The molecular basis of fertilisation in coral *Acropora* and its role in speciation

Thesis submitted by

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in October 2007

Thesis submitted in fulfillment of the requirements of the degree of

Doctor of Philosophy in the School of Pharmacy and Molecular Sciences

at James Cook University

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Acknowledgements

First, I would like to give my many thanks to my supervisor Prof. David J. Miller for his extensive help during my PhD project. I would also like to thank all of the DJM lab members, Hiroki, Chuya, Nishikawa (Japanese guys), Brent (Integrin guy), Lubna (Lab manager), Hugo and his wife Griselda, Zoe (Coral expert), Alejandro, Francois, Lucija, Marcelo, Bin, Tara, Alycia, and Yvonne, as well as Danielle, Eldon, David Hayward, and Lauretta from the Australian National University. I also thank my family, cousins, and friends for their continued encouragement. I gratefully acknowledge Mr. Shinichi Iguchi for his substantial assistance and advice. I wish to acknowledge receipt of a scholarship from the Okinawa International Exchange & Resources Development Foundation.

Finally, I dedicate this thesis to the memory of my dear grandmother, Ms. Sada Arakaki, and my aunt, Ms. Umeko Shinzato, who have greatly encouraged me in pursuing my future.

Abstract

To shed light on diversification of the genus *Acropora* (Scleractinia, Cnidaria), one of the most widespread, abundant, and species-rich genera of hard corals (113–180 species), I searched for fertilisation-related genes in a model coral, *Acropora millepora*, and examined variations in these genes among *Acropora* species. First, by focusing on the ADAM–integrin interaction, which is involved in sperm–egg binding and membrane fusion in mammals, 15 ADAM–integrin interactions related to candidate genes obtained from an expressed sequence tag (EST) database of *A. millepora* were screened using hierarchical strategies (gene structures, gene expression patterns, fertilisation inhibition experiments). However, no evidence was found that these genes, other than integrin betacn1, are involved in *Acropora* fertilisation. To identify fast-evolving genes from *Acropora* species as fertilisation candidates, I then performed direct comparative sequence analysis with EST datasets from two acroporid species: *A. millepora* and *A. palmata* from the Caribbean Sea. Comparison of selected 849 independent genes from the *A. palmata* EST database (4,017 ESTs) to 10,232 ESTs from *A. millepora* resulted in the identification of 513 putative homologues. Within 163 homologous pairs in which dN and dS were examined, 93 homologous pairs had dN/dS ratios significantly <1, which suggests that these genes are under selection pressure associated with functional constraints. Six independent genes showed dN/dS ratios >1, and two of these had a significant deviation from one, suggesting that they are fast-evolving genes. It was unclear whether these fast-evolving genes are involved in fertilisation. Finally,

variations of integrin betacn1, which is involved in coral fertilisation, were compared among some *Acropora* species. Interestingly, comparison of integrin betacn1 sequences demonstrated that there are some mutations around the DxSxS motif, and two combinations of eight different clones showed significant possibilities of positive selection. However, it is unclear whether these variations are related to *Acropora* speciation. The next step is to characterise proteins making molecular complexes with integrin betacn1 or fast-evolving genes, and to compare these amino acid sequences among *Acropora* species.

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Chapter 1. General introduction

1.1. Reproductive systems of corals

Many reef-building corals participate in synchronous mass spawning events (Babcock et al. 1986; Harrison et al. 1984; Hayashibara et al. 1993), which represent opportunities for hybridisation among different species. In *Acropora* (Scleractinia, Cnidaria), which dominates coral reefs in the Indo-Pacific and is one of the most widespread, abundant, and species-rich (113-180 species) genera of corals (Veron 2000; Wallace 1999), in vitro cross-fertilisation experiments (Willis et al. 1997; Hatta et al. 1999), molecular data demonstrating allele sharing between species (Odorico and Miller 1997; Hatta et al. 1999; van Oppen et al. 2000, 2001, 2002; Vollmer and Palumbi 2002), and karyotyping (Kenyon 1997) support the hypothesis that natural hybridisation is likely to have played a major role during evolution. Interspecific hybridisation has been documented in other corals such as *Montipora* (Willis et al. 1997), *Madracis* (Diekmann et al. 2001), *Platygyra* (Miller and Babcock 1997; Miller and Benzie 1997; Willis et al. 1997), and the *Montastraea annularis* complex (Szmant et al. 1997), and appears to have been a major factor in the evolutionary success of *Acropora* (Willis et al. 2006). These results correspond to the reticulate evolution hypothesis for corals postulated by Veron (1995).

However, the overall significance of interspecific hybridisation for coral evolution remains contentious. Vollmer and Palumbi (2002) disputed the reticulation hypothesis, essentially arguing that hybridisation is an evolutionary dead end, with no long-term

evolutionary potential. In addition, the application of population genetic approaches (Márquez et al. 2002a, b) has demonstrated that while hybridisation is significant on evolutionary time scales, it occurs only rarely on ecological time scales even between species such as *A. hyacinthus* and *A. cytherea*, which are highly cross-fertile in in vitro trials (mean = 50%; Willis et al. 1997), occur in sympatry, and spawn synchronously. This implies that substantial, albeit incomplete, prezygotic barriers exist to hybridisation between coral species, although the nature of these isolating mechanisms and the molecular basis of gamete interactions remain unknown. One approach to answer these questions in *Acropora* is to unravel the fertilisation mechanism at the molecular level. However, our current knowledge of this area is very limited. In some animals (Vertebrata, Mollusca, Echinodermata), several molecules mediating fertilisation have been already identified.

1.2. The molecular basis of fertilisation in mammals

Mammalian fertilisation has been the subject of intense investigation, and a number of proteins mediating sperm–egg binding and membrane fusion have been identified (Evans 2002; Kaji and Kudo 2004; Snell and White 1996; Vacquier 1998). In mammals, fertilisation can be broadly divided into three stages: sperm attraction, penetrating the zona pellucida (ZP; a large extracellular matrix), and sperm–egg binding and membrane fusion at the egg plasma membrane (microvillar region; Figure 1.1). After sperm attraction, sperm bind to the ZP surrounding the egg, and the acrosome reaction follows. The acrosome reaction is an essential step for sperm to fertilise most animals (Dan

1967). In the mouse, the acrosome reaction is induced by ZP3 (a ZP glycoprotein), and sperm binding to ZP3 activates calcium ion channels, which triggers the exocytosis of the acrosomal vesicle (Vacquier 1998). Enzymes released from the acrosome of the sperm allow it to penetrate the ZP. After penetrating the ZP, the sperm can enter the perivitelline space. Sperm then bind to and fuse with the egg plasma membrane.

During the process of sperm–egg binding and membrane fusion in the mouse, sperm ligands belonging to the A Disintegrin and Metalloprotease (ADAM) family and integrins, which act as ADAM receptors on the egg plasma membrane, may be critical (Figure 1.2). In a classic series of experiments, Almeida et al. (1995) showed that integrin alpha6beta1 likely plays a critical role on the egg side in the gamete interaction in the mouse, the likely ligand on the sperm side alphabelonging to the ADAM family. Candidate ADAM molecules in this context are ADAM1 (fertilin alpha), ADAM2 (fertilin beta), and ADAM3 (cyritestin; Evans 2002). ADAM1, ADAM2, or ADAM3 fusion proteins can bind to the mouse egg plasma membrane and inhibit fertilisation (Bigler et al. 2000; Eto et al. 2002; Evans et al. 1997a, b; Takahashi et al. 2001).

One tetraspanin, CD9, is essential for sperm–egg membrane fusion in the mouse and acts as an integrin co-factor (Kaji et al. 2000; Le Naour et al. 2000; Miyado et al. 2000). A major feature of tetraspanins is that they can be associated with other proteins, including integrins, and form a network of multimolecular complexes (tetraspanin web; Boucheix and Rubinstein 2001). CD9 and other tetraspanins (CD63, CD81, CD151) can

interact with integrin alpha6beta1 (Boucheix and Rubinstein 2001; Miyado et al. 2000). Thus, not only CD9 but also CD63, CD81, and CD151 may be involved in fertilisation as members of the tetraspanin web. Ziyyat et al. (2006) recently reported that a monoclonal antibody against CD151 partially inhibited sperm–egg membrane fusion in humans. CD81 involvement in sperm–egg membrane fusion of the mouse has also been reported (Takahashi et al. 2001; Rubinstein et al. 2005).

In summary, ADAM–integrin interactions (ADAM, integrin, tetraspanin) are thought to provide an essential molecular complex in mammalian fertilisation. Despite some discrepancies between results obtained using antibodies or fusion proteins and results based on knockout mice (He et al. 2003; Miller et al. 2000), the evidence supporting roles for ADAM–integrin interactions during sperm–egg binding and membrane fusion is compelling (Almeida et al. 1995; Ziyyat et al. 2006). Other molecules involved in fertilisation of mammals, epididymal protein DE (sperm side; Cuasnicu et al. 1984) and glycosylphosphatidylinositol (GPI)-anchored protein (egg side; Alfieri et al. 2003), are related to sperm–egg membrane fusion in the rat and mouse. Izumo, a member of the immunoglobulin superfamily protein (sperm side), is an essential molecule for sperm–egg membrane fusion in the mouse and in humans (Inoue et al. 2004). However, the relationship between these molecules and ADAM–integrin interactions remains unknown.

1.3. The molecular basis of fertilisation in marine invertebrates

Many marine organisms, including corals, release their gametes into the water column, where fertilisation occurs. Before fertilisation, the sperm acrosome reaction must be induced. In starfish, egg jelly components, ARIS, Co-ARIS and asterosap, are involved in the sperm acrosome reaction (Hoshi et al. 1999). In the sea urchin, fucose sulphate polymer (FSP) of egg jelly induces the acrosome reaction (Vacquier and Moy 1997), and sperm receptors for egg jelly components (suREJs) have been characterised (Galindo et al. 2004). The eggs of invertebrates are surrounded by a vitelline envelope (VE), the outer layer of the egg (Evans 2000), and sperm must penetrate this envelope after the acrosome reaction. In ascidian, sperm have a chymotrypsin-like protease which is thought to be used for the sperm penetration of the VE (Saitoh et al. 1993). In the VE of ascidian, HrVC70 is a sperm receptor (Sawada et al. 2002), and this molecule is also an allorecognition one because self-sterile mature oocytes become self-fertile ones after the release of the HrVC70 was induced from the VE (Sawada et al. 2004). In abalone, the sperm protein lysin interacts with a VE receptor for lysin in the VE (VERL; Galindo et al. 2002), and solubilises the VE by non-enzymatic means and a species-specific manner (reviewed by Kresge et al. 2001; Lyon and Vacquier 1999; Swanson and Vacquier 1997). After penetration of the VE in abalone, an 18-kDa protein, which is secreted by the sperm during the acrosome reaction, is thought to mediate sperm–egg membrane fusion (Swanson and Vacquier 1995). In the sea urchin, fertilisation is mediated in a species-specific manner by the sperm protein bindin and a glycoprotein receptor for bindin on the egg surface (Glabe and Vacquier 1977, 1978). Although the

receptor for bindin had not been identified because of difficulties in purification, the VE receptor for bindin, EBR1 (a novel ADAMTS protein), has recently been characterised (Kamei and Glabe 2003). Bindin is also thought to be involved in sperm–egg membrane fusion in the sea urchin (Ulrich et al. 1998). As reviewed above, lysin and bindin play analogous roles on the sperm side for gamete interactions, but these proteins are likely to have independently evolved in different animal groups (Vacquier 1998). Therefore, it is unlikely that strict homologues of these proteins fulfil analogous roles in other animals.

1.4. The molecular basis of fertilisation in corals

As described above, the molecular bases of fertilisation in some animals have been clarified, but that of corals still remains unclear. The difficulty to examine the molecular basis of fertilisation is because fertilisation related molecules may be subject to convergent evolution and the different proteins have independently evolved in different animals (Vacquier 1998). For example, the homologs of lysin in abalone and bindin in sea urchin have not been identified in other taxa. However, it is likely to be hypothesised that ADAM–integrin interactions, known to occur during sperm–egg binding and membrane fusion in mammals, may play analogous roles in the sclelactinican coral *Acropora*. This hypothesis is based on the following data:

(1) The absence of an egg outer layer corresponding to the ZP, including the mammalian ZP proteins, or to the VE, including the VERL and bindin receptors of molluscs and echinoderms (Harrison and Wallace 1990).

(2) The presence of integrin betacn1 (discovered by Brower et al. 1997) mRNA in unfertilised *A. millepora* eggs.

(3) The ability of antibodies raised against integrin betacn1 to inhibit both sperm–egg binding and fertilisation in *A. millepora* (Iguchi et al. 2007; Márquez 2002).

(4) ZP deleted eggs in mammals show polyspermy (Yanagimachi 1988). This means that ADAM–integrin interactions have comparatively low levels of species specificity and may contribute to the ease of hybridisation in corals.

Considering these data, it is hypothesised that ADAM–integrin interactions may underlie fertilisation in *Acropora*. Justifications for extrapolating across such a great evolutionary distance include the remarkable similarity between the anthozoan and vertebrate gene repertoires (Kortschak et al. 2003; Kusserow et al. 2004; Technau et al. 2005) and striking parallels in patterns of expression of some genes (reviewed by Ball et al. 2004). Thus, characterising the ADAM–integrin interaction-related genes (ADAM, integrin, tetraspanin) in *Acropora* may shed light on the molecular basis of fertilisation in corals.

1.5. Fast-evolving genes in fertilisation

An important characteristic of genes with roles in fertilisation is that of rapid divergence driven by positive selection (Swanson and Vacquier 2002). Positive selection can be inferred from the number of nonsynonymous substitutions per nonsynonymous site (amino acid replacement changes; dN) significantly exceeding the number of

synonymous substitutions per synonymous site (silent changes; dS), which implies that amino acid changes are highly accumulated (so-called "fast-evolving genes"). Abalone lysin and sea urchin bindin are typical examples of fast-evolving genes (Lee et al. 1995; Metz and Palumbi 1996). Recently, to detect positive selection, a more sensitive method based on maximum likelihood analysis has also been established (Yang et al. 2000). With this new method, Swanson et al. (2003) suggested that positive selection is pervasive in mammalian fertilisation proteins. Also, in ADAM–integrin interaction-related genes, ADAM and CD9 were shown to be subject to positive selection (Civetta 2003; Glassey and Civetta 2004; Swanson et al. 2003). Thus, it is possible to look for genes with potential for fertilisation by focusing on high rates of evolutionary change in the candidate genes.

1.6. Role of fertilisation-related genes in the *Acropora* speciation process

Although *Acropora* is a highly diversified coral genus (113–180 species: Wallace 1999; Veron 2000), a cause for this diversification may be related to a change in the gamete recognition system, because it is difficult to explain such diversification in synchronously spawning animals by only postulating geographic isolation, such as a change in water currents. As described above, Willis et al. (1997) and Hatta et al. (1999) showed that hybridisation in *Acropora* is possible by performing in vitro cross-fertile experiments. On the other hand, they also clarified several cases of low fertilisation rates among some *Acropora* species that spawn synchronously. This suggests that species-specificity in the gamete recognition system among some *Acropora* species

must exist, because the in vitro cross-fertilisation experiment was performed in conditions suitable for the sperm to definitely encounter the eggs. Therefore, it seems that some variations in fertilisation-related genes may exist among *Acropora* species. In fact, in abalone and sea urchins, extensive amino acid substitutions have been found in fertilisation-related genes (e.g. lysin, bindin) among closely-related species, leading to reproductive isolation between them (Lee et al. 1995; Metz and Palumbi 1996). In addition, it has been proposed that speciation would have been caused by a change in fertilisation-related genes (Galindo et al. 2003; Lee et al. 1995; Swanson and Vacquier 2002). By examining variations in fertilisation-related genes of *Acropora*, it should be possible to clarify the reproductive isolation and speciation mechanism.

In summary, I proposed to investigate candidate genes for fertilisation using both functional (fertilisation inhibition experiments) and structural (fast-evolving genes) approaches. Characterising genes involved in fertilisation of *Acropora* and comparing the gene sequences among *Acropora* species may provide new insights into the molecular mechanisms underlying the rapid and extensive evolutionary diversification of this important coral genus.

1.7. Aim of this study

As described above, it is expected that ADAM–integrin interactions are responsible for fertilisation in *Acropora*. The first aim of this study was to clarify the molecular basis of fertilisation in *A. millepora* based on the ADAM–integrin interaction hypothesis. For

this, 14 objective genes were selected from an expressed sequence tag (EST) database of *A. millepora* (Kortschak et al. 2003) as candidates for roles in ADAM–integrin interactions (ADAM, integrin, tetraspanin). The nucleotide and amino acid sequences of these genes were determined and their structures were analysed (Chapter 2). Second, the gene expression patterns of six candidates were examined, based on the results of Chapter 2, to determine which genes are involved in the fertilisation of *A. millepora* (Chapter 3). Third, fusion proteins and antibodies of three candidates selected in Chapter 3 were made, and fertilisation inhibition experiments were performed to examine whether these genes are involved in the sperm–egg interaction in *A. millepora* (Chapter 4).

I also searched for genes with potential roles in fertilisation by focusing on fast-evolving genes (Chapter 5), because positive selection is widely observed in fertilisation-related genes (Swanson and Vacquier 2002; Swanson et al. 2003). To pursue this aim, two EST datasets from *A. millepora* and *A. palmata* (Kortschak et al. 2003; Schwarz et al. 2005) were used. It was then necessary to judge whether fertilisation-related candidates are associated with the speciation process in *Acropora* (Chapter 6). For this, the sequences of candidates selected in Chapters 4 and 5 were compared. Finally, all results are summarised and concluding remarks are made in Chapter 7.

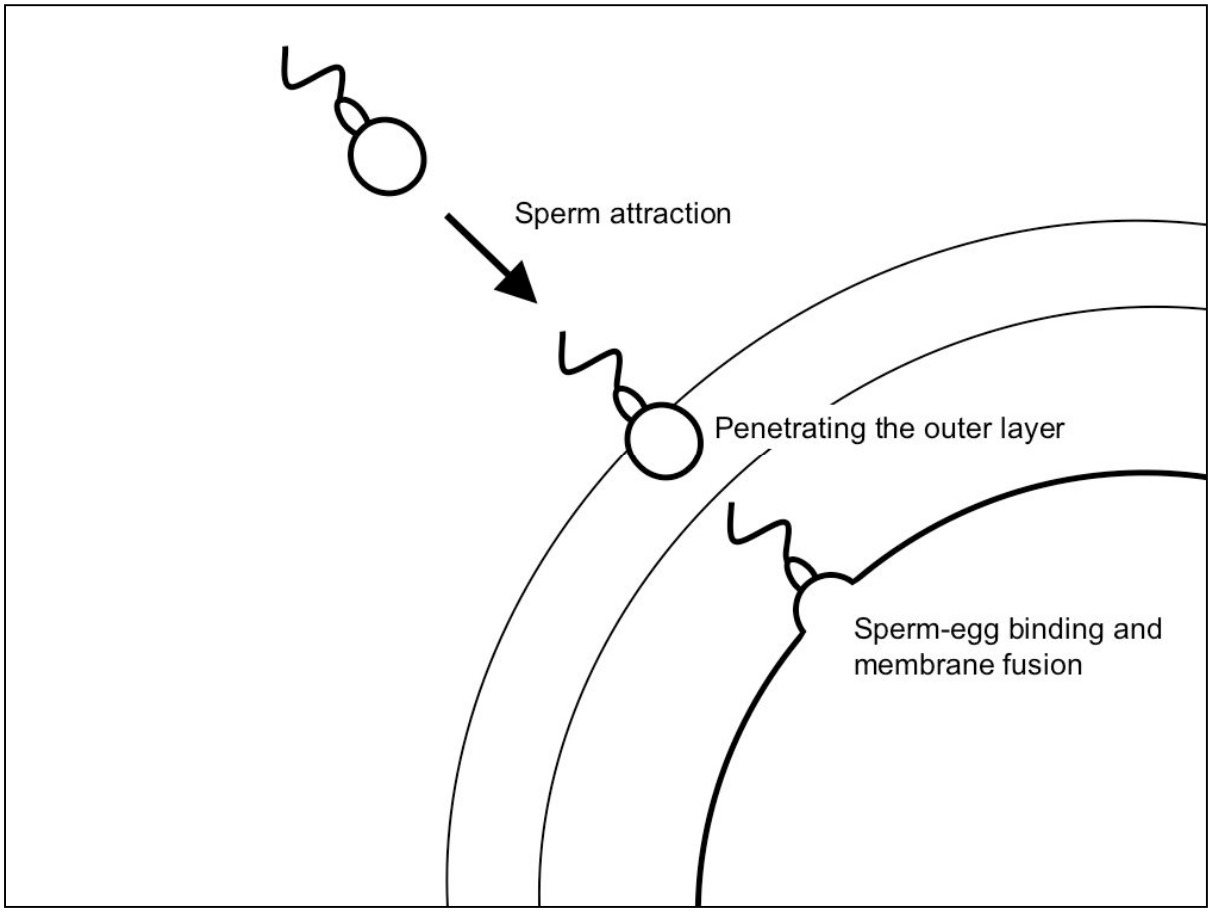

Figure 1.1. Schematic view of main steps in fertilisation.

