The Diversity and Bleaching Responses of Zooxanthellae in Kenyan Corals

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Abstract

Zooxanthellae of the genus *Symbiodinium*, the dinoflagellate endosymbionts of many benthic cnidarians, are phylogenetically diverse. Molecular analyses of ribosomal RNA genes indicate multiple *Symbiodinium* species in 7 known phylotypes, A-G. The diversity of *Symbiodinium* in corals from Kenya and sea anemones from the Mediterranean Sea was investigated by molecular methods. *Symbiodinium* in Kenya comprise phylotype A, C and D zooxanthellae that occur pan-tropically. The majority of Mediterranean *Symbiodinium* comprise a distinct group of 'temperate A' zooxanthellae that may be regionally endemic. The zooxanthellal chloroplast *psb*A gene, encoding the D1 protein of photosystem II, was sequenced. The *psb*A and nuclear 24S rRNA gene trees were congruent.

Resilience, i.e. the capacity for zooxanthellae to recover after bleaching, to bleaching induced by elevated temperature and darkness was investigated in *Porites cylindrica*. Resilience was assessed by changes in zooxanthellal densities on termination of stressor. Resilience was influenced by the nature and duration of stressor. Zooxanthellae in corals subjected to relatively long durations of darkness were more resilient than those in corals treated for shorter durations. The opposite trend was evident for zooxanthellae in corals exposed to elevated temperature. The basis for these contrasting results may lie in different endodermal processes during treatment with the two stressors. The recovery profile of corals that bleached on the reef was similar to those experimentally bleached using elevated temperature. No detectable changes in the molecular identity of zooxanthellae occurred on recovery.

Porites cylindrica recently recovered from experimentally induced bleaching and bleaching induced by natural stressors were subjected to a repetition of bleaching stressors to explore their capacity for acclimation, i.e. the development of resistance to bleaching stressors under laboratory conditions. Bleaching responses were not significantly affected by prior experience of bleaching stressor.

The relevance of these experiments on coral resilience and acclimation to field bleaching events is discussed.

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Except where otherwise acknowledged, the material presented in this thesis is the product of my own research, and has not been published elsewhere.

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Dedication

This work is dedicated to Dr. Conrad Clausen and Dr. Venus Clausen. Their passion for the subject, and devotion to the students of Africa are a tremendous blessing for those they have taught, and a great source of inspiration for me ...

...And to my three-year-old nephew, Hunter Qais Oaten, who is a great source of joy to our family. I look forward with great anticipation to fishing, diving and camping with you...

Farewell, Farewell! but this I tell
To thee, thou wedding guest!
He prayeth well, who loveth well
Both man and bird and beast.

He prayeth best, who loveth best
All things both great and small;
For the dear God who loveth us,
He made and loveth all.

Coleridge, 'Rime of the Ancient Mariner'

Chapter 1
Introduction

1.1 Coral-Zooxanthellal Symbiosis

Coral reefs are renowned for their outstanding beauty and biological diversity. They are often described as oases of productivity in the midst of low nutrient seas with rates of carbon fixation estimated at 40 g C m^{-2} day^{-1} for corals (Hatcher 1988) versus 0.01 g C m^{-2} day^{-1} in the open waters surrounding reefs (Hatcher 1988). An astonishing array of organisms finds shelter and food on coral reefs. For example, it is estimated that 32 of 34 recognized animal phyla are represented on eoral reefs compared with 9 phyla in tropical rainforests (Wilkinson 2002). Coral reefs are of immense value to the coastal communities adjacent to them, providing benefits that range from food, building materials, shoreline protection and income from tourism and fisheries.

Scleractinian corals are the very heart of a coral reef, their caleified carbonate skeletons are responsible for the structural framework. The exceptionally high rates of primary production exhibited by corals are almost entirely due to symbiotic algae that live as photosynthetic endosymbionts within their tissues. These endosymbionts are gymnodinioid dinoflagellates of the genus *Symbiodinium* and are colloquially termed zooxanthellae owing to their golden-brown colouration (Trench 1993). Zooxanthellae form symbiotic associations with many Cnidaria (e.g. sea anemones, gorgonians, soft corals, zoanthids, hydrozoans) on shallow water reefs. In addition, zooxanthellae have been found in a wider range of host species that include Foraminifera (Pawlowski *et al.* 2001, Pochon *et al.* 2001), tridacnid clams (Carlos *et al.* 1999, Baillie *et al.* 2000) and sponges (Hill & Wilcox 1998, Carlos *et al.* 1999). The zooxanthellae provide their hosts with nutrients, thereby promoting the growth and reproduction of the host, as well as enhancing calcification rates in hermatypic (reef-building) corals (Treneh 1993, Gattuso *et al.* 1999). It is their central role in the carbon and energy budget of corals, and the photosynthetic enhancement of calcification rates (through photosynthetic consumption of carbon dioxide) that underlies the success and dominance of stony corals in shallow water tropical seas (Muscatine & Porter 1977, Muscatine 1990, Gattuso *et al.* 1999).

In corals, zooxanthellae are located in the endoderm layer where they are enclosed by a host-derived membrane, the symbiosome (Douglas 1994). The symbiosome is derived from the original phagocytic vesicle that captured the zooxanthella, and undergoes

extensive modification to facilitate the transfer of nutrients and photosynthate between host and symbiont (Rands *et al.* 1993). In common with other dinoflagellates, zooxanthellae possess the photosynthetic pigments chlorophyll *a* and c_2 (Jeffrey & Humphrey 1975). The characteristic golden-brown colouration is derived from carotenoids and the light harvesting complexes comprise peridinin-chlorophyll *a*-proteins and peridinin-chlorophyll c_2-proteins (Loeblich 1984). Zooxanthellae utilise a C3 method of carbon fixation, i.e. carbon dioxide is directly assimilated by the enzyme ribulose biphosphate carboxylase/oxygenase (RuBisCO) and not by phosphoenolpyruvate carboxylase (Streamer *et al.* 1993). As in other dinoflagellates, zooxanthellae possess an unusual Form II RuBisCO that has a low affinity for carbon dioxide relative to oxygen (Morse *et al.* 1995). A large proportion of the photosynthetically fixed carbon is translocated across the symbiosome to the cytoplasm of the host. Translocation may exceed 90% of the carbon fixed by photosynthesis (Douglas *et al.* 1993, Trench 1993) and satisfies all or a large part of the host's respiratory requirement (Muscatine 1990). Compounds released to the host include glycerol (Grant *et al.* 1997, Douglas *et al.* 1993), lipids (Douglas *et al.* 1993) and glucose (Markell & Trench 1993, Gates *et al.* 1995). Zooxanthellae isolated from symbiosis release very little photosynthate to the incubation medium unless stimulated by a homogenate prepared from host tissues (Trench 1971). It is thought that a component(s) of host homogenate, often referred to as host release factor, induces the release of photosynthate in the intact symbiosis. The release factor may be a mixture of amino acids (Gates *et al.* 1995) or the non-protein amino acid taurine (Wang & Douglas 1997).

Zooxanthellae have been implicated in nitrogen recycling, although evidence for this is ambiguous and comes primarily from studies that show that Cnidaria experimentally deprived of zooxanthellae have elevated concentrations of ammonium in their tissues (Szmant-Froelich & Pilson 1977, Wilkerson & Muscatine 1984). This has been interpreted widely as evidence for the zooxanthellal assimilation of animal waste ammonium into amino acids that are transferred back to the animal. However, an alternative interpretation is that the receipt of zooxanthellal photosynthate promotes the host's capacity for ammonium assimilation and reduces the production of ammonium by the degradation of amino acids (Wang & Douglas 1998). Zooxanthellae do provide their hosts with essential amino acids (Swanson & Hoegh-Guldberg 1998, Wang & Douglas 1999), although several corals (Fitzgerald & Szmant 1997) and the tropical sea

anemone *Aiptasia pulchella* (Wang & Douglas 1999) can synthesize some essential amino acids.

Symbiodinium can grow and divide at much higher rates than their Cnidarian hosts (Hoegh-Guldberg *et al.* 1987). Despite this, the association is stable, i.e. under defined conditions (e.g. season, depth) the relative volume or biomass ratios of the symbiotic partners is predictable (Douglas 1994). Regulation of the zooxanthellal populations in corals occurs at two levels, firstly by the suppression of zooxanthellal growth and division predominantly though nitrogen limitation (Falkowski *et al.* 1993, Muscatine *et al.* 1998) and space constraints (Smith & Muscatine 1999, Jones & Yellowlees 1997), and secondly by the expulsion of excess symbionts (Baghdasarian & Muscatine 2000, Hoegh-Guldberg *et al.* 1987). Seasonal changes in light and temperature are also known to affect the density of zooxanthellae and the core of the photosynthetic pigments (Fagoonee *et al.* 1999, Fitt *et al.* 2000, Brown *et al.* 1999b), with lowest zooxanthellal densities occurring in late summer, at the time of (or soon after) the seasonal maximum in seawater temperatures.

1.2 Diversity of Zooxanthellae

After the initial description of the cultured zooxanthellae from the jellyfish *Cassiopia xamachana* as *Symbiodinium microadriaticum* Freudenthal (Freudenthal 1962), it was widely believed that all corals (and allied zooxanthellate animals) harboured the same species of symbiont. However, fundamental differences in the properties of isolated zooxanthellae of different origin (hosts and geographical locations) were subsequently reported. This variation included differences in growth rates (Chang *et al.* 1983), ability to infect (Schoenberg & Trench 1980a) and promote growth of host (Kinzie & Chee 1979), morphological characteristics (Schoenberg & Trench 1980b), chromosome numbers (Blank & Trench 1985), and photosynthetic responses to light (Igesias-Prieto & Trench 1994) and temperature (Warner *et al.* 1996). These observations challenged the historical perspective that all *Symbiodinium* belonged to the same species. Taxonomic studies however, were hindered by the paucity of morphological data (e.g. absence of thecal plates, flagella) in the symbiotic state and the intractability to *in-vitro* culture for most zooxanthellae. We now know that most cultured zooxanthellae are not the dominant algae in the symbiosis (Santos *et al.* 2001). This led to the development of genetic methods for identifying zooxanthellae that utilised a combination of restriction fragment length polymorphism (RFLP) (Rowan & Powers 1991a, 1991b) and sequence

analysis of nuclear genes encoding ribosomal RNA (rRNA genes or rDNA) (Rowan & Powers 1992). These methods are now routinely used for investigating the molecular diversity of *Symbiodinium* (e.g. LaJeunesse & Trench 2000, Toller *et al.* 2001a, Loh *et al.* 2001, Diekmann *et al.* 2002, Lajeunesse *et al.* 2003) The molecular evidence reveals that zooxanthellae comprise a diverse and monophyletic group of organisms, and verify conclusions based on morphological studies that there is neither a single *Symbiodinium* species utilized by all host taxa, nor a unique species in any one host taxon (Blank & Trench 1985, Blank & Trench 1986). Molecular studies have revealed 2 clades, one known as phylotype A, and the other containing phylotypes B-G (D was previously referred to as E) (Rowan 1998, Wilcox 1998, LaJeunesse 2001, Baker 2003). Although sequence variation in genes encoding ribosomal RNA is not predicted to have a direct impact on the zooxanthellal phenotype, there has been a strong impetus towards determining how rRNA gene sequences vary with physiological differences between symbionts. As there is now strong evidence for substantial within-phylotype variation in a number of phenotypic properties [e.g. photosynthetic responses to light (Savage *et al.* 2002)], ascribing phenotypic traits to an zooxanthellal phylotype must be done with caution.

The genetic approach has been crucial in identifying patterns that relate to the distribution of zooxanthellae in different hosts and in different physical environments (i.e. depth, latitude etc.). Some fundamental features have emerged:

1. There is no discernible relationship between the type of alga with which a host associates, and its own phylogenetic position. For example, a scleractinian host may form an association with an alga that is indistinguishable (at the level of ribosomal RNA gene sequence) with an alga hosted by a jellyfish (Rowan & Powers 1992).
2. The host-alga association is non-random. A particular host species commonly associates with a uniform zooxanthellal type(s) (defined by its ribosomal RNA gene sequence) over a wide geographical range (Rowan & Powers 1991a, 1991b, 1992). This pattern is referred to as *specificity*. This does not necessarily imply an inability for the host to form a partnership with other zooxanthellae, as associations with non-native zooxanthellae has been demonstrated in laboratory studies (Perez *et al.* 2001). However, the latter are usually transient and confer less benefit to their host than is the case with native zooxanthellae (Davy *et al.* 1997, Kinzie & Chee 1979). Under these circumstances, many symbioses are

more appropriately described as being *selective* rather than specific. Specificity between partners may involve an internal recognition process (Schoenberg & Trench 1980a, Colley & Trench 1983) and is established early in the life cycle of the host (Coffroth *et al.* 2001).

3. While many hosts are specific for one alga, others commonly form associations with two or more zooxanthellal types. Such associations are described as *polymorphic* infections (*mixed* infections are a special case of polymorphic infections in which two or more zooxanthellae occur simultaneously in a host). Polymorphic infections are more prevalent than was recognized in early studies. One of the best-characterised polymorphic systems in corals occurs in the *Montastraea annularis* complex of species. These corals are ecologically dominant on Caribbean reefs, and form symbiosis with *Symbiodinium* of phylotypes A, B, C and D (Toller *et al.* 2001a, Rowan *et al.* 1997), commonly as mixed infections. In the *Montastraea* sp. complex, the distribution of zooxanthellal phylotypes is strongly influenced by gradients of light, with phylotype C zooxanthellae restricted to deep water or low-irradiance micro-environments, while zooxanthellae of phylotypes A and B predominate shallow water or high-irradiance micro-environments (Rowan & Knowlton 1995, Rowan *et al.* 1997). Shifts in host-alga associations have also been reported between near-shore and offshore reefs (Toller *et al.* 2001a) and along latitudinal gradients (Rodriguez-Lanetty *et al.* 2001, LaJeunesse & Trench 2000). These observations have occasionally invited speculation on the physiological attributes of the zooxanthellal phylotypes. However it is not currently known if they have any functional basis in symbiosis, and must be interpreted with caution.

1.3 Coral Bleaching

During periods of environmental perturbation, the stability (i.e. regulation) of the zooxanthellal-cnidarian symbiosis is disrupted. This leads to a drastic reduction in the zooxanthellal component (e.g. Hoegh-Guldberg & Smith 1989) and/or the loss of photosynthetic pigments (e.g. Kleppel *et al.* 1989, Szmant & Gassman 1990). The resultant paling or 'whitening' of tissues (as corals take on the colour of underlying skeleton) is referred to as coral bleaching. Comparable paling of tissues linked to the loss of zooxanthellae or their pigments occurs in hosts other than corals and therefore the term 'coral bleaching' is actually a misnomer.

The loss of zooxanthellae during bleaching occurs in three principal ways: the degradation of zooxanthellae *in situ*, the loss of zooxanthellae (e.g. by exocytosis) into the gastric cavity, and the release of intact endoderm cells with their complement of zooxanthellae (reviewed in Brown 1997).

1.3.1 Environmental Triggers

Bleaching has long been recognized as a generalised response of zooxanthellate symbiosis to stress. As such, it is elicited by a variety of environmental stressors. Localized bleaching in the field has been reported in response to sedimentation (Bak 1978), oil pollution (Guzman *et al.* 1991), reduced salinity (Goreau 1964), decreased seawater temperature (Kobluk & Lysenko 1994) and aerial exposure (Yamaguchi 1975). In addition to these factors, bleaching has also been induced in laboratory studies in response to metal pollution (Harland & Nganro 1990), prolonged darkness (Titlyanov *et al.* 2002) and high salinity (Nakano *et al.* 1997). Bleaching has also been attributed to bacterial (Ben-Haim *et al.* 2003, Ben-Haim *et al.* 1999) and viral (Wilson *et al.* 2001) infections. The role of infective microbial agents in bleaching appears to be confined to a few symbioses and is temperature dependent.

Glynn (1983) first described the phenomenon of extensive coral bleaching across large areas of the Pacific Ocean. Extensive bleaching reports have since been a recurrent feature in coral reef literature, with numerous reports from locations in the Caribbean Sea, the Pacific and Indian Oceans (reviews in Brown 1997, Glynn 1993). Ascertaining cause and effect for the phenomenon of extensive coral bleaching (mass bleaching *events*) was not always straightforward due to the complex nature of the shallow-water coral reef environment and the lack of suitable long-term environmental data. Nevertheless, a consensus emerged attributing elevated seawater temperature as the primary cause for widespread coral bleaching events. Evidence from the field in support of this conclusion is now overwhelming (e.g. Lasker *et al.* 1984, Cook *et al.* 1990, Gleeson & Strong 1995, Podesta & Glynn 1997, Winter *et al.* 1998, Glynn *et al.* 2001). Frequently, bleaching thresholds for corals in any location are reached when sea surface temperatures (SST) exceed the long-term summer mean (Jokiel & Coles 1990). The term *bleaching hotspot* was therefore coined to describe SST anomalies approximating or exceeding by 1°C the long term monthly average for the warmest month of the year. These anomalies are estimated from the NOAA's satellite observations, and are an essential tool in forecasting coral bleaching events (Goreau & Hayes 1994).

As cited in the review by Glynn (1993), coral bleaching is often reported to occur during periods of calm seas and low wind velocity. These are conditions that favour radiant heating of shallow waters and maximum penetration of solar radiation. During bleaching episodes, corals are frequently observed to bleach only on surfaces exposed to high doses of solar radiation (Rowan *et al.* 1997, Brown *et al.* 2000a), indicative of the major contribution of light in the bleaching response. A synergistic interaction between elevated temperature and solar radiation in eliciting bleaching is now widely accepted. However, the wavelength band of light thought to contribute most significantly is in contention. Whereas some workers have favoured ultra violet radiation (UVR; 290-400 nm) (Lesser *et al.* 1990, Gleason & Wellington 1993), others consider photosynthetically active radiation (PAR; 400-700 nm) to play a more prominent role (Brown *et al* 2000b, Dunne & Brown 2001).

1.3.2 Physiological Determinants

The identification of elevated seawater temperatures (exacerbated by solar radiation) as the principal trigger for extensive bleaching prompted a shift in focus towards understanding the underlying ***mechanisms***, i.e. "the fundamental processes defining the response of the symbiosis to the bleaching triggers and resulting in the observed symptoms (of bleaching)"- as defined by Douglas (2003).

Despite considerable research in this area, information on bleaching mechanisms remains fragmentary. The emphasis has almost entirely been related to triggers that are the most significant in the field. Coral bleaching, as induced by elevated seawater temperature and solar radiation, is associated with damage to the photosynthetic apparatus of zooxanthellae. Considerable evidence for this comes from chlorophyll fluorometry studies, which assess photosynthetic function in symbionts during periods of exposure to thermal/solar stressors, and relate the decline in photosynthetic efficiency with elevated rates of expulsion of zooxanthellae from host tissues. Photosynthetic function is generally evaluated by an index known as the quantum or fluorescence yield of photosystem II (PSII) (F_v/F_m ratio: variable/maximal fluorescence ratio). The value of this index is indicative of the potential photochemical capacity of PSII (ability for zooxanthellae to utilise light energy for photochemical reactions in the reaction centre of PSII). Its measurement can be made non-invasively *in situ* by a device known as a pulse amplitude modulation (PAM) fluorometer. At optimum efficiency, F_v/F_m ratios of zooxanthellae typically range from 0.5-0.7 (Warner *et al.* 1996, Warner *et al.* 1999). In

their investigation of a natural bleaching event on the corals *Montastraea annularis* and *Montastraea franksi* from Florida, Warner and colleagues (1999) reported a significant decline in F_v/F_m in residual symbionts from bleached colonies, with greater damage to PSII in deeper waters, where bleaching was most pronounced. Observed in association with loss of PSII activity was an elevation in the rate of D1 reaction centre protein degradation, but not accompanied by an increase in D1 synthesis. Temperature dependent loss in PSII activity with attendant decline of D1 was also demonstrated in laboratory studies of symbiotic and cultured zooxanthellae (Warner *et al.* 1999). The D1 protein is essential in binding the components required for photosynthetic charge separation and electron transport in PSII (Schnettger *et al.* 1994) and exhibits rapid turnover involving light-dependant inactivation coupled with enhanced re-synthesis (Anderson *et al.* 1997). A perturbation in the rate of its turnover is therefore predicted to result in the loss of PSII function and subsequent expulsion of symbionts during thermal bleaching (Warner *et al.* 1999). Similarly, using PAM fluorometry, Jones *et al.* (2000) demonstrated the decline in F_v/F_m in symbionts from the coral *Plesiastrea versipora* exposed to elevated seawater temperature. In association with a decline in quantum yield of PSII, there was a significant decline in zooxanthellal density. The same pattern was evident in bleached corals from the field during a bleaching event on the southern Great Barrier Reef, Australia.

