grosberg, 1988), fusion between genetically different entities is commonly thought to be rare (Jackson, 1986) and low numbers of chimeras are typically expected in natural populations. However, fusion of genetically distinct corals has been observed on multiple occasions (Heyward & Stoddart, 1985; Resing & Ayre, 1985; Willis & Ayre, 1985). Furthermore, the occurrence of chimeras in natural populations of various colonial marine invertebrates (Sommerfeldt & Bishop, 1999; Ben-Shlomo *et al.*, 2001; Sommerfeldt *et al.*, 2003; Rinkevich, 2005; Ben-Shlomo *et al.*, 2008; Puill-Stephan *et al.*, 2009) and under experimental conditions (Amar *et al.*, 2008) indicates that their allorecognition systems at least occasionally allow the fusion of genetically non-identical entities.

The formation of chimeric entities in colonial marine invertebrates is believed to be facilitated during early ontogeny (Rinkevich, 2004c), as maturation of the allorecognition system may require a few months (or days depending on the organism). For example, a study using juveniles of the brooding corals *Seriatopora caliendrum* and *Seriatopora hystrix* revealed that fusions between grafted allogeneic colonies only occurred during the first four months post-settlement, suggesting that complete maturation of the allorecognition system requires about four months in these corals (Nozawa & Loya, 2005). Lack of an allorecognition system in the early stages of post larval settlement was also documented in four species of soft corals: *Nephthea sp.*,

Heteroxenia fuscescens, *Parerythropodium fulvum* and *Clavularia hamra* (Barki *et al.*, 2002). In these species, co-settlement of planula larvae facilitated high frequencies of allogeneic fusions, but these chimeras did not remain stable during longer term monitoring (up to 450 days) and many detrimental effects of fusion were noticed (such as the death of 1 or more partners, morphological resorption, slower growth, etc.). For the brooding coral *Stylophora pistillata,* three distinct stages in the maturation of the allorecognition system were defined within four months of settlement (Frank *et al.*, 1997). When larvae were younger than 2 months, almost all allogeneic colonies fused to form morphologically stable chimeras. Between 2 and 4 months post-settlement, fusions were transitory and culminated in tissue separation or death of a partner by the age of 4 months. After 4 months, no fusions were recorded for allografts, indicating that the allorecognition system had matured (Frank *et al.*, 1997). Similarly, for the planulating coral *Pocillopora damicornis*, fusions were observed when juveniles originating either from the same colony or from different colonies were brought into contact from 7 days to 3 month after planulation (Hidaka *et al.*, 1997). However, contact reactions between juveniles originating from the same source colony (potentially full or half siblings) remained as fusions for up to 7 months, whereas juveniles derived from different colonies fused in only a few cases and contact reactions subsequently resulted in non-fusion or incompatible fusion (Hidaka *et al.*, 1997). Overall, fusions or rejections appear to be linked to the timing of contact, i.e. whether the contact happens before or after allorecognition systems are mature, but the outcomes of contacts are also strongly influenced by the relatedness (siblings versus non-siblings) of different entities in the contact interaction. However, the maturation of allorecognition has not been studied in broadcast spawning corals, which typically acquire their algal endosymbiont, *Symbiodinium,* through uptake from the environment (horizontal uptake, Harrison &

Wallace, 1990), in contrast to brooding corals that generally acquire symbionts maternally (vertical transmission). Furthermore, allorecognition maturation with respect to fine-scale genetic relatedness, i.e. the capacity to distinguish between half siblings versus full or non-siblings, has not been investigated for any coral species.

Here I assess the maturation of allorecognition within *Acropora millepora,* a widespread and abundant broadcast spawning coral species on the Great Barrier Reef. Specifically, I investigated if the outcomes of contact reactions between juveniles of a broadcast spawning coral vary with different levels of relatedness or with time. Corals immunity still being at its premises and in the light of increasing occurrence of threats and diseases for coral reefs, there is a real need to better understand the complex immune system of corals. Therefore, investigating at which degree spawning coral juveniles discriminate self from non-self during ontogeny may provide insights into factors contributing to its vulnerability.

3.2 Materials and methods

3.2.1 Coral species and study site

This project investigated maturation of allorecognition in *Acropora millepora*, a broadcast spawning coral that is both abundant and ubiquitous on the Great Barrier Reef. This species is currently the best characterized coral at the molecular level and it is also easy to manipulate for experiments with eggs and larvae. Thus, *Acropora millepora* represents a good study species for immunity experiments involving early ontological stages.

Mature colonies of *A. millepora* were collected from reefs adjacent to Magnetic Island and south-west Pelorus Island, which are both located in the central region of the Great Barrier Reef, prior to the predicted spawning dates in Oct. 2007 at Magnetic Island and

in Nov. 2007 at Pelorus Island (Willis *et al.*, 1985; Babcock *et al.*, 1986). Colonies from Magnetic Island were transferred to the Australian Institute of Marine Science and those from Pelorus Island to the Orpheus Island Research Station for spawning and gamete collection. Colonies were maintained in 1000 L tanks supplied with running 1µm filtered sea water (FSW) at 28.5°C. The genotype of every colony (n=8 colonies per site) was determined prior to collection based on 3 microsatellite loci (Amil2_006, Amil5_028, and Amil2_022; (van Oppen *et al.*, 2007) to ensure corals were genetically distinct, thereby avoiding crosses between clone mates.

3.2.2 Rearing larvae

On the day of spawning, colonies were isolated in individual 70 L aquaria filled with 1µm filtered sea water (FSW) and kept isolated until they had finished spawning. Gametes from 6 colonies were collected from the water surface of respective isolated aquaria and mixed with gametes from a second colony in a new 70 L aquarium filled with FSW according to the experimental design shown in Figure 3.1. In summary, gametes from each pair-wise combination of the six colonies at each site were mixed to create one of four crosses, which resulted in the production of four larval batches (A, B, C & D) with different kinship levels: full siblings, half siblings and non-siblings (Fig. 3.1). Pair types and resulting larvae A-D were replicated for colonies from the two sites, and an asterisk (*) used to denote pairings originating from Pelorus Island crosses.

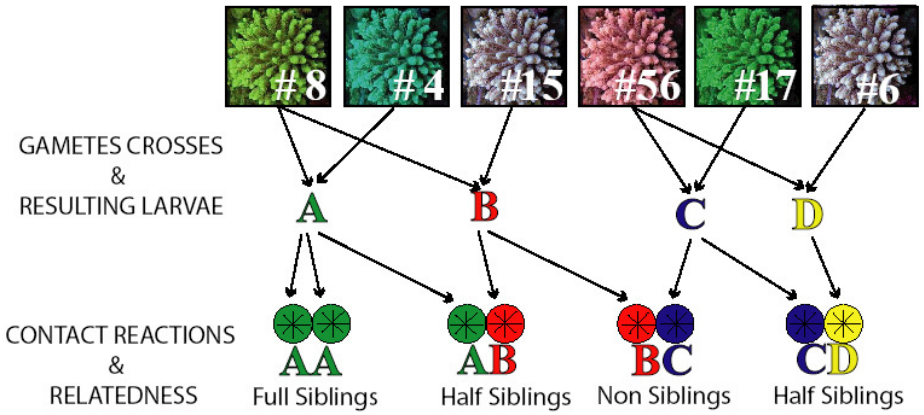

Fig. 3.1. Schematic diagram showing how gametes from different colonies (identified by a number) were crossed to produce larvae (A, B, C and D) and how contact reactions were set up between juveniles that differed in their kinship.

Gametes were allowed to fertilise for at least 1.5-2 hours, after which a small subset of eggs was sampled for microscopic confirmation of fertilisation and initiation of embryogenesis. Embryos were cleaned by performing three consecutive water changes of the 70 L tanks filled with FSW, which involved draining ~90% of the water from the bottom and slowly filling from the top. Embryos from each cross were then transferred into separate 500 L tanks supplied with running FSW at 28.5°C in a temperature controlled room and kept at a density of approximately one larva per mL. Embryos were checked microscopically in order to assess their development until ~48 hours after fertilisation, when the fully ciliated planula larva stage was reached. Four days after spawning, when swimming larvae had became elongated and had started to search the substratum for suitable settlement sites, the bottom of each tank was covered with underwater writing paper (previously rinsed and soaked in FSW for 24 hr) as settlement surfaces.

3.2.3 Establishing contact reactions between *A.millepora* juveniles

Ten days after spawning, contact reactions (Fig.3.1 & Table 3.1) were set up between coral juveniles settled on underwater writing paper by cutting out settled juveniles and pasting them next to each other on plastic tiles using MrSticky's ® underwater glue. Contacts were established so that juveniles were either just touching (immediate contact), 2 mm away from each other, or 5 mm away from each other (Table 3.1). Juvenile pairings established with increasing distances between coral recruits were designed to create contacts at a series of time points as the juveniles grew into contact with each other to test whether contact reactions (fusion or rejection) changed with age of juveniles. Contacts between half and non-siblings were established between juveniles that had settled solitarily. Juveniles that had settled in aggregations (two or more juveniles settled adjacently) in the settling tanks were designated as immediate contact reactions between full sibling juveniles. Because larvae from different crosses were maintained and settled separately, immediate fusion at settlement occurred only between full siblings reared in the same tank.

Table 3.1. Forced contact reactions between *Acropora millepora* juveniles

Larvae	A			B			C			D		
	Immediate Contact (n=10)	2 mm (n=10)	5mm (n=10)	Immediate Contact (n=10)	2 mm (n=10)	5mm (n=10)	Immediate Contact (n=10)	2 mm (n=10)	5mm (n=10)	Immediate Contact (n=10)	2 mm (n=10)	5mm (n=10)
A	AA1,AA2,AA3 AA4, AA5,AA6 AA7,AA8 AA9,AA10	AA11,AA22,AA33 AA44, AA55,AA66 AA77,AA88 AA99,AA1010	AA111,AA222,AAA333 AA444, AA555,AA666 AA777,AA888 AA999,AA101010	AB1 | AB10	AB11 | AB1010	AB111 | AB101010	AC1 | AC10	AC11 | AC1010	AC111 | AC101010	AD1 | AD10	AD11 | AD1010	AD111 | AD101010
B				BB1 | BB10	BB11 | BB1010	BB111 | BB101010	BC1 | BC10	BC11 | BC1010	BC111 | BC101010	BD1 | BD10	BD11 | BD1010	BD111 | BD101010
C							CC1 | CC10	CC11 | CC1010	CC111 | CC101010	CD1 | CD10	CD11 | CD1010	CD111 | CD101010
D										DD1 | DD10	DD11 | DD1010	DD111 | DD101010

Contact reactions between juveniles were named according to the relatedness of the paired juveniles, thus AA, BB, CC, and DD represented pairings of juveniles that were full siblings, i.e., each pair comprised juveniles originating from the same two parent colonies (see Fig. 3.1 for relatedness between juveniles). Contact reactions named AB and CD represented pairings between half siblings, i.e. the two juveniles in contact pairings shared one parent. Contact reactions named AC, BC, AD and BD represented pairings between non-siblings, i.e., the two juveniles in each contact pairing had different parents. Ten replicate contact reactions were established for each type of sibling pairing at each of the three time points. Juveniles which were just touching were numbered from 1 to 10. Juveniles which were 2 mm away from each other were numbered as 11, 22, 33, 44, 55, 66, 77, 88, 99, and 1010. Finally, juveniles 5 mm away from each other were numbered 111, 222, 333, 444, 555, 666, 777, 888, 999, and 101010 (Table 3.1). Thus, the contact reaction named BD5 represented a non-sibling pairing between a juvenile from cross B and a juvenile from cross D (see Fig. 3.2D), the number being the replicate number for an immediate contact.

Eleven days after spawning, the laboratory-reared juvenile corals in contact reaction pairings were placed in the field at 5m depth in Nelly Bay (Magnetic Island), an inshore reef (Babcock & Mundy, 1996; Anthony *et al.,* 2004) with a gentle slope down to approximately 10m. The plastic tiles were skewered on rods through a hole in the centre of the tiles, with spacers (2-3 cm long) between each tile. The rods were suspended between two star pickets, which had been driven into dead substratum on the reef, so that tiles were maintained in a vertical orientation to minimise accumulation of sediment on tile surfaces. Tiles were labelled, tagged and photographed prior to deployment on the reef.

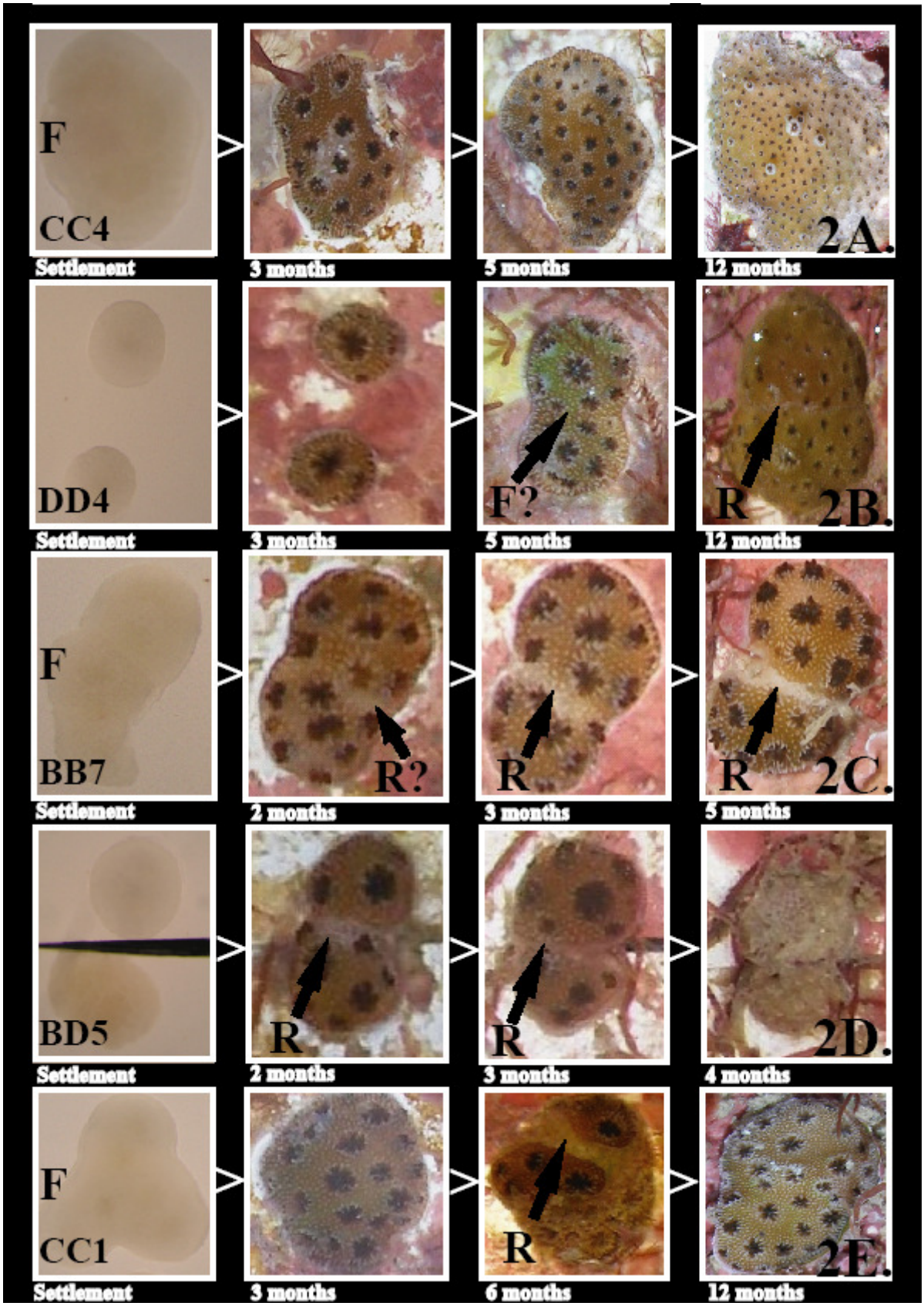

Fig. 3.2. Summary of the different scenarios observed during *Acropora millepora* juveniles settlement, growth, and contact reactions (F=Fusion, R=Rejection).