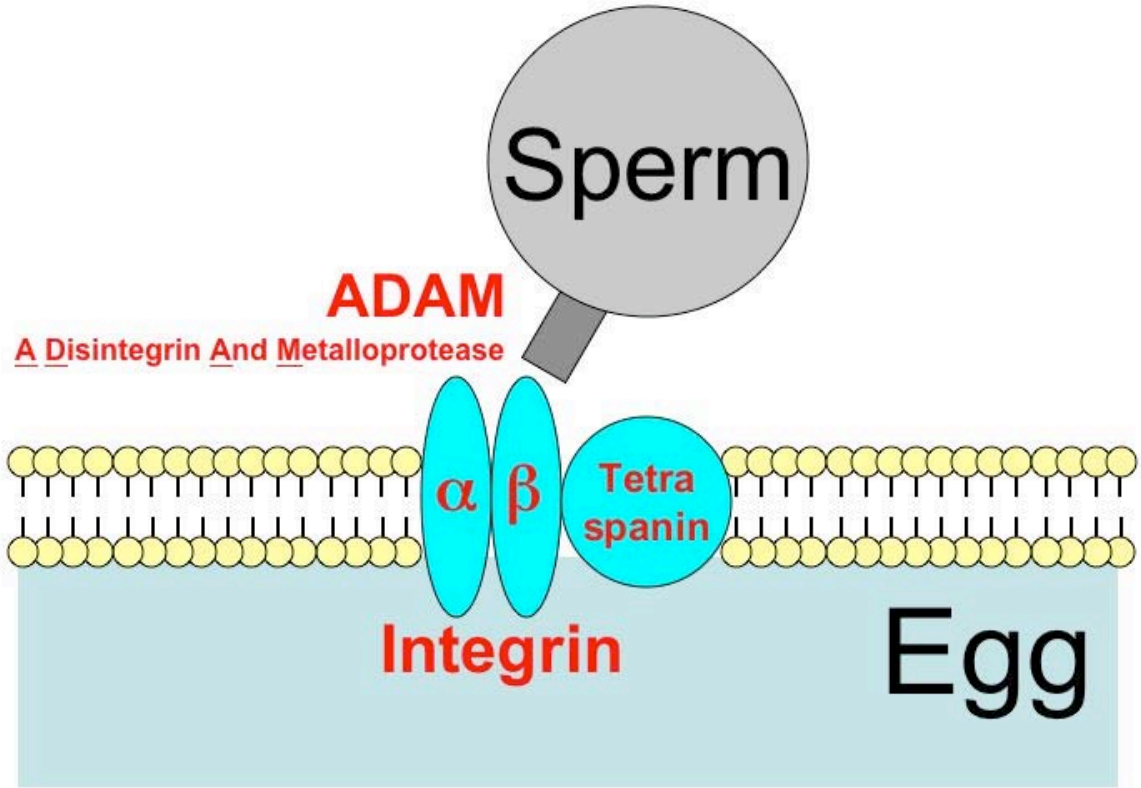

Figure 1.2. Schematic view of the ADAM–integrin interaction in mammals.

Table 1.1. List of fertilisation related molecules.

Animal	Molecules	Sites
Mammals	ZP3	Sperm
Mammals	ADAM1	Sperm
Mammals	ADAM2	Sperm
Mammals	ADAM3	Sperm
Mammals	DE	Sperm
Mammals	Izumo	Sperm
Mammals	integrin alpha6	Egg
Mammals	integrin beta1	Egg
Mammals	CD9	Egg
Mammals	CD81	Egg
Mammals	CD151	Egg
Mammals	GPI-anchored protein	Egg
Starfish	ARIS	Egg
Starfish	Co-ARIS	Egg
Starfish	asterosap	Egg
Ascidian	Chemotrypsin-like protease	Sperm
Ascidian	HrVC70	Egg
Abalone	lysin	Sperm
Abalone	18-kDa protein	Sperm
Abalone	VERL	Egg
Sea urchin	bindin	Sperm
Sea urchin	suREJs	Egg
Sea urchin	glycoprotein receptor for bindin	Egg
Sea urchin	EBR1	Egg

Chapter 2. Structures of the ADAM–integrin interaction-related genes (ADAM, integrin, tetraspanin) of *Acropora millepora*

2.1. Introduction

This chapter focuses on ADAM–integrin interaction-related genes (ADAM, integrin, tetraspanin) to search for genes with potential roles in the fertilisation of *Acropora millepora*. Fifteen target genes are introduced (Table 2.1), which may be related to the ADAM–integrin interaction and play important roles in the fertilisation of *A. millepora*. Six ADAM-like genes, three integrin-like genes, and five tetraspanin-like genes were found in an expressed sequence tag (EST) database (Kortschak et al. 2003), and were named AmADAM1-6, AmIntegrin alpha1, alpha2, beta2, and AmTetraspanin1-5. Integrin betacn1 (discovered by Brower et al. 1997) is also a target gene. These gene structures were analysed using bioinformatic methods (BLAST, alignment, phylogenetic analysis, conserved domain searches, SOSUI analysis). Based on the information gained, the potential roles in the fertilisation of *A. millepora* of these genes constitute the discussion in this chapter.

2.2. Materials and Methods

Cloning of ADAM–integrin interaction-related genes from *Acropora millepora*

Fourteen putative ADAM–integrin interaction-related genes from an EST database of *A. millepora* (constructed from cDNA libraries of early stage embryos and an adult colony)

were selected (Table 2.1). Clones were obtained from soaked Whatman papers and recovered from the Whatman papers as inserts in pBluescript SK (-) (Stratagene). Sequencing was performed with gene- and vector-specific primers through MACROGEN (Korea).

cDNA library screening

To gain full length fragments of the 5' flanking region-deficient gene (AmADAM4, AmIntegrin alpha1), cDNA library screening was performed. The cDNA library used was constructed from adult coral branches with the Lamda ZAP system (Stratagene). A radiolabelled probe was made by PCR amplification with EST cDNA clones as templates, and the probe was used to screen phage libraries using standard methods (Sambrook et al. 1992). Positive clones were recovered as inserts in BluescriptII SK(-) plasmids and grown in XL1-Blue MRF' cells.

Homology, conserved domain searches, and SOSUI analysis

To search for the highest homology of the target genes and to check for the existence of N-terminal methionine, BLAST analysis was performed based on the nucleotide sequences of the target genes. Domains of target genes were identified using the NCBI conserved domain search service (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). To infer the TM and EC domains of AmTetraspanins, the 'SOSUI' program (Hirokawa et al. 1998) was used.

Alignment and phylogenetic analysis

Multiple sequence alignments were generated using Clustal W (Thompson et al. 1994), and then shaded (black: identity; grey: conservative substitutions) using BOXSHADE 3.21 (http://www.ch.embnet.org/software/BOX_form.html). A maximum likelihood tree was produced using MolPhy version 2.3 (Adachi and Hasegawa 1996) and the Dayhoff substitution matrix with the local rearrangement search mode.

2.3. Results and Discussion

Gene structure of AmADAM1-6

From the EST database of *A. millepora*, six ADAM-like genes (AmADAM1–6; Table 2.1) were found. BLAST analysis with sequences of EST cDNA clones found that some AmADAMs lacked the anterior part of the open reading frame (ORF). To isolate the full-length AmADAM4 cDNA clone, *A. millepora* cDNA library screening was carried out. Approximately 50,000 plaques were screened, and five clones were positive. The phagemid containing AmADAM4 cDNA was isolated by in vivo excision. However, it was not possible to confirm the existence of the N-terminal methionine of AmADAM4 from the new cDNA clone. Finally, full-length sequences of AmADAM2 and 3 and partial length sequences of AmADAM1, 4, 5, and 6 were determined.

Based on these nucleotide sequences, BLAST analysis was performed. The highest homology of each gene is listed in Table 2.1. With the exception of AmADAM3, all AmADAMs belong to the ADAM-TS family. ADAM contains some characteristic

domains (Figure 2.1), and the disintegrin domain is important as a ligand for the integrin receptor (Wolfsberg et al. 1995). Therefore, an attempt was made to search for the disintegrin domain of each AmADAM. TSP domains were found in all AmADAMs, other than AmADAM3, using an NCBI conserved domain search (Figure 2.1), but it was not possible to confirm the existence of the disintegrin domain in these AmADAMs belonging to the ADAM-TS family. In addition, there are no reports that some genes belonging to the ADAM-TS family are involved in fertilisation in mammals, although Kamei and Glabe (2003) reported a novel ADAMTS protein, EBR1, characterised as a receptor for bindin in the sea urchin. This protein is a component of the vitelline envelope of the egg. Coral eggs lack the VE (Harrison and Wallace 1990); thus it is unlikely that AmADAMs belonging to the ADAM-TS family are involved in coral fertilisation.

On the other hand, AmADAM3 included the disintegrin domain, and it is similar to mouse ADAM10 according to BLAST analysis. Eto et al. (2002) suggested that the RX6DLPEF motif in the disintegrin domain of the ADAM family is important as a ligand for the integrin receptor, but mouse ADAM10 and AmADAM3 do not have this motif. Although it is unknown whether ADAM10 is related to fertilisation in mammals, it is impossible to judge only from the structure whether AmADAM3 is involved in fertilisation of corals.

Gene structure of AmIntegrin alpha1, alpha2, and beta2

Three integrin-like genes (AmIntegrin alpha1, alpha2, beta2; Table 2.1) were found in the EST database of *A. millepora*. BLAST analysis with the sequence of the EST clone of AmIntegrin alpha1 indicated that this clone lacks the anterior part of the ORF. To isolate the full-length AmIntegrin alpha1 cDNA clone, an *A. millepora* cDNA library screening was done. Approximately 100,000 plaques were screened, and nine clones were positive. The phagemid containing AmIntegrin alpha1 cDNA was isolated by in vivo excision, and successful library screening of AmIntegrin alpha1 confirmed the existence of the N-terminal methionine. Full length sequences of AmIntegrin alpha1, alpha2, and beta2 were determined. Based on these nucleotide sequences, BLAST analysis was performed. The highest homology for each gene is listed in Table 2.1. AmIntegrin alpha1 is similar to mouse integrin alpha9. Comparison of AmIntegrin alpha1 to other relatives demonstrated some characteristics common to α integrins: FG-GAP repeats, cation-binding sites, and a trans-membrane region are highly conserved (Figure 2.2). AmIntegrin alpha1 contains three DxD/NxD/NxxxD motifs used for cation binding (Tuckwell et al. 1992). The KxGFFKR motif in the cytoplasmic region is completely conserved in AmIntegrin alpha1. After determining the full sequence of AmIntegrin alpha EST clones, BLAST analysis was performed based on the nucleotide sequences. Contrary to expectations, AmIntegrin alpha1 was not similar to any integrin but was similar to Hydra collagen-like protein.

In AmIntegrin beta2, major features of integrin beta (signal sequence, putative I domain,

cysteine-rich stalk, transmembrane region, and cytoplasmic domains) are highly conserved (Figure 2.3). The DxSxS motif in the MIDAS domain used for cation binding (Tozer et al. 1996) is completely conserved, whereas the DDL motif, which is implicated in binding to RGD peptides (Pasqualini et al. 1995), is changed into EDL (Figure 2.2). The existence of 56 cysteines is a major characteristic of most beta integrins (Brower et al. 1997); however, AmIntegrin beta2 contains only 54 cysteines, which is similar to jellyfish integrin beta (Reber-Muller et al. 2001). BLAST analysis also confirmed that AmIntegrin beta2 is highly similar to jellyfish integrin beta. Reber-Muller et al. (2001) reported low-amino acid identity between jellyfish integrin beta and integrin betacn1, even though they are homologous. However, the present results demonstrate that AmIntegrin beta2 is a clear homologue of jellyfish integrin beta. This means that at least two beta integrins exist in basal cnidarians.

Considering the BLAST results for AmIntegrins, AmIntegrin alpha1 can be selected as a fertilisation candidate for *A. millepora* because this gene is similar to mouse integrin alpha9, which has been suggested to be related to fertilisation (Eto et al. 2002). On the other hand, whether AmIntegrin beta2 is involved in fertilisation is unclear, although another beta integrin (integrin betacn1) has already been shown to be involved in fertilisation (Iguchi et al. 2007; Marquez 2002).

Gene structures of AmTetraspanin1–5

From the EST database of *A. millepora*, five tetraspanin-like genes (AmTetraspanin1–5;

Table 2.1) were recovered. BLAST analysis with the sequences of the EST clones of AmTetraspanin1–5 confirmed the existence of N-terminal methionine in all five clones. Therefore, full length AmTetraspanin1–5 sequences were determined. The ORFs of AmTetraspanin1–5 comprise 241, 242, 228, 236, and 279 amino acids, respectively. Four TM domains and EC1 and EC2 loops were confirmed in all AmTetraspanins, except for AmTetraspanin3. AmTetraspanin3 lacks the fourth TM domain and the EC2 loop (Table 2.2). Alignment of the AmTetraspanins and other representatives was performed (Figure 2.4). In the EC2 region, the CCG motif, which is highly characteristic for tetraspanins (Hemler 2005), is absolutely conserved in all AmTetraspanins and other representatives, and three other cysteine residues that contribute to two crucial disulphide bonds within the EC2 region (Seigneuret et al. 2001) are also conserved, except for AmTetraspanin3 and human TM4SF13 (Figure 2.4). These data support the proposal that AmTetraspanins, other than AmTetraspanin3, are bona fide tetraspanins. AmTetraspanin3 and human TMSF13 should be termed 'Tetraspanin-like' genes.

Based on full sequences of AmTetraspanins, BLAST analysis was performed. The highest homology for each gene is listed in Table 2.1. In addition, a phylogenetic tree of the AmTetraspanins and their relatives (Figure 2.5) was built. Some AmTetraspanin-like sequences were obtained from *Nematostella vectensis* and *Hydra magnipapilla* from a local comparative genomics analysis platform (http://www.compagen.org), and these were added to the phylogenetic analysis. Putative homologues of AmTetraspanin 1, 3,

and 4 of *H. magnipapilla* were not added in this analysis because of the lack of anterior sequences for these genes. Each cluster basically reflects the results of BLAST analysis, except for the CD151 group. The CD63 group (cd63_frog, AmTet4, AmTet4-like_nv) and the CD151 group (cd151_mouse, AmTet1, AmTet1-like_nv, AmTet2, AmTet2-like_nv, AmTet2-like_hm) are closely related. The monophyly of the CD63 group is highly supported, whereas that of the CD151 group is ambiguous. Huang et al. (2005) were unable to judge whether a cnidarian CD151-like molecule from *H. magnipapillata* is an orthologue of CD63 or CD151, because this gene shares 40% identity with frog CD151 based on BLAST, but is clustered with vertebrate CD63 in phylogenetic analysis. This may be because of a very close relationship between the CD63 and CD151 groups.

Considering the results of BLAST and phylogenetic analysis of AmTetraspanins, AmTetraspanin1, 2, and 4 may be involved in the fertilisation of *A. millepora* because these homologous genes, CD63 and CD151, can interact with fertilisation-related integrins (alpha6beta1; Almeida et al. 1995) in mammals (Boucheix and Rubinstein 2001). In addition, Ziyyat et al. (2006) recently reported that a monoclonal antibody against CD151 partially inhibited sperm–egg fusion in humans, although the role of CD63 in fertilisation remains unknown.

In summary, AmADAM3, AmIntegrin alpha1, and AmTetraspanin 1, 2, and 4 were determined as potential candidate genes involved in the fertilisation of *A. millepora*, based on their structural data. Chapter 3 focuses on gene expression patterns of these

genes to examine whether they are expressed and play roles in the fertilisation of *A. millepora*.