Jones *et al.* (1998) have proposed that the primary site of damage to the photosynthetic machinery during thermal/light stress are the dark reactions of photosynthesis, following the point at which light-driven electron transport exceeds the capacity for the Calvin cycle. Subsequent damage to the light reactions would arise as a secondary effect following the collapse of photoprotective mechanisms by excess light energy.

In the laboratory, one of the observed symptoms of temperature-mediated bleaching has been the detachment of animal endoderm cells with their complement of zooxanthellae (Gates *et al.* 1992). This was proposed to result from temperature-induced dysfunction in host cell-adhesion. As phosphorylation of cell adhesion molecules was known to control adhesion of cells in other systems (Volberg *et al.* 1991, Hamaguchi *et al.* 1993), Sawyer & Muscatine (2001) altered patterns of intracellular protein phosphorylation in the tropical sea anemone *Aiptasia pulchella* by the administration of caffeine (which enhances tyrosine phosphorylation of proteins) to the incubation medium. Their results showed that host cell release was triggered by changes in temperature (cold and heat

shock) as well as by treatment with caffeine. Furthermore, alterations to patterns of protein phosphorylation was detected in both cold-shocked and caffeine treatments. Based on these results, Sawyer & Muscatine (2001) have proposed that changes in cellular patterns of protein phosphorylation with resultant dysfunction in host cell adhesion is an important mechanism for bleaching.

As a direct result of photosynthesis, symbiotic cnidarians are routinely exposed to an elevated partial pressure of oxygen within their tissues (Richier *et al.* 2003). Oxygen is a natural acceptor of electrons from the electron transport chain, with consequent formation of the superoxide (O_2^-) anion (Alscher *et al.* 1997, Fridovich 1978). This is in turn reduced to hydrogen peroxide (H_2O_2) and hydroxyl (OH^-) radicals as intermediate and final products (Alscher *et al.* 1997, Fridovich 1978). These species are often referred to as *toxic* or *reactive* oxygen species (ROS) as they have the potential to inflict substantial damage to cellular components (Alscher *et al.* 1997, Asada & Takahashi 1987, Fridovich 1986). Photosynthetic organisms are well equipped to protect themselves from oxygen toxicity by way of antioxidant enzymes such as superoxide dismutases (SODs) and peroxidases (which scavenge O_2^- and H_2O_2 respectively) (Alscher *et al.* 1997, Asada & Takahashi 1987). In cnidarians, both the animal and alga possess an impressive array of SODs (Richier *et al.* 2003). However, during exposure to elevated temperature/light, increased photosynthetic evolution of oxygen and its subsequent reduction may feasibly exceed the capacity for the antioxidant defence system to neutralize enhanced levels of ROS. The resultant physiological state of oxidative stress has been postulated to be a causative factor in coral bleaching (Lesser 1996, 1997). This has been tested with *in vitro* cell cultures of *Symbiodinium* sp., where significantly higher cellular concentrations of ROS were detected on exposure to elevated temperatures, accompanied by decline in the quantum yield of PSII (Lesser 1996). The addition of exogenous scavengers of ROS improved photosynthetic performance indicating that oxidative stress might play a role in the inhibition of photosynthesis during thermal/light stress. This study was extended to investigate the role of oxidative stress in the thermal bleaching response of the coral *Agaricia tenuifolia* (Lesser 1997). As predicted, exposure of corals to elevated temperature resulted in a decline in photosynthetic performance and zooxanthellal density. However, when corals were subjected to heat stress in the presence of exogenous antioxidants, neither loss in photosynthetic function nor bleaching of zooxanthellal symbionts was detected.

Results from the study by Downs *et al.* (2002) also support the 'Oxidative Theory of Coral Bleaching', i.e. bleaching is a coral's last line of defence against oxidative stress. The coral *Montastraea annularis* was sampled along a depth transect at a site that exhibited a pattern of increased bleaching at greater depth, during a season characterized by elevated SSTs. Assays comprised quantifying products associated with oxidative stress (protein carbonyl, lipid peroxide) and host antioxidant enzymes (Cu/Zn and Mn SOD). As water temperatures increased seasonally, so too did levels of oxidative damage products. Corals at depth accumulated significantly higher levels of these damage products, and significantly lower levels of antioxidant enzymes, preceding the onset of bleaching.

During thermal bleaching, necrotic and programmed cell death pathways have been indicated in host and alga (Dunn *et al.* 2002). These pathways were investigated using the sea anemone *Aiptasia* sp. subjected to elevated seawater temperature. A suite of techniques (which involved staining of paraffin wax embedded tissue sections, in situ end labelling of fragmented DNA, gel electrophoresis and electron microscopy) was employed to differentiate different cell death pathways. Necrotic host endoderm tissues were detected after a treatment period of 4 days. Tissue necrosis was associated with the release of apparently healthy zooxanthellae into the gastric cavity. On sustained treatment for another 3 days, degradation of zooxanthellae ensued. This involved two forms of cell death, namely programmed cell death and cell necrosis. The defining features of programmed cell death included condensation of cytoplasm and organelles, shrinkage of cells and DNA fragmentation. Cell necrosis was characterised by dilation of organelles and cytoplasm, cell swelling and lysis, dispersion of cell debris and DNA fragmentation. Histological examination of tissues from corals that underwent thermal/solar bleaching in the field have also indicated necrosis of host tissues (Glynn *et al.* 1985, Lasker *et al.* 1984), with the retention of zooxanthellae of normal appearance in all but the most necrotic samples (Glynn *et al.* 1985)

Symbiotic interactions between zooxanthellae and their hosts are likely to be disrupted during bleaching. These interactions involve the translocation of photosynthetic products by alga to host (Trench 1993), and an analogous (but unknown) exchange of signalling molecules. Damage to zooxanthellal photosynthetic machinery during bleaching implies the diminished capacity to supply host with fixed carbon compounds. It has therefore been suggested that functional symbioses are maintained through the

sustained production of (a) signal(s) that prevents the initiation of host defences against invading organisms (repression of bleaching; Douglas 2003). Zooxanthellal-derived photosynthate is a candidate for this putative signal. Support for this theory comes from the freshwater *Chlorella*-hydra symbiosis, where strains of *Chlorella* with relatively low rates of photosynthate release are expelled from the host at elevated rates (Douglas & Smith 1984). Alternately, damaged or impaired zooxanthellae might release compounds (or leak cytoplasm contents) that stimulate host defences (induction of bleaching; Douglas 2003).

1.3.3 Consequences of Bleaching

The consequences of bleaching may be considered on three levels:

1.3.3.1 Consequences to the Bleached Symbiosis

Not surprisingly, the loss or decline of alga-derived energy during bleaching has strong implications for the affected host. These impacts range from impaired healing and regeneration of damaged tissues (Mascarelli & Bunkley-Williams 1999, Fine *et al.* 2002), reduced tissue biomass (Szmant & Gassman 1990), reduced fecundity (Szmant & Gassman 1990, Omori & Hatta 2001) and diminished rates of growth and calcification (Glynn 1993). Additionally, bleached corals suffer from an increased susceptibility to reef sediments and macroalgal invasion (Mascarelli & Bunkley-Williams 1999) and are more prone to disease (Glynn 1983). Frequently, bleached corals fail to recover their zooxanthellal populations and die (Glynn 1983, Goreau *et al.* 2000).

1.3.3.2 Consequences to the Reef Community

Goreau and colleagues (2000) compiled reports from published studies and personal communications from individuals relating to a global bleaching event in 1997-1998. Their synthesis summarises changes to the community structure of reefs following major bleaching episodes. Immediately on mortality, coral surfaces are overgrown by filamentous algae. The long-term outcome is dependent on intensity of fishing pressure and input of nutrients (Goreau *et al.* 2000). High nutrient input reefs remain alga dominated; algal turf replaces filamentous forms where fishing effort is low, and fleshy macroalgal forms emerge on reefs with high fishing pressure. Encrusting calcareous red algae replaced filamentous forms on reefs with low nutrient inputs and low fishing effort.

Bleaching susceptibility is not uniform across different taxa. In the scleractinian corals, it is widely recognized that corals with branching morphologies, for example the Acroporids and Pocilloporids, are generally more sensitive (i.e. prone to bleach) and suffer high mortality (Marshall & Baird 2000, Hueerkamp *et al.* 2001, Goreau *et al.* 2000). In Indo-Pacific reefs, this pattern of differential susceptibility after a global bleaching event has resulted in a change in the dominant corals from branching species to the major surviving corals, the massive *Porites* species (Goreau *et al.* 2000). Mortality of corals has strong implications for associated fish and invertebrate populations. There is also growing concern that certain temperature-sensitive coral species may become extinct.

1.3.3.3 Consequences to Worldwide Economies

It is estimated that coral reefs provide US$ 30 billion each year in net benefits in goods and services to worldwide economies (Cesar *et al.* 2003). It is virtually impossible to caleulate the extent to which coral bleaching contributes to the global trend in eoral reef degradation. Nevertheless, one study estimates that the net present value of future losses from bleaching over the next 50 years ranges from US$ 21 billion to US$ 83 billion (Cesar *et al.* 2003). Although such estimates are far from certain, they highlight the profound impact of coral bleaching on the livelihoods of millions of people worldwide.

1.3.4 Variation in Bleaching Susceptibility

Interspecific and intraspecific variation in bleaching susceptibility is a common feature of bleaching. This is partly due to the varying extents to which individual symbioses have the capacity to safely divert or dissipate excess solar energy from the reaction centre of PSII, thereby protecting the photosynthetic apparatus from damage.

The diversion of solar energy away from PSII can arise from host pigments; the fluorescent pigments (known as pocilloporins) of corals can alter the light environment of host tissues by re-emitting excess light at wavelengths of low photosynthetic activity (Salih *et al.* 2000, Dove *et al.* 2001). In addition, mycosporine-like amino acids synthesized by zooxanthellae absorb UVR (Banaszak *et al.* 2000). Once solar energy strikes PSII, part can be dissipated from the reaction centre as heat [i.e. the non-photochemical quenching (qN) component of chlorophyll fluorescence studies]. This occurs due to a group of carotenoid pigments known as the xanthophylls (Demmig-Adams & Adams 1996). The pH-dependent interconversion of xanthophylls

(xanthophyll cycling) results in the safe dissipation of excess light as heat and is an important element in photosynthetic control. In dinoflagellates, the xanthophylls are diadinoxanthin and diatoxanthin (Ambarsari *et al.* 1997).

In an experiment designed to investigate the contribution of zooxanthellae in temperature bleaching (Perez *et al.* 2001), aposymbiotic *Aiptasia pallida* from Bermuda were infected with zooxanthellae from other sea anemones. Re-infected hosts were incubated at different temperatures. At each of these temperatures, the expulsion rate and photosynthetic rate of freshly isolated zooxanthellae was measured. The decline in photosynthetic performance with increasing temperature varied between the symbionts used to re-infect the host, and furthermore this was related to the differential release of zooxanthellae. This led the authors to conclude that zooxanthellae play a major role during temperature bleaching.

The study of Rowan *et al.* (1997) on the Caribbean corals *Montastraea annularis* and *Montastraea faveolata* is indicative of genetic variation in the susceptibility of *Symbiodinium* to bleaching. These corals bear zooxanthellae of phylotypes A, B and C, often as mixed infections. During the elevated temperature/solar bleaching in late summer 1995, only those corals bearing greater than 35% C showed visible signs of bleaching, and those dominated by B did not bleach. The proportion of C in colonies that had mixed infections with C and A and/or B declined significantly during bleaching. These observations point towards higher thermal/solar bleaching susceptibility of the phylotype C alga in the *Montastraea* species complex.

Brown *et al.* (2002a), working with the coral *Goniastrea aspera*, in which parts of the colony exposed to high solar radiation are less susceptible to bleaching than areas subject to less exposure (Brown *et al.* 2000a), investigated a suite of molecular biomarkers in host and alga in order to determine the underlying basis of thermotolerance. Their results showed that surfaces exposed to high light had lower levels of oxidative stress, and higher levels of host heat-shock proteins (Hsp60 and Hsp70) and host antioxidant-enzyme CuZn SOD. Zooxanthellal defences (antioxidant enzymes, xanthophyll cycling, etc.) on 'high light' surfaces, however, were not enhanced. These results demonstrate the significance of host tissues in determining overall bleaching susceptibility. The experiments conducted by Bhagooli & Hidaka (2003) add indireet support for the same conclusion over a wider range of corals. These

studies compared the photochemical efficiency (F_v/F_m) of zooxanthellae *in hospite* and freshly isolated from a number of coral species. Measurements were carried out at different combinations of temperature and light levels. High light produced a more pronounced decline and diminished recovery of PSII activity following high temperature treatment in isolated zooxanthellae than for zooxanthellae *in hospite*, possibly indicating that the host environment ameliorates the impacts of environmental adversity by offering photoprotection for intracellular zooxanthellae. The observed order of bleaching susceptibility in the field among the corals studied was markedly different to that inferred by photosynthetic responses of isolated zooxanthellae to temperature and light. Zooxanthellae least affected by high temperature when *in hospite* were the most susceptible when isolated.

1.3.5 Coral Bleaching: Links with Increasing SSTs and El Niño

Mass bleaehing events are a relatively recent phenomenon. The frequency and geographical seale of bleaching reports in the scientific literature has risen dramatically since the 1980's (Hoegh-Guldberg 1999, Glynn 1993, Brown 1997). Prior to this, reports were infrequent and often anecdotal (Hoegh-Gudberg 1999). After the causal link between elevated SST and coral bleaching was firmly established, researchers turned to historical records of SST to explain the rise in the incidence of bleaching events. The records unambiguously demonstrate a significant warming of tropical SST during the last century (Hoegh-Guldberg 1999). Regressions on contemporary (1979-1999) SST data blended from three sources, ships, buoys and satellites (Integrated Global Ocean Services System; IGOSS-nme blended weekly SST data), generated highly significant ($p < 0.001$) increasing trends in excess of 2°C per century in many tropical seas (Hoegh-Gudberg 1999). These data are supported by other studies using independent datasets and dating further back [e.g. Brown (1997): MOHSST 6 dataset 1946-1996 = 1.26°C per century at Phuket, Thailand versus Hoegh-Guldberg (1999): IGOSS-nme blended dataset 1979-1999 = 2.30°C per century at same location]. Thus, the increase in incidence of bleaching events since the 1980s is set against a background of rising SSTs during the same period.

Rising SSTs are not in themselves adequate to fully explain the bleaching events of the past two decades. As pointed out by Stone *et al.* (1999), major bleaching events in the Pacific Ocean during 1982-83, 1986-87, 1991, 1994 and 1997-98 were all periods of heightened El Niño activity. El Niño is a disruption of ocean-atmosphere interactions in

the tropical Pacific initiated by a slackening of the westward blowing trade winds across the central Pacific Ocean (A strong El Niño in the Pacific Ocean projects climatic anomalies globally due to an integration of the world's ocean and atmosphere systems; i.e. climatic teleconnections). Using two indices correlating with El Niño activity, Stone and colleagues (1999) determined that the probability of occurrence of an El Niño increased markedly in the 1970's, indicating what appears to represent a 'climate shift'. It appears that the heightened incidence of extensive coral bleaching events since the 1980's are the result of enhanced El Niño activity riding on a platform of ever increasing tropical SSTs (Stone *et al.* 1999, Hoegh-Guldberg 1999). The El Niño of 1997-98 initiated a coral bleaching event unprecedented in the primary literature both in its scale and intensity (Goreau *et al.* 2000).

What of the future of the world's coral reefs? Using a number of existing Global Circulation Models (GCMs), Hoegh-Guldberg (1999) sought to predict future changes to SSTs and how this would impact the frequency of coral bleaching events. Data from published studies and Internet postings were used to estimate thermal bleaching thresholds of corals at the study sites. Results were grim. Regardless of the simulation model used, the frequency of bleaching events per decade was predicted to increase sharply at all study sites (7 tropical locations were studied). Disturbingly, most locations were predicted to experience bleaching conditions at least once every year within 30-50 years.

Sheppard (2003) has pointed out that forecast SSTs frequently fail to integrate seamlessly with historical temperature records. Additionally, predictions have sometimes misjudged the amplitude of seasonal temperature oscillations, thereby erroneously predicting when SSTs that proved lethal to corals during the 1997-98 bleaching event will recur. Forecast SST data (HadCM3 model: 1950-2099) at 33 sites in the Indian Ocean were transformed to merge without seam with preceding historical data (HadISST1. 1871-1999), and at each site, predictions were made on the probability of recurrence of SSTs that were lethal to the vast majority of shallow corals (> 90%) during the 1997-98 bleaching event. To compare between sites, an *extinction date* was selected as the date when the probability of the warmest month (or warmest 3 months or warmest quarter) equalling 1997-98 lethal temperatures was 0.2. This value was based on the estimated age at which many corals reach sexual maturity, i.e. 5 years. The extinction dates from three north-south transects in the Indian Ocean were plotted on a

graph, and results predict that reefs 10-15°S will be first affected, with lethal temperatures recurring on average once every 5 years by the years 2010-2025.

1.3.6 Adaptation and Acclimation

Predicting recurrences of bleaching from models forecasting SST changes often infers bleaching thresholds of corals based on prior bleaching events. This threshold is sometimes considered to be approximately 1°C above the mean summer maximum (Goreau & Hayes 1994). However, recent studies (Sheppard 2003, Hughes *et al.* 2003) point out that such predictions should not disregard the ongoing evolution of bleaching resistance (i.e. adaptation) and/or physiological acclimatisation by corals and their zooxanthellae (thus raising bleaching thresholds). Circumstantial evidence for adaptation is provided by variation in bleaching thresholds within coral species that have wide latitudinal (and hence temperature) ranges (Hughes *et al.* 2003, Sheppard 2003). The primary concern among reef scientists is that the current rate of climate change is faster than the evolutionary capacity for the coral-alga symbiosis to adapt.

The best recent evidence for physiological acclimatisation of corals to elevated temperature/light comes from a study by Brown *et al.* (2000a) on the shallow water Indo-Pacific coral *Goniastrea aspera*. At their study site in Thailand, west-facing surfaces are annually exposed to a greater dose of solar radiation during the months of January-March than are east-facing surfaces, and undergo solar bleaching. Sea surface temperatures are maximal in May, and in 1991 and 1995 positive temperature anomalies were recorded during this month. *G. aspera* exhibited an unusual pattern of temperature bleaching in May of both years. Only the east-facing surfaces of corals bleached, and the west-facing surfaces, which had undergone solar bleaching earlier in the year, were resistant to bleaching. This observation demonstrated that acclimatisation associated with recent history of high solar radiation (accompanied by solar bleaching) protected the west-facing surfaces to subsequent thermal/solar bleaching.

The 'Adaptive Bleaching Hypothesis' (ABH) was formally conceptualised by Buddemeier and Fautin (1993) who postulated that the expulsion of sub-optimal zooxanthellae (during bleaching) facilitated the incorporation of new types of zooxanthellae. This would change the physiological properties of the symbiosis and better equip it to cope with emerging environmental challenges. The ABH has been received with interest for a number of reasons. That zooxanthellae comprise a diverse

group of organisms is now firmly established (Rowan 1998), and furthermore some corals can associate with multiple zooxanthellal partners (Rowan & Knowlton 1995). More significantly, the type of symbiont with which a coral associates is sometimes correlated with its susceptibility to bleaching (Rowan *et al.* 1997). Incorporation of zooxanthellae from an external source has been demonstrated in experiments where aposymbiotic sea anemones were returned to their natural habitat (Kinzie *et al.* 2001), and furthermore, changes in zooxanthellal types have been reported in corals (Baker 2001, Toller *et al.* 2001b, Glynn *et al.* 2001), sponges (Hill & Wilcox 1998) and octocorals (Lewis & Coffroth 2004) on recovery from bleaching.

The ABH is unproven and controversial. Fundamental difficulties include the fact that replacement of symbionts whilst stress is ongoing has never been demonstrated. Furthermore, change in zooxanthellal types on recovery does not necessarily imply an adaptive/acclimatory response for the coral, i.e. changes in zooxanthellal types on recovery are not necessarily advantageous to the host.