3.2.4 Establishing contact reactions between *A.millepora* adults

Contact reactions were set up between branches of the parental colonies (i.e., colonies from which gametes were collected) from Magnetic Island. Approximately 30 branches were snapped from each of the six colonies, glued so that they were maintained in an upright position on terracotta tiles with marine epoxy paste, and kept in an outdoor 1000 L tank (shaded, light levels~250µE) supplied with running 1µm FWS at 28.5°C (5L/min flow + pump for water movement). Branches were acclimated to aquarium conditions for two days prior to initiating contact (Fig. 3.3). Feeding could not be performed because access to planktonic food was not possible. Four replicate adult pairings (see examples in Fig. 3.3) were set up between branches from each of the six colonies (labelled 1-6), for every adult contact reaction pairing possible. Thus isogenic adult pairings comprised 1-1, 2-2, 3-3, 4-4, 5-5, and 6-6 branch pairings; and allogenic adult pairings comprised 1-2, 1-3, 1-4, 1-5, 1-6, 2-3, 2-4, 2-5, 2-6, 3-4, 3-5, 3-6, 4-5, 4-6, 5-6 branch pairings. Control branches (i.e. single branches with no contact) were also monitored in order to follow survival of branches in the absence of contact with another branch. Coral branches were kept in aquaria for the entire study period (2 weeks). The study was terminated because control branches started to suffer mortality.

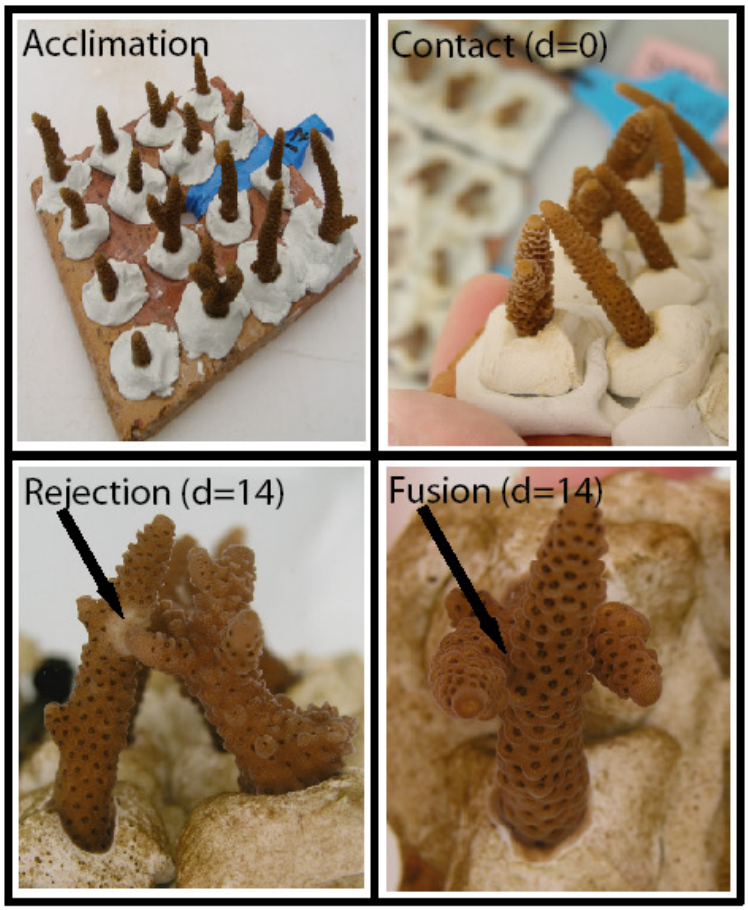

Fig. 3.3. Branches from adult *Acropora millepora* colonies, attached with epoxy paste, during acclimation time (acclimation), and once contact was established (contact, d=0). Contacts resulted in Fusion or Rejection reactions after 14 days of contact (d=14) under laboratory conditions.

3.2.5 Assessing contact reactions between *A.millepora* juveniles

In order to investigate the fate of contact reactions between coral juveniles, tiles were monitored and photographed at the following time points: in the laboratory prior to deployment in the field (10 days after spawning), every month up to May 2008 (i.e. for 6 and 5 months post-settlement for corals from Magnetic Island and Pelorus Island, respectively), and then on the 30th of October 2008 (i.e. almost 12 and 11 months post-settlement for corals from Magnetic Island and Pelorus Island, respectively). Tiles were kept in the field, at 5m depth, in Nelly Bay (Magnetic Island).

The outcomes of contact reactions were scored microscopially as fusions (F), identified when tissues appeared to be continuous across the contact area and new polyps appeared along the contact margin (Fig. 3.2A & 2E), or rejections (R), which were characterised by discontinuity of tissues along the line of contact (i.e. no tissue fusion and/or no addition of new polyps, Fig. 3.2C) and a white line (sometimes very thin, Fig 3.2B) along the contact zone. The rejection category was equivalent to the non-fusion categories of Hidaka *et al.* (1997) and Nozawa & Loya (2005). A third type of contact reaction, incompatible fusion, has been described by Hidaka (1997) and Nozawa & Loya (2005) and characterised as apparent fusion of tissues of paired corals, however the presence of a distinct white border zone along the contact area clearly separates the two juveniles. For simplicity and because tissues were clearly incompatible, these reaction outcomes were included in the rejection category. In order to maximize the number of replicates surviving through to the end of the study, we did not sample any juveniles for genetic analysis or histological confirmation of fusions.

3.2.6 Assessing contact reactions between *A.millepora* adults

To assess the outcome of contact reactions between branches of adult colonies of *A. millepora*, photographs of the contact reactions were taken one and fourteen days after contact (see Fig. 3.3). We classified the outcome of contact reactions between adults as either fusion (F) or rejection (R) according to the descriptions above for juvenile pairings. Rejections involved the production of a pad of undifferentiated skeleton between the two branches (white zone, see Fig. 3.3). However, due to the brevity of the experiment, fusions (F) did not involve continuous tissue across the contact area nor new polyps appearing along the contact margin, but they were characterized by the absence of rejection signs.

3.3 Results

3.3.1 Contact reactions between *A.millepora* juveniles

Every contact reaction between non-siblings ultimately resulted in a rejection event (Fig. 3.4). More than ninety three percent of non-sibling interactions (n=15) displayed rejection reactions after initial contact. At approximately 1.5 months post-settlement, the first two non-sibling juvenile pairs (BD5 and AD4) grew into contact. The first signs of rejection were observed two months post-settlement in pair BD5 (Fig. 3.2D & Fig. 3.4). Then, from 3 months post-settlement onwards up to the end of the experiment, 100% of non-sibling juveniles in contact were displaying signs of rejection (Fig. 3.5A). AD4 was the only non-sibling pairing that resulted in a fusion reaction, which was visible at approximately two months post-settlement (Fig. 3.4). However, this initial fusion was reversed one month later (three months after settlement), with the signs of rejection observed three months post-settlement (AD4 on Fig. 3.4).

Seventy-five percent of contacts between half siblings (n=8 pairings) resulted in rejection reactions after initial contact (Fig. 3.4). Only two out of the eight (i.e., 25%) half sibling pairings resulted in fusion, one at 3 months and one at 4 months post-settlement. However, in one case (CD4), the fused colony died within one month and in the other case (AB8*), signs of rejection were visible within a month of observing the initial fusion reaction (Fig. 3.4). From 5 months post-settlement onwards, and up to the end of the experiment, 80% or more of half sibling juveniles in contact displayed signs of rejection (Fig. 3.5A).

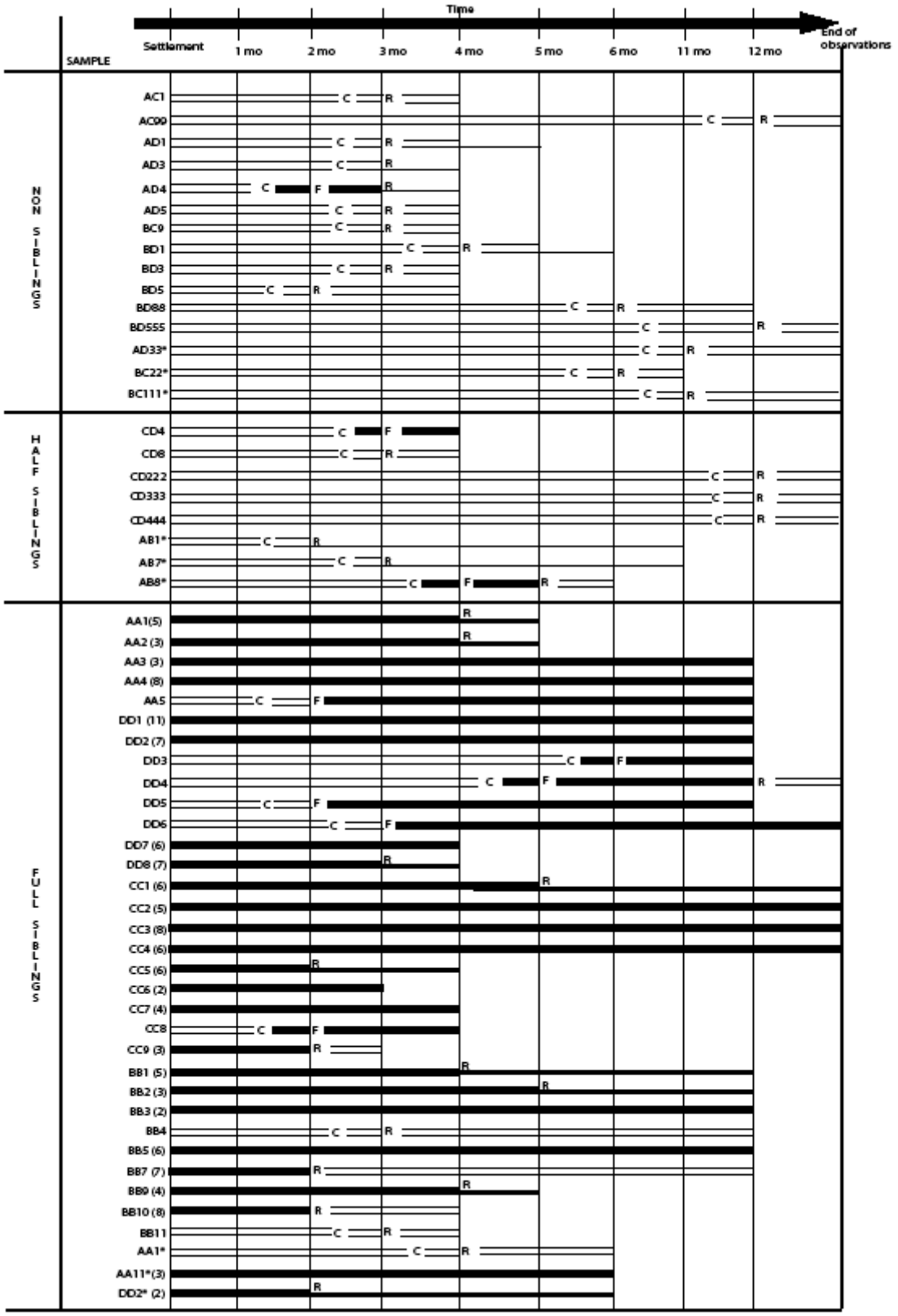

Fig. 3.4. Observation of contact reactions between *A.millepora* juveniles from settlement until 12 months post-settlement. Bold lines represent fused juveniles. Thin lines represent non fused juveniles. End of lines represent either the death of the corals or the end of the observation period. C = contact between different juveniles. F = Fusion. R = Rejection. When juveniles were fused at settlement, numbers of fused recruits are indicated in brackets.

Fusion was far more prevalent between full siblings than between half siblings or non-sibling pairings. Approximately 91% of contact reactions between full siblings (n=34 pairings) resulted in fusion following the initial contact (Fig. 3.4). Only three full sibling contact interactions did not result in fusion after the initial contact (AA1*, BB4, and BB11, Fig. 3.4). Most of the fusions (25 of 31) occurred at settlement, when two or more juveniles settled in aggregations. However, more than 40 percent of all fused colonies (13 of 31) eventually showed signs of rejection. The first signs of rejection within fused aggregations were observed at two months post-settlement for pair BB7, BB10, CC5, CC9, and DD2* (Fig. 3.2C & Fig. 3.4). Nevertheless, 11months post-settlement approximately 73% of full sibs in contact and alive (n=19) were still fused and did not shown signs of rejection (Fig. 3.5B), and 66% of them were still fused at 13 months post-settlement (n=6).

Mortality rates reached 73.3% for non-siblings, 62.5% for half-siblings, and 64.7% for full siblings. No significant differences in mortality rates were detected among groups, likely due to low replicate numbers. Rejection reactions caused or contributed to mortality rates, with 100% mortality occurring after rejection between non-siblings, 80% between half-siblings, and 50% between full siblings.

