UNIVERSITY OF SOUTHAMPTON

FACULTY OF NATURAL AND ENVIRONMENTAL SCIENCES

Ocean and Earth Sciences

Are specific coral-*Symbiodinium* partnerships associated with survival in extreme temperature environments of the Persian/Arabian Gulf?

by

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ABSTRACT

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ARE SPECIFIC CORAL-*SYMBIODINIUM* PARTNERSHIPS ASSOCIATED WITH SURVIVAL IN EXTREME TEMPERATURE ENVIRONMENTS OF THE PERSIAN/ARABIAN GULF?

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Coral populations of the Persian/Arabian Gulf (hereafter referred to as the Gulf) survive thermal regimes more extreme than any other coral-reef ecosystem in the world. The harbouring of thermally-tolerant symbiotic algae of the genus *Symbiodinium* has been proposed as a mechanism for Gulf corals to survive these extreme thermal regimes. However, coral-*Symbiodinium* associations of the Gulf region are poorly characterised.

This thesis investigates whether specific coral-*Symbiodinium* partnerships are associated with survival in the Gulf.

A precursory method optimisation study investigated an infestation of corallivorous *A. acroporae* (identified through 28S rDNA sequencing) at the Coral Reef Laboratory, Southampton (CRL). Direct uptake of coral-derived fluorescent proteins and photocompetent *Symbiodinium,* hypothesised to advance the worms' camouflage were demonstrated. In addition, its global distribution in the aquaristic industry was resolved, highlighting its invasive potential through anthropogenic introductions.

In four Gulf-derived *Porites lobata* colonies aquaria-cultured for more than 14 months at the CRL, maintenance of thermal tolerance *ex situ*, regulation of host fluorescence by heat and growth rates approximate to those in the field were demonstrated. In addition, harboured *Symbiodinium* were phylotyped using novel PCR primers designed to amplify the ITS region of *Symbiodinium* ribosomal DNA with consideration given to low host-complementarity at annealing sites. *Symbiodinium* ITS2 subclade C3 were predominant in all four Gulf colonies, a symbiont previously associated with thermal sensitivity. This association was further explored in seven species of corals off the coast of Abu Dhabi where C3 predominance was also demonstrated.

P. lobata, P. lutea and *P. harrisoni* coral-*Symbiodinium* associations from the western UAE coast, to Muscat in the Gulf of Oman were characterised using a novel denaturing gradient gel electrophoresis protocol. The occurrence of only C3 associations in the Gulf (133 colonies; 6 sites) contrasting with mixed associations of C3, D1 and C15 in the Strait of Hormuz and Gulf of Oman (108 colonies; 7 sites) are reported. This suggests C3 partnerships are associated with survival in the Gulf and whilst they may be exported, their advantage – a product of their unique environment - over other partnerships appears to be lost, limiting their potential to restore reefs external to the Gulf through assisted migration.

The *Symbiodinium* rDNA sequences collected during this thesis are used to critically asses the use of rRNA secondary structures and the ITS regions in defining subcladal types. The results advocate taxonomic analysis using the entire ITS region and evaluate rRNA secondary structure screening as bias towards the underestimation of diversity.

Contents

ABSTRACT i

Contents i

List of tables vii

List of figures ix

Declaration of authorship xi

Acknowledgements xiii

Abbreviations xiv

Chapter 1: Introduction 1

1.1 Elucidating resilience mechanisms of Persian/Arabian Gulf corals to better predict the effects of warming seas on coral reef ecosystems 1

1.2 Coral-*Symbiodinium* associations in the Persian/Arabian Gulf 6

1.3 Thesis hypothesis 8

1.4 Thesis structure and overview 9

1.4.1 Chapter 2 - Widespread distribution of corallivorous *Acropora-eating flatworms* (*AEFW*) in aquaria: Implications for coral husbandry and natural populations 9

1.4.2 Chapter 3 - Primer design for amplification of the ITS and ITS2 region of the *Symbiodinium* rDNA operon for molecular cloning and denaturing gradient gel electrophoresis 10

1.4.3 Chapter 4 - Corals from the Persian/Arabian Gulf as models for thermotolerant reef-builders: Prevalence of clade C3 *Symbiodinium*, host fluorescence and *ex situ* temperature tolerance 11

1.4.4 Chapter 5 - Contrasting coral-symbiont associations within, and external to, the Persian/Arabian Gulf: The potential of 'internal' coral-*Symbiodinium* associations to act as 'saviours' of Indo-Pacific reefs 13

1.4.5 Chapter 6 - A critical appraisal of current methods utilised to estimate *Symbiodinium spp*. diversity and the implementation of a novel 'ITS region' definition of subcladal variants 14

Chapter 2: Widespread distribution of corallivorous *Acropora eating flatworms* (*AEFW)* in aquaria: Implications for coral husbandry and natural populations 17

2.1 Abstract 17

2.2 Introduction 18

2.3 Methods 19

2.3.1 Collection and fixation 19

2.3.2 Preparation of genomic DNA 20
2.3.3 Molecular identification of AEFW and *Symbiodinium* 20
2.3.4 Phylogenetic analysis of 18S and ITS2 sequences 20
2.3.5 Photographic documentation, fluorescence measurements and microscopy 21
2.3.6 Physiological experimentation 21
2.4 Results and Discussion 22
2.4.1 Infestation with *AEFWs* in aquaria 22
2.4.2 The camouflage strategy of *AEFWs* involves incorporation of host proteins and photosynthetically active symbionts 23
2.4.3 Molecular identification of the *AEFW* - Implications for a global distribution of a corallivorous species 26
2.4.4 Using coral fluorescence to examine infestation with *Amakusaplana acroporae* 28
2.5 Conclusion 29
Chapter 3: Primer design for amplification of the ITS and ITS2 region of the *Symbiodinium* rDNA operon for molecular cloning and denaturing gradient gel electrophoresis 31
3.1 Abstract 31
3.2 Introduction 32
3.3 Primer assessment and design for the amplification of the complete ITS1-5.8S-ITS2 region of *Symbiodinium spp.* rDNA 36
3.4 Primer assessment and design for use in DGGE 38
3.5 Contrasting melting profiles of the SYM_VAR_5.8S/CLAMP and ITSintfor2/ITSCLAMP amplicons 39
3.6 Assessment of melting profile differences between the ITSintfor2/ITSCLAMP and SYM_VAR_5.8S/CLAMP amplicons 40
3.7 Empirical assessment of the SYM_VAR_5.8S/CLAMP pair – resolution of subclades A1, C3, C15 and D1 43
3.8 Empirical assessment - resolution of subcladal variants 44
3.9 Diversity identified as part of the spatial characterisation of coral-*Symbiodinium* associations in the Persian/Arabian Gulf, Strait of Hormuz and Gulf of Oman using the novel DGGE system 45
3.10 Evaluation of multiple band 'fingerprints' 49
3.11 Notes on voltage, run time, gradient formation, comb usage and the D-Code system, Bio-Rad 50
3.12 Summary 52
Chapter 4: Corals from the Persian/Arabian Gulf as models for thermotolerant reef-builders: Prevalence of clade C3

Symbiodinium, host fluorescence and _ex situ_ temperature tolerance 53

4.1 Abstract 53
4.2 Introduction 54
4.3 Materials and methods 58
4.3.1 Coral material, collection and culture 58
4.3.2 Identification of algal symbionts 60
4.3.3 Determination of growth rates 61
4.3.4 Photographic documentation and spectroscopic analyses 61
4.3.5 Heat stress treatment 62
4.4 Results 63
4.4.1 Algal symbionts of Abu Dhabi corals 63
4.4.2 Culture of *P. lobata* from the Gulf in a closed aquarium system under controlled conditions 64
4.4.3 Identification of algal symbionts in laboratory-cultured *P. lobata* 65
4.4.4 Patterns of host fluorescence in *P. lobata* 66
4.4.5 Heat stress responses of *P. lobata* from different geographical origins 67
4.5 Discussion 70
4.5.1 Differences in algal symbiont identity 70
4.5.2 Laboratory cultures of *P. lobata* 71
4.5.3 Fluorescent proteins 72
4.5.4 Temperature challenge experiment 72
4.6 Conclusion 73

Chapter 5: Contrasting coral-_Symbiodinium_ associations within, and external to, the Persian/Arabian Gulf: The potential of 'internal' coral-_Symbiodinium_ associations to act as 'saviours' of Indo-Pacific reefs 75

5.1 Abstract 75
5.2 Introduction 76
5.3 Methods 81
5.3.1 Meta-analysis of *Porites-Symbiodinium* associations 81
5.3.2 Sampling and collection of corals 81
5.3.3 Genomic DNA extractions 82
5.3.4 PCR-DGGE analysis 83
5.3.4.1 Sample preparation for DGGE by PCR 83
5.3.4.2 Running of samples 84

5.3.4.3 DGGE band characterisation 84
5.3.4.4 Validation of novel protocol 85
5.4 Results 86
5.4.1 Meta-analysis of *Porites-Symbiodinium* associations 86
5.4.2 Spatial analyses of predominant *Symbiodinium* ITS2 type 87
5.5 Discussion 91
5.6 Conclusion 98
Chapter 6: A critical appraisal of current methods utilised to estimate *Symbiodinium spp*. diversity and the implementation of a novel 'ITS region' definition of subcladal variants 101
6.1 Abstract 101
6.2 Introduction 102
6.3 Methods 107
6.3.1 Note on terminology: 'Subclade' and 'Variant' 107
6.3.2 Relative resolving power of the ITS1, 5.8S, ITS2 and complete ITS region of the *Symbiodinium* rRNA gene 108
6.3.3 Critical assessment of secondary structure screening 109
6.3.4 Estimating diversity and defining subcladal types in the genus *Symbiodinium* 112
6.4 Results and Discussion 112
6.4.1 Relative resolving power of the ITS1, 5.8S, ITS2 and complete ITS region 112
6.4.2 Critical assessment of secondary structure screening 116
6.4.3 Estimating diversity and defining subcladal types in the genus *Symbiodinium* 119
6.5 Conclusions 122
Chapter 7: General conclusions and future work 125
7.1 General conclusions 125
7.2 Future work 129
Appendices 133
Appendix 1: Widespread distribution of corallivorous *Acropora eating flatworms* (*AEFW)* in aquaria: Implications for coral husbandry and natural populations 133
Appendix 2: Primer design for amplification of the ITS and ITS2 region of the *Symbiodinium* rDNA operon for molecular cloning and denaturing gradient gel electrophoresis, respectively 137
Appendix 3: Contrasting coral-symbiont associations within, and external to, the Persian/Arabian Gulf: The potential of 'internal' coral-*Symbiodinium* associations to act as 'saviours' of Indo-Pacific reefs 143

List of References 149

List of tables

Table 4.1 Temperature regimes of coral habitats in the Gulf and selected regions elsewhere 55

Table 4.2 *Symbiodinium* clades in corals from the Abu Dhabi coastline 63

Table 4.3 *Symbiodinium* clades in *P. lobata* before and after 15 months of laboratory culture 66

Table 5.1 Corals sampled per study site and predominant ITS2 subclade type hosted as determined by PCR-DGGE. 88

Table 6.1 Comparison of various regions of the rDNA operon 113

Table 6.2 Sequence support of mutation positions 119

Table 6.3 ITS1 and ITS2 types as defined from matches to sequence accessions in the GenBank database 121

Table 7.1 Documented incidences of AEFW 133

Table 7.2 Primers used in the AEFW study 134

Table 7.3 References and associated data used in the meta-analysis of *Porites spp.* associations 143

List of figures

Figure 2.1 Recorded cases of *AEFWs* 18

Figure 2.2 *AEFW* infest acroporids in aquaria 22

Figure 2.3 Camouflage strategy of *AEFW*..... 24

Figure 2.4 Genotypic analysis of *AEFWs* 27

Figure 2.5 Effect of *AEFW* infestation on host fluorescence. 29

Figure 3.1 Schematic of the rDNA Operon..... 33

Figure 3.2 Tm50% melt profiles 42

Figure 3.3 Annotated DGGE gel images - subcladal resolutions 43

Figure 3.4 Annotated DGGE gel image – subcladal variant resolution 45

Figure 3.5 Annotated DGGE gels – Gulf variants 46

Figure 3.6 Tm50% melt profiles – Gulf Variants 48

Figure 3.7 DGGE gels representative of the work flow involved in validating bands in multiple band profiles 50

Figure 4.1 Sea Surface Temperature (SST) profiles characteristic for the coral communities in the Gulf (data from Abul Thama, Bahrain) and Fiji (data from Bega, Fiji). 57

Figure 4.2 Growth of replicate colonies of *P. lobata* from Abu Dhabi in the laboratory. 65

Figure 4.3 Fluorsecence of GFP-like proteins in the host tissue of *P. lobata*... 67

Figure 4.4 Differential responses of *P. lobata* to experimental heat stress treatments. 69

Figure 5.1 Map of the Persian/Arabian Gulf region 79

Figure 5.2 Sampling locations. 82

Figure 5.3 Meta-analysis of *Symbiodinium* spp. associations with corals in the genus *Porites*. 87

Figure 5.4 Location of sampling sites within and outside of the Gulf 89

Figure 5.5 Annotated PCR-DGGE gels inferring subcladal ITS2 *Symbiodinium* type. 90

Figure 6.1 Parsimony networks calculated using individual and entire sections of the ITS region 115

Figure 6.2 Alignment of variant representative C3 sequences..... 117

Figure 6.3 Secondary structure of the *Symbiodinium sp*. type C3 ITS1-5.8S-ITS2 region 118

Figure 7.1 Effect of AEFWs infestation on host corals' pigments 134

Figure 7.2 Macroscopic observation of *AEFW*..... 135

Figure 7.3 Consensus identity plot 139

Figure 7.4 Partial image of a multiple sequence analysis of the complete ITS region of *Symbiodinium* rDNA 140

Figure 7.5 Code written in Visual Basic to manipulate the .OUT file produced by the Poland program 141

Declaration of authorship

I, Benjamin C C Hume

declare that the thesis entitled

Are specific coral-*Symbiodinium* partnerships associated with survival in extreme temperature environments of the Persian/Arabian Gulf?

and the work presented in the thesis are both my own, and have been generated by me as the result of my own original research. I confirm that:

- this work was done wholly or mainly while in candidature for a research degree at this University;
- where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated;
- where I have consulted the published work of others, this is always clearly attributed;
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Abbreviations

AEFW *Acropora-eating flatworm*

bp Base pair

CFP Cyan fluorescent protein

CRL Coral Research Laboratory

CTAB Cetyl trimethylammonium bromide

DGGE Denaturing gradient gel electrophoresis

DNA Deoxyribonucleic acid

dNTP Deoxyribonucleotide triphosphate

FP Fluorescent protein

GBR Great Barrier Reef

gDNA Genomic deoxyribonucleic acid

GFP Green Fluorescent Protein

ICC Iranian coastal current

ICE Iranian coastal eddies

IPCC Intergovernmental Panel on Climate Change

ITS Internal transcribed spacer

LSU Large subunit

MP Maximum parsimony

my Million years ago

NOCS National Oceanography Centre, Southampton

NYU New York University

PAM Pulse amplitude modulated

PCR Polymerase chain reaction

RFLP Restriction fragment length polymorphism

ROS Reactive oxygen species

RNA Ribonucleic acid

rDNA Ribosomal deoxyribonucleic acid

rRNA Ribosomal ribonucleic acid

SST Sea surface temperature

SSU Small subunit

UAE United Arab Emirates

Chapter 1: Introduction

1.1 Elucidating resilience mechanisms of Persian/Arabian Gulf corals to better predict the effects of warming seas on coral reef ecosystems

Coral reefs provide a range of resources that are not only important to the human populations who depend directly on them for sources of protein and trade (estimated at one billion people in Asia alone;UNEP 2006), but as ecological keystone species, supporting many species of fish and acting as seeding populations (Hughes et al. 2003). In addition, coral reefs provide a range of abiotic services such as coastal protection and economic enrichment through tourism (Moberg and Folke 1999). Minimum estimates of reef building corals range upwards from a minimum of 835 species (Veron 1995) with an estimate for reef-dependent species between one and nine million (Reaka-Kudla 1997).

Until the last few decades the predominant factors concerning the coral reef research community, with regards to the decline in reef ecosystems, were over fishing and exploitation by local populations (Hughes 1994). With the increase in the ability to store and transport goods, local markets had become global with associated demands increasing - from servicing a local tropical population to supplying the world with a full range of reef-derived services and goods (Sadovy and Vincent 2002). Coral reefs have suffered as a result of human activity for centuries if not millennia but they have been experiencing a radical acceleration in the degree of exploitation in the last fifty years (Pandolfi et al. 2003; Wilkinson 2008). Ecological shifts from coral- to macroalgae-dominated reefs are becoming common place as herbivorous fish are removed and agricultural run-off supplements nutrients to an otherwise low-nutrient system (Hughes 1994).

The effects of greenhouse gases and climate change in relation to widespread coral bleaching were considered tenuous as little as 20-30 years ago (Glynn 1993). The evidence is incontrovertible now that the relation between coral physiology and environmental factors are better understood (Brown 1997) with

past reef biodiversity crises correlating with periods of rapid environmental change (Brown 1997; Kiessling and Simpson 2011).

Whilst anthropogenic changes in climate are causing environmental modulations that are devastating coral reef ecosystems (predominantly ocean acidification and increases in sea surface temperatures [SSTs]), there are certain factors that may act in favour of coral reefs. For example, wide dispersal of larvae and broadcast spawning (McKinney 1998) provide protection from local extinction and aid the redistribution of populations. Despite these resistant characteristics, recent records of mass coral bleaching (events where the obligate symbiosis between coral host and dinoflagellate symbionts of the genus *Symbiodinium* break down leading to the expulsion of the algal symbiont, and if repopulation is not timely, death of the host) such as the global 1998 event when more than 90% of all shallow water tropical corals died (Sheppard 2003) show that corals, and in proxy the ecosystems that they support, are being forced past their limits of acclimatisation and recovery. (Acclimatisation may be defined as the process of an individual modifying its phenotype to better suit an environment, during which no genotypic changes occur. A version of acclimatisation, acclimation, is the process of acclimatisation occurring in a non-natural setting, such as in response to thermal challenges in an aquaria-based experimental setting. Acclimatisation should not be confused with adaptation, an individual's phenotypic change to better suit an environment due to modification of the genotype.)

Due to the physiological limits of most reef-supporting corals being narrow (Sale 2013), these limits are often reached with little or no warning. As such, widespread coral bleaching has been increasing in frequency over the last few decades (Baker et al. 2008) as these physiological limits are exceeded due to an increase in frequency of SSTs above historically encountered maxima. Large-scale mortalities during these events cause reductions in coral reef growth (Baird and Marshall 2002) and depression of reproductive capability (Mendes and Woodley 2002). Nineteen per cent of coral reefs have already been lost globally and a further 35% are seriously threatened (Wilkinson 2008). Globally, there are no pristine reefs left (Jackson et al. 2001; Pandolfi et al. 2003).

The raised sea-level and SSTs that are predicted as a result of climate change within the next century have occurred previously and repeatedly throughout the past two million years and in the more distant past. In contrast, the magnitude and rate of increase of CO_2 levels, have not (Pandolfi and Greenstein 2007). Coral reef communities were regularly decimated by climatic changes (increases in SST and ocean acidification; Kiessling and Simpson 2011) before the Quaternary Period (0-2588 my) and recovery intervals ranged from 4 to 100 my. Due to these climatic events, framework building organisms were scarce amongst coral reef communities that existed before the Quaternary Period (Newell 1971). Within the Quaternary Period, however, coral reef development occurred relatively unaffected by climatic changes or, recovered so quickly as to leave no record of a negative effect (Greenstein et al. 1998; Pandolfi 1996; Pandolfi and Greenstein 2007). Studies of contrasting spatial differences in reef coral communities during successive high stands of sea level demonstrated that local environmental variation associated with runoff from the land had greater influence on reef coral community composition than variation in global climate and sea level (Pandolfi 1999). The relative lack of effect of climatic events during the Quaternary Period is in contrast to the global scale decline in coral reef communities currently occurring. Whilst the coral reefs of the Quaternary Period successfully survived higher SSTs than are currently found in the majority of coral harbouring Indo-Pacific waters, with little discernible negative effect, current coral communities face potentially higher rates of change, both in SST and CO_2 levels (Pandolfi 2011). Modern reefs have also been preconditioned by human impacts and it is hypothesised that the frequency of anthropogenic disturbances may decrease their resilience to further perturbations (Hughes et al. 2003).

Current modelling efforts to predict the effects of climate change – predominantly ocean acidification and sea surface temperature increases – predict a range of outcomes. The most pessimistic of these project global-scale loses of coral reefs within the next few decades (Hoegh-Guldberg 1999; Hoegh-Guldberg et al. 2007; Donner 2009). Projections that see the maintenance of coral cover to 2100 and beyond rely on assumptions of aggressive emission reductions and varying degrees of thermal adaptation by corals (Baskett et al. 2009). Many modelling efforts predict a reduction in abundance of thermally susceptible coral species, such as those in the genus *Acropora*. In place of

these species a move towards more thermally resilient species such as the massive corals, for example corals within the Faviidae or Poritidae families, are predicted (Hughes et al. 2003). However, recent evidence may suggest that the often faster growth rate and therefore recovery rate (from perturbations) of the more thermally susceptible coral species may provide a competitive advantage over relatively slower growing thermally resilient corals (Pandolfi et al. 2011). Indeed, faster growth rates and therefore shorter generation times will offer an advantage to the rate at which increases in bleaching thresholds may occur through evolutionary adaptation (Baird et al. 2009). In this way, the future direction of community shift, either towards a dominance of slower growing thermally resilient corals or, towards more susceptible but faster growing corals, will be dependent on local physical and biotic environmental parameters.

Modelling the responses of coral reef systems to climate change often creates estimates of a homogenous nature when undertaken at a global resolution. However, recent work elucidating the variability in corals at both an ecosystem and individual level has shown differences in: calcification ability under conditions of increasing acidity, susceptibility to bleaching and associated recovery, and adaptability to rapid warming (Pandolfi et al. 2011). These disparities in the ability of corals to adapt and withstand perturbations suggest that global variation will be more heterogeneous in nature. In addition, warming of seas previously uninhabitable to tropical coral populations will facilitate a widening of inhabitable latitudes and create novel habitats (Greenstein and Pandolfi 2007). These new niches may be seen as thermal refugia and may be likened to deeper cooler waters in their ability to escape at least some of the negative effects of climatic change. Instead of the complete decimation of coral reef ecosystems globally, it is therefore likely that a community shift towards those species and ecosystems best suited to acclimatise and adapt, or mitigate change, will be realised.

As with all models, projections are limited by their understandings of the interactions of coral reef ecosystems with their environment. In order to understand to what extent coral reef ecosystems will be able to

adapt/acclimatise and survive given future prediction of global climate change, it is therefore fundamentally important to elucidate the mechanisms available to them by which they might adapt/acclimatise to current climate fluctuations. In understanding this we can infer how these organisms will accommodate predicted future conditions.

Tropical corals have adapted over time to inhabit a wide range of environments in the tropical and subtropical waters in which they survive (Veron 2000). Different environments pose different challenges, both abiotic and biotic, to the coral-symbiont relationship and therefore different survival strategies exist (Buddemeier and Smith 1999; Hoegh-Guldberg et al. 2007). The investigation of coral-symbiont relationships and survival strategies from, and in, environments that represent an unequal predominance of a given stress, e.g. high or low thermal stress, may offer the opportunity to infer (through comparison with a baseline coral stasis) how given stressors are mitigated. In this way, corals from the physically extreme Persian/Arabian Gulf (hereafter referred to as 'the Gulf') may be seen as ideal candidates for study in the context of elucidating if, and by which mechanisms, coral populations of the world may adapt/acclimatise to future predicted increases in sea surface temperatures over the next century (Feary et al. 2013).

In addition to the use of these coral populations in elucidating survival mechanism, the potential for coral populations of the Gulf to act as seeding (natural larval export) or donor (assisted migration; translocation of resilient associations in reef restoration projects) populations (of coral-symbiont associations that are resistant to specific or combinations of environmental stressors) to coral reef ecosystems that are in decline due to the breakdown and loss of relatively 'sensitive' coral-symbiont associations has been proposed (Coles and Riegl 2012). Strengthening of coral reef ecosystems in decline due to increases in environmental perturbations by influx of tolerant coral-symbiont associations, may occur either through direct replacement/out competition of less tolerant associations or through the integration of genetic material enabling more tolerant or plastic phenotypes. The potential ability of the Gulf populations to provide this service however is limited by their ability to be exported from the Gulf and their ability to survive in waters external to the Gulf. Along with the mechanisms by which the Gulf coral populations

mitigate the extreme physical stressors of their environments, these two abilities are undetermined.

1.2 Coral-*Symbiodinium* associations in the Persian/Arabian Gulf

Sea surface temperature (SST) maxima in the Gulf can reach up to 36°C and waters can chill to as low as 12°C in the winter (Sheppard et al. 2010). The corals of the Gulf experience annual thermal variations larger than any other coral community in the world. In contrast, the majority of tropical coral populations experience thermal maxima in the range of ~29°C with seasonal variations of ~4°C. In parallel to extremes in temperature, corals of the Gulf may experience hyper-saline conditions ranging from 37-43; the highest salinities of the Gulf are found in the southern waters off the UAE coast (Johns et al. 2003). Despite the apparent adaptation/acclimatisation to such extremes in temperature and salinity, bleaching occurrences in Gulf waters are documented due to thermal minima and maxima indicating that, like the majority of coral populations, the corals of the Gulf are surviving on the edge of their narrow physiological limits and have a relative inability to survive even small deviations (e.g. 1°C increase in SST above summer average maximum) above and below these limits (Riegl 2002,2003).

The adaptation/acclimatisation of coral populations of the Gulf demonstrates the large capacity for adaptation/acclimatisation of the coral holobiont as a whole. Corals of the Gulf are already surviving temperatures higher than the majority of corals in the Indo-Pacific are predicted to experience by the end of the century according to the latest climate change predictions (rises of 1-3°C by 2099 as predicted by the IPCC committee; IPCC 2007). Time however plays a significant limiting factor. The Gulf is considered a relatively young water body and its coral populations may only have had as little ~6ka (Purkis et al. 2010) to gain their unique resistance (the time since the Gulf became joined with the Gulf of Oman, a shallow sill being responsible for the previous isolation and uninhabitable conditions). Given the speed of predicted changes in climate, coral populations will likely have insufficient time to adapt in a

similar way. Rather, acclimatisation mechanisms may be exploited, such as modifications to the predominant symbiont genotypes (Sampayo et al. 2008).

Harbouring different genotypes of *Symbiodinium* may confer advantages in energetic supply (Little et al. 2004; Mieog et al. 2009; Jones and Berkelmans 2010) or an ability to withstand environmental extremes and perturbations to the coral host (Rowan 2004; Berkelmans and van Oppen 2006; Mieog et al. 2009). The six clades of *Symbiodinium* identified in scleractinian corals (clades A-D, F and G) are further divided into subclades. Of these subclades, differences in the conferring of host tolerances and energetic advantages have been demonstrated (Rowan et al. 1997; Berkelmans and van Oppen 2006; Jones et al. 2008; Lesser et al. 2010; Cooper et al. 2011). The modification of the predominant symbiont genotype hosted by coral populations to coral-symbiont associations that may offer an advantage in a particular environment may therefore represent a means of acclimatisation. This modification of symbiont type may occur either through natural selection of better-suited associations, uptake of new symbionts after a bleaching event or by changing the relative abundance of symbiont types already present in the host (symbiont shuffling) (Rowan et al. 1997; Baker 2001; Baker et al. 2004; Berkelmans and van Oppen 2006; Jones et al. 2008).

Previous analyses of coral symbionts in the Gulf are limited to three studies covering two separate regions to date: Baker et al. 2004, collected samples from 9 coral genera off the Saudi Arabian coast; and Mostafavi et al., 2006 and Shahhosseiny et al., 2011 sampled 8 and 5 genera of corals, respectively, in waters off the Iranian coast and in the Strait of Hormuz. Each of these studies found a predominance of Clade D *Symbiodinium*, a clade of *Symbiodinium* that is often found in partnership with stress tolerant coral-symbiont associations (Baker et al. 2004). The thermal resilience of coral-symbiont associations of the Gulf were therefore thought to be, at least in part, due to associations with these stress-resistant symbionts.

Low sampling numbers (i.e. one or two colonies) and minimal subcladal identification (the majority of associations are identified to a cladal level; thermal tolerances of *Symbiodinium* spp. are resolved at a subcladal level) in the Iranian waters and Strait of Hormuz, limit our understanding of the symbiont-host communities in these areas. Furthermore, these waters are

closest to the inflow from the Gulf of Oman and therefore cannot be considered representative of the majority of the Gulf in their physical properties such as thermal regimes and salinities. Whilst sampling frequencies are higher off the Saudi Arabian coast, all associations are resolved to a cladal level. Therefore whilst initial sampling attempts may suggest a predominance of clade D coral-*Symbiodinium* associations, the associations of the Gulf are poorly characterised. To date, the associations found in waters external to the Gulf (e.g. the eastern Strait of Hormuz and the Gulf of Hormuz) are uncharacterised.

By determining whether the unique resilience of coral-symbiont associations of the Gulf is due to specific coral-*Symbiodinium* associations we can better understand whether such acclimatisation strategies may be available for other coral populations to mitigate increase in thermal stress due to climate change.

1.3 Thesis hypothesis

This thesis tests the hypothesis: **Specific coral-*Symbiodinium* partnerships are associated with survival in the extreme temperature environment of the Persian/Arabian Gulf.**

To test this hypothesis and to assess the potential of coral-symbiont associations of the Gulf to mitigate further degradation of thermally stressed reefs external to the Gulf, this thesis aims to:

- **Establish and characterise Gulf-derived, aquaria-cultured, *Porites lobata* colonies as models for thermotolerant reef-builders.**
- **Characterise coral-*Symbiodinium* associations within Gulf corals through phylotyping (using the internal transcribed spacer region, ITS1-5.8S-ITS2, of the rDNA operon) the algal complements of corals sampled from UAE coastal waters.**
- **Characterise the distribution of coral-*Symbiodinium* associations within the Strait of Hormuz and in the external waters of the Gulf of Oman.**

Chapters 2, 4, 5 and 6 are presented in the format of scientific papers containing abstract, introduction, methods and results and discussion sections. Due to the methodological nature of chapter 3 the methods, results and discussion sections are not utilised and instead the contents of the chapter

are divided into subsections of the methods development chapter. The conclusion section is replaced with a summary section.

Each chapter contains a detailed, fully referenced introduction. As such, the remainder of this thesis introduction will provide an overview of the structure of the thesis and each of the chapters within with only a brief, partially referenced introduction to the topic included.

1.4 Thesis structure and overview

1.4.1 Chapter 2 - Widespread distribution of corallivorous *Acropora-eating flatworms* (*AEFW*) in aquaria: Implications for coral husbandry and natural populations

This project was conducted for training purposes as an introduction to coral husbandry and molecular techniques that would be used in future work. Experimental aquaria at the Coral Reef Laboratory, Southampton (CRL) were infested with a corallivorous flatworm that showed an affinity for corals of the genus *Acropora*. Such flatworms have been documented in popular literature and are commonly referred to as *Acropora eating flatworms* (*AEFWs*). These flatworms may quickly destroy entire colonies of aquaria cultured corals and no natural predator has been identified. In this study we tested the hypothesis that *AEFWs* not only constitute a widespread threat to coral husbandry but may also become an invasive species to natural coral reefs ecosystems through anthropogenic introductions. To investigate this hypothesis survival strategy, functional symbiont assimilation and host prey preference were examined in detail. Additionally, an international compendium of popular and scientific literature accounts of the *AEFW* was made to assess their distribution. The AEFW was genetically identified in order to compare the CRL infestation with other scientific reports.

This study genetically identified the *AEFW* infesting the CRL experimental aquaria as *Amakusaplana acroporae* thereby establishing its international distribution within the aquaristic industry. Popular literature accounts of this flatworm in areas in close proximity to natural coral reef ecosystems, a lack of known predators and an adaptive camouflage necessitate the consideration of

A. acroporae as a potentially damaging invasive species to vulnerable ecosystems through anthropogenic introductions.

1.4.2 Chapter 3 - Primer design for amplification of the ITS and ITS2 region of the *Symbiodinium* rDNA operon for molecular cloning and denaturing gradient gel electrophoresis

This chapter details the design and evaluation of two sets of related polymerase chain reaction (PCR) primers for use in the characterisation of coral-*Symbiodinium* associations of the Gulf; the first for use in molecular cloning and the second for use in PCR denaturing gradient gel electrophoresis (DGGE). For a detailed introduction to the topic please refer to chapter 3.

In the context of taxonomically resolving *Symbiodinium spp.* (phylotyping), molecular cloning is used to create a bacterial library of PCR amplicon fragments. The desired region of DNA is amplified by PCR. Individual amplicon fragments are placed into bacterial hosts via recombinant vectors where the vector (and therefore PCR fragment) is copied. The bacterial plasmid (vector) can then be extracted from individual bacterial colonies. In this way a large number of identical PCR fragments can be isolated and sequenced. Whilst this technique allows an excellent quantification of symbiont diversity harboured within a coral host, only limited by the number of individual bacterial colonies sampled, it is both time and cost intensive compared to phylotyping by DGGE.

DGGE involves the PCR amplification of a region of DNA, the sequence of which is in question. This amplicon has a region high in concentration of GC bases attached to it known as a GC clamp. This fragment is then electrophoretically run on an acrylamide gel, along which, denaturing conditions increase. These denaturing conditions will cause bifurcation of the double stranded DNA molecule causing the DNA fragments migration to slow or stop. The denaturant conditions at which this bifurcation occurs will be a product of the DNA fragments sequence. Therefore DGGE may be used to resolve sequence differences (for a more detailed introduction to the topic please refer to Myers et al. 1985; Muyzer and Smalla 1998; LaJeunesse 2002 or chapter 3 of this thesis). DGGE, in contrast to traditional molecular cloning techniques is less costly in time and resources. However, its ability to resolve intra-host diversity

is limited. Generally speaking DGGE is most effective in identifying predominant subclades within a host.

The genetic identification of *Symbiodinium* to a subcladal level through taxonomic analysis of the internal transcribed spacer region (ITS) 2 region of the rDNA were undertaken in Chapter 1 using the primer pair msg2 and msg3 (Savage et al. 2002). However, using these primers to amplify from a mixed genomic DNA template containing *Symbiodinium* and *Porites spp*. DNA led to the undesired amplification of *Porites* DNA in preference to *Symbiodinium* DNA. Therefore alternative primers were designed. The design of novel primer pair SYM_VAR_FWD and SYM_VAR_REV is covered in this chapter including the consideration of an annealing site with low host-complementarity that maintains effective amplification across multiple clades of *Symbiodinium.*

The second primer pair detailed in this chapter was designed to anneal within the PCR product of SYM_VAR_FWD and SYM_VAR_REV. This second primer pair, SYM_VAR_5.8S and SYM_VAR_CLAMP were designed to amplify a fragment for use in DGGE. Currently, one set of PCR primers, ITSintfor2 and ITSCLAMP are predominantly used in the literature to assess *Symbiodinium* phylotype through DGGE (LaJeunesse 2002). Amplification of host DNA has been documented using these primers. As such low host-complementarity was also considered in the design of these primers. The effectiveness of the novel primers to successful amplify and resolve different clades and subclades of *Symbiodinium* spp. is critically evaluated in this chapter, firstly through analysis of theoretical melting curves and then empirically through the running of DNA previously phylotyped through traditional molecular cloning techniques.

1.4.3 Chapter 4 - Corals from the Persian/Arabian Gulf as models for thermotolerant reef-builders: Prevalence of clade C3 *Symbiodinium*, host fluorescence and *ex situ* temperature tolerance

The first aim of this thesis is to:

- **Establish and characterise Gulf-derived, aquaria-cultured, *P. lobata* colonies as models for thermotolerant reef-builders.**

In order to do this, four colonies of Gulf-derived *P. lobata* were imported and introduced to the CRL experimental aquaria system (D'angelo and Wiedenmann 2012). It was critical to demonstrate that these colonies would retain their unique resilience *ex situ*, and that they contained *Symbiodinium* genotypes representative of the coral populations they were sampled from (advantages and disadvantageous may be conferred to coral hosts dependent on which *Symbiodinium* genotype they harbour; please see chapter 5 for more detail). Furthermore it was an objective of this study to assess the use of coral host fluorescence to monitor coral health.

The establishment of such a model coral population in an experimental aquaria setup allows great potential to test the response of the corals to controlled modulations of their environments.

To demonstrate thermal resilience *ex situ*, Middle Eastern specimens were compared to Fijian specimens from thermally milder and less variant waters in response to a heat challenge representative of temperatures found in Gulf waters.

To demonstrate that aquaria-based *P. lobata* contained *Symbiodinium* genotypes representative of the region, 37 coral specimens representing 7 species from the Saadiyat reef in the southern Gulf off the coast of Abu Dhabi were analysed.

The results establish the Gulf-derived *P. lobata* specimens as powerful tools in the study of the physical resistance of Gulf corals. Maintenance of *ex situ* thermal tolerance was demonstrated after long-term culture and results from the genotyping of *Symbiodinium* harboured by these corals proved unexpected (all colonies harboured Symbiodinium subclade C3, commonly associated with thermally sensitive associations). This unexpected result further highlighted the uniqueness of the coral-symbiont associations of the Gulf and provided the premise for the next section of research; the characterisation of coral associations from the western UAE coast through the Strait of Hormuz and south towards Muscat, Oman.

This study documents the successful establishment of Gulf-derived *P. lobata* colonies as models for thermotolerant reef-builders in the CRL.

1.4.4 Chapter 5 - Contrasting coral-symbiont associations within, and external to, the Persian/Arabian Gulf: The potential of 'internal' coral-*Symbiodinium* associations to act as 'saviours' of Indo-Pacific reefs

The investigation detailed in this penultimate chapter accomplishes the thesis aims to:

- **Characterise coral-*Symbiodinium* associations within Gulf corals through phylotyping (using the internal transcribed spacer region, ITS1-5.8S-ITS2, of the rDNA operon) the algal complements of corals sampled from UAE coastal waters.**
- **Characterise the distribution of coral-*Symbiodinium* associations within the Strait of Hormuz and in the external waters of the Gulf of Oman.**

In completing these aims, this study also discusses the potential of thermally resilient Gulf coral associations to be exported from the Gulf, and their ability to act as 'saviours' of Indo-Pacific reefs through their incorporation and associated benefits in thermal resilience.

The change in physical environment between the southern Gulf and the Gulf of Oman over a relatively small distance (as small as 100km) is a unique phenomenon. In the southern Gulf waters are characterised by extreme thermal variability (>20^{o}C annually), and hyper salinities (~43). Moving towards the entrance of the Gulf, towards the Strait of Hormuz, the salinities quickly become more oceanic and the thermal variability begins to diminish. Once in the Gulf of Oman moving south towards the Muscat coast, salinities are considerably reduced (~36) from those of the hyper-saline Gulf waters and high temperature stress is greatly reduced (~7^{o}C annually). In place of the extreme high thermal stress faced by Gulf corals, corals in this region may experience drops of up to 10^{o}C over a single 24 hour period due to fluctuations in the relatively shallow (between 5 and 10m) and extreme thermocline found in these waters (Coles 1997).