1.4 Project Description

This project investigated the diversity of zooxanthellae in Kenya and in European locations from the Mediterranean Sea. In addition, experiments on bleaching responses of the symbiosis were conducted in Kenya. Following is a brief description of the main study sites, definitions of key responses, and an outline of the thesis including statements of the major hypotheses tested.

1.4.1 Primary Study Site

The Kenya coastline on the Western Indian Ocean extends slightly over 500 km between latitudes 1-5°S. The prevailing current along the coastline is the warm, north flowing East Africa Coastal Current (EACC). This is a deflection of the South Equatorial Current after it contacts the continent in southern Tanzania. The northern coastline seasonally receives cool, upwelling water from the Somali Current System (Obura 2001a). An almost continuous Fringing Reef is the dominant marine feature along the majority of the Kenya coastline, interrupted in the north by the discharge of sediment and freshwater from two major rivers. Studies carried out by Hamilton & Brakel (1984) provide the most comprehensive list to date of scleractinians from Kenya and Tanzania, listing 112 species in 50 genera. Lemmens & Smeets (1987) reported an additional 43 species in 4 genera from the northern periphery of Kenya's fringing reef

system. However, taxonomic errors in the identification of scleractinian species from the region cannot be ruled out (Sheppard 1998). The coastal climate is strongly influenced by the Monsoon winds, which blow from the northeast (NEM, December-March) and the southeast (SEM, May-October). The interim between NEM and SEM, usually in April, is characterised by calm winds and clear water visibility This favours the maximal penetration of solar radiation and heating of shallow waters [sea surface temperatures in Kenya range from 25-31°C (Obura 2001a)]. During this period, corals have low zooxanthellal densities and many are visibly pale. The diversity of zooxanthellae in Kenyan corals is unknown.

Interannual variability in SST in the western Indian Ocean is strongly influenced by the El Niño (Chambers & Tapley 1999). Additionally, the quasi-periodic Indian Ocean dipole mode exerts an independent influence on SST variability in the Indian Ocean (Saji *et al.* 1999). Both the El Niño signal and the Indian Ocean dipole mode produce anomalously warm SSTs in the western Indian Ocean basin. In 1998, the El Niño and the Indian Ocean dipole modes were in phase, producing the warmest SSTs on record for the region (Goreau *et al.* 2000). Mortality from the ensuing bleaching event was unprecedented in its scale and severity. Bleaching was most extensive and severe in the Indian Ocean (Goreau *et al.* 2000) and Kenya's reefs suffered 50-80% mortality rates (Obura 2001a). In Kenya, coral community structure was greatly altered, with near-complete mortality recorded for a number of dominant species including *Acropora* spp., *Pocillopora* spp. and branching *Porites* spp. (Obura 2001a).

Zooxanthellate shallow water corals are generally restricted to low latitudes (25°N-25°S) and are entirely absent from the European Coast of the Mediterranean Sea (35-43°N) (Spalding *et al.* 2001). However, a number of sea anemone species from the region are zooxanthellate. The diversity of zooxanthellae in benthic Cnidaria from this region is unknown. Clues may come from previous studies on the diversity of zooxanthellae at high latitudes. Such studies have demonstrated that both the temperate sea anemone *Anthopleura elegantissima* and the wide-ranging Indo-Pacific coral *Plesiastrea versipora* host *Symbiodinium* of Phylotype B at the high latitude limits of their ranges (LaJeunesse & Trench 2000. Rodriguez-Lanetty *et al.* 2001). In addition, the temperate sea anemone *Anemonia viridis* from the northeast Atlantic Ocean hosts *Symbiodinium* of Phylotype A (Bythell *et al.* 1997).

1.4.2 Terminology

Terms used to define host-zooxanthellal responses to bleaching stressors in experiments for this project may have different meanings when applied in different contexts. Therefore, key responses are shown in Figure 1.1, and are defined as follows:

- The term *coral bleaching* is used in its strictest sense; i.e. the decline in zooxanthellal density in a coral colony in response to an external stressor (e.g. prolonged darkness or elevated seawater temperatures). Pigment changes were not quantified during this study.
- *Resistance*: The property of zooxanthellal populations in a coral colony to maintain a stable population density when subjected to an external bleaching stressor
- *Susceptibility*: The property of zooxanthellal populations in a coral colony to undergo a significant decline in population density when subjected to an external bleaching stressor.
- *Resilience*: The capacity of zooxanthellal populations in a bleached coral colony to recover from a decline in population density after the application of an external bleaching stressor is terminated. A quick onset of recovery is characteristic of greater resilience whereas a delayed onset of recovery is a feature of diminished resilience.
- *Temperature Tolerance*: The range of temperatures over which a coral colony can physiologically function. On either side of the lower or upper temperature tolerance limits, vital physiological processes are disrupted resulting in mortality of the coral colony. It is important to note that upper temperature tolerance limit is not applied as a proxy for bleaching threshold.

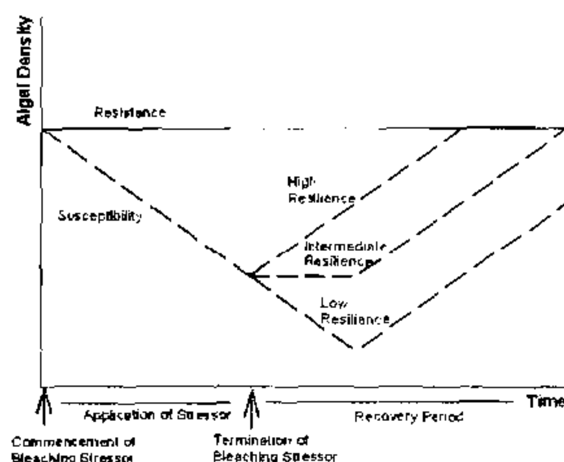

Figure 1.1: Key responses of the zooxanthellal populations of coral colonies to bleaching stressors.

1.4.3 Outline of Thesis

1.4.3.1 Research Described in Chapter 3

Symbiodinium populations are genetically diverse. The genetic identity of zooxanthellae from the western Indian Ocean is unknown. *Symbiodinium* populations in a number of stony coral species from Kenya's reefs were therefore identified by restriction analysis and sequence analysis of rRNA genes. Kenya's reefs lie at low latitudes (1-5°S). As a comparative study, the diversity of *Symbiodinium* populations in a number of sea anemone species from the European coast of the Mediterranean Sea (35-43°N), also currently unknown, was investigated. In order to determine how closely related the zooxanthellae in Kenya and in the Mediterranean Sea are to zooxanthellae elsewhere in the world, sequences from this study were compared with *Symbiodinium* rRNA gene sequences from the Genbank database, and phylogenetic trees were constructed. (H_0: *Symbiodinium* from Kenya and the Mediterranean Sea are closely related to *Symbiodinium* elsewhere in the world, i.e. *Symbiodinium* are cosmopolitan).

1.4.3.2 Research Described in Chapter 4

The mechanisms by which elevated SSTs induce coral bleaching include damage to the photosynthetic apparatus of zooxanthellae. However, field studies have implicated a variety of environmental triggers for localized bleaching events. Mechanisms by which these different stressors induce bleaching are poorly understood. Recovery processes are likely to be influenced by the direct impacts of different bleaching stressors on the host and resident zooxanthellae. Two bleaching stressors, darkness and elevated seawater temperature, were therefore used to induce bleaching in a stony coral from Kenya, *Porites cylindrica*, and population densities of zooxanthellae were monitored during the recovery period. (H_0: Resilience is not dependent on the nature of the bleaching stressor). Furthermore, as it is not known what influence the duration of bleaching stressors exert on recovery, corals were subjected to different durations of the two bleaching stressors. (H_0: Resilience is not dependent on the duration of the bleaching stressor).

Mild to moderate bleaching occurred in April 2003 along the Kenyan coastline and in northern Tanzania. Colonies of *P.cylindrica* at Kanamai Reef bleached to varying extents in the field. Fragments from colonies classified as bleached (pale yellow), partially bleached (tan) and unbleached (chocolate brown), were transferred from the field to the laboratory, and population densities of zooxanthellae in these fragments

were monitored during the recovery period. (H_0: Resilience is not dependent on the extent to which natural bleaching has occurred).

Changes in the identity of zooxanthellae in corals have been known to oecur on recovery from bleaching. This is thought by some to be an adaptive/acclimatory response. The zooxanthellae in experimental corals before, during and after recovery from bleaching, were therefore identified by molecular methods. (H_0: There are no changes to the type of zooxanthellae hosted by *Porites cylindrica* on recovery from bleaching).

1.4.3.3 Research Described in Chapter 5

The frequency with which coral bleaching will recur has been predicted to increase sharply. However, physiological acclimatisation has been known to occur in corals and/or their zooxanthellae, thereby preventing corals from bleaching as predicted during adverse environmental conditions. To investigate acclimatory responses, corals with zooxanthellal populations recently recovered from experimental and natural bleaching were subjected to a repetition of the bleaching stressor. (H_0: Reeent experience of bleaching does not confer resistance to bleaching on repetition of the bleaching stressor).

Chapter 2
Materials and Methods

2.1 Molecular Analysis of Zooxanthellae

2.1.1 Sources of Chemicals and Experimental Materials

Unless otherwise stated, Sigma-Aldrich Co. Ltd., Dorset, UK, supplied all inorganic and organic chemicals and reagents. Scleractinians (stony corals) sampled for this study were collected from reef sites along the Kenya coastline, shown on the map in Figure 2.1. Tissue sampling, and the experiments described in section 2 2, were conducted with authorization from the Office of the President, Government of Kenya, under Research Permit Number MOEST 13/001/32C14 Actinarian (sea anemone) hosts were obtained from European locations in the Mediterranean Sea The origin of samples used for this study are summarised in Table 2.1.

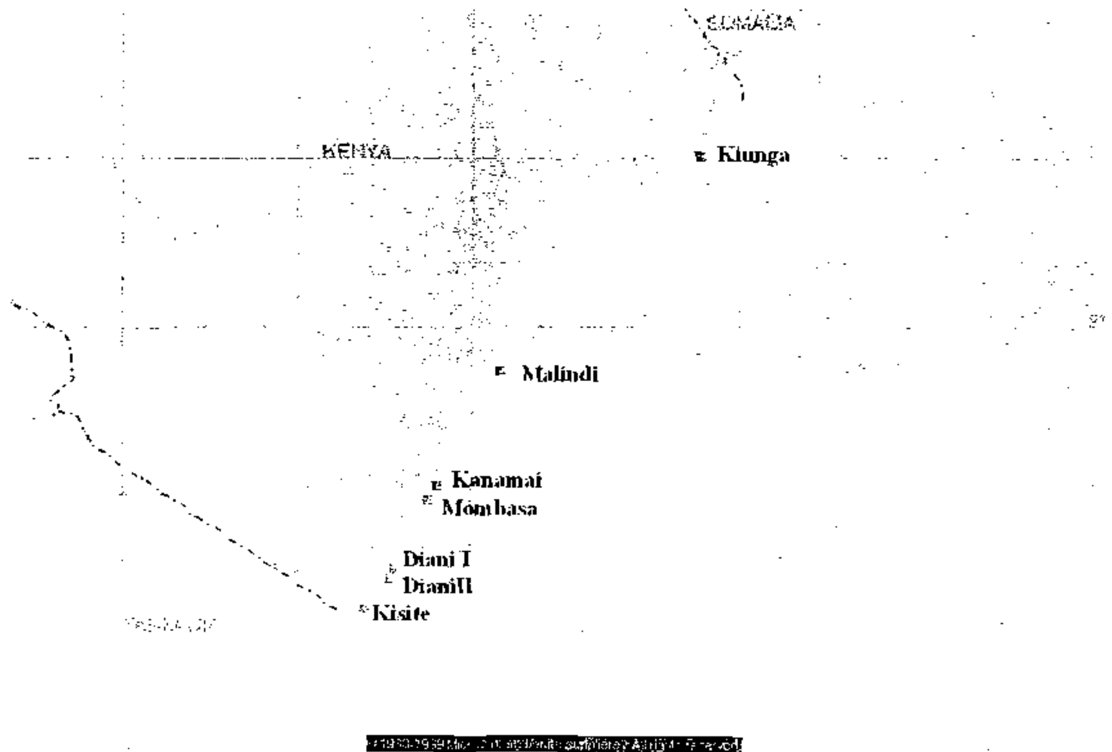

Figure 2.1: The reef sites in Kenya where corals were sampled during February 2001 and February 2002.

Table 2.1: Sampling locations in **a)** the Mediterranean and **b)** Kenya. Numbers specify sample sizes for each species, from locations indicated, identified by PCR-RFLP of rRNA genes (rDNA). Known sampling sub locations in the Mediterranean are indicated in brackets. The sample of *Anemonia sulcata* from Germany was collected from an aquarium, whereas all other sea anemone samples were collected from their natural habitat. Marine Reserves in Kenya are areas in which the Government approves traditional fishing methods. Marine Parks are no-take areas.

a) MEDITERRANEAN	Location (Country)			
Species	Italy	France	Spain	Germany
Aiptasia diaphana		1 (Colliure)		
Anemonia rustica	3			
Anemonia sulcata	3	10		1
Balanophyllia europaea			1 (Cadaques)	
Bunodeapsis strumosa		1 (St. Cyprien)		
Caryophyllia smithi		1 (Banyuls)		
Cereus pedunculatus		2 (Colliure)	1 (Cadaques)	
Cladocora cespitosa		1 (Banyuls)		
Cotylorhiza tuberculata		1 (Le Dramont)		
Cribinopsis crassa		1 (Banyuls)		

b) KENYA	Location (Grid coordinates, MPA status)						
	Kiunga	Malindi	Kanamai	Mombasa	Diani I	Diani II	Kisite
	1.97°S 41.31°E	3.25°S 40.15°E	3.97°S 39.58°E	4.06°S 39.58°E	4.36°S 39.56°E	4.41°S 39.55°E	4.72°S 39.38°E
	Reserve	Park	Unprotected	Park	Unprotected	Unprotected	Park
Acropora hyacinthus	2	1					1
Acropora palifera						4	3
Acropora valida				4			
Coscinarea mcneilli	3	3		3	1		
Galaxea fascicularis	4	2		2	2		3
Pocillopora damicornis		4		4			
Porites cylindrica			4				

2.1.2 Sampling

Sampling in Kenya was carried out in February 2001, when *Acropora valida* and *Pocillopora damicornis* were collected, and in February 2002, during which the remaining species were collected. Corals were sampled from shallow water reefs (depths ranging from 0.3 – 8.0 metres at low tide) whilst snorkelling or using SCUBA. Small fragments were broken off the parent colony using a hammer and chisel. Coral tissue was scraped off these fragments with a clean knife blade, and preserved in vials containing NaCl-saturated dimethyl sulphoxide (DMSO) buffer (20% DMSO, 0.25 M Na_2EDTA, saturated NaCl, pH 7.5). Jöerg Wiedenmann, University of Ulm, Germany, provided Mediterranean material.

2.1.3 DNA Extraction

Sea anemones collected from the Mediterranean Sea were provided as DNA samples. Two different protocols were used to extract DNA from DMSO-fixed Kenyan coral tissues. The second protocol was adopted in favour of the first, as it did not involve working with hazardous chemicals:

I. Fixed coral tissues were centrifuged at 2200 *g* for 2 minutes, and the pellets suspended in 200 µl DNA Isolation Buffer (DNAB; Rowan & Powers 1991a; 0.4 M NaCl, 50 mM Na_2EDTA, pH 8). The tubes were re-centrifuged at 2200 *g* for 2 minutes before suspending pellets in 200 µl DNAB containing 1% sodium dodecyl sulphate (SDS), and incubating in a water bath at 65°C for 30 minutes. Proteinase K, to a final concentration of 0.5 mg/ml, was added to the samples and the mixtures were incubated overnight in a water bath at 45°C. The following day, 400 µl cetyltrimethylammonium bromide (CTAB; 1 M NaCl, 1.5 µg/ml glycogen), that binds organic compounds at salt concentrations greater than 0.7 M, was added to each sample tube. Following incubation at 65°C for 30 minutes, 600 µl chloroform was added to each tube to extract DNA. The tubes were gently shaken for 20 minutes and allowed to stand for 5 minutes at room temperature to clear the aqueous layer, before a centrifugation step of 14250 *g* for 5 minutes. DNA was precipitated with 95% ethanol and 0.3 M sodium acetate. Extractions were suspended in 20 µl T0.1E (10 mM Tris-HCl, 0.1 mM Na_2EDTA) with 20 µg/ml Rnase A added.

II. DNA was extracted from DMSO fixed coral tissues using the DNeasy® Plant Mini Kit (Qiagen) according to the manufacturer's instructions

The quality and quantity of DNA extracted by the above methods was estimated by gel electrophoresis of 2 μl of each extraction on a 1% agarose gel in 1 x Tris acetate EDTA (TAE) buffer (40 mM Tris, 1 mM Na_2EDTA). Agarose gels were stained in 0.5 μg/ml ethidium bromide for 15 minutes, and visualised under ultra violet (UV) light. Comparison of the brightness of bands with that of the 1 kilobase (kb) DNA stepladder (Promega) enabled an estimation of the quantity of DNA extracted.

2.1.4 PCR Amplification

2.1.4.1 Ribosomal Genes

Polymerase Chain Reaction (PCR) was used to amplify 18S rRNA and 24S rRNA genes (rDNA). 18S rDNA was amplified using the primers ss3z (5'-AGC ACT GCG TCA GTC CGA ATA ATT CAC CGG- 3') and ss5z (an equimolar mix of two primers 5' –GCA GTT ATA ATT TAT TTG ATG GTC ACT GCT AC– 3' and 5' –GCA GTT ATA GTT TAT TTG ATG GTT GCT GCT AC– 3') (Rowan & Powers 1991a). The 18S primers used were specific for zooxanthellae, and bind to DNA inside the universal eukaryotic primers, amplifying a product of approximately 1600 base pairs (bp). 24S rDNA was amplified with the universal primers 24D1F51 (5' –TTA AGC ATA TAA GTA AGC GGA GGA- 3') and 24D23R1 (5' –CTC CTT GGT CCG TGT TTC AAG ACG- 3') (Baker *et al.* 1997a), which amplify a 650 bp region at the 5' end of 24S rDNA, including two hypervariable regions, D1 and D2. A copy of the ribosomal RNA gene, and the annealing position for the primers used in this study is shown in Figure 2.2.

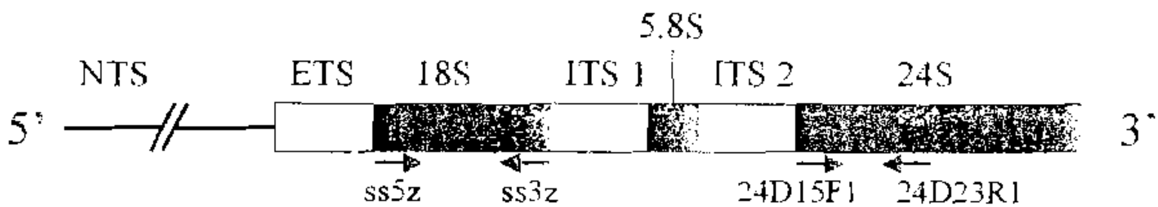

Figure 2.2: Organisation of a copy of the tandemly repeated eukaryotic ribosomal RNA gene (adapted from Hillis & Dixon 1991). Arrows indicate the annealing positions and the direction of PCR amplification for the primers used in this study. NTS: non-transcribed spacer, ETS: external transcribed spacer, ITS: internal transcribed spacer.

A typical PCR reaction mix contained 1.5 mM (3 mM for 24S rDNA) $MgCl_2$, 0.2 mM (0.5 mM for 24S rDNA) dNTPs, 0.2 µM ss3z or 24D1F51, 0.2 µM ss5z or 24D23R1, 1x Taq Polymerase buffer, and 2.5 U Taq DNA Polymerase, making a volume of 48 µl to which 1-2 µl template DNA was added. Promega supplied all PCR reagents except for the primers, which were obtained from MWG Bioteeh. PCR-Amplifieation was carried out in a thermal cycler [Techne Genius, Techne (Cambridge) Ltd.] with the following conditions: 28 eycles (30 eycles for 24S rDNA) of denaturing (94°C, 45 seconds), annealing (56°C, 45 seconds) and extension (72°C, 2 minutes), followed by a final extension (72°C, 6 minutes) before cooling and holding at 4°C. Five µl of PCR amplified products were loaded onto wells on a 1% agarose gel in 1 x TAE (40 mM Tris, 1mM Na_2EDTA) buffer, and stained with 0.5 µg/ml ethidium bromide, prior to viewing under UV light.