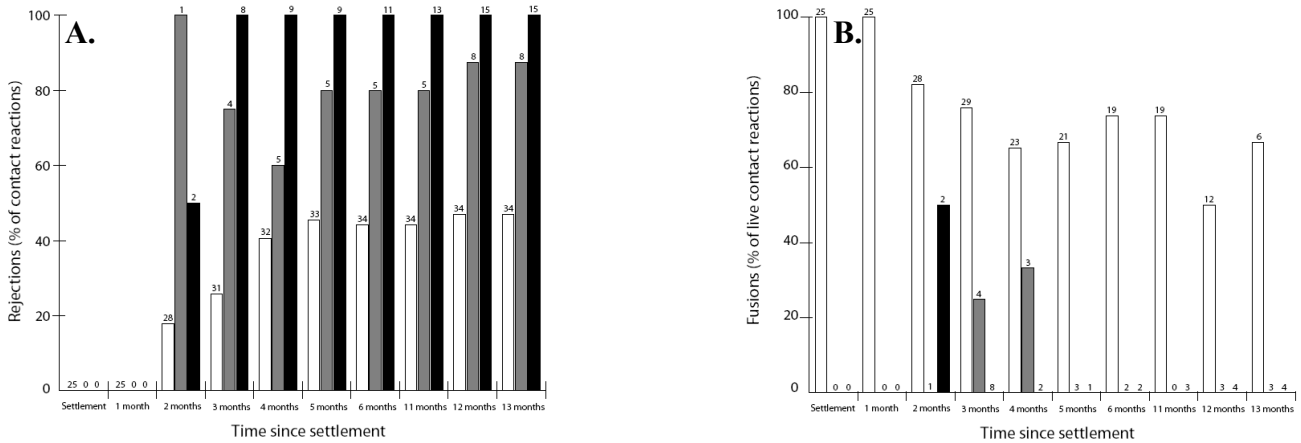

Fig. 3.5. Percent of rejection (**A.**, in % of corals in contact) or fusion (**B**, in % of corals alive in contact) for contacts between full sibling (□), half sibling (▒), and non sibling (■) *A.millepora* juveniles, from settlement up to 13 months. Numbers of contacts are indicated above each histogram.

3.3.2 Contact reactions between *A.millepora* adults

There were striking differences in the frequency of fusion and rejection events between allografts (contacts between branches from different colonies of the same species) and isografts (contacts between branches from the same colony) involving paired branches from adult colonies of *Acropora millepora*. After two weeks in contact, almost 50% of allografts displayed signs of rejection (Fig. 3.6). Rejection reactions were characterised by bleached tissue or skeleton at the contact area (i.e. white tissue, see Fig. 3.3). Fusion reactions, which were characterised by a lack of signs of rejection, but also by a lack of signs of complete fusion (see Fig. 3.3), were observed in just 28% of allografts (Fig. 3.6). In contrast, none of the isografts showed signs of rejection (Fig. 3.6). Seventy-five percent of isografts displayed signs of fusion two weeks after contact was established (Fig. 3.6). The proportion of pairings in which one or both branches in the contact interaction died was similar between isografts and allografts (25 and 23 percent respectively, Fig. 3.6).

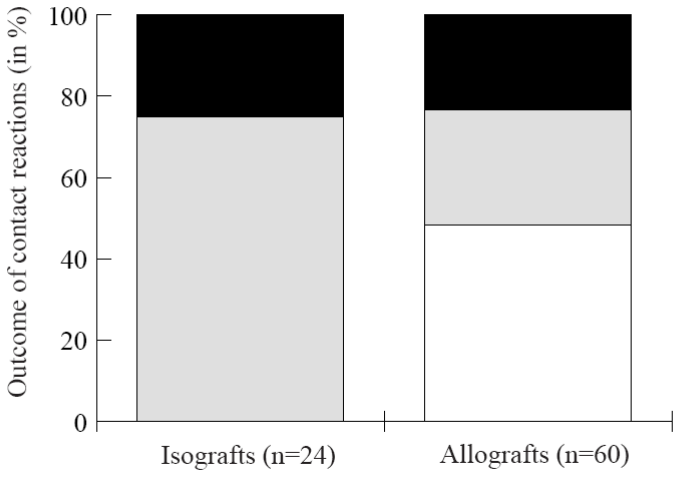

Fig. 3.6. Outcome of contact reactions established between branches of adult *A.millepora* from 6 different colonies after 14 days of contact. Contact resulted in non fusions (□), non rejections (▒), or the death of one or both branches (■). Isografts were contacts between branches from the same colony (n=24 contact reactions). Allografts were contacts between branches from different colonies (n=60 contacts).

3.4 Discussion

This study indicates that juveniles of the broadcast spawning coral *Acropora millepora* lack precision in non-self recognition early in ontogeny, with maturation of the allorecognition system beginning at approximately two months post-settlement. Increasing numbers of rejections in full sibling contact reactions, from 18% at 2 months (n=28 contact reactions) to approximately 47% at 12 months (n=34 contact reactions), suggest that development of the allorecognition system is gradual in this coral species (Fig. 3.5A). In combination with findings of step-wise maturation of allorecognition systems by 3-4 months in juveniles of the brooding corals *Stylophora pistillata* (Frank *et al.*, 1997) and *Seriatopora* (Nozawa & Loya, 2005), my results highlight an emerging pattern of delayed maturation of allorecognition systems in juvenile corals, with much longer delays occurring in a broadcast spawning coral. The level of genetic relatedness between juveniles coming into contact in the first few months strongly influences the outcome of contact reactions, with fusion generally occurring when juveniles are full siblings, whereas rejection reactions generally occur between unrelated juveniles.

My study is the first to investigate allorecognition maturation in corals at fine genetic scales, by comparing the outcomes of contact reactions between half siblings versus full- or non-siblings. More than 90% of full sibling pairings (n=34 pairings) of *A. millepora* resulted in fusion when juveniles first came into contact, including contact reactions that were initiated between 5 and 6 months post settlement. In contrast, only 25% of half sibling pairings (n=8 pairings) and 7% of non-sibling pairings (n=15 pairings) resulted in fusion at first contact. Two of these fused half- and non-sibling colonies survived for more than 2 months following fusion and in both cases, initially fused colonies showed signs of rejection within 1 month of fusion. In contrast, fusions persisted in half of the full sibling pairings that were still alive at 12 months (n=6

juveniles out of 12 alive, or 17% of all full sibling pairings), indicating that once fused, there may be selective advantages in maintaining chimeric colonies when partners are full siblings. The comparatively low levels of fusion in half- and non-sibling contact reactions indicate that relatedness strongly influences the outcome of the contact.

Mortality rates did not differ significantly among groups, but rejection influenced death in the majority of contact reactions.

Indiscriminate fusion of juveniles in the first month post-settlement followed by the first signs of rejection at two months, in contact reactions involving non-, half- or full-siblings, suggests that allorecognition maturation may occur in a step-wise manner in *A. millepora,* as has been found for *Stylophora pistiallata* (Frank *et al.*, 1997). However, whereas development of allorecognition through three stages of maturation was achieved rapidly within the brooding *S. pistillata,* i.e. 1) the formation of morphologically stable chimeras at less than two months, 2) transitory fusion between two and four months ending in tissue separation or death of a partner at the age of four months, and 3) rejections after four months, final maturation of allorecognition was not detected in the broadcast spawning *A. millepora,* even at 13 months, which is when the study ended. Early maturation of allorecognition (by four months post-settlement) in juveniles of two other brooding species, *Seriatopora caliendrum* and *Seriatopora hystrix* (Nozawa & Loya, 2005), further corroborates these differences between brooding and broadcast spawning corals.

The late onset of rejection reactions in allogeneic pairings of the broadcast spawning coral *A. millepora,* which was at least double the 4 months that were required for the onset of rejection reactions in the brooding corals *Styllophora* and *Seriatopora*, may be related to differences in the acquisition of endosymbiotic *Symbiodinium* between corals with these two reproductive modes. One of the differences between most broadcast

spawning and brooding corals is the acquisition of algal symbionts from the environment by the larvae or recent recruits by the former, while brooded larvae acquire *Symbidinium* from their mother colony. The uptake of *Symbiodinium* by coral larvae and juveniles during early ontogeny is relatively non-selective. During the first few months post-settlement, it has been observed that juveniles of *Acropora tenuis* and *A. millepora* are able to take up various *Symbiodinium* types, regardless of the type present in parental colonies (Abrego *et al.*, 2009). Although there is no proof that recognition of allogeneic coral tissues and xenogeneic *Symbiodinium* cells involve the same immune pathways, the non-selectivity of *Symbiodinium* uptake in the first few months post-settlement provides further support that acroporid corals lack a mature non-self recognition system during this time. After an initial flexible uptake (Little *et al.*, 2004), corals become dominated by one symbiont type (Abrego *et al.*, 2009), reflecting the possible maturation of non-self recognition. Therefore, the lack of an efficient non-self recognition system in the first few months may be a factor contributing to initial flexibility in symbiont uptake.

My results demonstrate that, once mature, allorecognition systems of adult colonies of *A. millepora* are capable of discriminating genetically different conspecifics in as little time as two weeks. In the majority of contacts between adult branches, clear signs of non fusion were observed between genetically different conspecifics after two weeks of contact. However, a few allografts did not show any clear signs of rejection, but due to the short observation time (see methods for reasons of short experiment time); it could not be concluded whether fusion was established. On the other hand, contact between branches from the same colonies (i.e., isografts) did not show any signs of rejection after two weeks of contact. These contrasting results compared to the observations on juveniles reinforce the notion that coral juveniles lack a mature allorecognition system

in their early life. Such results corroborates observations on *Pocillopora damicornis* corals where fusion was only observed in isografts between adult colonies, and newly settled larvae produced by presumed genetically different colonies (in this case, colonies belonging to different colour morphs) from the same coral species were able to fuse (Hidaka, 1985b). These results also suggested that newly settled recruits of corals lack a functional allorecognition system as expressed by the adult colonies (Hidaka, 1985b). While it is clear that adult corals need an efficient non-self recognition system to respond to various external assaults, such as diseases, bacteria, and competition with other animals or plants, the reasons for the apparent lack of efficient allorecognition early in ontogeny are less obvious.

The lack of a mature allorecognition system during early ontogeny is likely to facilitate the fusion of coral juveniles in their early life. However, after the allorecognition maturation period, only very closely related conspecifics are able to fuse or remain associated within the same colony. The formation and persistence of entities involving associations of genetically different juveniles (i.e. chimeras) could either represent a case of allorecognition "failure" or an acceptance and co-habitation (i.e. tolerance) of very closely related genotypes. However, except for acknowledging tolerance as an immunological phenomenon, I feel that further discussion of the tolerance literature is secondary to the focus of my thesis. Hence, the mature alloimune system of corals might still allow fusion between compatible or closely related genotypes, and once compatible genotypes are associated within a chimera, they co-habitate and constitute a stable chimera. Evidence that chimeras occur and persist on the reef has been found through genetic analysis of wild populations of *A. millepora* on the Great Barrier Reef (Puill-Stephan *et al.*, 2009). Interestingly, the possibility for genetically different but very closely related corals of the same species to fuse and remain fused in chimeric

entities might explain fusion events observed between different adult corals of the same species in self recognition bio assays in earlier studies (Willis & Ayre, 1985).

To summarize, I observed that juvenile *A.millepora* juveniles lack the efficient allorecognition system expressed by the adults. It is only later during ontogeny that *A. millepora* juveniles showed signs of rejection from two months post-settlement onwards while in contact with conspecifics of the same age. The level of genetic relatedness strongly influenced the outcome of contact reactions between individual corals, as all non-sibling juveniles rejected each other from 3 months post settlement onwards while full siblings could still fuse 6 months post-settlement and remain fused until the end of the experiment. The initial lack and the slow maturation of allorecognition could eventually be beneficial for spawning corals, as it enables a flexible uptake of symbionts but it also allows conspecifics to settle together, fuse and form chimeras in order to increase size more rapidly than through growth alone. However, if juvenile corals would completely lack an allorecognition system in their early life stages, their vulnerability to external stressors such as pathogens would make survival almost impossible. Therefore, investigating at which degree spawning coral juveniles discriminate self from non-self during ontogeny would help elucidating aspects of the complex immune system of corals.

Chapter 4.0 Expression of putative immune response genes during early ontogeny and allorecognition maturation in the coral *Acropora millepora*

4.1 Introduction

Many marine invertebrates lack a mature allorecognition system in their earliest life history stages, but are able to distinguish non-self within days to months. For example, allorecognition reaches a mature state within the first two weeks after metamorphosis in the hydrozoan *Hydractinia symbiolongicarpus* (Wilson & Grosberg, 2004), but requires more than two weeks in the bryozoan *Celleporella hyalina* (Hughes *et al.*, 2004), and up to four months post-settlement for the corals *Stylophora pistillata* (Frank *et al.*, 1997) and *Seriatopora* spp. (Nozawa & Loya, 2005). The lack of an efficient allorecognition system is most likely universal among both scleractinian and alcyonacean corals (Barki *et al.*, 2002; Nozawa & Loya, 2005). In agreement with this hypothesis, recent observations of fusion among juveniles of the coral *Acropora millepora* following aggregated larval settlement, as well as after contact through growth for up to 11 months post-settlement, suggest that this broadcast spawning coral species lacks a mature allorecognition system in its early life (Chapter 3). Moreover, some juveniles originating from fused aggregations remained alive for almost two years, with no signs of rejection among interacting genotypes. Genetic analysis showed that these colonies were chimeras (i.e., different genotypes within the same colony, Chap. 2). In some colonies arising from fusion, the first signs of rejection were detected at two months post-settlement, suggesting that juveniles were not able to recognize genetically different conspecifics prior to this (Chap. 3).

Allorecognition in invertebrates is commonly thought to rely on pathways involved in innate immunity, thus PAMPs (pathogen-associated molecular patterns; Janeway Jr & Medzitov, 2002) may be involved in activating innate immune responses in corals. Such detection is believed to be achieved by proteins called Pattern Recognition Receptors (PRRs), which are expressed by innate immune cells and have the ability both to

recognize PAMPs and to subsequently initiate an immune response (Janeway Jr. & Medzhitov, 2002). However, there is not yet enough data on the underlying mechanisms of innate immunity to link allorecognition with detection of PAMPs in corals. Additionally, some studies have highlighted criteria for an adaptative immunological response in corals rather than an innate response (Salter-Cid & Bigger, 1991).

PRRs receptors include C-type lectins (Robinson *et al.*, 2006) according to recent studies that have highlighted their involvement in immunity, allorecognition and non-self recognition in corals (Wood-Charlson *et al.*, 2006; Grasso *et al.*, 2008; Kvennefors *et al.*, 2008). Coral lectins bind to glycans on the surface of *Symbiodinium* cells, the endosymbiotic microalgae that form an important association with corals (Wood-Charlson *et al.*, 2006), and it has been shown that intact glycans are crucial for the recognition and successful acquisition of *Symbiodiunium* in the coral *Fungia scutaria* (Wood-Charlson *et al.*, 2006). Thus lectin type genes are good candidate genes for molecular studies of non-self recognition in corals.

The ability of lectins to recognize and bind to various types of bacteria and to *Symbiodinium* cells was described for Millectin, a mannose binding lectin gene in *A. millepora* (Kvennefors *et al.*, 2008). Similarly, CEL III lectin types described in the sea cucumber *Cucumaria echinata* (Kouzuma *et al.*, 2003) are able to recognize cell surface carbohydrate chains on non-self tissues (i.e., PAMPs), but in addition, they are also responsible for the formation of ion permeable pores in target cell membranes (for lysis of target cells) after conformational change and oligomerization. High sequence similarity between the *Acropora* CELIII proteins (CELIII A036-E7 & A049-E7) and the CEL III described in *Cucumaria echinata* suggests that they may have similar roles (Grasso *et al.*, 2008). Additionally, lectins have been linked to complement C3 in the innate immune system of a solitary ascidian (Sekine *et al.*, 2001), and are potentially

involved in a lectin-dependent complement system (Sekine *et al.*, 2001). Moreover, the recent description of coral complement C3 (Dishaw *et al.*, 2005; Miller *et al.*, 2007) suggests complement C3 could be a potential key gene in allorecognition, and its activation could rely on lectins, as described in ascidians (Sekine *et al.*, 2001). Finally, apextrin encodes a perforin domain containing protein, which contains no identifiable domains other than the complement MAC/PF (Miller *et al.*, 2007). Apextrin could also be involved in immune responses through the complement pathway and represent another gene potentially involved in allorecognition.