Sampling a range of corals over this extent (from the western coast of the UAE, north east along the coast to the Strait of Hormuz, and finally south along the eastern coast of the UAE and Oman to Muscat) allows not only a better spatial characterisation of host-symbiont partnerships in the Gulf and Gulf of Oman waters (in which coral-symbiont partnerships have not been assessed to date) but also an insight into which physical parameters may define the unique

symbiont communities found in the Gulf. By contrasting the associations found inside and external to the Gulf, the potential for the export and propagation of Gulf associations to the external waters are assessed.

The results of this study demonstrate a strong correlation between coral-symbiont associations and the physical oceanography of the Gulf region. A complete predominance of subclade C3 within the Gulf but mixed complements in the Strait of Hormuz and Gulf of Oman are described. These results suggest an export of thermally tolerant coral-symbiont associations from the Gulf into the Gulf of Oman. However, the thermally tolerant associations appear to lose what competitive advantage they gain within the Gulf, externally, in the waters of the Strait of Hormuz and the Gulf of Oman, suggesting the unique physical parameters of the gulf are responsible for the unique thermally resilient associations found within.

This study successfully characterises coral-associations in the Gulf, the Strait of Hormuz and the Gulf of Oman and demonstrates the successful export of thermally resilient Gulf associations. These associations are however unlikely to spread far from the Gulf, as demonstrated by their out competition by other association. Therefore, thermally resilient corals-symbiont associations of the Gulf are unable to act as 'saviours' of Indo-Pacific reefs.

1.4.5 Chapter 6 - A critical appraisal of current methods utilised to estimate *Symbiodinium spp.* diversity and the implementation of a novel 'ITS region' definition of subcladal variants

The collection of more than 500 complete and partial *Symbiodinium* rDNA operon sequences acquired through the course of this PhD represent a considerable resource, especially considering the lack of subcladal taxonomic resolution of coral-*Symbiodinium* associations in the Gulf Region. As such, it was the initial aim of this final results chapter to classify all of the sequences to a subcladal level (using the ITS2 region of the rDNA operon in concurrence with the literature standard) and to identify, phylogenetically, any novel sequences found as part of this course of work. However, during the initial processing of the sequence collection several areas of concern became apparent including:

- The identification of ITS2 types according to defining sequence divergences outside of the strictly defined ITS2 region;
- The discounting of sequence divergences that were strongly suggested to be due to genuine ecological diversity, as possible pseudo-gene representatives, by current widely used rRNA secondary structure screening techniques;
- And, a lack of subcladal taxonomic definitions utilising the entire ITS1-5.8S-ITS2 region.

Due to these issues, this chapter critically assesses current methods of *Symbiodinium spp*. diversity estimation and subcladal identification. It also offers an alternative method for the definition of subcladal *Symbiodinium* variants. This is accomplished through:

- Characterising and quantifying the phylogenetic resolving power of the individual internal transcribed region 1 (ITS1), 5.8S and ITS2 regions of the *Symbiodinium* rDNA operon in contrast to use of the entire region (ITS1-5.8S-ITS2)

- Critically assessing of the use of rRNA secondary structure screening to infer nucleotide consensus divergences as possible representatives of pseudo-genes

- Offering a novel, alternative system for the taxonomic placement of *Symbiodinium* rDNA operon sequences containing the entire ITS region in relation to both ITS1 and ITS2 types. The six most frequently sampled subcladal variants within the *Symbiodinium* clade C acquired during this PhD are defined accordingly through an 'ITS type'.

The conclusions of this chapter supports the return to the sequencing of, and subcladal characterisation by, the use of the entire ITS region as opposed to defining an ITS2 type (or to a lesser extent ITS1 type) that has become common practice. Furthermore, the results do not support the use of secondary structural analysis, in its current form, as an appropriate means of inferring consensus divergences in sequences as possible representatives of pseudo-genes. Finally, the six most frequently occurring Symbiodinium clade C subcladal types/variants found in the Gulf region are defined according to their ITS region and in relation to both the ITS1 and ITS2 type definitions.

Chapter 2: Widespread distribution of corallivorous *Acropora eating flatworms* (*AEFW)* in aquaria: Implications for coral husbandry and natural populations

Note: The content of this chapter is a manuscript in preparation for submission to Coral Reefs' "note" format:

Contributing Authors: Hume BCC, D'Angelo C, Cunnington A, Smith E, Wiedenmann J.

Contribution to chapter by BCCH: Collection and fixation of *A. acroporae* specimens; preparation of genomic DNA; molecular identification of *A. acroporae* and *Symbiodinium spp.*; white light/fluorescence photography and documentation of egg clusters; white light/fluorescence photography of bite marks and 'bleached' worms; joint contribution to manuscript preparation.

2.1 Abstract

The infestation of corals in captivity with *Acropora-eating flatworms* (*AEFWs*) is a known phenomenon within aquaria and the marine hobbyist industry. In this report the phenotypic changes associated with *AEFW* infestation of corals kept in aquaria in the UK are evaluated. It is demonstrated that the successful camouflage strategy adopted by the flatworms involves incorporation of photosynthetically active *Symbiodinium spp.* as well as host pigments, both derived from the coral prey. Finally, the responsible flatworms are identified as identical to a new species of corallivorous, ectoparasitic polyclad (Polycladida: Prothiostomidae): *Amakusaplana acroporae* recently isolated from Acroporids kept in aquarium conditions in the USA and thus evaluate a putative widespread distribution of this particular organism. Our results indicate that *AEFWs* needs to be considered as a potentially damaging invasive species via anthropogenic introductions to vulnerable coral reef ecosystems.

2.2 Introduction

Coral reefs, one of the most biodiverse and productive ecosystems in the world, are sensitive to a range of environmental perturbations including the potentially devastating effects of corallivory (defined as the direct assimilation of live coral tissue) (Moberg and Folke 1999; Hughes et al. 2003). At least 165 diverse corallivorous organisms have been described, including for instance *Acanthaster planci,* capable of reaching high population densities with individuals consuming up to 5-6 m^2 yr^{-1} of live coral (Chesher 1969; Rotjan and Lewis 2008).

Infestation of Acroporid corals kept in aquaria by a particular type of corallivorous flatworms known as *Acropora-eating flatworms* (*AEFW*s) has been reported both in the scientific and hobbyist literature (Table 7.1Appendix 1; Figure 2.1). Although the natural occurrence of *AEFWs* is not yet defined, individuals sharing several morphological characteristics have been found attached to healthy coral colonies in two different geographical locations (Matsushima et al. 2010; Rawlinson and Stella 2012). Genotypic analyses have identified the specimens found on *Acropora humillis* in Okinawa, Japan as belonging to the genus Waminoa whereas those isolated from *A. valida* in Lizard Island, GBR, corresponded to *Amakusaplana acroporae*, a newly described species within the order polycladida (Matsushima et al. 2010; Rawlinson et al. 2011).

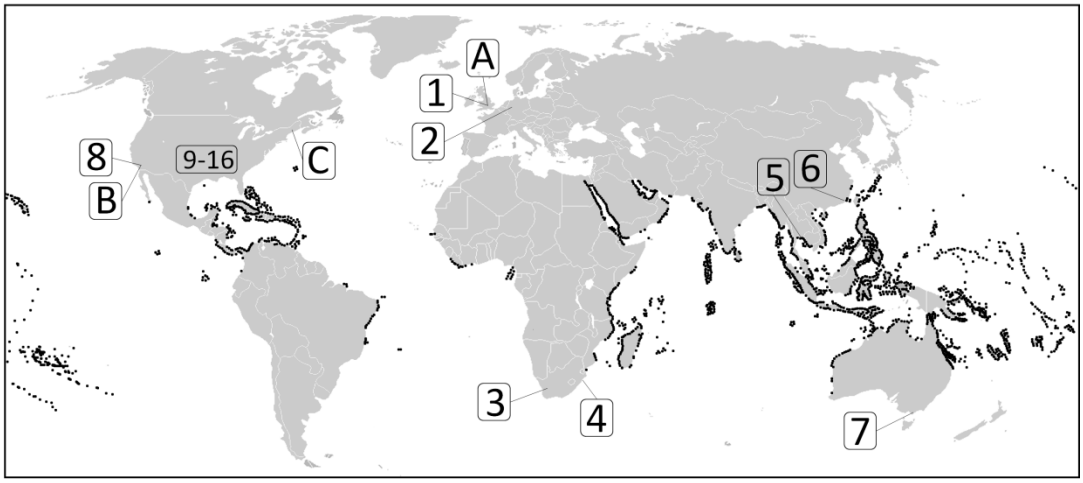

Figure 2.1 Recorded cases of *AEFWs* in popular (numbers 1-16) and scientific (letters A-C) literature. Black spots show the global distribution of tropical coral reefs.

The introduction of a foreign species into a natural ecosystem may have dramatic consequences. For example, the escape of *Caulerpa taxifolia* from an aquarium into the Mediterranean Sea has led to the wide-spread invasion of this macroalgae native to the Indian Ocean, out competing indigenous species and disrupting ecological balances (Meinesz et al. 1993; Wiedenmann et al. 2001). Also, *Pterois volitans and P. miles*, native to the Indo-Pacific were accidentally introduced off the coast of Florida in the early 1990s (Semmens et al. 2004; Schofield 2009). The commonly known lionfish is currently spreading throughout the Caribbean region posing a threat to natural coral reef ecosystems.

Aquaria and ornamental species constitute the majority of those documented as invasive in aquatic ecosystems (Lowe et al. 2000; Padilla and Williams 2004). Thus, *AEFWs*, for which natural predators are not known, might not only constitute a widespread threat to coral husbandry but could also become an invasive species to natural coral reefs ecosystems. To study this hypothesis the survival strategies of the flatworms in aquaria, including camouflage strategy, functional symbionts assimilation and host prey preference were examined in detail. The *AEFWs* associated with infestations in the aquarium trade in Southampton, UK were identified genotypically and the data was analysed comparatively with reports from the hobbyist and scientific literature, obtaining a thorough picture of the knowledge related to this organism. Finally, the effect of *AEFWs* on coral host fluorescence was assessed to establish light emission as a tool to monitor early infestation of corals.

2.3 Methods

2.3.1 Collection and fixation

Individuals of *A. acroporae* were detached and collected from the tissue of different acroporid corals maintained in isolated compartments in the Coral Laboratory Research Aquarium at the NOCS, Southampton, UK. Worms were dislodged from the corals using a water stream and fixed with absolute ethanol.

2.3.2 Preparation of genomic DNA

Genomic DNA (gDNA) was prepared from a group of 6 flatworms and from infested colonies of *A. millepora* (from which the worms were collected) using a published methodology (Hume et al. 2013).

2.3.3 Molecular identification of AEFW and *Symbiodinium*

The genotypic identification of the S*ymbiodinium spp*. in the corals and in the worm was conducted by amplifying the ITS1-5.8S-ITS2 ribosomal DNA using the zooxanthelae specific primers msg2 and msg3 (Table 7.2 Appendix 1) (Savage et al. 2002). The PCR reaction was performed using 100-250ng of gDNA, 200µM of each dNTP, 0.5 µM of each primer and 0.02 Units of Phusion DNA polymerase (Finnzymes). All PCR products were purified, incubated with taq polymerase (for 20mins at 72°C) to add adenine tails and cloned using StrataClone PCR Cloning Kit (Agilent Technologies). Sequencing services were provided by Eurofins MWG Operon.

ITS1-5.8S-ITS2 rDNA *Symbiodinium* sequences were deposited in Genbank with accession numbers JN711475-JN711486 (isolated from *A. millepora*) and JN711487-JN711498 (isolated from AEFW).

The 18S rDNA region was amplified and sequenced (in two different fragments) using primers (LHP Fwd / LHP Rev and RHP Fwd / RHP Rev) designed according to an alignment of Polyclad 18S sequences. A novel primer pair (BC Fwd and BC Rev) to amplify the 28S region of *AEFW* was designed from an alignment of polyclad 28S sequences available in GenBank (Table 7.2 Appendix 1). Advantage 2 PCR Enzyme System (Clontech) was used with a thermal cycle of: 95°C for 1 min, 35 cycles of 95°C for 30s, 58°C for 30s, 68°C for 1min, followed by a final step at 68°C for 1min. The amplified fragments were purified, cloned and sequenced as described above. The sequences were deposited in Genbank with accession numbers JN711500 (28S) and JN711499 (18S).

2.3.4 Phylogenetic analysis of 18S and ITS2 sequences

Phylogenetic analysis was performed using MEGA 5 (Tamura et al. 2011). Sequences were aligned using ClustalW and phylogenetic trees were

constructed using maximum likelihood (ML) methods. Analysis was performed to infer an optimal nucleotide substitution method. For 18S analysis a Tamura-Nei with gamma distribution and 5 rate categories was selected based on Akaike Information Criterion. ITS2 sequences were analysed using the Hasegawa-Kishino-Yano model with equal rates. The certainty of ML nodes was tested with bootstrap analysis (100 replications).

2.3.5 Photographic documentation, fluorescence measurements and microscopy

Photographs of coral colonies were taken with a Camedia C-730 Ultra Zoom digital camera (Olympus, Germany) as described in D'Angelo et al. 2008. Close-up images of coral branches and flatworms (adult specimens and egg clusters) were obtained with a Leica DFC420C video camera fitted to a Leica MZ10 stereomicroscope as detailed in Smith et al. 2013.

Photosynthesis efficiency of *Symbiodinium* was determined by pulse amplitude modification (PAM) fluorometry using a Diving PAM (Walz) in a collection of specimens removed from coral hosts.

Fluorescence spectra were measured on the animal using a Varian Cary Eclipse fluorescence spectrometer (Varian, Palo Alto, USA), equipped with a fibre optic probe.

2.3.6 Physiological experimentation

The flatworm's dependence on the coral hosts was evaluated by maintaining specimens released from coral branches, in a separate tank with no access to corals. Flatworms attached on coral colonies maintained in neighbour aquaria under the same conditions served as controls. Survival of the *AEFW* was monitored over a 10 days period. The survival of the worms was followed by inspecting their attachment to the aquarium glass.

To analyse thermal stress response, fragments of *Acropora sp*. infested with *AEFW* were placed in a separate tank where the water temperature was increased from 25˚C to 31˚C over a period of 5 days. The survival of the colonies and the attachment of the worms to the coral tissue were examined

over a 3 day period. The symbiont content in coral tissue and AEFW was monitored by microscopy observation.

2.4 Results and Discussion

2.4.1 Infestation with *AEFWs* in aquaria

Visual examination of infested acroporids revealed that the flatworms were always attached onto the shaded side of the coral branches (Figure 2.2, Figure 7.2 Appendix 1). Microscopical imaging under blue light detected red light emission of photosynthesis-associated pigments associated with the presence of zooxanthellae on the coral surface (Figure 2.2b). Red light fluorescence was also observed in the area where the *AEFWs* were present.

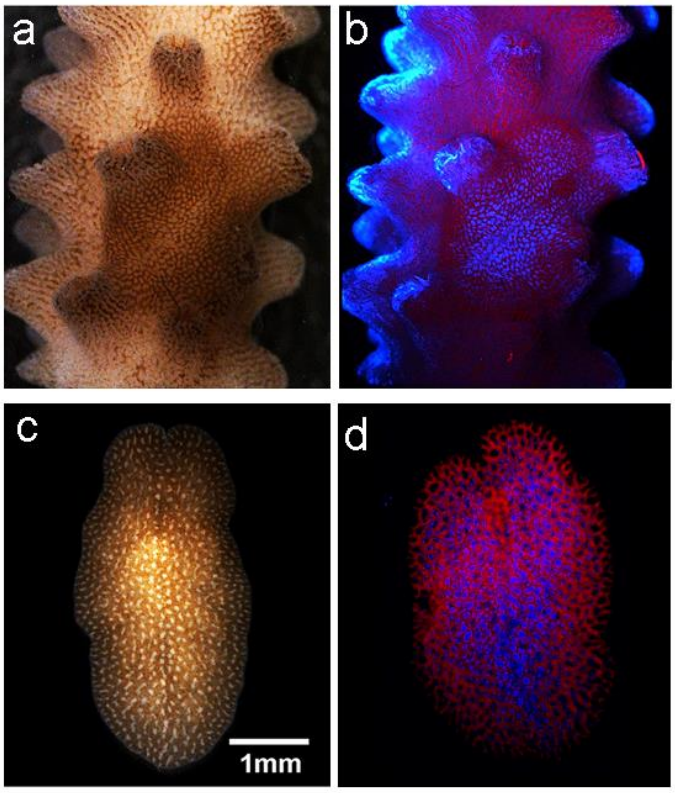

Figure 2.2 *AEFW* infest acroporids in aquaria. (a-b) Photographs of a live *A. pulchra* branch where a flatworm is attached. The pictures were taken under the microscope with day light (a) or under the microscope using a cyan/dsRed filter (b). (c-d) Images, taken as above, of an *AEFW* specimen that was mechanically detached from the coral prey.

AEFW specimens could be mechanically dislodged from the coral branch with a water jet, and when examined under the microscope, they displayed the brownish appearance of the animal body under day light (Figure 2.2c). The released flatworms were of difference sizes reaching up to 3 mm in length and 1 mm in width. The presence of algal cells inside the worms was indicated by red light emission detected under blue light excitation (Figure 2.2d). Notably, the emission of cyan light, most likely related to host fluorescence was detected in the coral tissue including the region occupied by the flatworm as well as inside the isolated specimens (Figure 2.2c and d). Egg clusters (formed of between 5 and 90 individual eggs) were found only on bare coral skeleton with close proximity to live coral tissue (Figure 7.2 Appendix 1).

Although several different coral species were incubated in the same aquarium, infestation with flatworms was related exclusively to acroporid corals. To define a host prey preference of the *AEFWs* within this genus eight different species of acroporids were incubated side by side and visually monitored infestation over a period of approximately 4 months. The highest to lowest preference displayed was as follows: *A. valida > A. millepora > A. pulchra > A. polystoma>A. yongei >A. gemmifera > A. microphthalma > A. tortuosa*.

2.4.2 The camouflage strategy of *AEFWs* involves incorporation of host proteins and photosynthetically active symbionts

The feeding-associated bite marks of the flatworms on the coral were observed preferentially on the shaded side of the branches. The microscopic inspection of these under white and blue light corroborated a complete digestion of the coral tissue (Figure 2.3a-b).

Fluorescent spectra obtained directly on the coral surface identified a cyan fluorescent protein with maximum emission at 483nm in the animal host (Figure 2.3c; D'Angelo et al. 2008). Similar measurements performed on isolated flatworms revealed an overlapping fluorescence pattern, displaying the emission of a cyan fluorescent protein (λ_{max} 483 nm) and the emission of photosynthetic pigments (λ_{max} 680 nm). These results demonstrate that *AEFWs* incorporate putatively functional host proteins from the coral prey that aid in their hiding approach on the coral surface.

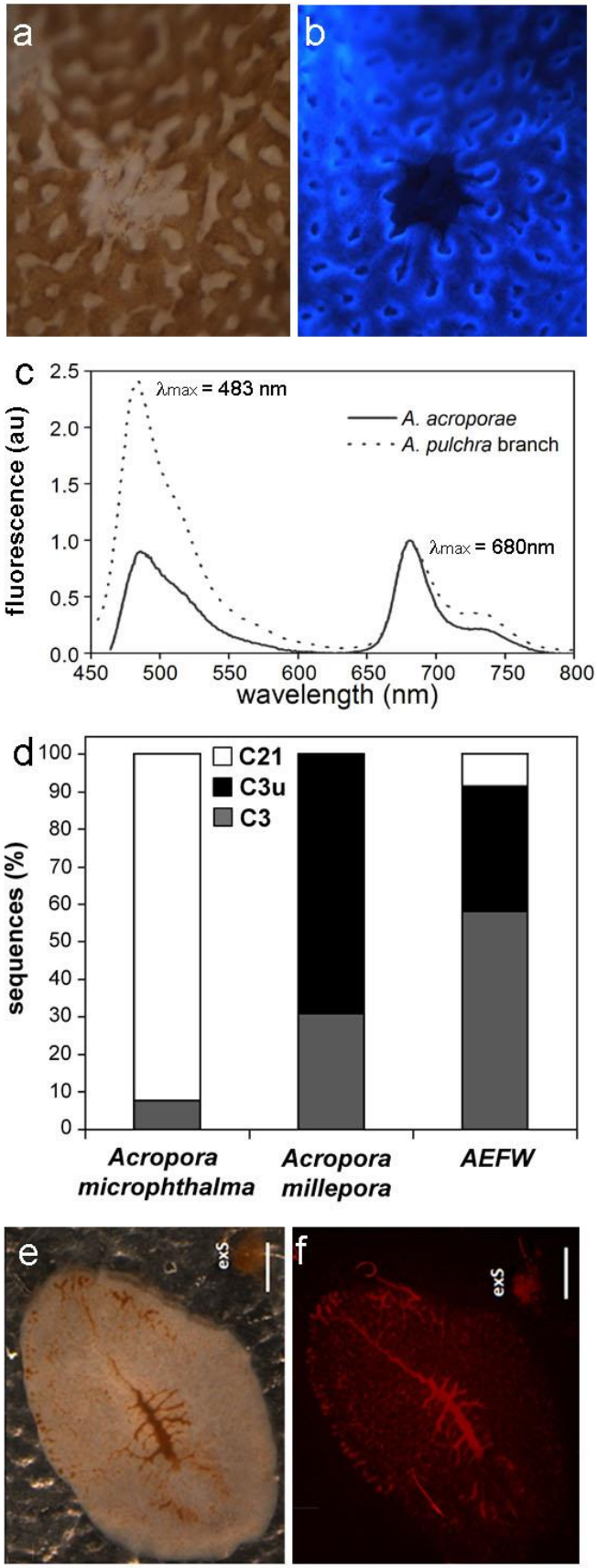

Figure 2.3 Camouflage strategy of *AEFW*. Close-up photographs of a bite mark of the flatworm on the coral tissue taken under white light (a) or with blue light using a cyan/dsRed filter (b). (c) Fluorescence emission spectra (λ_{exc} = 420 nm) recorded directly on the coral tissue (solid line) and on the isolated flatworm (dotted line). (d) Comparison of the Symbiodinium subclades identified in an infested colony of *A. millepora*, in detached *AEFWs* and in a healthy colony of *A. microphthalma* incubated in the same aquarium. Photographs of *AEFW* detached from coral animals exposed to thermal stress were taken under white light (e) and with a ds/Red filter (f). The *Symbiodinium* (exS) cells expelled from the flatworms are visible in the pictures.

Additionally, as described above and in agreement with previous reports (Rawlinson et al. 2011; Rawlinson and Stella 2012), the brown appearance of the flatworms was due to their incorporation of algal cells most likely from the coral prey. However, when photosynthetic efficiency was measured in detached *AEFW* specimens, values of Fv/Fm>0.5 were obtained. This demonstrated that the incorporated *Symbiodinium* cells are photosynthetically active and might thus also provide the flatworm with an energetic advantage through their association (Jokiel and Townsley 1974). This possibility was evaluated by comparing the survival capability of flatworms attached to coral branches and exemplars isolated from the coral prey. When incubated in the same aquaria but without access to the prey, all specimens died within 5 to 7 days, suggesting that the incorporation of algal cells does not provide them with an alternative nutritional resource. Rather, the comparatively short starvation durations from this study suggest that their primary purpose is to offer camouflage to the flatworm specific to the prey coral.

Phylotyping of the zooxanthellae through the analysis of ITS2 sequences identified two different subclades of *Symbiodinium* (C3u and C3) in infested colonies of *A. millepora* (Figure 2.3d). Identical zooxanthellae subclades were detected in *AEFWs* isolated from the coral colonies, demonstrating that the algal symbionts within the flatworm cells are incorporated directly from their prey. Additionally, the *AEFWs* contained subclade C21symbionts. The presence of a genetically distinct *Symbiodinium* within the flatworm may suggest an additional host-source of algae. In agreement with this hypothesis, subclade C21 was found in a colony of *A. microphthalma* growing in the same aquarium. Our analyses, however, cannot rule out the possibility that *Symbiodinium* C21 might result from a selective association of the flatworms towards a particular subclade that might constitute a small proportion of *A. millepora's* symbiont assemblage.

The expulsion of zooxanthellae from coral tissue is a well-studied phenomenon that characterises their response to increased temperature (Brown 1997; Hoegh-Guldberg 1999). Whether a comparable mechanism would be defined by the association between *Symbiodinium* and *AEFWs* was examined. Infested *Acropora sp.* colonies were exposed to thermal stress over a period of 8 days. At 31˚C, bleaching was visible in the coral host and some of the flatworms started to detach. Close observation of these flatworms

demonstrated the expulsion of intact zooxanthellae into the surrounding water (Figure 2.3e-f).

2.4.3 Molecular identification of the *AEFW* - Implications for a global distribution of a corallivorous species

The characteristics outlined so far in section 2.4 are similar to those reported from corals infested with different species of flatworms both in aquaria and in the wild (Jokiel and Townsley 1974; Barneah et al. 2007; Matsushima et al. 2010; Rawlinson et al. 2011; Rawlinson and Stella 2012).

Sequence analysis of the 18S region of the SSU rDNA demonstrated that our isolated *AEFW* belongs to the polycladida and groups within the cotylea clade (Figure 2.4a). Only recently, detailed morphological and histological examination together with molecular analyses of the 28S rDNA identified *AEFW* isolated from aquaria in the United States as a novel species within the order polycladida (Rawlinson et al. 2011). This species, *Amakusaplana acroporae*, exhibited similar characteristics as our isolated *AEFW*. Comparative analyses of a 618-nucleotide fragment of the 28S rDNA sequence from AEFW and the three sequences available from *A. acroporae* show a 99-100 % identity among them (Figure 2.4b). *A. acroporae* was also suggested to be the flatworm responsible for the loss of *Acropora* sp. colonies at the Birch Aquarium at Scripps, USA in 2008 (Nosratpour 2008).

A review of the hobbyist literature indicates that infestation with flatworms is a recurring phenomenon in the aquaculture of corals (Table 7.1, Appendix 1). Reported observations include an exclusive preference for *Acroporids*, camouflage of the worms particularly in the shaded side of infested colonies, bite marks on the coral tissue visualized in advanced stages of infestation and egg clusters deposited on bare dead skeletons. These observations and inspection of the worms and egg clusters from photographic evidence are in close agreement to the characteristics of the infestation displayed in our experiments.

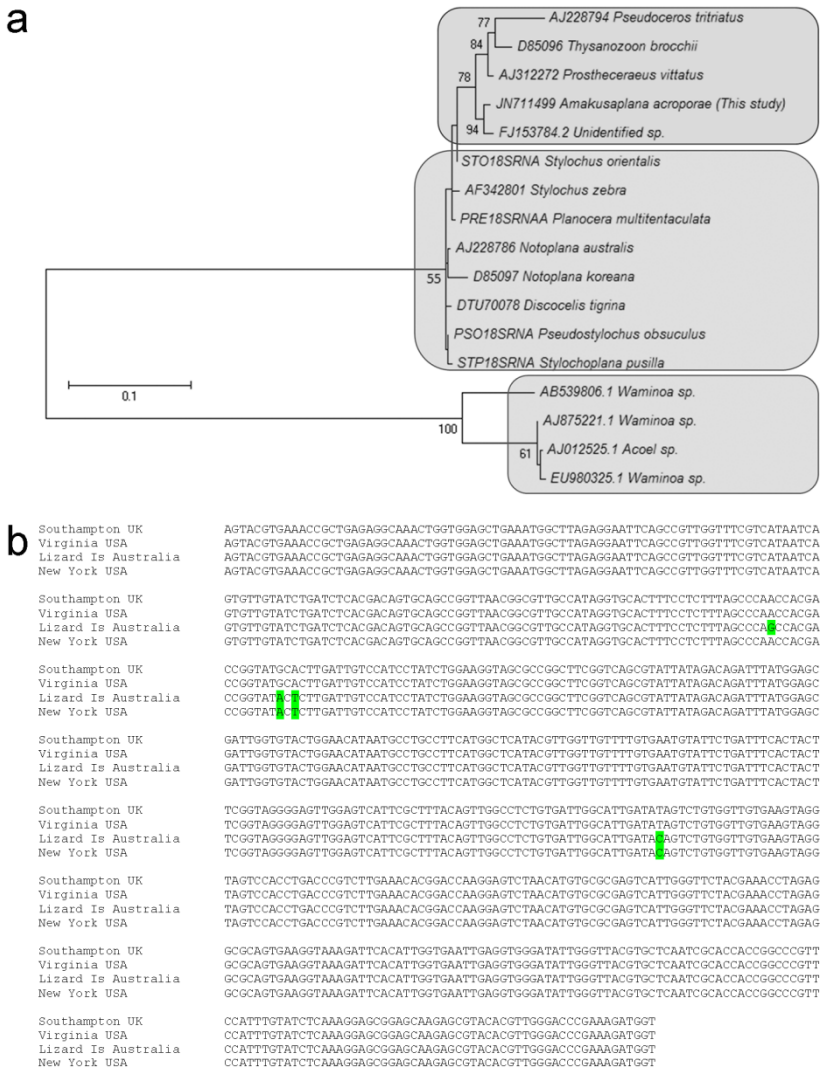

Figure 2.4 Genotypic analysis of *AEFWs*. (a) Phylogenetic analysis of 18S rDNA sequences from *A. acroporae* by Maximum Likelihood method. Numerals are bootstrap values for 100 replicates. The three defined groups are Cotylea (above), Acotylea (centre) and Acoela (below). (b) Sequence comparison of a 28S fragment amplified from *AEFW* isolated in aquaria in Southampton, UK, Virgina, USA; and New York, USA; or in the wild in Lizard Island, GBR, Australia (Genbank accession numbers are: JN711500, HQ659011, HQ659010 and JQ791553, respectively)

In agreement with our results it seems most likely that the *AEFW* responsible would correspond to *A. acroporae*. Infestation has been recorded in several different countries including those (such as the USA, Thailand and Australia) that might have a direct access to natural coral reefs (Figure 2.1). These results demonstrate that *A. acroporae* is present in aquaria from a range of geographical locations indicating a global distribution of this species and thus constituting an actual threat to the coral aquaristic industry. Moreover, the presence of a voracious corallivore (for which the natural predators are not known) in the coral trade and in aquaria maintained in close proximity to naturally occurring reefs represents a risk to the natural ecosystems in the event of an introduction into reef waters (Lowe et al. 2000; Wiedenmann et al. 2001).

2.4.4 Using coral fluorescence to examine infestation with *Amakusaplana acroporae*

In corals, the expression of pigments similar to the green fluorescent protein (GFP) is regulated in response to changes in environmental conditions (Dove et al. 2006; D'Angelo et al. 2008; D'Angelo et al. 2012). Among these pigments, the fluorescent proteins (FPs) constitute ideal markers of coral health (D'Angelo et al. 2008; D'Angelo et al. 2012) that could provide a non-invasive optical technique to detect infestations with *AEFWs*. During photoacclimation experiments, a high light exposed *A. millepora* replicate colony infested with a single *A. acroporae* failed to produce the same amount of amilFP597 (Cunnington, D'Angelo, Wiedenmann unpublished). Three colonies of *A. millepora* are shown in Figure 2.5b. The leftmost is healthy and the host green fluorescence is visualized with blue light excitation. The middle colony is infested with flatworms that are not apparent in the upper side of the branches however, the green light emission of the animal tissue is dramatically reduced. The rightmost colony has died after severe infestation. Measurement of fluorescence spectra directly on the surface of the coral demonstrated that light emission (with a maximum at 516 nm) is more than three times lower in infested animals compared to healthy colonies (Figure 7.1 Appendix 1, Figure 2.5c). These results indicate an observable phenotypic change in the corals that could be monitored *in situ*, even when the worms are not readily detectable. When coral fragments cut from infested and healthy colonies of

Acropora spp. are examined under the microscope, the bite marks indicative of *AEFWs* feeding on the coral tissue become evident. In addition infestation is accompanied by a reduction in cyan/green fluorescence emission by the coral host (Figure 2.5b-c).

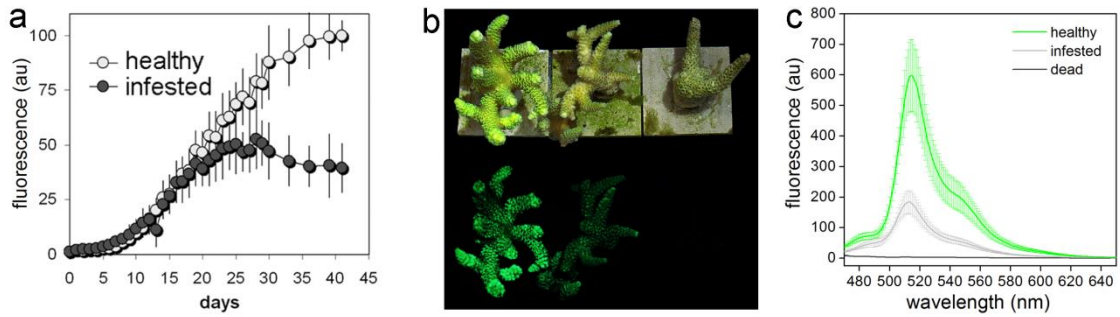

Figure 2.5 Effect of *AEFW* infestation on host fluorescence.(a) Time course of host fluorescence emission (λ_{ex} 530nm, $\lambda_{max\ em}$= 597 nm) in colonies of *A. millepora* after being moved (at time=0) from low light to high light exposure. Infestation of one of the animals is recognisable due to its lack of response to the new environment after 18 days and subsequent gradual loss of fluorescence. (b) Photographs of *A. millepora* colonies in day light (above) and under blue light (below). The left colony is healthy, the middle colony is infested with AEFW and the right specimen has died from the infestation. (c) Fluorescence spectra measured directly on the upper side of the colonies displayed in (b).

2.5 Conclusion

The global distribution of the *AEFW A. acroporae* in the aquaristic industry through the genetic identification of specimens from the Coral Reef Laboratory, Southampton, UK has been demonstrated as identical to specimens found in the USA. Mechanisms for host-specific camouflage in *A. acroporae* have been elucidated through examining the extraction and incorporation of host-derived fluorescent proteins, and photosynthetically active host- derived *Symbiodinium* dinoflagellate algae. Prey-preference of *A. acroporae* across eight *Acropora spp.* has also been documented, and coral infestations have been successfully examined through the use of coral fluorescence.

Evidence presented in this and other studies including the ability of *A. acroporae* to rapidly destroy aquaria-cultured *Acroporid* colonies, its host-specific camouflage, the laying of egg clusters capable of releasing up to several thousand hatchlings within approximately 21 days (Rawlinson et al.

2011), a genus-wide prey preference for *Acroporids*, a lack of known predators, and its documented global distribution in the aquaristic industry, necessitate *A. acroporae* to be considered a potentially damaging invasive species to vulnerable ecosystems through anthropogenic introductions.

Chapter 3: Primer design for amplification of the ITS and ITS2 region of the *Symbiodinium* rDNA operon for molecular cloning and denaturing gradient gel electrophoresis

Note: The content of this chapter is the sole work of BCCH.

3.1 Abstract

The 18S (3' end), 5.8S and 28S (5' end) regions of the rDNA operon are highly similar in coral and their symbiotic algae of the genus *Symbiodinium*. With regards to accurate phylotyping of the symbionts this presents challenges in designing PCR primer sets to successfully amplify the complete internal transcribed region 1 (ITS1)-5.8S-ITS2 region of the rDNA operon for use in phylogenetic analyses. Amplifications of host DNA have occurred when using primers designed to amplify symbiont DNA (in the course of this PhD, and documented in the literature). This co-/mis-amplification is due to insufficient consideration of similarity in coral host binding sites during design.

To improve current techniques used for phylotyping analysis and to minimise confounding electrophoresis analyses, specifically in denaturing gradient gel electrophoresis-PCR (DGGE-PCR), novel primers were developed. The design and evaluation of two primer sets are described here: SYM_VAR_REV and SYM_VAR_FWD, annealing in the 18S and 28S regions and amplifying the complete ITS region; and, SYM_VAR_5.8S and SYM_VAR_CLAMP, annealing in the 5.8S and 28S regions and amplifying the complete ITS2 region, used in DGGE-PCR analysis. Dissimilarity to host DNA of the novel primers and previously used primers was assessed. Primer pair SYM_VAR_5.8S and SYM_VAR_CLAMP were designed specifically for use in DGGE-PCR analysis. As such, the set-up and evaluation (through theoretical and empirical means) of a novel DGGE protocol is also described here.

Both primer sets demonstrate successful and correct amplification of *Symbiodinium* DNA without the amplification of coral DNA across *Symbiodinium* clades, A, C and D. The novel DGGE protocol demonstrates the successful resolution of differences between ITS2 regions as small as a single nucleotide. Advances in gel electrophoresis run time and lengths of regions successfully assessed are described.

3.2 Introduction

Coral reef ecosystems are formed and supported by species of hermatypic (stony/reef-building) corals (Moberg and Folke 1999). Corals form an obligate symbiotic relationship with unicellular dinoflagellate algae of the genus *Symbiodinium* (Baker 2003). The algal symbiont can be responsible for fulfilling up to 90% of the energetic needs of the host coral through translocation of photosynthates (primarily in the form of glycerol with other compounds including alanine, glucose, fumaric acid, succinic acid and glycolic acid; Muscatine et al. 1984; Davies 1991; Mccloskey et al. 1994; Davy et al. 1996). Depending on the genotype of the harboured symbiotic algae advantages in energetics or resilience may be conferred (Rowan 2004; Berkelmans and van Oppen 2006; Mieog et al. 2009). The ability to identify the symbiotic genotype through phylogenetic analyses (phylotyping) is therefore an essential part of understanding the biology of coral-*Symbiodinium* partnerships.

The genus *Symbiodinium* (Eukaryota: Chromalveolata: Alveiolata: Dinoflagellata: Dinophyceae: Suessiales: Symbiodiniaceae) is highly diverse with eight major clades (A to H; Pochon et al. 2004; Pochon et al. 2006). When the effect of differences in *Symbiodinium* genotype on the ability to confer advantage to a coral host were first being investigated, advantages such as an increased resilience to heat were associated at a cladal level (Gates and Edmunds 1999). Cladal level analyses are commonly performed using restriction fragment length polymorphisms (RFLP) analyses of the small subunit (SSU) and large subunit (LSU) of the rRNA gene (rDNA operon; Figure 3.1). Further research has improved our understanding of these relationships. Intra-cladal differences in ability to confer resistance have been demonstrated (e.g. in the most diverse clades to associate with hermatypic corals, clade C; Little et

al. 2004; Cantin et al. 2009; Jones and Berkelmans 2010). As a function of this, *Symbiodinium* genotypes are now commonly identified to a subcladal level.

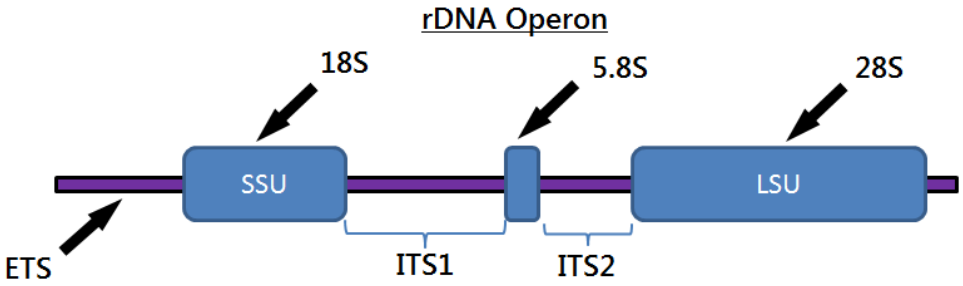

Figure 3.1 Schematic of the rDNA Operon including the external transcribed spacer (EST), the small subunit (SSU; 18S), the internal transcribed spacers 1 & 2 (ITS1 & ITS2), the 5.8S region and the large subunit (LSU; 28S).