Success with PCR amplification of *Symbiodinium* 18S rRNA genes was almost always achieved when DNA was extracted with protocol I (section 2.1.3) but almost never achieved with protocol II. This may have been the result of shearing of DNA during extraction with the DNeasy® Plant Mini Kit (DNA extraction protocol II). On collection from the field, corals were immediately incubated in DMSO fixative. Perhaps 18S rDNA PCR amplification rates with protocol II would have been more successful had tissue samples been processed by the centrifugal separation of zooxanthellal pellets from host material (especially skeletal components).

The use of universal primers often resulted in co-amplification of host 24S rDNA, particularly for Kenyan material, at 850 bp. No additional bands were observed. When host bands occurred, PCR amplified products were separated by gel electrophoresis, and the zooxanthella 24S rDNA PCR product was cut out of the agarose gel using a clean surgical blade, and purified using the protocol specified in the QIAquick® Gel Extraction Kit (Qiagen). 24S rDNA PCR was then carried out, substituting gel extractions for DNA template. Second round of PCR generally resulted in successful amplification of zooxanthellal 24S rDNA minus any host 24S rDNA bands.

2.1.4.2 Chloroplast (*psb*A) Gene

Genomic DNA of host tissues sampled from Kenya, the Mediterranean, and aquarium specimens of *Anemonia* sp. housed at the University of York were sent to Christopher Howe and Adrian Barbrook, who sequenced partial *psb*A in their laboratories at the Department of Biochemistry, University of Cambridge. In addition, DNA from Bermudan

hosts, provided by Anne Savage, was also sent to Cambridge for sequencing. The templates were prepared from the following hosts:

Kenya – *Acropora valida*: Mombasa, *Porites cylindrica*: Kanamai, *Coscinarea mcneilli*: Diani I, *Galaxea fascicularis*: Diani I, *Galaxea fascicularis*: Kiunga, *Acropora palifera*: Kisite.

Mediterranean – *Balanophyllia europaea*: Spain, *Cereus pedunculatus*: Spain.

UK – two samples of *Anemonia viridis* collected from Llewyn Peninsula, Wales and housed in aquariums at the University of York.

Bermuda – *Porites astreoides*, *Cassiopia xamachana*, *Diploria labyrinthiformis*, *Madracis decactis*, *Scolymia* sp.

Primers used for PCR amplification of *Symbiodinium psb*A were:

- Forward TGGYTWTATAACGGTGGWACWTAYC psba1f
  Reverse CCARATWCCRATWACWGGCCA psba2r
- Forward GGATGGGTAGAGAATGGGAATTACG psba3f
  Reverse CGAGAGTTATTRAAGGAACCATATTG psba4r

All samples were sequenced directly from purified PCR products with the exception of *Cereus pedunculatus* and the two samples of *Anemonia* sp., which were first cloned prior to sequencing.

2.1.5 Restriction Enzyme Analysis

2.1.5.1 Restriction Analysis of PCR-Amplified 18S rDNA

In order to identify the phylotype of zooxanthellae from 18S rDNA, *Taq*I (Promega) and *Dpn*II (New England Biolabs) restriction enzymes were used to digest 18S rDNA PCR products in separate digestion reactions (Rowan & Powers 1991a). [Note that the phylotype designations (A-G) discussed in this thesis report are as reviewed by Baker (2003).] A typical digestion reaction mix comprised 3 U of restriction enzyme (*Taq*I or *Dpn*II in separate reaction tubes), 1.5 µl 10 x enzyme buffer, 0.15 µg bovine serum albumin (BSA) and 8-10 µl of 18S rDNA PCR product. The reaction tubes were incubated overnight at 65°C (for *Taq*I) or 37°C (for *Dpn*II). Digested products were then run on 1% agarose gels in 1 x TAE buffer before staining with ethidium bromide and viewing under UV light. The approximate size, in base pairs, of diagnostic fragments produced by restriction analysis of PCR-amplified rRNA genes is shown in Table 2.2.

Table 2.2: Approximate sizes, in base pairs, of diagnostic fragments produced by the restriction enzyme digestion of PCR-amplified zooxanthellal rRNA genes. 18S rRNA genes were digested with *Taq*I and *Dpn*II, and the enzymes used to analyse 24 rRNA genes were *HpyCh4*IV and *Dde*I. Due to faster electrophoretic mobility, fragments smaller than the ones indicated are not visible on the agarose gel. Temperate A (denoted as A') and standard A zooxanthellae have identical 18S PCR-RFLP banding profiles. Likewise, temperate A and phylotype B zooxanthellae are indistinguishable by 24S PCR-RFLP with the enzyme *HpyCh4*IV.

	18S PCR-RFLP		24S PCR-RFLP	
Phylotype	*Taq*I	*Dpn*II	*HpyCh4*IV	*Dde*I
A	700, 600	850, 580	350, 180, 120	270, 180
A'	700, 600	850, 580	650	380, 170
B	850, 500	750, 500	650	
C	880, 700	860, 500	400, 250	
D	720	860, 500	520	

2.1.5.2 Restriction Analysis of PCR-Amplified 24S rDNA

With the exception of *Acropora valida* and *Pocillopora damicornis*, the majority of samples (>90%) from Kenya failed to yield zooxanthellal 18S rDNA PCR products. This necessitated the development of a method, alternative to PCR-RFLP of 18S rDNA, to quickly distinguish between the phylotypes of zooxanthellae hosted by Kenyan corals. A 24S rDNA PCR-RFLP assay was developed as follows-

Zooxanthellal 24S rDNA was amplified by PCR, and the products were digested with the restriction enzyme *HpyCh4*IV (New England Biolabs). A typical digestion reaction mix comprised 7 U *HpyCh4*IV, 1.5 μl 10 x enzyme buffer and 7 μl of 24S rDNA PCR product in 15 μl reaction mix. The reaction tubes were incubated overnight in a water bath at 37°C. Digested products were then run on a 1% agarose gel in 1 x TAE buffer before staining with ethidium bromide and viewing under UV light. The sizes of diagnostic fragments produced by this assay are indicated in Table 2.2.

All Mediterranean samples were first analysed by PCR-RFLP of 18S rDNA. However, 24S rDNA sequences from the symbionts of *Anemonia viridis* and *Cereus pedunculatus* place them within a distinct subgroup of 'temperate' A zooxanthellae (Savage *et al.* 2002). These symbionts are indistinguishable from 'standard' A zooxanthellae when assayed by the 18S rDNA PCR-RFLP described in section 2.1.5.1. In order to distinguish between the two

phylotypes, 24S rDNA PCR products from Mediterranean samples were digested with *Dde*I restriction enzyme (Promega). A typical digestion reaction mix comprised 3 U *Dde*I, 2.0 µl 10 x enzyme buffer, 0.20 µg bovine serum albumin (BSA) and 8-10 µl of 24S rDNA PCR product. The reaction tubes were incubated overnight at 37°C and digested products were then run on 1% agarose gels in 1 x TAE buffer, stained with ethidium bromide and viewed under UV light. Diagnostic bands produced by this assay are indicated in Table 2.2.

2.1.6 Cloning and Sequencing *Symbiodinium* 24S rDNA

The 24S rDNA PCR products from Mediterranean *Anemonia* spp., and Kenyan *Pocillopora damicornis* and *Acropora valida*, were cloned into a plasmid vector prior to sequencing. All remaining samples were sequenced directly from PCR products. In addition zooxanthellal 18S rDNA from *Pocillopora damicornis* was also cloned in order to discriminate between mixed PCR products indicated by RFLP banding patterns.

2.1.6.1 Ligation with Plasmid Vector

The PCR products were cleaned using QIAquick™ PCR Purification Kit columns (Qiagen) following the manufacturer's instructions, prior to ligation to the pGEM®–T Easy plasmid vector (Promega). This vector has 3'-T overhangs at the insertional site, which improves the efficiency of ligation by preventing circularisation of vector, and by providing compatible overhangs with PCR products generated by Taq DNA Polymerase, which adds deoxyadenosine to the 3' end of amplified fragments (Promega Life Science Catalog 2002, p189). Insertional inactivation of the gene that encodes the α-peptide for β-galactosidase, allows for blue/white screening of recombinant colonies on indicator plates. Typical ligation reactions comprised 25 ng pGEM®–T Easy, 2 U T4 DNA Ligase, 1 x T4 DNA Ligase buffer, and 50-150 ng PCR product, making a total volume of 10 µl Reactions were incubated at 4°C for at least 24 hours.

2.1.6.2 Transformation of Bacterial Competent Cells

Five µl of the ligation reaction was added to a 50 µl aliquot of *E. coli* DH5-α competent cells. After mixing gently and incubating on ice for 30 minutes, the cells were heat-shocked at 42°C, and then placed on ice for 2 minutes. A volume of 900 µl of warm LB broth was added to the transformed cells, and the tubes were then incubated at 37°C for approximately 2 hours. LB agar plates with 50 µg/ml ampicillin were spread with a solution of 0.01% X-Gal and 50 mM IPTG (isopropyl-β-thiogalactopyranoside) to make

up the selection plates. Between 100 and 300 μl of the transformation cultures were spread out on each selection plate. These were then incubated overnight at 37°C.

2.1.6.3 Screening Recombinant Colonies

Colonies that grew on the selection plates had successfully been transformed with the plasmid vector, as they were resistant to ampicillin. To check that recombinant colonies bore an insert of the predicted size, a sterile tip was used to make slight contact with a white bacterial colony growing on the surface of the selection plate, then dipped into small volume of standard 24S rDNA PCR mix before finally being dipped into a 100 μl aliquot of LB broth containing 50 μg/ml ampicillin. The 24S rDNA PCR reaction was carried out, and the amplified products were visualised on a 1% agarose gel. The aliquots of LB Broth containing those colonies that yielded a 650 bp PCR product were grown overnight, agitating at 37°C, in 3.5 ml LB broth containing 50 μg/ml ampicillin.

2.1.6.4 Isolation and Purification of Recombinant Plasmid DNA

Glycerol stocks were prepared from the overnight cultures by adding 500 μl of each culture to 500 μl 30% glycerol. Recombinant plasmid DNA was isolated from the remaining volume of transformation culture using QIAprep® Spin Miniprep Kit columns (Qiagen), following the manufacturer's instructions. A small volume of isolated plasmid was digested with *Eco*RI (Promega) to cut out the gene insert for quantification. The digestion reaction comprised 2 U *Eco*RI, 1 x enzyme buffer, 1 x BSA and 1 μl of isolated plamid preparation making a volume of 20 μl which was incubated overnight in a water bath at 37°C. Plasmid concentration was estimated by comparing intensity of bands with known concentrations of DNA [e.g. the bands produced by known quantities of 100 bp DNA ladder (Promega)] on a 1% agarose gel stained with ethidium bromide.

2.1.7 Big Dye Terminator Sequencing

24S rDNA PCR products were sequenced directly using the primer 24D1F51 (5' –TTA AGC ATA TAA GTA AGC GGA GGA- 3') or the primer 24D23R1 (5' –CTC CTT GGT CCG TGT TTC AAG ACG- 3') (Baker *et al.* 1997a). PCR products were cleaned using the QIAquick™ PCR Purification Kit columns (Qiagen) prior to sequencing. Cloned 24S rDNA PCR products were sequenced from the pGEM®-T Easy plasmid vector using the primer T7 (5' –TAA TAC GAC TCA CTA TAG GG- 3') or SP6 (5' –ATT TAG GTG ACA CTA TAG AA- 3') (Promega) which bind on either side of the multiple cloning site

on the vector. Each purified PCR product, or cloned product, was sequenced twice, once using 24D15F1 (or SP6), and once using 24D23R1 (or T7), to generate two sequences running in opposite directions. The two sequences were aligned to resolve ambiguities. Sequencing reaction mixtures utilised a BigDye^{TM} Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems). A typical reaction mixture comprised 4 µl terminator ready reaction mix (TRRM), 4 µl sequencing buffer (200 mM Tris-HCl, 5 mM $MgCl_2$), 3.2 pmol/µl primer (24D15F1 / SP6 or 24D23R1 / T7) and 200-500 ng template DNA. The thermal cycler conditions consisted of an initial denaturising step (95°C, 30 seconds), followed by 25 cycles of denaturising (95°C, 30 seconds), annealing (50°C, 15 seconds) and extension (60°C, 4 minutes), before cooling and holding at 4°C.

An isopropanol precipitation protocol was used to purify sequencing reactions. Each reaction was mixed with 80 µl 75% isopropanol before they were incubated at room temperature for 15 minutes. Centrifugation for 20 minutes at 21000 *g* resulted in the precipitation of DNA. The supernatant was removed and the pellet was gently washed with 250 µl 75% isopropanol. The tubes re-centrifuged at 21000 *g* for 5 minutes before the supernatant was carefully removed. The resulting pellet was dried in an oven at 30°C for 40 minutes. The dry pellets were suspended in a loading buffer (EDTA/Blue Dextran in formamide) and run on a 4.25% polyacrylamide gel in an ABI Automated Sequencer (PE Applied Biosystems) by staff at the DNA Sequencing Facility located in the Department of Biochemistry, University of Oxford.

2.1.8 Sequence Data and Phylogenetic Analysis

Sequence electropherograms were aligned and analysed using the program SEQMAN (DNASTAR Ltd., UK). Resolved sequences were first checked against the Genbank database using BLAST search (Altschul *et al.* 1990) to ensure that they corresponded to *Symbiodinium* 24S rDNA (or *Symbiodinium psbA*). Sequences were then exported in FASTA format to the program ClustalX (Thompson *et al.* 1997), in which multiple alignments were made. Alignments were visually inspected and refined in GENEDOC (http://www.psc.edu/biomed/genedoc/), then converted to NEXUS format. The NEXUS file was opened in the program PAUP* version 4 (Swofford 1998) where alignment datasets were tested for the optimal model of nucleotide evolution using Modeltest version 3.06 (Posada & Crandall 1998). The likelihood settings from the model selected were implemented prior to construction of Neighbor-Joining (NJ) distance trees (Saitou & Nei 1987). Maximum Parsimony (MP) trees were constructed using a heuristic search

algorithm and weighted with default settings, with gaps treated as missing data. Bootstrap analysis with 1000 replicates was conducted to assess relative support for trees. The *Symbiodinium* 24S rRNA gene sequences from Genbank used in a comparison with those from this study are outlined in Table 2.3.

The Neighbour-Joining tree for *Symbiodinium psb*A utilised uncorrected distance settings (number of nucleotide substitutions divided by the length of alignment), as the tree constructed with the likelihood settings recovered by Modeltest was not congruent with the phylogeny constructed from corresponding *Symbiodinium* 24S rRNA gene sequences.

Table 2.3: Details of 24S rRNA gene sequences from Genbank that were used for phylogenetic analyses. Continued on following page.

Accession	Dinoflagellate Species	Host Species	Host Location	Phylotype	Source
AY074939	*Symbiodinium* sp.	*Anemonia viridis*	UK	A'	Savage *et al.* 2002
AY074940	*Symbiodinium* sp.	*Anemonia viridis*	UK	A'	Savage *et al.* 2002
AY074945	*Symbiodinium* sp.	*Cereus pedunculatus*	France	A'	Savage *et al.* 2002
AF060896	*Symbiodinium microadriaticum*	*Cassiopia xamachana*		A	Wilcox 1998
AF349556	*Symbiodinium* sp.	*Acropora longicyathus*	Australia	A	Loh *et al.* 2001
AJ308903	*Symbiodinium* sp.	*Porites nigrescens*	Reunion	A	Pochon *et al.* 2001
AJ311947	*Symbiodinium* sp.	*Acropora* sp.	Israel	A	Pochon *et al.* 2001
AY074941	*Symbiodinium* sp.	*Bartholomea annulata*	Bermuda	A	Savage *et al.* 2002
AY074949	*Symbiodinium* sp.	*Condylactes gigantea*	Bermuda	A	Savage *et al.* 2002
AY074967	*Symbiodinium* sp.	*Porites astreoides*	Bermuda	A	Savage *et al.* 2002
AY074968	*Symbiodinium* sp.	*Porites porites*	Bermuda	A	Savage *et al.* 2002
AF427455	*Symbiodinium* sp.	*Tridacna gigas*	Indo-Pacific	A	Santos *et al.* 2002
AF170139	*Symbiodinium* sp.	*Diploria strigosa*	Bahamas	B	A. Baker (unpublished)
AF170144	*Symbiodinium* sp.	*Plesiastrea versipora*	Australia	B	A. Baker (unpublished)
AF331858	*Symbiodinium* sp.	*Madracis mirabilis*	Curaçao	B	Diekmann *et al.* 2002
AY074946	*Symbiodinium* sp.	*Favia fragum*	US Virgin Islands	B	Savage *et al.* 2002
AY074950	*Symbiodinium* sp.	*Condylactes gigantea*	Bermuda	B	Savage *et al.* 2002
AY074966	*Symbiodinium* sp.	*Montastraea annularis*	Bermuda	B	Savage *et al.* 2002
AY074969	*Symbiodinium* sp.	*Siderastrea radians*	Bermuda	B	Savage *et al.* 2002
AF060890	*Symbiodinium* sp.	*Montastraea franksi*	Florida Keys	C	Wilcox 1998
AF170129	*Symbiodinium* sp.	*Pavona gigantea*	Panama (Pacific)	C	A. Baker (unpublished)

Table 2.3: continued from previous page

Accession	Dinoflagellate Species	Host Species	Host Location	Phylotype	Source
AF170133	*Symbiodinium* sp	*Acropora nasuta*	Australia	C	A. Baker (unpublished)
AF170135	*Symbiodinium* sp.	*Montastraea cavernosa*	Bahamas	C	A. Baker (unpublished)
AF170142	*Symbiodinium* sp.	*Pocillopara damicornis*	Galápagos	C	A. Baker (unpublished)
AF170145	*Symbiodinium* sp.	*Pavona varians*	Australia	C	A. Baker (unpublished)
AJ308893	*Symbiodinium* sp.	*Acropora* sp.	Reunion	C	Pochon *et al.* 2001
AJ311942	*Symbiodinium* sp.	*Acropora* sp.	Israel	C	Pochon *et al.* 2001
AY074948	*Symbiodinium* sp	*Montastraea annularis*	US Virgin Islands	C	Savage *et al.* 2002
AY074970	*Symbiodinium* sp.	*Scolymia* sp.	Bermuda	C	Savage *et al.* 2002
AY074971	*Symbiodinium* sp.	*Stephanocoenia intersepta*	Bermuda	C	Savage *et al.* 2002
AF308900	*Symbiodinium* sp.	*Pavona decusata*	Guam	D	Pochon *et al.* 2001
AF349546	*Symbiodinium* sp.	*Seriatopora hystrix*	Malaysia	D	Loh *et al.* 2001
AJ308901	*Symbiodinium* sp.	*Goniopora fruticosa*	Guam	D	Pochon *et al.* 2001
AJ308902	*Symbiodinium* sp.	*Acropora palifera*	Guam	D	Pochon *et al.* 2001
AY074957	*Symbiodinium* sp.	*Goniastrea aspera*	Thailand	D	Savage *et al.* 2002
AF060899	*Gymnodinium varians*		New Zealand	E	Wilcox 1998
AJ291535	*Symbiodinium* sp.	*Amphisorus* sp.	Maldives	F	Pawlowski *et al.* 2001
AJ308895	*Symbiodinium* sp.	*Marginopora kudakajimaensis*	Guam	F	Pochon *et al.* 2001
AJ291536	*Symbiodinium* sp.	*Marginopora* sp.	Guam	G	Pawlowski *et al.* 2001
AJ291537	*Symbiodinium* sp.	*Amphisorus* sp.	Guam	G	Pawlowski *et al.* 2001
AF060900	*Gymnodinium beii*			Outgroup	Wilcox 1998
AF060901	*Gymnodinium simplex*			Outgroup	Wilcox 1998

2.2 Experimental Analysis of Recovery from Coral Bleaching

2.2.1 Collection of Corals and Sampling Location

Experiments were conducted in Kenya, in April-August 2002, and April-August 2003. The coral species utilised was *Porites cylindrica* Dana 1846, collected at low tide at a depth of 0.3 metres from Kanamai Reef (3.97°S, 39.58°E; see Figures 2.1 and 2.3). After pilot studies with a number of species, including *Pocillopora damicornis* and *Acropora* spp., *P. cylindrica* was selected because of its tolerance to indoor aquaria; its known susceptibility to bleaching in Kenya (D. Obura, personal communication); amenability to PCR-based molecular analysis of zooxanthellae; and its regular cylindrical morphology and shallow calices of corallites which are suitable for zooxanthellal density measurements. In April 2003, a number of *P. cylindrica* colonies bleached at Kanamai Reef, providing an opportunity for examining the in-laboratory recovery rates of corals that had bleached to varying extents in the field.