To evaluate the roles of putative immune response genes in coral innate immunity, expression levels of lectins, complement C3 and apextrin were compared during allorecognition maturation in juveniles of the coral *Acropora millepora.* In addition, differential expression of these genes during the onset and establishment of *Symbiodinium* endosymbiosis provides further insights into their roles in recognising non-self during the establishment of coral-algal symbiosis.

4.2 Materials and methods

4.2.1 Obtaining and rearing coral larvae

Mature colonies of *Acropora millepora* were collected from Nelly Bay, Magnetic Island, in the central Great Barrier Reef prior to the predicted spawning in October 2007. Colonies were transferred into 1000 litre aquaria with running, temperature-controlled (28.5°C), 1 µm filtered sea water (FSW) at the Australian Institute of Marine Science. The genotype of each colony collected was determined based on analyses of 3 microsatellite loci: Amil2_006, Amil5_028, and Amil2_022 (van Oppen *et al.*, 2007) prior to spawning to ensure corals were genetically distinct and to avoid crosses between clone mates.

On the anticipated night of spawning, colonies were isolated in individual 70 L aquaria filled with FSW, and kept isolated until at least 45 minutes after spawning had commenced. Gametes were collected from the water surface and mixed with gametes from a second colony in separate 70 L fertilization aquaria filled with FSW. Four gamete crosses were performed, giving rise to four different gamete cultures (Fig. 4.1). Gametes were allowed to fertilise for at least 1.5-2 hours, after which a small subset of eggs was sampled for microscopic confirmation of fertilisation and development. Embryos were cleaned by three consecutive water changes of the 70 L fertilisation aquaria, draining ~90% of the water from the bottom and slowly refilling from the top each time. Embryos were then transferred into 500 L settlement aquaria supplied with running (1L/min), temperature-controlled (28.5°C) FSW in a controlled environment facility, at a density of approximately one larva per mL. One 500L settlement aquaria was established for each of the larval cultures A-D, thus each culture derived from one set of parents and comprised full sibling larvae (Figure 4.1). Embryos were checked microscopically in order to assess their development until ~48 hours following fertilisation, when the fully developed, ciliated planula stage was reached. Once swimming larvae were elongated and had started to search the substratum for settlement sites, the bottom of each tank was covered with 30 autoclaved terracotta tiles (15 cm x 15 cm).

Eleven days after spawning, the laboratory-reared juvenile corals that had settled on the terracotta tiles were placed in the field. The terracotta tiles were tagged and skewered on rods through a hole in the centre of the tiles, with spacers (2-3 cm long) placed between each tile. The rods were suspended horizontally between two star pickets that had been driven into dead substratum at 5m depth in Nelly Bay (Magnetic Island), an inshore reef

(Babcock & Mundy, 1996; Anthony *et al.,* 2004) with a gentle slope down to approximately 10m.

To compare expression of immune genes throughout larval and juvenile development, 3 samples (20-30 polyps per sample) from each larval batch (A, B, C, and D) were collected at six time points between spawning and six months post-settlement. Sampling was performed one month after spawning (December 2007) and every month to May 2008. Each sampling was performed at midday (between 11am and 1pm) using sterile scalpel blades. Each sample consisted of approximately 20-30 polyps per cryotube which was immediately snap frozen in liquid nitrogen and subsequently kept at -80°C. Three replicate tubes were sampled for each sibling group of juveniles (i.e., A, B, C & D) per time point (3 x 4 = 12 samples per sampling time). Consequently, every "sample" represented a pool (20-30 individuals per sample) of closely related coral juveniles defined as full sibling groups (see Fig. 4.1). Therefore, for ease of reference, larvae and juveniles from tank A are referred to as full siblings A, larvae from tank B are as full siblings B, and so on (see Fig. 4.1).

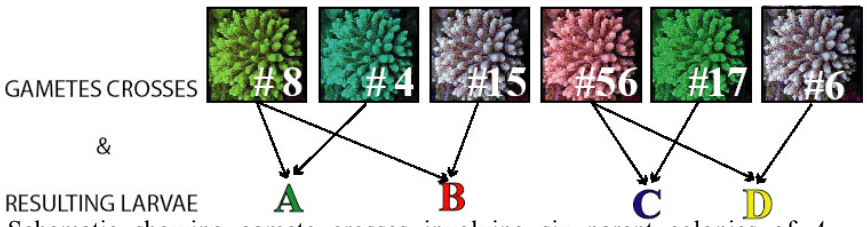

Fig. 4.1. Schematic showing gamete crosses involving six parent colonies of *Acropora millepora* (identified by a number #) that were performed to establish four larval cultures referred to as full sibling groups A, B, C, & D.

4.2.2 mRNA extraction, treatment, and cDNA synthesis

Messenger RNA (mRNA) was extracted from coral samples using the Dynabeads® mRNA direct kit (Invitrogen), following the protocol detailed in (Császár *et al.*, 2009). mRNA was eluted in 10mM Tris-HCl buffer (pH=7.5), and kept at -80°C. Following

extraction, mRNA was treated for genomic DNA contamination using Turbo DNA free™ (Ambion). Then, mRNA concentration was estimated using a NanoDrop ND1000 (Nanodrop technologies). Finally, the quality of the mRNA was verified using a 2100 BioAnalyzer (Agilent). Subsequently, mRNA concentrations were re-calculated by taking into account rRNA contamination.

Complementary DNA (cDNA) synthesis was performed using SuperSript™ III first-strand synthesis supermix (Invitrogen). Samples for cDNA synthesis were prepared using a CAS 1200 robot (Corbett Robotics) by adding 70 ng of mRNA per reaction, 10 µL of 2x RT reaction mix, 2 µL of RT enzyme mix, and DEPC treated water (Ambion) in order to reach a final volume of 20 µL/reaction. Samples with mRNA concentrations <8.75 ng/µL were not used for cDNA synthesis. The cDNA synthesis was performed on a Gradient Mastercycler (Eppendorf) and the protocol consisted of the following steps: 25°C for 10 min, 50°C for 30 min, 85°C for 5 min, and the reaction was terminated by placing tubes on ice. All samples were prepared and processed during the same cDNA synthesis run. Subsequently, samples were diluted in DEPC treated water (Ambion) in order to reach a final concentration of 0.5 ng/µL, and were kept at -20°C.

4.2.3 Primer design

Primers were designed using OligoPerfect™ designer (Invitrogen), using the following parameters: primer size from 18 to 27 bases, melting temperature from 68 to 72°C, primer GC content from 50 to 57 %, and amplicon size from 148 to 152 bp. Subsequently, melting temperatures, primer quality and primer dimer were checked using the primer test section of FastPCR (http://www.biocenter.helsinki.fi/bi/Programs/download.htm).

Selection of Internal Control Genes (ICGs) for standardisation of relative gene expression was based on previous studies of gene expression in *A. millepora* (Seneca *et al.*, 2009),

and primers were designed for: ribosomal protein genes S7, L13 and L9 (RpS7, RpL13 and RpL9 respectively), Ctg1913 and GAPDH (Table 1). Genes of interest (GOI) were chosen because of their suspected involvement in *A. millepora* immunity (Miller *et al.*, 2007; Grasso *et al.*, 2008; Kvennefors *et al.*, 2008), and primers were designed for apextrin, complement C3, and 2 CEL III type lectins: A036-E7, and A049-E7 (Table 1). Primers (Sigma-Genosys) were then re-suspended in 1xTE in order to reach a concentration of 200 µM. Subsequently 10 µM aliquots were made for every primer by diluting primers in DEPC treated water (Ambion).

Table 4.1. Internal Control Genes (ICGs) and Genes of Interest (GOI) and associated primers used in qRT-PCR reactions. * Internal Control Genes for *A.millepora* (Seneca *et al.*, 2009)

Gene name	Primer sequence	Amplification region	Tm (°C)	GC (%)
RpS7*	F: t c t t c c c t g c c a c a t c a a c c t c c t R: g a a a c c c c a a g a t g c g g g t g a a c	503-631	F: 70.4 R: 69.8	F: 54.2 R: 56.5
RpL13*	F: a c t a t g c g g g c a a c g g a t g g t t c R: g g a t g g a g c a c g g a a a t g a a a t g g	94-244	F: 70.4 R: 67.5	F: 56.5 R: 50.0
RpL9*	F: g c c g c a t t c t c a c a c g c c t a a t g R: t g a t c a a g g g g g t c g t c t a t g g c t a	278-427	F: 69.6 R: 69.1	F: 56.5 R: 52.0
Ctg1913*	F: a g a t t g t g g c g t t g g g g a a t g c t R: c g c a c a g a a g c a g c a a g c a a t g a	206-356	F: 70 R: 69.4	F: 52.2 R: 52.2
GAPDH*	F: t g t t c c a a a g a a g c g c g c a t a a c c R: t t c c c t g g g a g a a g t t c g g t g g a	1066-1213	F: 69.1 R: 70.2	F: 50.0 R: 56.5
Apextrin	F: c g g g a c g c a a a c g t t t t g g a g t t R : c a g g a a a c a t c t t c g g g g c c a a c	1915-2062	F: 69.4 R: 69.6	F: 52.2 R: 56.5
C3	F: t c a a g t g g a a g g t c g c g t g g a a a R: g c c t c c t t t t g g a a c c g g a a g t g	199-351	F: 69.3 R: 69.5	F: 52.2 R: 56.5
A036-E7	F: c t c a t t g c a t t g c t g g g g t c c t g R: t t g a g a g g c t g c t g t g g g g a a g a	44-194	F: 69.6 R: 70.7	F: 56.5 R: 56.5
A049-E7	F: t g t c c g a g g a t g c a t g t g g c a a t R : g c a a t c c t c a t c c a g g c a t c g t g	1380-1530	F: 69.6 R: 69.2	F: 52.2 R:56.5

4.2.4 Real time quantitative (qPCR)

GOIs and ICGs were amplified in 20 µL reactions (3 replicate/sample/gene), each containing 10 µL QuantiTect SYBR green (Qiagen), 6 µL DEPC treated water (Ambion), 1 µL Forward primer (at 10 µM), 1 µL Reverse primer (at 10 µM), and 2 µL of cDNA (at 0.5 ng/µL). All reactions were prepared using a CAS 1200 robot (Corbett Robotics), in order to decrease pipetting error. qPCRs were performed on a Rotor Gene 3000A (Corbett research), with the following steps: 15 min at 95°C, 50 cycles at 95°C during 10 sec and 65°C during 120 sec, with a final melting step from 60°C to 95°C. Gain was set to 10x for all runs. Data were acquired at the end of each cycle and each data point represented a mean of 20 fluorescence readings from each tube. Because of the Rotor Gene limited capacity (only 72 samples per run), samples were run for each of the full sibling groups (i.e., A, B, C or D) separately, but included all time points for 2 or 3 genes per run. Every sample was run 3 times for each gene.

4.2.5 Primer specificity and efficiency

Primer specificity was checked at the end of every run with melting curves for detection of any non-specific amplification or primer dimerization. Melting curves were obtained by heating starting at 60ºC and a rate of 0.1ºC per second up to 95ºC with continuous measurement of fluorescence. Data were checked using the analysis section (Melting A.FAM) of the Rotor Gene software.

In order to calculate the primer efficiencies (E), the dilution method was used on a pool of cDNA (Rebrikov & Trofimov, 2006). A dilution series was performed (1/1, 1/2, 1/5, and 1/10) on pooled cDNA from all samples. Data were obtained using the analysis section (Cycling A.FAM, Standard Curve) of the Rotor Gene software.

4.2.6 Analysis

Ct values were obtained using the analysis section (Cycling A.FAM) of the Rotor Gene software. Noise slope correction was activated in order to take into account the background fluorescence level of every sample during the run. Also, dynamic tube normalization option was activated in order to determine the average background level of each individual sample before amplification commenced. Ct values were obtained after setting the threshold at 0.03 for all runs, and were then entered into Excel. Ct values were transformed in relative quantities (Q), using the delta Ct formula (Vandesompele *et al.*, 2002), and taking the first time point (per full sibling group) in the time series as a reference:

$Q = E^{(refCt - sampleCt)}$ with E being the amplification efficiency of the gene.

From relative quantity data, we were able to determine the best ICGs using GeNorm 3.5 (http://medgen.ugent.be/~jvdesomp/genorm/), for every full sibling group. Subsequently, normalization factors (NF) were calculated per sample:

NF = geometric mean (Q_{ICG1},Q_{ICG2},Q_{ICG3})

Finally, Normalized expression (NE) levels were calculated per gene and per sample.

$NE = NF/Q_{sample}$

Statistical analyses were performed for each full sibling group (biological replicates) and for each gene using Statistica 6.0, testing first for data normality. After Log10 transformation, the normality assumption was validated and ANOVAs were performed for the factors: time (6 levels maximum), gene (4 levels maximum) and sample wich was nested within time but orthogonal with gene (i.e, sample(gene*time) in Statistica). A supplementary Post Hoc Tukey HSD test was performed if significant differences in gene expression were detected.

4.3 Results

4.3.1 Internal Control Genes

Using GeNorm, the best ICGs and the appropriate number of ICGs per genotype were determined. Three ICGs were used for each full sibling group: RpS7 and RpL13 for all, plus GAPDH for group A, RpL9 for groups B and C, and Ctg1913 for group D samples.

4.3.2 Genes Of Interests

The ANOVA for the factors SAMPLE(GENE*TIME) revealed that each full sibling group had a significant variance of gene expression over time: full siblings A ($SS=0.21$, $F=27.62$, $df=39$, $p<0.01$), full siblings B ($SS=0.09$, $F=23.18$, $df=25$, $p<0.01$), full siblings C ($SS=0.33$, $F=57.26$, $df=32$, $p<0.01$), and full siblings D ($SS=0.15$, $F=14.49$, $df=40$, $p<0.01$).

Post-hoc Tukey HSD tests revealed that gene expression levels of the gene A036-E7 were significantly higher in March (i.e., 4 months post-settlement, Figs. 4.2A, 4.2B and 4.2C) than at any other time for full siblings groups A, B, and C ($p<0.01$). Additionnally, post-hoc Tukey HSD tests revealed no significant difference in expression levels of the gene A036-E7 between samples within the same full sibling groups A, B, and C in March ($p>0.98$).