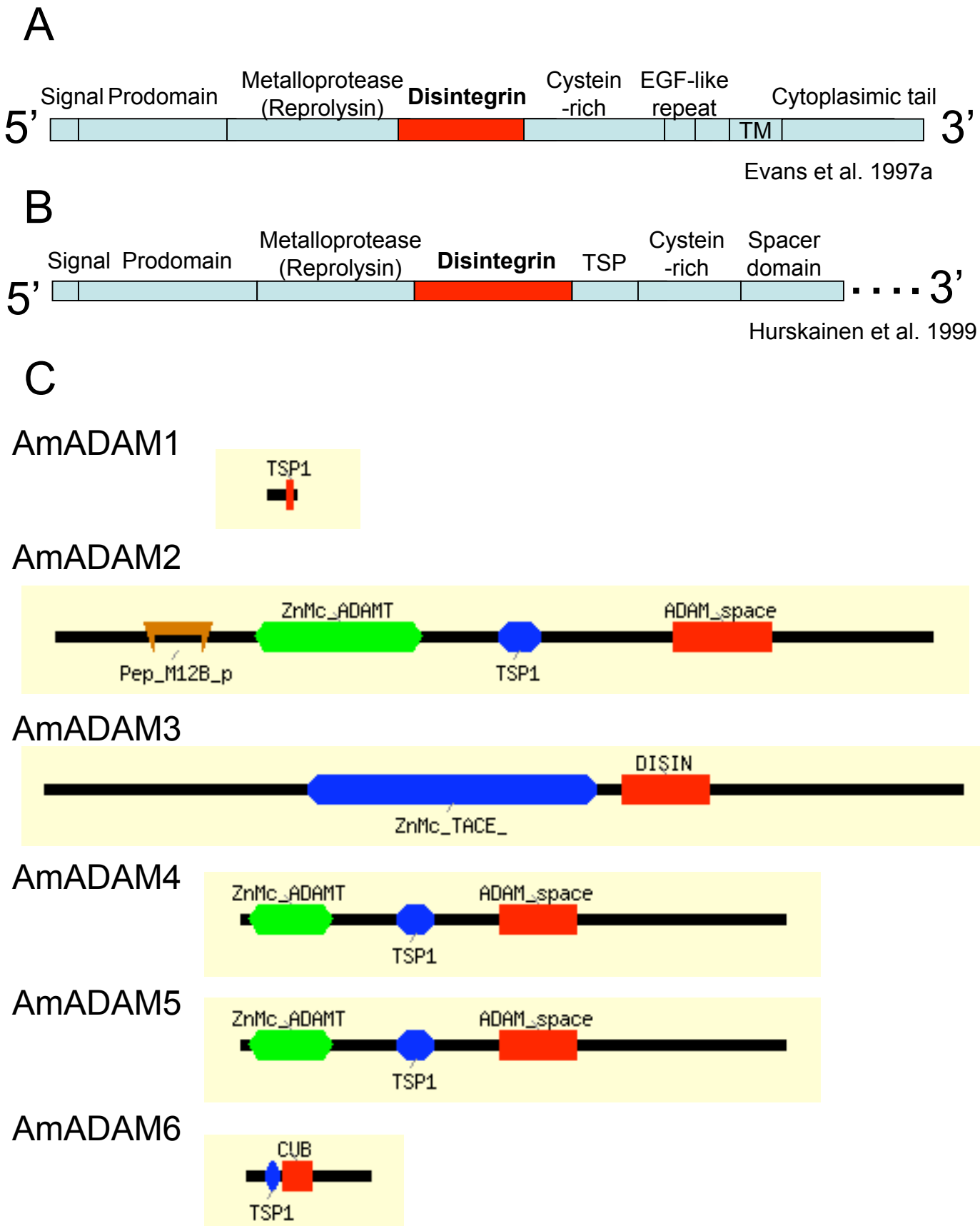

Figure 2.1. (A) and (B) Schematic representation of ADAM and ADAM-TS, respectively. (C) Results of the NCBI conserved domain search of AmADAMs.

```
AmItga1   -------------------MLFTSITCLLVLSLSLPS--YGFNLDLES-ALIFSPPPGSESKYFGYSVALHSFR-------DKYWAIVG
ItgaPS2   --------MSGDSIHRRRMALHCPITSLILLLIAMS--AHGYNIDLPS-YVRFRQSSNS---MFGFSIAMHKGRSGFYGNQNNVSLIVG
ItgaV     MAFPPRR-----RLRLGPRGLPLLLSGLLLPLCR------AFNLDVDS-PAEYSGPEGS---YFGFAVDFFVP-----SASSRMFLLVG
ItgaPat2  -----------MREGSFPRRIGLLLGLLGLLAG-----VATFNIDTKN--VVVHHMAGN---YFGYSLDFYHE------QKGMPVLVVG
PcItga    --------------------MGLTIFINLLLAVN---IVLGFNVDTKSPLYYSNNEPGS---FFGYSAAFNLY---------YKSVIVG
MmItga9   -------MGGPAGLRTGAGGLRALLLALVAAGVPAG----AYNLDAQR-PVRFQGPSGS---FFGYAVLEHFH-------ENTRWVLVG
                                  1         2                                   #1                3
AmItga1   PLSNGSSG-AEKFTRYGAVYRCEYT---GSQSC-SSIDVDYTYPDILNGD-----FIEEKIGQWLGAVVY-STGKGGKAMACAPRYIQL
ItgaPS2   PKFDTSRY-QQGVTEAGGVFKCSLNDDD----C-KLVPFDSKGNNRNVDK----EVVDRKSYQWLGATVA-TGRDSDLVVACAPRYVFH
ItgaV     PKANTT---QPGIVEGGQVLKCDW--SSTRR-C-QPIEFDATGNRDYAK----DDPLEFKSHQWFGASVR-SKQ--DKILACAPLYHWR
ItgaPat2  PEAESNNPNLAGIRRPGAVYACSVN----RATC-REVHVDKMKGNLKKLNGSHLVPIEEKSHQFFGATVR-SNDKHDKIVVCAPKYTYF
PcItga    PLSKQSN-----IGSGGAAYQCSYSSTDPKV-CTVMANADVKKGEGDDLT-----------NQWFGATVS---SDDRNTLVCAPRFAN-
MmItga9   PKADSKY--STSVKSPGAVFKCRVH-TNPERRC-TELDMARGRTRGAPCG---KTCRGDRDDEWMGVSLARQPRADGRVLACAHRWKNI
                          4                   5                   6                   #2
AmItga1   NYNSEITLRWLLGRCFLLKDDLSGHLNIKGILNPCEY---EPKGTRDYGY-CEAGFSAEYMKDPP--YNVILGTVGSRRWTGAVMMVD-
ItgaPS2   MTPSRAFRIDPVGTCFTSHN-FEEFYEVSP----CRTNN---WGYHRQGS-CQAGFSAAINGNGS---RLFIGAPGSWYWQGQTYSIPP
ItgaV     EM---KQEREPVGTCFLQDG--TKTVEYAP----CRS---QDIDADGQGF-CQGGFSIDFTKAD----RVLLGGPGSFYWQGQLISDQV
ItgaPat2  SK---FEVIEPVGTCFYAENGFDNAEEFSS----CKQEP-ARHGRHRLGYG-QCGFSAAVPGKKNQ-NRVFIGAPGVWYWQGAMFSQN-
PcItga    NGA-DGKEYYLLGKCVNVIESTNFAWKP------CAARH-----------CQAGFSAGYYGDNPQTAKIVSNVVSGVPTYSLSDNKQ-
MmItga9   YEAD---QILAHGFCYLIPSNLQAKG-KVLIP--CYEE-YKKKYGEEHGS-CQAGIAGFFTEEL-----VVMGAPGSFYWAGTLKVLN-
                                                                          #3
AmItga1   ------------------PDNPFVSPIKTT------QDQIDLHEYNGYSIASG-LFYVQSRADVVSGAPRADSIKGKALIYKYESGQFS
ItgaPS2   AKFPFKPPLYQPFGTGGMASSHDVTRPENQVFSTSESASVNDDSYLGYSMVTG-DFDGDRSEDVAIGMPRGGNLVGRIVVNRWNMANIF
ItgaV     EIVSK------------YDPNVYSIKYNNQ-LATRTAQAIFDDSYLGYSVAVG-DFNGDGIDDFVSGVPRAARTLGMVYIYDGKNMSSL
ItgaPat2  -----------------IKNQTDRPNTEYG-------SKEYDHDMMGYSTATG-DFDGDGIDDIVAGVPRGNDLHGKLVLYTSKLKMMI
PcItga    -----------------FEGRIALAPGGGGNTAFTNNKHLNYSDYMGYAVTSGKFSKDKNTLVVVGGSPRGNELAGKVIMYKADATSGY
MmItga9   -----------------LTDNTYFKLNDEA-------IMNRRYTYLGYAVTAG-HFSHPSITDVVGGAPQDEGIGKVYIFRADRRSGTL
                                   ...  ......                #4
AmItga1   RSQLPQPKDL-HTGSYFGSVVCAVDLD-KNGYADVLVGAPYFTDVLDE--------------GRVYIYLND-GKGILNLLDTVLWGDKK
ItgaPS2   ---NITGR---QIGEYFGYSLATSDVD-GDGLDDLLIGAPMYTDPDNVEGKY--------DVGRVYILLQGGPTEEKRWTTEHIRDGYH
ItgaV     ---NFTGE---QMAAYFGFSVAATDIN-GDDYADVFIGAPLFMDRGSDGKLQ--------EVGQVSVSLQ--RASG--DFQTTKLNGFE
ItgaPat2  -LTDEVST---QHGQYCGGSVAVADVN-KDGRDDIIMGCPFYTDYGSVKDAKTQERKPQYDVGKVIVMLQT---APGVFGKQIAVVGDD
PcItga    VFSTVIQDPSKKTGTYFGSALAAVAIEKAKEYSDLVIGAPYYSEDAMN-------------AGAVYVYKNS--QQDTLELKQTLYGDKT
MmItga9   KIFQASGK---KMGSYFGSSLCAVDLN-MDGLSDLLVGAPMFSEIR--------------DEGQVTVYLNQGHGALEEQLT--LTGDAA
                        ..........#5
AmItga1   NAHFGQAIASVGDLNGDGFNDVAIGAPMEGEDGSGAVYIYHGSSNGIELPYSQKIVG---SFIQPG--IKMFGISLAG-----------
ItgaPS2   KGRFGLALTTLGDVNGDGYGDFAVGAPYDGPEGRGVVYIFHGSPMGPLAKPSQIIKSEQ-LVEGAPY-PRTFGFALSG-----------
ItgaV     FARFGSAIAPLGDLDQDGFNDIAIAAPYGGEDKKGIVYIFNGRSTGLNAVPSQILEG---QWAARSM-PPSFGYSMKG-----------
ItgaPat2  WGRFGHAVAAAGDLNLDGYNDVIVGAPYAGKNKQGAVYVIHGSKDGVRELPTQKIEG---ANIGHGN-IKSFGFSLTG-----------
PcItga    GGLFGYSVVSVGDLNSDSFNDVAVGAPWGGPDGKGAVYLYYGTAGDEPFLLKQKLYPKDLGGPSAG-SLDGFGFSIAEFFEYSTRYARN
MmItga9   NAHFGESIANLGDIDDDGFPDVAVGAPKE-EDFAGAVYIYHGDANGIVPKYSMKLSG---RRLNPT--LRMFGQSISG-----------
  ..........                  #6                                  7                   8                   #7   9
AmItga1   VDVDQNGYPDIVVGAYASDKAVLIRTKSVVSVRVAMTLSEQHITVEGNDSS-CDLDGKRYK--CVNVTVHLRYLDAIS-------STAT
ItgaPS2   LDMDGNTYPDLAVGAYSSDQVFIFKSRPVAAVNAETSFASNSKLISLDDRS-CQLVRDHKKVPCMLLTT---------CWSYT--GRYL
ItgaV     TDIDKNGYPDLIVGAFGVDRAILYRARPVITVNAGLEVYPSI--LNQDNKT-CSLPGTALKVSCFNVRF---------CLKAD--GKGV
ItgaPat2  EDVDGNGMPDIAVGAWKSGNAAVLLTKPVVTVTGQTEPESAL--ISVEDKN-CDVDGKLGKQACKHINT---------CFKYE--GKGD
PcItga    NDIDRNGYPDLAIGAYRSNQLVLFKSRPVISVKNNIDYDRNLKIDLYSNTNMCINPADGKKYKCIKKF-DV-------CFTIITPGD-K
MmItga9   IDMDGNGYPDVTIGAFLSDSVVLLRARPVITVDVSIFLPGSINITAPQ----CHDGQQP--VNCLNVTV---------CFRFH--GKNV
                                                                                  10
AmItga1   D---LHLTFTFDLD----------KDIDVQLRRMFFYDEVTKSRTVSLRQNTTLAKPNTTYEVLRKTAYIKSKDELGDLTSLLPFDVTL
ItgaPS2   E--QLDFDVSWL----------LDAKKLLNPRMFFLRDEGKNIRNQTIRLN-----YGQKYCLNETVYLLD--KVQDKLTPLEVEARY
ItgaV     PRK-LNFQVELLLD---------KLKQKGAIRRALFLYSRSPSHSKNMTISR-----GGLMQCEELIAYLRDESEFRDKLTPITIFMEY
ItgaPat2  PND-LEFDLRFNLDDHSPEPRAYFLQKDVKSDRSIKVAQGSKTRDHPSSIEQRVRLEKGRQKCFRHRFFASST--MKDKLSPIHWSVNY
PcItga    D---VTFTVDID------------KETTGGKRGFVVEGTQQKSVFSGVVTEI----AQVAKCQPYTIYLRNN--TKDVFREMIIKISW
MmItga9   G--EIGLNYNLTAD---------VAQKEKGQLPRVYFVLFGETAGQVSERLQLS---HMDEVCHHYVAHVKRR--VQDVISPIVFEAAY
                                                                  11        12
AmItga1   QPQP--------PCANNTCPILNDRIATSLRVTIPFAKK--CRKRI--ICEPDLVLSAQPVFNPKSD---VLRLGVVKDFTIELTVENK
ItgaPS2   LRSSRPLDPMVRHRRSILEPVIDQNREIVLRDAINIQKN--CGPDN--ICEPDLKLKVS-TVDK-------YLFGSPEPLVIEVFISNT
ItgaV     LDYR------TAADTTGLQPILNQFTPANISRQAHILLD--CGEDN--VCKPKLEVSVDSDQKK-------IYIGDDNPLTLIVKAQNQ
ItgaPat2  YVES----KTGKLRGDKLEPAIDTTVPLSFQNKINIANN--CGKDD--LCVPDLKVTAVADREK------FLLGTQDNTMLINVTVQNG
PcItga    LKQP-------TNCQDNLCPITNQMSQLTNLVRIPYIKG--CSDDGNPECNSDLSIVMQKQLAGDFKT---IQVGITKTLNIVAKITNK
MmItga9   LDEHVMGE--EDRELPDLTPVLRWKKGQRISQKNQTVFERNCQSED---CAADLQLRGKLLLSSVDEKTPHLALGAVKNISLNISISNL

AmItga1   EDSAYTSRITLKYPEALNYIGTDEGVE---
ItgaPS2   ED-AFEAAFYMVTPPDLQFRKLQQLGEKKD
ItgaV     EG-AYEAELIVSIPLQADFIGVVRNNE-AL
ItgaPat2  ED-SYETKLYFDVPQGFEYGGIESVGGDGS
PcItga    EN-AFINTLKIVYPKEVNPNKVVIKDF-AG
MmItga9   DD-AYDANVSFNVSRELFFINMWQKEE---
```

Figure 2.2. Multiple sequence alignment of Acropora millepora integrin alpha (AmItga1) with representative integrin alpha amino acid sequences from fly (ItgaPS2, accession no. P12080), human (ItgaV, accession no. Q6QAF3), nematode (ItgaPat2, accession no. P34446), jellyfish (PcItga, accession no. Q9GSF4), and mouse (MmItga9, accession no. Q91YD5). The seven conserved FG-GAP repeats are shown by black lines. Squares indicate cation binding motifs (DxD/NxD/NxxxD). The 18 cysteine residues in the extracellular domain are numbered. Putative cleavage site (RRRR) of AmItga1 is shown by asterisks. The predicted transmembrane domain is underlined with double lines. The highly conserved consensus motif KxGFFKR is shown by circles.

```
                    13                  14
AmItga1   ------CDRKPSDNNTQ---TATCYIGNPLLAK-SMKSFGIKFSPGRVKED-----FVVQVETSSQDEDANIEDNKKTISVAVKYEADV
ItgaPS2   TPIT--CSPPTPENNH----TLKCDIGNPLESG-KIAHFKISLVPEEKYGSSSSYDFYWEANSTNLEKPGSEYDNKIRQSVGIWVDTDL
ItgaV     ARLS--CAFKTENQTR----QVVCDLGNPMKAG-TQLLAGLRFSVHQQSEMDTSVKFDLQIQSSNLFDKVS---PVVSHKVDLAVLAAV
ItgaPat2  KSAPA-CSPTSDEPDSDGKWTFACDLGNPLPAN-KVVSSVVRVTASSDKPPLAPISINAHVNSSNDEEAHTVADNKVTFTIPVDFKNQL
PcItga    TPVW-DPDSTKDGNEK----TFSVTLASPIGPN-KHTTVTVEFGIASVAKGTKKLTFIGRIETLSVEMVPAD--NVYTLEIPVSLLANL
MmItga9   MGIS--CELLESD-------FLKCSVGFPFMRSKSKYEFSVIFDTSHLSGEEEILSFIVTAQSGNLERSEALHDNTLTLTVPLVHEVDT

AmItga1   ITGTTKQDQVVYQGPVRS-----KEEVKKDLDSIGPEIVQTLSVRNNGPSDIRSSELVVSFPKAYS-SSKPDSYLLYLLLVELDGAS--
ItgaPS2   IKGTSLPDYQLYKADDYK-ELENATKE----DDIGPQVVHIYEIRNNRPSIIEEAEVFIHLPY----ETIVGDPLMYLLNQPETGG---
ItgaV     IRGVSSPDHVFLPIPNW-----EHKENPETEEDVGPVVQHIYELRNNGPSSFSKAMLHLQWPY----KYN-NNTLLYILHYDIDGP---
ItgaPat2  LNGRSNPEQVDFSMTNK------TRVDAFDDNEIGPVVSHLYQISNRGPSEVDSATLDIFWPS----FSTEGGHLLYIITEPVVNPP--
PcItga    VTGNVEPEQLKWNKDASP-------------KDVGAEIIHTFYFQNLGPSTVDTSDVVIQFPER-----FDGSVMFNIFKADLKGTDKS
MmItga9   ITGIVSPTSFVYGESVDASN----FIQLDDQECHFQPVNITLQVYNMGPSTLPGSSVSISFPS---RLSPGGAEMFQVQDMVVSQ----
                15                                                                          ****
AmItga1   --GIC-------------------------------------------------------DASVNPLKIKPRNETATESTPSRRRRD---
ItgaPS2   -KIQCDDVAFNEYNLLLDEKLVKKSYLQAQGAIWNSAQVSGQSSSSSSSGGASVHIEKARGEGFVRGVLVSNSTDAGDKLSPKQVEQRR
ItgaV     --MNC-----------------------------------------------------TSDMEINPLRIKISS-LQTTEKNDTVAGQ-GE
ItgaPat2  NKGRCR-----------------------------------------------------VKQLQNVNPLNLRITNEHVPTEPPVAKTPNEYS
PcItga    SVGTCT-----------------------------------------------------YGKLNTLGLNVTTEEVGGVNSTAKKLRARR
MmItga9   EKGNC-----------------------------------------------------SLQRNPTPCIIPQEQ--ENIFHTIFAFFSKSG

AmItga1   ------------------------------------------------------------------------------------------
ItgaPS2   EDTLEALGDASFV-----------------------------------------HRD-RASQAVQEPQVNQTSFTTYSTSSSSSGSGAP
ItgaV     DHLITKRD-------------------------------------------------------------------------------
ItgaPat2  EEDDESYEDETTTQSQSTRHQSTQHQTHHQSGPVHVYEKDEEKIRQNTGNWQYVEDKKKKGDYEYIPDDQEYDGDDFEEEDDEDFDRAG
PcItga    AD--------------------------------------------------------------------------------------
MmItga9   K-------------------------------------------------------------------------------------

AmItga1   ------------------------------------------------------------------------------------------
ItgaPS2   AQLRGHSTQGHIQMAGPVQHTSSSSSSNYRSWPAQQQQQHQQLLLAGSGGSGLGSPVTFNDKSQFGGRNNNFHTGTLDLGTLNRGNVDN
ItgaV     ------------------------------------------------------------------------------------------
ItgaPat2  KRVKRNPTPKKKKKGGEHRGEPRSDKARFSDLREAVKLSKEAGGVVDYKG-------------------------------------
PcItga    ------------------------------------------------------------------------------------------
MmItga9   ------------------------------------------------------------------------------------------

AmItga1   ------------------------------------------------------------------------------------------
ItgaPS2   LYRSQGQYQNPSQSLGQSQGQFQANANQGHYQGQNQAQFQARNPGFQGQTSYQGQTQYSGQPGGYQTHHVTYSSGSKPYYGRENEDFYD
ItgaV     ------------------------------------------------------------------------------------------
ItgaPat2  ------------------------------------------------------------------------------------------
PcItga    ------------------------------------------------------------------------------------------
MmItga9   ------------------------------------------------------------------------------------------
                                                                  16        17    18
AmItga1   ----------------------------------------TGNLVLSC--RQAASQTFKCQLGQLKAGDKAN-IKMTFR--FWEN-TLL
ItgaPS2   DNLQQATPGHWSSSSSSSSSSGTRRLRRSNDKDGATEKPLQIDLNSPCQSAR--CKSIRCVVTNLGTEDGDA--AFVAIRARMVAKI-M
ItgaV     -------------LALSEG----------------------DIHTLGC--GVAQCLKIVCQVGRLDRGKSA--ILYVK-SLLWTE-IFM
ItgaPat2  ---------------------------------------PLSRASVDC--NSLRCTHIECDIYDLKEDEFV--LVEIFSR-LYTN-TLV
PcItga    ----------------------------------------TIPQLGC-GGSIKCREIKCTLGLLQKGAGAT-VKITSSLVDYTFQQLI
MmItga9   ----------------------------------------VLDCEKPGSFCLTLHCNLSALPKEESR-TINLYML----LNTEIL

AmItga1   ELDS---PKAVELVTSANVKVSDDITQSNYENDDTEIKIKARPASTAAQKKKTPWWIILLSVLGGLLLVAAVIVILYKVGFFKRKQIKD
ItgaPS2   KLASNVPLNVSTLAVANVTLLPFIGAPKDAIVKTHEIFYKAEPEPLQ-VPDVVPLWVVVLAACAGALIFLLLVWLLYKCGFFNRNRPTD
ItgaV     KENQNHSYSLKSSASFNVIEFPY-KNLPIEDITNSTLVTTNVTWGIQPAPMPVPVWVIILAVLAGLLLLAVLVFVMYRMGFFKRVRPPQ
ItgaPat2  EKNP-G-GDISSLALARVTSTKYNLPHKPTLI---TAVSTNMNAIASEEGRDLPWWLYLLAILIGLAILILLILLLWRCGFFKRNRPPT
PcItga    D------SSTINVQAKFTSTAVDKPKTAPPDTVVIGFTAISPNLTKEGESSTVEWWIIFICILVAILIIAVIVFIMYKKGFFKRKKMGE
MmItga9   KDSS---SVIQFMARAKVKVEPALRVVEIANGNPEETLVVFEALHNLEPRGYVVGWIIAISLLVGILIFLLLAVLLWKMGFFRRRYKEI
                                                                 ●  ●●●●●

AmItga1   SAPDTTETTAL-------------------------
ItgaPS2   SQERQPLRNGYHGDEHL-------------------
ItgaV     EQ-EREQLQPHENGEGNSET----------------
ItgaPat2  HAELRADRQPNAQYADSQSRYTSQDQYNQGRHGQML
PcItga    EEEEELRKGDPEE-----------------------
MmItga9   EAEKNRKENEDGWDWVQKNQ----------------
```

Figure 2.2. Continued.