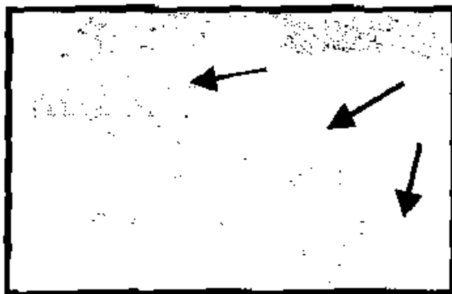

Figure 2.3: Kanamai Reef, Kenya. Inset, *Porites cylindrica*, marked by arrows, is one of the dominant coral species at this site.

Colonies selected for study were separated by at least 5 metres. Fragments were broken off parent colonies with a twisting motion of a knife blade targeted at the base of finger-like projections. The approximate length of a fragment harvested in this manner was 4-5 centimetres. Colony morphology usually prevented sunlight from penetrating to the base of these fragments, resulting in a ring of dead skeleton, colonised by sponges in some instances (see Figure 2.4a). The implication of this was two-fold: first that each individual fragment collected constituted an independent sample, as second, that collection caused

minimal tissue damage at the site of breakage. Fragments were collected from the mid-section of parent colonies, and never from the base of parent colonies, where there was minimal light penetration, particularly to the undersides of fragments (see Figure 2.4b). Fragments were transported submerged in a shallow tub of seawater and the average time taken from field to laboratory was 45 minutes.

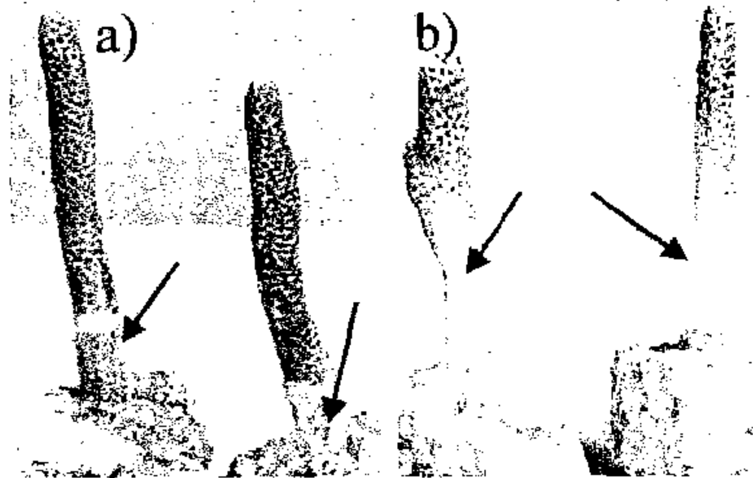

Figure 2.4: *Porites cylindrica* fragments collected from Kanamai Reef (I). Arrows mark aspects of coral biology important to note during collection; **a)** dead tissue at base, sometimes covered by sponges **b)** pale colouration along the undersides of fragments collected from the base of parent colonies.

2.2.2 Maintenance of Coral Fragments

Coral fragments were glued (Superglue, Alpha Techno Co., Japan) at their base onto dead coral stones. Fragments were held indoors, under natural light (12 hour dark/light cycle) in plastic seawater tanks, as shown in Figure 2.5, each with a capacity of 10-12 litres. Seawater was aerated continually using Ghost II air pumps (Waterlife, UK). In the event of power failures, the power supply was integrated with electrical output from a petrol generator. Seawater was exchanged daily by charging black PVC reservoirs with seawater collected from a nearby beach. Flow-through was achieved by gravity feed, and the daily rate of exchange approximated three quarters of the capacity of each tank. When discharging, the reservoir would generally empty over a four-hour period. Flow rates were controlled by a plastic tap at the head of the pipe connecting the reservoir to the tank housing corals. None of the plumbing involved the use of metallic components, which were found to be toxic to the corals, and which could have resulted in bleaching not directly due to intended experimental manipulation (Harland & Nganro 1990, Brown 2000). Filamentous and turf algal growth were periodically scraped off coral fragment bases using a soft bristle toothbrush, and the walls and bottoms of all experimental tanks

were similarly cleaned on a weekly basis. The positions of tanks were changed once a week in order to minimise problems associated with simple segregation of treatments (Hurlbert 1984), and particularly the possible influence of a sunlight gradient in the laboratory. All fragments were maintained in these conditions for at least one week before experiments commenced, in order to allow corals to acclimate to laboratory conditions, and to identify any diseased or damaged fragments, which were removed.

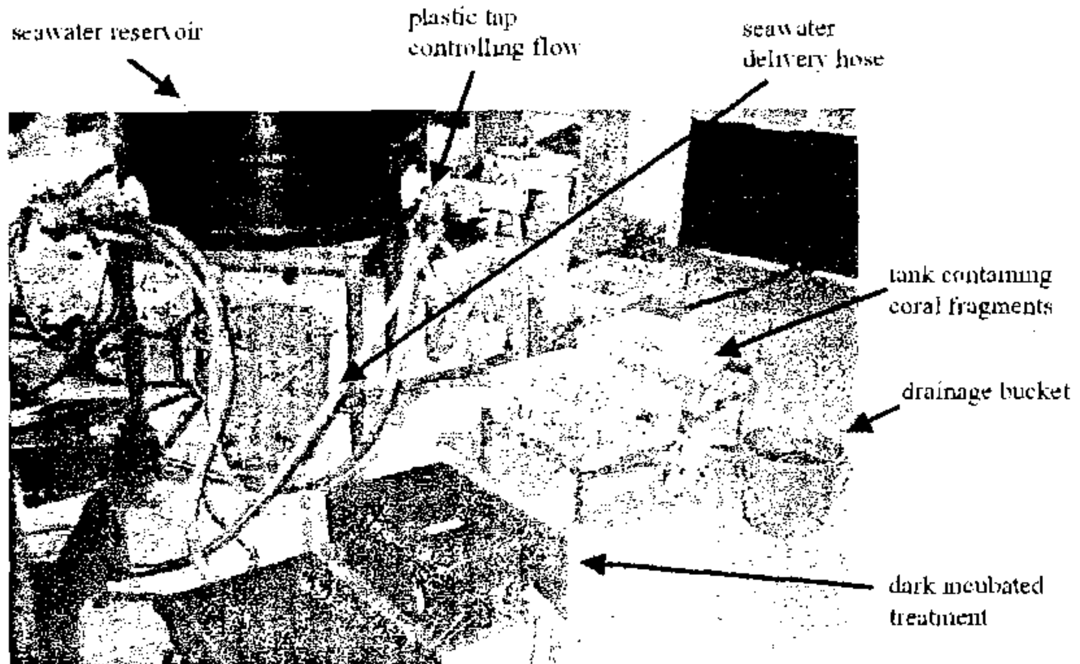

Figure 2.5: The laboratory in which corals were housed, and where experiments were conducted.

2.2.3 Zooxanthellal Density Measurements

The density of zooxanthellae, expressed as number of cells per square centimetre of coral skeleton, and the sole index by which recovery from bleaching was assessed, was determined using the 'aluminium foil' technique of Marsh (1970). Fragments were removed from experimental tanks, and cut at the mid-section using a metal bolt cutter. Owing to colony growth at the base of fragments after several weeks in indoor tanks, and at the tips of fragments under field conditions, (see Figure 2.6a and b), zooxanthellal and pigment density was considered to be lowest in these areas, and therefore measurements were always obtained from the mid-section of fragments. A metal bolt-cutter was used to section the fragment, resulting in a cylindrical sample from which coral tissue was removed with a fine jet of GF/C (Whatman) filtered (pore size 1.2 μm) seawater pressurised through a nozzle with a dental water-pik. Filtered seawater was added to the coral blastate to a final volume of 200-900 ml, then homogenised using a hand-held

homogeniser. One millilitre of homogenized blastate was loaded onto a Sedgwick Rafter counting chamber (PhycoTech Inc., USA), and the number of zooxanthellae per quadrant was counted in 10 randomly selected quadrants using a tally counter and a compound binocular light microscope (magnification x 100). The Sedgwick Rafter chamber holds precisely one millilitre fluid when fully loaded, and comprises 1000 quadrants. Thus, the average number of cells per quadrant is multiplied by 1000 to determine the number of cells per millilitre of fluid. Two consecutive counts were made as above, and the average of the two counts was calculated. The two counts differed from their overall mean by 0 – 23%, with 72% of counts lying within 10% of the mean.

The average number of dividing cells for two separate counts of 500 cells was made in order to determine the percentage of dividing cells. The area of coral skeleton exposed by water piking was determined by carefully wrapping the exposed skeletal area with aluminium cooking foil, and then weighing the foil paper obtained in this manner. By comparing the weight of foil paper with that of standards of known area prepared from the same batch of foil paper, the area of skeleton exposed was calculated using regression analysis.

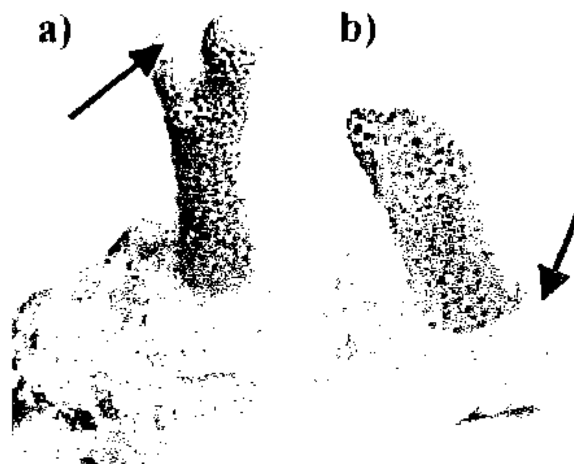

Figure 2.6: *Porites cylindrica* fragments collected from Kanamai Reef (II). Arrows mark aspects of coral biology important to note during zooxanthellal density measurements: **a)** pale tissues at tips of fragments when collected from the reef **b)** growth at base of fragment after several weeks in the laboratory.

Measurements of zooxanthellal density and division rates were generally commenced at 08:00, approximately two hours after dawn. Each coral fragment generally took approximately 30 minutes to process.

2.2.4 Experimental Designs:

For ease of referral, experimental protocols (outlining pretreatments/treatments and recovery periods) are summarised in Table 2.4a (experiments 1-4) and 2.4b (experiments 5-8).

Table 2.4: A summary of the pretreatments/treatments and recovery periods for experiments 1-4 (a) and experiments 5-8 (b).

(a)

Exp.	Treatment/Durations	Recovery (days)
1	24 hr dark: 5,10, 15, 20, 25 days	84
2	24 hr dark: 7, 14, 21 days	42
3	32.5°C: 48, 96 hr	63
4	Field Stressors: bleached, partially bleached, unbleached	63

(b)

Exp.	Pretreatment/Duration	Recovery (days)	Treatment/Duration
5	24 hr dark: 21 days	42	24 hr dark: 21 days
6	24 hr dark: 21 days	42	32.5°C: 72 hr
7	32.5°C: 96 hr	63	32.5°C: 24 hr[1]
8	Field Stressors: bleached, partially bleached, unbleached	63	32.5°C: 72 hr

[1] The intended duration of treatment was 72 hours. Treatment was terminated prematurely owing to the onset of coral mortality.

2.2.4.1 Dark-Treatment of Corals: Experiments 1 and 2

Experiment 1 was carried out in April–August 2002. On April 16 2002, 120 fragments from each of two *P. cylindrica* colonies were collected from the field and distributed to 12 tanks, with 10 randomly selected fragments of each colony in each tank. The experiment was started after an initial acclimation period of 7 days. Two tanks were selected as 'controls', and two tanks were dark-treated for each of 5, 10, 15, 20 and 25 days (treatment tanks). Dark-treatment comprised enclosure in lightproof heavy-duty black polythene, with no alteration to seawater exchange and aeration. The experimental layout is shown in Figure 2.7. For each treatment tank, one randomly selected fragment of each colony was

assayed immediately upon removal of the polythene sheet (day 0), and on days 7, 21, 42, 63 and 84. One fragment per colony in the control tanks was assayed at the start of the experiment, and at four-week intervals thereafter. The assay comprised measurements of zooxanthellal density and the percentage of dividing cells.

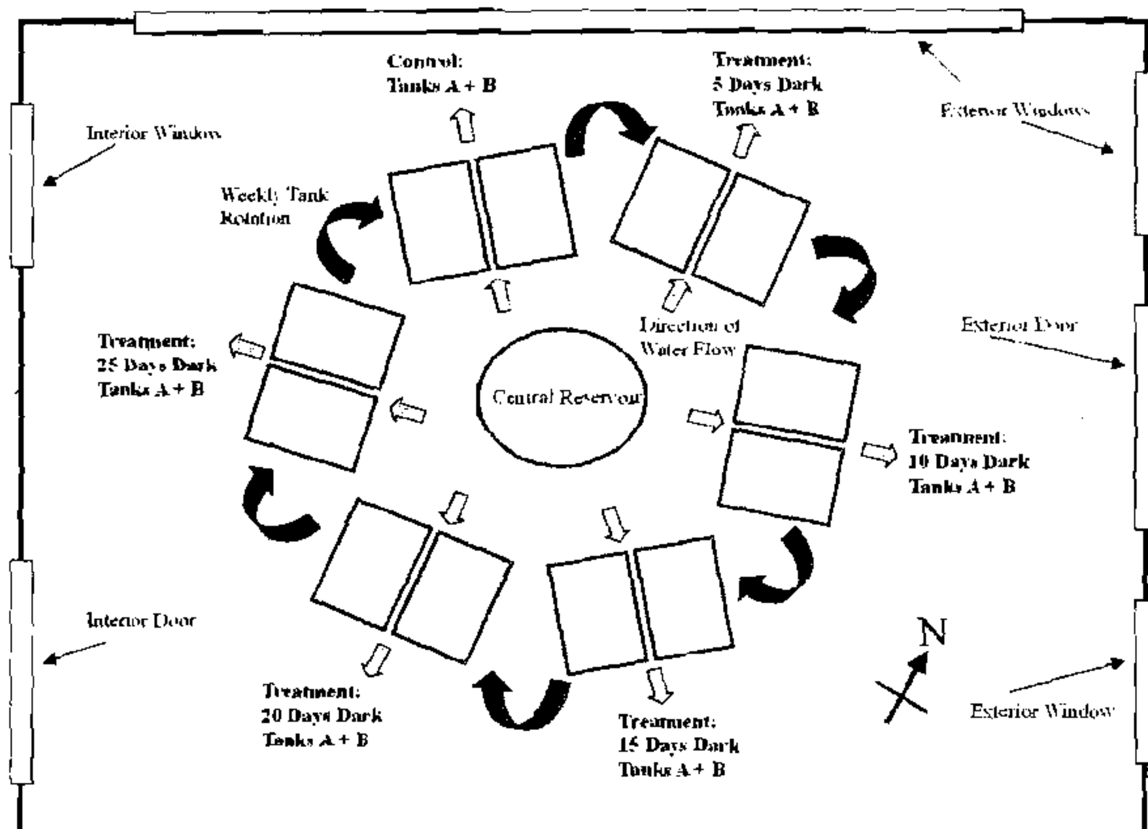

Figure 2.7: Layout for Experiment 1. All treatment and control tanks housed 10 fragments of each of 2 coral colonies.

Experiment 2 was carried out in May–July 2003. The experiment was a modification of Experiment 1 in which all treatment tanks were enveloped by black polythene on the same day. In Experiment 2, dark-treatments were commenced on different days, but terminated on the same day, which allowed for simultaneous measurements to be made on designated days post-exposure to light on fragments from all treatment tanks. Eighty fragments of each of two *P. cylindrica* colonies were collected from Kanamai on May 9 2003. Ten randomly selected fragments of each colony were divided into 8 tanks. The experiment was started after an initial acclimation period of 7 days. Two tanks were selected as 'controls', and two tanks were dark-treated for 21 days. Seven days and 14 days later, two tanks were dark-treated for each of 14 days and 7 days respectively. Dark treatment comprised enclosure in lightproof heavy-duty black polythene, with no changes to aeration and seawater exchange. The experimental layout is shown in Figure 2.8. Immediately upon

removal of the polythene sheet, 1 fragment from each colony in each treatment tank was assayed for zooxanthellal density and the percentage of dividing cells (day 0) and again on days 7, 21 and 42. One fragment per colony in each control tank was assayed at the start of the experiment, and at four-week intervals thereafter. This experiment was conducted in parallel with Experiment 3, in which treatments comprised elevated temperature-treatment, and which is also outlined in Figure 2.7.

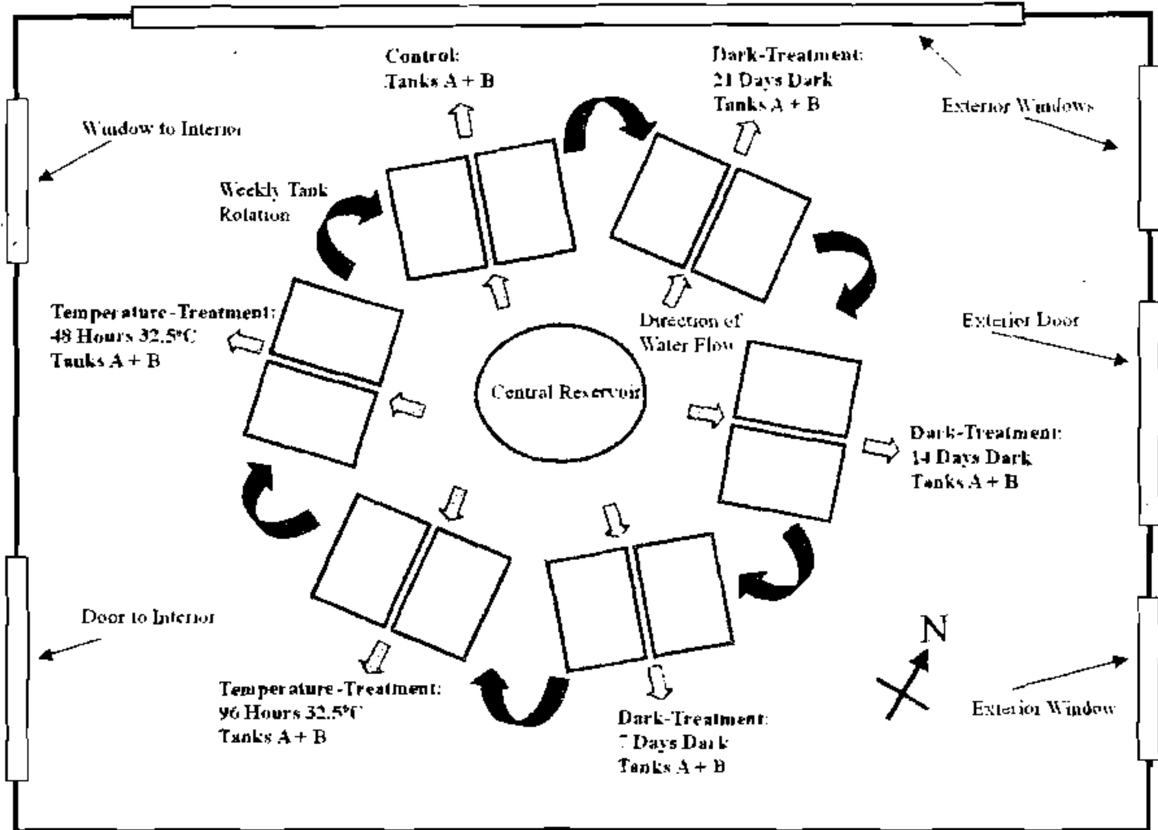

Figure 2.8: Experimental layout for experiments 2 and 3. All treatment and control tanks housed 10 corals from each of 2 coral colonies. The same colonies were used for both experiments.