Expression levels of apextrin were also significantly higher for full sibling group A in March (Fig. 4.2D) than at all other sampling times in the six months post-settlement ($p<0.01$), but samples at the same time point, differed significantly from each other ($p=0.037$). Juveniles from full sibling group A showed the same trend for complement C3 gene, i.e., higher levels of expression in March (data not shown); however these levels were not significantly different from those at all other time points ($p>0.05$).

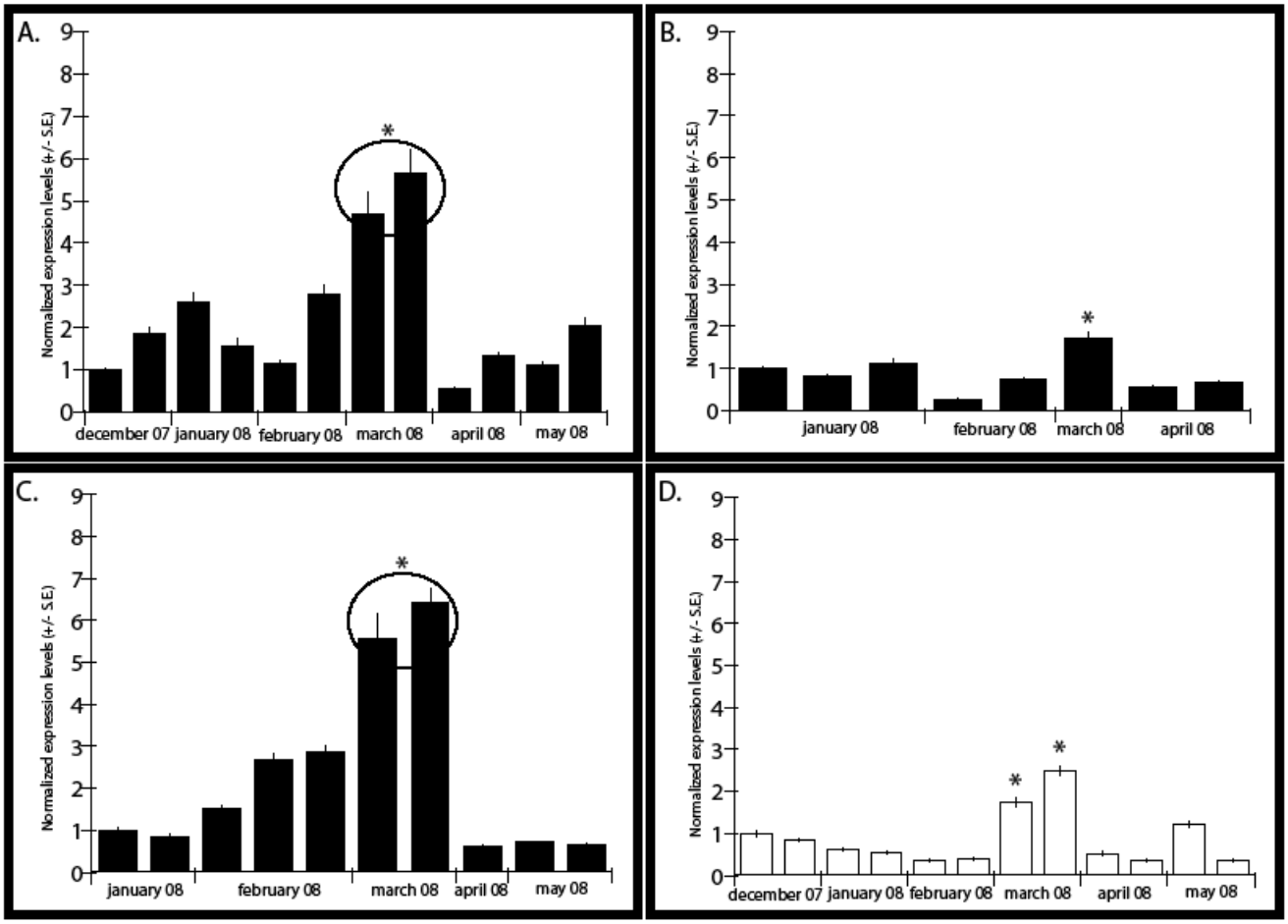

Fig. 4.2. Comparison of mean (±SE) normalized expression levels for the genes A036-E7, a CELIII type lectin gene, (black histograms) or apextrin (white histograms), from December 2007 to 6 months post-settlement (May 2008) for: **A)** full sibling group A; **B)** full sibling group B; **C)** full sibling group C; **D)** full sibling group A. Data represent normalized expression levels (normalization performed with 3 ICGs). Circles: no significant difference between sample replicates for the same time point. *: significant difference with all other samples ($P<0.05$, Tukey HSD). n=3 expression levels per sibling group per sample.

4.4 Discussion

Juveniles of the coral *Acropora millepora* showed differential expression of putative immune response genes during a six month period following settlement, raising the possibility that the candidate genes tested may be involved in the maturation of allorecognition systems during early life history stages of corals. The most striking result was that expression of one of the CELIII type lectin genes, A036-E7, was significantly greater during the fourth month post-settlement for juveniles in three of the four full sibling groups tested. CELIII type lectin genes are known to be involved in non-self recognition in a number of marine invertebrates (Kouzuma *et al.* 2003; Kvennefors *et al.* 2008). Evidence that *A. millepora* juveniles from the same cohort showed the first signs of rejection in contact reactions at two months after settlement (see chapter 3) indicates that allorecognition systems are non-functional in *A. millepora* prior to two months. Although fusions ceased to occur between non-sibling juveniles growing into contact after two months, it took five months before fusions between half-siblings ceased (chapter 3), suggesting that maturation of allorecognition systems involved in distinguishing half-siblings requires five months. The corresponding peak in expression levels of the CELIII type lectin gene at four months post-settlement provides corroborative evidence that it is likely to be involved in allorecognition in *A. millepora*.

The two-month delay in peak expression for the CELIII type lectin gene in comparison to the appearance of the first signs of rejection between corals in contact reactions (chapter 3) might reflect a potential dual role of CELIII lectins in *A. millepora*. For example, the CEL III type lectin isolated from the sea cucumber *Cucumaria echinata* is involved in both the recognition of cell surface carbohydrate chains (i.e., PAMPs) and the lysis of non-self tissues (Kouzuma *et al.*, 2003; Uchida *et al.*, 2004). The dual role is possible because CELIII consists of 3 domains: the N-terminal carbohydrate binding

domains (1 and 2) and the C-terminal domain (#3) (Kouzuma *et al.*, 2003; Uchida *et al.*, 2004). The N-terminal region binds to specific carbohydrates for recognition (Kouzuma *et al.*, 2003), which then triggers a conformational change in the C-terminal region, inducing oligomerization and resulting in the formation of ion-permeable pores on the target cell membrane (Kouzuma *et al.*, 2003). The peaks in CELIII A036-E7 and A049-E7 at four months post settlement, rather than two months when the first signs of allorecognition were observed in *A.millepora* (chapter 3), may relate to increased activity associated with the lysis of non-self tissues. Moreover, high sequence similarities between the CELIII *Acropora* proteins (CELIII A036-E7 & A049-E7) and the CEL III described in *Cucumaria echinata* suggest that they could have similar roles (Grasso *et al.*, 2008). Consequently, the peak in the expression of the CELIII type lectin A036-E7 observed at 4 months post-settlement might be related to maturation of mechanisms involved in the recognition of half-siblings and/or the lysis of non-sibling tissues, both of which would be consistent with the hypothesis that lectins play a crucial role in coral allorecognition.

It is important to note, however, that results also show a drop in the expression of CELIII type lectin after the peak in the fourth month. If expression levels were only associated with immuno-competence, one would expect the levels of lectins to remain high after the fourth month, rather than drop to levels similar to those in the pre-competency period.

Therefore, in addition to enhanced immuno-competence, the peak and then the drop in expression of lectins might be linked to strong non-self recognition challenges occurring around four months post-settlement, given that high expression levels would be expected until the end of the study if immuno-competence were the only explanation. I hypothesize that the initiation of non-self and allorecognition maturation (~2 months)

could be followed by the recognition of incompatible symbionts, either *Symbiodinium* types and/or coral-associated bacteria. Studies of the establishment of *Symbiodinium* symbioses reveal that lack of specificity in initial *Symbiodinium* uptake is followed by a winnowing of *Symbiodinium* types present within coral juveniles (Little *et al.*, 2004; Abrego *et al.,* 2009).

During the first few months post-settlement, juveniles of *Acropora tenuis* and *A. millepora* are able to take up various *Symbiodinium* types, following which they became dominated by one type, although the period of winnowing may be quite extensive (Abrego *et al.*, 2009). Lectins are suspected to play a role in the recognition of *Symbiodinium* in corals (Wood-Charlson *et al.*, 2006) and the temporal expression pattern observed here for CEL III A036-E7 is consistent with this notion. For symbiont recognition and successful acquisition, intact glycans on symbiont cell surfaces must bind to coral lectins (Wood-Charlson *et al.*, 2006). Additionally, the ability of lectins to recognize various types of PAMPs on non-self was described for Millectin, a mannose binding lectin gene in *A. millepora* (Kvennefors *et al.*, 2008). The binding region of this functional mannose binding lectin showed high sequence diversity, suggesting that Millectin could recognize and bind to various bacterial species and *Symbiodinium* types (Kvennefors *et al.*, 2008). In addition, lectin type genes show high levels of expression in alloimmune challenged ascidians (Oren *et al.*, 2007), with the highest expression levels occurring when signs of rejection appeared between incompatibly paired ascidians. Moreover, ascidians potentially have a lectin-based opsonisation system (non-self tissues marked with lectins for destruction), with lectins playing a role in opsonisation at the point of rejection (Oren *et al.*, 2007).

Lectins have also been linked to complement C3 in the innate immune system of a solitary ascidian and are potentially involved in a lectin-dependent complement system

(Sekine *et al.*, 2001). Although links between lectins and complement genes were not demonstrated in corals, in combination, the presence of complement C3 (Dishaw *et al.*, 2005; Miller *et al.*, 2007) and the involvement of lectins in coral immunity (Kvennefors *et al.*, 2008) suggest the possible involvement of a lectin-dependent complement system in allorecognition pathways of corals. Although expression of complement C3 did not differ significantly throughout the 6 month period, nevertheless, it was highly expressed in the fourth month in juveniles of sibling group A, when both apextrin and CELIII type lectin A036-E7 were also highly (and significantly) expressed. The co-expression of these three genes during development raises the possibility that they could influence the immune system of corals through a common pathway or system, but further studies investigating the expression of apextrin, complement C3 and lectin type genes in alloimmune challenged corals are needed to elucidate and clarify the emerging picture of a complex system of innate immunity in corals. Other complement genes, such as complement C2 and C5 recently identified (based on ESTs and BLAST hits only) in *Aiptasia*, could also be relevant key genes in cnidarian immunity (Sunagawa *et al.*, 2009).

To summarize, my results show that one CELIII type lectin, A036-E7, was expressed at significantly higher levels four months post-settlement in juveniles of *A. millepora*. Together with findings from other recent studies of *A. millepora*, these results indicate that lectins are likely to play a crucial role in allorecognition and more generally in the innate immune system of corals, which is likely to have greater complexity than commonly thought. I hypothesize that high expression levels of A036-E7 were linked to allorecognition maturation and/or non-self recognition challenges, such as algal symbiont recognition and the winnowing of algal symbiont diversity. Some caution should be exercised in interpreting my results, as peaks in the expression of genes studied were

followed by a drop, while high expression levels would be expected until the end of the study if immuno-competence were the only explanation for the observed peak. Also, during this study, the expression of a limited number of genes (4 genes only, no large scale genomic screening) in *A. millepora* was assessed at one location for a limited batch of coral juveniles. It is therefore possible that a range of uncharacterised environmental or physiological factors may have influenced patterns of gene expression. Further, in future studies focusing on allorecognition and non-self recognition in juveniles that are challenged, for example by contact reactions or symbiont infection, validation of the roles of CELIII type lectins, complement C3 and apextrin in allorecognition and non-self recognition responses of corals is required.

Chapter 5.0 Chimersim in wild populations of the broadcast spawning coral *A. millepora* on the Great Barrier Reef

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All the data was collected and analyzed by E. Puill-Stephan, who also wrote the chapter and manuscript after intellectual contributions by all co-authors.

5.1 Introduction

Chimeras are organisms containing tissues or cells of two or more genetically distinct individuals (Rinkevich & Weissman, 1987) which typically arise through fusion. Fusion of genetically distinct individuals has been documented in at least nine phyla of protists, plants and animals (Buss, 1982; Rinkevich, 1999), including cnidarians in experimental allorecognition studies (Heyward & Stoddart, 1985; Resing & Ayre, 1985; Willis & Ayre, 1985). However, the extent to which natural chimeras occur in populations of reef corals is currently unknown.

Natural chimerism provides both benefits and costs for genetically heterogeneous organisms (Pineda-Krch & Lehtila, 2004b). A major benefit of chimerism is that colonies may have a greater store of genetic variability and hence a wider range of physiological qualities and characteristics compared to non-chimeric colonies (Ben-Shlomo *et al.*, 2001). Also, fusion provides a mechanism to increase in size more rapidly than growth alone, and could thus enhance chances of survival (Sammarco, 1982; Jackson, 1986). Conversely, costs include potentially decreased growth rate and decreased reproductive output of fused colonies (Rinkevich & Loya, 1985). The occurrence of two (or more) genotypes within the same individual or colony could also lead to cell linage competition for position in the germ line (Buss, 1982), which has been identified as a potentially severe cost associated with the chimeric state (Buss, 1982). The costs and benefits associated with chimerism have provoked considerable debate. While it is known that chimeras exist, their importance appears to be under-rated, primarily because chimerism challenges evolutionary theory developed for genetically homogeneous individuals and chimeras are commonly thought to be rare in natural populations (Pineda-Krch & Lehtila, 2004a).

Mutation within cell lineages (i.e., somatic mutation) is a second mechanism leading to the presence of genetically distinct tissues within individuals (Buss, 1982). Somatic mutations are relatively common in plants, but usually only affect a portion of the meristem. Strictly speaking, such plants are chimeras as they are composed of two or more genetically distinct tissues and are indeed often referred to as "chimeras" (Whitham & Slobodchikoff, 1981). To avoid confusion, however, we will refer to the latter as mosaicism rather than chimerism because of the various characteristics that clearly differentiate their respective origins, particularly the origin of chimeras through fusion versus the origin of mosaics through somatic mutation (Strassmann & Queller, 2004).

Natural chimeras usually originate from allogeneic fusions (i.e., fusions between different individuals of the same species). Although chimerism occurs in a wide range of organisms (Buss, 1982) and has even been recorded in humans and other mammals (Rinkevich, 1998; Rinkevich, 2001), it has been reported much more frequently from the marine environment, primarily from benthic organisms with planktonic larvae or propagules, such as red algae (Santelices *et al.*, 1999) or colonial marine animals including corals, bryozoans and ascidians (Santelices, 2004). Thus, this phenomenon may be most common in species in which fragmentation and fusion are normal features of the life cycle (Hughes, 1989). Therefore, the occurrence of chimerism in colonial marine animals further challenges notions of genetic uniqueness within clonal organisms (Santelices, 1999) and the commonly held view that clonality is a mechanism for maintaining well-adapted lineages (Strassmann & Queller, 2004).