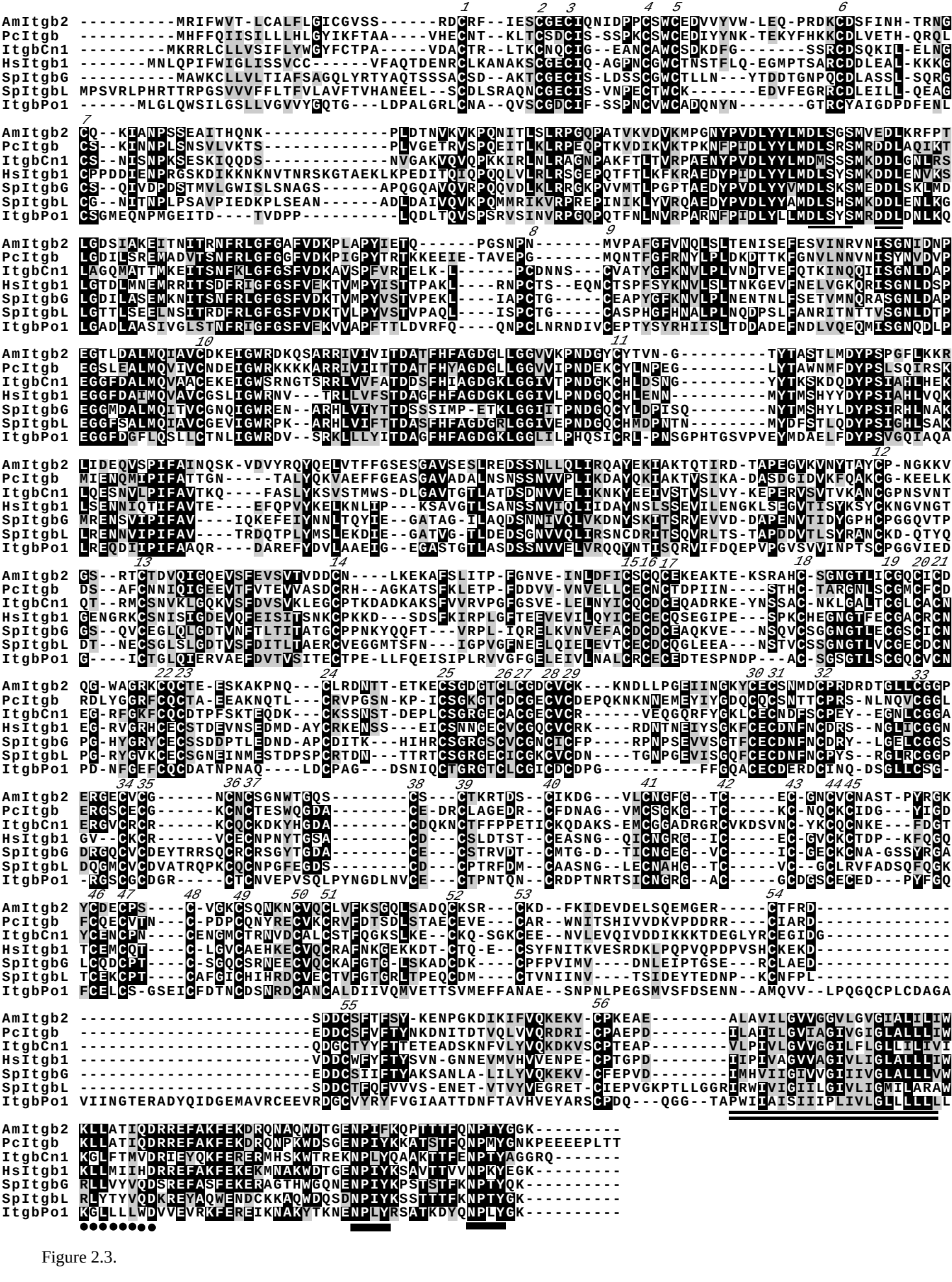

Figure 2.3.

Fig. 2.3. Multiple sequence alignment of *Acropora millepora* integrin beta2 (AmItgb2) with representative integrin beta amino acid sequences from jellyfish (PcItgb, accession no. Q9GSF3), coral (ItgbCn1, accession no. AF005356-1), human (HsItgb1, accession no. Q8WUM6), sea urchin (SpItgbG, accession no. P92163; SpItgbL, accession no. O76727), and sponge (ItgbPo1, accession no. O18482). The 56 cysteine residues in the extracellular domain are numbered. The highly conserved motifs DxSxS and DLL are shown by lines. The predicted transmembrane domain is underlined with double lines. Circles show the conserved membrane proximal sequence KLLxxxxD. Two NPxY/F motifs are underlined with bold lines.

```
AmTet1            1 ------------MEGGAKIIKYLVVFFNFLFFIFGCVLVGVGAWSLVKIGDFNDLTEGS-
AmTet2            1 ------------MCG-ITCIKRLLFAFNFIFWLAGAAILGIGIWTEIDPGQFYAFIGSTG
cd151_mouse       1 ---MGEFNEKKATCG-TVCLKYLLFTYNCCFWLAGLAVMAVGIWTLALKSDYISLLASST
AmTet4            1 -----------MASGGMVLVKYLLFFFNFIFWISGIVLIVVGAVAKNNYGNFTLVSEKEL
Cd63_frog         1 ----------MAVEGGMKCVKFLMFFFNFVFWVCGIALIAIGIYVQIQL-NHTLIMKNAA
AmTet3            1 ---MYSPKYSKAQSCAYFCSKNVLVALNTLYIFIALVLIGVAVYAKASAKITSLPILGG-
TM4SF13_human     1 -----------MVCGGFACSKNCLCALNLLYTLVSLLLIGIAAWGIGFGLISSLRVVGV-
AmTet5            1 ---------MAQEGKVSRFLKYTLFVFNLLYWCIGGVMIGVGLWAVTQKSDYNTFSSIS-
Tspan15_human     1 MPRGDSEQVRYCARFSYLWLKFSLIIYSTVFWLIGALVLSVGIYAEVERQKYKTLESAF-

AmTet1           48 YSSASTVMIVAGVLIALIAFFGCCGAWKESRCLLIMFFICLLVILIIEITFAVLGYINRN
AmTet2           48 YSLPAKLLMAAGGFVMIVGFLGCCGAIKESRLLLGLYFACLLLIFAAEAVAGILGFLYRE
cd151_mouse      57 YLATAYILVVAGVVVMVTGVLGCCATFKERRNLLRLYFILLLIIFLLEIIAGILAYVYYQ
AmTet4           50 TTG-PVFLIAIGVIVSFIGFLGCCGAYKENYCMVTTFAILLGIIFVLEIAAGAYAYSRRD
Cd63_frog        50 SSGAPIVVIVVGVVIFLIAFFGCCGALKENYCMVTTFAVVLVLIFLVEIAAAIAAYVYKD
AmTet3           57 -------VIACGVFLLLVAVLGVVGAVRHSQVILFFYMVILFLVFVIQISVSIGAVAISH
TM4SF13_human    49 -------VIAVGIFLFLIALVGLIGAVKHHQVLLFFYMIILLLVFIVQFSVSCACLALNQ
AmTet5           51 -TDPAAVMVAVGGFIFIISFFGTVGALRENICFLKTYMIVMIIIVILEVIAGLLAFAFWP
Tspan15_human    60 -LAPAIILILLGVVMFMVSFIGVLASLRDNLYLLQAFMYILGICLIMELIGGVVALTFRN

AmTet1          108 KVDDAFDKGMENIIQQKYGASDMKAITDSVDKLQQQEMCCGWINFTDWYISNFT------
AmTet2          108 KIDDEITNRLRDEIRTKYGVTIDATTDQVVDNLQIRLKCCGIVNSTDWADSKWQDK----
cd151_mouse     117 QLNTELKENLKDTMVKRYHQSGHEGVTSAVDKLQQEFHCCGSNNSQDWQDSEWIHSG--E
AmTet4          109 KLDEYATKALKKAVDN---YSNDK---ETIDKVQREFKCCG---AESYKDYFGG------
Cd63_frog       110 KLRTAFEDSFKNGMSK---YNTTKDITESIDLLQKEFKCCGAFNATDWKQYPPF------
AmTet3          110 NRQADLMK---------AGWQKIPNKSSIKEQIQSARNCCGFQNKSLPVD----------
TM4SF13_human   102 EQQGQLLE---------VGWN---NTASARNDIQRNLNCCGFRS----------------
AmTet5          110 EVQKSVDSKFKTAIER---YRDDVDLQNAIDAVQENFQCCGSTDLNDWDINRYFKCGGRS
Tspan15_human   119 QTIDFLNDNIRRGIEN---YYDDLDFKNIMDFVQKKFKCCGGEDYRDWSKNQYHDCSAPG
                                                        ***

AmTet1          162 NGEHKVPDSCCKE----------QSENCGEAADTNN-NIYKVGCKTKLEEFIKDKLYHIG
AmTet2          164 NPQLKVPLSCCKEGG--------NTTTCNSKAEIDASKINQEGCLEKLKEFVNNHLFILG
cd151_mouse     175 ADSRVVPDSCCKT----------MVAGCGKRDHASNIYKVEGGCITKLETFIQEHLRVIG
AmTet4          154 DNNATFPTSCCKNET--------DCPATVGAAKDAESSLFREGCVKAVESFLKKHLIVVG
Cd63_frog       161 NGTDAVPDSCCK-------------TIIAGCGKDPSATINTDGCATGIDQWVKKNIGIVA
AmTet3          151 -VPLGHPDCRALP-------------CCENSDDWSCPKCQT--CYDTLEDVINHLLKVAG
TM4SF13_human   134 ----VNPNDTCLA-------------SCVKSD----HSCSP--CAPIIGEYAGEVLRFVG
AmTet5          167 PEQCGVPYSCCVRKLNERPNVQCGWHAREQHRLALKGKIYITGCLDAVLEWFRNHLVIVA
Tspan15_human   176 PLACGVPYTCCIRNTTEVVNTMCGYKTIDKERFSVQDVIYVRGCTNAVIIWFMDNYTIMA
                              **                                  *

AmTet1          211 ALGITVVVIQILGMIFALVLACKIESEGKYA-----------------------------
AmTet2          216 VVGVAVAGIQLIGMIFACCLFCSIEVD--------------------------------
cd151_mouse     225 AVGIGIACVQVFGMIFTCCLYRSLKLEHY-------------------------------
AmTet4          206 GVGVGIAFIQVIGIVFACCLMRSIKKEYEVM-----------------------------
Cd63_frog       208 GVALGIALFEILGIIFACCLMKGIRSGYEVM-----------------------------
AmTet3          195 GIGLFFSFSLLFGVCMTYRYRHQRDPRANPGAFL--------------------------
TM4SF13_human   171 GIGLFFSFTEILGVWLTYRYRNQKDPRANPSAFL--------------------------
AmTet5          227 AVAVAFAFPEVVGIIMTHLFIKQIKEQIEAWKNPQTFKYRPSQEVNGRSGPFY------
Tspan15_human   236 GILLGILLPQFLGVLLTLLYITRVEDIIMEHSVTDGLLGPGAKPSVEAAGTGCCLCYPN
```

Figure 2.4. Alignment of amino acid sequences of AmTetraspanins. Some conserved motifs are indicated by an asterisk, *, below the block. Accession number of sequences other than AmTetraspanins: cd151_mouse, Q921J7; TM4SF13_human, Q6FGK0; cd63_frog, Q7SY95; Tspan15_human, O95858.

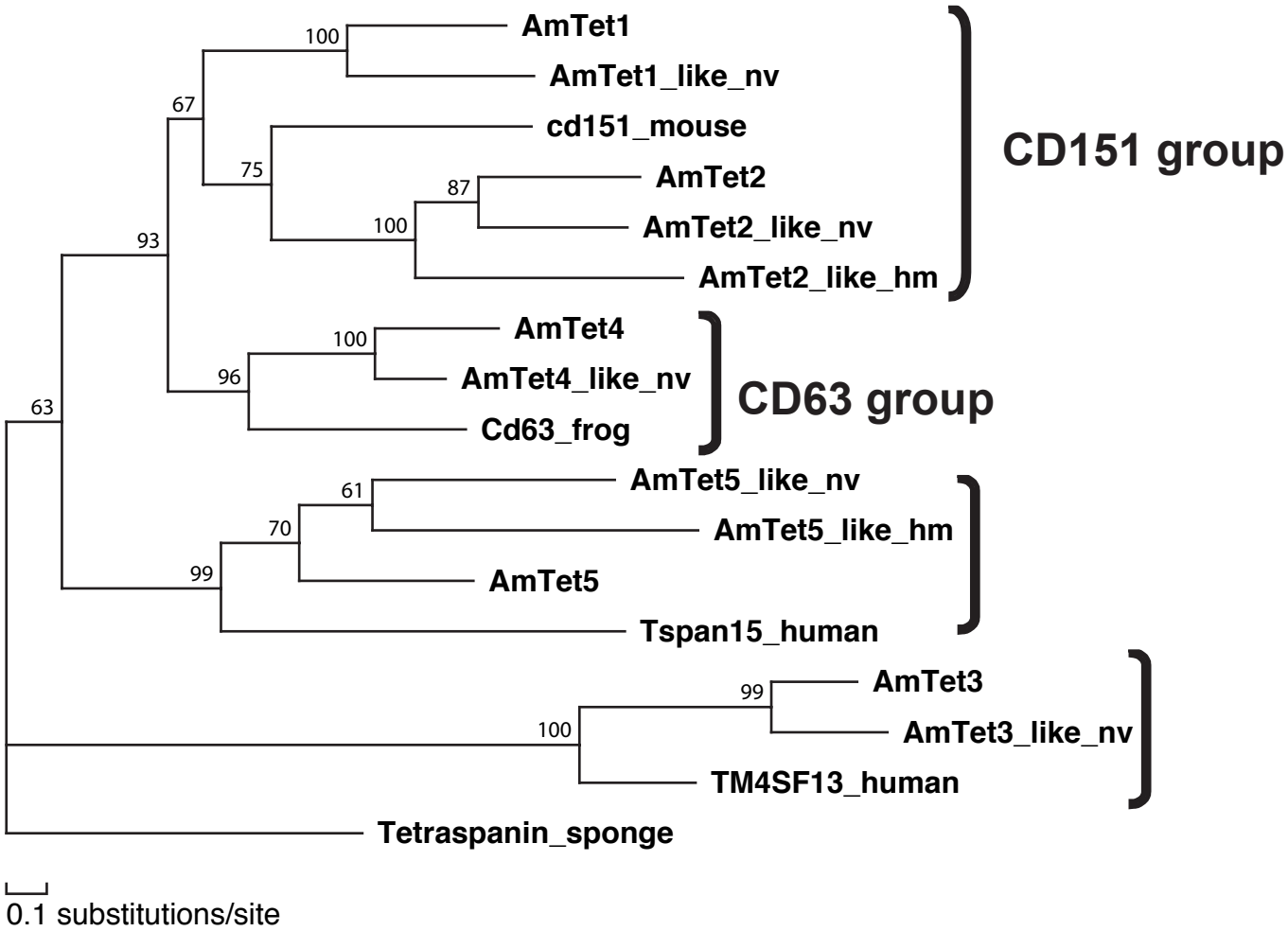

Figure. 2.5. Phylogenetic tree of AmTetraspanins in relation to other tetraspanins. The bootstrap probability for each node is indicated adjacent to the node. Sponge tetraspanin was added as an outgroup (accession number: Y18100-1).

Table 2.1. List of 15 target genes.

Gene	EST code	Stage	GeneBank homologue	Length	Accession No.
AmADAM1	B032-D4	Prawnchip	human ADAM-TS16	Partial	N/A
AmADAM2	B016-D12	Prawnchip	human ADAM-TS18	Full	N/A
AmADAM3	B018-D12	Prawnchip	human ADAM10	Full	N/A
AmADAM4	A006-B9	-	human ADAM-TS6	Partial	N/A
AmADAM5	A039-B9	Post-settlement	human ADAM-TS5	Partial	N/A
AmADAM6	A039-B9	Adult colony	human ADAM-TS17	Partial	N/A
AmIntegrin alpha1	B030-E3	Adult colony	mouse integrin alpha9	Full	N/A
AmIntegrin alpha2	D038-F8	Adult colony	hydra collagen-like protein	Partial	N/A
Integrin betacn1	-	-	human integrin beta1	Full	AF005356-1
AmIntegrin beta2	D012-H9	Adult colony	jellyfish integrin beta	Full	N/A
AmTetraspanin1	A003-G11	Post-settlement	mouse CD151	Full	N/A
AmTetraspanin2	A035-E3	Post-settlement	mouse CD151	Full	N/A
AmTetraspanin3	C005-D12	Prawnchip	human TM4SF13	Full	N/A
AmTetraspanin4	C008-C11	Pre-settlement	frog CD63	Full	N/A
AmTetraspanin5	A045-A2	Adult colony	human Tspan15	Full	N/A

Table 2.2. The position of TM and EC regions of AmTetraspanins.