Subcladal identification is predominantly inferred from the sequencing of the first or second internally transcribed region of the ribosomal RNA gene (ITS1 or ITS2 rDNA fragments; LaJeunesse 2001; Figure 3.1); whether the subcladal ITS1 or ITS2 type characterisation of *Symbiodinium* genotype represents a sufficient resolution in the context of conferring advantage to the coral host remains undetermined. Characterisations of *Symbiodinium* to higher taxonomic resolutions (through the analysis of microsatellite sites) than the afore mentioned ITS1 or ITS2 types are becoming common and perform a particular function in highly resolute molecular ecological studies (Santos et al. 2003; Santos et al. 2004). In the context of inferring fitness of a given host-symbiont pairing to a given environment, analyses to subcladal level through the analysis of the ITS1 or ITS2 region of the rRNA gene are generally considered appropriate (LaJeunesse 2001).

Analysis of the ITS1 or ITS2 type (from here on in referred to as the subcladal type) will generally follow one of three routes. Firstly, after amplification by PCR and subsequent purification, amplicons may be directly sequenced. Sequences returned from this method will represent the majority sequence in the PCR amplicon. As such no inference of diversity may be made. Investments in time and cost are minimal and therefore dependent on the context of the analysis, this method may suffice. Alternatively, traditional molecular cloning approaches may be followed whereby individual amplicon fragments may be introduced to bacterial hosts via recombinant vectors thus creating a clone library. These bacteria may then be grown and the vector and therefore PCR

fragment copied many times. A culture derived from a single bacterium may then be used to harvest large copies of the recombinant vectors (plasmids). Subsequent targeted sequencing allows the sequence of one of the original PCR amplicon fragments to be inferred. In terms of diversity estimates, molecular cloning is limited only by the number of bacterial colonies cultured and processed and as such this method represents the best means of providing thorough diversity estimates (Thornhill et al. 2007). This method does however represent a significant investment in time and resources. Both of these methods rely solely on the sequencing of the PCR amplicon either as a majority or with reference to individual fragments. In contrast, denaturing gradient gel electrophoresis (DGGE) allows visual inference of subclade type without costly sequencing of every sample (LaJeunesse 2002; Thornhill et al. 2006b; Sampayo et al. 2007; Frade et al. 2008; Goulet et al. 2008).

Similar to molecular cloning or direct sequencing methods, DGGE relies on the amplification of an appropriate amplicon using PCR (Muyzer and Smalla 1998). Unlike the other two methods one of the primers used in the amplification has a GC rich sequence, known as a 'clamp', attached to it (Sheffield et al. 1989). The resulting amplicon will therefore have a corresponding clamp. This amplicon is then run through a polyacrylamide matrix that is made up with a constant matrix concentration but an increasing gradient (in the direction the amplicons are run; made from formamide and urea) of denaturants. The amplicon is loaded onto the matrix through pre-made wells and run electrophoretically. The double stranded fragments are initially free to migrate through the gel, however, as the denaturant concentration increases amplicons encounter denaturant conditions that cause the disassociation of the double stranded amplicon in the areas within the lowest melting domain (Myers et al. 1985). Due to the high melting temp of the GC rich end of each amplicon this region will never be within the lowest melting domain and therefore should not experience disassociation. Disassociation of the lowest melting domain results in an arrest in mobility due to bifurcation of the fragment (Fischer and Lerman 1983). The degree of migration of a given amplicon sequence will be defined by its sequence and length, due to these characteristics defining melting temp and migration speed, respectively. In this way DGGE may be used to assess diversity, a typical mixed profile taking the form of multiple bands in a single lane. In order to identify given bands of a DGGE profile, bands may be

extracted, purified and sequenced either directly or by molecular cloning. By running previously identified (through sequencing) subcladal types as markers in adjacent matrix lanes, similarities between matching profiles or even single bands may be used to infer matching sequences visually without the need for sequencing.

DGGE is best suited to identifying predominant symbiont types within a host. Its ability to resolve minor subcladal contributors is limited. Several studies have noted an inability of DGGE to resolve certain *Symbiodinium* ITS2 rDNA amplicons (Apprill and Gates 2007; Pochon et al. 2007; Sampayo et al. 2009); similar issues have been observed in other microbial systems (Sekiguchi et al. 2001). Indeed, amplicons with identical melting domains will not be resolved through DGGE-PCR. The main advantage to be gained from the use of DGGE is the relatively small investment in time and resources compared to traditional molecular cloning methodologies. Once optimised (representing a considerable investment in time initially) DGGE can be a relatively high throughput method of subcladal *Symbiodinium* analysis.

There are currently two DGGE systems/apparatus widely used for this purpose (DGGE-2001 [Scientific], DCode System [Bio-Rad], see review; Thornhill et al. 2010). Large variability is seen in the results produced by each system and in the times/voltages required for clearest results. Reference to the use of DGGE in the literature without specific reference to setups is therefore unhelpful. Within the literature, in the context of identification of *Symbiodinium* subcladal type, one protocol is predominantly used (LaJeunesse 2002). This setup uses a specific set of primers (ITSintfor2 and ITS2CLAMP) that bind in the 5.8S and 28S region of the rRNA gene in order to amplify the entire ITS2 region. Other protocols may also use these primers but make use of different run times and voltages. The amplification of coral host products using these primers has been documented (LaJeunesse 2002); such amplification may confound analyses of *Symbiodinium* diversity.

The erroneous amplification of coral host DNA in PCRs used in the assessment of symbiotic algae diversity is clearly not ideal and alone would represent sufficient motivation to re-design primers for this purpose. However, in addition to this, amplicons previously identified using the primer pair SYM_VAR_FWD and SYM_VAR_REV from earlier studies could not be used as

templates by the primer pair of LaJeunesse 2002 due to the latter mentioned primer pair annealing outside of the SYM_VAR_FWD/REV PCR product. The extensive array of Gulf-derived sequences collected through previous sampling efforts would therefore not be available as controls to use in the optimisation and correct setup of the Bio-Rad Dcode system available to the Coral Reef Laboratory.

In this chapter the development of a novel DGGE system (including primer redesign and empirical optimisation of the DGGE methodology using the D-Code system, Bio-Rad) is described and validated as a means to:

- Establish a high throughput system for sample analysis
- Allow the incorporation of previously characterised *Symbiodinium* DNA, specifically plasmids containing previously sequenced ITS2 rDNA for use in identification of novel samples
- Develop a primer set specifically designed to resolve between host and symbiont DNA
- Optimising methodological procedures specifically for the Bio-Rad Dcode system through empirical trials to maximise throughput without compromising on resolution

Also described in this chapter is the novel design of the SYM_VAR_FWD and SYM_VAR_REV primers, mentioned previously (Hume et al. 2013). These primers were designed to avoid *Porites spp*. coral host amplification, which was a problem whilst using the primers msg2 and msg3 (Savage et al. 2002). Given the importance of *Porites spp*. coral in this thesis, this SYM_VAR pair was designed specifically to differ from *Porites sp*. DNA whilst still maintaining robust amplification of *Symbiodinium*-derived DNA.

3.3 Primer assessment and design for the amplification of the complete ITS1-5.8S-ITS2 region of *Symbiodinium spp*. rDNA

Due to unwanted host amplification of *Porites spp*. DNA using the primer pair msg2 and msg3 (designed for the amplification of the ITS1-5.8S-ITS2 region of the ribosomal RNA gene of *Symbiodinium spp.*; Savage et al. 2002), a re-assessment of these primers was required. In order to assess possible regions of complimentary sequence between the msg2/msg3 primer pair and the rRNA gene of *Porites spp.*, a multiple alignment of sequences (*Porites spp*. host and *Symbiodinium* spp. symbiont) available in the NCBI (Sayers et al. 2012)

database was made. Due to a limitation in the number of *Porites spp*. sequences covering a sufficient area of the 5.8S region, the alignment was limited to only 2 host sequences (one each of *P. lobata* and *P. lutea*; FN565161.1 and JQ911592.1, respectively) that covered the area of the 18S region to which the msg2 forward primer was complimentary (Figure 7.3 Appendix 2). Similarly, one sequence fully, and one sequence partially, spanned the area of the 28S region in which the msg3 reverse primer was complimentary (AY320328.1 and JQ911592.1; Figure 7.3 Appendix 2). Analysis of this alignment demonstrated a 100% match of the msg2 sequence and an 84% match (22 out of 24 bp) of the msg3 sequence in the *P. lutea* 18S and 28S regions, respectively. Although the primers were close matches to the *Symbiodinium* clades also assessed as part of the alignment (100% and 96-100% complementation of msg2 and msg3, respectively), the presence of host and algal genomes in the total DNA extracts that were used as templates for PCRs utilising the primers meant host amplification was unsurprising.

Given the lack of suitability of the msg2/msg3 primer pair to the *Porites*-related molecular work to be carried out, primer redesign was required. The novel primer pair would fulfil the following characteristics: a lack of *Porites spp.* host complementation and the ability to amplify the complete ITS1-5.8S-ITS2 region of multiple clades of *Symbiodinium* (specifically clades A-D; Clades commonly found in scleractinian corals). The 18S-ITS1 and ITS2-28S boundaries of the *Symbiodinium* rRNA gene were empirically determined through assessment of a multiple sequence alignment across a range of clades and subclades (including representatives of clade E, an exclusively free-living clade, to assess sequence variability) spanning the desired diversity of *Symbiodinium* (Figure 7.4 Appendix 2). Five regions were clearly defined in this alignment through their contrasting conserved or un-conserved character. These regions were used to define the minimum extent of the amplicon amplified by the novel primers and to identify appropriately conserved regions in the 18S and 28S region where primers could potentially anneal. To ensure a lack of *Porites* host DNA amplification, through minimising sequence similarity, host sequences were considered in parallel with symbiont. Primer 3 software (Rozen and Skaletsky 2000) was used to identify suitable oligonucleotide pairs in the defined suitable regions. Primers SYM_VAR_FWD and SYM_VAR_REV (Hume et al. 2013) were created, binding in the conserved 18S and 28S regions

and showing a 50% and 64% similarity to host DNA, respectively. Similarities of 96-100% and 100% to symbiont DNA sequences were demonstrated by SYM_VAR_FWD and SYM_VAR_REV, respectively.

3.4 Primer assessment and design for use in DGGE

Accounts of coral host DNA amplification by primer pair ITSintfor2 and ITS2CLAMP (ITS-Reverse with the addition of a 39 bp GC clamp; Sheffield et al. 1989) designed for the amplification of the complete ITS2 region, warranted further investigation before proceeding with the setup of experimental methodologies novel to the Coral Reef Laboratory. Similar to the assessment of primer pair msg2/msg3, alignments were constructed assessing similarities between the primers, host and symbiont DNA (Figure 7.3 Appendix 2). Primers ITSintfor2 and ITS2CLAMP demonstrated similarities to host DNA of 89 and 88% and similarities to Symbiont DNA of 94-100% and 76-84%, respectively. Host amplification products are therefore not surprising given this high similarity to host DNA and imperfect *Symbiodinium* DNA alignment. Novel primer design was undertaken in a similar way to the design of the SYM_VAR_FWD/REV primer pair. However, assessment of the potential amplicon for a suitable melting/denaturing profile confounded the design (consideration of amplicon melt profiles is considered later in this chapter). Furthermore, it was desirable to produce a primer set that could use the SYM_VAR amplicon as a template (so that PCR-amplicon-containing plasmids from previous sample analyses may be used in the setup and optimisation of the DGGE protocol). As such, a suitable forward primer (annealing in the 5.8S region) was designed to complement the primer SYM_VAR_CLAMP (SYM_VAR_REV with the addition of a 39 bp GC clamp). The resulting primer pair, SYM_VAR_5.8S (ATCTTGGCTCGAGCACCTATGAAGG), SYM_VAR_CLAMP ((CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCC)CGGGTTCTCTTGTTTGACTTCATGC), demonstrated similarities to host DNA of 72% and 60%, and similarities to *Symbiodinium* DNA of 88-100% and 92-100%, both respectively.

3.5 Contrasting melting profiles of the SYM_VAR_5.8S/CLAMP and ITSintfor2/ITSCLAMP amplicons

Differences in annealing sites of both sets of DGGE primers may cause changes to the migration patterns of the respective amplicons. Theoretical melting profiles may be used to predict the migration characteristics of amplified fragments run in DGGE. As such theoretical melting profiles were calculated for *Symbiodinium* amplicons representative of clades A, B, C and D (subcladally identified sequences for A1, B1, C3, C15 and D1) using both primer sets. Theoretical melting profiles of amplicons were created using stacked Tm50% probabilities (the temperature at which a given base stack within a sequence has a 50% chance of being in an open state) based on the algorithms of Poland 1974 including modifications by Fixman and Freire 1977 and used in the implementation described by Steger 1994 . The Poland program (Steger 1994) was used to generate stacked probabilities under temperatures of 75-90^{o}C and a step size of 0.04^{o}C, thermodynamic parameters of Klump 1988, dissociation constant (β) of 1.0 X 10^{-3} and strand concentration of 1.0 X 10^{-6}. The .OUT file created by the program was converted to a plain .txt file and imported into Microsoft's Excel in a space delimitated format where the Tm50% temperatures corresponding to each nucleotide were extracted using a short program written in Microsoft's Visual Basic (Figure 7.5 Appendix 2), and plotted.

The resolving power of the DGGE methodology is based on the retardation of amplicon migration through the acrylamide gel. This retardation is caused due to the bifurcation of part of the sequence once a melting temperature of a melting domain has been reached (Myers et al. 1985). Melting temperatures of any given sequence region are defined by the sequence. Therefore changes in sequences, may lead to changes in the melting temp of a region. However, the change in melting temperature is dependent on the force of attraction between the sense and anti-sense nucleic acid strands and therefore nucleotide alterations that do not modify this force of attraction may not induce a change in melting temp. Therefore, while some nucleotide changes as small as a single base pair may be resolved using DGGE, others may go unresolved. The mobility of any given fragment is greatly limited once any proportion of the sequence has become bifurcated. Some texts refer to a complete arrest in

migration once partial bifurcation has been realised, but through empirical evidence the present study found this is not the case and migration will continue through the gel, albeit at a significantly reduced rate (data not shown). In contrast to the continuous theoretical Tm50% profiles that are calculated (as shown in Figure 3.2), the bifurcation of a sequence occurs in discrete melting domains (proximal regions of the sequence that have similar Tm50% temperatures). Thus, as an amplicon migrates through an increasing denaturant gradient the part of the sequence with the lowest melting domain will bifurcate first. Once this has happened migration will be retarded and the time taken for further migration along the denaturant concentration, until a concentration that may cause a second melting domain melting temperature to be met (if present), will be a function of the difference in Tm50%s of the two lowest melting domains. As such, the melting profiles of a given amplicon may be used to assess which areas of a given fragment may be resolved for variations in sequence (Fixman and Freire 1977). Ideally, only one melting domain should be present (other than the high melting domain created artificially by the GC-clamp attached to the reverse primer) and the regions of interest (5.8S, ITS2 in this case) should be situated in it. If several melting domains are present (other than the GC-clamped region) the difference in melting temperature between these domains should be minimised so that the fragment migration may be a function of the sequence in both domains.

3.6 Assessment of melting profile differences between the ITSintfor2/ITSCLAMP and SYM_VAR_5.8S/CLAMP amplicons

To assess the capability of the novel DGGE primers to resolve sequence differences between amplified fragments, melt curve analyses from *Symbiodinium* subclades A1, B1, C3, C15 and D1 were contrasted with those created by the amplification using ITSintfor2 and ITSCLAMP (Figure 3.2). Melting domains across the four clades are predicted to have higher melting temperatures when amplified with the novel primers compared to the primers of LaJeunesse 2002. Melt profiles of subclade A1 would suggest only a single melting domain when using the primers of LaJeunesse 2002. However, assessment of the novel amplicon revealed the presence of two domains,

which could be problematic especially considering that the lower domain is the smaller of the two and incorporates the later part of the ITS2 sequence and some of the 28S and primer sequence. The majority of the ITS2 sequence appears to be present in the higher melting domain. However, given that the melting temperatures of the domains lie within 1°C of each other, and there is no discrete jump between them, the migration of this clade of amplicon may still be a function of the entire ITS2 region. Although A1 sequences have been recovered from previous sequencing work during this PhD, no other subcladal variants have been detected in any of the Indo-Pacific/Gulf region coral samples analysed. Should a sequencing effort be undertaken involving a larger variation of clade A *Symbiodinium* genotypes (e.g. in the Caribbean) then further intra-cladal melt curve analyses should be carried out to better understand the capabilities of these novel primers to detect change in amplified sequences from this clade.

The melt curves of all other cladal representatives amplified with the novel primers demonstrate lowest melting domains that incorporate the entire ITS2 region, similar to that of the LaJeunesse 2002 primer set amplicon. Melting temperatures of these domains are approximately 0.5-0.7°C higher than those of the LaJeunesse 2002 primer set amplicon representatives and the larger sequence length of the novel fragment appears not to hinder the formation of suitable melting profiles.

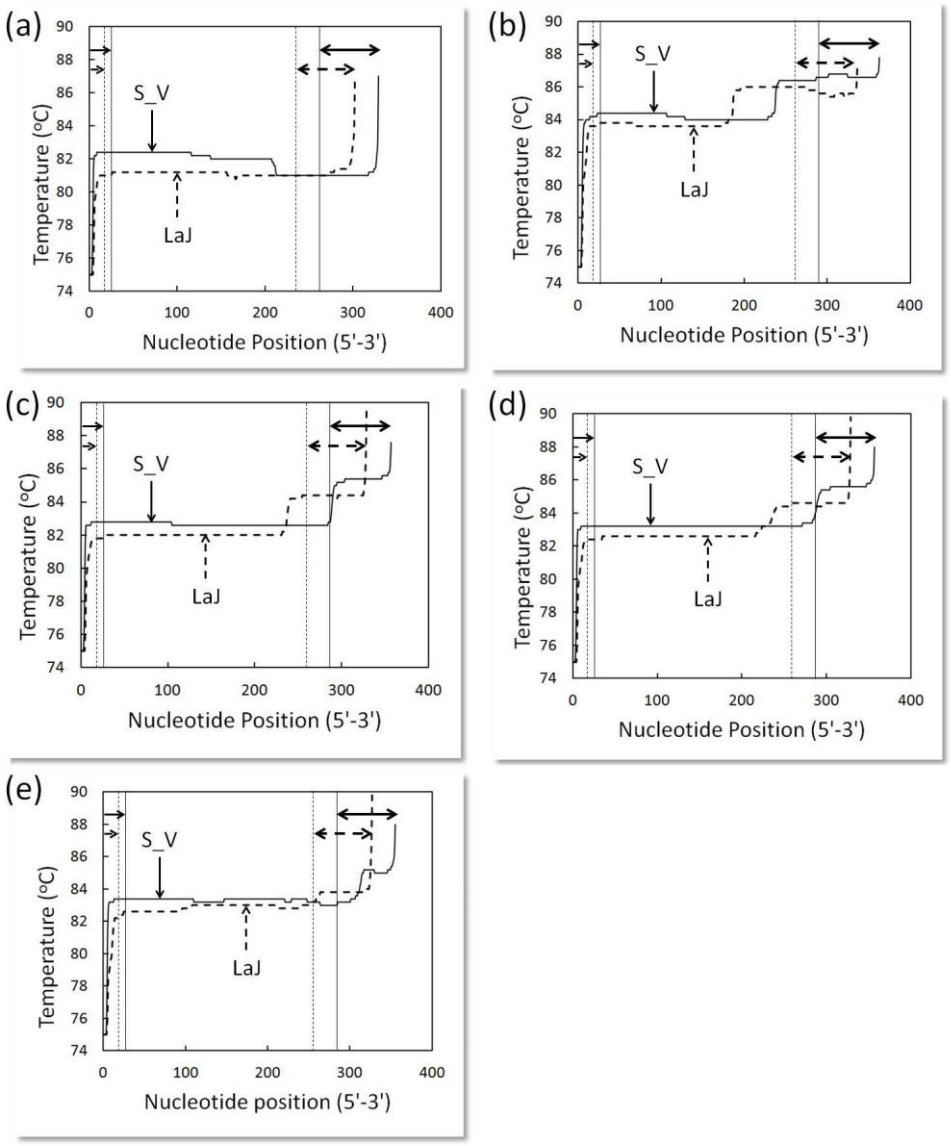

Figure 3.2 Tm50% melt profilesof the partial 5.8S, complete ITS2 and partial 28S –containing amplicons produced using the primer pairs SYM_VAR_5.8S/SYM_VAR_CLAMP (this study; solid line) and ITSintfor2/ITS2CLAMP (LaJeunesse 2002; dashed line) in *Symbiodinium* subclades A1, B1, C3, C15 and D1, (a), (b), (c), (d) and (e) respectively. Primer annealing sites relative to the nucleotide position are defined by the two headed (clamped primers) and one headed (unclamped, forwards primers) arrows and corresponding vertical lines; dashed arrows representing primers ITSintfor2/ITS2CLAMP, and solid arrows, SYM_VAR_5.8/CLAMP.

3.7 Empirical assessment of the SYM_VAR_5.8S/CLAMP pair – resolution of subclades A1, C3, C15 and D1

Given the predominance of clade B in the Western Atlantic and its rarity in the Indo-Pacific waters, its resolution was not tested empirically. The similarity of the theoretical melting profiles of the SYM_VAR- and LaJeunesse-amplicons of this clade's representative (B1) give further assurance that resolutions of, and within, this clade should be successful.

The different migrations patterns, and therefore successful resolution, of subclades A1 and C3 are demonstrated in Figure 3.3a. Multiple identical samples were loaded in this gel to assess the stability of the gradient formed. Identical migration distances of all related samples demonstrate a stable gradient throughout the gel. Figure 3.3b demonstrates the different and relative migration distances of A1, C3, C15 and D1. The migration distances concur with the theoretical melting profiles calculated, A1 having the lowest melting temperature domain and D1 the highest. B1 would be expected to run to a position below D1.

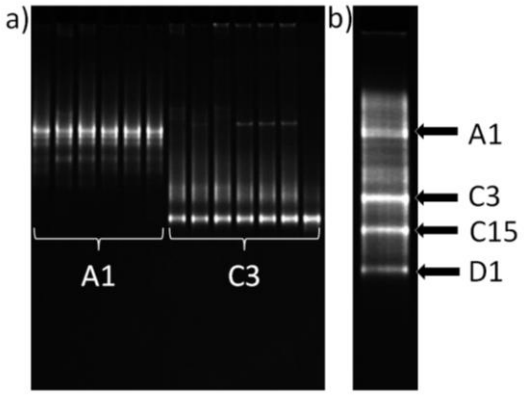

Figure 3.3 Annotated DGGE gel images - subcladal resolutions. (a) Migration differences between subclade A1 and C3. The first six lanes are run with *Symbiodinium* subclade A1 amplicons. The following seven lanes contain subclade C3. (b) A single well loaded with four separate amplicons one of each of the subclades A1, C3, C15 or D1. All amplicons are generated from PCR-amplicon-containing plasmid-based DNA.

3.8 Empirical assessment - resolution of subcladal variants

The ability of this DGGE system to detect sequence variants with differences as small as single nucleotide substitutions, deletions or insertions was tested through the use of previously identified (through previous sampling efforts of the PhD) and novel (not found in the NCBI database) variants of the generalist symbiont C3. Variants of C3 were chosen due to their predominance in the Gulf region as determined through previous studies (Hume et al. 2013). Figure 3.4 demonstrates the migration pattern of four of these variants as well as their corresponding sequences. The migration distances cover a range above and below the basal C3 sequence. Whilst the variant sequences are clearly separated from each other, the proximity of bands produced by C3.8 and C3.4 to the C3 band may prove difficult to resolve. Identification of predominant subclade(s) in circumstances such as these will likely be achieved through the combined consideration of major band migration position and secondary bands unique to a given symbiont diversity, termed a 'fingerprint' (see LaJeunesse 2002). These secondary bands are produced in environmental samples due to secondary amplifications and formations of different 2D structures of the predominant amplicon that will in turn lead to additional band formations. It should be noted that amplicons produced in the use of assessing the resolution capabilities of this DGGE system are based on PCR amplicon-containing-plasmid DNA templates rather than total DNA coral extracts from environmental samples.

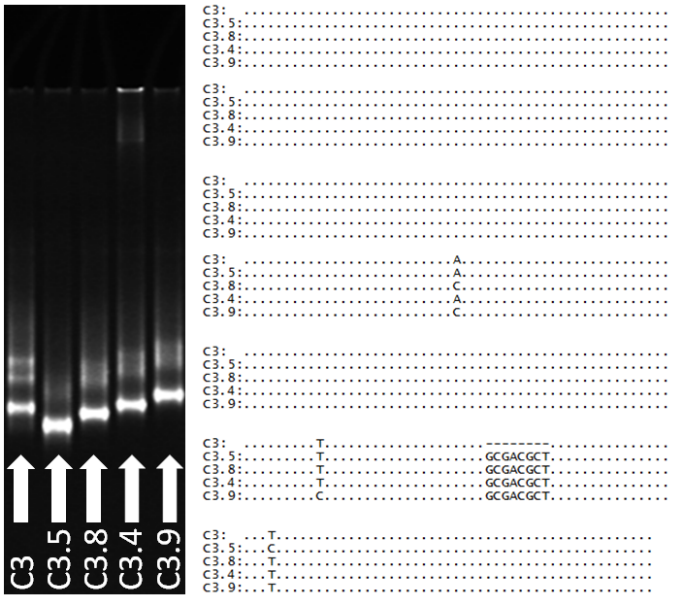

Figure 3.4 Annotated DGGE gel image – subcladal variant resolution. Lanes loaded with C3 and novel C3- subcladal variants, as annotated (left). Multiple sequence alignment of the five novel C3 variant sequences displaying only divergences from the basal C3 sequence, correlating the difference in migration pattern to unique sequence (right). A nucleotide in place of a dot (.) denotes a substitution from the C3 'type'. A dash (-) denotes an insertion/deletion (indel).

3.9 Diversity identified as part of the spatial characterisation of coral-*Symbiodinium* associations in the Persian/Arabian Gulf, Strait of Hormuz and Gulf of Oman using the novel DGGE system

Four dominant phylotypes of *Symbiodinium* were identified in the coral-*Symbiodinium* characterisation of the Gulf region: C3, C15, C15L and D1 (Figure 3.5a). All of these phylotypes with the exception of C15L had been previously identified in scleractinian corals. Phylotype C15L (named according to its migration characterisations amongst the novel C15 phylotypes identified in this study) accounted for 42% of the C15-cluster sequences (LaJeunesse 2005; Stat et al. 2009) identified in this study. Of the four variants of C15 identified in this study, three were previously unidentified and each of the C15 variants demonstrated different migration characteristics. In addition to the C15L variant, C15HM and C15LM were also named according to their migration (Figure 3.5b). Variants C15HM and C15L were defined by two and one

nucleotide substitutions in the 5.8S region, respectively (Figure 3.6b). However, no difference was seen between C15, C15HM and C15L in the less conserved ITS2 region. Notably, the nucleotide substitution that defines C15L as a variant from C15, lay within the annealing region of the ITSintfor2 primer. As such, this variant would not have been resolved had the DGGE analysis been run using the amplicon produced by the ITSintfor2/ITSCLAMP primer pair. The C15LM variant was also defined by a single nucleotide substitution – this lay in the ITS2 region. The differences in sequence that defined the different variants were responsible for changes to estimated melting profiles of as little as 0.1°C. This small change was apparently sufficient to cause a difference in migration and demonstrate the extreme sensitivity of this system.

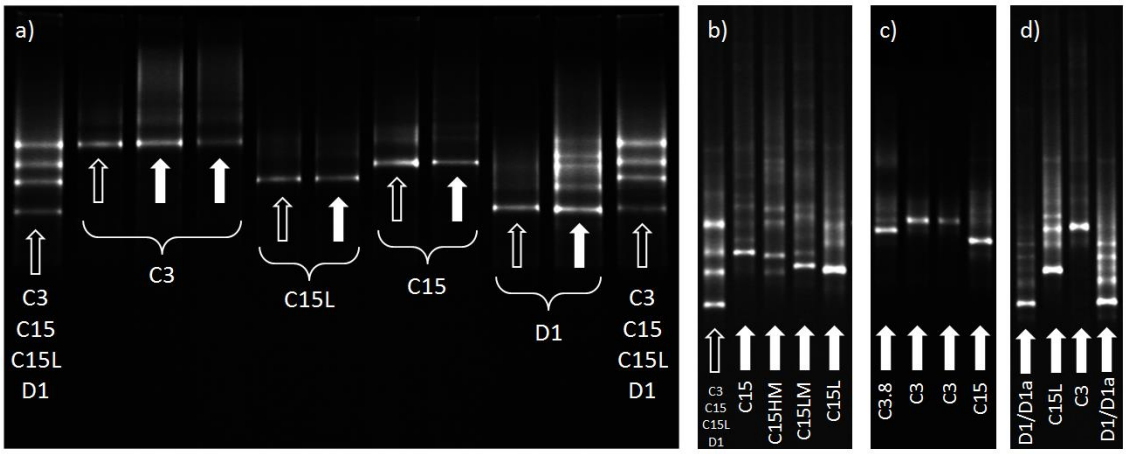

Figure 3.5 Annotated DGGE gels – Gulf variants. (a) Gel shows four differing amplicons representing the four predominant phylotypes identified in the Gulf. Lanes either side of the phylotypes contain a marker used for correlation of migration position to phylotype. (b) The four amplicon migration patterns representing the four phylotypes identified in the Gulf of Oman and Strait of Hormuz that belong to the C15-cluster. (c) Amplicon migration pattern of the only C3 derivative identified in Gulf region, C3.8 relative to basal C3 and C15. (d) The relative migration patterns and presence of mixed symbiont association D1/D1a compared to subclades C15L and C3. It should be noted that the D1a band in (d) is faint and runs below the brighter D1 ban. In all gels, hollow arrows represent amplicons amplified using PCR-containing-plasmid DNA as template whilst white filled arrows represent amplicons with template from environmental sample.

The waters of the gulf were characterised by a complete predominance of C3 coral-*Symbiodinium* associations. No C3 variants were identified as part of this study despite the presence of variants being observed in previous studies (in different species of corals; see chapter 4). Outside of the Gulf in the waters of the Strait of Hormuz, one single C3 variant sequence was resolved by the DGGE methodology, variant C3.8 (first identified in a previous sampling effort in waters of Abu Dhabi carried out as part of the present PhD body of work). This variant showed a distinctly different migration pattern to the ubiquitous C3 (Figure 3.5c), an advancement on the results demonstrated when empirically testing the ability of the DGGE system to resolve known variants of the C3 phylotype (Figure 3.2). The ability of the DGGE system to resolve this predicted 'problematic variant' provides support for the system's ability to discern other closely related variants, and in doing so, strengthens findings of a lack of C3 variation in the most recent study within the Gulf. The difference in migration patterns seen between the C3 and C3.8 act as supporting evidence for the ability of this DGGE system to resolve changes that occur not only in the lowest melting domain but in other domains characterised by higher melting temperatures – as demonstrated by the resolution of this variant despite the defining sequence difference being in a region of the 3' end of the ITS2 region, an area predicted to be defined by a higher temperature melting domain (Figure 3.6a).

The final subcladal variant identified as part of the spatial study was the D1a variant of the D1 phylotype. This phylotype produced a band that ran below the D1 type. The D1a variant was only identified as part of a mixed compliment, representing up to half of the symbiont compliment (inferred from band intensity; Figure 3.5d). Similar to the resolution of C15 variants, the single nucleotide substitution that defines the D1a phylotype causes an estimated rise in Tm50% melting temperature of approximately 0.2°C, a change apparently sufficient to modify the migration adequately to be resolved visually on a DGGE gel.

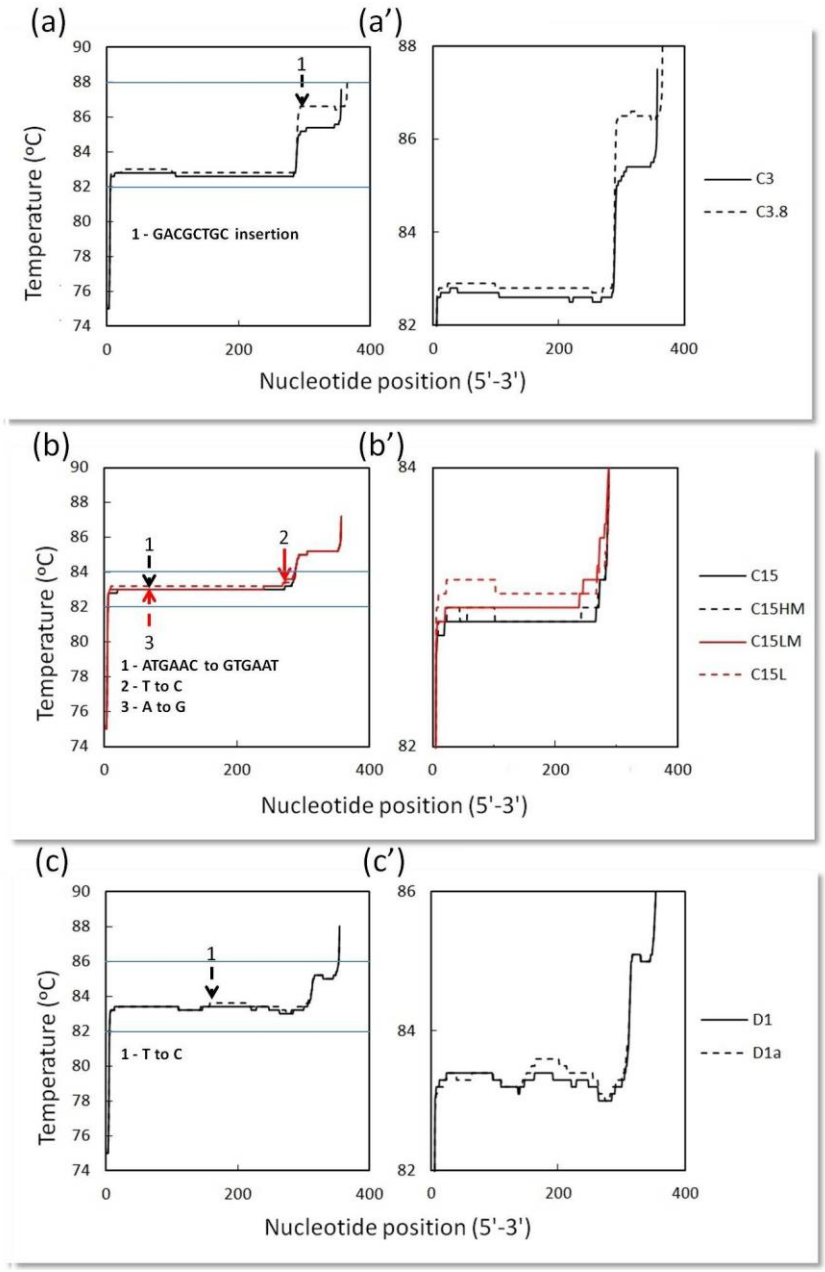

Figure 3.6 Tm50% melt profiles – Gulf Variants- of the partial 5.8S, complete ITS2 and partial 28S region of *Symbiodinium* rDNA as amplified by the SYM_VAR_5.8S/CLAMP primer pair. Several variants of each basal subclade type (left-hand set of plots; a) C3, b) C15, c) D1 are plotted. Each plot a-c has a corresponding plot a'-c' displaying the same data but with a more resolute Y-axis. The bounds of the Y-axis of the second plot are represented by horizontal blue lines on the first. Positions of sequence nucleotide differences relative to the basal 'type' amplicon fragment are represented by numbered arrows. The changes to the sequence at each arrow are listed on the corresponding plot. The colour and form of the arrow (e.g. red, dashed) correspond to the subclade variant listed in the corresponding plot key.

3.10 Evaluation of multiple band 'fingerprints'

The majority of samples analysed as part of the spatial characterisation contained only one predominant phylotype of *Symbiodinium*. However, several samples did harbour mixed phylotype compliments (predominantly D1/D1a). These profiles were distinguishable by the presence of single bands corresponding to migration characteristics of other phylotypes. Diversity assessment through 'traditional' molecular cloning of PCR amplicons displaying migration patterns attributable to mixed phylotypes confirmed the presence of multiple symbiont phylotypes.

Phylotype D1 produced four faint bands in addition to the stronger identifying band that ran at the same distance as the D1 marker, amplified from a PCR-fragment-containing plasmid. Multiple, fainter, additional bands were also seen in certain samples that had to be amplified using an extended number of PCR cycles (more than 30) due to low template amount and quality (due to polysaccharide contamination). These additional bands were generally 'doublets' found a small distance above the subclade identifying band (e.g. Figure 3.7). Both in this case and in the case of the D1 phylotypes, the additional bands were investigated to identify whether they might represent sequence variants from the main phylotypes. To do this, samples were initially run on a DGGE gel, secondary bands were excised, re-amplified and a further DGGE run performed on the resultant amplicon (Figure 3.7). In all investigated cases, 're-amplification amplicons' ran with the same profiles as the initial samples displaying the additional bands with the same migration properties. Intensities of the additional and phylotype identifying bands were also comparable. Given the identical appearance of the re-amplified profiles, the additional bands are likely caused by 2D conformations of the DNA sequences that lead to modifications of the physical properties and therefore migration characters of the sequences. A correlation between number of PCR cycles and intensity/presence of additional bands would further support this theory.

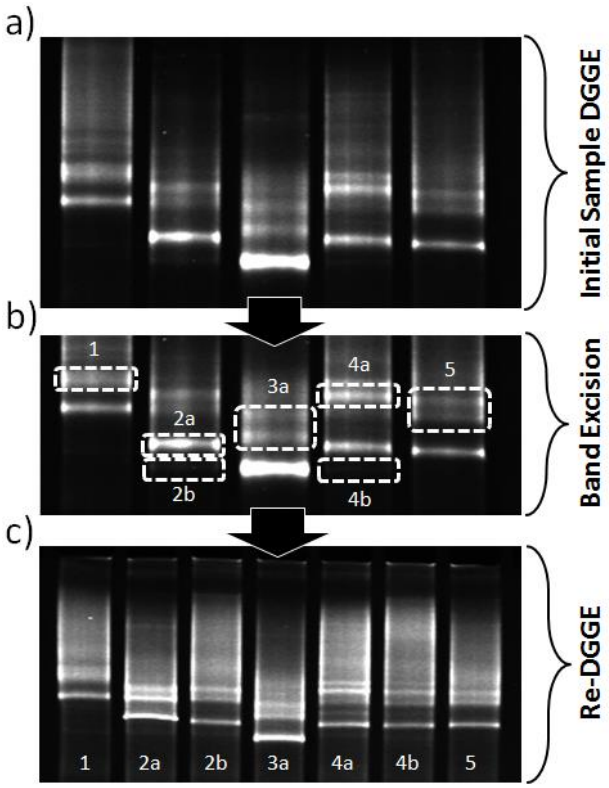

Figure 3.7 DGGE gels representative of the work flow involved in validating bands in multiple band profiles. In this example, five samples in question are run and imaged (a). Bands in question are excised (b) and re-amplified. The new amplicons are then re-run on a second DGGE gel (c) and the profiles re-examined. Labels and regions surrounded by a dashed box in (b) represent bands excised and re-run in (c).