Colonial, modular organisms, including most sponges, cnidarians, bryozoans, and many terrestrial and marine plants, are composed of repeated building units (modules such as polyps, zooids, etc.) that replicate through budding, and which lead to vegetative growth

of colonies (Hall & Hughes, 1996). Within sessile, modular, marine invertebrates (e.g. sponges, bryozoans, ascidians, and cnidarians), chimeras can originate from the fusion of larvae that settle adjacently, or from the fusion of colonies that come into contact through growth or movement (Sommerfeldt *et al.*, 2003). Because the allorecognition systems of adult colonial marine invertebrates generally effectively discriminate between clone mates and non-clone mates (Grosberg, 1988), low proportions of chimeras are typically expected in natural populations. However, studies have clearly demonstrated the possibility of genetically distinct corals fusing (Heyward & Stoddart, 1985; Resing & Ayre, 1985; Willis & Ayre, 1985). Also, the sometimes high occurrence of chimeras in natural populations of various colonial marine invertebrates other than corals (Sommerfeldt & Bishop, 1999; Ben-Shlomo *et al.*, 2001; Sommerfeldt *et al.*, 2003; Rinkevich, 2005; Ben-Shlomo *et al.*, 2008) and under experimental conditions (Amar *et al.*, 2008) indicates that their self-nonself recognition systems at least occasionally allow the fusion of genetically non-identical entities.

Chimeras have been widely observed in natural populations of colonial marine ascidians (Sommerfeldt & Bishop, 1999; Ben-Shlomo *et al.*, 2001; Sommerfeldt *et al.*, 2003; Rinkevich, 2005; Ben-Shlomo *et al.*, 2008); however, surprisingly little is known about the extent of chimerism in natural populations of adult corals. The majority of studies about chimerism in corals have focused on juveniles and particularly on larvae during the settlement phase when they come into contact with conspecifics. To date, aspects of chimerism have only been assessed in brooding corals (Neigel & Avise, 1983; Hidaka, 1985a; Hidaka, 1985b; Stoddart *et al.*, 1985; Chadwick-Furman & Rinkevich, 1994; Frank & Rinkevich, 1994; Frank *et al.*, 1997; Hidaka *et al.*, 1997; Frank & Rinkevich, 2001; Barki *et al.*, 2002; Rinkevich, 2004a; Nozawa & Loya, 2005; Amar *et al.*, 2008), whereas coral reefs are dominated by broadcast spawning species of coral. Here we

explore the extent of genetic chimera occurrence (i.e., the cohabitation of different genotypes within a single coral colony) within two populations of *Acropora millepora*, a common broadcast spawning coral on the Great Barrier Reef (Australia), using genetic characterization of coral tissues at 12 polymorphic DNA microsatellite loci.

5.2 Materials and methods

5.2.1 Sampling

To estimate the frequency of occurrence of chimeras in natural populations of the branching coral *Acropora millepora*, 65 colonies were tagged, photographed and sampled at south-west Pelorus Island (Pelorus Island, Palm Island group, S 18°33.030' E 146°29.316'), and 59 colonies in Nelly Bay at Magnetic Island (Magnetic Island, S 19°10.115' E 146°51.006'). Colonies between 15 and 40 cm in diameter were selected haphazardly for tagging from within an area of ~10 m x 400 m in Nelly Bay (Magnetic Island) and ~10 m x 200 m in south-west Pelorus (Pelorus Island). Fifteen cm was selected as the minimum size because colonies needed to be sexually mature for reproduction experiments and 40 cm was the maximum size sampled due to permit restrictions (Great Barrier Reef Marine Park Authority permit # G07/22554.1). Colonies that showed visual evidence of genetic differences, such as rejection lines or different morphological types or colors within an apparently single colony were excluded from sampling because it could not be discounted that such colonies represented two separate colonies in close association. Such colonies would have been scored as chimeras, whereas they represented cases of 2 (or more) incompatible colonies in close contact (e.g., swp#68 and 69, see below). The application of these conservative criteria means that it is likely that we missed some chimeras. Hence, this study provides a minimum estimate of the frequency of chimeras in natural populations of *A. millepora*. Sampling of one apparently fused colony, which appeared to be a single colony but had two

clearly distinct colored sections separated by a rejection line, provided an opportunity to estimate what level of genetic difference resulted in rejection between two closely associated colonies. We considered this colony as two different colonies: Swp68 and Swp69.

In order to increase the likelihood of detecting genetic variability at the colony level, branches were sampled as far away from each other as possible across the colony. Because of permit restrictions, the maximum sample size per colony could not exceed eight branches. Samples were named according to (1) their site of origin and called Mag or Swp for Magnetic Island or south-west Pelorus Island respectively, (2) their colony number (1 to 59 in Magnetic Island, and 1 to 69 in south-west Pelorus Island), and (3) the branch replicate (from A to H). Once sampled, coral fragments were preserved and stored in 100% ethanol for down-stream DNA extraction and genotyping.

5.2.2 DNA extraction and genotyping

DNA was extracted using 'Wayne's method' (Wilson *et al.*, 2002). DNA pellets were re-suspended in 200 mL of 10 mM Tris (pH=9) and stored at 4°C. Prior to amplification, DNA was diluted at 1:10 in MilliQ-water. Microsatellite loci were amplified in 10 µL multiplex PCR reactions, in PTC-100 Peltier Thermal Cyclers. Four different primer mixes (MP2, MP3, MP5, and MP9 see Table 5.1) each amplifying two, three or four microsatellite loci were used. Eleven microsatellites were specifically designed for *A. millepora* (van Oppen *et al.*, 2007; Wang *et al.*, 2008). Another locus (Apam3_166) previously developed for an acroporid species from the Caribbean, *Acropora palmata*, was also used because of its successful amplification in *A. millepora* and its high level of polymorphism (van Oppen *et al.*, 2007). These loci are unlinked (van Oppen *et al.*, 2007; Wang *et al.*, 2008). Reactions contained 1 µL DNA template, 1 µL 10x primer mix, 5 µL 2x Qiagen multiplex PCR mix, and 3 µL milliQ-water. The cycling protocol was: 1 x

95°C (15 min), 35 x (30 sec at 94°C, 90 sec at 50°C, and 60 sec at 72°C), 1 x 60°C (30 min), and 4°C for ever. PCR products were diluted in Sample Loading Solution (SLS from Beckman Coulter) at 1:10. Then, 2.5 µL of the diluted PCR products were loaded into a Genetic Analysis System CEQ 8800, together with 37.25 µL of SLS and 0.25 µL of 400 bp size standard (Beckman Coulter), for separation and subsequent PCR product size determination.

Table 5.1. Primer mix, associated microsatellites and dyes, concentrations, and number of alleles (Na) per population.

Primer mix name	Micorsatellite loci	Repeat motif	Associated WellRED dye	Concentration in 10x primer mix	*Na* in Magnetic Island population	*Na* in south-west Pelorus population
MP2	Amil2_006	$(CA)_4TA(CA)_4$	D2	0.8 µM	6	6
MP2	Amil5_028	$(TCACA)_7TCAC$ $(TCACA)_4$ TCACTCACTCACA	D3	0.8 µM	8	7
MP2	Amil2_002	$(TG)_{10}$	D4	0.28 µM	6	5
MP3	Apam3_166	$(AAT)_{28}$	D2	1.5 µM	16	15
MP3	Amil2_22	$(AC)_{10}$	D3	1.0 µM	13	14
MP3	Amil2_23	$(AG)_7$	D4	0.6 µM	6	5
MP5	Amil2_010	$TA(TG)_{11}$	D2	0.5 µM	17	14
MP5	Amil2_012	$GA(CA)_6GA(CA)_2$	D3	0.3 µM	3	3
MP9	Wgs_152*	$(AT)_9$	D4	1.0 µM	12	8
MP9	Wgs_035*	$(GTAT)_6(GTTT)_8$	D3	1.5 µM	6	7
MP9	Wgs_189*	$(ATCT)_7$	D2	2.0 µM	7	7
MP9	Wgs_134*	$(GATA)_6$	D2	2.0 µM	5	4

* locus amplified only with chimeric samples

5.2.3 Scoring

Once samples were run through the CEQ 8800, data were analyzed with the Fragment Analysis software from the Genetic Analysis System CEQ 8800 from Beckman Coulter (400FragmentAnalysisParameter). All results were scored manually. Based on No Template Controls peak values, peaks under 5000 RFU were not scored. Fragment sizes were recorded into Microsoft Excel for further analysis.

Eight loci (from primer mixes MP2, MP3 and MP5, see Table 5.1) were amplified and scored for all samples. In order to minimize scoring errors, all chimeric samples were processed twice. Four loci from primer mix MP9 (see Table 5.1) were only amplified and scored for chimeric samples.

5.2.4 Chimerism

Several mutational models have been developed for microsatellites (Ellegren, 2000): the Infinite Allele Model, Stepwise Mutation Model, Two phase model and Generalized stepwise model, and the K-allele model (Estoup *et al.*, 2002). However, stepwise mutations, consisting of the addition or subtraction of one single repeat unit, are the most common mutations in microsatellite loci in plants, birds and humans (O'connel & Ritland, 2004). Thus, in our study, when genotypes within a single colony differed by one allele at only one locus, we assumed alleles with a single repeat difference were probably produced by a somatic mutation, and therefore the colony could not be classified as a chimera (e.g., Swp64, Table 5.3). This approach provided a lower estimate of chimera proportions within the studied samples. Estimates of the rate of somatic mutations per locus per cell generation (10^{-7}) for multicellular clonal organisms (e.g., *Goniastrea aspera, G. favulus,* and *Platygyrus sinensis*) (Orive, 2001) suggest it is far less likely that two independent somatic mutations would have occurred in the same tissue. We are therefore confident that if genotypes within a single colony displayed at

least two non-shared alleles, the colony was chimeric. Consequently, a second, more conservative estimate of chimerism was calculated, where a colony had to display at least two non-shared alleles to be classified as a chimera. The percentage of chimerism was calculated within each population and overall, by determining the number of chimeric colonies compared to the total number of sampled colonies.

5.2.5 Analysis

Microsatellite locus polymorphisms were calculated with GenAlEx 6.1 (Peakall & Smouse, 2006) within each population. Relatedness between all genotypes (based on eight loci) was calculated with the Queller and Goodnight estimator in GenAlEx 6.1 (Peakall & Smouse, 2006). Queller and Goodnight's pairwise relatedness estimator (QG) values are expected to be equal or higher than 0.5 (i.e. QG≥0.5) for full sibs. Half sibs are expected to have values around 0.25, and QGs of unrelated individuals are expected to be close to 0 (Queller & Goodnight, 1989). Relatedness analysis was performed on genotypes based on eight loci (see Scoring section) because these loci were amplified for all samples, while four extra loci were amplified only for chimeric samples, and resulting relatedness data were not comparable to the population level.

5.3 Results

5.3.1 Proportion of chimerism in natural populations of *Acropora millepora*

A total of 984 samples, representing 124 colonies collected from populations of *A. millepora* at Magnetic Island and south-west Pelorus Island were genotyped using up to 12 microsatellite loci. All microsatellite loci used were highly polymorphic in both populations, and displayed up to 17 alleles (Table 5.1). Using conservative criteria (i.e., genotypes within colonies displayed two or more non-shared alleles), we estimate that 2% and 5% of *A. millepora* colonies in the Magnetic Island and Pelorus Island

populations, respectively, are chimeras. In total, six chimeric colonies were observed. In the Magnetic Island population, 2 out of 59 colonies (colonies 56 and 59) displayed two genotypes that differed by one allele, and one colony (24) consisted of two genotypes differing by at least two alleles (Tables 5.2 & 5.3). In the south-west Pelorus (Pelorus Island) population, three colonies (colonies 1, 44, and 15) displayed two genotypes that each differed by two or more alleles (Tables 5.2 & 5.3). Overall, chimeras represent 3% of all sampled corals according to this conservative criterion (Table 5.2).

Table 5.2. Number and proportion (%) of chimeric colonies in two wild populations (Magnetic and south-west Pelorus Islands) of *Acropora millepora*, after excluding likely somatic mutations under the assumption of a stepwise mutation model. Potential mosaics (**) with a single allele difference potentially arising from a stepwise mutation are also shown (last column)**.**

Population	Number of colonies with 1 allele difference, excluding potential somatic mutations	Number of colonies with ≥2 allele differences	Number of chimeric colonies	Proportion of chimeras within the population (if different at 1 allele, excluding potential somatic mutations)	Proportion of chimeras within the population (if different at ≥2 alleles)	Number of colonies with 1 allele difference potentially from somatic mutation = mosaic **
Magnetic Is (N=59)	2	1	3	5 %	2 %	1 **
south-west Pelorus Is (N=65)	0	3	3	5 %	5 %	0 **
All (N=124)	2	4	6	5 %	3 %	1 **

A less conservative estimate, based on counts of all colonies with more than one distinct genotype within a colony, including those that displayed just one non-shared allele (excluding single allele difference by one mutational step), indicates that 5% of colonies in both populations were chimeras. Mosaics arise from somatic mutations while chimeras originate from the fusion of genetically different individuals. Because the most common somatic mutation is a single mutational step, a colony displaying a single non-shared allele differing by only one mutational step, is more likely to be a mosaic (e.g. Swp 64, Table 5.2 & 5.3). Based on this criterion, only 0.8% of the sampled colonies were potential mosaics (i.e., 1 colony out of 124). However, non-single step mutations may also arise through somatic mutations, thus colonies with a single non-shared allele could also represent cases of somatic mutations (e.g., Mag 56 & 59). If all single allelic differences were assumed to arise through somatic mutations, 2.4% of the sampled colonies potentially represent mosaics. Overall, however, we consider it highly unlikely that two somatic mutations could arise in relatively young coral colonies (<40cm in diameter), and thus our conservative estimate of chimerism based on two non-shared alleles should avoid scoring colonies as chimeras that were in fact mosaics.

Table 5.3. Twelve loci genotypes of chimeric colonies for two wild populations (Pop): south-west Pelorus (Swp) and Magnetic Island (Mag). The most common genotype per colony is indicated in **bold**. The proportion (in %) of each genotype within the colony is estimated based on the number of branches with the same genotype. Samples are named according to: their site of collection (Mag or Swp), their colony number (1 to 59 in Magnetic Island, and 1 to 69 in south-west Pelorus Island), and the branch replicate (from A to H).* non shared alleles (i.e., allelic differences). ** single allelic variation potentially arising from somatic mutations (N.B. Colony Swp 64 is not considered to be a chimera because the only allelic variation potentially arises from somatic mutation).