	TM1	TM2	TM3	TM4	EC1	EC2
AmTetraspanin1	10-32	52-74	80-102	211-233	33-51	103-210
AmTetraspanin2	10-32	49-71	82-104	210-232	33-48	105-209
AmTetraspanin3	19-41	51-73	82-104	-	42-50	-
AmTetraspanin4	8-30	50-72	81-101	201-223	31-49	102-200
AmTetraspanin5	13-35	53-75	85-107	217-239	36-52	108-216

Chapter 3. Gene expression pattern of ADAM–integrin interaction-related genes of *Acropora millepora*

3.1. Introduction

Chapter 2 described the structures of target genes, which allowed selection of AmADAM3, AmIntegrin alpha1, and AmTetraspanin1, 2, and 4 as potential candidate genes for fertilisation in *A. millepora*. However, at this stage, it is impossible to judge whether these genes are truly involved in fertilisation only from examining their structures. To judge whether these genes, apart from AmADAM3, are related to fertilisation in *A. millepora*, their expression patterns were examined in unfertilised eggs of *A. millepora* using reverse transcriptase-PCR (RT-PCR), Western blotting, in situ hybridisation, and immunocytochemisty. For AmADAM3, RT-PCR was performed with seasonal adult branch RNA (before and after spawning) because detecting gene expression in sperm is difficult. Checking the expression pattern of these genes would facilitate the prediction of which genes are involved in fertilisation of *A. millepora*. Almeida et al. (1995) performed RT-PCR and immunocytochemistry with unfertilised mouse eggs to find which integrins are involved in fertilisation. In particular, comparing the expression of integrin betacn1, which has already been cloned (Brower et al. 1997), and expression of target genes will be informative because integrin betacn1 has already been shown to be involved in the fertilisation of *A. millepora* (Marquez 2002). This chapter describes results on the expression patterns of target genes (AmADAM3, AmIntegrin alpha1, AmTetraspanin1, 2, and 4) and discusses which genes are involved

in the fertilisation of *A. millepora*.

3.2. Materials and Methods

Sample collection and RNA extraction

Unfertilised eggs were collected from a single colony of *A. millepora* (Spawned on 01 November 2004). Adult branches were collected from a single colony of *A. millepora* (September, October, November, and December 2004). Total RNA from unfertilised eggs was isolated using the RNeasy Mini Kit (QIAGEN, Chatsworth, CA, USA) according to the manufacturer's protocol. Total RNA of adult branches was isolated using RNAWIZ (Ambion, Austin, TX, USA) according to the manufacturer's protocol. To check the quality, RNA was visualised with ethidium bromide on 1% agarose gels under ultraviolet light.

RT-PCR analysis

RNA was treated with DNase (Fermentas, Glen Burnie, MD, USA) to remove contaminating genomic DNA. Single-stranded cDNA (sscDNA) was synthesised using the First-strand cDNA Synthesis Kit (Amersham Biosciences, Piscataway, NJ, USA) with about 1 µg of total RNA. One microlitre of the sscDNA was used per 20 µl of the PCR. For AmADAM3, the primer pair AmADAM3RT-F1 (5'-CAAATCCAGCGAATGAGTG-3') and AmADAM3RT-R1 (5'-GCAACAGAGAAGGCATTG-3') was used to amplify a fragment of 984 bp. For AmIntegrin alpha1, the primer pair AmItga1RT-F1 (5'-GCCAATGAAACAGCTACG-

3') and AmItga1RT-R1 (5'-TTGTCTCCAGCCTTCAAC-3') was used to amplify a fragment of 130 bp. For integrin betacn1 (Brower et al. 1997), the primer pair ItgB1RT-F1 (5'-CTTGTGTTGCCACTTATGGCTT-3') and ItgB1RT-R1 (5'-CTGCTACTTGCATTAACGCATC-3') was used to amplify a fragment of 144 bp. The primer pairs for AmTetraspanins were as follows; AmTetraspanin1: AmTet1RT-F1 (5'-TGTAAATTGCAGCCTGCG-3') and AmTet1RT-R1 (5'-GGAAGAGTATGATCCTTCAGTCAG-3'; 226 bp); AmTetraspanin2: AmTet2RT-F1 (5'-ATTGATGCTACGACCGAC-3') and AmTet2RT-R1 (5'-AGAAGACGACTAGCCCAGTC-3'; 404 bp); AmTetraspanin4: AmTet4RT-F1 (5'-GTGCGGATTTTTGTGCAG-3') and AmTet4RT-R1 (5'-CCACAGCACCAACAACAATTAG-3'; 151 bp). The PCR profile was as follows: 1 min at 94 ^{o}C, 40 cycles of 0.5 min at 94 ^{o}C (denaturation), 0.5 min at 50 ^{o}C (annealing), 2 min at 72 ^{o}C (extension), followed by an additional extension for 2 min at 72 ^{o}C. The PCR products were visualised with ethidium bromide on 1% agarose gels under ultraviolet light. As a positive control for fidelity and efficiency of the amplification, the partial cDNA of coral actin was amplified by PCR with actin-F1 (5'-CGAACACGGAATCGTAACCAACTGG-3') and actin-R1 (5'-CAAATCCAGACGTAAGATGGCATGG-3'). These primers were designed based on sequence of the actin EST clone (unpublished data).

Western blotting

To examine protein expression patterns of integrin betacn1 and AmIntegrin alpha1,

Western blotting with unfertilised eggs of *A. millepora* was undertaken. Polyclonal antibodies of integrin betacn1 and AmIntegrin alpha1 were used. Eggs were homogenised with 500 µL of 10 mM CHAPS including 10 mM EDTA, 1 µM leupepsin, and 1 µM pepstatin, and centrifuged at 10,000 x *g* for 15 min. The supernatant (5 µg protein) was separated by SDS-PAGE on 10% polyacrylamide gels under reducing conditions (sample buffer containing 20 mM DTT). Protein concentration was determined with the Bradford protein assay. Five micrograms of proteins were electrically transferred to a nitrocellulose membrane (Amersham Biosciences, Piscataway, NJ, USA). The membrane was blocked with 3% BSA in TBS (150 mM NaCl, Tris-Hcl 50 mM) and then incubated overnight at room temperature with polyclonal antibodies diluted at 1:100 in 3% BSA in TBS. Polyclonal antibodies raised in rabbit to integrin betacn1 and AmIntegrin alpha1 (refer to Chapter 4 for details of antibody production of AmIntegrin alpha1) were used for Western blotting. The membrane was then incubated with a peroxidase-conjugated swine anti-rabbit IgG (DAKO) for 60 min at room temperature. After three washes with TBS, signals were detected with an enhanced chemiluminescent (ECL) HRP substrate according to the manufacturer's instructions (Amersham Biosciences, Piscataway, NJ, USA).

Whole mount in situ hybridisation and immunostaining

To examine the mRNA expression of AmTetraspanin1 and 4, integrin betacn1, and AmIntegrin alpha1, in situ hybridisation with unfertilised eggs of *A. millepora* was carried out. In addition, immunostaining with unfertilised eggs of *A. millepora* was done

to examine the protein expression of integrin betacn1 and AmIntegrin alpha1. Polyclonal antibodies of integrin betacn1 and AmIntegrin alpha1 were used for immunostaining. The basic procedures for fixation and hybridisation of coral eggs were carried out as previously described (Hayward et al. 2001). Photographs were captured directly with a Spot digital camera. Digitised images were processed with Adobe Photoshop.

3.3. Results

RT-PCR analysis demonstrated that mRNAs of integrin betacn1 and AmIntegrin alpha1 are expressed in unfertilised eggs of *A. millepora*. AmTetraspanin1 and 4 are clearly expressed in unfertilised eggs of *A. millepora*, whereas expression of AmTetraspanin 2 was not detected (Figure 3.1). The expression of AmADAM3 disappears just before spawning, although integrin betacn1 is expressed in all seasons (Figure 3.2).

In situ hybridisation also demonstrated that AmTetraspanin1 and 4 are expressed in unfertilised eggs of *A. millepora* (Figure 3.3), but expression of integrin betacn1 and AmIntegrin alpha1 was not detected. Western blotting and immunostaining were performed with coral integrin polyclonal antibodies, but no signal could be detected in unfertilised eggs of *A. millepora*.

3.4. Discussion

As can be seen from RT-PCR experiments (Figure 3.1), mRNAs encoding integrin

betacn1 and AmIntegrin alpha1 are present in unfertilised *Acropora* eggs. Maternal integrin mRNAs have also been reported in a wide variety of other animals (e.g. Whittaker and DeSimone 1993), and in the hydrozoan *Podocoryne*, a homologue of integrin betacn1 and a putative orthologue of AmIntegrin alpha1 are similarly maternal (Reber-Muller et al. 2001). These results indicate that AmIntegrin alpha1 may exist and play a role (e.g. fertilisation), together with integrin betacn1, in unfertilised eggs of *A. millepora* because integrins exist as alpha-beta heterodimers and are involved in a broad range of functions in cell–cell adhesions and signalling pathways (Hynes 2002).

AmTetraspanin1 and 4 are expressed in unfertilised eggs of *A. millepora* (Figure 3.1). The association of integrins with various tetraspanins has often been documented (e.g., Boucheix and Rubinstein 2001). In mammals, tetraspanins are central players in gamete interactions, acting as receptors or co-receptors for the integrin ligand (Le Naour et al. 2000; Miyado et al. 2000). Therefore, AmTetraspanin1 and 4 may make a molecular complex with coral integrins and may be involved in the fertilisation of *A. millepora*.

The expression of AmADAM3 disappears just before spawning, although integrin betacn1 is expressed in all seasons (Figure 3.2). The same expression pattern is detected in AmTat1 cloned from *A. millepora* and is similar to human T-type amino acid transporter (hTAT1) by BLAST analysis (Go 2005). AmTat1 is thought to be a target gene that AmDM1, the *A. millepora* homologue for Dsx, down-regulates (Go 2005). The disappearance of AmADAM3 before spawning may prevent sperm protein production

in preparation for spawning. However, AmADAM3 involvement in fertilisation cannot be unambiguously clarified from these expression results.

Western blotting and immunostaining with coral integrin polyclonal antibodies could not detect signals in the unfertilised eggs. This may be related to the design of the selected domains for coral integrin antibody production. The selected ligand binding domains of integrins for antibody production (see Chapter 4) may be more variable than those of the stem region. During fixation of unfertilised eggs of *A. millepora*, a change in ligand binding domains of integrins may have occurred that inhibited the access of antibodies. To overcome this possibility, integrin antibody against the stem region should be produced.

In summary, AmIntegrin alpha1 and AmTetraspanin 1 and 4 were selected as candidate genes for fertilisation in *A. millepora* on the basis of their expression patterns described above. In Chapter 4, the results of fertilisation inhibition experiments with gene antibodies or fusion proteins are used to judge whether the above genes are involved in the fertilisation of *A. millepora*.

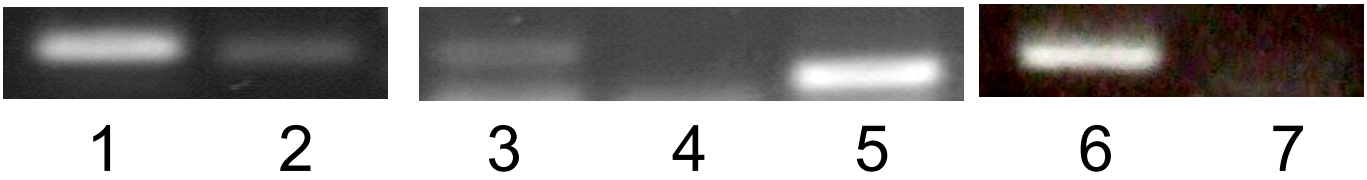

Figure 3.1. Expression of integrin betacn1 (lane 1), AmIntegrin alpha1 (lane 2), AmTetraspanin1 (lane 3), AmTetrspanin2 (lane 4), and AmTetraspanin4 (lane 5) in unfertilised eggs of *Acropora millepora*. Lane 6 corresponds to the positive control (actin), and lane 7 corresponds to the negative (no template) control. The lower bands in lane 3 and 4 are primer dimmers.

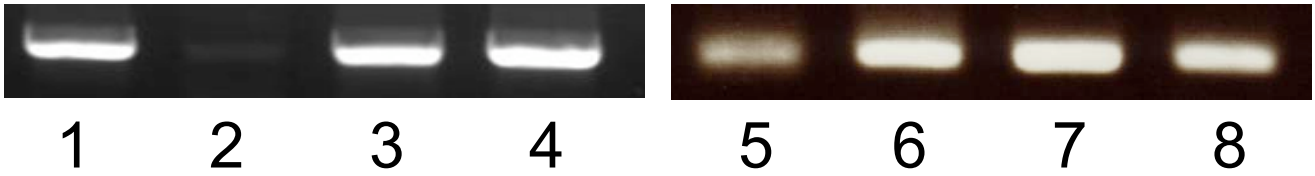

Figure 3.2. Expression of AmADAM3 and integrin betacn1 in a seasonal adult colony of *Acropora millepora*. Lane 1-4: AmADAM3; lane 1 September; lane 2 October; lane 3 November; lane 4 December. Lane 5-8: integrin betacn1; lane 5 September; lane 6 October; lane 7 November; lane 8 December.

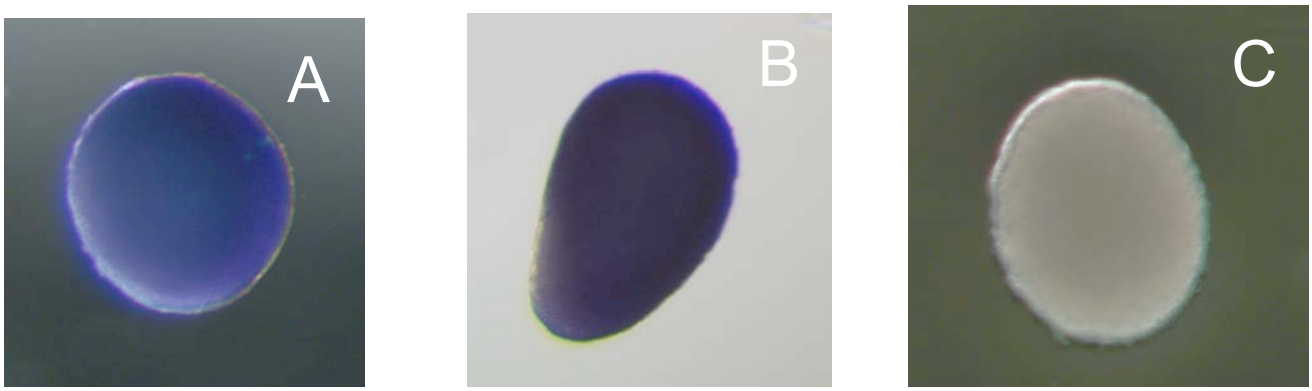

Figure 3.3. Expression of AmTetraspanin1 (A) and AmTetraspanin4 (B) in unfertilised eggs of *Acropora millepora*. (C) corresponds to the treatment with a sense primer.

Chapter 4. Preparation of fusion proteins and antibodies, and fertilisation inhibition experiments

4.1. Introduction

In Chapter 3, it was predicted that AmIntegrin alpha1 and AmTetraspanin1 and 4 are likely to be involved in the fertilisation of *A. millepora*. In mouse, Almeida et al. (1995) used antibodies against integrins to judge which integrin is associated with fertilisation. This method is also applicable to corals. Fertilisation inhibition experiments have been carried out with antibodies against integrin betacn1 and showed that integrin betacn1 is involved in the fertilisation of *A. millepora* (Iguchi et al. 2007; Márquez 2002). AmTetraspanin1 and 4 may be associated with the fertilisation of *A. millepora*.

Tetraspanin CD9 is critical for sperm–egg membrane fusion in knockout mice (Kaji et al. 2000; Miyado et al. 2000; Le Naour et al. 2000), and fusion protein of EC2 of CD9 inhibits the fertilisation in the mouse (Higginbottom et al. 2003; Zhu et al. 2002). In AmTetraspanins, therefore, a similar method can be used to examine the role of these genes in fertilisation by using the fusion proteins including the Extracellular Matrix 2 (EC2) region in a fertilisation inhibition experiment.

Thus, to examine whether AmIntegrin alpha1 and AmTetraspanin1 and 4 are involved in the fertilisation of *A. millepora*, fertilisation inhibition experiments were performed with these antibodies or fusion proteins. I treated only eggs with fusion proteins and

antibodies because the target genes above should be expressed in eggs based on ADAM-integrin interaction hypothesis. In addition, cross-fertilisation experiments were conducted using sperm exposed to eggs for different time periods to find the optimal fertilisation period. This chapter describes the preparation of fusion proteins and antibodies for genes potentially involved in the fertilisation of *A. millepora*, and the results of fertilisation inhibition experiments in coral spawning events.

4.2. Materials and Methods

Creation of constructs containing DxD/NxD/NxxxD motifs of AmIntegrin alpha1

A DNA fragment containing 144 amino acids from the head region beta-propeller repeats 5, 6, and 7, and including the three DxD/NxD/NxxxD motifs of AmIntegrin alpha1 (AmItga1), was generated by PCR from the AmIntegrin alpha1 cDNA plasmid with a 5' primer engineered with a *Bam*HI restriction site (5'-GGATCCAGTGTTGTTTGTGCTGTG-3') and a 3' primer with a *Xho*I restriction site (5'-CTCGAGCATACGCGCCGACAACAAT-3'). This fragment was inserted into *Bam*HI and *Xho*I restriction sites of the pGEX-4T-2 vector (Amersham). Restriction enzyme digestion, ligation, and transformation were performed according to standard protocols (Sambrook et al. 1992).

Creation of constructs containing the EC2 domain of AmTetraspanin1 and 4

A DNA fragment of 108 amino acids encoding the EC2 domain of AmTetraspanin1 was generated by PCR from cDNA plasmids with a 5' primer engineered with a *Bam*HI

restriction site (5'-GGATCCTATATCAATCGTAATAAG-3') and a 3' primer engineered with a *Xho*I restriction site (5'-CTCGAGCACCAATGTGGTACAATTT-3'). A DNA fragment containing 99 amino acids encoding the EC2 domain of AmTetraspanin4 (AmTet4-EC2) was generated via PCR from the cDNA plasmids with a 5' primer engineered with a *Bam*HI restriction site (5'-GGATCCTATGCATATTCCAGGCGA-3') and a 3' primer engineered with a *Xho*I restriction site (5'-CTCGAGCGTGTTTCTTGAGGAAAGA-3'). These fragments were inserted into *Bam*HI and *Xho*I restriction sites of the pGEX-4T-2 vector (Amersham). Other procedures used are as described above.

Expression of GST fusion proteins

AmIntegrin alpha1 fusion protein (AmItga1-GST) was expressed in *Escherichia coli* XL1 Blue cells. The cultures were grown at 37°C to an OD_{600} of 0.4, before induction by adding isopropyl-β-d-thiogalactopyranoside (IPTG) to a final concentration of 1 mM, and then incubating for a further 4 h. The cells were then lysed by sonication and separated into soluble and insoluble fractions by centrifugation. AmItga1-GST protein was recovered in membrane (rather than soluble) fractions and could not be purified using affinity chromatography; therefore, the AmItga1-GST protein was purified by washing the insoluble fraction with 50 mM Tris-HCl (pH 9.0) and 2% Triton X-100. GST fusion proteins of AmTetraspanin1 (AmTet1-GST) and AmTetraspanin4 (AmTet4-GST) were induced in *E. coli* XL1 Blue cells with IPTG to a final concentration of 0.1 mM. Soluble fractions including fusion proteins were purified using an affinity

chromatography procedure on glutathione beads, according to the manufacturer's instructions (Amersham). The concentration of protein was measured using the Bradford assay and optical density. Protein purity was assessed using Coomassie staining of SDS-PAGE gels.

Antibody production

For antibody production, rabbits were immunised using four aliquots of the purified AmItga1-GST protein (300 to 400 ug/dose) in Freund's complete (first injection) or incomplete (all other injections) adjuvant. Injections were given at intervals of 2 weeks. One week after the final injection, an aliquot of serum was evaluated for antibody titre by Western analysis, and the rabbits were bled out within 24 h. The blood was allowed to clot at room temperature for 3 h and stored overnight at 4°C to allow the clot to contract, after which the clot was manually removed and the serum was collected. Antibody production was performed by IMVS Veterinary Services (South Australia).