3.11 Notes on voltage, run time, gradient formation, comb usage and the D-Code system, Bio-Rad

Differences between DGGE apparatus (Thornhill et al. 2010), and now primers, lead to variations in the voltages, runtimes and methodologies required to produce informative and reproducible results. Furthermore, the poor design and build quality of the D-Code system cause many additional technical challenges. An extensive investment in time and resources was made to undertake a thorough empirically-based optimisation of the Bio-Rad D-Code system as part of setup for the CRL. A summary of the optimal parameters produced is given here, as well as a discussion of several technical

considerations to aid others in the setup/consideration of the D-Code DGGE system.

Comb insertion depth (depth to which the bottom of the comb teeth inserted into the gel) affected the run time and denaturant gradients required due to the insertion of the PCR amplicon into different denaturant conditions. The relatively deep 20 well comb produced by Bio-Rad required running times and conditions significantly divergent from other combs and as such was not utilised in the study. The use of the 16 and 32-well combs produced by Bio-Rad were successfully used in combination with a 3hrs run time at 150v. Run times as short as 2.5hrs were successfully used with the 32-well comb but produced poor results with the 16 well comb.

The D-Code system is provided with two spacer sets, one grooved and one un-grooved. The use of both sets is directed in the user manual. However, use of the grooved set without the application of grease to the spacer will result in leakage and improper setting of the gel. It is advised that only spacers without a groove are used in the DGGE application.

The D-Code system requires the tight seal of the glass plate sandwich against a rubber gasket forming the upper reservoir chamber that is separated from the main body of running buffer, hence creating a potential difference. The sub-optimal seal of the plates against this gasket are due to poor build of the system and leaks are common, to the extent that it was not possible to achieve a complete seal in the system. The proper sealing of this aspect of the system is essential to creating sharp gel images. When improper seals are formed gel images appear blurry and undefined. Application of grease to the edges at either side of the gasket prevents leakages and allows proper use of the system.

The D-Code system allows the simultaneous running of two gel sandwiches. However, unequal heating at the back of the D-Code system (due to the proximity of the heating element; known problem, discussed in online forums) produces a gel with blurry bands. These gels are borderline in their ability to infer phylotypes, unless the differences in migration patterns are large. No solution is currently available to overcome this issue.

A denaturant gradient of 35-65% (40 ml deionised formamide and 42g urea per 100ml of 100% denaturant) was found most suitable based on theoretical followed by empirical testing. Theoretically the narrower the gradient range, the higher the resolution the DGGE system can produce. However, use of gradients with a range of less than approximately 25% caused uneven gradients to be produced through the use of the gradient former (model 475 Gradient Delivery System) provided with the D-Code system. Upper gradient parameters considerably below 65% resulted in the complete migration of bands out of the gel.

3.12 Summary

The creation of SYM_VAR_REV/SYM_VAR_FWD and the consideration of potential host amplification in their design, as well as their amplification of the entire ITS1-5.8s-ITS2 region (as opposed to merely the ITS1 or ITS2) position them as a useful resource for the coral-reef scientific community to allow for accurate phylotyping of *Symbiodinium*. The associated DGGE primer set SYM_VAR_5.8S/SYM_VAR_CLAMP that have been characterised and tested, theoretically and empirically, demonstrate advancements on previous systems including: consideration of host genotype in their design, length of sequence screened and the availability of an associated ITS1-5.8s-ITS2 primer set.

Chapter 4: Corals from the Persian/Arabian Gulf as models for thermotolerant reef-builders: Prevalence of clade C3 *Symbiodinium*, host fluorescence and *ex situ* temperature tolerance

Note: This chapter is published in Marine Pollution Bulletin:

Hume BCC, D'Angelo C, Burt J, Baker AC, Riegl B, Wiedenmann J (2013) Corals of the Persian/Arabian Gulf as models for thermotolerant reef-builders: Prevalence of clade C3 *Symbiodinium*, host fluorescence and *ex situ* temperature tolerance. Marine Pollution Bulletin, http://dx.doi.org/10.1016/j.marpolbul.2012.11.032

Contribution to chapter by BCCH: Joint contribution to coral husbandry; measurement of growth rates; genomic DNA extractions; *Symbiodinium* phylotyping; primer design; taxonomic analyses; joint contribution to collection and fixation of samples in the field; white light/fluorescence imagery of coral samples; thermal challenge experiment and documentation through fluorescence spectroscopy; contribution to manuscript preparation.

4.1 Abstract

Corals in the Persian/Arabian Gulf endure summer temperatures of up to 36°C, making them ideal subjects to study the mechanisms underlying thermal tolerance. Unexpectedly, we found the ''generalist'' *Symbiodinium* clade C3 to be the prevalent symbiont among seven coral species from Abu Dhabi (UAE) waters. Moreover, C3 represented the only dominant symbiont type in *Porites spp.* from this region. The ''thermotolerant'' symbionts D1a and C15 were not encountered, indicating that the association with these symbionts cannot be the sole reason for the heat tolerance of Gulf corals. The association of *Porites lobata* with specific symbiont types (C3 vs. C15) in samples from habitats with very different temperature regimes (Abu Dhabi vs. Fiji) remained unaffected by laboratory culture. During temperature stress experiments specimens from

both locations strongly downregulated green fluorescent protein (GFP)-like pigments. However, the Abu Dhabi samples were less prone to bleaching and showed lower mortality.

4.2 Introduction

Hermatypic corals are primarily responsible for the formation of modern reefs that succeed in oligotrophic waters due to the light dependent symbiosis with unicellular dinoflagellate algae (zooxanthellae) belonging to the genus *Symbiodinium* (Muscatine and Porter 1977). Several environmental factors, such as changes in light levels and seawater temperature can trigger the loss of zooxanthellae from the host (Brown 1997; Douglas 2003; Baker et al. 2008). When the visual appearance of the coral becomes dominated by the white colour of the skeleton showing through the tissue, the phenomenon is known as ''bleaching''. Bleaching due to thermal stress is considered to be induced by the algal production of reactive oxygen species (ROS), particularly H_2O_2 (Smith et al. 2005; Lesser 2006; Suggett et al. 2008; Tchernov et al. 2011) Although the animal host might survive and recover, mass mortality is frequently observed among bleached corals (Glynn 1996; Baker et al. 2008). Over the past decades, mass bleaching events associated with elevated seawater temperatures have become more frequent and have contributed to the rapid degradation of coral reefs (Hughes et al. 2003; Baker et al. 2008; Burt et al. 2011). Currently, an estimated 30% of reefs are severely damaged and >50% may be lost within the next two decades according to climate predictions (Hughes et al. 2003; Sheppard 2003).

In general, temperatures as little as 1°C above the average annual maximum can trigger bleaching, but the actual bleaching threshold depends also on the duration of temperature stress and the stress history of the corals (Glynn 1996; Baker et al. 2008). Furthermore, the threshold for heat-induced bleaching can be lowered by unfavourable concentrations of inorganic nutrients in the water column (Wiedenmann et al. 2013). Particularly, elevated levels of dissolved inorganic nitrogen can increase bleaching susceptibility (Wooldridge 2009; Wagner et al. 2010; Wiedenmann et al. 2013) and this may be exacerbated by higher densities of symbionts in nutrient-enriched corals (Wooldridge 2009).

Symbiotic corals can acclimatise or adapt to temperature stress(Coles and Brown 2003). While many coral reefs experience maximal temperatures of ~29°C and annual temperature fluctuations of ~4°C some coral communities within the Gulf of Oman and the Gulf can cope with exceptionally high seasonal temperature maxima (32–36°C) as well as daily (up to 9°C) and annual (~20°C) fluctuations (Kinsman 1964; Coles 1997; Figure 4.1 and Table 4.1; Riegl et al. 2011). The existence of these coral communities makes it evident that at least some hermatypic corals can survive under conditions that are predicted elsewhere as a result of global warming within the next centuries. Gulf corals have been subjected to a hotter climate than other corals for only ~6 ka years (Purkis et al. 2010), over which period the observed heat resistance presumably developed. The understanding of the mechanisms underlying this acclimatisation/adaptation potential is of crucial importance to accurately forecast the global fate of coral reefs.

Table 4.1 Temperature regimes of coral habitats in the Gulf and selected regions elsewhere

Region	**Site**[2]	**Max T**[1]	**Min T**[1]	**Annual ΔT**[1]
Persian Gulf	Abu Dhabi, UAE[3]	36. 0	16.0	20.0
	Abul Thama, Bahrain	32.0	19.2	12.8
Gulf of Oman	Muskat, Oman	30.2	23.0	7.2
Western Indian Ocean	Mahe, Seychelles	29.4	25.4	4.0
	Chagos Archipelago	29.2	26.8	2.4
	Mombasa, Kenya	29.2	25.0	4.2
	Blue Bay, Mauritius	27.7	23.0	4.7
	Lakshadweep, India	29.6	27.3	2.3
	Ningaloo, Australia	28.2	23.4	4.8
Indo-Pacific	East coast, Taiwan	29.0	23.9	5.1
	Apo Reef, Philippines	29.7	27.0	2.7
	Wakatobi, Indonesia	29.6	27.0	2.6
Pacific Ocean	Beqa, Fiji	28.0	24.6	3.4
	Heron Island, Australia	27.3	21.5	5.8
	Davies Reef, Australia	28.5	24.0	4.5

	Oahu, Hawaii, USA	27.0	24.2	2.8
Atlantic Ocean	US Virgin Islands, USA	28.5	25.8	2.7
	Abrolhos Reefs, Brazil	28.0	24.6	3.4
	Bermuda	27.0	19.6	7.4
	Palm Beach, USA	29.0	24.2	4.8

[1] MaxT: Maximum mean Temperature, Min T: Minimum mean Temperature, Annual DT: Annual Temperature Oscillation. All values given in degrees C.

[2] NOAA Coral Reef Watch 50-km Satellite Virtual Station http://coralreefwatch.noaa.gov/satellite/vs/index.html

[3] Data from: Coles S.L. (2003) Coral species diversity and environmental factors in the Arabian Gulf and the Gulf of Oman: A comparison to the Indo-Pacific region. Atoll Research Bulletin. p1-p19.

The heat stress tolerance of corals could potentially be supported at different levels, for instance by the protection of cellular components through an enhanced antioxidant defence (Lesser 1997) or elevated level of heat shock proteins (Choresh et al. 2004) or by higher levels of photoprotective substances such as mycosporine-like amino acids (Dunlap and Shick 1998) or green fluorescent protein (GFP)-like pigments (Salih et al. 2000; Dove et al. 2001). Yet another strategy might be to host symbiont communities dominated by thermotolerant symbionts, either by the uptake of new symbionts after a bleaching event or by changing the relative abundance of symbiont types already present in the host (symbiont shuffling) (Rowan et al. 1997; Baker 2001; Baker et al. 2004; Berkelmans and van Oppen 2006; Jones et al. 2008). However, it is not yet established whether this observed acclimatisation will allow the corals to survive in the long run since only 23% of coral species may be able to change their symbionts (Goulet 2006; Goulet 2007). A study conducted in 2000–2001 found that ~60% of the sampled coral colonies from the Gulf were associated with *Symbiodinium* Clade D, a zooxanthellae strain considered to be thermotolerant (Baker et al. 2004). Clade D symbionts were also detected in corals off the Iranian coast (Mostafavi et al. 2007; Shahhosseiny et al. 2011). Hence, the resilience of corals from the Gulf was considered to be at least partially due to the association with this particular *Symbiodinium* clade.

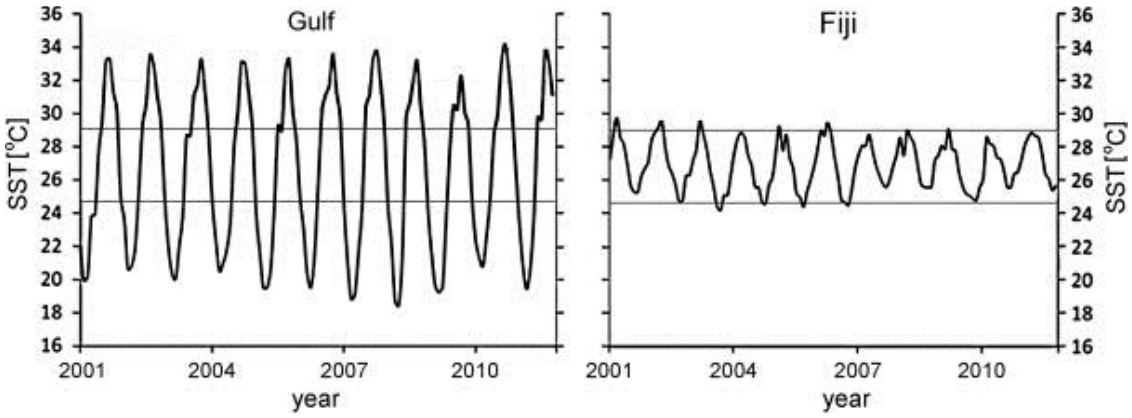

Figure 4.1 Sea Surface Temperature (SST) profiles characteristic for the coral communities in the Gulf (data from Abul Thama, Bahrain) and Fiji (data from Bega, Fiji).The graphs show the SST values from the beginning of December 2000 to mid-October 2011. The typical fluctuation range of the Fiji location is marked by horizontal lines. Source data retrieved from NOAA Coral Reef Watch (http://coralreefwatch.noaa.gov/satellite/intex.html) for 50km Satellite Virtual Stations.

Here, we have examined the dominant symbiont clades in seven corals species (*Cyphastrea microphthalma, Favia pallida, Platygyra daedalea, Porites harrisoni, Porites lobata, Porites lutea and Acropora clathrata*) growing off the Abu Dhabi coast (UAE) to extend the data set on zooxanthellae associated with heat tolerant corals. We report that the 'generalist' C3 is the prevalent *Symbiodinium* in these corals, rather than members of clade D, indicating that the association with these symbionts cannot be the sole reason for the heat tolerance of Gulf corals. Hence, the more complex mechanisms underlying the thermal tolerance of Gulf corals call for further experimental studies. Molecular and physiological analyses under tightly controlled laboratory conditions hold great promise to improve the knowledge about the mechanisms underlying the heat tolerance of reef corals (D'angelo and Wiedenmann 2012). While other fields of (molecular) biology rely heavily on the thorough analysis of a few model organisms, at the moment there are not many coral models for laboratory studies (Weis et al. 2008). However, recent progress in coral culture enables the propagation of these organisms in excellent physiological conditions over many years or even decades (D'angelo and Wiedenmann 2012) and experiments with laboratory strains have already yielded important insights into physiological processes in reef corals (Allemand et al. 2004; D'Angelo et al. 2008; D'Angelo et al. 2012).

Corals from the Gulf region, for instance *P. lobata*, are of great importance as reef builders because they have a wide global distribution (Veron 2000). They offer the opportunity for comparative studies and thus represent excellent models to study the capacity of corals to adapt to increasing water temperatures. Here, we characterised *P. lobata* from Abu Dhabi (UAE) reefs adapted to maximal monthly mean temperatures of ~34°C as laboratory model for heat stress tolerance. For comparison, we analysed conspecifics from Fijian reefs (Pacific Ocean) where maximal monthly mean temperatures usually do not exceed 28°C (Coles 2003) (Figure 4.1). We succeeded in establishing laboratory cultures of *P. lobata* from these two regions and have propagated them by fragmentation over 2 years in the coral mesocosm at the Coral Reef Laboratory at the University of Southampton, UK (D'angelo and Wiedenmann 2012). Furthermore, we examined the effects of long-term aquarium culture on the community structure and stability of algal symbionts in these corals.

Tissue levels of GFP-like proteins respond to a variety of stimuli such as altered light levels, mechanical damage or the presence of epibionts; which makes these pigments easily accessible biomarkers of environmental stress (D'Angelo et al. 2008; D'Angelo et al. 2012). Hence, we have tested how GFP-like host pigments respond to elevated temperatures and evaluated their suitability as intrinsic heat stress markers in *P. lobata*. We present a thorough spectrometric characterisation of the GFP-like complement in *P. lobata* that establishes their downregulation in response to temperature stress. The well characterised laboratory models presented in this paper will facilitate future studies of the mechanisms underlying thermal tolerance of hermatypic corals.

4.3 Materials and methods

4.3.1 Coral material, collection and culture

Field identification relied on local experts. Fragments of UAE and Fiji samples that were identified as *P. lobata* during field collection were bleached in sodium hypochlorite solution and inspected under a microscope (MZ10, Leica). Analysis of the corallite structure (Veron 2000) confirmed the field identification of these samples. The samples were distinguishable from *P. lutea* by the lack of a protruding ring of five tall pali, and from *P. solida* by the lack of a flattened columella. However, the identifications need to be considered

within the limits set by the taxonomic value of skeletal features among *Porites spp*. (Forsman et al. 2009).

Samples for *Symbiodinium* analyses were removed from colonies of *C. microphthalma, P. daedalea, F. pallida, P. lobata, P. harrisoni and A. clathrata* located in 4–6 m depth on Saadiyat reef and Dhabiya reefs, 15 km northeast and 30 km southwest of Abu Dhabi city, respectively, on 6 October 2010. Material was collected from colonies that were situated at least 10 m apart from each other, frozen and stored at -80°C. Subsamples were shipped in RNA later to the University of Southampton for further analyses. Additional samples of *P. lobata, P. harrisoni* and *P. lutea* were collected on 17 September 2012 on Saadiyat reef and fixed in EtOH (p.a.) immediately after collection.

Specimens of *P. lobata* were collected live from 4 m depth on Saadiyat reef in Abu Dhabi, United Arab Emirates (24°35'56.4''N, 54°25'17.4''E) in May 2010. Fragments (~10 x 10 cm) of individual colonies were removed with a hammer and chisel. Parental colonies were approximately 60 cm in diameter, typical for colonies of this species in the southern Gulf.

The colonies were taken immediately to New York University – Abu Dhabi and maintained for 15 days in individual aquaria at 32°C and a salinity of 42 ppt on a 10:14 h light:dark regime under metal halide lights. Afterwards, they were transported submerged in ~500 ml of seawater in individual vessels in an insulated container to the University of Southampton, UK, where they arrived within 48 h. After arrival, the colonies were introduced to a 500L unit of the experimental mesocosm of the Coral Reef Laboratory, in which the salinity was adjusted to 42 ppt, the value measured in the transport water. Over the first week, light levels were kept low (50 µmol photons/m^2/s) to reduce stress. Afterwards, the light intensity was increased to ~150 µmol photons/ m^2/s over a period of 2 weeks. Lamps were operated on a 10 h/ 14 h light/dark cycle. The temperature in the tanks was kept at 27.5°C.

P. lobata collected from Fiji reefs in the vicinity of Galao Village were acquired through the ornamental trade (Tropical Marine Centre, London, UK) in November 2009. The corals were received approximately 2 weeks after collection. Small fragments were fixed in EtOH immediately after arrival for symbiont phylotyping.

The choice of conspecifics from these two disparate geographical areas as material for comparative physiological experimentation was driven by considering the markedly different annual temperature profiles that characterise these regions (Figure 4.1). The Fijian corals were kept in a separate compartment of the mesocosm at a salinity of 33 and a constant temperature of 24.5°C. A detailed description of the aquarium system is provided in (D'angelo and Wiedenmann 2012).

To examine the effects of aquarium culture on the stability of the zooxanthellae symbiosis in *P. lobata* from both locations, fragments were removed from the colonies 15 months after their introduction in our mesocosm for *Symbiodinium* re-identification.

4.3.2 Identification of algal symbionts

The genetic identification of *Symbiodinium* at the subclade level was performed by analysis of the ITS2 sequence region of genomic DNA samples. DNA was prepared from coral tissue using a modification of a described method (Sokolov 2000). Coral tissue was scraped off the skeleton with a scalpel, quickly frozen in liquid nitrogen and crushed to a fine powder which was resuspended in one volume of lysis buffer containing 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 10 mM EDTA (pH 8.0) and 1% SDS and an equal volume of Tris-buffered phenol. After phase separation by centrifugation (14,000 rpm, 4°C, 5 min), the aqueous phase was removed and extracted twice with phenol:chloroform:isoamyl-alcohol (25:24:1) and once with chloroform:isoamyl-alcohol (24:1). A last purification step was performed by adding 0.1 volumes of saturated KCl and incubating on ice for 5 min. After centrifugation, the supernatant was transferred to a new tube and the DNA was precipitated from the supernatant by the addition of one volume of isopropanol, recovered either using a pipette tip or by centrifugation (14,000 rpm, 4°C, 20 min), washed once with 70% ethanol, air dried and resuspended in milliQ water.

The DNA region spanning the 18S, ITS1, 5.8S, ITS2, 28S region (Savage et al. 2002) of the *Symbiodinium spp.* ribosomal DNA was amplified with the primers SYM_VAR_FWD (5'CAGCTTCTGGACGTTGYGTTGG3') and SYM_VAR_REV (5'CGGGTTCWCTTGTYTGACTTCATGC3'), designed specifically to anneal in the

18S and 28S regions of the *Symbiodinium spp.* (clades A–E) rDNA. The suitability of these primers was confirmed by the successful amplification of the correct fragments from the *Symbiodinium* subclades A, C and D (data not shown).

Amplification was accomplished in the presence of 200 μM of each dNTP, 0.5 μM of each primer, 50ng of gDNA and 0.5 μl Advantage 2 polymerase mix (Clontech) per 50 μl final reaction volume. The PCR encompassed an initial denaturation step of 1 min at 98°C followed by 35 cycles of denaturation (95°C/30s), annealing (62°C/30s), and elongation (68°C/1min). A final elongation step of 2 min was performed at 68°C. PCR products were purified using the Jetquick gel extraction spin kit (Genomed), incubated with taq polymerase for 20 min at 72°C to incorporate A-overhangs, and cloned using StrataClone (Stratagene). Plasmid DNA was prepared from *E. coli* colonies using a QIAprep Spin Miniprep Kit (Qiagen). Sequencing services were provided by Macrogen.

Identification of *Symbiodinium* types was achieved by comparison of the ITS2 sequence (220 bp) with reference sequences available in Genbank using the BLAST tool (www.ncbi.nlm.nih.gov/ blast).

4.3.3 Determination of growth rates

After 6 months of acclimation to the tank conditions, colonies of Fijian and Arabian *P. lobata* were fragmented to obtain the experimental replicate colonies and the fragments were attached to glass tiles using cyanoacrylate glue. At four time points over the experimental period of 11 weeks, the wet weight of the replicate colonies was determined. Before each weighing, algal films were removed from the surface of the glass tiles using a toothbrush whilst keeping the colony submerged. Colonies were gently inverted to remove excess water and the base of each glass tile was blotted dry for 10s before weighing.

4.3.4 Photographic documentation and spectroscopic analyses

Fluorescent and white light photographs of coral tissue were taken using a Leica DFC420C camera connected to a Leica MZ10F fluorescence microscope as described in (D'Angelo et al. 2012). Excitation and emission spectra were

recorded on the live animal tissue of colonies submerged in a small temperature controlled transport aquarium using a fibre optic probe connected to a Varian Cary Eclipse fluorescence spectrophotometer as previously described (Leutenegger et al. 2007; D'Angelo et al. 2008). The excitation spectra of cyan and red light emitting pigments were obtained with emission wavelengths set at 550 nm and 630 nm, respectively.

4.3.5 Heat stress treatment

Experimental corals were separated in control and treatment groups. Geographical origin was represented by 6 replicate colonies which were derived from two distinct mother colonies. Each group was maintained in a 40 l tank. The tanks received identical light levels (photon flux of 150µmol/m^2/s) and the same water movement was created with turbelle pumps (Tunze). Water changes (~15%) were performed daily using water from the main compartment of the mesocosm in which the Abu Dhabi corals were cultured at a salinity of 42 ppt. For the Fiji coral tanks, the salinity was adjusted to 33 ppt by dilution with demineralised water.

Prior to the experiment, the corals were acclimated to 27.5°C for 1 week. Subsequently, the temperature was gradually increased to 31.5°C (ramping 0.5°C/day). The temperature was then kept constant at 31.5°C for a period of 21 days. Afterwards, the corals were incubated at lower temperatures (27.5°C) to allow recovery. Two tanks were used as controls for each geographical location: the control tank for Fijian corals was maintained at 24.5°C; whereas the Arabian corals were kept at 27.5°C.

The effect of heat stress on the coral holobiont was monitored by recording the number of colonies that appeared completely bleached under the microscope. Additionally, coral fluorescence was documented microscopically and by measuring the fluorescence emission of the host tissue.

4.4 Results

4.4.1 Algal symbionts of Abu Dhabi corals

To help identify a model containing symbionts representative of the Abu Dhabi region, we analysed the symbiont communities in 36 colonies representing six common Gulf coral species.

Sequencing of 146 ITS2 fragments revealed *Symbiodinium* C3 as the most common symbiont type in these Gulf coral species, namely *P. lobata, P. lutea, P. harrisoni, C. microphthalma, F. pallida, P. daedalea* and *A. clathrata* (Table 4.2). This symbiont was detected either exclusively (in *P. lobata* and *P. lutea*) or in combination with other clades, predominantly with A1. In *C. microphthalma*, both colonies analysed harboured a mixture of C3 and A1. The same combination was found also in one colony each of *P. daedalea* and *P. harrisoni*, whereas all other colonies of these species were dominated by C3 symbionts. C3 was also prevalent in different colonies of *A. clathrata*. Among the analysed coral species, the greatest diversity in symbiont association was detected in *Favia pallida*, where types C3, A1 and C15 were found in one single colony. The other samples of this species yielded A1 or a mixture of C3 and A1. The types C15, C21 and C36 were only represented by a single sequence each, indicating the background presence of these symbiont strains in *F. pallida*, *P. daedalea,* and *A. clathrata*, respectively. None of the samples contained any detectable clade D symbionts.

Table 4.2 *Symbiodinium* clades in corals from the Abu Dhabi coastline

Coral species (collection year)	Analysed colonies	Sequences per individual colony	Clades (sequence ratio) per individual colony
Cyphastrea microphthalma (Oct. 2011)	2	11	C3:A1 (2:9)
		5	C3:A1 (3:2)
Favia pallida (Oct. 2011)	2	5	C3:C15:A1 (1:1:3)
		9	C3:A1 (1:8)
		6	A1 (6:0)
Platygyra daedalea (Oct. 2011)	2	10	C3:A1 (3:7)
		8	C3:C36 (7:1)
Porites harrisoni	3	7	C3:A1 (3:4)
		8	C3 (8:0)

(Oct. 2011)		7	C3 (7:0)
Porites harrisoni (Sept. 2012)	5	4	C3 (4:0)
		1	C3 (n.a.)
		3	C3 (3:0)
		3	C3 (3:0)
		3	C3 (3:0)
Porites lobata (Oct. 2011)	3	5	C3 (5:0)
		5	C3 (5:0)
		5	C3 (5:0)
Porites lobata (Sept. 2012)	5	5	C3 (5:0)
		3	C3 (3:0)
		3	C3 (3:0)
		3	C3 (3:0)
		1	C3 (n.a.)
Porites lutea (Sept. 2012)	5	4	C3 (4:0)
		4	C3 (4:0)
		4	C3 (4:0)
		3	C3 (3:0)
		3	C3 (3:0)
Acropora clathrata (2010)	8	1	C21x1, C3x7
total	**35**	**146**	**C3:A1:C15:C21:C36 (104:39:1:1:1)**

4.4.2 Culture of *P. lobata* from the Gulf in a closed aquarium system under controlled conditions

We established a laboratory culture of *P. lobata* from Abu Dhabi waters in the experimental mesocosm of the Coral Reef Laboratory at the University of Southampton (D'angelo and Wiedenmann 2012). All colonies survived removal from the reefs, transport and acclimation to aquarium conditions. After 6 months, the mother colonies were fragmented and glued to colour coded tiles. The growth of the replicate colonies was followed over a period of 11 weeks. By the end of the study period, the replicates had visibly extended over the tiles by encrusting growth (Figure 4.2a). The wet weight of the colonies was determined at 3 week intervals. The growth could be best described with an exponential function, resulting in an average increase in wet weight of 50% by the end of the monitoring period of 11 weeks (Figure 4.2b). Normalised to the tissue covered surface area of the replicate colonies at the beginning of the experiment, this increase corresponds to a weight gain of ~1.3 ± 0.2 g/cm^2/year.

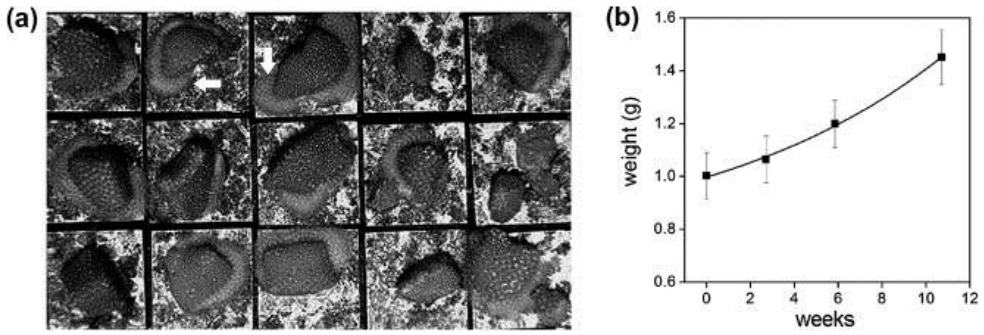

Figure 4.2 Growth of replicate colonies of *P. lobata* from Abu Dhabi in the laboratory.(a) Photograph of replicate colonies growing in the experimental mesocosm at the University of Southampton. Areas of encrusting growth can be distinguished by the light colour of the tissue. (b) Data points represent the mean weight of 60 replicate colonies produced by fragmentation of 4 individual mother colonies. An exponential fit (r^2 = 0.998) illustrates the average weight gain over a time course of 11 weeks. Error bars denote the standard error of the means.

4.4.3 Identification of algal symbionts in laboratory-cultured *P. lobata*

Understanding the potential effect of laboratory culture on algal symbiont community composition is of crucial importance for interpreting the responses to experimental treatments. Therefore, we investigated whether the transfer of *P. lobata* from Abu Dhabi and Fiji waters to the aquarium resulted in changes to the dominant symbiont, e.g. due to transport stress, changes in environmental conditions, or due to the exchange of symbionts (Baker et al. 2004; Berkelmans and van Oppen 2006) with other tank inhabitants. We determined the dominant *Symbiodinium* associated with *P. lobata* after 15 months of laboratory culture and compared the composition with those from samples fixed immediately after collection in the field. The analysis of four genotypes of *P. lobata* derived from 4 individual colonies from Abu Dhabi waters after 15 months of laboratory culture found C3 as the only symbiont type (Table 4.3). In contrast, *P. lobata* samples from Fiji harboured exclusively C15, both after collection and after 15 months of growth in captivity (Table 4.3).

Table 4.3 *Symbiodinium* clades in *P. lobata* before and after 15 months of laboratory culture

Origin	Analysed colonies	Sequences	Clades
Abu Dhabi	8	30	C3
NOCS mesocosm (Abu Dhabi origin)	4	16	C3
Fiji	4	14	C15
NOCS mesocosm (Fiji origin)	3	9	C15

4.4.4 Patterns of host fluorescence in *P. lobata*

GFP-like proteins can potentially serve as indicators of physiological changes in coral-zooxanthellae association (Dove et al. 2006; D'Angelo et al. 2008; Smith-Keune and Dove 2008; D'Angelo et al. 2012). Therefore, we examined the specific complement of host pigments for the model corals, determining the excitation and emission spectra *in situ* after 15 months of aquarium culture. When excited with blue light, *P. lobata* from Abu Dhabi exhibited cyan fluorescence in the polyps and the coenosarc (Figure 4.3a). Similarly, emission in the cyan spectral region was detected in animals originating from Fiji, although in these corals the intensity tended to be stronger in the polyps compared to the coenosarc (Figure 4.3b). In agreement with a detailed study of the expression patterns of GFP-like proteins (D'Angelo et al. 2012), the red fluorescence was regularly observed in the growing margins in samples from both origins.

Spectroscopic analyses performed *in situ* in corals from Abu Dhabi, revealed the presence of a cyan fluorescent protein (CFP) with excitation and emission maxima at 466 nm and 490 nm respectively (Figure 4.3c). The excitation/emission maxima of the CFP for Fiji samples were located at 486 nm/498 nm (Figure 4.3d). A red fluorescent protein characterised by excitation

maximum at 574 nm and emission maximum at 610 nm was detected in the margins of representative colonies from both locations (Figure 4.3e and f).

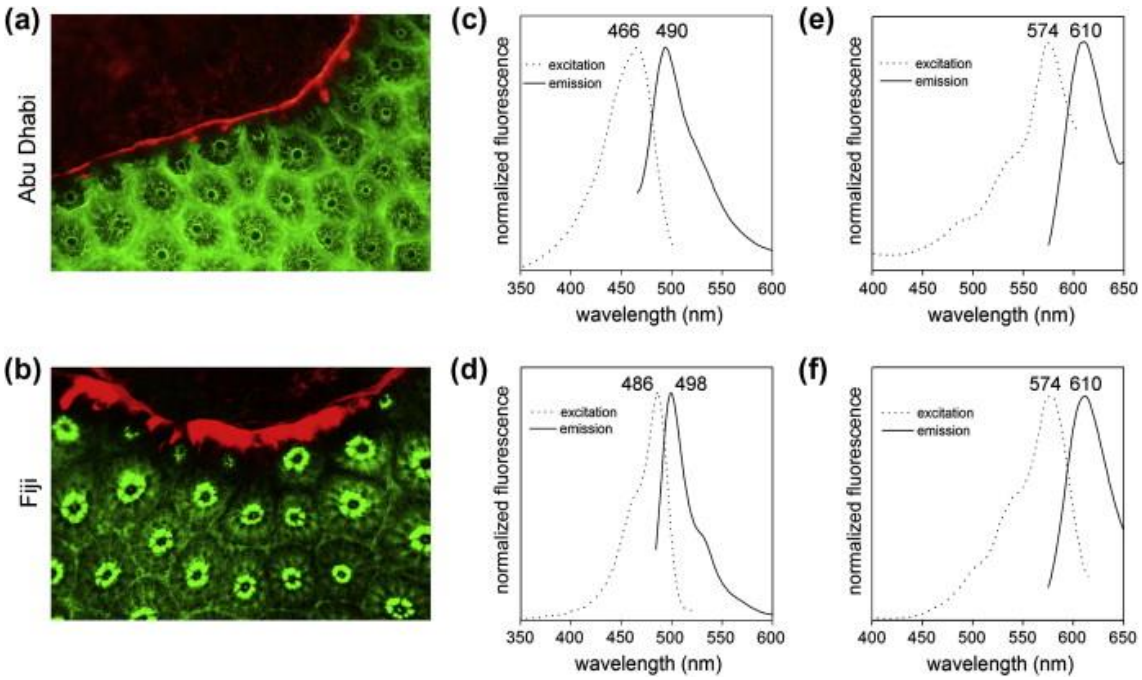

Figure 4.3 Fluorsecence of GFP-like proteins in the host tissue of *P. lobata*. Fluorescence photographs of representative colonies from Abu Dhabi (a) and Fiji (b) taken under the fluorescence microscope using a CFP/DsRed dual band pass filter. Images were produced using the green and red channel data. Cyan fluorescence is emitted from polyps and the coenosarc. The red fluorescence is restricted to the growing margin of the colonies (visible as the upper colony border in panels a and b). The normalised excitation and emission spectra of cyan (c and d) and red (e and f) fluorescent proteins were recorded directly on the expressing tissue of representative colonies from Abu Dhabi (c and e) and Fiji (d and f). The positions of the fluorescence maxima (nm) is given by the numbers next to the peaks.

4.4.5 Heat stress responses of *P. lobata* from different geographical origins

We tested whether the thermal tolerance of Abu Dhabi corals is retained under laboratory conditions. For this purpose, we examined the response of *P. lobata* from Abu Dhabi and from Fiji to a 21-day heat stress treatment. We used a fibre optic probe coupled to a spectrometer to quantify changes in the cyan fluorescence emission as an indicator of physiological response of the host tissue. Samples from both locations responded to the increased temperatures by a dramatic reduction of cyan fluorescence. After 10 days at 31.5°C, the cyan

fluorescence of both Arabian and Fijian corals was essentially lost (Figure 4.4). Fluorescence microscopic inspection revealed that the red fluorescence of the colony margins also vanished within this period (Figure 4.4).

Over the course of the treatment, the replicate colonies were inspected under the microscope at weekly intervals to monitor changes to the yellow-brown colouration derived from zooxanthellae pigments visible through the tissue. Colonies were considered to be completely bleached as soon as no yellow-brown colour was visible in the tissue under the microscope (Figure 4.4). We note that photographic images of such colonies showed areas which appeared darker (e.g. left margin in the bleached colony in Figure 4.4c). This colouration was contributed by pigmentation anomalies of the skeleton, possibly indicating the presence of endolithic algae. The heat-treated colonies of *P. lobata* from Abu Dhabi showed a paler appearance compared to the controls but none of them bleached completely (Figure 4.4c). In contrast, ~27% of all Fijian colonies were completely bleached already after 5 days of treatment. This value increased to 50% after 2 weeks of heat exposure and, at day 21, all Fijian corals were affected. At this stage, most of the remaining coral tissue was lost. Subsequently, all colonies were transferred to ambient temperatures to monitor the recovery potential over a 2-month period. Fifteen days after being returned to control conditions, host tissue fluorescence was clearly detected under the microscope in the Abu Dhabi samples, particularly in the polyps (Figure 4.4d). The cyan fluorescence emission increased over time in all replicates of Abu Dhabi origin, except for one sample which showed no increase in fluorescence and ultimately died. After 56 days of recovery, the surviving colonies displayed tissue fluorescence comparable to the control samples when inspected under the microscope. This observation was confirmed by spectrometric recordings of the tissue fluorescence (Figure 4.4d). In contrast, only a few polyps of the Fiji corals exhibited cyan fluorescence under the microscope at the beginning of the recovery period. However, this local emission was below the detection limits of the spectrofluorometic measurements (Figure 4.4d). None of these samples recovered their initial fluorescence and eventually all of them died completely.