2.2.4.2 Temperature-Treatment of Corals: Experiment 3

Experiment 3 was carried out in May – August 2003. The combined experimental layout for experiments 2 and 3 is shown in Figure 2.8. On May 9 2003, 60 fragments from each of 2 coral colonies were collected from the reef. Ten randomly selected fragments from each colony were divided into 6 tanks. The experiment was started after an acclimation period of 24 days. Two tanks were selected as 'controls', and the coral fragments in two 'treatment' tanks were transferred to a water bath for 96 hours. The temperature in the water bath was gradually raised over 4-6 hours from ambient (approximately 28°C) to 32.5°C using a filament immersion heater in combination with an external temperature

controller with inbuilt thermostat (Electronics Workshop, Department of Biology, University of York). Aeration and seawater exchange was maintained throughout treatment. Two days after the experiment started, the coral fragments in the remaining pair of 'treatment' tanks were temperature-treated in the water bath at 32.5°C for 48 hours. Treatment was terminated simultaneously for both durations of temperature-treatment, and the fragments were transferred back to their respective 'treatment' tanks. Immediately upon termination of temperature-treatment, one fragment from each colony for each treatment tank was assayed for the density and percent of dividing zooxanthellae (day 0), and again on days 7, 21, 42 and 63. Experiment 3 was conducted in parallel with experiment 2, with the use of the same coral colonies. Control tanks for both experiments were identical, and measurements of zooxanthellal density and percentage of dividing cells for control tanks were made as described for experiment 2.

A pilot study prior to experiment 3 eliminated the need for 'procedural' controls (Hurlbert 1984) as it revealed that no significant differences in zooxanthellal density was generated by the procedure of moving coral fragments from experimental tanks to the water bath, holding these in the unheated bath for a minimum of 5 days, before moving them back to their original tanks.

2.2.4.3 Naturally Bleached Corals: Experiment 4

In April 2003, mild to moderate bleaching was observed along the Kenyan coastline and in northern Tanzania. In Kenya, the combination of anomalously high temperature, calm weather conditions and spring low tides immediately prior to bleaching suggest a combined effect of all these factors may have elicited bleaching. Bleaching of a small proportion (< 10%) of *P. cylindrica* colonies at Kanamai Reef provided an excellent opportunity to study the recovery profiles in zooxanthellal density for corals that had bleached to varying extents in the field. It was considered important to monitor recovery of corals in isolation from the reef, as this eliminated the need to measure a whole suite of environmental variables that potentially influence zooxanthellal density and ensured a relatively stable environment during recovery. Corals were visually inspected and assigned to one of three bleaching states according to coloration; bleached (pale yellow), partially bleached (tan) and unbleached (chocolate brown). Paling of coral tissues can also result from loss of zooxanthellal pigments (Fitt & Warner 1995), and therefore, before collection of coral fragments, it was first verified that the zooxanthellal density in 'bleached' fragments was lower than that of 'partially bleached' and 'unbleached' fragments. Twenty

fragments from each of three coral colonies for each of the three bleaching categories were collected and transported to the laboratory. Collections were made on April 16 2003, soon after the onset of the southeast monsoon winds, known locally as 'Kusi', and after cessation of the environmental conditions that were believed to have elicited bleaching. For each of the three bleaching categories, 10 randomly selected fragments from each colony were held in each of two replicate tanks. This made a total of 30 fragments in each of 6 tanks, as shown in Figure 2.9. Each tank was supplied with seawater from a separate reservoir. Zooxanthellal density and percentage of dividing cells were measured for one randomly selected fragment for each colony for each tank, to give six readings per bleaching category immediately upon collection from Kanamai (day 0), and thereafter on days 7, 21, 42 and 63.

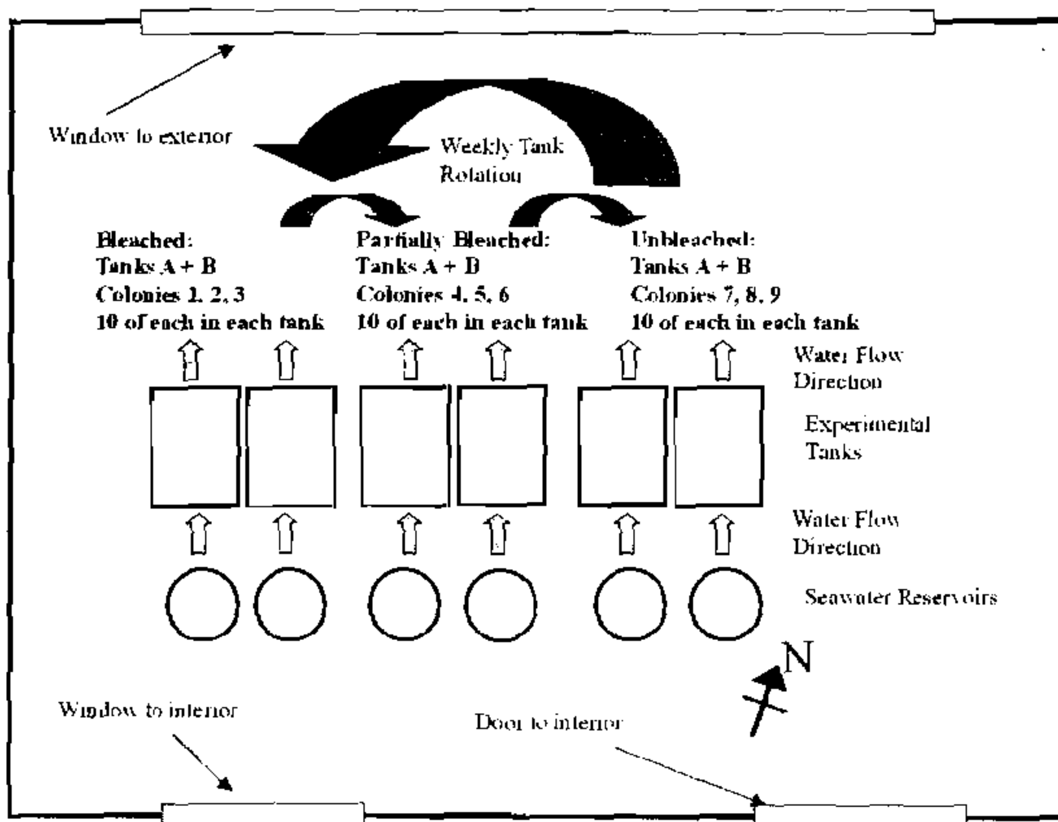

Figure 2.9: Experimental layout for experiment 4. All experimental tanks housed 10 fragments from each of three colonies.

2.2.4.4 Experimental Bleaching of Corals with Zooxanthellal Populations Recently Recovered from Experimental Bleaching: Experiments 5, 6 and 7

Three experiments, 5, 6 and 7, explored the impact of previous experimentally induced bleaching, and subsequent recovery of zooxanthellal populations, on the bleaching response of corals to dark-treatment or to elevated temperature-treatment:

Experiment 5 was carried out in July-August 2003. The experiment involved the pre-treatment of corals (to induce bleaching) by incubating coral fragments in absolute-darkness for 21 days (controls were kept under the ambient light regime), followed by 42 days under the ambient light regime. (The '21 days dark-treatment' in experiment 2 comprised the pre-treatment for experiment 5; the same coral colonies were used) This allowed for recovery of the zooxanthellal population in pre-treated (bleached) fragments. Treatment consisted of incubation in absolute-darkness for 21 days (controls were kept under the ambient light regime). There were 2 fragments from each of 2 coral colonies for each of the 4 experimental groups generated by the 2 x 2 design of this experiment, making a total of 16 fragments, each of which was assayed for zooxanthellal density immediately upon termination of treatment.

Experiment 6 was carried out in July-August 2003. The design involved pre-treating coral fragments (to induce bleaching) by incubation under conditions of absolute-darkness for a period of 21 days (controls were kept under the ambient light regime), before maintaining fragments for 42 days under the ambient light regime, which allowed for the recovery of the zooxanthellal population in pre-treated (bleached) fragments. (As in experiment 5, the '21 days dark-treatment' in experiment 2 comprised the pre-treatment for experiment 6). Treatment consisted of exposure to elevated temperature of 32.5°C for 72 hours (controls were kept under the ambient temperature regime). There were 2 fragments from each of 2 coral colonies for each of the 4 experimental groups resulting from the 2 x 2 design of this experiment, making a total of 16 fragments for which zooxanthellal density measurements were obtained immediately upon suspension of treatment.

Experiment 7 was carried out in August 2003. Corals were pre-treated by exposure to seawater at 32.5°C for 96 hours, which induced bleaching (controls were kept at the ambient temperature regime). (The '96 hour temperature-treatment' in experiment 3 comprised the pre-treatment for experiment 7). Following pre-treatment, fragments were kept for 63 days under the ambient temperature regime to allow for the recovery of the zooxanthellal population in pre-treated (bleached) fragments, before treatment, which comprised exposure to elevated temperature of 32.5°C for 72 hours, but discontinued after 24 hours for reasons provided in chapter 5 (controls were maintained under the ambient temperature regime). There were 2 fragments from each of 2 coral colonies for each of the 4 experimental groups generated by the 2 x 2 design of this experiment, making a total of 16 fragments for which zooxanthellal density was assayed on suspension of treatment.

2.2.4.5 Experimental Bleaching of Corals with Zooxanthellal Populations Recovered from Natural Bleaching: Experiment 8

Experiment 8, conducted in June 2003, investigated the impact of recovery of zooxanthellal populations from natural bleaching on the bleaching response to elevated temperature. Coral fragments that had been collected from Kanamai Reef in a 'Bleached' and 'Partially Bleached' state (and 'Unbleached' controls) were kept for 63 days under the ambient temperature regime to promote recovery of zooxanthellal populations in bleached corals before treatment commenced. (The same coral colonies were used as in experiment 4). Treatment comprised exposure to elevated temperature of 32.5°C for 72 hours (controls were maintained under the ambient temperature regime). There were 2 fragments from each of 3 coral colonies for each of the 6 experimental groups resulting from the 3 x 2 design of this experiment, making a total of 36 fragments, each of which was assayed for density of zooxanthellae immediately upon termination of treatment.

2.2.5 Molecular Analysis of Zooxanthellae Before/After Recovery from Bleaching

To determine whether corals bear new zooxanthellal phylotypes after recovery from experimental bleaching, coral tissues were sampled from each of the 2 colonies used in experiments 2 and 3. Samples were taken from treatment and control tanks, and were obtained at the start and at the end of the experiment. In addition, samples were also obtained from treatment tanks immediately on termination of dark or temperature treatment. The times at which tissue sampling was carried out are summarised in Table 2.5.

Tissue samples were also obtained from each of 3 coral colonies collected from the reef in bleached and unbleaehed states (these colonies were used in experiment 4). Samples were obtained immediately on collection, and after 63 days in the laboratory and 84 days in the field as summarised in Table 2.5.

The zooxanthellae from these samples were identified by PCR-RFLP and sequence analysis of 24S rDNA, as described in section 2.1. Coral tissues were DMSO-fixed, and transported to England endorsed on CITES Export Permit Number 006823 [CITES Security Stamp No. KE9118108, issued by the Kenya Wildlife Services (KWS)].

Table 2.5: The time points at which tissue samples of corals were obtained for molecular analysis of their zooxanthellae. The arrows mark the times relative to treatment and recovery at which sampling was conducted, and numbers below the arrows indicate the number of samples obtained. For experiment 4, sampling was conducted both in the field and laboratory after the recovery period.

Exp.	No. Samples ↓	Treatment	No. Samples ↓	Recovery	No. Samples ↓
2	2	24 hr dark: 21 days	2	42	2
3	2	32.5°C: 96 hr	2	63	2
2 & 3	2	Treatment controls			2
4		Field stressors:			
		bleached	2 [1]	84	1 field [2]
		unbleached (controls)	3		3 field [3]
					3 lab [3]

[1] The sample from the third colony did not yield a PCR product.

[2] Two colonies were dead in the field. Data from laboratory samples were not available due to lack of PCR-amplification.

[3] Sampling was conducted at 84 days for field samples, and 63 days for laboratory samples.

2.2.6 Statistical Analysis

The Anderson-Darling and Bartlett's Tests were used to test data for assumptions of 'normal distribution' and 'homogeneity of variances' respectively, before applying the two-sample T-Test or ANOVA. This was followed by post-hoc analysis with Fisher's LSD test. Percentage data were arcsine-square root-transformed prior to use of ANOVA. Significant differences were tested for at the $p = 0.05$ level of significance. Statistical analyses were performed with the MINITAB (Version 10.1) software.

Chapter 3

The Diversity of Zooxanthellae in Kenya and the Mediterranean Sea

3.1 Introduction

The lack of observed sexual reproduction in *Symbiodinium* precludes the use of the 'biological species concept' to delineate species boundaries in this diverse group. Hence, of the 11 currently named species, 10 were characterised by morphological criteria. These comprise four *in vitro* cultures that have been described formally, namely *Symbiodinium microadriaticum*, *S. pilosum*, *S. kawagutii* and *S. goreaui* (Freudenthal 1962, Trench & Blank 1987), and six cultures without formal description, namely *S. cariborum*, *S. bermudense*, *S. californium*, *S. pulchorum*, *S. meandrinae* and *S. corculorum* (Banaszak *et al.* 1993, Trench 1993, McNally *et al.* 1994, Banaszak & Trench 1995a, b).

Only a small subset of zooxanthellae has successfully been brought into culture (Rowan 1998, Santos *et al.* 2001), thereby imposing severe limitations to the application of morphological criteria to identify species. Molecular methods, and particularly DNA sequence data, have provided us with the best tools with which to investigate diversity in zooxanthellae. Since the inception of molecular methods to characterise zooxanthellae, two species of dinoflagellate, *Gymnodinium linuchae* (Trench & Thinh 1995) and *G. varians*, have been reclassified as belonging to *Symbiodinium* (LaJeunesse 2001, Wilcox 1998, LaJeunesse & Trench 2000). In addition, *Symbiodinium muscatinei* has been named as a species based entirely on DNA sequence data (LaJeunesse & Trench 2000).

The starting point for molecular investigations into the diversity of zooxanthellae has traditionally been to utilise restriction fragment length polymorphism (RFLP) in nuclear genes encoding ribosomal RNA (rRNA) (Rowan & Powers 1991a). Restriction enzyme analysis involves firstly amplifying a gene of interest by polymerase chain reaction (PCR). When the gene under consideration is known or predicted to vary in its restriction enzyme motif between different zooxanthellae, then digesting the PCR products with the appropriate restriction enzyme would generate differentially sized fragments. The migratory pattern of these fragments observed on an agarose gel during electrophoresis (RFLP profile) identifies the zooxanthella(e) being studied. This enables the researcher to construct an overview of the main *Symbiodinium* lineages present in

samples being analysed. The major limitations of this method, however, are that it only identifies *Symbiodinium* with known or predicted RFLP profiles, and that it merely identifies a zooxanthella by the phylotype it belongs to but does not provide detailed phylogenetic resolution, i.e. uncover genetic differences between zooxanthellae at sites other than restriction enzyme motifs. Nonetheless, restriction enzyme analysis has proven to be a relatively inexpensive and efficient way to determine zooxanthellal phylotypes, especially when working with large sample sizes.

The vast majority of *Symbiodinium* phylogenies constructed to date have been based on sequences of nuclear-encoded rRNA genes. These have included sequences of small subunit (18S) (e.g. Carlos *et al.* 1999, Darius *et al.* 2000), partial large subunit (24S) (e.g. Loh *et al.* 2001, Pawlowski *et al.* 2001) and internal transcribed spacers (ITS 1 / ITS 2) and 5.8S regions (e.g. LaJeunesse *et al.* 2003) of rRNA genes. Of these, phylogenies constructed with partial 24S rRNA genes are the most comprehensive to date (Pawlowski *et al.* 2001, Pochon *et al.* 2001, Baker 2003). The phylogenies recovered with each of these datasets have been remarkably congruent, revealing seven distinct lineages (A-G) (Rowan & Powers 1991a, Carlos *et al.* 1999, LaJeunesse & Trench 2000, Pochon *et al.* 2001, Rodriguez-Lanetty 2003) that are often called clades (for monophyletic clade), or phylotypes as in this study. The DNA sequence variation encompassed by *Symbiodinium* is in excess of that separating many recognized species (and even genera and families) of free-living dinoflagellates (Rowan & Powers 1992). This has led to the consensus view that there are multiple species within each phylotype.

The major aim of phylogenetic studies on *Symbiodinium* has been to distinguish species based on sequences of individual genes. However, the topology of gene trees may differ from that of species trees owing to genetic polymorphism in the ancestral species (Gaur & Li 2000). In order to avoid errors of inference between gene trees and species trees, one needs to use a number of unlinked genes in the reconstruction of a phylogeny. Yet despite the abundance of phylogenetic studies on zooxanthellae, relatively few have employed a multiple marker approach, allowing for the direct comparison between trees or for composite phylogenies to be constructed. Fewer still have used molecular markers that are inherited independently of nuclear-encoded rRNA genes. A notable exception to this was the study by Santos *et al.* (2002), which made use of chloroplast-encoded partial large subunit rRNA genes to reconstruct the phylogeny of *Symbiodinium*. The topologies of trees from this study were strikingly similar to

phylogenetic studies on zooxanthellae.

Chloroplast genomes generally consist of a large circular DNA molecule on which the entire complement of between 100 and 200 genes is located (Sugiura 1992). Studies have recently brought to light the anomalous organisation of chloroplast genomes in several species of peridinin-containing dinoflagellates (Zhang *et al.* 1999, Barbrook & Howe 2000, Barbrook et al. 2001, Howe *et al.* 2003), including *Symbiodinium* (Takishita *et al.* 2003, Barbrook & Howe, personal communication). Several small (2-3 kbp) circular DNA molecules, called minicircles, that each carry one or two genes, have replaced the standard chloroplast genome in these species. Minicircles also contain a 'core' region whose sequence is highly conserved between minicircles of a given species carrying different genes (Howe *et al.* 2003), but very different between even closely related species. Thus far, only a handful of genes have been identified on these minicircles (Zhang *et al.* 1999, Barbrook & Howe 2000, Barbrook et al. 2001, Howe *et al.* 2003). These include the genes for the small and large subunits of ribosomal RNA and the gene encoding the D1 protein of photosystem II (PSII), called *psb*A. The latter, *psb*A, is a particularly well-suited candidate for phylogenetic studies on zooxanthellae on at least three counts:

1. *Psb*A is carried on the chloroplast. This allows for an independent test of evolutionary relationships inferred from sequences of nuclear-encoded rRNA genes.
2. Sequence data from the highly conserved core regions of chloroplast minicircles may prove essential in unravelling species boundaries in the genus *Symbiodinium*.
3. Ribosomal RNA genes are under strict purifying selection and as such must abide by very specific structural and functional constraints. Variation in these genes is not predicted to have a direct impact on the zoooxanthellal phenotype. The D1 protein has previously been implicated in susceptibility to bleaching during periods of elevated temperatures (Warner et al. 1999). Variation in *psb*A may potentially provide us with information relating to variation in susceptibility to bleaching in *Symbiodinium*.

A study on the functional diversity of zooxanthellae is beyond the scope of the current study. However, as results from parallel studies on functional differences between zooxanthellae continue to unfold, the implications for a particular host of housing particular zooxanthellae under defined environmental conditions will become clearer. An accurate and comprehensive picture of zooxanthellal diversity in a given location may prove crucial in predicting the responses of zooxanthellate symbioses to predicted changes in climate. For instance, the study of Rowan *et al.* (1997) strongly indicated genetic variation in susceptibility of *Symbiodinium* to coral bleaching. The work undertaken for this chapter was aimed at investigating the diversity of zooxanthellae in corals from Kenya and in sea anemone from the European coast of the Mediterranean Sea. This is currently unknown for both areas.

The specific objectives of this study were.

1. To identify the phylotypes of zooxanthellae in samples of corals from Kenya (1-5°S) and sea anemone from the Mediterranean Sea (35-43°N) by PCR-RFLP of nuclear-encoded 18S and 24S rRNA genes.
2. To sequence nuclear-encoded partial 24S rRNA genes of the zoooxanthellae identified by PCR-RFLP above (objective 1). Additionally, to compare these with sequences from Genbank to determine the phylogenetic relationship of zooxanthellae from Kenya and the Mediterranean to that elsewhere in the world.
3. To construct phylogenies with sequences of chloroplast-encoded *psb*A from samples of zooxanthellae for which 24S rRNA gene sequences were known (corresponding samples from objective 2) In so doing, to independently test established phylogenies and to assess the suitability of *psb*A for phylogenetic studies on zooxanthellae.

3.2 Results

3.2.1 PCR Amplification of *Symbiodinium* rRNA Genes

3.2.1.1 Amplification of 18S rRNA Genes

Small subunit (18S) ribosomal RNA gene PCR was carried out with genomic DNA as template using the zooxanthellal-specific primers ss3z and ss5z (Rowan & Powers 1991a). A single product of approximately 1600 bp was amplified as shown in Figure 3.1. No additional PCR products were observed.