Fertilisation inhibition experiments using antiserum against the AmItga1-GST fusion protein

Gravid coral colonies were collected from fringing reefs in Nelly Bay, Magnetic Island, off Townsville (Queensland, Australia; December 2006). Gametes were collected and prepared following Willis et al. (1997). The reciprocal of each sperm–egg combination was considered to be a separate cross. Three crosses using three colonies of *A. millepora* were performed. All crosses were performed in 1-ml volumes in 3-ml vials and

replicated three times. Between 10 and 100 eggs were incubated at room temperature for at least 15 min with each treatment (seawater, antiserum, antibody-free serum) before the sperm were added. An optimal concentration of 10^6 sperm/ml in usual condition (Omori et al. 2001; Willis et al. 1997) was used for each cross. Eggs were fixed with formalin-beta-glycerophosphate 6 h after addition of sperm, and the number of unfertilised eggs and developing embryos was counted under a dissecting microscope.

Fertilisation inhibition experiments with AmTetraspanin1-GST fusion protein

Gravid coral colonies were collected from the NE Reef on Orpheus Island of the Great Barrier Reef (December 2005). Two crosses using three colonies of *A. millepora* were performed. Other procedures followed those described above.

Fertilisation experiments to estimate optimal fertilisation period

Gravid coral colonies were collected from a fringing reef near Oku fishery port on Okinawa Island, Japan. Five crosses using six colonies of *A. digitifera* were performed at Sesoko Island, Okinawa, Japan (June 2006 and June 2007). Gametes were collected and prepared following Willis et al. (1997). The reciprocal of each sperm–egg combination was considered to be a separate cross. All crosses were performed in 1-ml volumes in 3-ml vials and replicated three times. Between 24 and 250 eggs were used for this experiment. An optimal concentration of 10^6 sperm/ml (Willis et al. 1997) was used for each cross. To remove sperm completely, gametes were washed with filtered

seawater for 10 min, 30 min, 1 h, 3 h, and 6 h after adding sperm. Detachment of sperm from the eggs was confirmed by microscopic observation. Eggs were fixed with 3–4% formalin 6 h after addition of sperm, and the number of unfertilised eggs and developing embryos was counted under a dissecting microscope.

Fertilisation inhibition experiments using fusion proteins (AmTetraspanin1 and 4-GST) with *Acropora digitifera*

Gravid coral colonies were collected from a fringing reef near Oku fishery port on Okinawa Island, Japan. One cross using two colonies of *A. digitifera* was performed at Sesoko Island, Okinawa, Japan (June 2006). The reciprocal of each sperm–egg combination was considered to be a separate cross. Fertilisation conditions were the same as those described above. One hour after adding sperm, all eggs were washed with filtered seawater to remove sperm, based on results of the fertilisation experiment described above (fertilisation is almost completed within 30 min in these experimental conditions). The other procedures followed those described above.

4.3. Results

Protein expression and antibody production

Fusion proteins of AmIntegrin alpha1 (AmItga1-GST), AmTetraspanin1 (AmTet1-GST), and AmTetraspanin4 (AmTet4-GST) were successfully expressed (Figure 4.1). Production of antibodies against AmItga1-GST protein also succeeded. The sensitivity and specificity of the antibodies against AmItga1-GST protein were confirmed by

probing Western blots of whole *E. coli* lysates with or without the recombinant antigen, and signals were detected as a single band.

Fertilisation inhibition experiments using antiserum against AmItga1-GST protein

The antiserum against AmItga1-GST protein did not affect the fertilisation rates of *A. millepora* in treatments at higher antiserum concentrations (0.5 dilution; 95.7 ± 0.99%) compared to seawater (96.8 ± 1.36%; Mann–Whitney, $p>0.05$) and pre-immune serum controls (95.8 ± 0.46%; Mann–Whitney, $p>0.05$; Figure 4.2).

Fertilisation inhibition experiments using AmTetraspanin1-GST fusion protein

AmTet1-GST protein did not affect fertilisation rates of *A. millepora* in the treatment with the higher fusion protein concentration (200 ug/ml; 96.8 ± 5.00%) compared to the GST fusion protein control (200 ug/ml; 95.1 ± 4.59%; Mann–Whitney, $p>0.05$; Figure 4.3). Moderate fertilisation rates were detected in some self crosses (Figure 4.3), which may be a consequence of the artificial conditions or of self-fertilisation.

Fertilisation experiments to estimate the optimal fertilisation period

Very low fertilisation rates (<2%) were detected in some self crosses, probably as a consequence of the artificial conditions (Figure 4.4). In the treatment removing sperm within 10 min, a clear decrease in fertilisation rate (68.1 ± 8.21%) was found compared to leaving the sperm for 6 h (90.8 ± 4.01%; Mann–Whitney, $p=0.0011$; Figure 4.4). Except for 3 h and fixing after 6 h, significant differences in fertilisation rates were

detected between other treatments (30 min vs 6 h: Mann–Whitney, p=0.018; 1 h vs 6 h: Mann–Whitney, p=0.048; Figure 4.4). A significant difference in fertilisation rates was not detected between the 3-h and 6-h treatments (Mann–Whitney, p=0.41; Figure 4.4).

Fertilisation inhibition experiments using fusion proteins (AmTet1 and 4-GST) with *Acropora digitifera*

In this experiment, one step of washing eggs 1 h after adding sperm was added, because it was previously confirmed that fertilisation was almost completed within 30 min in the experimental conditions used (Figure 4.4). Between treatments with fusion proteins (AmTet1 and 4-GST) and the control without fusion proteins, differences in fertilisation rates could not be detected (Figure 4.5).

4.4. Discussion

Involvement of integrins in coral fertilisation

Whereas anti-integrin betacn1 clearly affected sperm–egg interactions (Iguchi et al. 2007; Márquez 2002), the antibody against AmIntegrin alpha1 did not appear to have any significant effects on either sperm binding or fertilisation rates under the same conditions. Results from sperm-binding experiments in the presence of antibody were indistinguishable from preimmune serum controls (Figure 4.2). Sperm motility was normal under all antiserum treatments (data not shown).

Although the possibility that the antibody against AmIntegrin alpha1 recognises

epitopes that are inaccessible prior to gamete interactions (thereby unable to block the binding site) cannot yet be ruled out, these experiments suggest that, despite the fact that both integrins are maternally expressed, AmIntegrin alpha1 may not be involved in coral fertilisation. Integrin beta1-type molecules have been shown to dimerize with a number of different alpha subunits (Hynes 2002), so it appears likely that additional alpha integrins are probably present in *Acropora*. Consistent with this finding, a preliminary investigation of the integrin complement of a related anthozoan, the sea anemone *Nematostella vectensis* (for which a whole genome sequence is now available), suggests the presence of at least three distinct alpha integrin subunits (Knack et al. unpubl. data).

The alpha subunit of the integrin heterodimer directly implicated in mammalian fertilisation is alpha6 (Almeida et al. 1995), which confers specificity for laminin as a ligand (Hynes 2002). Although AmIntegrin alpha1 is most similar in sequence to the mammalian alpha9 type (see Chapter 2), its ligand binding properties remain unresolved (Knack et al. unpubl. data). Among the suggested coral integrin complements are members that bind individual laminin and RGD peptides preferentially. Priority should be placed on characterising the ligand binding properties of the full complement of *Acropora* integrin alpha subunits and assessing their potential roles in gamete interactions.

The role of tetraspanins in coral fertilisation

AmTetraspanin1 and 4 were expressed in unfertilised eggs (see Chapter 3), but it was not possible to prove whether these genes are involved in the fertilisation of *Acropora*. A fertilisation inhibition experiment with AmTet1-GST fusion protein was performed on Orpheus Island, but the fusion protein did not appear to inhibit the fertilisation of *A. millepora* (Figure 4.3). A further fertilisation inhibition experiment was done with AmTet1 and 4-GST fusion proteins using *A. digitifera* in Okinawa and removing sperm within 1 h to avoid excessive exposure of eggs to sperm (see below). However, these proteins could not inhibit the fertilisation of *A. digitifera* (Figure 4.5), although statistical analysis could not be performed due to the low number of replicates. In conclusion, it is unclear whether AmTetraspanin 1 and 4 are involved in the fertilisation of *Acropora*.

Possible overestimation of fertilisation rates in coral cross-fertilisation experiments

The results suggest that fertilisation is almost completed within 30 min under the experimental conditions used. In previous cross-fertilisation experiments, some cases of interspecific hybridisation have been observed (e.g. *A. pulchla–A. millepora*: mean 45%; *A. nasuta–A. formosa*: 45.1 to 76.5%; Hatta et al. 1999; Willis et al. 1997). However, these experiments allowed sperm to remain for 4 to 8 h. Consequently, fertilisation rates among *Acropora* species may have been overestimated. In the present study, fertilisation inhibition experiments using polyclonal antiserum raised against recombinant AmIntegrin alpha1 showed that the antiserum did not affect coral fertilisation (Figure 4.2), while antiserum against integrin betacn1 dramatically affected coral fertilisation.

However, the possibility of AmIntegrin alpha1 involvement in coral fertilisation cannot be completely ruled out, because the fertilisation rates were examined 6 h after addition of sperm, without excluding them at earlier time points. In these fertilisation experiments, a decrease in sperm binding of even 99.99% should allow fertilisation to occur; therefore, considering the present results, future experiments should exclude sperm completely at least 1 h after their addition in order to evaluate accurate fertilisation rates in corals.

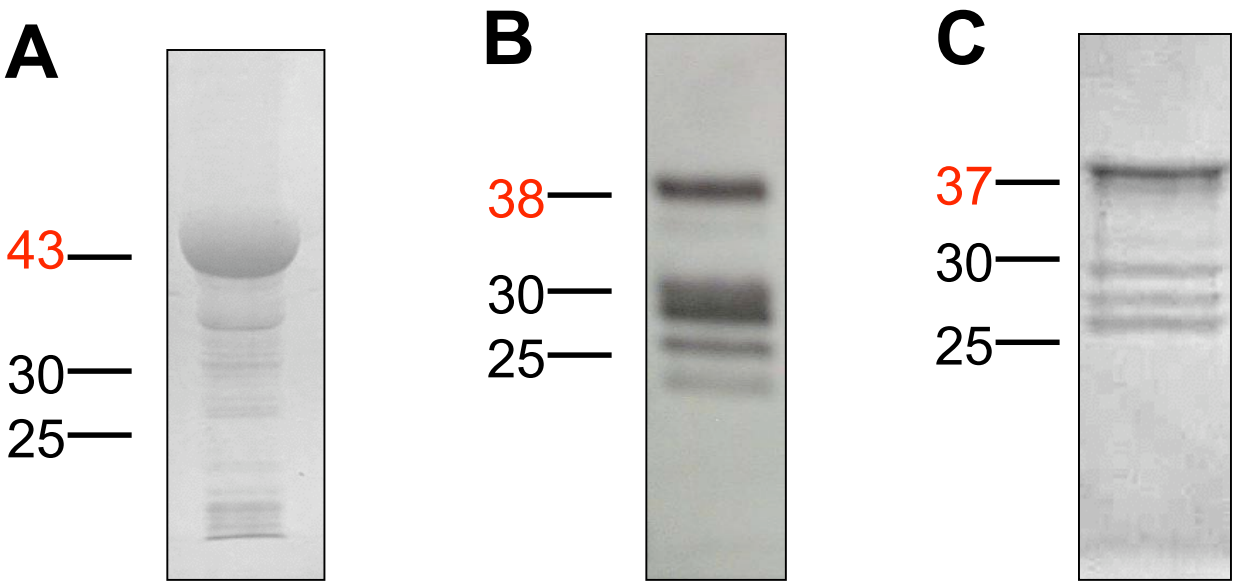

Figure 4.1. Expression of GST fusion proteins of AmIntegrin alpha1 (AmItga1-GST), AmTetraspanin1 (AmTet1-GST), and AmTetraspanin4 (AmTet4-GST). (A): AmItga1-GST. (B): AmTet1-GST. (C): AmTet4-GST. The red numbers show the expected protein size of each fusion protein.

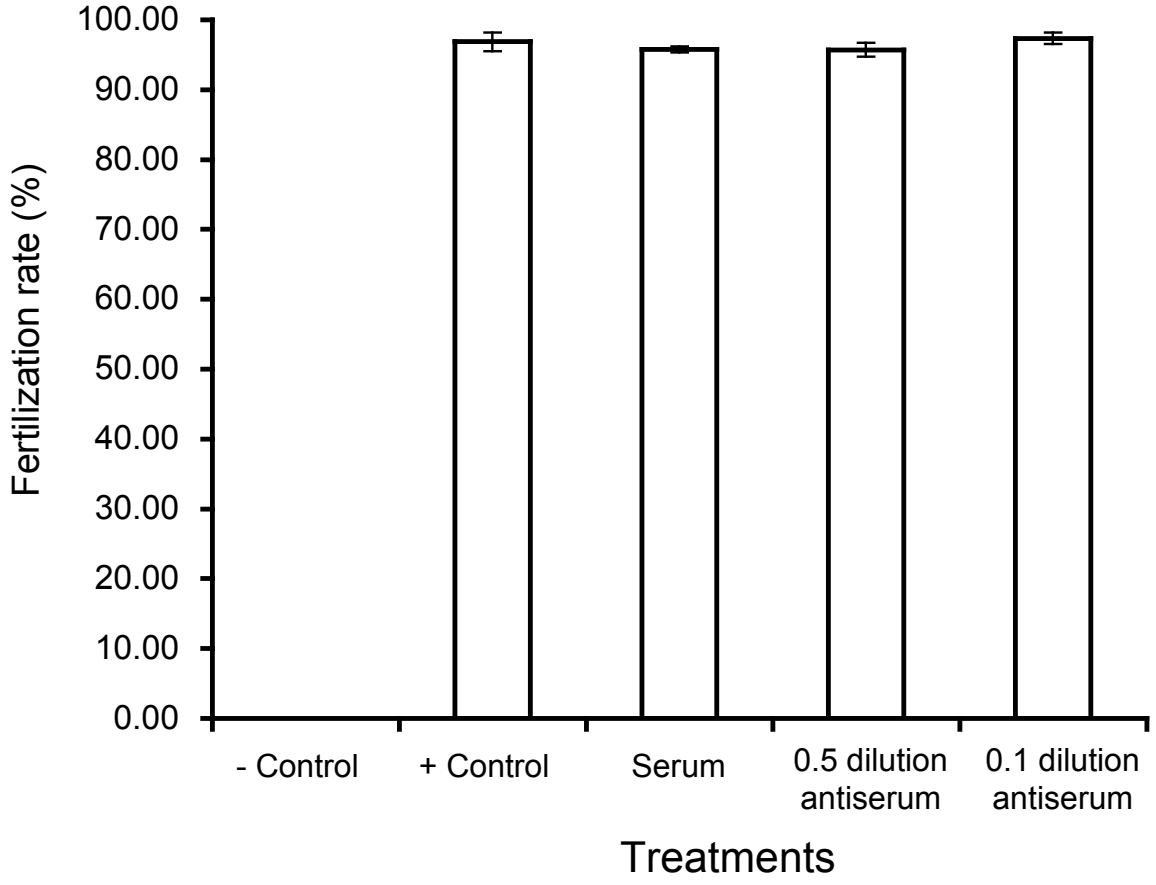

Figure 4.2. Fertilisation rates (%) of *Acropora millepora* eggs treated with different doses of antiserum against AmItga1-GST protein. Bars show average fertilisation rates and standard errors for three crosses (n=9). Each treatment was repeated three times per cross. Sperm were added at a concentration of 10^6 sperm/ml. - Control: only eggs and no sperm in 1x artificial seawater; + Control: eggs and sperm in 1x artificial seawater; Serum: eggs and sperm in a 0.5 dilution of rabbit serum in 1x artificial seawater; 0.5 dilution antiserum: eggs and sperm in a 0.5 dilution of rabbit serum containing antibodies against *A. millepora* AmItga1-GST protein in 1x artificial seawater; 0.1 dilution antiserum: eggs and sperm in a 0.1 dilution of rabbit serum containing antibodies against *A. millepora* AmItga1-GST protein in 1x artificial seawater.

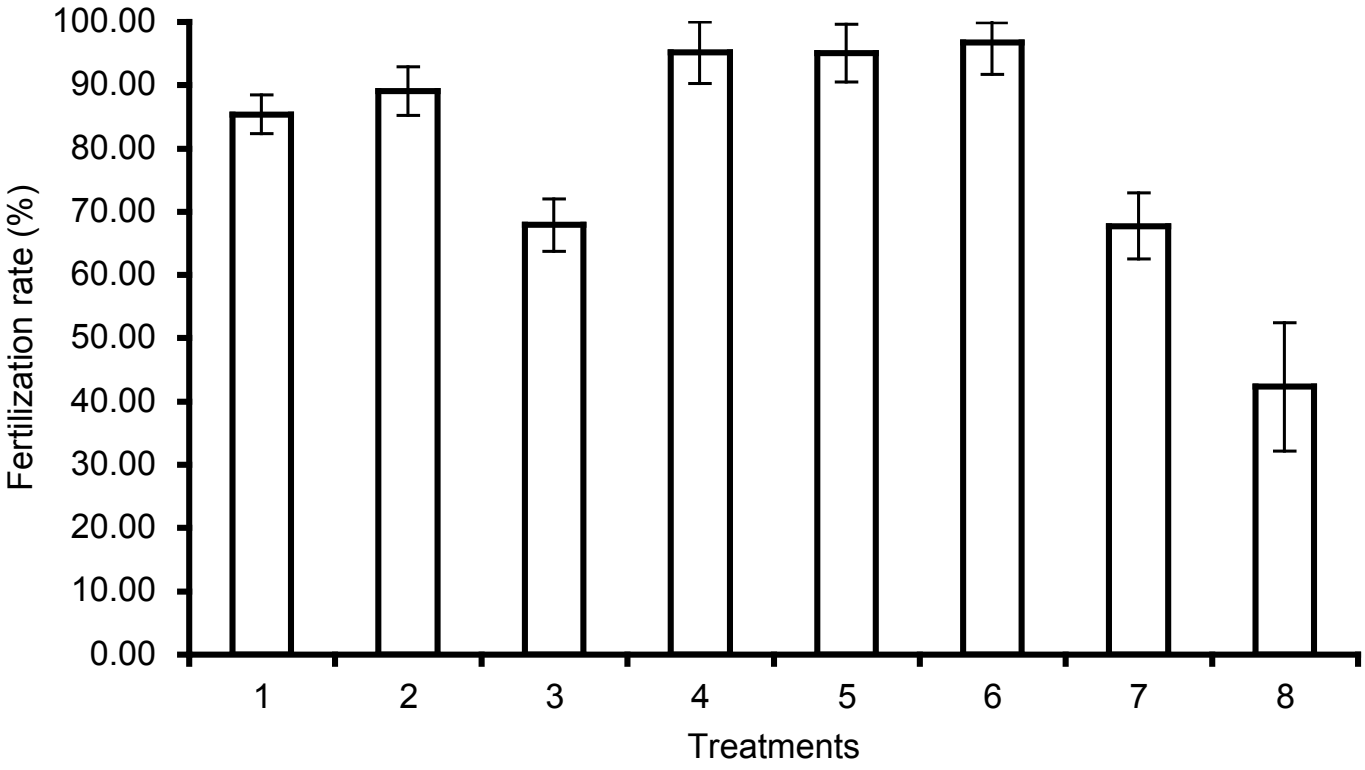

Figure 4.3. Fertilisation rates (%) of *Acropora millepora* eggs treated with AmTet1-GST fusion protein. Bars show average fertilisation rates and standard errors for two crosses (n=6). Each treatment was repeated three times per cross. Sperm were added at a concentration of 10^6 sperm/ml. 1: eggs and sperm in 50 ug/ml GST fusion protein; 2: eggs and sperm in 50 ug/ml AmTet1-GST fusion protein; 3: eggs and sperm in 100 ug/ml GST fusion protein; 4: eggs and sperm in 100 ug/ml AmTet1-GST fusion protein; 5: eggs and sperm in 200 ug/ml GST fusion protein; 6: eggs and sperm in 200 ug/ml AmTet1-GST fusion protein; 7: eggs and sperm in 1x artificial seawater; 8: only eggs and no sperm in 1x artificial seawater.