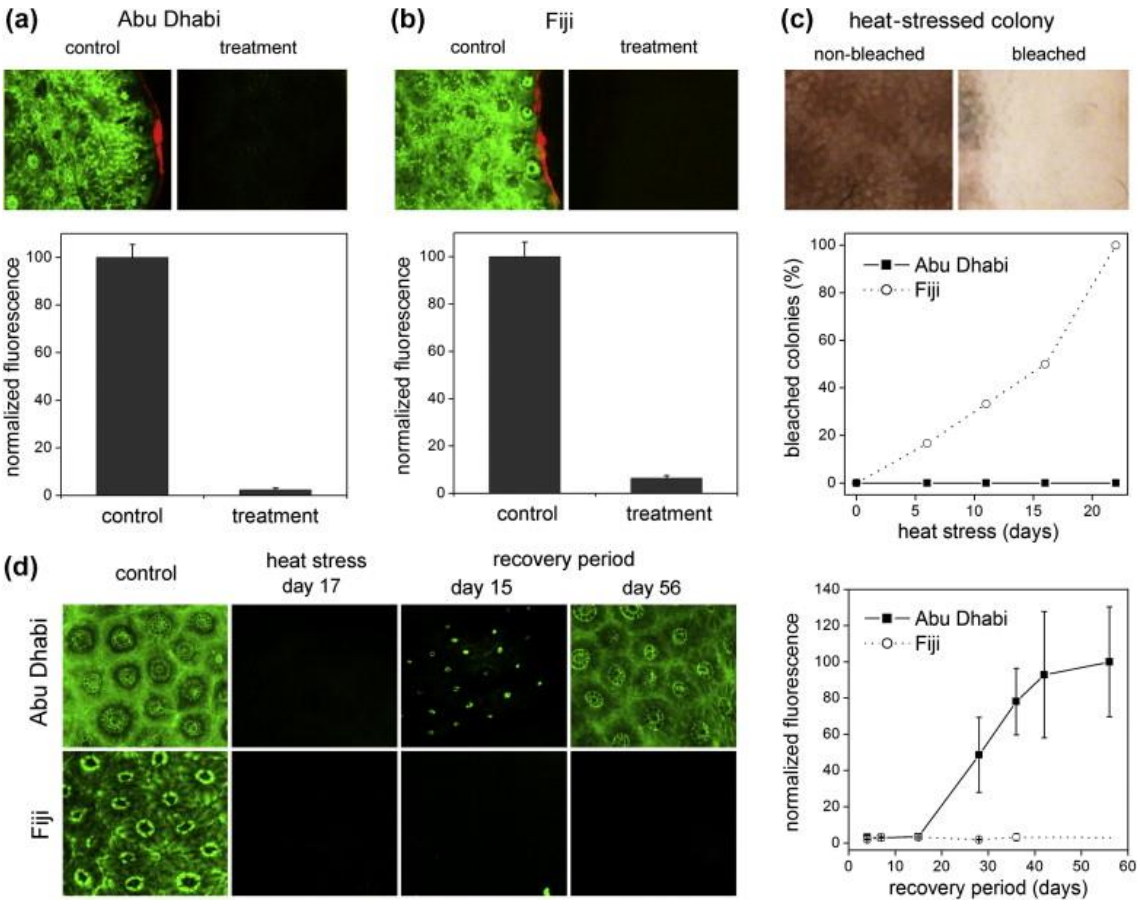

Figure 4.4 Differential responses of *P. lobata* to experimental heat stress treatments. The upper fluorescence photographs show the essentially complete loss of green and red fluorescence after a 10-day exposure of colonies from Abu Dhabi (a) and Fiji (b) to 31.5°C. Images were acquired using identical exposure times. The cyan tissue fluorescence of the relevant colonies was quantified directly by spectrometric measurements of the coral tissue. The bars show the mean of six measurements and error bars represent standard error of the means. (c) Bleaching in response to heat stress. The upper panels show representative photographs of *P. lobata* from heat stress treated colonies that were considered to be non-bleached (left) and completely bleached (right). The amount (%) of completely bleached colonies was recorded upon weekly microscopic inspections over the course of the exposure to 31.5°C. (d) Fluorescence microscopic images documenting the intensity changes of cyan tissue fluorescence from control conditions, after 17 days of incubation time at 31.5°C, and during the recovery period 15 and 56 days after returning the corals to reduced temperature. Right graph: spectrometric quantification of cyan tissue fluorescence during the recovery period. The data points represent mean values of six measurements and error bars denote standard error of the means.

4.5 Discussion

4.5.1 Differences in algal symbiont identity

In *P. lobata* and six other common coral species from the coast of Abu Dhabi, *Symbiodinium* in clade C were the dominant symbionts. This clade accounted for ~73% of all the ITS2 sequences recovered and was detected in all species studied (Table 4.2). Most sequences (~71%) were C3, whereas sequences of C15, C21 and C36 were only detected once. Remaining sequences (~27%) were all A1, which was the second most abundant symbiont type, and which was mostly found in combination with C3 in *C. microphthalma, F. pallida, P. daedalea* and *P. harrisoni*.

Interestingly, *Symbiodinium* clade D was not represented among the 146 sequences analysed in this study. The preponderance of this clade was first reported from coral species including representatives of the taxa *Cyphastrea, Favites* and *Platygyra* from the southern Gulf, off the Saudi coast in the aftermath of the 1998 mass bleaching event (Baker et al. 2004). Likewise, in the northern Gulf, off the coast of Kish, Hengam and Larak Islands (Iran), clade D was found in several species including colonies of *A. clathrata, C. microphthalma, F. pallida* and *P. daedalea* (Mostafavi et al. 2007; Shahhosseiny et al. 2011). It was thus suggested that the dominance of clade D might partially explain the unusual heat tolerance of Gulf corals. The absence of clade D symbionts in corals from Abu Dhabi suggests that the dominance of this clade might represent a regional or temporary phenomenon, promoted by local environmental conditions or recovery from extreme events such as the mass coral bleaching of 1998. Further sampling would help discern the spatial and temporal distribution of symbionts in the Gulf. The prevalence of *Symbiodinium* C3 in Abu Dhabi corals is surprising, given that this type is considered a ‘‘generalist’’ on Indo-Pacific reefs (LaJeunesse et al. 2003). The fact that Abu Dhabi corals routinely experience summer temperature of up to 36°C (Foster et al. 2012) indicates that this *Symbiondinium* clade can also support symbiotic associations with an exceptional thermotolerance. Therefore, *Symbiondinium* C3 in Abu Dhabi corals represent an ideal model system for in-depth analyses of the role of algal symbionts in the thermal tolerance of the coral holobiont.

We found *Symbiodinium* C15 to be the major symbiont associated with corals from the comparably benign temperature regime of Fijian waters. This type has been suggested as contributing to the resistance of *Porites spp*. to temperature anomalies (LaJeunesse et al. 2003) and it is the characteristic symbiont found in *Porites* from the Pacific and Indo-Pacific (LaJeunesse 2005; Barshis et al. 2010). Noteworthily, C15 contributed almost 95% of the sequences isolated from 31 colonies of *P. lobata* from American Samoa (Barshis et al. 2010).

We showed that the symbiotic association of *P. lobata* from both Abu Dhabi (C3) and from Fiji (C15) is stable under culture conditions in the laboratory. These results are in agreement with previous observations for other corals and sea anemones growing in long-term aquarium culture (Smith et al. 2009; Hartle-Mougiou et al. 2012). The stability of the coral-algal association makes *P. lobata* an excellent model for laboratory experimentation to study the role of C3 and C15 *Symbiodinium* in the thermal tolerance of this species in different geographical regions.

4.5.2 Laboratory cultures of *P. lobata*

We successfully established laboratory strains of *P. lobata* from Abu Dhabi, UAE. Encrusting growth contributed significantly to the weight gain of replicate colonies after fragmentation (Figure 4.2a). Therefore, the growth characteristics are different from larger, hemispherical colonies, that extend mostly along vertical and horizontal growth axes (Lough and Barnes 2000). However, a rate of weight gain of ~1.3 g cm^{-2} $year^{-1}$ determined over the first 3 months after fragmentation agrees well with the range of 1.38 – 1.63 g cm^{-2} $year^{-1}$ measured previously for *P. lobata* from various locations on the Great Barrier Reef (Lough and Barnes 2000). These data suggest that growth rates in laboratory culture are similar to those in the field. Moreover, the data from the growth experiment indicate that the number of replicate colonies can be doubled in less than 12 months, providing sufficient material for sustainable experimentation. Aside from *P. lobata* from Abu Dhabi, conspecifics from Fiji could be established as a model for comparative studies of corals originating from habitats with dramatically different temperature regimes.

4.5.3 Fluorescent proteins

A recent study showed that *P. lobata* from different geographical regions express a similar complement of GFP-like proteins. Typically, cyan FPs can be found in the polyps and the coenosarc and a red FP in the growth zones (D'Angelo et al. 2012). These host pigments belong to the family of GFP-like proteins (Dove et al. 2001; Wiedenmann et al. 2004; Oswald et al. 2007; Alieva et al. 2008; Wiedenmann et al. 2009) of which several (but not all) are regulated at transcriptional level in reef corals in response to changes in environmental conditions; in particular by blue light (Leutenegger et al. 2007; D'Angelo et al. 2008; Desalvo et al. 2008; Smith-Keune and Dove 2008; Bay et al. 2009). Only recently, the expression of red FPs in the growth zones, wounded or epibiont-infested colony parts of *P. lobata* was correlated with areas characterised by accelerated cell proliferation (D'Angelo et al. 2012). Thus, once the spatial expression pattern of GFP-like proteins and their regulation in a particular species is defined, these pigments constitute powerful biomarkers to monitor physiological responses of corals by non-invasive optical techniques (D'Angelo et al. 2008; Wiedenmann et al. 2009; D'Angelo et al. 2012). Here, we show that the typical expression patterns of FPs are retained after >15 months of laboratory culture, suggesting that the aquarium conditions sustain the natural pigmentation of this species.

4.5.4 Temperature challenge experiment

We compared the responses of *P. lobata* from Abu Dhabi and Fiji to increased temperatures to evaluate whether adaptation to temperature regimes at their origin is retained in the laboratory. Microscopic and fluorescence spectrometric measurements of the coral colouration were used to monitor responses of host and symbionts to elevated temperatures.

We found that the FP-derived host tissue fluorescence of *P. lobata* from both geographical regions was dramatically reduced when temperatures were increased. These observations are in agreement with previous observations that also showed a loss of fluorescence under heat stress in other species (Desalvo et al. 2008; Smith-Keune and Dove 2008). Our results demonstrate clearly that FP fluorescence of *P. lobata* can be used for non-invasive optical monitoring of the stress response of the host tissue. Regarding the putative

photoprotective function of GFP-like proteins (Salih et al. 2000; Dove et al. 2001), the consistent downregulation under heat stress does not support any superior FP-mediated protection of the experimental Abu Dhabi specimens.

Interestingly, microscopic inspections revealed that only *P. lobata* samples from Fiji displayed complete loss of zooxanthellae pigment in the coral tissue in response to the increased temperatures. Although the Abu Dhabi samples showed a visible paling, none of the colonies bleached completely.

Cyan fluorescence emission of the host tissue was fully regained in heat-treated *P. lobata* from Abu Dhabi after approximately 2 months of recovery at reduced temperatures. In contrast, replicates from Fijian colonies did not show comparable signs of recovery after the temperature was reduced and eventually all of the heat treated colonies died. These findings suggest that adaptation to different thermal environments has a long-term influence on the potential of the *P. lobata* to respond to episodes of high temperature and that the samples from Abu Dhabi are more tolerant of the high temperatures in experimental treatments. Since Gulf corals are increasingly endangered in their native habitat (Burt et al. 2008; Burt et al. 2011; Burt et al. 2012), it was suggested that these uniquely thermally adapted genotypes should be conserved, or perhaps even transplanted, for the benefit of other reefs that are expected to experience similar temperature extremes by 2099 as those currently experienced by the Gulf (Riegl et al. 2011). Such a strategy would only make sense if the thermal tolerance of Gulf corals was indeed retained *ex situ*.

The temperature used in our experimental treatments was well below the maximum temperature that Abu Dhabi corals experience in their natural habitat. Therefore, it is not yet possible to judge the extent to which the observed heat tolerance of Gulf corals is the result of thermal tolerance on the part of the corals or their algal symbionts, by transient thermal acclimatisation, or by the unique water chemistry of the Gulf, including its exceptionally high salinity.

4.6 Conclusion

We characterised *P. lobata* from two locations with strongly differing temperature regimes. We found that neither association with *Symbiodinium* in

clade D, nor a particular expression pattern of photoprotective host pigments could be solely responsible for the exceptional thermal tolerance of corals from the Gulf. The prevalence of the ‘‘generalist’’ C3 *Symbiodinium* in Abu Dhabi corals calls for a further evaluation of the role of different symbionts in heat resistance. We demonstrate that our laboratory models can be used in long term experiments under controlled conditions that will greatly facilitate future efforts to decipher the role of the host, symbionts and the abiotic environment in defining thermal tolerance.

Knowledge gained from these experiments should also help evaluate whether Gulf corals could potentially be used for assisted migration in reef restoration projects outside their natural habitat (Riegl et al. 2011).

Chapter 5: Contrasting coral-*Symbiodinium* associations within, and external to, the Persian/Arabian Gulf: The potential of 'internal' coral-*Symbiodinium* associations to act as 'saviours' of Indo-Pacific reefs

Note: This chapter is a manuscript in preparation for submission, contributing authors: Hume BCC, D'Angelo C, Smith E, Burt J, Wiedenmann J.

Contribution to chapter by BCCH: Joint contribution to sample collection and fixation; majority contribution to genomic DNA extraction; primer design; DGGE setup and optimization; DGGE analysis of samples; image preparation; Porites-*Symbiodinium* meta-analysis; manuscript preparation.

5.1 Abstract

Scleractinian corals of the physically extreme Gulf predominantly harbour subcladal C3 *Symbiodinium*, associations that have demonstrated exceptional thermal tolerance. Whilst coral associations have been characterised inside the Gulf, very little knowledge exists pertaining to the character of associations external to the Gulf in the Strait of Hormuz and the Gulf of Oman. As such, it is unclear whether these tolerant associations are exported from the Gulf waters, and if so, whether they are viable in the Gulf of Oman and other external waters where they might act as thermally tolerant replacements to thermally sensitive coral-symbiont associations under threat from climate change-induced warming waters. Here 133 and 108 colonies of *Porites spp.* (*P. lutea, P. lobata and P. harrisoni*) were sampled inside, and externally to, the Gulf, respectively. Dominant *Symbiodinium* complements were identified using DGGE analyses. This spatial analysis demonstrates the complete association of internal corals with subclade C3 *Symbiodinium*, in contrast to associations in the Strait of Hormuz and Gulf of Oman waters harbouring subclades, C3, C15-cluster and D1/a. Results identify the export of thermally tolerant associations out of the Gulf to surrounding waters and demonstrate their ability to survive in these waters. However, their gradual out competition with increasing

distance from the Gulf suggests the competitive advantage they have within the Gulf is lost externally. The dominance of these resilient associations is therefore likely constrained to the Gulf's waters limiting their ability to replace thermally sensitive associations in thermally stressed reefs to confer an increase in thermal resilience.

5.2 Introduction

The degradation of coral reef ecosystems is occurring on a global scale. The breakdown in relationship between the hermatypic corals, primarily responsible for coral reef formation, and their symbiotic unicellular dinoflagellate algae of the genus *Symbiodinium* are largely culpable for this degradation. The expulsion of these symbiotic algae by the coral host, coral bleaching, may be linked to a range of physical perturbations not limited to high or low temperature, light or salinity. Globally, an increase in bleaching intensity and frequency is being realised throughout tropical hermatypic coral populations. These trends, evident from the past decades are intrinsically linked to increases in environmental perturbations – predominantly rising sea surface temperatures as part of the climate change phenomenon (Hoegh-Guldberg 1999). There is currently no evidence for the slowing of reef degradation and given that sea water temperature increases as little as 1°C above annual average maxima can elicit a bleaching response, projections of future climate change increases of several degrees Celsius towards the end of this century (IPCC, 2007) have the potential to increasingly degrade coral populations.

There is evidence to suggest an ability of certain corals to adapt and acclimatise to short- and long-term physical perturbations. However, the underlying mechanisms are unclear (Coles and Brown 2003). The adaptive mechanisms that may serve to mediate long-term increases in sea surface temperature are most likely inadequate when weighed against the rate at which thermal increases are occurring (Hoegh-Guldberg 1999,2009). Mitigation of increasing environmental pressures may be more effective through acclimatisation strategies.

Harbouring different genotypes of *Symbiodinium* may confer advantages in energetic supply (Little et al. 2004; Mieog et al. 2009; Jones and Berkelmans

2010) or an ability to withstand environmental extremes and perturbations to the coral host (Rowan 2004; Berkelmans and van Oppen 2006; Mieog et al. 2009). The six clades of *Symbiodinium* identified in scleractinian corals (clades A-D, F and G) are further divided into subclades. Of these subclades, differences in the conferring of host tolerances and energetic advantages have been demonstrated (Rowan et al. 1997; Berkelmans and van Oppen 2006; Jones et al. 2008; Lesser et al. 2010; Cooper et al. 2011). For example, the comparison of the thermally sensitive 'generalist' ITS2 type C3 and purported thermally tolerant C15 subclade in the conferring of heat tolerance (Fisher et al. 2012). A comparative conferring of high- (Rowan 2004; Berkelmans and van Oppen 2006) and low- (LaJeunesse et al. 2010a) temperature tolerance has also been demonstrated in Clade D *Symbiodinium*; yet higher thermal tolerances of subclade C1 have been reported (Abrego et al. 2008; Jones et al. 2008). Demonstrated decreases in coral lipid content, size and number of eggs (Jones 2011); growth rates (Little et al. 2004); and symbiont-produced carbon photosynthate incorporation (Cantin et al. 2009) in corals harbouring clade D *Symbiodinium* compared to clades C1 or C2, suggest that hosting such thermally tolerant symbionts may come at a cost. The demonstration of species-specific interactions between host and symbiont that define bleaching susceptibility (Abrego et al. 2008; Fitt et al. 2009) and differing symbiont stress responses between freshly isolated and *in hospite Symbiodinium* cells (Bhagooli and Hidaka 2003; Goulet et al. 2005) together highlight the complexity of interactions between these symbiotic partners and the potential of host factors to determine physiological performance. Coral communities may change their symbiont compliment either via selective death of individuals with ill-suited symbiont-host combinations, or by changing the dominant symbiont type after a bleaching event, either by uptake of novel symbionts from the environment or by changing the relative abundance of symbionts already present within the coral (symbiont shuffling; Rowan et al. 1997; Berkelmans and van Oppen 2006; Jones et al. 2008). The mitigation potential of changing symbiont type may, however, be limited due to the relatively high fidelity of most coral species (Goulet 2006).

The majority of tropical corals experience maximal temperatures of ~29°C with annual fluctuation of ~4°C. In contrast, coral communities of the Gulf may experience thermal maxima of 32-36°C, and annual fluctuations of ~20°C; the

largest of any major tropical reef- harbouring waters. The Gulf corals have been subjected to these extreme thermal regimes for ~6ka (Purkis et al. 2010) over which time their thermal tolerance has evolved. Coral communities experiencing these extremes are therefore already surviving in thermal regimes equal to or greater than those predicted by current forecasts for the end of the century (IPCC 2007). These corals have the potential to provide insight into the adaptation/acclimatisation potential of coral reef ecosystems. Understanding the mechanisms responsible for their ability to survive in such extreme conditions is crucial if we are to accurately predict the adaptive potential of global coral reefs under forecasts of increasing sea surface temperatures.

The Gulf, orientated east-west, has a maximum depth of approximately 60m with ~30m depths across the majority of its area. There is only one opening on the eastern side where water enters through the Strait of Hormuz, a feature approximately 50km across at its narrowest point (Coles and Riegl 2012; Figure 5.1). Water circulation in the gulf is seasonally variable, dependent on water heating. It is weakest in winter months due to a lack of stratification and density driven outflow-inflow through the Strait of Hormuz. The north-westerly flowing Iranian Costal Current (ICC) carries water from the Strait of Hormuz to the north of Qatar during March-June before becoming unstable forming mesoscale eddies (the Iranian Current Eddies; ICE) that carry water far into the northern Gulf from July onwards. Remnants of ICE are seen until November at which time dissipation occurs due to winter cooling and thermocline collapse. The Gulf waters are therefore characterised by an overall counter clockwise flow with a deep high salinity outflow leaving after a 3-5 year residence time (Thoppil and Hogan 2010). The relatively enclosed nature of the Gulf, extremes of temperature and scarce precipitation and freshwater inputs (the Tigris-Euphrates being the only significant source of freshwater input) are responsible for extreme salinities >39 throughout the majority of the Gulf (Sheppard et al. 2010) and ranging between 40-43 in surface waters off the southern UAE coast (Johns et al. 2003).

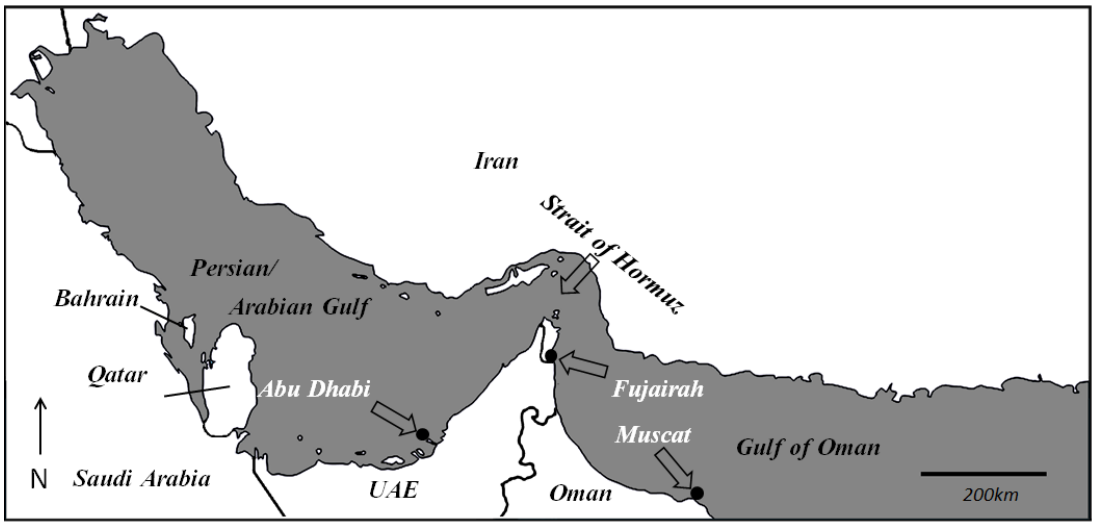

Figure 5.1 Map of the Persian/Arabian Gulf region

Coral reef assemblages within the Gulf are commonly dominated by corals of the genus *Porites,* e.g. Dubai, UAE,(Riegl 1999; Riegl et al. 2001; Burt et al. 2011) , Kuwait (Coles and Fadlallah 1991), Iran (Kavousi et al. 2011), as well as several other dominant genera including *Acropora*, *Platygyra* and *Favia*. Due to bleaching episodes in the Gulf, a shift in dominant genera to more thermally tolerant corals is occurring. This shift often occurs in favour of the massive corals including *Porites* spp. (Coles and Fadlallah 1991; Riegl 1999; Riegl 2002; Burt et al. 2011) of which there are four predominant species found in the Gulf: *P. lutea, P. lobata, P. nodifera and P. harrisoni* (previously named *P. compressa*; Veron 2000). Their significant contribution to reef structure and relative bleaching tolerance implicate the *Porites spp.* as an increasingly important genus in relation to the maintenance of Gulf reefs. *P. lobata* and *P. lutea* are found throughout the Indo-Pacific whereas the distributions of *P. nodifera* and *P. harrisoni* are limited to the Gulf, Gulf of Oman, Arabian Sea, Red Sea and the east coast of Africa. Throughout the Indo-Pacific, corals of the genus *Porites* are known to demonstrate a high fidelity with purportedly thermally tolerant subclade C15 (LaJeunesse 2005; Putnam et al. 2012). However, no attempt has been made to quantify the extent of this fidelity.

There is an extreme contrast in the physical environment of the Gulf compared to the neighbouring Gulf of Oman where conditions are less extreme in terms of salinity (<37 year round; Johns et al. 2003) and annual thermal range (temperatures ranging from 23.0-30.2°C; Muscat, Oman; Hume et al. 2013).

However, due to the influences of upwelling from the Arabian Sea, corals in the Gulf of Oman may experience thermal changes as large as 8°C over a period of 24 hours (off Fahal Island, Muscat Capital Area; Coles 1997). More northerly sites in the Gulf of Oman may however remain relatively unaffected by such upwelling events (Coles 1997). This contrast between the Gulf and the Gulf of Oman over such a small distance represents a unique opportunity to assess how the obligate coral-symbiont partnerships differ between the less thermally extreme and less saline waters of the Gulf of Oman to the more physically extreme waters of the Gulf. Understanding any environmentally influenced modifications to this relationship will improve our understanding of the mechanisms behind how corals are able to adapt/acclimatise and survive in what are the most thermally extreme coral-populated waters in the world.

The export of Gulf coral-symbiont associations into the Gulf of Oman and Indo-Pacific waters, either through natural processes such as larval export or through artificial translocations (reef restorations; Riegl et al. 2011) has been proposed as a means to potentially mitigate, at least in part, the further degradation of thermally stressed reefs through the incorporation of thermally resilient, Gulf-derived associations. However, it is unknown whether Gulf associations are exported and if so, whether these associations may survive in the less extreme environments of the Gulf of Oman. The predominant symbiont subclade(s) harboured by three species of coral in the genus *Porites* (*P. lobata, P. lutea and P. harrisoni*) at six sites inside and four sites outside of the Gulf (as well as three sites in the Strait of Hormuz) are characterised in order to identify any differences in the host-symbiont partnerships and to assess whether Gulf associations are exported and survive in the surrounding waters. We report stark contrast in the symbiont complements found inside the Gulf (ITS2 type C3 in all corals sampled) compared to those outside (sites contained a mixture of C15, C3 and D1 predominant ITS2 types). This divergence away from the strict fidelity of *P. lobata* and *P. lutea* with symbiont C15 (as quantified in this study) suggests a strong environmentally driven shift in host-symbiont partnerships.

5.3 Methods

5.3.1 Meta-analysis of *Porites-Symbiodinium* associations

In order to quantitatively characterise the globally observed fidelity between corals of the genus *Porites* and *Symbiodinium* ITS2 type C15-cluster (LaJeunesse 2005; Stat et al. 2009) a meta-analysis was undertaken of available literature. Literature containing information on harboured *Symbiodinium* at a subcladal level was obtained from peer-reviewed scientific papers and the GeoSymbio database (Franklin et al. 2012) (Table 7.3 Appendix 3). Symbiont associations were categorised by clade except for associations in clade C that were categorised as either 'C15-Cluster' or 'Other Subcladal C'. A list of studies used in the meta-analysis is available in Table 7.3, Appendix 3.

5.3.2 Sampling and collection of corals

Corals were collected at five locations (some containing several reefs/sites) within the Arabian/Persian Gulf (from west to east: Dalma Island, Saadiyat Island, Ras Ghanada, Umm Al Quwain and Ras Al-Khaimah [two reefs]), in the Strait of Hormuz (Musandam region; 3 sites) and three locations in the Gulf of Oman off the coast of Fujairah (north to south: Dibba and Al Aqah) and Muscat in September 2012 and March 2013 (Figure 5.2). Where possible samples were all collected at similar depths and samples were taken from the side of corals to ensure similar light regime histories. Where possible three species of *Porites* were sampled at each site: *P. lobata, P. lutea and P. harrisoni*. Small sections of coral tissue and underlying skeleton (approximately 10cm^2 and 1cm in depth) were removed using a hammer and chisel and placed into individual plastic containers. Once brought to the surface samples were drained of seawater and fixed in 99.9% ethanol and placed directly on ice. Alternatively, samples were placed directly on dry ice immediately after removal from the sea.

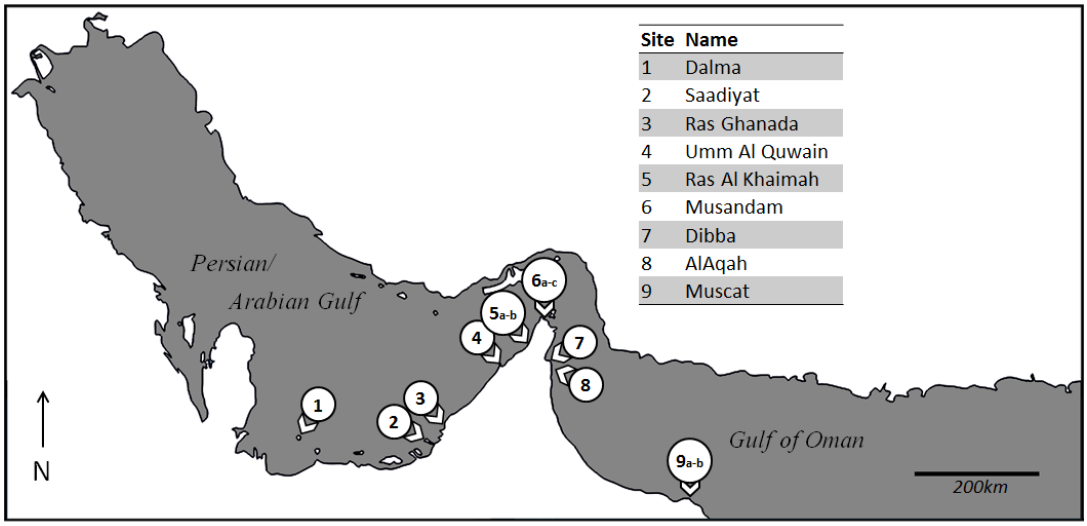

Figure 5.2 Sampling locations. Table insert site numbers correspond to labelled sampling sites 1 – 9 shown on the map. Sites at which several reefs were sampled at one location are denoted with letters after the site number (e.g. 5a-b; two reefs sampled at site 5).

5.3.3 Genomic DNA extractions

For samples collected in September 2012, genomic DNA extractions were performed using a modified version of the standard Wizard genomic DNA purification kit protocol. Coral symbionts were extracted from between 0.5 and 2cm^2 of coral tissue (dependent on symbiont density) into a 50ml tube using a water pick. The symbionts were then pelleted by centrifugation at 4000g for 5 mins. Following centrifugation the pellet was transferred to a 1.5ml microcentrifuge tube and 600 ml of nuclei lysis buffer was added. The sample then underwent homogenisation with micropestle followed by further centrifugation and homogenisation. The lysate was incubated with 0.1 mg/ml proteinase K for 1 hour at 65°C followed by incubation with 6 mg/ml RNase at 38°C for 10mins. Protein precipitation buffer (250 ml) was then added and the extract was inverted several times. After centrifugation at maximum speed for 5mins, 600µl of supernatant was transferred to a second 1.5ml microcentrifuge tube that contained 700 ml of 100% isopropanol. After incubation at room temperature overnight the DNA was pelleted by centrifugation at maximum speed for 5 mins. The supernatant was removed and the pellet was washed with 600µl of 70% ethanol. The DNA was centrifuged once more at maximum speed for 5 mins, the supernatant

removed and the pellet dried. The DNA was resuspended in 50µl of TE (10 mM Tris-HCL pH 8.0, 1mM EDTA pH 8.0).

Genomic DNA extractions were performed on samples collected in March 2012 using a modified CTAB extraction as described in (Hartle-Mougiou et al. 2012).

5.3.4 PCR-DGGE analysis

5.3.4.1 Sample preparation for DGGE by PCR

Symbiodinium spp. were characterised to subclade level using the ITS2 region of the rRNA gene. Novel primers were developed for use in denaturing gradient gel electrophoresis that could amplify from the product of the primer pair SYM_VAR_FWD and SYM_VAR_REV as used in previous phylogenetic analyses to amplify the ITS1-5.8S-ITS2 region of the rRNA gene (see Hume et al. 2013). SYM_VAR_FWD and SYM_VAR_REV were originally designed due to unwanted amplification of host ITS2 rRNA genes when using the msg2, msg3 primers; (Savage et al. 2002) in particular, when attempting to amplify *Symbiodinium* DNA from *Porites spp.* samples. Amplification of host DNA is also reported in LaJeunesse 2002 when using primers ITSintfor2, ITS2Clamp (LaJeunesse 2002). The novel DGGE primers designed for this study aimed to resolve host and symbiont sequences effectively whilst still maintaining a single melting domain that effectively spans the entire ITS2 region of the rRNA gene (Figure 3.1). In addition, the use of these novel primers allowed *Symbiodinium spp*. samples previously characterised to the subcladal ITS2 type level by molecular cloning methodologies using the SYM_VAR_FWD and SYM_VAR_REV pair to be amplified as markers to aid in DGGE optimisation and verification of correct amplification and identification by the DGGE setup. The novel primers designed were SYM_VAR_5.8S (priming in the 5.8S region of the ITS2 rRNA gene) 5'-ATCTTGGCTCGAGCACCTATGAAGG-3' and SYM_VAR_Clamp (identical priming site to SYM_VAR_REV and with addition of a 40bp GC clamp) 5'(CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCC)CGGGTTCTCTTGTTTGACTTCATGC3'. PCR amplification for DGGE analysis was run using 200µM of each dNTP, 0.5µM of each primer, 2.5mM MgCl2, 1X GoTaq Flexi buffer, 2.5U of GoTaq DNA Polymerase and approximately 50ng of template DNA. PCRs were conducted using a MyCycler thermocycler (BioRad) with an initial denaturation at 95°C for 30 s followed by 35 cycles of 95°C for 30 s, 56°C for

30 s, 72°C for 30 s with a final extension step of 72°C for 5 mins. When amplifying from plasmid template (i.e. when creating DNA to use as a marker) the annealing temperature was raised to 66°C and the number of cycles was reduced to 30.

5.3.4.2 Running of samples

DGGE analysis was conducted using a BioRad DCode System for DGGE with a model 475 gradient former. The gels and apparatus were prepared to the manufacturer's specifications. Samples were run on 8% polyacrylamide gels with a gradient of 35-65% where a 100% denaturant solution contained 40% (v/v) of formamide (deionized) and 42% (w/v) of urea. Immediately before pouring the denaturant solutions were degassed using a sonic water bath for 3 minutes under vacuum at 65°C. Run times and voltages as well as sample loading volumes were dependent on the gel comb used. When using a 16 well gel comb, samples were run at 150V for 3 hrs and 10µl of PCR sample was loaded with 10µl of 2x gel loading dye; with the 32 well comb, samples were run at 130V for 2.5 hrs and 5µl of PCR sample was loaded with 5µl of 2x gel loading dye. Gels were stained for 15 mins in 200 ml of TAE running buffer with an end concentration of 50 µg/ml of ethidium bromide and destained in water for a further 15 mins. Markers for ITS2 types were created by amplifying plasmids that contained previously sequenced and characterised 18S-ITS1-5.8S-ITS2-28S rRNA genes from previous molecular cloning based phylogenetic analyses (see Hume et al. 2013).

5.3.4.3 DGGE band characterisation

Samples that ran with an identical DGGE 'fingerprint' were grouped and prominent bands of one representative were excised, reamplified and re-run on a further DGGE gel. If these amplicons ran with an identical finger print to the original, no further analysis was undertaken. In cases where a different profile was produced, the amplicons were sequenced to screen for novel ITS2 types. The dominant *Symbiodinium* ITS2 subcladal type was identified for each sample. Prominent bands to be assessed were excised from gels using a clean and sterile scalpel, placed in 500µl of water and homogenised with a micropestle before being incubated at 4°C overnight. After incubation, samples were centrifuged at maximum speed for one minute and 4µl of this supernatant was used as template for a further PCR using the same GC

clamped primers as the initial analysis. This PCR amplicon was then run on a further DGGE gel in order to characterise the excised band as either a true ITS2 subcladal representative, a heteroduplex or in some cases a structural conformation of an identical sequence running a different distance in the gel. True ITS2 bands once excised and re-run would run at an identical height to the original excised band. In contrast, excised bands that were characterised as either heteroduplexes or conformations (i.e. part of the unique DGGE 'fingerprint' for a given subclade) would produce multiple bands identical to the original PCR amplicon loaded. Bands to be sequenced were excised and reamplified with the primers SYM_VAR_5.8S and SYM_VAR_REV using Advantage 2 Polymerase mix (Clontech) with 4µl of excised band template and the same concentrations of primers and dNTPs as the previous PCR. The PCR was cycled as 95°C for 1 min followed by 30 cycles of 95°C for 30 s, 56°C for 30 s and 68°C for 30 s with a final extension of 2 mins at 68°C. The PCR products were cloned using StrataClone (StrataGene) and plasmid DNA was prepared from *E. coli* colonies using a Fermentas GeneJET Plasmid Miniprep Kit (Thermo Scientific). Sequencing services were provided by Macrogen.

5.3.4.4 Validation of novel protocol

The DGGE system developed in this study demonstrated successful resolution at a cladal (resolution of clades A, C and D) and subcladal (demonstrated through the resolution of different C15 and C3 derivative ITS2 types) level similar to previous methods (E.g. LaJeunesse 2002). Resolution of fragments with single nucleotide differences was also clear. In contrast to previous methods (i.e. Thornhill et al. 2010) the gels were run over significantly shorter times. The redesign of primers to specifically differentiate between coral and *Symbiodinium* DNA (a confounding problem with previously used DGGE methodologies) has proved successful so far with no host-based sequences identified to date. However, amplification of further samples, in particular from other coral genera, will verify this result in a more robust manner.

5.4 Results

5.4.1 Meta-analysis of *Porites-Symbiodinium* associations

Due to the separate evolutionary radiations of symbionts from the Atlantic and Indo-Pacific water bodies (LaJeunesse 2005), distribution analyses are presented accordingly. Within the Indo-Pacific, a total of 369 *Porites* associations were identified in the literature. Of these, 83% were identified as subclade C15-cluster associations, 17% as Other Subcladal C and less than 1% as clade D. Of the 17% of associations that fell into the 'Other Subcladal C' category 73% were from a single high-latitude reef study in the Gulf of California (an association with *P. panamensis*). No clade A or B associations were identified. The Atlantic was characterised by a 39% association with Other Subcladal C, 53% clade A and 8% clade B making up a total of 151 associations identified from the literature. No C15-cluster or clade D associations were identified. As part of the analysis 154 associations of *P. lobata* and *P. lutea* were identified. All colonies had origins in the Indo-Pacific basin and 100% of the colonies associated with symbionts belonging to the C15-cluster. The global distributions of all 520 *Porites-Symbiodinium* associations identified in the analysis are illustrated in Figure 5.3A. The specific distributions of P. lobata and P. lutea are illustrated in Figure 5.3B.

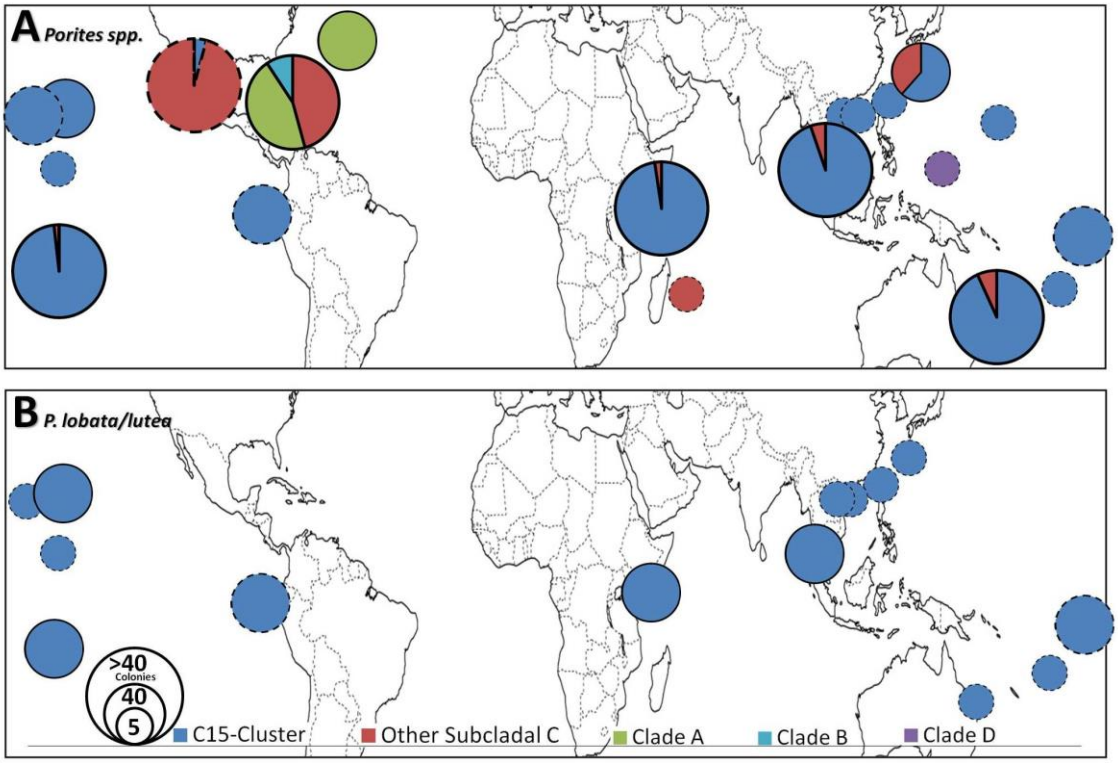

Figure 5.3 Meta-analysis of *Symbiodinium* spp. associations with corals in the genus *Porites*.The analysis includes data from 34 studies covering 520 *Porites-Symbiodinium* associations. Corals from the Gulf and Gulf of Oman are excluded from the meta-analysis. Pie charts may represent a single study or the summation of several studies of close proximity with dashed outline charts containing only one species and solid outlines more than one species. Symbiont associations are identified to the cladal level for clades A, B and D. Clades C associations are divided into the subcladal resolutions of the C15-cluster and 'Other Subcladal C' (containing clade C associations not belonging to the C15-Cluster). Figure 5.3A illustrates coral-symbiont association across the entire *Porites* genus whereas figure Figure 5.3B includes analyses relating to *P. lobata* or *P. lutea* associations only.