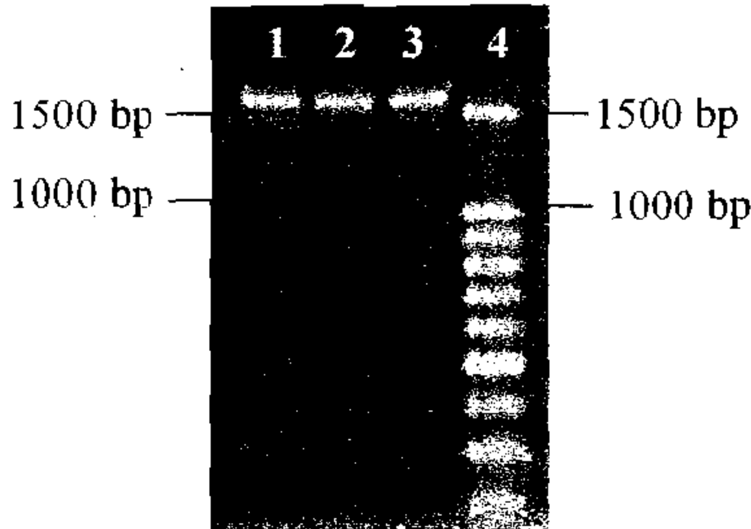

Figure 3.1: Zooxanthellal 18S rRNA gene PCR products using the primers ss3z and ss5z. Lanes 1-3 contain PCR products of approximately 1600 bp length from the Kenyan corals *Acropora valida*, Mombasa, *Acropora palifera*, Kisite and *Porites cylindrica*, Kanamai, respectively. The DNA ladder is in lane 4.

3.2.1.2 Amplification of 24S rRNA Genes

The primers 24D15F1 and 24D223R1 (Baker *et al.* 1997b) were used to amplify large subunit (24S) ribosomal RNA genes, with genomic DNA as template. These primers are designed to amplify two of the hypervariable regions of 24S rRNA gene (D1 and D2) and the conserved core region between them. Zooxanthellal PCR products, which sometimes varied markedly in intensity as shown in Figure 3.2, were approximately 650 bp in length. In many instances (>50%), particularly with coral samples, an additional band that corresponds with the host 24S rRNA gene was observed at approximately 850 bp. No other products were produced.

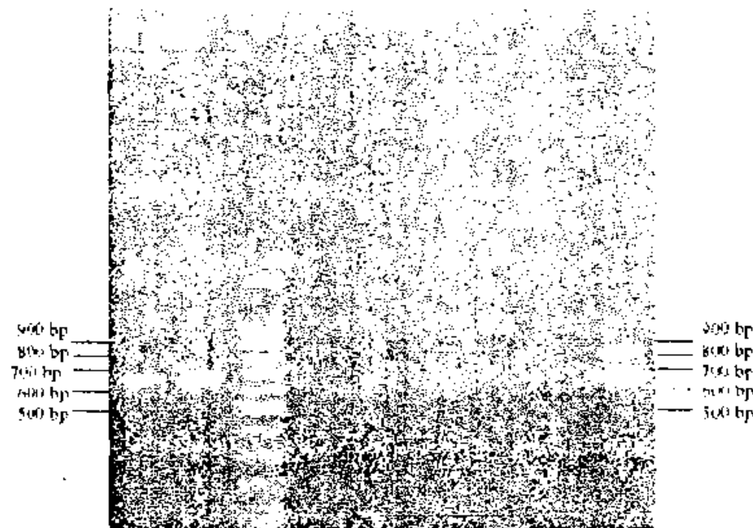

Figure 3.2: Zooxanthellal 24S rRNA gene PCR products from samples of Kenyan corals using primers 24D15F1 and 24D223R1 Zooxanthellal products are approximately 650 bp in length. Lane 9 shows an additional band at approximately 850 bp corresponding with host 24S rDNA PCR product. Lane 1: *Coscinarea mcneilli*, Kiunga, lane 2: *Porites cylindrica*, Kanamai, lane 4: *Acropora hyacinthus*, Kiunga (weak products at 650 bp and 850 bp, not visible in the image above), lane 5: *Acropora hyacinthus*, Malindi (weak product at 650 bp), lane 6: *Acropora palifera*, Diani II, lane 7: *Acropora palifera*, Kisite (weak product at 650 bp), lane 8: *Acropora hyacinthus*, Diani I (no visible product), lane 9: *Galaxea fasicularis* Mombasa. The DNA ladder (1500 bp & 1000 to 100 bp in 100 bp units) is in lane 3.

3.2.2 PCR-RFLP of *Symbiodinium* rRNA Gene Fragments

Zooxanthellae from Kenyan corals were assigned to one or more phylotypes by 18S PCR-RFLP (enzymes *Taq*I and *Dpn*II) or, in the event of failure to amplify 18S rRNA genes (see section 2.1.4.1; chapter 2), by 24S PCR-RFLP (enzyme *HpyCh4*IV). The zooxanthellae from Mediterranean anemones were first identified by 18S PCR-RFLP (enzymes *Taq*I and *Dpn*II), followed by 24S PCR-RFLP (enzyme *Dde*I) to diseriminate between temperate A and standard A zooxanthellae.

3.2.2.1 PCR-RFLP of 18S rRNA Genes

The banding patterns produced by restriction analysis of PCR-amplified 18S rRNA genes from *Symbiodinium* in Kenyan corals revealed algae belonging to the previously described phylotypes A, C (Rowan & Powers 1991a) and D (Toller *et al.* 2001a). Diagnostic banding profiles for 18S PCR-RFLP are shown in Figure 3.3.

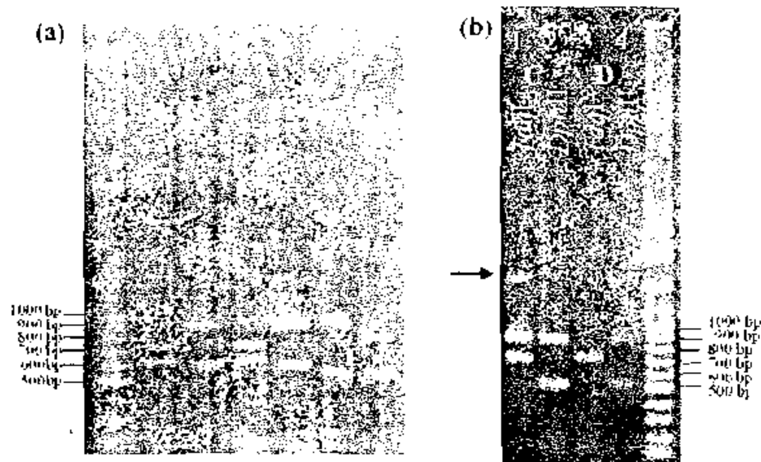

Figure 3.3: Diagnostic-banding patterns for different phylotypes of *Symbiodinium* produced by the digestion of PCR-amplified 18S rRNA gene products with the restriction enzymes *Taq*I and *Dpn*II. **(a)** Temperate A (denoted A') zooxanthellae from *Anemonia sulcata*, France (lanes 2 and 3), standard A zooxanthellae from *Acropora valida*, Mombasa-Kenya (lanes 4 and 5) and phylotype B zooxanthellae from *Bunodeopsis strumosa*, France (lanes 6 and 7). Temperate A and standard A zooxanthellae have indistinguishable 18S PCR-RFLP banding patterns. The DNA ladder is in lane 1. **(b)** Phylotype C zooxanthellae from *Porites cylindrica*, Kanamai-Kenya (lanes 1 and 2) and phylotype D zooxanthellae from *Galaxea fasicularis*, Kiunga-Kenya (lanes 3 and 4). The PCR products in lane 1 were only partially digested and the arrow points towards a band of undigested 18S rDNA PCR product. The DNA ladder is in lane 5.

Two of 4 samples of algae from *Acropora valida*, Mombasa, housed mixed infections with phylotypes A and C as shown in Figure 3.4a. The *Dpn*II enzyme digestion of 18S rRNA genes from each sample of algae from *Pocillopora damicornis*, shown in Figure 3.4b, produced four bands- two of which are diagnostic of phylotype C zooxanthellae, and two additional bands at approximately 550 bp and 650 bp. Restriction analysis of PCR-amplified fragments of cloned *Symbiodinium* rRNA genes from *P. damicornis*, shown in Figure 3.4c, confirmed this previously undescribed banding pattern.

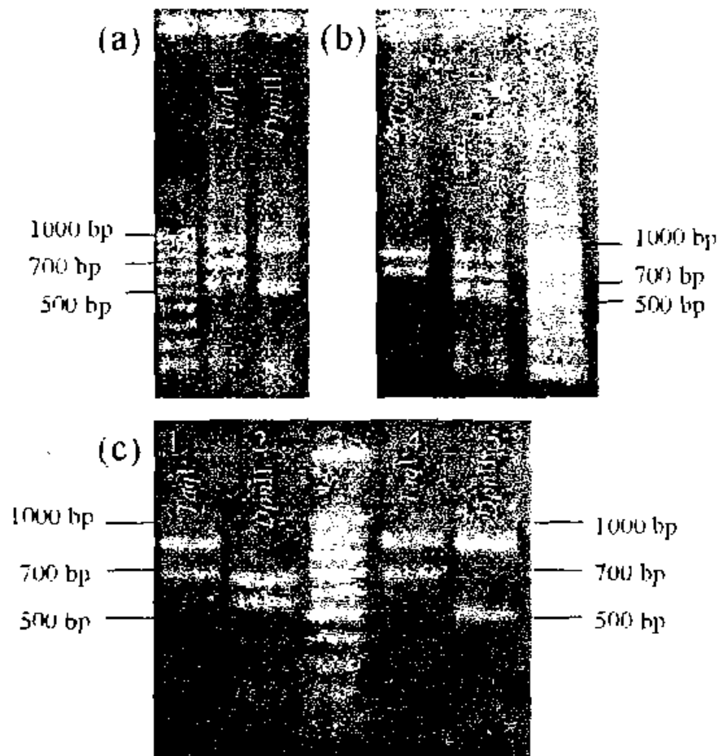

Figure 3.4: Banding patterns produced by restriction analysis (enzymes *Taq*I and *Dpn*II) of PCR-amplified 18S rRNA gene products from zooxanthellae in corals from Kenya that housed mixed infections with: **(a)** Phylotypes A and C (lanes 2 and 3; *Acropora valida*, Mombasa). The DNA ladder is in lane 1. **(b)** Phylotypes C and an unidentified 18S rDNA PCR amplicon (lanes 1 and 2: *Pocillopora damicornis*, Mombasa). Lane 3 carries the marker. **(c)** RFLP banding profiles produced by *Taq*I and *Dpn*II digestion of PCR-amplified fragments from two 18S rRNA gene clones from *Symbiodinium* in *Pocillopora damicornis*, Mombasa (clone 1: lanes 1 and 2, clone 2: lanes 4 and 5). The DNA marker is in lane 3.

With the exception of the algae hosted by *Bunodeopsis strumosa*, France, restriction analysis of PCR-amplified *Symbiodinium* 18S rRNA genes from Mediterranean hosts produced banding patterns indicative of infection with phylotype A zooxanthellae. The algae hosted by *B. strumosa* belonged to phylotype B (Figure 3.3a, lanes 6 and 7).

3.2.2.2 PCR-RFLP of 24S rRNA Genes

Restriction analysis (with enzyme *HpyCh4*IV) of PCR-amplified *Symbiodinium* 24S rRNA genes from Kenyan corals produced bands indicative of algae belonging either to phylotype C or phylotype D, with no mixed infections. Diagnostic banding patterns for 24S PCR-RFLP are shown in Figure 3.5

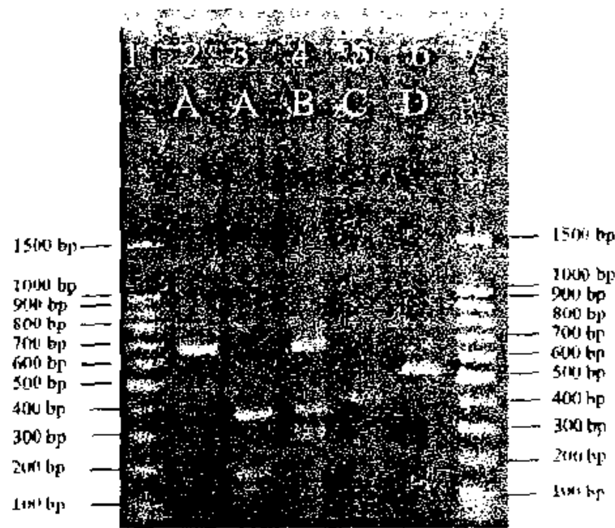

Figure 3.5: PCR-RFLP banding patterns for different phylotypes of *Symbiodinium* produced by the digestion of PCR-amplified zooxanthellal 24S rRNA genes with the enzyme *HpyCh4*IV. Lane 2: *temperate* A zooxanthellae from *Anemonia sulcata*, France (denoted A'), lane 3: *standard* A zooxanthellae from *Acropora valida*, Mombasa-Kenya, lane 4: phylotype B zooxanthellae from *Bunodeopsis strumosa*, France (note diagnostic band at approximately 650 bp plus two additional bands at 350 bp and 300 bp produced by the digestion of an unidentified 24S rDNA PCR amplicon), lane 5: phylotype C zooxanthellae from *Porites cylindrica*, Kanamai- Kenya, lane 6: phylotype D zooxanthellae from *Galaxea fasicularis*, Kiunga- Kenya The DNA markers are in lanes 1 and 7. Temperate A and phylotype B zooxanthellae have indistinguishable 24S PCR-RFLP banding patterns.

With the exception of *B. strumosa*, restriction analysis of PCR-amplified *Symbiodinium* 18S rRNA genes from all Mediterranean samples indicated infection with phylotype A zooxanthellae. As shown in Figure 3.3, the 18S rRNA gene PCR-RFLP utilised in this study fails to distinguish between standard A and temperate A zooxanthellae. A 24S rRNA gene PCR-RFLP assay (with the enzyme *Dde*I) was therefore employed to discriminate between standard and temperate A (Savage *et al.* 2002). A diagnostic 24S rRNA gene PCR-RFLP gel is shown in Figure 3.6. Results revealed that with the exception of *B. strumosa*, France, all Mediterranean anemones sampled for this study housed temperate A zooxanthellae. No further studies were undertaken to elucidate the unidentified 24S rRNA PCR product from *B. strumosa*, and this is not considered any further.

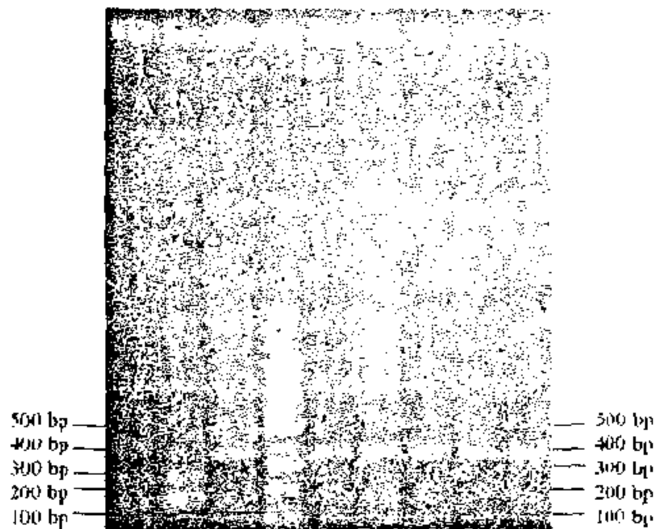

Figure 3.6: Diagnostic-PCR-RFLP to discriminate between *temperate* A and standard A zooxanthellae. Zooxanthellal 24S rRNA gene PCR products from Mediterranean *Anemonia* spp. were digested with the restriction enzyme *Dde*I. Lanes 1, 2 and 3 are positive controls for standard A (lanes 1 and 2: *Acropora valida*, Mombasa- Kenya) and *temperate* A (lane 3: *Anemonia sulcata*, France) zooxanthellae. Lane 5: *Anemonia rustica*, Italy, lane 6: *Anemonia sulcata* var. *rufescens*, Italy, lane 7: *Anemonia sulcata* var. *smaragdina*, France, lane 8: *Anemonia sulcata* var. *viridis*, France, lane 9: *Anemonia sulcata* var *vulgaris*, Italy. The DNA ladder is in lane 4.

3.2.2.3 Summary of PCR-RFLP Results

The overall results of restriction analysis on rRNA genes from zooxanthellae in Kenya and in the Mediterranean Sea are outlined in Table 3.1. Kenyan corals housed algae from the previously described phylotypes A, C and D. Two of four samples of *Acropora valida* had mixed infections with algae belonging to phylotypes A and C. Restriction analysis of *Symbiodinium* 18S rRNA genes in *Pocillopora damicornis* suggested two PCR amplification products, one of which was characteristic of phylotype C zooxanthellae, and one of which was previously uncharacterised. All remaining species of Kenyan corals housed monomorphic (single phylotype) infections throughout the range from which they were sampled, with the exception of *Acropora hyacinthus* which hosted phylotype D zooxanthellae in Kiunga, and phylotype C elsewhere. The dominant algal phylotype in Kenya is C, occurring in five of the seven coral species studied.

Temperate A zooxanthellae are the dominant phylotype in samples from the Mediterranean, housed by nine of ten species studied. The symbionts in *Bunodeopsis strumosa*, France are phylotype B zooxanthellae.

Table 3.1: Phylotypes of zooxanthellae from locations in **(a)** the Mediterranean and **(b)** Kenya, as identified by restriction analysis of rRNA genes. Zooxanthellae from the Mediterranean were identified by 18S PCR-RFLP, followed by 24S PCR-RFLP and those in Kenyan corals were identified by 24S PCR-RFLP, or, as indicated in the table, by 18S PCR-RFLP. Numbers are the number of samples identified, and letters denote phylotype designations. Temperate A zooxanthellae are denoted as A'. The '?' specifies an unidentified rRNA gene PCR amplicon.

a) MEDITERRANEAN		Location (Country)					
Species	Italy	France	Spain	Germany			
Aiptasia diaphana		1 A'					
Anemonia rustica	3 A'						
Anemonia sulcata	3 A'	10 A'		1 A'			
Balanophyllia europaea			1 A'				
Bunodeopsis strumosa		1 B					
Caryophyllia smithi		1 A'					
Cereus pedunculatus		2 A'	1 A'				
Cladocora cespitosa		1 A'					
Cotylorhiza tuberculata		1 A'					
Cribinopsis crassa		1 A'					
b) KENYA				Location (Site)			
	Kiunga	Malindi	Kanamai	Mombasa	Diani I	Diani II	Kisite
Acropora hyacinthus	2 D	1 C					1 C
Acropora palifera						4 D [1]	3 D [1]
Acropora valida				2A [2], 2 A + C [2]			
Coscinarea mcneilli	3 C	3 C		3 C	1 C		
Galaxea fascicularis	4 D	2 D		2 D	2 D		3 D [1]
Pocillopora damicornis		4 C + ? [3]		4 C + ? [3]			
Porites cylindrica			4 C [2]				

[1] one sample by 18S PCR-RFLP

[2] two samples by 18S PCR-RFLP

[3] four samples by 18S PCR-RFLP

3.2.3 Sequence Data

A total of 15 Mediterranean sequences comprising 9 haplotypes, and 36 Kenyan sequences comprising 16 haplotypes were processed. A haplotype is defined here as a unique string of nucleotides in a DNA sequence that can be distinguished from all other haplotypes. Multiple sequences with the same haplotype were truncated to the shortest for phylogenetic reconstruction. Aligned sequences are shown in Figure 3.7, coded M1-M15 and K1-K36 respectively for ease of referral. The haplotype of each sequence is outlined in Table 3.2 along with the phylotype to which it belongs, as predicted by restriction analysis. Sequences ranged from 549 bp (sequence M1: *Cribinopsis crassa*, France) to 648 bp (sequence K28: *Pocillopora damicornis*, Mombasa). Cloned sequences were typically 646 bp or 647 bp. All sequences were first run through BLAST searches (Altschul *et al.* 1990) to check for closely related sequences in Genbank. In each case, the nearest matches in Genbank were *Symbiodinium* 24S rRNA gene sequences, confirming that sequences from this study were those of the symbiont. The base composition (G + C content) of sequences varied between 47.8% (sequence M10: *Anemonia sulcata* var. *viridis*, France; M13: *Anemonia rustica*, France) and 50.6% (sequence K3: *Galaxea fascicularis*, Mombasa; K7: *Galaxea fascicularis*, Malindi), which is within the range reported for dinoflagellate 24S rRNA genes (Lenaers *et al.* 1989).