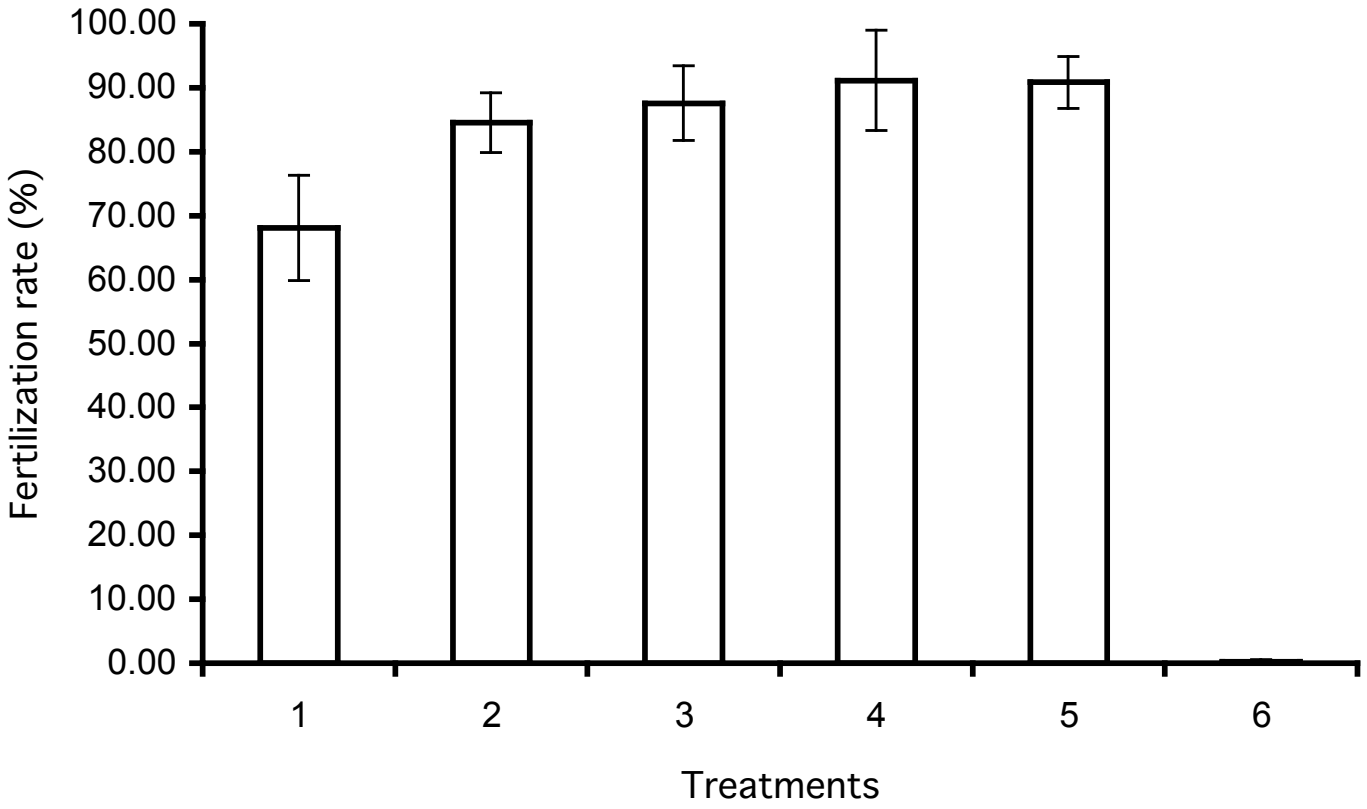

Figure 4.4. Fertilisation rates (%) of *Acropora digitifera* eggs washed each time after addition of sperm. Bars show average fertilisation rates and standard errors for five crosses (n=15). Each treatment was repeated three times per cross. Sperm were added at a concentration of 10^6 sperm/ml. 1: eggs and sperm washed for 10 min after addition of sperm; 2: eggs and sperm washed for 30 min after addition of sperm; 3: eggs and sperm washed for 1 h after addition of sperm; 4: eggs and sperm washed for 3 h after addition of sperm; 5: eggs and sperm fixed for 6 h after addition of sperm; 6: eggs without addition of sperm.

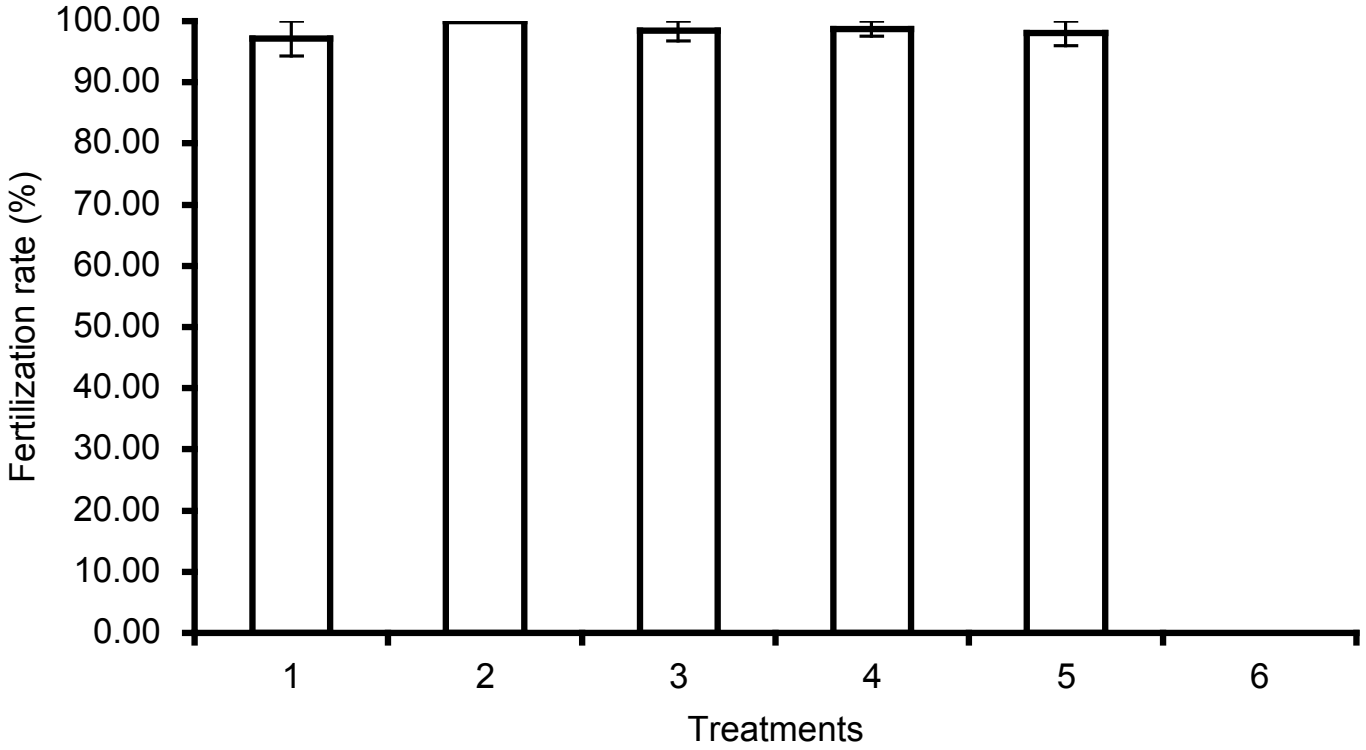

Figure 4.5. Fertilisation rates (%) of *Acropora digitifera* eggs treated with AmTet1-GST and AmTet4-GST fusion proteins. Bars show average fertilisation rates and standard errors for one cross (n=2). Each treatment was repeated twice per cross. Sperm were added at a concentration of 10^6 sperm/ml. 1: eggs and sperm in 50 ug/ml AmTet1-GST fusion protein; 2: eggs and sperm in 100 ug/ml AmTet1-GST fusion protein; 3: eggs and sperm in 50 ug/ml AmTet4-GST fusion protein; 4: eggs and sperm in 100 ug/ml AmTet4-GST fusion protein; 5: eggs and sperm in filtered seawater; 6: only eggs and no sperm in filtered seawater.

Chapter 5. Identification of fast-evolving genes in the scleractinian coral *Acropora* using comparative EST analysis

5.1. Introduction

Chapters 2 to 4 only focused on ADAM–integrin interaction-related genes to search for genes with potential roles in the fertilisation of *A. millepora*. However, other EST candidates may play key roles in animal gamete interactions. A recent EST project on *A. millepora* (Kortschak et al. 2003) provided an opportunity to search for many new candidates for roles not only in fertilisation but also in other developmental events (e.g. Hayward et al. 2004). However, even if fertilisation-related candidate genes other than ADAM–integrin interaction-related ones can be found, and it is difficult to examine all possible candidates through the hierarchical strategies described in Chapter 2-4.

As described in Chapter I, it is possible to pursue candidate genes for fertilisation by focusing on whether they are fast-evolving genes (genes subjected to positive selection). In corals, fluorescent proteins are under positive selection due to a coevolutionary race at the molecular level through symbiosis (Field et al. 2006). However, information for fast-evolving genes in corals is restricted to this one report. Recent EST projects on two *Acropora* species (*A. millepora*, *A. palmata*; Kortschak et al. 2003; Schwarz et al. 2005) have provided an opportunity to perform comparative EST analysis to undertake extensive searches for fast-evolving genes in this genus (Forêt et al. 2007). Here, the first extensive search for fast-evolving genes in the coral *Acropora* is presented, using

direct comparative sequence analysis based on two large-scale *Acropora* EST datasets.

5.2. Materials and Methods

Selection of EST sequences and BLAST analysis

In total, 849 non-redundant, independent genes, annotated as unknown, unnamed, hypothetical proteins, membrane, and immunity-related candidates (lectin; Fujita et al. 2004) from the *A. palmata* EST database (4,017 ESTs) were selected. These sequences were compared using the TBLASTX algorithm (Altschul et al. 1990) with the *A. millepora* EST database (10,232 ESTs) through a comparative genomics platform for basal metazoa (http://compagen.zoologie.uni-kiel.de/blast/datasets.html). Genes with an E value $<10^{-10}$ were selected as homologous. The similarities of amino acid sequences were obtained from the results through TBLASTX.

Selection analysis

Correct frame positions were judged based on amino acid sequences that were translated correctly in each of the two species. Where this was successful, a positive selection for possible dN and dS values with the nucleotide sequences of the ORF from both species was examined. To assess whether the dN/dS ratio was significantly different from 1, the Nei–Gojobori Jukes–Cantor method (Nei and Gojobori 1986) was implemented, which uses a Z-test to determine whether dN/dS is significantly different from zero. The dN/dS ratio was evaluated through MEGA ver. 3.1 (Kumar et al. 2004).

5.3. Results and Discussion

A large number of independent genes annotated as unknown, unnamed, and hypothetical proteins were selected, because these genes can be found often enough to obtain a large number of candidates (818 genes: 20%) from the *A. palmata* EST database (4,017 ESTs). In addition, 29 independent genes annotated as membrane candidates and two lectins annotated as immunity-related candidates were selected from the *A. palmata* EST database, because several genes located on membranes, as well as immunity-related genes, evolve at accelerated rates (Jordan et al. 2001; Hughes and Nei 1988; Tanaka and Nei 1989). From the 849 independent genes of *A. palmata*, 513 homologous genes were found in the *A. millepora* EST database. Amino acid similarities of 450 gene pairs for which correct translation positions are available were examined. The similarities of amino acids of these homologous pairs ranged from 27.6% to 100% (average 83.6%; Figure 5.1). Most of the homologous pairs demonstrated high amino acid similarities (337: over 80%), but several homologous pairs showed relatively low amino acid similarities (Figure 5.1). These lower similarities may be attributed to 'dirty (inaccurate)' nucleotide sequences, because the nucleotide sequence of a specific EST from each of the two species was not translated properly. On the other hand, several homologous pairs are available for correct translation from both species but also have very low amino acid similarities. These latter homologous pairs may not be orthologous with each other. To examine this possibility, BLAST analysis among selected ESTs of *A. palmata*, the EST dataset of *Hydra magnipapillata* (163,221 ESTs) and that of *Nematostella vectensis* (146,976 ESTs) was performed. In this analysis, 53 *A. palmata*

ESTs were used that had low amino acid similarities against *A. millepora* ESTs (under 80%) and for which the correct translation from both *Acropora* species ESTs was available. As a result, many putative homologues of *H. magnipapillata* and *N. vectensis* demonstrated higher amino acid similarities than those of *A. millepora* (Table 5.1). This suggests that some putative homologues of *A. palmata* could not be found in *A. millepora* ESTs due to the comparatively low number of ESTs of *A. millepora*. Therefore, some comparisons among sequences from multiple gene families would have been reflected in the lower amino acid similarities found in our comparative sequence analysis between the two *Acropora* EST datasets.

To identify potential candidate genes under positive selection, dN and dS were calculated based on 164 homologous pairs that are available for correct alignments and have higher amino acid similarities (over 80%). The distribution of dN and dS from the 164 homologous pairs, except for one, is shown in Figure 5.2. One of the 164 homologous pairs had a very high dS (>1) and was not included in Figure 5.2. Almost all homologous pairs (158) had a dN/dS ratio <1. Among these, 93 homologous pairs had dN/dS ratios significantly <1, which suggests that these genes are under purifying selection associated with functional constraints. Six independent homologous pairs had dN/dS ratios >1 and two showed a significant deviation from one (Table 5.2). One of the homologues significantly under positive selection is lectin. Lectin is associated with innate immune responses (Fujita et al. 2004). In corals, some studies on the relationship between lectin and symbiosis have already been reported (Jimbo et al. 2000; Wood-

Charlson et al. 2006). BLAST analysis demonstrated that the *Acropora* lectin under positive selection shown in this study is highly similar to hemolytic lectin CEL-III of the sea cucumber, *Cucumaria echinata* (E value=4e-98). Sea cucumber lectin is a Ca^{2+}-dependent and galactose-specific lectin (Hatakeyama et al. 1994). Wood-Charlson et al. (2006) suggested that the α-galactose residue is one of the carbohydrates constituting potential recognition ligands for lectin/glycan interactions in symbiosis of coral larvae. Therefore, the *Acropora* lectin reported here may also be subject to positive selection through symbiosis.

The dN/dS ratios of most homologous pairs was <1, but it cannot be ruled out that these homologues are non-fast-evolving gene candidates, because Caribbean and Indo-Pacific *Acropora* species are clearly distinct and deeply branched (van Oppen et al. 2001), hence the trace for positive selection might be silenced by neutral nucleotide changes. More sensitive approaches with multiple species sequences would be useful to judge whether the genes showing higher dN/dS ratios are under positive selection or relaxation through negative selection (Yang et al. 2000). The next step is to clarify functional aspects (e.g. fertilisation) of the candidate genes under positive selection that have been identified in this study.

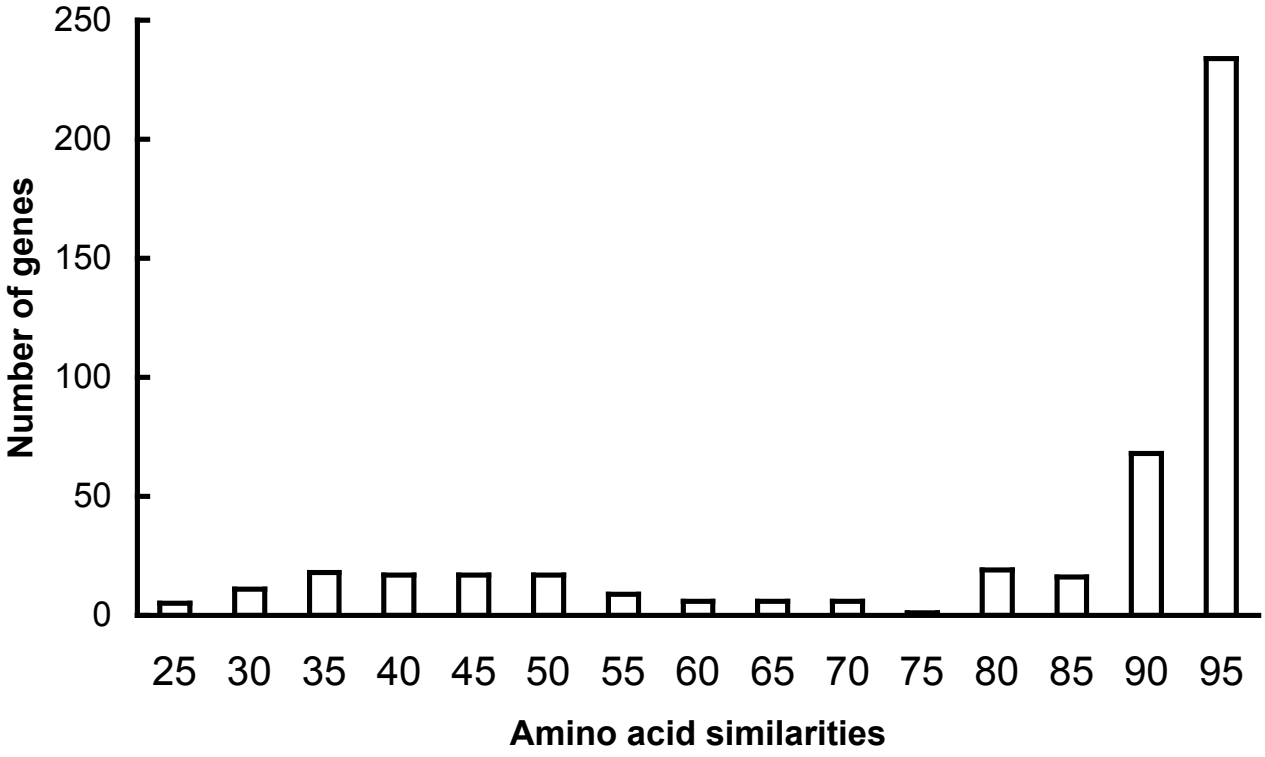

Figure 5.1. Distribution of the number of amino acid similarities in 450 *Acropora millepora_A. palmata* homologous pairs.