5.4.2 Spatial analyses of predominant *Symbiodinium* ITS2 type

Of the 303 coral samples analysed; 133 corals were collected at the 'internal' Gulf sites: Dalma, Saadiyat Island, Ras Ghanada, the Ras Al Khaimah region and Umm Al Quwain; 108 in the Strait of Hormuz (Musandam region); and 62 at the 'external' sites Dibba, Al Aqah and Muscat (Table 5.1).

Table 5.1 Corals sampled per study site and predominant ITS2 subclade type hosted as determined by PCR-DGGE.

. Site	Species	Predominant Subclade
Dalma (Gulf)	*P. lobata*	-
	P. lutea	C3(4)
	P. harrisoni	C3(12)
Saadiyat (Gulf)	*P. lobata*	C3(12)
	P. lutea	C3(11)
	P. harrisoni	C3(11)
RasGhanada (Gulf)	*P. lobata*	C3(3)
	P. lutea	C3(5)
	P. harrisoni	C3(5)
Umm Al Quwain (Gulf)	*P. lobata*	-
	P. lutea	C3(18)
	P. harrisoni	-
Ras Al-Khaimah A (Gulf)	*Porites spp.*	C3(30)
Ras Al-Khaimah B (Gulf)	*Porites spp.*	C3(22)
Musandam A (SoH)	*P. lobata*	C3(7) C15H(7) C15L(1) D1(1)
	P. lutea	C3(8) C15H(1)
	P. harrisoni	C3(1)
Musandam B (SoH)	*P. lobata*	C3(2) C15H(10) C15L(2)
	P. lutea	C3(1) C15H(5) C15L(2) D1(1) D1/D1a (1)
	P. harrisoni	C3(3) C15H(4) C15L(2)
Musandam C (SoH)	*P. lobata*	C3(19) C15H(4) C15L(3) D1(5) D1/D1a(1)
	P. lutea	C3(4) C15H(1) C15L(1) D1(4)
	P. harrisoni	C3(2) D1(7)
Dibba (GoO)	*P. lobata*	C15H(9) C15L(4) D1(1)
	P. lutea	C15L(2)
	P. harrisoni	-
AlAqah (GoO)	*P. lobata*	C3(6), C15i(3), D1/D1a(6)
	P. lutea	C3(1), C15i(2), D1/D1a(2)
	P. harrisoni	D1(2)
Muscat A (GoO)	*Porites spp.*	C3(1) C15H(5) C15L(5) C15LM(1) D1(4)
Muscat B (GoO)	*Porites spp.*	C15H(3) C15HM(1) C15L(3) D1(1)

Numbers in parentheses represent the number of corals sampled that contained the given ITS2 subclade type. Classing of sites as Gulf, SoH or GoO refer to sites in the Gulf, the Strait of Hormuz or the Gulf of Oman, respectively.

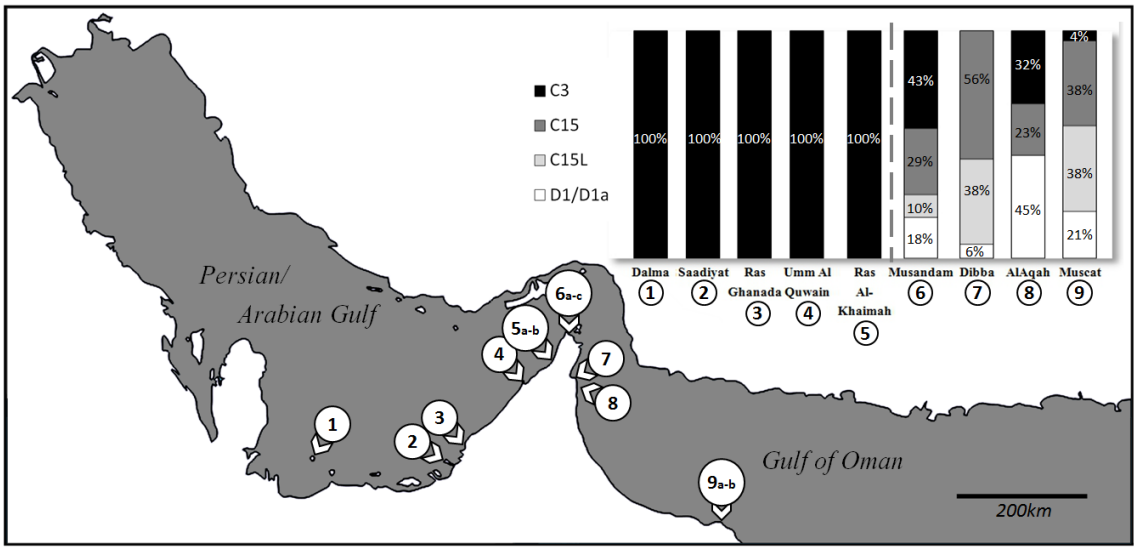

Figure 5.4 Location of sampling sites within and outside of the Gulf (map). Percentage abundance of the four subcladal *Symbiodinium* ITS2 types identified at each sampled site. The single occurrences of C15HM and C15LM were grouped with C15 and C15L, respectively (insert).

At all of the internal sites, ITS2 type C3 was predominant in every coral with no other predominant subclade present (Figure 5.4; Figure 5.5, top gel). The four external sites and three sites in the Musandam region were characterised by a mixed symbiont composition (Figure 5.4; Figure 5.5, bottom gel) with representatives of all four dominant subcladal types identified in the DGGE analysis: C3, C15, C15L (a novel C15 derivative named according to its lower running position in the DGGE gel than the basal C15 type; Figure 5.5, C15L) and D1. Three novel C15 derivative types (listed in order of migration, least to furthest migration) were identified through the DGGE analysis conducted and are referred to in this study according to their migration pattern relative to C15 as C15HM (one occurrence), C15LM (one occurrence) and C15L (approximately equal predominance to C15). Associations with clade D symbionts took the form of either a single dominant D1 subclade or a mixture of D1/D1a in approximately equal proportions. A gradual decrease in the proportion of C3 associations was seen from the internal sites (100%) through the Musandam sites (~43% predominance), across to the Fujairah sites (~15% predominance) and south to Muscat (only 1 C3 association in 24 samples). Concurrent with the decrease in C3 associations, an increase in C15-cluster associations (C15 and C15L) was demonstrated.

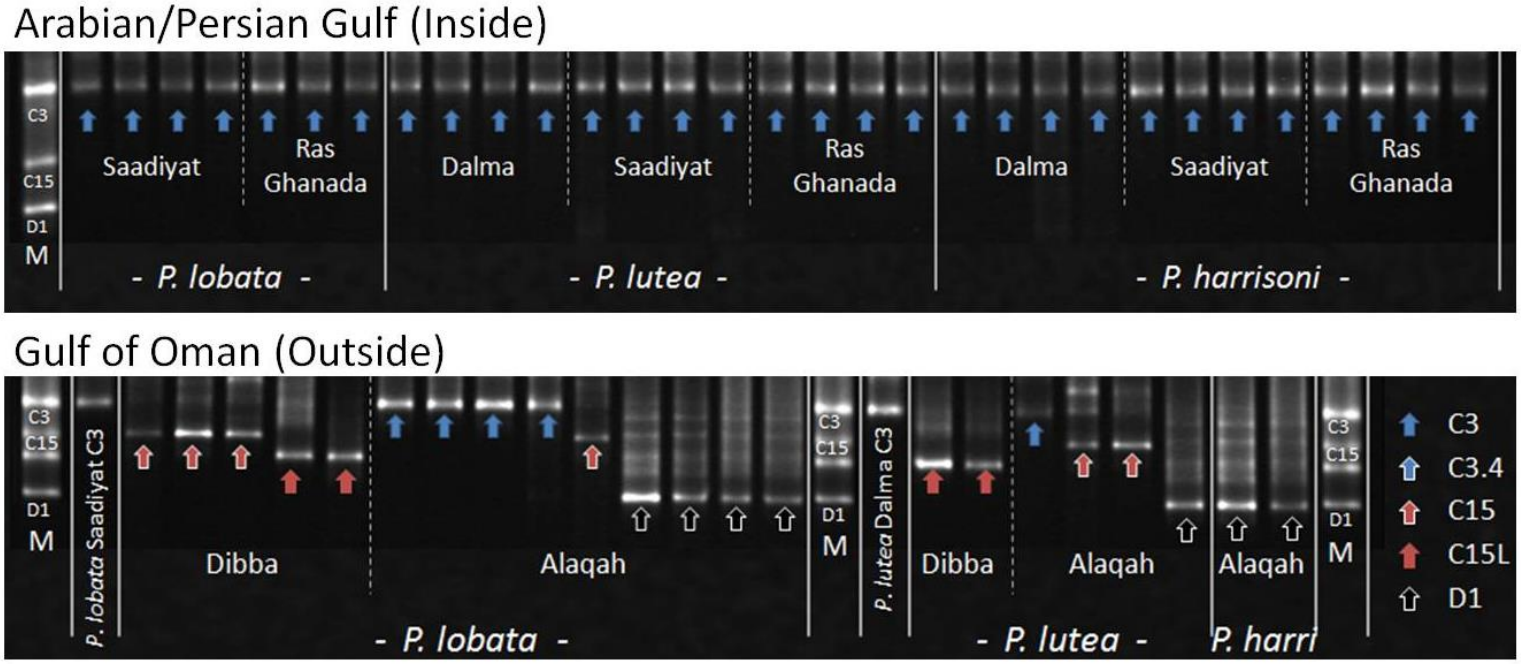

Figure 5.5 Annotated PCR-DGGE gels inferring subcladal ITS2 *Symbiodinium* type. A selection of corals sampled from three sites inside the Gulf are run in the top image and corals sampled at two sites outside the Gulf in the Gulf of Oman are run in the bottom image. Both images have samples run from left to right ordered by species and sub-ordered by site, as annotated. ITS2 type subclade markers are run in the left most lanes (annotated 'M') on both gels and subsequently as annotated in the bottom gel. *Symbiodinium* ITS2 type can be inferred from the distance that the predominant band has run and are annotated using the coloured arrows according to the key.

5.5 Discussion

The 133 corals sampled along the north and northwest UAE coastline at 6 sites within the Gulf showed a complete predominance of ITS2 type C3 *Symbiodinium*. A complete lack of any other *Symbiodinium* subclade was found. This homogeneity is in contrast with the mixed *Symbiodinium* complements (including C3, C15, C15L and D1/D1a) found at the three sites outside of the Gulf in the Gulf of Oman. The discrete difference between the internal and external *Symbiodinium* communities indicate that the difference in physical water parameters between the two water bodies (i.e. across the Strait of Hormuz) has a significant effect on host-symbiont partnerships. Furthermore, the complete homogeneity of symbiont complements between the internal sites across a spatial range of approximately 430km suggests that the same over-arching symbiont-host defining pressure(s) are acting on each site and that these pressure(s) are of greater influence than other pressures that may otherwise define the host-symbiont complements at each local scale.

The complete predominance of C3 associations at the internal sites closest to the Strait of Hormuz (e.g. Ras Al Khaimah region, Figure 5.4) would suggest that these sites have symbiotic associations defined by environmental pressures similar to the other internal sites. This finding is unexpected given the proximity of the Ras Al Khaimah region sites to the Strait of Hormuz. Studies characterising the symbiont complements off the Iranian coast (as little as 100Km north of the Ras Al Khaimah region sites) by contrast demonstrate a mixed diversity (clades C and D) similar to those identified in the Strait of Hormuz. This contrast in biogeographical distribution highlights the differences in environmental pressures between the Iranian and UAE coastal waters and may be explained by the water circulation of the Gulf. The Iranian Costal Currents (ICC) and the Iranian Current Eddies (ICE) transport cooler, less saline influx water from the Strait of Hormuz northwest along the Iranian coast. This water has a minimal effect on the more saline waters off the UAE coast moving in the opposite direction. The waters off the Iranian coast running NW-SE, and the UAE coast running SW-NE may therefore be interpreted as two separate water masses moving in opposite directions and characterised by different physical parameters (Johns et al. 2003) that in turn define the coral-symbiont associations they support. Corals surviving off the Iranian

coast are therefore influenced by the influx waters from the Gulf of Oman that will cause a dampening in their environmental conditions both in terms of salinity and thermal regime. By contrast, corals of the UAE coast are exposed to the more mature waters of the Gulf (towards the terminus of the counter clockwise circulation) in the form of higher salinities (40-43) and larger, more extreme thermal regimes.

Of the 144 *P. lobata* and *P. lutea* colonies identified in the literature with predominant *Symbiodinium* genotypes resolved to a subcladal level, 100% associated with the C15-cluster. The vertical transmittance (acquisition through maternal inheritance) of *Symbiodinium* in *P. lobata* and *P. lutea* links evolutionary fitness of the symbiont to growth and reproductive output of the host (Krueger and Gates 2012). Fidelity strengthening adaptations of the host (i.e. evolution of vertical transmittance in *Porites spp*.) in conjunction with symbiont adaptation to minimise alternative partnerships (i.e. loss of motility and therefore potential for horizontal transmittance in C15) are evolutionary traits associated with the reduction of antagonism in co-evolving long-term partnerships (Ewald 1987).

Associations of *Porites spp*. with C15 are found throughout the Indo-Pacific. The stability of these relationships has been demonstrated over large geographical ranges (See Figure 5.3), across temperature gradients as well as through bleaching events and after transplantations in the field (LaJeunesse et al. 2004a) and to long-term aquaria setups (Hume et al. 2013). Furthermore, the *Porites*-C15 association is purportedly heat tolerant with evidence implicating both host- and symbiont-instigated mechanisms (Fitt et al. 2009). The divergence from this high fidelity in the Gulf and Gulf of Oman waters demonstrated in this study is therefore geographically unique and unexpected, likely due to the extreme conditions. No cases of *P. lobata-/P. lutea*-C3 associations have been identified elsewhere, either as an acclimatisation and means to extort the advantage of such an association, or, due to the out competition of other associations in environmental conditions, similar to that of the Gulf (high salinity, extreme thermal regimes). Whether these unique Gulf associations are an adaptation or acclimatisation is indeterminable.

The lack of C3 associations in *P. lobata/lutea* elsewhere may suggest Gulf corals have adapted to disassociate from the C15-cluster fidelity that is

ubiquitous in Indo-Pacific *Porites spp*. to associate with C3 as part of a better suited coral-symbiont partnership. Whether all Indo-Pacific *P. lobata/lutea spp*. have the ability to associate with C3 *Symbiodinium* is unknown. Given the evolutionarily basal nature of the C3 *Symbiodinium* (LaJeunesse 2005), *Porites spp*. may have previously associated readily with this symbiont before evolutionary radiation of the C15-cluster. Therefore despite the long-term co-evolution of *P. lobata/lutea* with C15-cluster *Symbiodinium* that would suggest a limitation of these corals to readily change symbiont, *Porites spp*. may already have a genetic disposition to form stable C3 association. In this way, the *Porites*-C3 associations seen in the Gulf may be caused by the out competition of other associations due to the extreme environmental conditions.

Further analyses of C3 communities inside and outside of the Gulf to a higher genetic resolution (e.g. analysis of microsatellite sites) would be highly informative. Analysis at this level will enable a higher resolution understanding of the internal communities that appear homogenous at the ITS2 type level and elucidate whether C3 populations inside the Gulf are genetically similar or isolated from those of the Gulf of Oman. Genetic analyses of host populations in parallel will aid in resolving any separate evolution of the Gulf region corals in comparison to *Porites* populations across the Indo-Pacific region.

Previous analyses of coral symbionts in the Gulf were limited to four studies covering three separate regions. Three of these studies found a predominance of clade D *Symbiodinium* in the Strait of Hormuz, Iranian coastal waters and off the Saudi Arabian coast (Baker et al. 2004; Mostafavi et al. 2007; Shahhosseiny et al. 2011). The exception to this trend, Hume et al., 2013, found a complete absence of clade D *Symbiodinium* off the Abu Dhabi coast. Harbouring clade D *Symbiodinium* has been demonstrated experimentally to confer an increased thermal tolerance in its coral host (Berkelmans and van Oppen 2006). Coral associations with clade D symbionts (usually subclade D1/D1a) are commonly recognised in stressful environments including high and low temperature, low and high light levels, and waters impacted by turbidity (van Oppen et al. 2001; Chen et al. 2003; Ulstrup and Van Oppen 2003; Mostafavi et al. 2007; Shahhosseiny et al. 2011). Additionally, clade D symbionts are identified in corals that are able to withstand environmentally induced bleaching when corals at the same location harbouring different *Symbiodinium* genotypes have

succumbed (LaJeunesse et al. 2010b). Equally, accounts of stable relationships in areas difficult to assess as stressful also exist. It should be noted that the affinity of Clade D for a given environment appears host-specific (Cooper et al. 2011). Given the stress tolerant nature of associations with this clade it is surprising to find a lack of these associations in this Gulf study. Equally surprising is the ubiquitous presence of subclade C3, a symbiont considered a generalist and thermally sensitive (although see Hume et al., 2013 for experimental evidence of thermally tolerant Gulf coral associations with C3).

Together with ITS2 type C1 the *Symbiodinium* ITS2 type C3 forms an ancestral core of separate species radiations in the Atlantic-Caribbean and Indo-Pacific (LaJeunesse 2005). This evolutionarily basal nature of C3 underpins its host-unspecific or generalist nature. The apparent stasis in divergence of both the C1 and C3 subclades since at least the mid-Pliocene when the uplift of the Central American isthmus occurred (3.1 to 3.5 MYA; LaJeunesse 2005), in conjunction with the purported large host-generalist populations of the time, would suggest that these ancestral subclades were responsible for host-symbiont associations over a large geographical extent and therefore a multitude of diverse environmental niches. These subclades would therefore have been true generalists not only in the sense of host partnerships but in their ability to colonise such diverse environmental niches. This environmental flexibility may explain the ability of C3 to succeed in the extremely variable waters of the Gulf, where other subclades may not. The apparent selection of the Gulf's physical parameters towards C3 associations may therefore be seen as selection of a symbiont with a greater phenotypic plasticity.

Whilst no comparison has been made between ITS2 types C3 and D1 in their ability to supply the energetic demands of the coral host, comparisons between D1 and other C subclades (C1 and C2) demonstrate that corals harbouring clade D symbionts are at a disadvantage energetically (Little et al. 2004; Cantin et al. 2009; Mieog et al. 2009; Jones and Berkelmans 2010). The relative reduction in growth, larval survival and carbon photosynthate incorporation established experimentally in Clade D harbouring corals will have a significant impact on corals' survival in extreme and stressful environments, such as are found in the Gulf, through the reduced ability to mitigate physiological stress. The selection against energetically ineffective host-

symbiont combinations by the extreme conditions of the Gulf waters may be one explanation for the lack of clade D found in this study.

Given the reported tolerance of D1/D1a and C15 associations to high and low, and high temperature stress (Fitt et al. 2009; Barshis et al. 2010), respectively, it is unlikely that the lack of associations seen in this study are due to extremes of either cold or hot thermal stress alone. Alternatively, the large thermal range experienced in the Gulf, including annual minima as low as 16°C as well as extreme cooling events local to the southern Gulf region, may shape the symbiont communities. These low temperatures and large thermal ranges may be likened to other high-latitude coral populations that experience more variable thermal regimes than their tropical counterparts.

Annual temperature fluxes in the tropical Indo-Pacific may be as little as 2°C, a stark contrast to the flux of up to 20°C seen in the Gulf. High-latitude coral populations that may experience temperatures as low as 13°C in extreme cases (e.g. Iki Island, Japan) are characterised by high diversity and local endemicity of coral-symbiont pairings. *Symbiodinium* communities at high-latitude locations are relatively poorly studied, but from those studies that do exist, differences in host-specific symbiont partnerships from tropical locations are apparent. For example, colonies of *Porites heronensis* sampled at Lord Howe Island (31°S; 10°C annual range with temperatures ranging from 17-27°C) harboured subclades C111, C117 (both derivative of C3) and C3, not the C15 commonly found in tropical populations. Across 16 additional species predominance of generalists C1 and C3 as well as a collection of novel C3 derived symbionts were found. No clade D symbionts were discovered. This predominance of 'thermally sensitive' and 'generalist' symbionts as well as the lack of clade D may be likened to the Gulf communities found in this study. Similarly to Lord Howe Island, populations in Bermuda (32°N) experience a 17-28°C annual thermal range. Bermudan corals demonstrate a predominance of clades A, B and C, but again notably, a lack of clade D. Whilst the Gulf symbiont communities may show pairings with symbiont generalists and a divergence from host-specific fidelities similar to other high latitude populations, a low symbiont diversity is apparent, perhaps a factor of the relatively young age of the Gulf water body or the greater environmental pressures.

In addition to the thermal regimes, salinity also varies substantially from the Gulf of Oman (approximately 36) to the southern Gulf (up to 43). The relatively fresh waters of the Gulf of Oman extend into the Strait of Hormuz. The movement of these less saline waters NW along the Iranian coast correlates with the strength of the ICC and ICE; salinities are lowest during the summer when influxes are maximal. Sites sampled as part of this study cover a salinity gradient (east to west; from approximately 37 in the Ras Al Khaimah region to 42 at Dalma; Johns et al. 2003). Given that no change in coral-symbiont association is witnessed along this salinity gradient it is likely that salinity is not the sole defining pressure of symbiont associations.

Whilst the effects of hypo-salinities on the coral holobiont has received attention due to its environmental relevance with regards to the inundation of coral reefs by freshwater inputs, the effects of hyper-salinity are poorly studied with only a handful of studies to date. The use of short term exposures (Coles and Jokiel 1978; Porter et al. 1999) and environmentally inappropriate salinity and temperature challenges (Sakami 2000) in the majority of the studies limit their application in the consideration of the Gulf's physical environment. However, several studies assessing the effects of salinity on the holobiont (Muthiga and Szmant 1987) and isolated symbionts *ex hospite* (Rogers and Davis 2006) may suggest that corals are not as stenohaline as traditionally thought (Coles and Jokiel 1992) exhibiting acclimation/acclimatisation to salinities up to 45. The survival of entire reef ecosystems in the Gulf waters is evidence of at least certain corals' abilities to adapt. Whether long-term exposure and adaptation/acclimatisation to salinity plays a role in defining host symbiont associations by affecting the symbiont physiology (i.e. by selecting against associations with a given symbiont genotype, e.g. subclade D1 or C15) or by directing host adaptations that may modify otherwise common partnerships remains undetermined and warrants further investigation.

Moving from the four external sites in the Gulf of Oman, across the three sites within the Strait of Hormuz, to the six internal sites in the Gulf, a shift from C15- to C3-dominated communities occurs. The majority proportion of C15 associations (61%) found at the external sites is in accordance with the usual *Porites*-C15 fidelity found throughout Indo-Pacific populations. Whilst the sites sampled in the Gulf of Oman lie outside of the Gulf and are therefore not

subject to the same degree of extreme thermal regime and salinities, the physical parameters in these waters may still be considered extreme when compared to the parameters of coral-populated Indo-Pacific waters. Specifically, the sites sampled off the coast of Fujairah, approximately 100km south of the Strait of Hormuz experience annual SST (temperatures ranging from approximately 22.5-32°C; Foster et al. 2012) several degrees above and below those found in the majority of coral-harbouring tropical Indo-Pacific waters. The corals sampled at Muscat (the most southerly site; approximately 350Km SW of the Strait of Hormuz) experience similar annual thermal variability. These reefs are however exposed to an additional low temperature stress (drops of up to 8°C over a 24hr period; Coles 1997) due to a shallow thermocline between 5-10m and influences of upwelling from the Arabian Sea. Given the 'stressful' nature of these waters the presence of clade D associations would not be surprising. However, species specific *P.lobata/lutea*-D1 associations are still unique to the region and, similar to the minority proportion (14%) of *Porites*-C3 associations also found at the external sites, their survival external to the Gulf may be a factor of the still relatively large thermal range. Despite the fact that the majority of outflow from the Gulf through the Strait of Hormuz is in the form of a deep saline layer, the presence of these unusual associations external to the Gulf suggests their successful export. The host-symbiont associations found at the three sites sampled in the Strait of Hormuz unsurprisingly represented an intermediary between the internal and external sites with an equal majority of C3 and C15 associations (42 and 40%, respectively). This biogeographical gradient of associations seen from the Gulf (predominance of C3), through the Strait of Hormuz (relatively even proportion of C3 vs. other associations), down the Oman and UAE coast to Muscat (only one C3 in 24 associations), suggest that the internal, thermally tolerant associations gain a competitive advantage from the physical environment of the Gulf. Although the internal associations appear physiologically able to survive outside of the gulf their gradual replacement by associations representative of Indo-Pacific *Porites* associations i.e. C15-cluster, suggest their out-competition. This in turn would suggest that the internal environment of the Gulf offers a unique advantage to these thermally tolerant associations.

Whilst internal Gulf associations are able to survive externally to the Gulf in waters of significantly lower salinity, their gradual out competition with increasing distance from the Gulf would suggest that they are unable to act as 'saviours' for coral reefs that face an increase in thermal stress due to warming seas. Predicting whether these resilient internal associations may regain an advantage over other associations as seas continue to warm will be dependent on elucidating the properties of the Gulf that afford the resilient Gulf populations their advantage – whether it be a function of salinity, extreme thermal range or some other factor.

5.6 Conclusion

Here we have characterised host-symbiont associations in corals of the genus *Porites* inside and outside the Gulf. Our findings indicate that the differences in physical characteristics between the Gulf and the Gulf of Oman have a significant influence on coral-*Symbiodinium* associations. Given the exclusivity of the *P. lobata/lutea* –C3 associations to the Gulf and Gulf of Oman region demonstrated in this study (in contrast to the complete fidelity of these species with C15 throughout the rest of the Indo-Pacific) these unique association may either be an adaptation of Gulf corals to a better suited coral-symbiont partnership or a product of natural selection by the Gulfs unique environment. We suggest that the uniqueness of Gulf coral associations may be a product of the collective physical stresses of the Gulf's large annual thermal range and extreme salinities more than any single physical stressor alone.

The ability of global corals to survive the predicted increases in frequency and magnitude of thermal perturbations due to climate change through mechanisms similar to those in the Gulf is unlikely given the limited time available for adaptation to occur and the inability of the resilient Gulf associations to become ecologically dominant external to the Gulf.

The association of Gulf corals with the generalist C3 and the empirically proven thermal tolerance of this association highlight a considerable knowledge gap and misunderstanding in our classification of thermally-tolerant and –sensitive symbionts. Further investigations into the genetic similarities between coral hosts and symbionts of the Gulf and their Indo-Pacific conspecifics are essential, as is an improvement in our understanding of the effects of hyper-

saline environments on the coral holobiont, specifically in terms of thermal tolerance.

Chapter 6: A critical appraisal of current methods utilised to estimate *Symbiodinium spp*. diversity and the implementation of a novel 'ITS region' definition of subcladal variants

Note: The content of this chapter is the sole work of BCCH.

6.1 Abstract

The Internal Transcribed Spacer region 2 (ITS2) and to a lesser extent ITS1 regions are commonly used to assess diversity and to define subcladal types. However, differences in resolving powers between the two regions and the presence of type-defining sequence divergences outside of these regions cause problems when identifying strict ITS2 or ITS1 types. Additionally, the multi-copy nature and intra-genomic variance in the rDNA operon of *Symbiodinium* spp. presents problems when attempting to assess diversity due to the presence of pseudo-genes (genes that do not represent genuine ecological diversity).

Here, more than 300 sequences representing the entire ITS region (ITS1-5.8S-ITS2) of the rDNA operon from three clades of *Symbiodinium spp.* (A, C and D) are used to quantify and characterise the difference in taxonomic resolution of the ITS1, ITS2, 5.8S and ITS region. In addition, the common practice of screening sequence nucleotide divergences in relation to their influence on secondary structures of the pre-rRNA is critically assessed.

The investigations conducted in this chapter demonstrate the surprisingly low resolving power of the ITS2 region compared to the ITS1 and 5.8S regions within clade C. The 5.8S region is shown to have high primary sequence divergences from other *Symbiodinium* 5.8S sequences, despite its conservative secondary structure, and these divergences are demonstrated to have a negative effect on defining ITS2 types. Taxonomic resolution through use of

the entire ITS region rather than either of the ITS1 or ITS2 separately is demonstrated to offer advances in diversity resolution and support of subcladal variants. The use of secondary structural screening is demonstrated to discount a high proportion of sequence divergences that are otherwise strongly supported, even across geographical distribution, and as such is not supported in its current form. Finally, a new system is proposed for the phylogenetic definition of subcladal types based on the entire ITS region and the 6 most abundant subcladal types found in this study are defined accordingly.

6.2 Introduction

Of the eight genera of dinoflagellate algae reported to have symbiotic representatives, scleractinian corals predominantly harbour species of the genus *Symbiodinium*; a genus that also commonly associates with platyhelminths, molluscs, and protists (Muscatine and Porter 1977; Trench 1993; Glynn 1996; Rowan 1998; Lobban et al. 2002). The genotypically-defined variation in susceptibility of algal symbionts to physical environments (Little et al. 2004; Cantin et al. 2009; Jones and Berkelmans 2010; Jones 2011) have the potential to define the fitness of coral hosts and therefore implicate identifying symbiont genotype, at an appropriate genetic resolution, as important in the consideration of the physiology of coral-*Symbiodinium* associations. Through determining the biogeographical distributions of these associations and characterising their physiological implications, the fitness of coral reef ecosystems to current and predicted future environments may be better understood.

Initial estimates of diversity within the genus *Symbiodinium* were confounded by the lack of morphologically distinguishable features, as resolved through light microscopy (Freudenthal 1962). Diversity estimates were as low as a single species (*S. microadriaticum*; Freudenthal 1962) until investigations of cultured symbionts isolated from different hosts and geographical locations, using not only morphological but biochemical and genetic parameters, identified *Symbiodinium* as being a highly diverse genus (Schoenberg and Trench 1980a,b,c; Chang et al. 1983; Mcnally et al. 1994). Identification of differences in cell morphology, mobility, ultrastrucutre, circadian rhythms, growth rates, host infectability, photoacclimation and thermal tolerance in

isolated cultures support the diverse nature of the genus (Schoenberg and Trench 1980a,b,c; Fitt et al. 1981; Chang et al. 1983; Colley and Trench 1983; Trench and Blank 1987; Iglesias-Prieto and Trench 1994,1997; Little et al. 2004). Whilst it is generally accepted that the genus *Symbiodinium* contains many species, few have been formally described (Trench 1993; Rowan 1998).

The first genetic studies undertaken to assess the diversity within the genus *Symbiodinium* used DNA/DNA hybridisation and allozymes (Schoenberg and Trench 1980a; Blank and Huss 1989). In one of these studies the differences in binding between different *Symbiodinium* isolates were similar to the observed differences in binding between *Symbiodinium* and other algal representatives from different classes (Blank and Huss 1989). This diversity was unexpectedly high. Genetic resolution of taxonomy within the genus *Symbiodinium* was next undertaken through the analysis of the nuclear ribosomal DNA operon. This operon is made up of the small subunit (SSU; 18S), the internal transcribed spacer 1 (ITS1), the 5.8S region, the internal transcribed spacer 2 (ITS2) and the large subunit (LSU; 28S), as well as external spacers. The SSU, first analysed by Rowan and Powers (Rowan and Powers 1991), resulted in the identification of divergent lineages that are now commonly referred to as clades or types; clades A, B and C (Rowan and Powers 1991), D (Carlos et al. 1999), E (*S. californium;* Lajeunesse and Trench 2000; LaJeunesse 2001), F (LaJeunesse 2001), G (Pochon et al. 2001) and H (Pochon et al. 2004). The clades were later supported by phylogenies inferred through the use of chloroplast (Santos et al. 2002; Takishita et al. 2003) and mitochondrial genes (Takabayashi et al. 2004). Similar to early genetic investigations, the diversity identified in these studies was unexpectedly high. Investigations using the SSU and other genes started to characterise the biogeography of host-symbiont associations. However, due to the relatively conserved nature of the SSU and later analysed LSU (Lenaers et al. 1989; Wilcox 1998), as well as chloroplast and mitochondrial markers, a genetic distinction between symbionts displaying differences in physiological parameters within the clades was not possible.

In an attempt to resolve these intra-cladal types (now commonly referred to as sub-clades) LaJeunesse investigated the ITS1, 5.8S and ITS2 regions (LaJeunesse 2001). The ITS regions have been successfully used to resolve at or below the species level in protists, animals, plants, fungi and macrophyte algae (Gonzalez et al. 1990; Lee and Taylor 1992; Coleman et al. 1994; Goff et al.

1994; Baldwin et al. 1996). In this investigation, LaJeunesse (2001) detailed a subcladal resolution of symbionts within the genus *Symbiodinium* that closely approximated physiologically distinct populations. Identical 'ITS types' (defined through the phylogenetic analyses of the entire ITS1-5.8S-ITS2 region) identified in some species (i.e. *S. pilosum, S. meandrinae, S. corculorum* , type A1; *S. pulchrorum, S. bermudense*, type B1; *S. californium S. kawagutii*, type F1) bring into question the original identifications of some *Symbiodinium* species. The approximate correlation of the ITS resolved phylogenies with physiologically distinct populations suggest that this region may be appropriate for use in ecological studies. Further assessments of the ITS region have concluded that it may be a suitable candidate for the resolution of dinoflagellate species (LaJeunesse 2002; Sampayo et al. 2007; Sampayo et al. 2009). In competition to this, a lineage-based approach (Dean and Ballard 2004; De Queiroz 2007) was put forwards in which multiple sequences from mitochondrial (*Cyt b*), chloroplast (*cp23S*) and ribosomal (LSU & ITS) genes may be used to identify fundamental biological units (species) within *Symbiodinium* (Sampayo et al. 2009). Genetic markers such as the *psbA* minicircle, that resolves at a similar taxonomic level to the ITS2 region, but critically, lacks the confounding issues of being a muti-copy gene displaying high intra-genomic variation (such as is seen with the rRNA gene) have been proposed as a supporting or alternative marker to ITS rRNA analyses (LaJeunesse and Thornhill 2011). Despite this however, the majority of investigations into coral-S*ymbiodinium* associations still utilise the rDNA operon, specifically the ITS region. Analyses using microsatellite flanking regions or microsatellite alleles may resolve the *Symbiodinium* yet further, to a population or even clonal level (Santos et al. 2004).

The high divergence of the ITS1 and ITS2 regions between clades limit the uses of the ITS region to within clade diversity assessments. Contrary to LaJeunesse's original investigation (LaJeunesse 2001) into the use of the ITS region, the majority of contemporary studies utilise only the ITS2 region of the rRNA gene as a means to identifying subcladal *Symbiodinium* 'types' (the relatively less conserved ITS1 region is also used to define subclade albeit to a lesser extent) as opposed to the entire ITS1-5.8S-ITS2 region. As a rapid means to characterise coral-symbiont associations, the ITS2 type may be characterised through differential electrophoresis migration patterns using denaturing

gradient gel electrophoresis (DGGE; see chapter 3 for further introduction) as made popular by studies such as LaJeunesse 2002. The wide-spread use of this methodology throughout the literature has played a large part in establishing the ITS2 region as the default marker for *Symbiodinium* subcladal assessment.

The multi-copy character and demonstrated high intra-genomic variation of the rDNA operon confound accurate estimations of diversity. As well as these biologically-based issues, PCR and cloning artefacts may further complicate and increase diversity estimates. Furthermore, definition of ITS2 types where the defining nucleic divergence from the consensus does not lie in the strictly defined ITS2 region has now produced a situation whereby one given ITS2 sequence may have several identical matches that cover the full query and yet are defined as separate subclades in online databases (i.e. GenBank). For example, sequences for C15 derivatives: C15.2 (FJ461509; Stat et al. 2009); C15.6, C15.7, C15.8 (FN563472.1, FN563473.1, FN563474.1; Barshis et al. 2010); C15k (GU111873; LaJeunesse et al. 2010a); and, C15h, C15m (FJ646568, FJ646569; Smith et al. 2009) all differ from the C15 basal sequence in regions outside of the ITS2 region, in the 5.8S or 28S regions. Use of additional sequence regions in the definition of novel subcladal types may offer an additional resolving power, however, in these instances, the regions used in the definition must be strictly defined and accurately described to avoid defining pseudo-replicate ITS2 types as demonstrated above. The presence of these 'type-defining' divergences in the rDNA highlight the loss in resolving power that occurs when only the ITS2 region (as opposed to the originally used ITS1-5.8S-ITS2) is considered as part of the analysis. Analysis of the entire region would provide a greater ability to resolve subcladal types due to the analysis of two 'variable' regions in parallel.

Accurate assessment of subcladal diversity using the rDNA operon, be it through the analysis of the ITS1 or ITS2 regions independently or the entire ITS region, is dependent on the accurate identification of PCR and cloning artefacts, and pseudo-genes (genes that are harboured within a *Symbiodinium* cell but are non-functional, caused by intra-genomic variation of the DNA sequence; Thornhill et al. 2007). Screening for pseudo-genes and PCR artefacts within a collection of sequences produced through sequencing representatives of several bacterially cloned amplicon libraries may be done in several ways.

PCR artefacts such as chimeras and incorporation errors produced during amplification are commonly assessed through a comparative analysis of multiple libraries. If an insertion/deletion (indel) or substitution occurrence is not supported in sequences from one (or more depending on screening parameters) other bacterial library or from defined types found in the literature then the occurrence may be conservatively identified as a PCR artefact or a representative of a non-functional pseudo-gene. Divergences from the consensus that are found in multiple libraries may be further assessed by taking in to account their effect on the secondary structure of the RNA (or pre-RNA in the case of the ITS regions).

The three rDNAs (18S, 5.8S and 28S) as well as the internal and external transcribed spacers are transcribed by RNA polymerase I into a single precursor molecule (the 35-45S pre-rRNA; Hunter et al. 2007). After several further processing steps mature and fully functional rRNA is formed. These processing steps include the excision of the spacer regions; nucleotide modifications, such as methylation and pseudoridylation; terminal additions of nucleotides; and further cleavages and trimmings of the precursor molecule (Perry 1976; Venema and Tollervey 1999). The correct secondary structure of the ITS regions is essential for the correct processing of rRNA transcripts. Modifications to this secondary structure may inhibit or prevent the correct formation of mature product (Musters et al. 1990; Liu and Schardl 1994; van Nues et al. 1995; Michot et al. 1999; Cote and Peculis 2001). The secondary structure of the ITS1, 5.8S and ITS2 regions are made up of single stranded regions with helices in-between. These helices are generally formed of a base-paired stem capped with an apical loop of unpaired nucleotides. Modifications to the primary sequence that would result in a negative influence on the secondary structure of the ITS or surrounding regions may result in the improper formation of mature rRNA and therefore represent a non-functional gene (a pseudo-gene). Screening of nucleotides that diverge from the consensus in relation to their potential effect on secondary structures may therefore be used to conservatively identify potential pseudo-genes. Ambiguous nucleotides may be reverted to the consensus if deemed to have a potentially negative effect on secondary structure (if they disrupt base-paired regions in the helical stems; modifications to unpaired regions are considered to represent no change to the secondary structure).