Sample	Genotype proportion	Pop	Locus name	Amil2 012	Amil2 012	Amil2 010	Amil2 010	Amil2 002	Amil2 002	Amil5 028	Amil5 028	Amil2 006	Amil2 006	Amil2 023	Amil2 023	Amil2 022	Amil2 022	Apam3 166	Apam3 166	Wgs 152	Wgs 152	Wgs 134	Wgs 134	Wgs 189	Wgs 189	Wgs 035	Wgs 035
Swp1a-d	*50%*	*Swp*		*101*	*101*	*160**	*172**	*96*	*100**	*132**	*175*	*100*	*100*	*135*	*135*	*169*	*169*	*128**	*131**	*90*	*90*	*117*	*121**	*168**	*180**	*170**	*174**
Swp1e-h	*50%*	*Swp*		*101*	*101*	*166**	*168**	*96*	*104**	*175*	*175*	*100*	*102***	*135*	*135*	*165**	*169*	*158**	*167**	*90*	*102**	*117*	*117*	*164**	*196**	*166**	*194**
Swp15a-d,g,h	**75%**	**Swp**		**101**	**103**	**160***	**166**	**96**	**100**	**132**	**175**	**100**	**100**	**135**	**135**	**165**	**169**	**131**	**131**	**88**	**88**	**105**	**109**	**164**	**164**	**166**	**174**
Swp15e, f	*25%*	*Swp*		*101*	*103*	*164**	*166*	*96*	*100*	*132*	*175*	*100*	*100*	*135*	*135*	*165*	*169*	*131*	*131*	*88*	*88*	*105*	*109*	*164*	*164*	*166*	*174*
Swp44a-g	**87.5%**	**Swp**		**101**	**101**	**166**	**166**	**96**	**96**	**175**	**180**	**100**	**100**	**135**	**137**	**171**	**183**	**155***	**158**	**88**	**88**	**109**	**109**	**164**	**172**	**174**	**174**
Swp44h	*12.5%*	*Swp*		*101*	*101*	*166*	*166*	*96*	*96*	*175*	*180*	*100*	*100*	*135*	*137*	*171*	*183*	*158*	*161**	*88*	*88*	*109*	*109*	*164*	*172*	*174*	*174*
Swp64a,c-h	**87.5%**	**Swp**		**101**	**101**	**160**	**166**	**96**	**100**	**132**	**132**	**96**	**100**	**135**	**135**	**163**	**167**	**158**	**158**	**88**	**96**	**117**	**117**	**180**	**180**	**174**	**174**
Swp64b	*12.5%*	*Swp*		*101*	*101*	*160*	*166*	*96*	*100*	*132*	*137***	*96*	*100*	*135*	*135*	*163*	*167*	*158*	*158*	*88*	*96*	*117*	*117*	*180*	*180*	*174*	*174*
Mag24a-g	**87.5%**	**Mag**		**101**	**101**	**160**	**178***	**96***	**100**	**185***	**185**	**98***	**100**	**133**	**135**	**171***	**171**	**155**	**158**	**92***	**118***	**109**	**117**	**164**	**176**	**178**	**178**
Mag24h	*12.5%*	*Mag*		*101*	*103**	*160*	*166**	*100*	*100*	*137**	*137*	*100*	*102**	*133*	*135*	*173**	*179**	*155*	*158*	*90**	*104**	*109*	*117*	*164*	*176*	*178*	*178*
Mag56a	*12.5%*	*Mag*		*101*	*101*	*160*	*166*	*96*	*96*	*137*	*175**	*100*	*100*	*133*	*135*	*157*	*167*	*131*	*131*	*88*	*90*	*109*	*109*	*164*	*164*	*182*	*182*
Mag56b-h	**87.5%**	**Mag**		**101**	**101**	**160**	**166**	**96**	**96**	**137**	**137**	**100**	**100**	**133**	**135**	**157**	**167**	**131**	**131**	**88**	**90**	**109**	**109**	**164**	**164**	**182**	**182**
Mag59b-h	**87.5%**	**Mag**		**101**	**101**	**158**	**166**	**96**	**100**	**175**	**175**	**100**	**100**	**135**	**135**	**163**	**177**	**131**	**131**	**88**	**92**	**117**	**121**	**164**	**180**	**170**	**174**
Mag59a	*12.5%*	*Mag*		*101*	*101*	*158*	*166*	*96*	*100*	*132**	*175*	*100*	*100*	*135*	*135*	*163*	*177*	*131*	*131*	*88*	*92*	*117*	*121*	*164*	*180*	*170*	*174*

5.3.2 Relatedness

Based on genotypes from eight loci, genetic relatedness among samples within chimeric colonies was high (mean QG=0.654 ± 0.160, Table 5.4). In contrast to the high relatedness found for genetically distinct branches within chimeric colonies, the vast majority of colonies within each population were unrelated (mean QG = -0.007 ± 0.002). Two exceptions were neighboring clone mates in the Magnetic Island population (i.e. colonies Mag9 and Mag10, Mag16 and Mag17). Overall, fewer than 0.2% of paired samples (n=8515 pairs) showed relatedness indices greater than average relatedness indices found for branches within chimeras (QG≥0.654 ± 0.160, Table 5.4). Moreover, relatedness between rejecting colonies (Swp68 & Swp69) was very low, QG=0.08. Hence, visually incompatible genotypes displayed low relatedness and clear genetic differences. Note that only 2 rejecting colonies were sampled (i.e., only one QG calculated) and more sampling would be needed to confirm the low QG value calculated when colonies are incompatible.

Table 5.4. Comparisons of pairwise relatedness in chimeric colonies (**bolded**), in rejecting colonies (*italicized*), and in all samples. Pairwise relatedness estimators calculated according to Queller and Goodnight (1989).

Paired samples	Queller and Goodnight (1989) estimator - Mean
Swp1a-d / Swp1e-h	**0.289**
Swp15a-d, g-h / Swp15e-f	**0.884**
Swp44a-g / Swp44h	**0.898**
Mag24a-g / Mag24h	**0.026**
Mag56a / Mag56b-h	**0.920**
Mag59a / Mag59b-h	**0.907**
All chimeras (n=6 pairwise comparisons)	**0.654 ± 0.160**
Swp68a-d / Swp69a-d	*0.080*
All samples (n=8515 pairwise comparisons)	-0.007 ± 0.002

5.4 Discussion

High levels of chimerism (5% overall, or 3% according to a more conservative estimate based on the presence of at least two non-shared alleles) were found in two wild populations of the broadcast spawning coral, *Acropora millepora*, on the Great Barrier Reef. Both the Magnetic Island and the south-west Pelorus Island populations had similar levels of chimerism, i.e., 5% chimerism within each population based on genotypic differences at one allele, and 2% or 5%, respectively, based on genotypes displaying at least two non-shared alleles. These results indicate that chimerism is a common feature of this coral's biology.

Coral colonies that contain different genotypes may also arise through somatic mutation and therefore, based on this mode of origin, are best described as mosaics. Using the presence of a single non-shared allele differing by only one mutational step as the criterion for identifying mosaics, 0.8% of the sampled colonies were potentially mosaics while 3% were likely to be chimeras (with genotypes displaying at least two non-shared alleles). Thus chimeras represented a much greater proportion of colonies found to be genetically variable within the two study populations than mosaics.

Genetic chimerism has not been described for any wild population of coral prior to this study, but brooding corals under experimental conditions are known to have the potential to form genetic chimeras in their early life stages (Amar *et al.*, 2008). The application of molecular tools to studies of non-cnidarian colonial marine invertebrates has also revealed relatively high levels of chimerism within wild populations. Random Amplified Polymorphism DNA (RAPD) analysis assessed the presence and the extent of chimerism in the colonial ascidian, *Diplosoma listerianum* (Sommerfeldt & Bishop, 1999), and revealed that 34% of *Diplosoma listerianum* colonies in a wild population on the Langness Peninsula, Isle of Man (British Isles) possessed multiple genotypes (i.e.,

were chimeras). A similar study of one population from artificial settlement plates and seven natural populations of *Diplosoma listerianum* in the Isle of Man, North Wales, Cornwall and Devon (UK) also revealed high levels of chimerism (Sommerfeldt *et al.*, 2003). In this latter study, chimeric colonies were present in all populations studied, at frequencies ranging from 3% to 61%, and up to six different genotypes were present in some colonies. The use of highly polymorphic microsatellite loci in two different populations of the ascidian, *Botryllus schlosseri* (one native population from Caesarea (Israel) and one recently introduced population from Woods Hole marina (MA, USA)) revealed ~9% of colonies were chimeric in these two widely separated populations (Ben-Shlomo *et al.*, 2008). Molecular tools have been integral to investigate the presence of chimeras in natural populations of colonial marine invertebrates and sometimes reveal very high levels of chimerism. High levels of chimerism (up to 61%) in *D. listerianum* were probably uncovered due to the high intensity of sampling: 288 colonies, and 12 samples per colony, for relatively small sized colonies (Sommerfeldt *et al.*, 2003).

The proportions of chimerism in populations of *A. millepora* presented here are likely to underestimate the true extent of chimerism, as the sampling protocol was restricted to a maximum of eight branches per colony (Great Barrier Marine Park Authority permit limitations). Despite the small sample size per colony, we nevertheless documented up to two genotypes per colony (see Table 5.3). Given that an adult colony of *A. millepora* 40 cm in diameter consists of approximately 600 branches (pers. obs.), much larger sample sizes would have significantly increased our ability to detect chimerism. Moreover, if chimeric genotypes are cryptic within colonies, as our data suggest (see below), it is highly likely that our sampling missed a significant proportion of chimeras. Additionally, if chimerism reduces colony survival in the early stages of a coral's life,

sampling relatively large colonies (from 15 to 40cm in diameter) might have further underestimated the incidence of chimerism in the sample populations. On the other hand, if chimerism enhances colony survival, sampling relatively large colonies (from 15 to 40 cm in diameter) might have overestimated chimerism. Unfortunately, no data on the survival of chimeras are available to assess if the size class sampled is likely to have over- or under-estimated the occurrence of chimerism in the two populations. However, biases in our sampling protocol - i.e. avoiding multi-colour or non-uniform colonies, sampling only 8 branches per colony, and restricting sampling to relatively large colonies, and relatively small geographic scales within each population – is most likely to have under-estimated the extent of chimerism in the two populations of *A. millepora.*

Chimeric colonies of *A. millepora* had one dominant genotype and a second, cryptic genotype (Table 5.3). In the majority of the colonies (except colony Swp1), six or seven of the eight sampled branches were genetically identical and one or two were different. Such differences may reflect cell lineage competition where one genotype is morphologically resorbed, as described for *B. schlosseri* (Rinkevich, 2002). However, even after complete morphological resorption, the germ line and/or the somatic lineages of the inferior partner may still successfully parasitize the "winning" partner (Rinkevich, 2002; Rinkevich & Yankelevich, 2004). Although morphological resorption is a possible explanation for "dominant" genotypes within chimeras, it has only been observed in cytomictical chimeras, which are defined as chimeras in which some cells of the two parent organisms have become so mixed that they can no longer be separated into individuals (Rinkevich & Weissman, 1987). An example is the colonial tunicate *Botryllus schlosseri*, where fusion establishes a common circulation system which mixes blood cells from each partner of the chimera. In contrast, suspected coral

chimeras in the wild (e.g. *Stylophora pistillata*) show no evidence of mixed cellular elements, as evidenced for instance by each partner retaining its original colour (Rinkevich & Weissman, 1987). Coral chimeras are therefore more commonly referred to as "sectorial chimeras", where each partner within the chimera remains an individual (Rinkevich & Weissman, 1987).

While this and other studies (Ben-Shlomo *et al.*, 2008) may have underestimated the proportion of chimeras in wild invertebrate populations, strong theoretical arguments exist to support the hypothesis that chimeras are rare. Strassmann and Queller (Strassmann & Queller, 2004) highlighted the destructive genetic conflicts that can arise within chimeras. In particular, costs such as cell lineage competition are associated with the formation of chimeras (Rinkevich & Loya, 1985; Buss, 1990; Rinkevich & Weissman, 1992; Stoner & Weissman, 1996; Barki *et al.*, 2002; Chadwick-Furman & Weissman, 2003). However, other authors point out potential benefits associated with the chimeric state. Because chimeras harbour a greater genetic diversity than genetically homogeneous individuals, they can display "chimeric vigour", i.e., they may be able to use or cope with a wider range of environmental conditions. Other benefits of chimerism include developmental synergism (i.e., two aberrant forms are able to produce normal structures in a chimera), optimization of mate location, and the advantage of larger size in size-specific ecological processes (Buss, 1982). Specifically, fusion provides a mechanism for increasing size more rapidly than growth alone (Raymundo & Maypa, 2004), and could thus increase chances of survival (Sammarco, 1982; Jackson, 1986) for species where survivorship is size dependent (Rinkevich & Weissman, 1987). The benefit of harbouring higher genetic diversity and variability, and thus the ability to cope with more diverse environmental conditions, has been shown for the ascidian, *B. schlosseri* (Rinkevich & Yankelevich, 2004). In this species,

the somatic constituents of a chimera can be shifted from one genotype to another in response to environmental conditions (e.g., sea water temperature), indicating that some chimeras have the ability to "fine-tune" their genotype at critical times (Rinkevich & Yankelevich, 2004). Controversies over the potential costs and/or benefits of the chimeric state primarily reflect difficulties in studying chimeras in different organisms, as many studies have been laboratory based and laboratory "forced" chimeras could lead to associations between incompatible individuals (Buss, 1982). Future research should focus on investigating these questions in natural populations of chimeras.

Chimeras can originate from the fusion of larvae that settle close to each other or from the fusion of colonies that come into contact while growing or after movement (Sommerfeldt *et al.*, 2003). Recent studies have shown that juvenile cnidarians are able to form chimeras under experimental conditions (Amar *et al.*, 2008), and that fusion between allogeneic juveniles is promoted by the gregarious settlement of larvae (Rinkevich, 2004a; Amar *et al.*, 2008), which occurs commonly for a number of coral species (Lewis, 1974; Sammarco, 1982; Smith, 1997; Zilberberg & Edmunds, 2001; Barki *et al.*, 2002). Furthermore, if larvae of colonial marine invertebrates tend to aggregate with closely related individuals, they should be more prone to accept each other and fuse. The ascidian, *Botryllus schlosseri,* showed strong aggregation with sibling colonies, while unrelated colonies were significantly over-dispersed (Grosberg & Quinn, 1986). Larvae which shared a histocompatibility allele settled in aggregations and then promoted the formation of stable chimeric colonies in the field. Consequently, kin aggregations on limited available substrate could be one of the main causes of chimera formation in corals and other colonial marine invertebrates. Kin aggregations might be of even greater importance in broadcast spawning corals where thousands to millions of related juveniles are produced due to the often high synchrony in gamete

release of adjacent colonies (Babcock *et al.*, 1986). These related larvae may remain aggregated in the dense spawning slicks that form during still weather conditions (Oliver & Willis, 1987) and subsequently reach settlement competency at the same time. We found high relatedness between genotypes within chimeric colonies, while relatedness among neighbouring colonies within populations or between rejecting colonies (e.g. Swp68 and Swp69) was close to zero. The high relatedness between genotypes within chimeric colonies suggests that coral planulae settle in kin aggregations and may subsequently fuse and form chimeras. Alternatively, it is possible that non-related larvae settle and fuse to form chimeras, but that only closely related individuals survive and maintain a chimeric state.