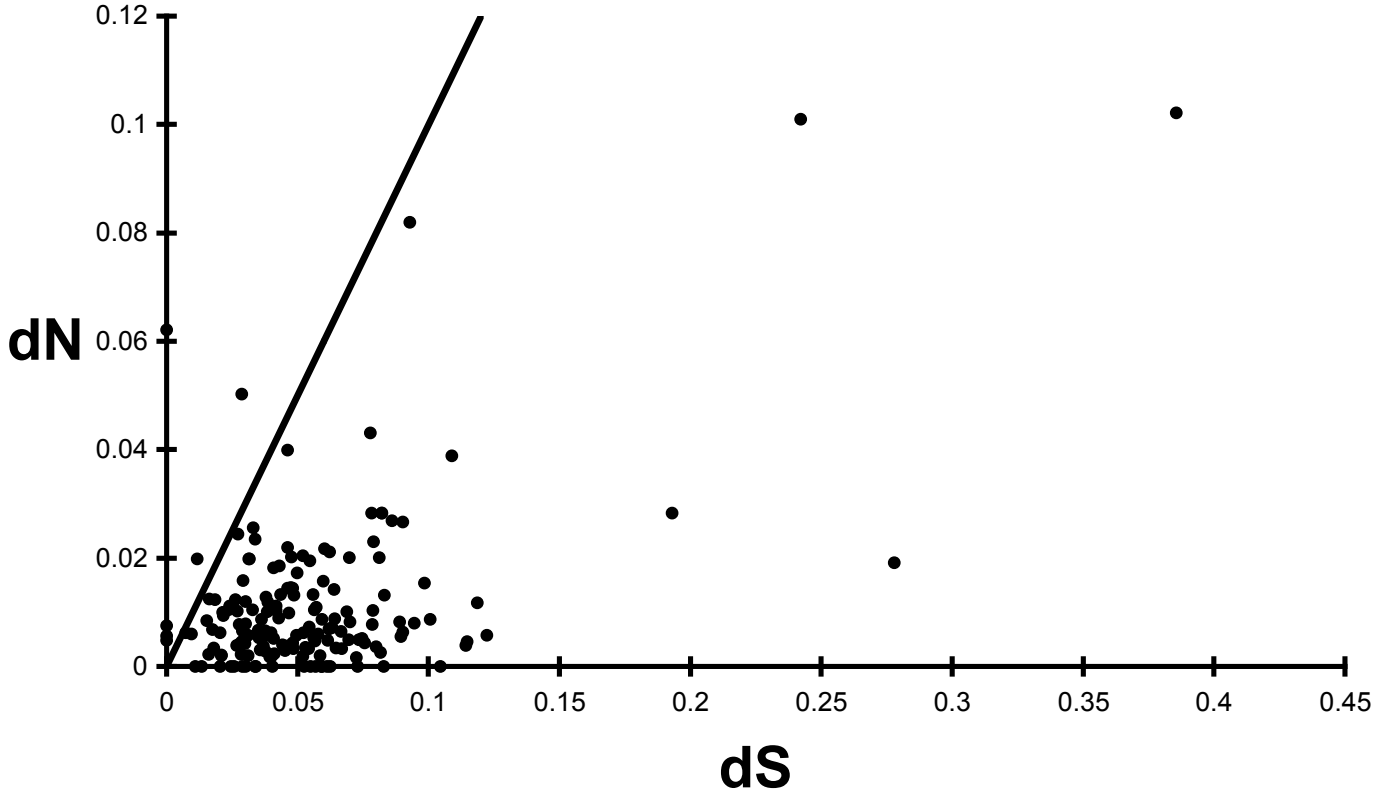

Figure 5.2. Number of nonsynonymous substitutions per nonsynonymous site (dN) plotted against the number of synonymous substitutions per synonymous site (dS) in 163 *Acropora millepora_A. palmata* putative homologous pairs. The line shows the neutral expectation of dN=dS.

Table 5.1. Amino acid similarity and E value of ESTs between *Acropora palmata* and *A. millepora* , *Hydra magnipapillata* , and *Nematostella vectensis* .

Cluster ID of *A. palmata* EST	*A. millepora*		*H. magnipapillata*		*N. vectensis*	
	Amino acid similarity (%)	E value	Amino acid similarity (%)	E value	Amino acid similarity (%)	E value
3740150	51	2.00E-55	**70**	8.00E-90	**84**	1.00E-99
3740342	38	2.00E-12	38	6.00E-27	38	2.00E-31
3740344	56	2.00E-17	N/A	N/A	45	4.00E-26
3740408	48	4.00E-27	**54**	2.00E-35	**65**	2.00E-41
3740423	30	1.00E-14	N/A	N/A	**32**	1.00E-14
3740434	37	1.00E-12	33	9.00E-10	**68**	6.00E-72
3740470	38	3.00E-13	**51**	3.00E-43	**71**	4.00E-66
3740486	41	1.00E-13	**53**	2.00E-35	**44**	1.00E-28
3740533	34	2.00E-17	**57**	2.00E-54	**59**	1.00E-130
3740662	68	3.00E-58	65	5.00E-32	**78**	1.00E-59
3741008	56	3.00E-46	43	4.00E-35	54	1.00E-33
3741055	43	5.00E-27	39	9.00E-21	**68**	2.00E-58
3741122	41	1.00E-37	N/A	N/A	**46**	6.00E-58
3741146	46	1.00E-45	**48**	5.00E-44	**91**	1.00E-112
3741173	33	5.00E-15	**45**	6.00E-39	**56**	2.00E-85
3741362	51	1.00E-23	**52**	2.00E-22	**71**	1.00E-47
3741397	34	2.00E-19	**60**	8.00E-59	**68**	2.00E-67
3741523	46	2.00E-28	**63**	2.00E-30	**83**	1.00E-105
3741610	51	4.00E-33	**55**	4.00E-39	**55**	2.00E-65
3741692	40	2.00E-20	N/A	N/A	**45**	2.00E-27
3741747	58	6.00E-55	41	1.00E-53	**85**	7.00E-86
3741763	70	1.00E-135	**82**	1.00E-134	**92**	0
3741776	48	3.00E-12	**53**	6.00E-23	**65**	4.00E-58
3741780	35	1.00E-12	**72**	5.00E-59	**75**	2.00E-79
3741867	36	3.00E-14	**42**	5.00E-17	**58**	1.00E-100
3741964	40	7.00E-20	**44**	6.00E-22	**62**	3.00E-30
3742102	65	9.00E-88	N/A	N/A	61	2.00E-67
3742108	41	1.00E-13	**43**	4.00E-11	**84**	2.00E-59
3742195	52	5.00E-51	52	7.00E-44	46	1.00E-51
3742272	34	3.00E-12	**41**	1.00E-14	**47**	9.00E-13
3742380	36	3.00E-33	**44**	2.00E-34	**43**	5.00E-33
3742451	66	3.00E-29	62	8.00E-27	**68**	2.00E-31
3742498	50	1.00E-46	**57**	3.00E-53	49	4.00E-49
3742501	72	1.00E-125	N/A	N/A	67	1.00E-104
3742654	48	1.00E-12	45	2.00E-19	**84**	1.00E-132
3742955	64	1.00E-49	**91**	3.00E-69	**93**	1.00E-71
3743017	48	5.00E-10	36	8.00E-12	46	2.00E-55
3743045	61	2.00E-55	**67**	1.00E-105	**64**	2.00E-58
3743083	36	2.00E-17	**39**	2.00E-15	**65**	4.00E-67
3743134	42	3.00E-11	N/A	N/A	**82**	1.00E-124
3743198	62	3.00E-48	57	6.00E-12	59	2.00E-80
3743269	40	1.00E-24	**48**	2.00E-38	**55**	7.00E-68
3743435	46	2.00E-12	**52**	4.00E-22	**54**	2.00E-72
3743666	31	1.00E-14	**63**	2.00E-63	**71**	2.00E-85
3743990	31	4.00E-21	**43**	6.00E-34	**74**	7.00E-72
3744128	56	4.00E-27	40	4.00E-18	**57**	7.00E-25
3744190	28	3.00E-12	**37**	4.00E-25	**33**	5.00E-19
3744315	44	2.00E-34	**79**	1.00E-86	44	3.00E-38
3744422	51	4.00E-26	**55**	5.00E-23	**55**	4.00E-32
3744681	30	4.00E-11	**44**	2.00E-42	**54**	5.00E-66
3744684	36	7.00E-20	**65**	6.00E-91	**89**	1.00E-121
3745390	51	6.00E-30	**52**	1.00E-33	44	5.00E-60
3745423	50	1.00E-10	**56**	5.00E-14	**77**	6.00E-23

N/A means that pulative EST could not be available due to an E value > 10^{-10}. Bold font shows higher amino acid similarity than that of *Acropora millepora* .

Table 5.2 Positive selection candidates of *Acropora* .

Cluster ID of *A. palmata* EST	BLAST annotation	Amino acid similarity (%)	dS	SE	dN	SE	dN/dS
3740301	hemolytic lectin CEL-III [Cucumaria echinata]	87	0	0	0.0621	0.0159	N/A**
3740353	Hypothetical protein FLJ13171, partial [Gallus gallus]	98	0	0	0.005	0.005	N/A
3741842	Hypothetical protein LOC494062 [Danio rerio]	93	0	0	0.007	0.004	N/A*
3742082	unknown [Solanum tuberosum]	96	0	0	0.006	0.006	N/A
3742940	unnamed protein product [Macaca fascicularis]	88	0.029	0.014	0.05	0.01	1.7241
3743383	Hypothetical protein LOC510680 [Bos taurus]	96	0.012	0.008	0.02	0.007	1.6667

Significance level: *0.05, **0.005

Chapter 6. Comparison of integrin betacn1 among *Acropora* species

6.1. Introduction

In Chapters 2 to 4, the priority was to find which candidate genes related to ADAM–integrin interactions are involved in the fertilisation of *Acropora millepora*. As a result, of the 15 target genes, it was only possible to gain direct evidence on the involvement of integrin betacn1 in *Acropora* fertilisation. In Chapter 5, searches for candidate genes with potential for fertilisation focused on fast-evolving genes. Several fast-evolving genes were found, but at this stage, there is no information about whether these genes are involved in fertilisation. On the other hand, it is clear that integrin betacn1 is involved in coral fertilisation (Iguchi et al. 2007; Márquez 2002). A comparison of integrin betacn1 among *Acropora* species would provide some insights into the role of this gene in the reproductive isolation and speciation of *Acropora*. Although integrin betacn1 includes many introns (26 introns), it is possible to select and compare a specific exon sequence because the positions of the introns are well characterised (Schmitt and Brower 2001). In particular, the sixth exon of integrin betacn1 is relatively large (132 bp) and includes the DxSxS motif which is important for ligand binding (Tozer et al. 1996). In this chapter, the results of comparisons of integrin betacn1 among *Acropora* species are provided, and its potential role in the reproductive isolation and speciation process is discussed.

6.2. Materials and Methods

DNA extraction, cloning, and sequencing procedures

DNA was extracted from branches of *Acropora digitifera*, *A. tenuis*, and *A. valida* from Magnetic Island, and from *A. palmata* from the Caribbean Sea. DNA extraction was performed as previously described (van Oppen et al. 1999). In addition, two DNA samples of *A. formosa* and *A. latistella* from the laboratory of David Miller (James Cook University) were used.

PCR was used to amplify 132 bp of the sixth exon of integrin betacn1 with ItgB1dmn-F1 (5'-GGAAATCCAGCCAAATTTACC-3') and ItgB1dmn-R1 (5'-CATTTGGCCGGCTAGACTTCG-3'). The PCR products were visualised with ethidium bromide on 1% agarose gel under ultraviolet light, excised, and purified with a QIAquick Gel Extraction Kit (Qiagen, Chatsworth, CA, USA). The DNA concentration was measured with a spectrophotometer at 260 nm, and the products were cloned into pGEM-T (Promega) following the manufacturer's instructions. Positive clones were confirmed by colony PCR using the primers described above and grown in LB medium followed by use of a Qiagen Plasmid Mini Kit (QIAGEN, Chatsworth, CA) according to the manufacturer's instructions. Sequencing was performed with vector-specific primers using MACROGEN (Korea).

Alignment and selection analysis

Multiple sequence alignments were generated using Clustal W (Thompson et al. 1994),

and then shaded (black: identity; grey: conservative substitutions) using BOXSHADE 3.21 (http://www.ch.embnet.org/software/BOX_form.html).

To assess whether the dN/dS ratio was significantly different from 1, the Nei–Gojobori Jukes–Cantor method (Nei and Gojobori 1986) was performed, which uses a Z-test to determine whether dN/dS is significantly different from zero. The dN/dS ratio was evaluated through MEGA ver. 3.1 (Kumar et al. 2004).

6.3. Results and Discussion

In total, 12 clones of the sixth exon of integrin betacn1 were available from six *Acropora* species, of which one to four clones from each species were obtained. Comparison of 13 amino acid sequences of the sixth exon of integrin betacn1, including the *A. millepora* clone, is shown in Figure 6.1. Interestingly, comparison of integrin betacn1 sequences showed some mutations around the DxSxS motif. In addition, two combinations of eight different clones (AmItgb1_1 vs ApItgb1_2, ApItgb1_2 vs AtItgb1_3) showed significant possibilities of positive selection (Table 6.1). The DxSxS motif of integrin beta is important for ligand binding, and substitution of the beta3 residue Asp119, Ser121, or Ser123 results in a loss of the ligand binding function of integrin alphaIIbeta3 (Tozer et al. 1996). Therefore, the mutation around the DxSxS motif might be under positive selection, which contributes to gamete specificities among *Acropora* species. However, several species shared an identical genotype. For example, *A. millepora* and *A. tenuis* share one identical genotype (AmItgb1_1 =

AtItgb1_1), although gamete incompatibilities have been reported between these two species (fertilisation rate: 0.3 %; Willis et al. 1997). In addition, positive selection signals were detected only between allopatric species from the Indo-Pacific region (*A. formosa*, *A. millepora*, *A. tenuis*) and the Caribbean (*A. palmata*). If *Acropora* speciation occurs by a change of fertilisation-related genes, as postulated in Chapter 1, clear fixed variations among sympatric species should be detected. However, this is not the case. In conclusion, it is unclear whether integrin betacn1, as a fertilisation-related gene, is important for the reproductive isolation and speciation process of *Acropora*. Integrin betacn1 expressions are detected in all developmental stages (Iguchi et al. unpublished data), which may lead to the high conservation of this gene among species because the mutations should influence this gene's role in all developmental stages.

```
AmItgb1_1     1  GNPAKFTLTVRPAENYPVDLYYLMDMSSSMKDDLGNLRSLAGQM
AfItgb1_1     1  GNPAKFTLTVRPAENYPVDLYYLMDMSSSMKDDLGNLRSLAGQM
AvItgb1_1     1  GNPAKFTLTVRPAENYPVDLYYLMDMSSSMKDDLGNLRSLAGQM
AtItgb1_1     1  GNPAKFTLTVRPAENYPVDLYYLMDMSSSMKDDLGNLRSLAGQM
AdItgb1_3     1  GNPAKFTLTVRPAENYPVDLYYLMDMSLSMNDDLGNLRSLAGQM
AdItgb1_4     1  GNPAKFTLTVRPAENYPVDLYYLMDMSLSMNDDLGNLRSLAGQM
AdItgb1_2     1  GNPAKFTLTVRPAENYPVDLYYLMDMSLSMNDDLGNLRSLAGQM
AdItgb1_1     1  GNPAKFTLTVRPAENYPVDLYYLMDMSLSMNDDLGNLRSLAGQM
AtItgb1_4     1  GNPAKFTLTVRPAENYPVDLYYLMDMSLSMNDDLGNLRSLAGQM
AtItgb1_3     1  GNPAKFTLTVRPAENYPVDLYYLMDMSLSMNDDLGNLRSLAGQM
AlItgb1_1     1  GNPAKFTLTVLPAEDYPVDLYYLMDMSLSMNDDLGNLRSLAGQM
ApItgb1_2     1  GNPAKFTLTVRPAENYPVDLHYLMDMSSSMNGDLGNLRSLAGQM
ApItgb1_3     1  GNPAKFTLTVRPAENYPVDLYYLMDMSSSMNGDLGNLRSLAGQM
```

Figure. 6.1. Comparison of 6th exon amino acid sequences of integrin betacn1 among *Acropora* species. Ad: *A. digitifera*; Af: *A. formosa*; Al: *A. latistella*; Am: *A. millepora*; Ap: *A. palmata*; At: *A. tenuis*; Av: *A. valida*. A number after the sample name indicates the clone number.

Table 6.1. Above diagonal:dN/dS ratio. Below diagonal:p value of the level of significance of positive selection

	AmItgb1_1	AvItgb1_1	ApItgb1_2	ApItgb1_3	AtItgb1_4	AtItgb1_3	AdItgb1_1	AlItgb1_1
AmItgb1_1		0	N/A	N/A	0.6090	N/A	0.6166	0.2916
AvItgb1_1	1.0000		0.9414	0.6292	0.2978	0.6166	0.3013	0.2274
ApItgb1_2	0.0358	1.0000		N/A	0.9181	N/A	0.9325	0.3674
ApItgb1_3	0.0784	1.0000	0.1584		0.6166	N/A	0.6254	0.2965
AtItgb1_4	1.0000	1.0000	1.0000	1.0000		0	0	0.2128
AtItgb1_3	0.0691	1.0000	0.0340	0.0740	1.0000		0	0.1428
AdItgb1_1	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000		0.1951
AlItgb1_1	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	

Note that AmItgb1_1 is identical to AfItgb1_1, AtItgb1_1, and AdItgb1_1 is to AdItgb1_2, AdItgb1_3, AdItgb1_4.

Chapter 7. General discussion

7.1. Are ADAM–integrin interactions involved in coral fertilisation?

Based on the ADAM–integrin interaction hypothesis, a search was undertaken for fertilisation-related genes by focusing on 15 ADAM, integrin, and tetraspanin genes from the EST database. In terms of gene structures, gene expression patterns, and fertilisation inhibition experiments, candidates involved in the fertilisation of *A. millepora* were pursued. From the results of fertilisation inhibition experiments (Chapter 4), there is no evidence that AmIntegrin alpha1 and AmTetraspanin1 and 4 are involved in the fertilisation of *A. millepora*. Therefore, whether ADAM–integrin interactions are related to fertilisation in *Acropora* is unclear at this stage. Only integrin betacn1 was found to be a fertilisation-related gene in *A. millepora* (Iguchi et al. 2007; Márquez 2002). It is possible that other fertilisation-related genes were not found due to the low number of ESTs of *Acropora millepora* and because the study focused on genes only from the EST database. This EST database originates from massive sequencing of a cDNA library, which provides gene candidates expressed at the mRNA level, not at the protein level. The next challenge is to directly characterise proteins making molecular complexes with integrin betacn1 in gamete interactions.

7.2. Is it possible to find fast-evolving genes as candidates for fertilisation?

As described in Chapter 5, fertilisation candidates were examined by focusing on fast-evolving genes because positive selection is pervasive in mammalian fertilisation

proteins (Swanson et al. 2003). As a result, several fast-evolving genes were found in *Acropora*. However, it was not confirmed whether these fast-evolving genes are in fact related to fertilisation. The next challenge is to judge whether these genes are involved in the fertilisation of *Acropora* by examining gene expression patterns and performing fertilisation inhibition experiments, as described in Chapters 3 and 4.

7.3. Are fertilisation-related genes involved in *Acropora* speciation?

As described in Chapter 6, it is unclear whether a fertilisation-related gene (integrin betacn1) is involved in *Acropora* speciation. Other genes associated with molecular complexes on the egg membrane (e.g., tetraspanins) may play some roles in coral gamete specificity. To confirm this possibility, proteins making molecular complexes with integrin betacn1 should be characterised, and a comparison of these amino acid sequences among *Acropora* species should be undertaken.

This study only focused on sperm–egg membrane binding and fusion processes in the fertilisation of *Acropora*, but fertilisation is divided into three main steps: sperm attraction by the egg, the penetration of the outer egg layer by sperm through the acrosome reaction, and sperm–egg membrane binding and fusion. Therefore, other steps in fertilisation should be considered to confirm whether fertilisation is related to reproductive isolation and speciation processes in *Acropora*. The absence of an egg outer layer in corals has been reported (Harrison and Wallace 1990). In relation to sperm attraction by the egg, a recent study demonstrated that sperm flagellar motility is

activated by an egg from the same species, but not activated by an egg from a different species in *Acropora*, which suggests that sperm attraction to eggs is species-specific (Morita et al. 2006). The species specificity at the level of sperm attraction and/or sperm–egg membrane binding and fusion may play important roles in the reproductive isolation and speciation process of *Acropora* although sperm motility may be helpless in sea strong currents and the attractants will be diluted soon after spawning in the natural field. At this stage, there is no information on how the change in gamete recognition is related to *Acropora* speciation processes. However, a comprehensive analysis of fertilisation would provide some insights into diversification processes in the genus *Acropora*.

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