However, whilst the modifications to secondary structures are better understood in organisms such as yeasts (*S. cerevisiae*; Musters et al. 1990), little knowledge of the exact effects in dinoflagellates exists. Therefore, whilst screening of this type has strong theoretical grounds, especially considering the conserved function and secondary structure of the rRNA across organisms (Mai and Coleman 1997; Coleman et al. 1998; Michot et al. 1999; Coleman 2003), it is still not an exact means of identifying potential pseudo-genes. Use of this method in the screening of sequences derived from bacterial cloning will under-estimate diversity and therefore may be viewed as a conservative approach. However, without a supporting or superior means available for screening, this method currently represents the most effective means of limiting the over-estimation of *Symbiodinium* diversity, if subcladal type definitions or functional gene quantifications must be made.

Through the course of this PhD, in excess of 500 sequences from more than 10 species of scleractinian coral have been collected through bacterial cloning of the rDNA operon. Of these 500 sequences, more than 300 of them cover the entire ITS1-5.8S-ITS2 region, with the remainder covering only the ITS2 region. It is the aim of this penultimate chapter to use the collection of sequences covering the complete ITS region and representing three clades of *Symbiodinium* (A, C and D) to:

- Estimate the relative resolving power/conservative character of the ITS1, 5.8S ,ITS2 and entire ITS region;
- Conduct a critical assessment of the effectiveness of the use of secondary structure analysis through the use of subcladal C3-derived sequences collected from 'inside' and 'outside' of the Gulf;
- Associate the most frequently found subcladal sequences (resolved over the entire ITS region) to an ITS1 and ITS2 type, and from this association identify these sequences according to their entire ITS region ('ITS type').

6.3 Methods

6.3.1 Note on terminology: 'Subclade' and 'Variant'

In this chapter the terms subclade and variant are referred to frequently. Whilst subclade is a term commonly used in the literature, variant is not. In the context of this chapter, it is important to define their meanings exactly.

'Subclade' refers to defined - either through the ITS1, ITS2, entire ITS region or some other genetic marker - genotypes of *Symbiodinium* that are resolved at a taxonomic level lower than clade. Examples are subclades C3, C1 and D1. The term subclade is also used to refer to genotypes that are genetically defined as being derivative from these basal subclades e.g.: C3cc, C1.3 and D1a.

'Variants' in this context refers to any genotype identified across any extent of the *Symbiodinium* rDNA operon that differs from other variants. They may be formally defined as subclades or not. For example, when the collection of *Symbiodinium* C3 sequences is processed in this study to identify the diversity of unique sequences, each of these unique sequences represents a variant. One of these sequences/variants may be identical to previously defined subclade C3. Therefore, in this context this clade C sequence variant is subclade C3. As such, a subclade is a variant, but not all variants may represent a subclade; Hence the term, 'subcladal variant'. The two are differentiated in this chapter because defined subclades should represent genuine ecological *Symbiodinium* genotypes, however, due to the multi-copy nature and intra-genomic variance of the rRNA operon, not all variants may relate to subcladal types. Therefore variant is used here in a methodological context to refer to the unique sequences/genotypes that may be defined as subcladal types.

6.3.2 Relative resolving power of the ITS1, 5.8S, ITS2 and complete ITS region of the *Symbiodinium* rRNA gene

The majority of sequences recovered from this PhD belonged to *Symbiodinium* clade C. Furthermore, the greatest rate of intra-genomic variability was seen in this clade (one variant, ITS2 type A1, was found in 30 clade A sequences and, 2 clade D variants, ITS2 types D1/D1a were found in 8 clade D sequences; data not shown). As such, relative resolving powers of the rDNA regions were tested in their ability to identify subcladal types within the clade C sequences. Multiple sequence alignments were made of all clade C sequences for which the entire ITS region was available (approximately 260 sequences). Code was written in Microsoft's C# language using the Microsoft Visual Express Suite 2012 to automate the removal of unsupported nucleotide substitutions and indels (defined as occurrences of divergence from the consensus that were only found in sequence representatives from one bacterial library) from the

alignment. Such unsupported occurrences were reverted to the consensus. Remaining divergences from the consensus were viewed as potential representatives of genuine subcladal variants. The ITS1, 5.8S and ITS2 regions were defined according to both the multi-clade alignment made in chapter 3 and the regions defined in several secondary structure analyses (Gottschling and Plotner 2004; Hunter et al. 2007; Thornhill et al. 2007; both the empirical- and literature-based definitions of the regions showed excellent agreement). Sequences representing potential subclades were defined as any sequence containing a novel combination of supported sequence divergences and therefore a unique sequence. Further code was written to identify supported nucleotide substitution/indels and assign sequences to unique variants potentially representative of unique subclades. All unique cladal variants identified through the use of the newly designed code were verified by eye. As well as quantifying the number of variants identified by each region, parsimony networks were created using the software Network (Fluxus Technology). Further code was generated to automate the process of converting fasta format alignments to strict nexus formats that represented an acceptable form of input to the Network software. Parsimony networks were computed using the default setting of the Network software and through the Median Joining methodology, with an epsilon value of 0 and the 'frequency > 1 criterion' active (this caused only the variants represented by more than one sequence to be used in the computation of the parsimony network, limiting complexity of the final network analyses). Post-processing MP calculations were performed and the networks were created by drawing the shortest trees as a free-standing network – no modification was made to the torso. Alignments were altered so that indels were assessed as a single change.

6.3.3 Critical assessment of secondary structure screening

A multiple sequence alignment of all sequences representative of, or close derivatives from, subcladal ITS2 type C3 from the Gulf region (the Gulf, Strait of Hormuz or Gulf of Oman) was made using the methods previously mentioned with unsupported substitutions or indels reverted to the consensus. C3 variants were then computed where a novel variant was defined as a sequence containing a unique combination of supported (found in one or more bacterially cloned libraries) substitutions or indels. A second multiple sequence

alignment was then made that contained one representative of each variant identified from the first alignment. Secondary structures of the ITS1 region were used as defined in Thornhill and Lord 2010. The secondary structure of the ITS2 region was inferred from the works of Hunter et al. 2007 using sequence AY686647. The secondary structure was manipulated and verified as energetically favourable using the Mfold server (Zuker 2003) and the 4sale software (Seibel et al. 2006; Seibel et al. 2008). The primary sequence of the 5.8S region of *Symbiodinium* rRNA used in Thornhill et al. 2007 showed considerable variation to the C3 5.8S sequence used in this study. The secondary structure was therefore re-estimated using the Mfold sever to determine energetically favourable folding patterns, taking into account regions of the 5' and 3' ends of the 5.8S region that form complimentary secondary structures with the LSU. The results of the re-estimation of secondary structure showed highly similar folding forms to the original dinoflagellate 5.8S region defined by Gottschling and Plotner 2004 and were therefore assumed as correct.

The ITS1, 5.8S and ITS2 folding structures were combined into a single structural schematic using the 4sale software. Sites of supported divergence (by one or more bacterial libraries; as described previously) from the consensus were identified on the secondary structure schematic and were assigned as either having a positive effect (extending the number of paired bases as part of a helix), a neutral effect (indel or substitution that occurred at a region of unpaired bases, either as part of an apical loop or as part of the single stranded section between helices), a weakened interaction (G-C to a G-U pairing) or a negative interaction (disturbance of one of more pairings within the structure without creating additional pairings). Any consensus divergences that did not result in either a positive or neutral effect were considered as a possible representative of a pseudo-gene and were reverted to the consensus. The number of variants in the alignment was then re-computed to calculate the decrease in estimated diversity due to secondary structure screening.

The likelihood of consensus divergences that were deemed to have a negative effect on secondary structure of rRNA to have been generated by random PCR/sequencing error or random intra-genomic variation was assessed. Frequency probabilities for PCR/sequencing error rates were conservatively estimated using 30 clade A *Symbiodinium* sequences. Within these sequences

0 supported mutations were found and therefore no variants were found. The rate of intra-genomic variation was distinctly low in this clade in comparison to clade C (data not shown). For the application of estimating error, the occurrences of all of the single nucleotide substitutions (singletons; no indels were apparent) were attributed to PCR/sequencing error. In this model, intra-genomic variation was assumed to be 0. By discounting variation due to intra-genomic variation this model of error frequency estimation will be bias in the direction of over estimating error due to PCR or sequencing error. Seventeen singleton consensus divergences occurred across the 30 611bp long sequences, producing a probability for any given nucleotide to have been produced by PCR/Sequencing error of 9e-4. The probability for two sequences, produced from two separate PCRs to have the same consensus divergence caused by PCR/sequencing error would therefore be approximately < 8.1e-7. Support of any mutational change/consensus divergence by additional sequences, especially from additional bacterially cloned PCR libraries would lower this probability value further.

The likelihood of consensus divergences to have been caused by random-occurrences of intra-genomic variation in the same sequence location not due to genuine ecological diversity was estimated, again conservatively, using the >260 clade C *Symbiodinium* sequences containing the entire ITS region. In this model, insertions and deletions were treated as single changes to the sequences and only consensus divergences that were supported (occurred in the same bp position of the sequence in other sequences) by five or less other sequences were counted as being due to random, and not genuine diversity caused, intra-genomic variation (In reality, a sequence divergence found in five other sequences may well be caused, dependent on the number of separate libraries, by genuine ecological diversity. This level of support is set deliberately high to ensure a conservative estimate). In this model the possibilities of consensus divergences being due to PCR/sequencing error were discounted. Four hundred and twenty six divergences (as defined above) were found in the 263 673bp long sequences. This gives a probability of any given nucleotide being caused by random intra-genomic variation alone of approximately 2.4e-3. Similar to above, the probability for two sequences, produced from two separate PCRs to have the same consensus divergence

caused by random intra-genomic variation would therefore be approximately 5.8e-5.

It should be noted that the above two models for estimating probability are not intended to be an accurate approximation, rather, they represent an excessively conservative estimation to demonstrate the approximate magnitude of the probabilities that the consensus divergences considered in this part of the study are due to chance alone and not an effect of genuine ecological diversity.

6.3.4 Estimating diversity and defining subcladal types in the genus *Symbiodinium*

Predominant clade C variants were identified from the ITS region parsimony network created as part of assessing the relative resolving power of the rDNA operon regions. Variants that were represented by at least 8 sequences were used in this part of the analysis (variants labelled with black arrows in Figure 3.3.1). Closest accession similarities that had a subcladal type defined were identified in GenBank for the ITS1 and ITS2 regions of one representative sequence from each variant. The quality of match of each of these literature-based sequences was noted and the type definition given by the database records were used as the ITS1 and ITS2 types. By considering the combination of both of these types, for each of the predominant ITS region sequences, an 'ITS type' was defined.

6.4 Results and Discussion

6.4.1 Relative resolving power of the ITS1, 5.8S, ITS2 and complete ITS region

The ITS1 region demonstrated the least conserved nature with the highest number of variants defined for a given region and the highest number of variants defined per bp of sequence (

Table 6.1). A similar number of variants supported by at least 8 sequences were defined by both ITS regions, however, the ITS1 region demonstrated a greater distance between the two majority variants than the ITS2 region (10 and 3 sequences changes as opposed to 3 and 2; Figure 6.1). These findings suggest

that in concurrence with the higher rate of intra-genomic variation, the ITS1 region is better suited to identifying lower level taxonomic diversity than the ITS2 region. These results would suggest that the predominant use of the ITS2 region over ITS1 in the literature may limit our ability to resolve *Symbiodinium* diversity in, at least, clade C.

Table 6.1 Comparison of various regions of the rDNA operon with regards to length, number of unique variants (a sequence containing a unique combination of supported consensus divergences [divergences that are found in sequences from more than one amplicon bacterial library]) defined and the relative number of variants defined normalised to length of the region.

Region	Length (bp)	Number of unique variants defined	Variants defined per bp
ITS1	221	62	.28
5.8S	158	33	.21
ITS2	204	26	.13
ITS1-5.8S-ITS2	583	114	.20

Unexpectedly, the 5.8S region defined a greater number of variants than the ITS2 region, despite its documented conserved character (Gottschling and Plotner 2004; Thornhill et al. 2007) and shorter sequence length. Accounts of the conserved nature of the 5.8S region generally refer to the secondary structure rather than the primary sequence. Thornhill et al. 2007 demonstrates a relatively conserved secondary structure of the 5.8S region amongst *Symbiodinium* representing several clades (including clades A, C and D), even with a difference of up to 16 substituted bp across the 161 bp region (subclade A4). Despite the large absolute number of sequence variants defined in the 5.8S region (

Table 6.1), only four of these are supported by more than 18 individual sequences and the remaining variants have a maximum support of 5 individual sequences with the majority only supported by one or two (Figure 6.1). Furthermore the relatively small distance between the unique variants (maximum two mutations) in comparison to variants defined in the other regions, further supports the conserved nature of this region, especially between subclades (given that all sequences from this analyses are within clade C). Due to its commonly considered conserved nature, the entire 5.8S

region is rarely used in the consideration of *Symbiodinium* phylogenies at a subcladal level, with the ITS regions taking preference. Results from this study support the literature findings that this conserved nature is at a higher order (structural). Importantly however, a considerable primary sequence variation is demonstrated. The substantial (higher than the ITS2 region) number of supported variants identified in the 5.8S region in this study highlight the potential of the use of this region in creating a more powerful resolution of taxonomy when used in conjunction with one of the more commonly used ITS regions.

One hundred and fourteen unique variants were identified through the use of the entire ITS region. Whilst a similar number of variants supported by 8 or more sequences were resolved (six; Figure 6.1) in comparison to the individual ITS regions, improvements in distances between 'outgroup' variants were apparent (15 and 6 sequence modifications; see Figure 6.1). The similar number of variants identified using the entire ITS compared to the ITS1 or ITS2 region that were supported by more than 8 sequences suggests that the predominant variants being defined in the ITS1 and the ITS2 region are the same subcladal type. If different subcladal types were being defined in the ITS1 and the ITS2 regions then the number of types defined using the entire ITS region would be considerably larger (closer to a product of the number of types defined in each of the individual regions) and variants identified by the entire regions would have low numbers of sequences supporting them. This relatedness can be identified in each of the separate region networks (i.e. blue, green and red groupings in Figure 6.1) and supports the use of the entire ITS region in determining subcladal diversities (as it will incorporate the resolving power of all three individual regions combined).

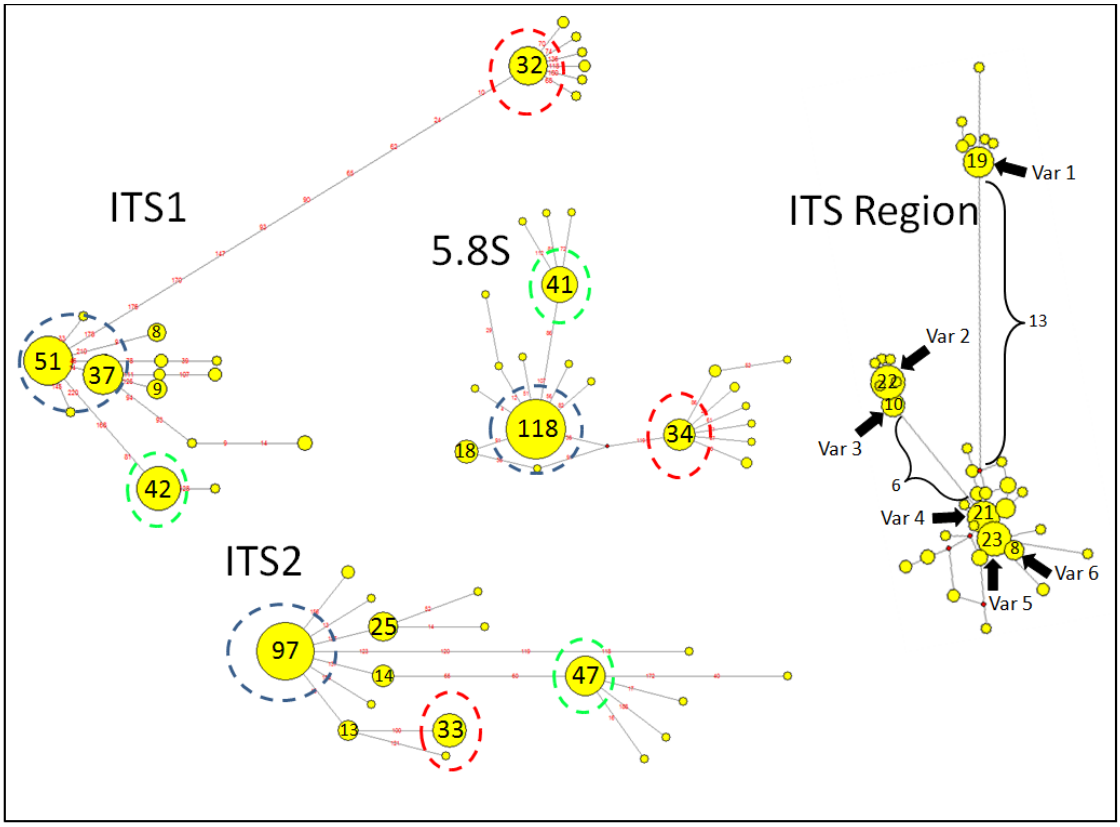

Figure 6.1 Parsimony networks calculated using individual and entire sections of the ITS region (as labelled) of the clade C *Symbiodinium* rDNA operon from 263 sequences. Numbers within circles represent the frequency of supporting sequences. Circles with less than 8 supporting sequences are unlabelled. Coloured and dashed circles represent rough groupings of sequences (grouped by colour). Grouping were verified manually as corresponding by identifying constituent sequences. Red marks on connecting lines represent one sequence change each. The 'ITS Region' network has no red mutation marks displayed due to the density of the network. To visually quantify mutational changes between sequence variants in this network, braces annotate the number of mutations in two given links. Black arrows labelled 'Var 1-6' correspond to variants representative of at least 8 sequences and used in the 'ITS type' subcladal identification in section 6.3.4

The use of parsimony analyses, similar to screening using estimated effects to RNA secondary structure, does not offer an exact means of identifying genuine sub-cladal diversity; however, by taking in to consideration the support (number of sequences that fall into a given sequence variant definition) that any given variant may have, that variant may be evaluated as likely to represent a genuine sub-cladal type or not. Increases in the number of sequences and the number of bacterial clone libraries used in analyses of this type will increase the power to resolve taxonomic diversities.

6.4.2 Critical assessment of secondary structure screening

Of the 84 ITS2 type C3 and C3-derivatives sequences identified from the Gulf region, 11 were an exact match for the basal C3 sequence. Within the remaining 73 sequences 25 C3 variants were identified from a combination of 18 substitutions or indels across the entire sequence length (Figure 6.2). Two of these consensus divergences were deemed to have a positive effect on the secondary structure of the rRNA, 7 a negative effect and 9 a neutral effect (Figure 6.2 and Figure 6.3). After reversion of the 7 consensus divergences deemed to have a negative effect on the rRNA secondary structure, the original identification of 25 C3 variants from 73 sequences was decreased to 9 variants, a 64% decrease.

All of the consensus divergences that were discounted as potential pseudo-gene representatives by the structural analysis, with the exception of divergence 15, had considerable support from other sequences, including sequences representative of separate clone library PCR amplicons (Table 6.2). Divergence 15 was only found in two sequences from two libraries. As such, it represented the least supported divergence. Even so, the probability of this divergence being due to chance, rather than a genuine ecological diversity, is small (Table 6.2). Divergences 4 and 5 were found solely in sequences attained from coral hosts external to the Gulf in the Gulf of Oman. The correlation between geographical location and sequence further support a genuine ecological association – one that would be discounted through the use of such structural screening despite the high support (9 sequences from 5 libraries). The data presented here would suggest that screening *Symbiodinium* rRNA sequences through analysis of secondary structure may not represent an effective means of inferring consensus divergences representing pseudo-genes. Whilst the methodology may be based on sound theory, unknown elements such as, the degree of change to the secondary structure required to functionally influence effective formation of mature RNA and the actual, as opposed to theoretical, folding structures of the primary sequences limit the exactness of this method.

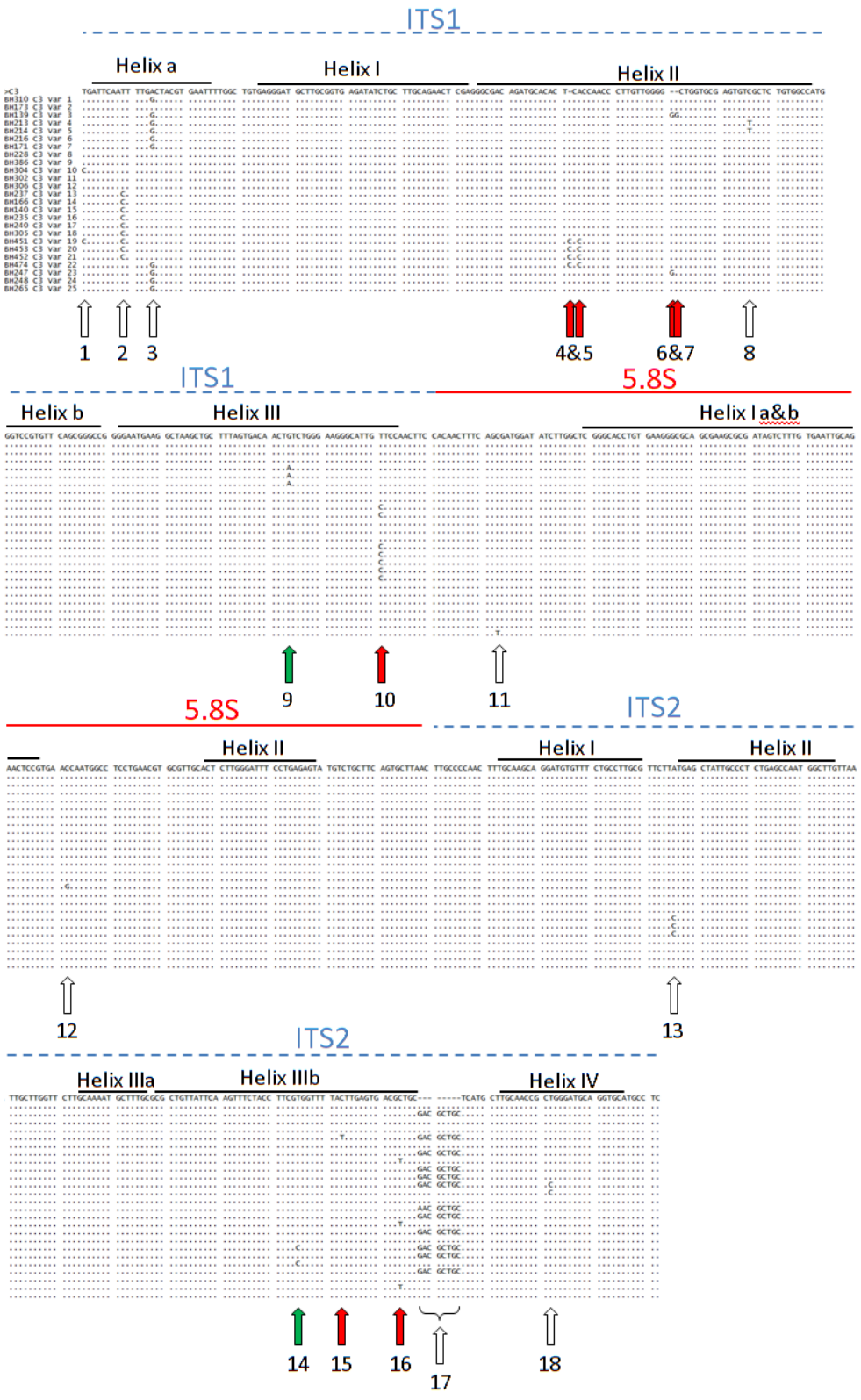

Figure 6.2 Alignment of variant representative C3 sequences obtained from bacterial cloning of PCR amplicons containing the complete *Symbiodinium* sp. ITS1-5.8S-ITS2 rRNA gene from corals of the Saadiyat region and Fujairah emirate, UAE. The alignment was created as described in chapter 3. Each aligned sequence is representative of at least two sequences obtained from separate PCR libraries (different bacterially cloned

PCR amplicons). A nucleotide in place of a dot (.) denotes a substitution from the C3 'type'. A dash (-) denotes an insertion/deletion (indel). Coloured dashed lines above each alignment section denote which rRNA region the sequence lies within and correspond to markings in Figure 3.3.3. Solid black lines denote nucleotides found in corresponding helices marked in Figure 3.3.3. Arrows below the alignment represent sites of sequence divergence from the type C3 sequence. The effects of these modifications on the secondary structure of the rRNA gene are represented by the colour of the arrows. Red arrow = negative influence, blue arrow = weakened interaction (G-U pairing), white arrow = no influence and green arrows = positive influence. 26 potential C3 variants are apparent.

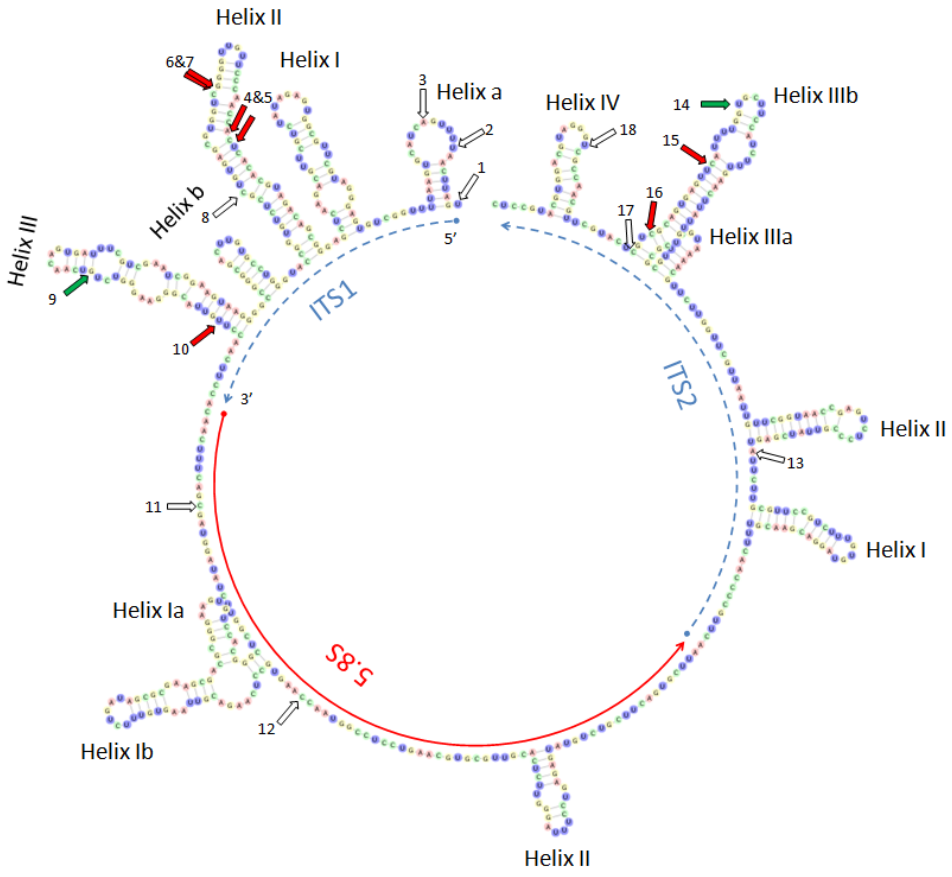

Figure 6.3 Secondary structure of the *Symbiodinium sp*. type C3 ITS1-5.8S-ITS2 regionof the rRNA gene. Regions and contained helices are labelled and correspond to Figure 3.3.2. Short arrows correspond to those of Figure 3.3.2 and represent sequence divergences from type C3 and are colour coded according to their effect on the secondary structure of the rRNA gene. Red arrow = negative influence, blue arrow = weakened interaction (G-U pairing), white arrow = no influence and green arrows = positive influence.

Table 6.2 Sequence support of mutation positions(consensus divergences) seen in the alignment from Figure 3.3.2.

Mutation position	Number of sequences containing mutation	From number of bacterially cloned PCRs	Probability caused by random PCR/sequencing error	Probability caused by random intra-genomic variation
4 + 5	9	5	≈< 8e-7	≈<5.8e-6
6 + 7	7	6	≈< 8e-7	≈<5.8e-6
10	11	8	≈< 8e-7	≈<5.8e-6
15	2	2	≈< 8e-7	≈<5.8e-6
16	5	5	≈< 8e-7	≈<5.8e-6

Mutation positions in column one refers to the sites marked with arrows in Figures 3.3.2 and 3.3.3. The probability of each of these mutations to have been caused by either PCR error/sequencing error or random intra-genomic variation are assessed according to the estimations described in the methods section of this chapter.

6.4.3 Estimating diversity and defining subcladal types in the genus *Symbiodinium*

More than four times as many subcladal variants were identified through the use of the entire ITS region compared to the ITS2 region (

Table **6.1**). Six variants were identified in the ITS region parsimony network as being supported by at least 8 sequences (Figure 6.1). For a representative of each of these variants, a match in the database was found with at least a 98% coverage and 99% sequence match (at the nucleotide level). The largest divergence from the queried sequence was 2 single base pair substitutions (Table 6.3). An ITS type for each of these variants was defined (Table 6.3).

Use of the complete ITS region in phylogenetic analyses will provide a greater estimate of diversity than ITS2 analyses. Furthermore, variants/types found in ITS analyses will be more resolutely defined and the increase in number of inferable sites will produce phylogenetic analyses with greater support (i.e. ML analyses with greater bootstrap support) - this lack of support is often an issue with the analyses of such closely related sequences (i.e. ITS2 sequence analyses). Whilst ITS2 analyses may not be ideal for the assessment of diversity and defining novel subcladal types/variants, their use in the quick identification of symbiont phylotypes is still of great importance, especially through the use of systems such as DGGE (LaJeunesse 2002). This chapter

should in no way be interpreted as a criticism of the use of the ITS2 region in coral-symbiont association analyses. These analyses have particular power in large scale sampling strategies and analyses (i.e. many species) that are able to identify important biogeographical and ecological trends (LaJeunesse et al. 2004b). The particular concern of this chapter is the trend towards using the ITS2 or ITS1 region as the predominant means to identify subcladal types. These identifications, are not as resolute as the rDNA operon may allow, and the lack of full rDNA operon sequence available for the majority of defined 'types' means that placing new sequences with the full ITS region sequence available are often limited to being identified to either one of the ITS regions.

Whilst some investigations already deposit their entire ITS region sequences, these sequences are often limited to being placed phylogenetically according to ITS2 or ITS2 regions (Wicks et al. 2010). In order to overcome this problem, phylogenetic analyses may be used to identify grouping of well supported sequences (i.e. the well supported variants in Figure 3.3.1noted by black arrows). The ITS2 and ITS1 'basal' types (i.e. C3/C1 [ITS1], C3, C1, D1, etc.) may then be associated to these groupings according to the ITS2 and ITS1 regions of the ITS sequences. In this way the entire ITS region sequence may be assigned a subcladal type already associated to the ITS1 or ITS2, here referred to as the 'ITS type'. Association in this manner is essential if the wealth of knowledge available referring to biogeographical distributions and physiological tolerances already available in the ITS1 and ITS2 types are to be transferred to the ITS region definitions.

Table 6.3 ITS1 and ITS2 types as defined from matches to sequence accessions in the GenBank database.

Variant	Closest Match ITS1	Match Quality	ITS1 Type	Closest Match ITS2	Match Quality	ITS2 Type	ITS Type
Var 1	AB207184[a]	98:100	C15	HM031110[b]	100:100	C15	C15
Var 2	AF380564[C]	100:100	C2	GU111904[d]	100:99:1	C3cc	C3cc.1
Var 3	AF380564[c]	100:100	C2	GU111904[d]	100:100	C3cc	C3cc
Var 4	EU333742[e]	100:100	C1/C3	JF834209[f]	100:100	C3	C3
Var 5	EU333742[e]	100:99:1	C1/C3	JF834209[f]	100:100	C3	C3.1
Var 6	EU333742[e]	100:99:2	C1/C3	JF834209[f]	100:100	C3	C3.2

Variants 'Var 1-6' refer to variants identified (black arrows) in the ITS parsimony analysis (Figure 3.3.1). The closest sequence accession matches (for the ITS1 and ITS2 regions of the sequences from this study representative of the variant types 'Var1-6') found in the database that were identified to a subcladal level are noted ('Best Match ITS1 and ITS2'). The quality of this match is described in the format 'query coverage : max identity : number of base pairs mismatching if max identity is not 100' in the columns 'Match Quality'. ITS types of the closest match sequence accessions are noted in the columns 'ITS1 & ITS2 type'. The definitions, unique to this study of the ITS types are defined in the final column. References in which each sequence accession are found are noted in superscript letters a – Reimer et al. 2006 b – Fisher et al. 2012 c – van Oppen et al. 2001 d – Finney et al. 2010 e – Reimer and Todd 2009 f – Wicks et al. 2010.

6.5 Conclusions

The multi-copy nature and high intra-genomic variation of the rDNA operon does confound diversity estimates and without suitable controls in place, diversity will undoubtedly be overestimated. This study highlights the ability to reduce the likelihood of including artefact variants (created due to PCR error, sequencing error or intra-genomic variation) through discounting/reverting divergences from the consensus that are not well supported by their presence in other libraries (parameters for support will vary depending on the characteristics of the investigation).

Parsimony network analyses have been demonstrated in this study to be a powerful tool in visually and quantitatively assessing the distribution of sequence variants. These networks, alongside further quantitative analysis, have demonstrated the significant benefits in taxonomic resolution available when the entire ITS region, rather than the ITS1 or ITS2 regions, are used in inferences of sub-cladal taxonomy. Use of groupings, such as shown in Figure 6.1, from parsimony network analyses, or any phylogenetic analysis (e.g. ML analysis), in the inference of biogeographical and ecological trends may avoid the necessity of identifying ecologically genuine subcladal diversity. Groupings of this type will most likely represent genuine subcladal types.

To avoid any screening for pseudo-genes or PCR/Sequencing errors (assuming these are constant, independent of sequence origin), given a sufficiently large sequencing strategy, the analyses of differences in variation (i.e. number of unique variants) between sampled groups (i.e. between different species, environmental parameters [low light, high light] or geographical locations) may be sufficient for inferring significant differences in symbiont populations. Only once significant differences have been identified might specific subcladal-types need to be inferred and linked to possible physiological acclimatisation/adaptation.

In order to define subcladal types at a greater resolution with greater support, a system of classifying rDNA ITS region sequence subcladal types/variants is established here which maintains the association with ITS1 and ITS2 types. The use of the entire ITS region will not only allow further resolution due to the use of both of the ITS sites, but also due to the incorporation of the 5.8S region

which has been shown through literature examples to further taxonomic identifications of diversity (e.g. within the C15-cluster) that were otherwise unresolved using only the ITS1 or ITS2 regions. Analysis and definition of subclades using the entire region as an industry standard will increase our understanding of *Symbiodinium* subcladal taxonomy and therefore allow us to better associate subcladal genotypes to ecologically relevant phenotypes.

Chapter 7: General conclusions and future work

7.1 General conclusions

The coral populations of the Gulf have been studied for decades and their extreme physical tolerances, in particular their thermal tolerance, are well documented (Feary et al. 2013). Research efforts in to the elucidation of the mechanisms underpinning their extreme resistance are, in contrast, relatively young, as is the concept of using our understanding of these coral populations to better inform us of the potential effects of warming waters due to climate change on coral populations globally (Coles and Riegl 2012; Feary et al. 2013). The timing of the course of work related to this thesis was opportune. The novel research angle, unexplored research potential and strong research collaborations meant this series of work had a large potential to answer many questions and provide novel insights into the world's most extreme coral populations.

Before undertaking the series of investigations making up this thesis, little was known of the mechanisms allowing the Gulf corals to survive in their unique environment. Prior investigations had put forward evidence of their tolerance being at least in part due to their harbouring of clade D *Symbiodinium*. These analyses were limited to four studies covering three separate regions. Three of these studies found a predominance of clade D *Symbiodinium* in the Strait of Hormuz, Iranian coastal waters and off the Saudi Arabian coast (Baker et al. 2004; Mostafavi et al. 2007; Shahhosseiny et al. 2011). This PhD work was the first to highlight the close-to-complete predominance of clade C *Symbiodinium* across multiple reef-building coral species within the Gulf, specifically C3, a subclade previously characterised as being a host-generalist and thermally sensitive. The association of Gulf corals with generalist C3 and the empirically proven thermal tolerance of this association highlight a considerable knowledge gap in our classification of thermally-tolerant and -sensitive symbionts as well as in our understanding of the possible synergistic effects of environmental parameters such as extreme salinities on the physiological tolerances of the coral holobiont.

The unusual nature of these associations offers insight into a potentially critical misunderstanding in the area of coral-*Symbiodinium* associations. Specifically, the classification of *Symbiodinium* types as being able to withstand certain degrees and types of physical stress (Rowan 2004; Berkelmans and van Oppen 2006; Mieog et al. 2009) or to provide energetic advantages to the coral host (e.g. Little et al. 2004; Mieog et al. 2009; Jones and Berkelmans 2010). These understandings are some of the most fundamental in coral biology. The reliance of management modes and future prediction models on such coral-*Symbiodinium* associations and relations emphasise the need for the correct understanding of these mechanisms.

The successful setup of colonies of *P. lobata* in aquaria based at the Coral Reef Laboratory, Southampton, provides a resource of great potential from which to further characterise the corals of the Gulf in tightly controlled experimental environments. Through the establishment of the Gulf-derived *P. lobata* colonies as models, this investigation has demonstrated the maintenance of their thermal resilience *ex situ* (particularly in comparison to conspecifics from relatively milder Fijian waters) and has established the monitoring of host-fluorescence as an effective non-invasive marker of host stress.

The ability of Gulf corals to maintain thermal resilience *ex situ* was previously unknown. However, this knowledge is of critical importance for two reasons. Firstly, in understanding by which mechanisms Gulf corals are able to mitigate thermal stress and whether these mechanisms are adaptive or acclimatisation responses to their environment we may better comprehend the ability of corals situated external to the Gulf to mitigate the increases in SST that will be faced over the next several hundred years. Secondly, the ability of Gulf corals to maintain thermal resilience external to their unique environment in cultured conditions is crucial if management options such as assisted migration (such as proposed by Riegl et al. 2011 and Coles and Riegl 2012) are to be considered as genuine options for maintaining coral reef ecosystems. The maintenance of resilience *ex situ* (as demonstrated in this thesis) may support the viability of Gulf corals to be used as part of reef restoration projects (Riegl et al. 2011). However, it should be noted that the differences in salinity and thermal profiles have not been taken into account as part of this study.