Another possible cause of chimera formation is the "window in ontogeny" as proposed by Rinkevich (Rinkevich, 2004c). Natural chimerism originates during pregnancy in humans (blood chimeras, whole body, foetal-maternal, germ cell, and tumor chimeras; Rinkevich, 2001). Similarly, a narrow window early in the ontogeny of colonial marine invertebrates, prior to the development of the allorecognition system, may allow the formation of chimeric entities (Rinkevich, 2004c). Many marine invertebrates require days to months to reach a mature state of allorecognition. For example, maturation of the allorecognition system occurs within the first two weeks after metamorphosis in the hydrozoan *Hydractinia symbiolongicarpus* (Wilson & Grosberg, 2004), but requires more than two weeks in the bryozoan *Celleporella hyalina* (Hughes *et al.*, 2004), and approximately four months post-settlement for the corals *Stylophora pistillata* (Frank *et al.*, 1997) and *Seriatopora* spp. (Nozawa & Loya, 2005). The lack of an efficient allorecognition system in the early stages of ontogeny in scleractinian and soft corals is believed to be universal, and juvenile chimeras may represent a case of allorecognition

"failure", promoted by the gregarious settlement of larvae that is characteristic of many cnidarians (Rinkevich, 2004a).

In summary, chimerism in corals may originate in their early life history stages. Indeed, kin aggregations of larvae have the potential to fuse, more so during the period when corals appear to lack an efficient allorecognition system. Following an initial chimeric state (bi- or multi-partner chimeras), maturation of the allorecognition system of corals could potentially lead to the death of the entire entity or of just some of the genotypes within the genetically heterogeneous individual. Alternatively, some genotypes could be rejected, or cohabitation of closely related individuals in a chimeric state could persist. In this study, we found high levels (3-5% overall) of chimerism in two wild populations of the spawning coral, *A. millepora*, in the central Great Barrier Reef. We also found that partners within a chimera were closely related in comparison to a lack of relatedness generally found among neighboring colonies.

These results constitute the first genetic proof of the occurrence of chimeras within wild populations of adult corals. One implication of these results is that multiple samples should be collected from coral colonies in studies characterizing the genetic structure of coral populations. In order to further elucidate current understanding about how chimerism arises and why it persists, future research should compare the fate of genetically homogeneous and chimeric corals exposed to various external stressors, such as increased water temperature, low salinity, or pathogens and microbes.

Chapter 6.0 General discussion, major findings, and future research

6.1 General Discussion

The present study demonstrates that chimerism is an integral part of the life history of the scleractinian coral *Acropora millepora* and is the first molecular demonstration of chimerism in a broadcast spawning coral. Given similarities in the life history traits of *A. millepora* and other broadcast spawning corals, such as aggregated larval settlement, chimerism is likely to be common in the life histories of other species within the spatially dominant family Acroporidae, and potentially in broadcast spawning corals in general. Evidence of chimerism in many sessile, modular, marine invertebrates, including sponges, bryozoans, ascidians, and other cnidarians (Sommerfeldt *et al.*, 2003), corroborates this prediction. Previous research has focussed on contact reactions among juveniles, particularly among larvae during the settlement phase. My research is the first to demonstrate that chimerism persists into adulthood in corals and significantly expands current knowledge of chimerism within the Scleractinia. A third novel outcome from my research relates to the origins and underlying molecular pathways of chimera formation in corals.

Knowledge gained from my research supports the emerging picture that chimerism is more widespread in corals than previously thought. In summary, analyses of genetic variation at 9 microsatellite loci revealed that 50% of experimentally produced juveniles of *A. millepora* harboured different genotypes within the same colony (Chapter 2). Manipulations of early juveniles resulted in the creation of chimeras for up to 13 months post-settlement, proving that broadcast spawning corals have high potential for chimera formation, at least in experimental conditions (Chapters 2 & 3). Evidence that 47% of juveniles originated from aggregated settlement, with 1.5-fold more *Acropora millepora* larvae settling in aggregations than solitarily, identified the importance of gregarious larval behaviour in chimera formation (Chapter 2). Exploration of the role of

kinship in the origins of chimerism revealed that genotypes within persisting chimeric colonies showed high relatedness, the majority of chimeric colonies being either full or half sibling associations (Ch. 2). Similar results were found for wild populations of *A.millepora,* with over 66% of chimeric adult colonies representing full sibling associations (Chapter 5). The high relatedness among genotypes within adult chimeric colonies suggests that adult chimeras are likely to originate from kin aggregations of coral larvae. Alternatively, it is possible that non-related larvae settle and fuse to form chimeras, but only closely related individuals survive and maintain a chimeric state.

The potential for corals to fuse depends on the capacity of their allorecognition system to distinguish self from non-self. Therefore, investigating allorecognition in *A.millepora* constituted the next step towards elucidating the origins of chimerism in this species (Chapter 3). Fusion among juvenile corals may be facilitated by a "window in ontogeny", as proposed by Rinkevich (2004), during which allorecognition mechanisms are either suppressed or undeveloped. Fusions among juveniles of *A.millepora* at settlement (Chapters 2 & 3), followed by the first signs of rejection around 6 months post-settlement (Chapter 2) are consistent with a prolonged period of allorecognition maturation. Further insight into the timing of allorecognition maturation was provided by more rigorous contact reactions and monitoring studies, which revealed differences in the development of incompatibilities among sibling types (Chapter 3). Relatedness governed the rate of allorecognition maturation, as all contact reactions between non-siblings resulted in rejections by three months post-settlement, whereas it was five months before half siblings no longer fused, and fusions were still possible at 13 months for full siblings. The comparatively slow maturation of allorecognition in broadcast spawning corals (more than 13 months) compared to brooders (4 months) constitutes a significant difference in their life history strategies.

I hypothesize that late maturation of allorecognition may be adaptive and may contribute to flexibility in *Symbiodinium* uptake in the early ontogeny of broadcast spawning corals. The acquisition of symbionts from the environment by most broadcast spawners is a major difference between brooding and broadcast spawning life history strategies (although there are exceptions, including vertical acquisition of *Symbiodinium* in the broadcast spawning *Montipora* species, and horizontal acquisition in the brooding *Isopora* species; van Oppen *et al.*, 2009). Evidence from experiments assessing the ability of coral juveniles to take up various types of *Symbiodinium* during early ontogeny suggests that recognition mechanisms that would allow selective uptake of *Symbiodinium* types are not fine-tuned for at least the first six months post-settlement (Abrego *et al.*, 2009). After an initial flexible uptake, corals become dominated by one symbiont type, reflecting the possible maturation of non-self recognition. Although I was unable to draw definitive conclusions about the possible involvement of immunity-related genes in non-self recognition and allorecognition, I showed that one CELIII type lectin, A036-E7, was expressed at significantly higher levels four months post-settlement in *A.millepora* juveniles. Such high expression levels could be linked to allorecognition maturation but also to alloimmune challenges such as symbiont (*Symbiodinium* or bacteria) recognition or winnowing of symbiont diversity. The significant increase in A036-E7 observed in my study could consequently be linked to enhanced allorecognition abilities of coral juveniles or non-self recognition challenges occurring around the period of four months post-settlement. However, the peak in gene expression was followed by a drop, whereas high expression levels would be expected until the end of the study if immuno-competence were the only explanation of the observed peak. Therefore, I hypothesize that the beginning of non-self recognition and allorecognition maturation (~2 months) could be

followed by the recognition of incompatible symbionts or bacteria within the coral juveniles creating such high expression levels.

Overall, my results corroborate emerging evidence that lectins play a key role in allorecognition and more generally in the complex innate immune system of corals, as highlighted by recent studies of *A.millepora* that link lectins to metamorphosis (Grasso *et al.*, 2008), immune system function (Miller *et al.*, 2007; Kvennefors *et al.*, 2008), and *Symbiodinium* recognition (Wood-Charlson *et al.*, 2006) in corals.

Finally, a microsatellite study of 124 colonies collected from populations of *A. millepora* at Magnetic Island and south-west Pelorus Island confirmed that observations of chimera formation under experimental conditions accord with what happens in the wild. Using conservative criteria (i.e., the presence of two or more non-shared alleles in genotypes within colonies), 3% of *A. millepora* colonies sampled were identified as chimeras (Chapter 5). These values are likely to be vast underestimates of the true extent of chimerism in wild populations, as the sampling protocol was restricted to a maximum of eight branches per colony, while most colonies consist of hundreds of branches. Three-fold greater levels of chimerism in two populations of the ascidian, *Botryllus schlosseri* (i.e., ~9% chimerism) (Ben-Shlomo *et al.*, 2008) is consistent with this prediction. Consequently, chimerism is likely to be a common life history trait in sessile colonial marine invertebrates.

6.2 Major findings of this thesis

- The potential for chimera formation among juveniles of broadcast spawning corals is high and facilitated by gregarious behaviour of conspecific larvae, followed by aggregated settlement and fusion (Ch. 2).

- Lack of a mature allorecognition system at settlement facilitates fusion among coral juveniles and is likely to represent an adaptive trait (Ch. 2 & 3).
- Experimentally-produced, juvenile chimeras can persist for up to 23 months post-settlement (Ch. 2).
- The majority of chimeras represent associations among full siblings, both for experimentally-produced juveniles (Ch. 2) and for wild adult populations (Ch. 5).
- Incompatibilities recorded between all sibling types from two months onwards heralded the commencement of allorecognition maturation, with relatedness governing the rate of allorecognition maturation (Ch. 3).
- Rejections in all contact reactions between non-siblings by three months post-settlement, in comparison to five months for half siblings and more than 13 months for full siblings is consistent with a step-wise maturation of the allorecognition system in *A. millepora*.
- The comparatively slow maturation of allorecognition in broadcast spawning corals (more than 13 months) compared to brooders (4 months) constitutes a significant difference in their life history strategies.
- Allorecognition maturation in *A. millepora* may activate molecular pathways involving lectin genes (Ch. 4).
- Chimerism is present in wild populations of broadcast spawning corals and is likely to constitute more than 3-5% of populations (Ch. 5).

6.3 Future research directions

The occurrence of chimerism within scleractinian corals and associated increased genetic diversity within coral colonies may have important implications for

their resilience, potentially enhancing their capacity to compete for space and enabling a greater range of responses to environmental stressors and pathogen invasion (Ben-Shlomo *et al.*, 2001). Translocation of organic products of algal photosynthesis between fused colonies (Rinkevich & Loya, 1983) could also provide benefits to newly formed chimeras and could have real ecological importance in the recovery of coral reefs after storm or cyclone damage (Rinkevich & Loya, 1983). However, further assessment of the occurrence of chimerism in various coral species in the wild is needed to clarify the relevance of theoretical predictions regarding the benefits of chimerism for corals. Sampling greater numbers of branches within coral colonies would provide a more accurate estimate of the occurrence of chimeras in nature, as demonstrated when intense sampling uncovered high levels of chimerism (up to 61%) within ascidians (Sommerfeldt *et al.*, 2003). Such intensive sampling could also provide information on the distribution of genotypes within chimeric colonies, and confirm or refute the likelihood of high levels of cryptic chimerism on coral reefs.

The nearly three-fold greater size of chimeras compared to solitary juveniles found from settlement through to at least three months in my study suggests that chimerism is likely to be an important strategy for maximizing survival of vulnerable early life history stages of corals. Other studies have predicted that immediate size increase is an obvious advantage of chimera formation (Raymundo & Maypa, 2004; Amar *et al.*, 2008). In support of this prediction, growth and survival were improved in colonies of *Pocillopora damicornis* that were comprised of multiple fused genotypes compared to colonies originating from individual larvae (Raymundo & Maypa, 2004). However, longer term studies are still needed to more fully evaluate size-related and other possible benefits, as well as costs, of chimerism. Experimental studies comparing stress tolerance between chimeras and non-chimeras in response to acute stressors such as

extreme heat, low salinity, or pathogens would yield important insights into how and why chimeras persist in the wild.

Although corals are able to fuse with genetically different conspecifics and take up different *Symbiodinium* clades during the first months post-settlement, corals show signs of non-self recognition in their early life. Indeed, the allorecognition system of the coral *Seriatopora* is functional during the period of allorecognition maturation (i.e., first four months post-settlement). Moreover, rapid rejection reactions among xenografts (compared to allografts), suggest that these corals identify genetically distant encounters better than closer ones (Nozawa & Loya, 2005). Similarly, *Symbiodinium* infection experiments in *Fungia scutaria* showed that larvae could successfully take up homologous *Symbiodinium* types (e.g., C1f from *Fungia*, *Leptastrea*, and C1 from *Cyphastrea*) 21h after inoculation, while heterologous types (e.g., C31 form *Montipora capitata*) failed to infect juveniles 5h after inoculation (Rodriguez-Lanetty *et al.*, 2006). These results suggest that corals may rapidly develop a nonself recognition mechanism that is able to recognize genetically different entities (heterologous symbionts or different coral species), while precise allorecognition that enables discrimination between homologous symbionts or conspecifics may take more time. Consequently, setting up contact reactions similarly to those described in Chapter 3 with additional xenogeneic contacts (contacts between different species) may highlight the potential for spawning corals to discriminate non self attributes more rapidly than through contacts with conspecifics. In parallel with such an experiment, gene expression studies of solitary recruits using the same batch of juveniles, from settlement up to 12 months post-settlement, could identify genes involved in allorecognition and in the acquisition of *Symbiodinium*.

Investigating molecular pathways involved in allorecognition in corals is an important area for future research, particularly given the accumulating stresses affecting coral reefs globally (Hughes *et al.*, 2003). Indeed, undertsanding and unveiling aspects of the corals' immune system could provide coral reef managers with tools to improve survival and recovery of corals. For example, establishing contact reactions between adult branches and between coral juveniles, in combination with monitoring gene expression when corals come into contact, would highlight the genes and pathways activated during alloimune challenges. The expression of apextrin, complement C3 and lectin type genes should be monitored in alloimmune challenged corals, as well as other complement genes, such as the recently identified complement C2 and C5 gene (based on ESTs and BLAST hits only) in *Aiptasia* (Sunagawa *et al.*, 2009). Such experiments could eventually reveal the basis of a lectin-dependent complement system in coral innate immunity. This would consequently reveal key genes that could be used to evaluate the level of health and stress on coral reefs.

A better future and more effective management for coral reefs resides in a better undertanding of mechanisms for coping with stress and disturbances, thus there is a crucial need for further research on the still poorly known immune sytem of corals. As well, the net benefits and costs of chimerism should be more deeply explored, as this aspect of coral life histories may have important applications in coral reef rehabilitation and recovery after stresses and disturbances.

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