The use of host fluorescence as a non-invasive marker of host stress (as demonstrated in this thesis) is in support of the limited number of previous studies that also advocate its use in monitoring host health (e.g. D'Angelo et al. 2012). Assessment of holobiont health and reaction to stress is commonly monitored through the use of Fv/Fm monitoring of the algal symbionts. This proxy provides an effective means of monitoring algal complement health in the majority of symbiotic scleractinian corals (e.g. Wiedenmann et al. 2013) and the well characterised relation between symbiont and host health (Muscatine 1990) mean that this proxy may be used to infer host health as well. The common use of this proxy to monitor host health is due to the lack of appropriate (non-invasive, physiologically relevant), alternative markers, specifically designed to monitor the host fraction of the holobiont. Therefore, the establishment of host fluorescence as a non-invasive marker of host health and stress is a vital advancement in coral biology in a time when stress monitoring of the coral holobiont is so relevant.

This thesis has answered one of the most important questions pertaining to the Gulf's coral populations: Do these populations have the potential to, at least partially, mitigate future degradation of Indo-Pacific reefs through the provision of thermally tolerant coral-symbiont associations? The results from this study suggest that the thermally resilient C3-harbouring corals of the Gulf are a product of the Gulf's unique environment (high salinity and extreme thermal regimes). As such, the ability of corals in other locations to undergo an increase in physiological tolerance through similar mechanisms may be limited. These results also suggest the export of thermally tolerant associations from the Gulf. However, these associations are currently out competed in waters external to the Gulf. Therefore these thermally tolerant associations will not be able to mitigate further degradations of Indo-Pacific reefs.

Modern reef management techniques are extremely diverse and their successful implementation and evaluation is complex (Mumby and Seneck 2008). These techniques are constantly adapting in correlation with increased scientific understanding. In relation to the growing threat of climate change, management bodies will increasingly have to rely on techniques that offer long-term advantages to climatic stress mitigation. Local management measures such as protections from over fishing may offer, by proxy, an

increased resilience of coral reef ecosystems to climatic stressors (such as increased SSTs) through increased connectivity or increasing the availability of metabolic resources. These measures do not however directly improve the ability of a coral reef ecosystem to withstand climatic stressors. In contrast, management techniques such as assisted migration, as proposed out of the Gulf waters (Coles and Riegl 2012), have the potential to dramatically increase the thermal resilience of a coral reef ecosystem offering a long-term advantage. The assessment of the viability of these techniques is therefore of critical importance, be the viability supported, or in the case of this study, not.

This thesis has presented method development studies. As part of an initial training study, the international distribution of *A. acroporae* and elucidation of the mechanisms behind its host-specific camouflage were documented. This new knowledge, in parallel with other survival strategies, emphasise its consideration as a potentially damaging invasive species through anthropogenic introductions to vulnerable ecosystems.

The damaging potential of both invasive species (e.g. *Pterois volitans and P. miles*; Semmens et al. 2004; Schofield 2009) and corallivores (e.g. *Acanthaster planci*; Pratchett 2005) on tropical and coral reef ecosystems is well document. *A. acroporae* represents both an invasive species and corallivore (Rawlinson et al. 2011) and therefore has a destructive potential to coral reef ecosystems. As such, it is critically important to understand its parasitic behaviour and survival strategies. Current research on the flatworm relating to its distribution and destructive potential is limited (e.g. Rawlinson et al. 2001; Rawlinson and Stella 2012). As such, the information provided in this study on its feeding behaviour and survival strategy represents a resource on which to build further research which will allow a better evaluation of the threat posed by *A. acroporae*.

This thesis presents two new sets of PCR primers that will advance the field of *Symbiodinium* taxonomy. The low-host complementarity of these primers minimises the confounding of taxonomic analyses through reducing incorporation of coral DNA. This is especially important when considering the relatively high host-complementarity of commonly used *Symbiodinium* primers such as msg2 and msg3 (Savage et al. 2002). The potential amplification of unwanted host DNA when using such primers will confound analyses as well as waste resources and time.

The associated novel DGGE protocol presented in this thesis resolves single base pair sequence differences whilst resolving larger sequence fragments over shorter run times than the predominantly used methodology (LaJeunesse 2002). This predominantly used DGGE protocol is widely accepted and used in the analyses of *Symbiodinium* ITS2 diversity within the coral biology field. The provision of a second set of primers and a set of run times and voltages represents an additional and alternative resource for the field, especially considering the successful amplification of a range of *Symbiodinium* clades and novel annealing sites of the new primers.

Finally, this thesis critically assesses the predominant methodology used to define symbiont genotypes and estimate diversity within the *Symbiodinium* genus and advocates a reversion to the definition of subcladal types using the complete ITS region rather than the ITS2 or ITS1 regions currently favoured. Adoption of this standard will increase our understanding of *Symbiodinium* subcladal taxonomy and therefore allow us to better associate subcladal genotypes to ecologically relevant phenotypes.

The use of the ITS2, and to a lesser extent, the ITS1 region of the rDNA operon is the most commonly used phylogenetic marker to resolve *Symbiodinium* genotypes (Thornhill et al. 2010). The recommendation of this thesis to revert to the use of the entire ITS region therefore represents a large change to the standard methodologies used throughout the literature. However, due to its widespread use, changing the ITS component analysed may offer considerable increases in our knowledge of subcladal distributions and in turn associations between subcladal types and physiological advantages conferred to coral hosts. As stated previously, the understanding of associations between subcladal type and holobiont phenotype are some of the most fundamental and important in coral biology and therefore these phylogenetic considerations should be evaluated with appropriate weight.

7.2 Future work

The establishment of aquaria models, the characterisation of coral-*Symbiodinium* associations, and the continued methodological refinements as covered in this thesis, represent a framework on which to further our understanding of Gulf corals. The experimental aquaria setup will play a large

part in future investigations, the first of which will include elucidating the effects of hyper-salinities on a range of corals of Gulf- and Indo-Pacific-origin in parallel to presented thermal challenges. Studies of this manner will elucidate whether the extreme salinity of the Gulf is responsible for the thermal tolerance of Gulf corals by thermally challenging Gulf corals maintained in environments of lower salinity. By culturing Indo-Pacific corals (that are not adapted to higher salinity conditions) in higher salinities we can better understand if the corals of the Gulf are adapted or whether any coral, independent of salinity history, may acclimatise to these conditions. Furthermore, documentation of *Symbiodinium* genotype harboured in these corals may elucidate whether corals associating with symbionts other than C3 are at a physiological disadvantage.

The coral communities of the Gulf are the most thermally resilient in the world. This thesis has already suggested that this resilience may be in part due to a novel association (Porites-C3). However, the specific or combination of component(s) of the unique Gulf environment responsible for this phenomenon remains undetermined. Previous studies on the effects of salinity on the coral holobiont are limited (Coles and Jokiel 1978; Muthiga and Szmant 1987; Porter et al. 1999; Sakimi 2000; Rogers and Davis 2006) and no studies have assessed the effects of salinity on the coral holobiont's ability to withstand heat stress as described above. If it is determined that increased salinity may cause such unique heat resilient coral-*Symbiodinium* associations, further investigations into the mechanisms of this acclimatisation may be undertaken and potentially applied to increasing thermal resilience in coral populations. This research has the potential to unlock an understanding of how any coral may have the potential to dramatically increase its thermal tolerance over a relatively short time span (acclimatisation response) as a means to mitigating heat stress. The results of such investigations could potentially change the predicted course of corals' global degradation.

Another investigation will isolate and further characterise Gulf- and non-Gulf-derived C3 *Symbiodinium*. A more genetically resolute analysis of the C3 symbionts found in the general Indo-Pacific community and those found in the Gulf will aid in assessing the similarity of the two populations and elucidate whether thermal tolerances of Gulf corals are due to harbouring a genetically and physiologically distinct strain of *Symbiodinium* C3.

External to the Gulf, coral-C3 associations are not associated with thermal resilience (Fisher et al. 2012). Recording these thermally resilient associations within the Gulf is therefore an exceptional finding. The unique nature of these associations and their thermal resilience question the current assumptions that: ITS2 genotyping of *Symbiodinium* associations represents an appropriate resolution with which to link holobiont phenotype to *Symbiodinium* genotype (LaJeunesse 2001) and, that a given genotype of *Symbiodinium* will – in the majority of associations – confer a given phenotype to the coral holobiont (e.g. ITS2 type D1 associations are known to be stress resistant whilst C3 associations are thought to be relatively stress intolerant; Baker et al. 2004; Fisher et al. 2012). The first steps towards investigating whether these assumptions hold true in light of the recent findings will be to investigate whether the ITS2 C3 populations found within the Gulf differ in genotype - at a higher genetic resolution – to populations outside of the Gulf. Results from these investigations will of course have to be assessed in concert with findings from the salinity investigations described previously.

As part of this more detailed analysis of potential differences between the 'internal' and 'external' Gulf coral populations, further sampling efforts will be undertaken in order to increase the resolution of the geographical coverage. This increase in sampling resolution along with the detailed analysis of variant diversity networks (as discussed in chapter 6) across multiple species will further identify the export, survival and propagation of the thermally tolerant associations to the waters of the Gulf of Oman and will aid in understanding the population dynamics within and external to the Gulf which are currently poorly documented.

Finally, an assessment of seasonal stability of the Gulf associations will be undertaken using samples collected over a span of 3 years across all seasons. Given that both the salinity and thermal environment of the Gulf is seasonally driven, corals will face different stressors accordingly. As the harboured symbiont genotype may define the physiological fitness of the coral holobiont, advantages may be gained through the modification of harboured symbiont(s). Whilst the degree to which corals are able to change symbiont type (referred to as shuffling; Rowan et al. 1997; Baker 2001; Baker et al. 2004; Goulet 2006; Goulet 2007) is debated, cases in which corals have changed dominant symbiont type after bleaching events have been documented (Jones et al.

2008). Such changes have been suggested to act as part of an acclimatisation mechanism for corals to modify coral-*Symbiodinium* associations to better suit their environment (Douglas 2003). Whether corals of the Gulf respond to the relatively extreme annual temperature difference - or to bleaching occurrences - through modifying predominant symbiont type is unknown. However, such a mechanism would have potential to confer large advantages to the corals through maintaining a plastic phenotype. Therefore, characterising coral-*Symbiodinium* associations (to at least a subcladal level) across the seasons will elucidate whether this is an additional survival strategy used by the Gulf coral populations.

Appendices

Appendix 1: Widespread distribution of corallivorous *Acropora eating flatworms* (*AEFW)* in aquaria: Implications for coral husbandry and natural populations

Table 7.1 Documented incidences of AEFW in scientific and hobbyist literature.

Location	Source	Reference	Fig. 1
England	AJ	This Study	A
California, U.S.A.	AJ	Nosratpour 2008	B
New York, U.S.A	AJ	Rawlinson et al. 2011	C
Virginia, U.S.A.	AJ	Rawlinson et al. 2011	D
Melbourne Australia	HR	http://www.masa.asn.au/phpBB3/viewtopic.php?t=167904&p=277873	7
Hong Kong, China	HR	http://www.disturbedreefers.com/viewtopic.php?t=879&postdays=0&postorder=asc&start=390	6
England	HR	http://www.ultimatereef.net/forums/showthread.php?t=422442	1
Germany	HR	http://www.liveaquaria.com/general/general.cfm?general_pagesid=362	2
Cape Town, S. Africa	HR	http://www.sareefkeeping.com/forum/showthread.php?t=481	3
Durban, S. Africa	HR	http://www.marineaquariumsa.com/showthread.php?t=2683	4
Thailand	HR	http://reefcentral.com/forums/showthread.php?p=13067877&highlight=coralrx#post13067877	5
California, U.S.A	HR	http://www.nano-reef.com/forums/index.php?showtopic=251441	8
Florida, U.S.A	HR	http://www.thereeftank.com/forums/f190/flat-worms-aefw-92251.html	9
Florida, U.S.A	HR	http://pet.solazylife.com/sps-keepers/26000.html	10
Georgia, U.S.A	HR	http://www.reefcentral.com/forums/showthread.php?t=874082&pp=25	11
Illinois, U.S.A	HR	http://www.3reef.com/forums/diseases/aefw-acro-eating-flat-worms-start-quaratine-42188.html	12
Missouri, U.S.A	HR	http://reefcentral.com/forums/showthread.php?p=15919606&highlight=coralrx#post15919606	13
New York, U.S.A	HR	http://www.manhattanreefs.com/forum/coral-farming/15834-possible-new-aefw-treatment.html	14
South Dakota,	HR	http://www.reefcentral.com/forums/showthread.php?s=&threadid=874082&perpage=25&pagenumber=2	15

U.S.A			
Texas, U.S.A	HR	http://www.reef2reef.com/forums/general-sps-discussion/52584-aefw.html	16

AJ: academic journal, HR reef aquarium hobby reference. Letters A-D and numbers 1-16 identify the locations marked in the map in Figure 2.2.1

Table 7.2 Primers used in the AEFW study

Name	Description	Sequence	Source
Msg2	Partial ITS1-5.8S-ITS2 of rDNA, *symbiodinium spp*. specific	5'-GTAGGTGAACCTGCGGAAGGA-3'	Savage et al. 2002
Msg3		5'-TCCTCCGCTTACTTATATGCTTAA-3'	Savage et al. 2002
BC-Fwd	Partial 28S of rDNA, *Polycladida spp*. specific	5'–AGTACGTGAAACCGCTGAGAG–3'	This study
BC-Rev		5'–ACCATCTTTCGGGTCCCAAC–3'	This study
LHP-Fwd	Partial 18S of rDNA, *Polycladida spp*. specific	5'-AGCTATGGTTCCTTGGATCG–3'	This study
LHP-Rev		5'–CCAGTTGGCATCGTTTATGG–3'	This study
RHP-Fwd	Partial 18S of rDNA, *Polycladida spp*. specific	5'–GGTTCTATTTTGTTGGTTTTCG–3'	This study
RHP-Rev		5'–CCTTGTTACGACTTTTACTTCC–3'	This study

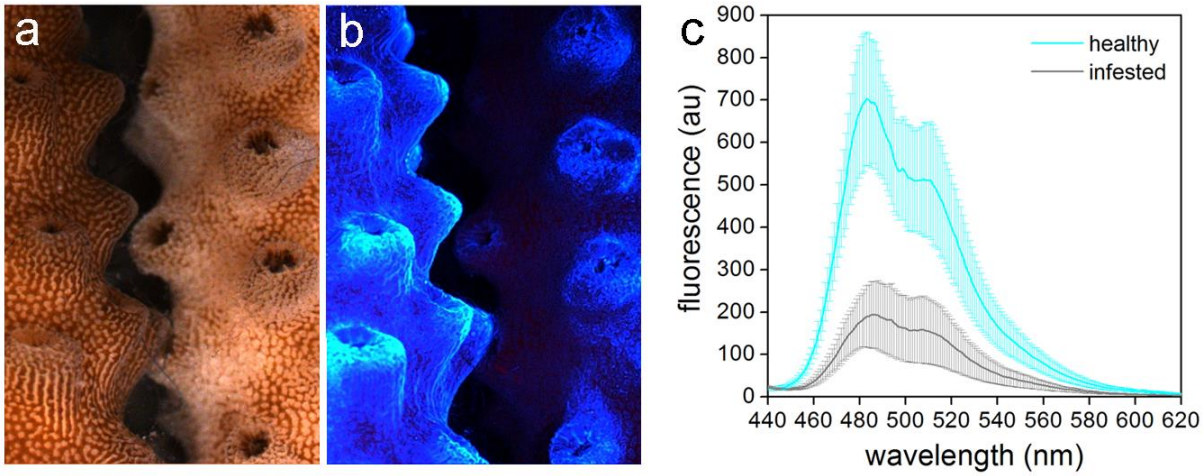

Figure 7.1 Effect of AEFWs infestation on host corals' pigments. (a-b) Microscopic images of *Acropora sp.* branches taken with day light (a), or under blue light, using a Cyan/dsRed filter (b). The branch on the left side of each panel is healthy, the right side one shows the feeding scars of *AEFWs*. (c) Fluorescence spectra measured directly on the surface of the branches shown in (a) and (b).

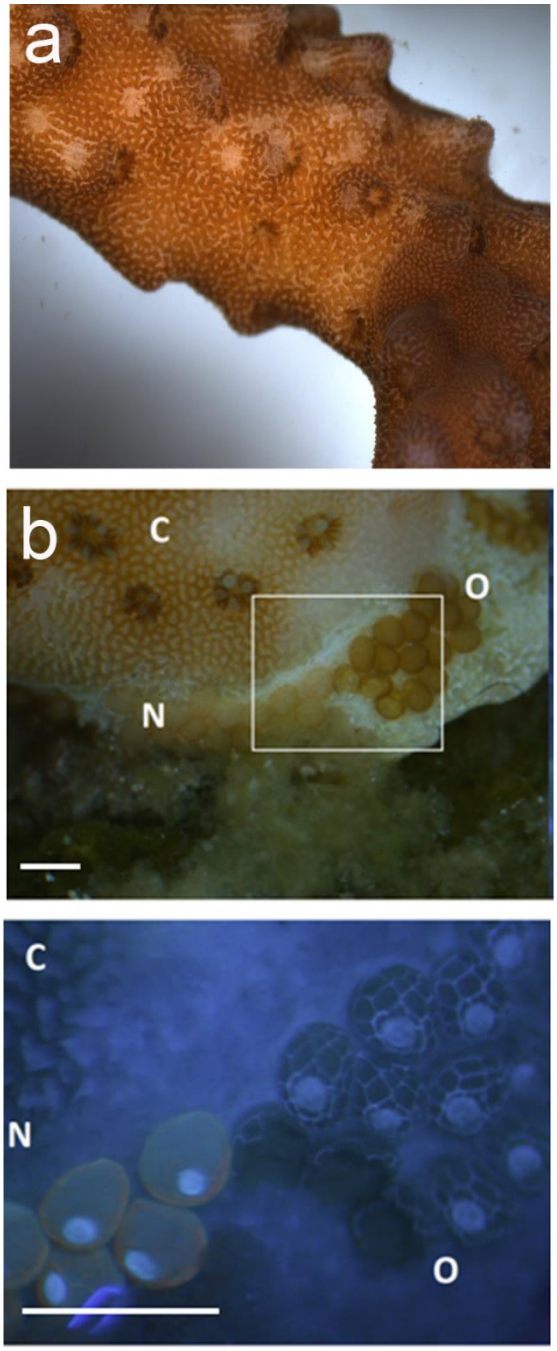

Figure 7.2 Macroscopic observation of *AEFW*. (a) Photograph of the shaded side of a branch from an *A.pulchra* colony infested with *AEFW*. The camouflaged worm can be observed on the right side of the picture. Bite feeding marks are evident along the branch. (b) *AEFW* egg clusters on a specimen of *A. millepora*. The image shows a cluster on the left approximately 2 days old (N), the older cluster on the right (O) is over a week in age. Both sets are laid on bare coral skeleton in close proximity to live coral tissue (C). The bottom image represents an enlargement of the white rectangle section. Fluorescent imagery reveals the segmented character of the eggs as well as differences in development. (Scale = 1mm)

Appendix 2: Primer design for amplification of the ITS and ITS2 region of the *Symbiodinium* rDNA operon for molecular cloning and denaturing gradient gel electrophoresis, respectively


18S
PARTIAL
ITS1
COMPLETE
5.8S
COMPLETE
ITS2
COMPLETE
28S
PARTIAL
SSU
LSU
1a msg2 740bp
msg3 1b
2a SYM_VAR_FWD 780bp
SYM_VAR_REV 2b
3a ITSintfor2 430bp
ITS-Reverse (CLAMP) 3b
4a SYM_VAR_5.8 450bp
SYM_VAR_REV (CLAMP) 4b
P. lobata
P. lutea
msg2
msg3
Clade A
Clade B
Clade C
Clade D
E2
SYM_VAR_FWD
SYM_VAR_REV
ITSintfor2
ITS-Reverse (CLAMP)
SYM_VAR_5.8
SYM_VAR_REV (CLAMP)

Figure 7.3 Consensus identity plot(top; spanning the partial 18S, complete ITS1, complete 5.8S, complete ITS2 and partial 28S regions of the rDNA operon from a multiple sequence alignment made from a range of *Symbiodinium spp*. in Clades A, B, C, D and E) with primer annealing sites denoted by arrow positions (below; relative to the consensus identity bp position). Arrows running left to right represent forward primers and vice versa for reverse primers. Red pairs represent primer pairs from the literature whilst blue represents novel primers designed as part of this study. A sub-alignment (four sets below consensus image and primer binding sites) of the main multiple sequence alignment used to create the consensus identity plot are provided for each primer annealing site. Each of these sub-alignments contains sequences from multiple clades of *Symbiodinium spp.* as well as all available *Porites spp.* sequences from the NCBI database. In these sub-alignments blue names represent coral sequences, green denotes primer sequences and red denotes *Symbiodinium spp.* sequences. Reverse primer sequences have been reverse complimented so that they may be matched to the same sequence as the forward primers. Rather than align clamped primers used in PCR-DGGE applications, the un-clamped version of these primers are aligned and 'CLAMP' is written in parentheses after their names to signify that these are the respective clamped primers for that DGGE pair.

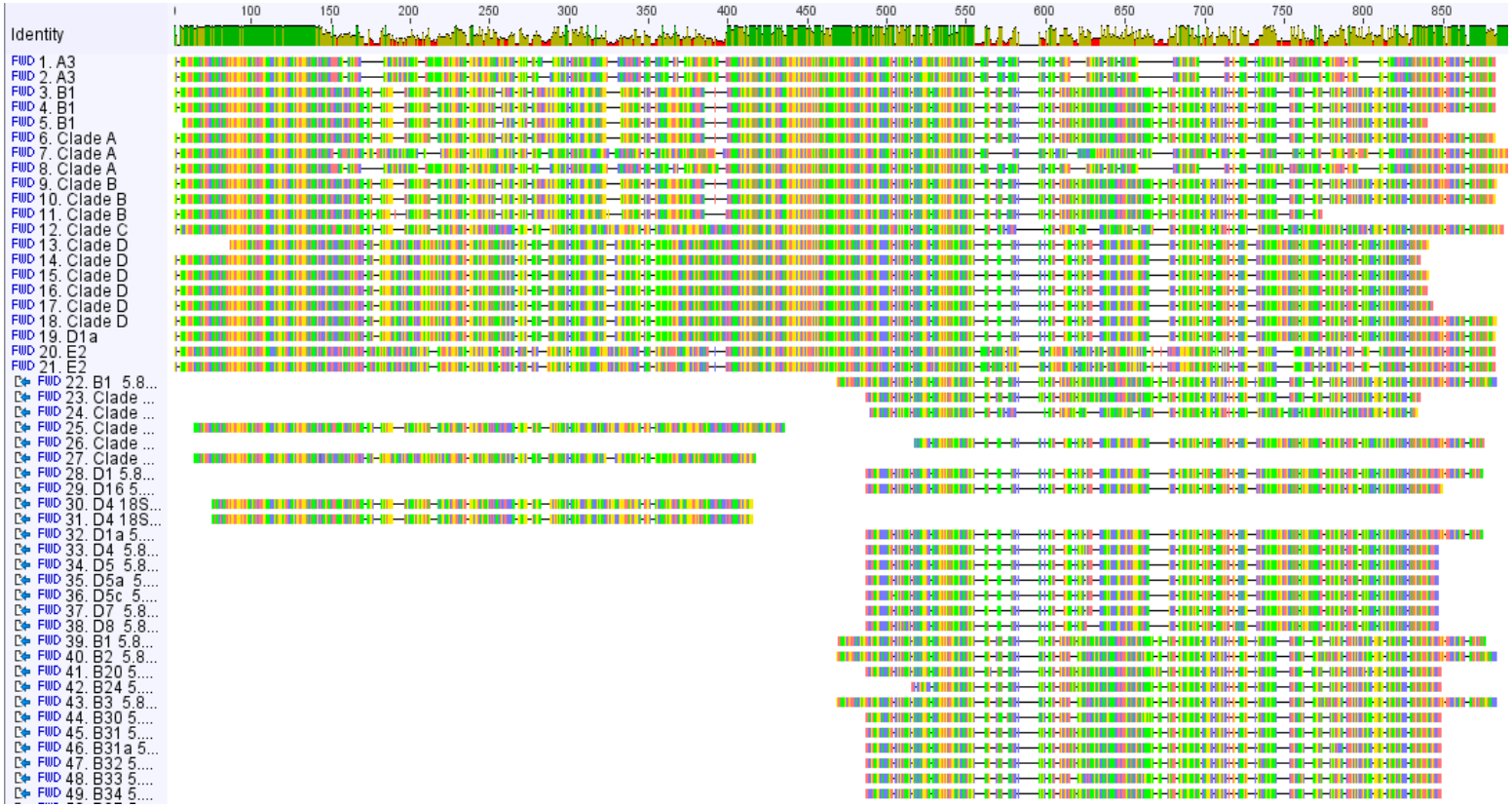

Figure 7.4 Partial image of a multiple sequence analysis of the complete ITS region of *Symbiodinium* rDNAoperon including partial sequences for the 18S and 28S regions used to determine the boundaries of the 18S-ITS1, ITS1-5.8S, 5.8S-ITS2 and ITS2-28S boundaries for primer design. *Symbiodinium* spp. representatives from clades, A, B, C, D and E are aligned. The discrete boundaries between areas of relatively high and low sequence conservation are apparent in the consensus identity plot (top).

```
Sub melttemp()

Dim row As Long
Dim temp As Single
Dim datarow As Integer
Dim nucleotide As Single
Dim dataArray(2000) As Single
datarow = 1
row = 1
temp = tempmake(row)
Label:
Range("C" & row).Select
Do While Not Selection.Value = 0
If Selection.Value < 0.5 Then
Range("B" & row).Select
nucleotide = Selection.Value
If checkArray(nucleotide, dataArray) = 1 Then
'do nothing
ElseIf checkArray(nucleotide, dataArray) = 0 Then
'add the nucleotide and the temp to the array
For x = LBound(dataArray) To UBound(dataArray)
If dataArray(x) = 0 Then
Dim arrayPlusOne As Integer
dataArray(x) = nucleotide
arrayPlusOne = x + 1
dataArray(arrayPlusOne) = temp
Exit For
ElseIf dataArray(x) <> 0 Then
End If
Next x
End If
datarow = datarow + 1
row = row + 1
Range("C" & row).Select
ElseIf Selection.Value > 0.5 Then
'increase row
row = row + 1
Range("C" & row).Select
End If
Loop
temp = tempmake(row)
If Selection.Value <> "Temperature," Then
GoTo Label
Else
End If
'function to write out the array in columns
writeOutArray (dataArray)
End Sub
Function tempmake(ByRef row As Long) As Single
Do While Selection.Value <> "Temperature"
If Selection.Value = "Temperature," Then
GoTo endfunc
Else
row = row + 1
Range("B" & row).Select
End If
Loop
Range("D" & row).Select
tempmake = Selection.Value
row = row + 3
endfunc:
End Function
Function checkArray(searchTerm As Single, myArray As Variant) As Integer
For x = LBound(myArray) To UBound(myArray)
If myArray(x) = searchTerm Then
checkArray = 1
Exit For
Else
checkArray = 0
End If
Next x
End Function
Function writeOutArray(dataArray As Variant)
Dim rownumber As Integer
rownumber = 1
For x = LBound(dataArray) To UBound(dataArray)
If x = 0 Then
'put code for first value as not sure how will handle 0
Range("J" & rownumber).Select
Selection.Value = dataArray(x)
ElseIf (x Mod 2) <> 0 Then 'odd number
'code for if it is an odd number here
If dataArray(x) <> 0 Then
Range("K" & rownumber).Select
Selection.Value = dataArray(x)
rownumber = rownumber + 1
ElseIf dataArray(x) = 0 Then
Exit For
End If
ElseIf (x Mod 2) = 0 Then 'even number
'code for if it is an even number here
If dataArray(x) <> 0 Then
Range("J" & rownumber).Select
Selection.Value = dataArray(x)
ElseIf dataArray(x) = 0 Then
Exit For
End If
End If
Next x
End Function
```

Figure 7.5 Code written in Visual Basic to manipulate the .OUT file produced by the Poland program(Steger 1994) after space delimitated import in Microsoft's Excel.

Appendix 3: Contrasting coral-symbiont associations within, and external to, the Persian/Arabian Gulf: The potential of 'internal' coral-*Symbiodinium* associations to act as 'saviours' of Indo-Pacific reefs

Table 7.3 References and associated data used in the meta-analysis of *Porites spp*. associations

***Porites* Species**	**(Sub)Clade**	**Origin**	**Qty**	**Reference**
P. compressa	C15	Hawaaii	1	(Rodriguez-Lanetty et al. 2004)
P. lobata	C15	Ofu, American Samoa	27	(Barshis et al. 2010)
P. lobata	C15	Ofu, American Samoa	6	(Smith et al. 2007)
P. lobata	KB1, 2, 3, 4, C15	Hawaaii	4	(Apprill and Gates 2007)
P. lutea	KB1, 2, 3, 4, C15	Hawaaii	3	(Apprill and Gates 2007)
P. compressa	C15, C15b, C15c	Hawaaii	7	(LaJeunesse et al. 2004a)
P. brighami	C15	Hawaaii	1	(LaJeunesse et al. 2004a)
P. lobata	Clade C	One Tree Island, South GBR	1	(Loh et al. 1998)
P. lobata	C15	Darwin island, Galapagos Archipelago	15	(Glynn et al. 2009)
P.lobata	C15	Fiji	3	(Hume et al. 2013)
P.lobata	C15	Hawaaii	4	(Thornhill 2003)
P.lobata	C15	Hawaaii	9	(Baker and Oliver)
Porites Spp.	C15 and deriv	Johnston atoll	5	(Stat et al. 2009)
P. lobata	C15	Johnston atoll	5	(Stat et al. 2009)
P. annae	C15 (2) C60(1)	Central GBR	3	(Schmidt et al. 2004)
P. cylindrica	C15(3) C60	Central GBR	4	(Schmidt et al. 2004)
P. lichen	C15(4)	Central GBR	4	(LaJeunesse et al. 2004a)
P. massive	C15(6)	Central GBR	6	(LaJeunesse et al. 2004a)
P. nigrescens	C15	Central GBR	1	(Schmidt et al. 2004)
P. rus	C15	Central GBR	1	(LaJeunesse et al. 2004a)
P. Vaughani	C15(2) C60	Central GBR	3	(Schmidt et al. 2004)
P. cylindrica	C56(a)	Okinawa, Japan	2	(Schmidt et al. 2004)
P. lichen	C56	Okinawa, Japan	2	(Schmidt et al. 2004)
P. lutea	C15	Okinawa, Japan	3	(Schmidt et al. 2004)
P. massive	C15	Okinawa, Japan	3	(Schmidt et al. 2004)
P. rus	C15	Okinawa, Japan	1	(Schmidt et al. 2004)
P. silimaniana	C15	Okinawa, Japan	1	(Schmidt et al. 2004)
P. lobata	C15	Ofu, American Samoa	6	(Smith et al. 2008)
P. annae	C28, C15	Heron Island, S	2	(LaJeunesse et al. 2003)

		GBR		
P. cylindrica	C15	Heron Island, S GBR	2	(LaJeunesse et al. 2003)
P. mayeri	C15	Heron Island, S GBR	1	(LaJeunesse et al. 2003)
P. nigrescens	C15	Heron Island, S GBR	1	(LaJeunesse et al. 2003)
P. rus	C15	Heron Island, S GBR	1	(LaJeunesse et al. 2003)
Porites sp.	Clade C	South Africa	1	(Sebastián et al. 2009)
P. lobata	Clade C	Kaledupa, Indonesia	10	(Hennige et al. 2010)
P. lutea	Clade C	Kaledupa, Indonesia	9	(Hennige et al. 2010)
P. compressa	C15	Hawaaii	3	(Pochon and Gates 2010)
P. cylindrica	C15	Hawaaii	1	(Pochon and Gates 2010)
P. lutea	C15 couple of derive	Moorea French polynesia	13	(Edmunds et al. 2012)
P. rus	C15 one C15.30	Moorea French polynesia	20	(Padilla-Gamiño et al. 2012)
P. irregularis	C15 and derive	Moorea French polynesia	2	(Putnam et al. 2012)
P. lobata	C15	Moorea French polynesia	5	(Putnam et al. 2012)
P. lutea	C15	Moorea French polynesia	5	(Putnam et al. 2012)
P. rus	C15	Moorea French polynesia	5	"(Putnam et al. 2012)
P. solida	C15	Moorea French polynesia	6	(Putnam et al. 2012)
P. lobata	Clade C	Hawaaii	49	(Apprill et al. 2007)
P. lutea	Clade C	Hawaaii	32	(Apprill et al. 2007)
P. asteroides	A4a, A4, B1	Yucatan peninsula, mexico	1	(LaJeunesse 2002)
P. colonensis	C1a	Yucatan peninsula, mexico	2	(LaJeunesse 2002)
P. divaricata	C9	Yucatan peninsula, mexico	1	(LaJeunesse 2002)
P. furcata	A4, B1, C4	Yucatan peninsula, mexico	7	(LaJeunesse 2002)
P. asteroides	A4, B1, C3	Cartagena, Columbia	20	(Camargo et al. 2009)
P. colonensis	Clade C	Cartagena, Columbia	10	(Camargo et al. 2009)
P. porites	Clade C	Cartagena, Columbia	5	(Camargo et al. 2009)
P. asteroides	C1a-j	Belize	2	(Finney et al. 2010)
P. divaricata	A3, C4	Belize	4	(Finney et al. 2010)
P. porites	A3, C4, C47	Belize	9	(Finney et al. 2010)
P. asteroides	A4, A4a,	Barbados	16	(Finney et al. 2010)

	C80, B1, C3,			
P. divaricata	C82	Barbados	1	(Finney et al. 2010)
P. porites	A4porites, C82, C82b	Barbados	10	(Finney et al. 2010)
P. asteroides	A4 and derive	St. John, U.S. virgin islands	20	(Green et al. 2010)
P. asteroides	A4a	Bahamas	6	(Thornhill et al. 2006a)
P. asteroides	A4a	Florida Keys	6	(Thornhill et al. 2006a)
P. asteroides	C1 and derive	Western Carribean, Belize/Yucatan	4	(LaJeunesse 2005)
P. asteroides	C1 and derive	Eastern Caribbean, Bahamas/Florida Keys	4	(LaJeunesse 2005)
P. divaricata	C44, C44a	Northern Caribbean	2	(LaJeunesse 2005)
P. porites	C45a, C1c.C45	Central Caribbean	2	(LaJeunesse 2005)
P. porites	C47	Western Caribbean	1	(LaJeunesse 2005)
Porites spp.	C10, C10a, C13, C14, C43	Eastern Caribbean	5	(LaJeunesse 2005)
Porites spp.	C15, C15a, C18	Eastern Pacific, Gulf California	3	(LaJeunesse 2005)
Porites spp.	C15, C15d	Central Pacific, Hawaii	2	(LaJeunesse 2005)
Porites spp.	C60	Western Pacific, Okinawa	1	(LaJeunesse 2005)
P. cylindrica	C15	S GBR	17	(Stat et al. 2008)
P. cylindrica	C15	Heron Island, S GBR	6	(Fitt et al. 2009)
P. annae	C15	Thailand, Cape Panwa region	2	(LaJeunesse et al. 2010a)
P. cylindrica	C15	Thailand, Cape Panwa region	2	(LaJeunesse et al. 2010a)
P. lobata	C15	Thailand, Cape Panwa region	5	(LaJeunesse et al. 2010a)
P. lutea	C15	Thailand, Cape Panwa region	14	(LaJeunesse et al. 2010a)
P. nigrescens	C15	Thailand, Cape Panwa region	3	(LaJeunesse et al. 2010a)
P. rus	C15	Thailand, Cape Panwa region	6	(LaJeunesse et al. 2010a)
P. spp.	C15	Thailand, Cape Panwa region	2	(LaJeunesse et al. 2010a)
P. stephensoni	C15, C114	Thailand, Cape Panwa region	4	(LaJeunesse et al. 2010a)
P. cylindrica	C15, C15j	Zanibar, Tanzania	9	(LaJeunesse et al. 2010a)
P. lobata	C15, C15i	Zanibar, Tanzania	6	(LaJeunesse et al. 2010a)
P. lutea	C15, C15i	Zanibar, Tanzania	9	(LaJeunesse et al. 2010a)

P. profundus	C15, C15j	Zanibar, Tanzania	4	(LaJeunesse et al. 2010a)
P. rus	C15	Zanibar, Tanzania	10	(LaJeunesse et al. 2010a)
Porites spp.	C15, C15j, C1	Zanibar, Tanzania	4	(LaJeunesse et al. 2010a)
P. panamensis	C66(10), C66a(13), C66b(2), C1(18), C75(2)	Southern gulf of California	45	(LaJeunesse et al. 2008)
P. rus	C15	Guam	1	(Pochon et al. 2001)
P. cylindrica	Clade C	Guam	1	(Pochon et al. 2001)
P. divaricata	Clade C	Guam	1	(Pochon et al. 2001)
P. nigrescens	Clade C	Guam	1	(Pochon et al. 2001)
P. asteroides	Clade A	Bermuda	11	(Savage et al. 2002)
P. porites	Clade A	Bermuda	11	(Savage et al. 2002)
P. lutea	C15	Heron Island, S GBR	7	(Fisher et al. 2012)
P. lutea	C15	Hainan, China	4	(Zhou and Huang 2011)
P. compressa	Clade C(2)	Kish Island, Hormuz strait	2	(Mostafavi et al. 2007)
P. compressa	Clade D	Larak	1	(Mostafavi et al. 2007)
P. lutea	C15	Kenting National Park Taiwan	1	(Wang et al. 2012)
P. porites	A2	Florida Keys	1	(Rogers and Davis 2006)
Porites spp.	A(1) or C(4)	Jana island, saudi coast	5	(Baker et al. 2004)
Porites Spp.	Clade A	Ras Abu Ali, Saudi coast	10	(Baker et al. 2004)
Porites Spp.	Clade A	Karan Island, Saudi coast	1	(Baker et al. 2004)
Porites Spp.	C(54), D(3)	Kenya	57	(Baker et al. 2004)
Porites Spp.	Clade C	Mauritius	4	(Baker et al. 2004)
Porites Spp.	C1	Mauritius	4	(McClanahan et al. 2005)
P. cylindrica	Clade C	Kenya	2	(Visram and Douglas 2006)
P. lobata	C15/b	Palmyra Atoll usa	2	(Wicks et al. 2012)
P. asteroides	A4a	Yucatan peninsula, mexico	1	(Banaszak et al. 2006)
P. colonensis	C1a	Yucatan peninsula, mexico	1	(Banaszak et al. 2006)
P. divaricata	C9	Yucatan peninsula, mexico	1	(Banaszak et al. 2006)
P. furcata	A4/B1, C4	Yucatan peninsula, mexico	2	(Banaszak et al. 2006)
P. lutea	C15	Halong Bay, Vietnam	4	(Faxneld 2011)
P. attenuata	Clade C	Palau	1	(Fabricius et al. 2004)
P. australensis	Clade C	Palau	1	(Fabricius et al. 2004)

P. cylindrica	Clade C(3) D(1)	Palau	4	(Fabricius et al. 2004)
P. lichen	Clade C(3)	Palau	3	(Fabricius et al. 2004)
P. monticulosa	Clade D	Palau	1	(Fabricius et al. 2004)
P. rus	Clade C	Palau	7	(Fabricius et al. 2004)
P. spp.	Clade C	Palau	2	(Fabricius et al. 2004)

The geographical location of *Porites spp.* with subcladal or cladal information is given along with the number of coral colonies sampled. Numbers in parentheses represent the number of subcladal types for a given collection of coral samples.

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