Table 3.2: A summary of sequence haplotypes. Phylotypes are as predicted by PCR-RFLP of 24S rRNA genes. The '?' denotes a novel PCR-RFLP profile.

Host species	Site of origin	Sequence code	Haplotype	Phylotype
Mediterranean				
Aiptasia diaphana	France	M2, M3	1	A'
Anemonia rustica	France	M13	7	A'
Anemonia sulcata var *rufescens*	Italy	M11	5	A'
Anemonia sulcata var *smaragdina*	France	M12	6	A'
Anemonia sulcata var *viridis*	France	M10	4	A'
Anemonia sulcata var *vulgaris*	Italy	M14	8	A'
Balanophyllia europaea	Spain	M5	2	A'
Bunodeopsis strumosa	France	M15	9	B
Caryophyllia smithi	France	M6	2	A'
Cereus pedunculatus	France	M4	1	A'
Cereus pedunculatus	France	M8	2	A'
Cereus pedunculatus	Spain	M9	3	A'
Cladocora cespitosa	France	M7	2	A'
Cribinopsis crassa	France	M1	1	A'
Kenyan				
Acropora hyacinthus	Kisite	K25	14	C
Acropora palifera	Kisite	K6, K10	11	D
	Diani II	K8	11	D
Acropora valida	Mombasa	K1	10	A
		K24	13	C
		K26	15	C
Coscinarea monilli	Kiunga	K20, K23	13	C
	Malindi	K17, K18	13	C
	Mombasa	K19, K21	13	C
	Diani I	K22	13	C
Galaxea fascicularis	Kiunga	K11, K14	11	D
	Malindi	K7, K9	11	D
	Mombasa	K2, K3	11	D
	Diani I	K4, K12	11	D
	Kisite	K5, K13	11	D
Pocillopora damicornis	Malindi	K29	18	?
		K32	21	?
		K34	23	?
		K35	24	C
		K36	25	C
	Mombasa	K27	16	?
		K28	17	?
		K30	19	?
		K31	20	?
		K33	22	?
Porites cylindrica	Kanamai	K15, K16	12	C

D1 Start

```
                 *        20         *        40         *        60         *        80
M1  : ??????????????????????????????????????CAGGATTCCCTTAGTAATGGCGAACGAACAGGGATCAGCTTGGGTTGGAAA :  51
M2  : ??????????????????????????????????????CAGGATTCCCTTAGTAATGGCGAACGAACAGGGATCAGCTTGGGTTGGAAA :  51
M3  : ???????????????????????????????????????????????????????GGCGAACGAACAGGGATCAGCTTGGGTTGGAAA :  33
M4  : ??????????TAAGTAAGCGGAGGAAAAGGAACTAAACAGGATTCCCTTAGTAATGGCGAACGAACAGGGATCAGCTTGGGTTGGAAA :  78
M5  : ??????????????????????????????????????CAGGATTCCCTTAGTAATGGCGAACGAACAGGGATCAGCTTGGGTTGGAAA :  51
M6  : ??????????????????????????????????????CAGGATTCCCTTAGTAATGGCGAACGAACAGGGATCAGCTTGGGTTGGAAA :  51
M7  : ??????????????????????????????????????CAGGATTCCCTTAGTAATGGCGAACGAACAGGGATCAGCTTGGGTTGGAAA :  51
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          *       100         *       120         *       140         *       160         *
M1  : CCAAGGCATCTGCCTTGGATTGTGGCCTTTAGACATTGTACCAACGGCGGCGCGAGCGCAAGCCTCTTGGAATAGAGCGTCTACCTGG : 139
M2  : CCAAGGCATCTGCCTTGGATTGTGGCCTTTAGACATTGTACCAACGGCGGCGCGAGCGTAAGCCTCTTGGAATAGAGCGTCTACCTGG : 139
M3  : CCAAGGCATCTGCCTTGGATTGTGGCCTTTAGACATTGTACCAACGGCGGCGCGAGCGTAAGCCTCTTGGAATAGAGCGTCTACCTGG : 121
M4  : CCAAGGCATCTGCCTTGGATTGTGGCCTTTAGACATTGTACCAACGGCGGCGCGAGCGYAAGCCTCTTGGAATAGAGCGTCTACCTGG : 166
M5  : CCAAGGCATCTGCCTTGGATTGTGGCCTTTAGACATTGTACCAACGGCGGCGCGAGCGCAAGCCTCTTGGAATAGAGCGTCTACCTGG : 139
M6  : CCAAGGCATCTGCCTTGGATTGTGGCCTTTAGACATTGTACCAACGGCGGCGCGAGCGCAAGCCTCTTGGAATAGAGCGTCTACCTGG : 139
M7  : CCAAGGCATCTGCCTTGGATTGTGGCCTTTAGACATTGTACCAACGGCGGCGCGAGCGCAAGCCTCTTGGAATAGAGCGTCTACCTGG : 139
M8  : CCAAGGCATCTGCCTTGGATTGTGGCCTTTAGACATTGTACCAACGGCGGCGCGAGCGCAAGCCTCTTGGAATAGAGCGTCTACCTGG : 139
M9  : CCAAGGCATCTGCCTTGGATTGTGGCCTTTAGACATTGTACCAACGGCGGCGCGAGCGTAAGCCTCTTGGAATAGAGCGTCTACCTGG : 139
M10 : CCAAGGCATCTGTCTTGGATTGTGGCCTTTAGACATTGTACCAACGGCGGCGCGAGCGTAAGCCTCTTGGAATAGAGCGTCTACCTGG : 175
M11 : CCAAGGCATCTGCCTTGGATTGTGGCCTTTAGACATTGTACCAACGGCGGCGCGAGCGCAAGCCTCTTGGAATAGAGCGTCTACCTGC : 175
M12 : CCAAGGCATCTGCCTTGGATTGTGGCCTTTAGACATTGTACCAACGGCGGCGCGAGCGTAAGCCTCTTGGAATAGAGCGTCTACCTGG : 175
M13 : CCAAGGCATCTGCCTTGGATTGTGGTCTTTAGACATTGTACCAACGGCGGCGCGAGCGTAAGCCTCTTGGAATAGAGCGTCTACCTGG : 175
M14 : CCAAGGCATCTGCCTTGGATTGTGGCCTTTAGACATTGCACCAACGGCGGCGCGAGCGTAAGCCTCTTGGAATAGAGCGTCTACCTGG : 175
M15 : CCGATACATCTGTGTCGGGCTGTAGCCTGCAGGCATAGCGCTATCGGCGGCTCGAGCGCAAGCCTCTTGGAATAGAGCGTGTGCCCGG : 165
K1  : CCGGAGCTTCTGCCTTGGATTGTGGCCTTTAGACATACTACCAACGGCGGCGCGAGCGTAAGCCTCTTGGAACAGAGCGTCTACGTGG : 175
K2  : CCAGGGCCCTGGTCCTGGGTTGTAGCCTGCAGACGTAGCGCTATCGGCGGCTTGAGCGAAAGCCTCTTGGAACAGAGCGTGTGCCCAG : 167
K3  : CCAGGGCCCTGGTCCTGGGTTGTAGCCTGCAGACGTAGCGCTATCGGCGGCTTGAGCGAAAGCCTCTTGGAACAGAGCGTGTGCCCAG : 166
K4  : CCAGGGCCCTGGTCCTGGGTTGTAGCCTGCAGACGTAGCGCTATCGGCGGCTTGAGCGAAAGCCTCTTGGAACAGAGCGTGTGCCCAG : 175
K5  : CCAGGGCCCTGGTCCTGGGTTGTAGCCTGCAGACGTAGCGCTATCGGCGGCTTGAGCGAAAGCCTCTTGGAACAGAGCGTGTGCCCAG : 175
K6  : CCAGGGCCCTGGTCCTGGGTTGTAGCCTGCAGACGTAGCGCTATCGGCGGCTTGAGCGAAAGCCTCTTGGAACAGAGCGTGTGCCCAG : 169
K7  : CCAGGGCCCTGGTCCTGGGTTGTAGCCTGCAGACGTAGCGCTATCGGCGGCTTGAGCGAAAGCCTCTTGGAACAGAGCGTGTGCCCAG : 166
K8  : CCAGGGCCCTGGTCCTGGGTTGTAGCCTGCAGACGTAGCGCTATCGGCGGCTTGAGCGAAAGCCTCTTGGAACAGAGCGTGTGCCCAG : 169
K9  : CCAGGGCCCTGGTCCTGGGTTGTAGCCTGCAGACGTAGCGCTATCGGCGGCTTGAGCGAAAGCCTCTTGGAACAGAGCGTGTGCCCAG : 176
K10 : CCAGGGCCCTGGTCCTGGGTTGTAGCCTGCAGACGTAGCGCTATCGGCGGCTTGAGCGAAAGCCTCTTGGAACAGAGCGTGTGCCCAG : 175
K11 : CCAGGGCCCTGGTCCTGGGTTGTAGCCTGCAGACGTAGCGCTATCGGCGGCTTGAGCGAAAGCCTCTTGGAACAGAGCGTGTGCCCAG : 175
K12 : CCAGGGCCCTGGTCCTGGGTTGTAGCCTGCAGACGTAGCGCTATCGGCGGCTTGAGCGAAAGCCTCTTGGAACAGAGCGTGTGCCCAG : 175
K13 : CCAGGGCCCTGGTCCTGGGTTGTAGCCTGCAGACGTAGCGCTATCGGCGGCTTGAGCGAAAGCCTCTTGGAACAGAGCGTGTGCCCAG : 175
K14 : CCAGGGCCCTGGTCCTGGGTTGTAGCCTGCAGACGTAGCGCTATCGGCGGCTTGAGCGAAAGCCTCTTGGAACAGAGCGTGTGCCCAG : 175
K15 : CTGAAGCTTCGGTTTCGGGATGTAGCCTGCAGGCATAGTGCTATCGGCGGCTCGAGCGTAAGCCTCTTGGAACAGAGCGTGTGCCCGG : 175
K16 : CTGAAGCTTCGGTTTCGGGATGTAGCCTGCAGGCATAGTGCTATCGGCGGCTCGAGCGTAAGCCTCTTGGAACAGAGCGTGTGCCCGG : 175
K17 : CTGAAGCTTCGGTTTYGGGATGTAGCCTGCAGGCATAGTGCTATCGGCGGCTCGAGCGTAAGCCTCTTGGAATAGAGCGTGTGCCCGG : 175
K18 : CTGAAGCTTCGGTTTYGGGATGTAGCCTGCAGGCATAGTGCTATCGGCGGCTCGAGCGTAAGCCTCTTGGAATAGAGCGTGTGCCCGG : 166
K19 : CTGAAGCTTCGGTTTCGGGATGTAGCCTGCAGGCATAGTGCTATCGGCGGCTCGAGCGTAAGCCTCTTGGAATAGAGCGTGTGCCCGG : 166
K20 : CTGAAGCTTCGGTTTCGGGATGTAGCCTGCAGGCATAGTGCTATCGGCGGCTCGAGCGTAAGCCTCTTGGAATAGAGCGTGTGCCCGG : 164
K21 : CTGAAGCTTCGGTTTCGGGATGTAGCCTGCAGGCATAGTGCTATCGGCGGCTCGAGCGTAAGCCTCTTGGAATAGAGCGTGTGCCCGG : 175
K22 : CTGAAGCTTCGGTTTCGGGATGTAGCCTGCAGGCATAGTGCTATCGGCGGCTCGAGCGTAAGCCTCTTGGAATAGAGCGTGTGCCCGG : 175
K23 : CTGAAGCTTCGGTTTCGGGATGTAGCCTGCAGGCATAGTGCTATCGGCGGCTCGAGCGTAAGCCTCTTGGAATAGAGCGTGTGCCCGG : 175
K24 : CTGAAGCTTCGGTTTCGGGATGTAGCCTGCAGGCATAGTGCTATCGGCGGCTCGAGCGTAAGCCTCTTGGAATAGAGCGTGTGCCCGG : 163
K25 : CTGAAGCTTCGGTTTCGGGATGTAGCCTGCAGGCATAGTGCTATCGGCGGCTCGAGCGTAAGCCTCTTGGAATAGAGCGTGTGCCCGG : 175
K26 : CTGAAGCTTCGGTTTCGGGATGTAGCCTGCAGGCATAGTGCTATCGGCGGCTCGAGCGTAAGCCTCTTGGAATAGAGCGTGTGCCCGG : 166
K27 : CTGAAGCTTCGGTTTCGGGATGTAGCCTGCAGGCATAGTGCTATCGGCGGCTCGAGCGTAAGCCTCTTGGAATAGAGCGTGTGCCCGG : 175
K28 : CTGAAGCTTCGGTTTCGGGATGTAGCCTGCAGGCATAGTGCTATCGGCGGCTCGAGCGTAAGCCTCTTGGGATAGAGCGTGTGCCCGG : 175
K29 : CTGAAGCTTCGGTTTCGGGATGTAGCCTGCAGGCATAGTGCTATCGGCGGCTCGAGCGTAAGCCTCTTGGAATAGAGCGTGTGCCCGG : 175
K30 : CTGAAGCTTCGGTTTCGGGATGTAGCCTGCAGGCATAGTGCTATCGGCGGCTCGAGCGTAAGCCTCTTGGAACAGAGCGTGTGCCCGG : 175
K31 : TTGAAGCTTCGGTTTCGGGATGTAGCCTGCAGGCATAGTGCTATCGGCGGCTCGAGCGTAAGCCTCTTGGAATAGAGCGTGTGCCCGG : 175
K32 : CTGAAGCTTCGGTTTCGGGATGTAGCCTGCAGGCATAGTGCTATCGGCGGCTCGAGCGTAAGCCTCTTGGAATAGAGCATGTGCCCGG : 175
K33 : CTGAAGCTTCGGTTTCGGGATGTAGCCTGCAGGCATAGTGCTATCGGCGGCTCGAGCGTAAGCCTCTTGGAATAGAGCGTGTGCCCGG : 175
K34 : TTGAAGCTTCGGTTTCGGGATGTAGCCTGCAGGCATAGTGCTATCGGCGGCTCGAGCGTAAGCCTCTTGGAATAGAGCGTGTGCCCGG : 175
K35 : CTGAGGCTTCGGTTTCGGGATGTAGCCTGCAGGCATAGTGCTATCGGCGGCTCGAGCGTAAGCCTCTTGGAATAGAGCGTGTGCCCGG : 175
K36 : CTGAAGCTTCGGTTTCGGGATGTAGCCTGCAGGCATAGTGCTATCGGCGGCTCGAGCGTAAGCCTCTTGGAATAGGGCGTGTGCCCGG : 179
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Figure 3.7: continued on next page

D1 End ↓

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          180         *         200         *         220         *         240         *         260
M1  : GTGAGAATCCTGTGTTTCGCTTGATGTCCGCCGTGCACGGTACTTGCTCACAGAGTCACGCTCCTCGGAATTGGAGCGTAAATTGGGT : 227
M2  : GTGAGAATCCTGTGTTTCGCTTGATGTCCGCCGTGCACGGTACTTGCTCACAGAGTCACGCTCCTCGGAATTGGAGCGTAAATTGGGT : 227
M3  : GTGAGAATCCTGTGTTTCGCTTGATGTCCGCCGTGCACGGTACTTGCTCACAGAGTCACGCTCCTCGGAATTGGAGCGTAAATTGGGT : 209
M4  : GTGAGAATCCTGTGTTTCGCTTGATGTCCGCCGTGCACGGTACTTGCTCACAGAGTCACGCTCCTCGGAATTGGAGCGTAAATTGGGT : 255
M5  : GTGAGAATCCTGTGTTTCGCTTGATGTCCGCCGTGCACGGTACTTGCTCACAGAGTCACGCTCCTCGGAATTGGAGCGTAAATTGGGT : 227
M6  : GTGAGAATCCTGTGTTTCGCTTGATGTCCGCCGTGCACGGTACTTGCTCACAGAGTCACGCTCCTCGGAATTGGAGCGTAAATTGGGT : 227
M7  : GTGAGAATCCTGTGTTTCGCTTGATGTCCGCCGTGCACGGTACTTGCTCACAGAGTCACGCTCCTCGGAATTGGAGCGTAAATTGGGT : 227
M8  : GTGAGAATCCTGTGTTTCGCTTGATGTCCGCCGTGCACGGTACTTGCTCACAGAGTCACGCTCCTCGGAATTGGAGCGTAAATTGGGT : 227
M9  : GTGAGAATCCTGTGTTTCGCTTGATGTCCGCCGTGCACGGTACTTGCTCACAGAGTCACGCTCCTCGGAATTGGAGCGTAAATTGGGT : 227
M10 : GTGAGAATCCTGTGTTTCGCTTGATGTCCGCCGTGCACGGTACTTGCTCACAGAGTCACGCTCCTCGGAATTGGAGCGTAAATTGGGT : 263
M11 : GTGAGAATCCTGTGTTTCGCTTGATGTCCGCCGTGCACGGTACTTGCTCACAGAGTCACGCTCCTCGGAATTGGAGCGTAAATTGGGT : 263
M12 : GTGAGAATCCTGTGTTTCGCTTGATGTCCGCCGTGCACGGTACTTGCTCACAGAGTCACGCTCCTCGGAATTGGAGCGTAAATTGGGT : 263
M13 : GTGAGAATCCTGTGTTTCGCTTGATGTCCGCCGTGCACGGTACTTGCTCACAGAGTCACGCTCCTCGGAATTGGAGCGTAAATTGGGT : 263
M14 : GTGAGAATCCTGTGTTTCGCTTGATGTCCGCCGTGCACGGTACTTGCTCACAGAGTCACGCTCCTCGGAATTGGAGCGTAAATTGGGT : 263
M15 : GTGAGAATCCTGCATTTCGCTCGATGTCCGCTGTCCACAGTGCTTGTTCTGAGAGTCACGCTCCTCGGAATTGGAGCGTAAATCAGGT : 253
K1  : GTGAGAATCCTGTGTTTCGCTTGATGCCCGCCGTGCACGGTACTTGCTCTCAGAGTCACGCTCCTCGGAATTGGAGCGTAAATTGGGT : 263
K2  : GTGAGAATCCTGTGTTTCGCTTGAAGTCCGCCGTGTACGGTGCTCGCTCTCAAAGTCACGCTCCTCGGAATTGGAGCGTAAATCAGGT : 255
K3  : GTGAGAATCCTGTGTTTCGCTTGAAGTCCGCCGTGTACGGTGCTCGCTCTCAAAGTCACGCTCCTCGGAATTGGAGCGTAAATCAGGT : 254
K4  : GTGAGAATCCTGTGTTTCGCTTGAAGTCCGCCGTGTACGGTGCTCGCTCTCAAAGTCACGCTCCTCGGAATTGGAGCGTAAATCAGGT : 263
K5  : GTGAGAATCCTGTGTTTCGCTTGAAGTCCGCCGTGTACGGTGCTCGCTCTCAAAGTCACGCTCCTCGGAATTGGAGCGTAAATCAGGT : 263
K6  : GTGAGAATCCTGTGTTTCGCTTGAAGTCCGCCGTGTACGGTGCTCGCTCTCAAAGTCACGCTCCTCGGAATTGGAGCGTAAATCAGGT : 257
K7  : GTGAGAATCCTGTGTTTCGCTTGAAGTCCGCCGTGTACGGTGCTCGCTCTCAAAGTCACGCTCCTCGGAATTGGAGCGTAAATCAGGT : 254
K8  : GTGAGAATCCTGTGTTTCGCTTGAAGTCCGCCGTGTACGGTGCTCGCTCTCAAAGTCACGCTCCTCGGAATTGGAGCGTAAATCAGGT : 257
K9  : GTGAGAATCCTGTGTTTCGCTTGAAGTCCGCCGTGTACGGTGCTCGCTCTCAAAGTCACGCTCCTCGGAATTGGAGCGTAAATCAGGT : 264
K10 : GTGAGAATCCTGTGTTTCGCTTGAAGTCCGCCGTGTACGGTGCTCGCTCTCAAAGTCACGCTCCTCGGAATTGGAGCGTAAATCAGGT : 263
K11 : GTGAGAATCCTGTGTTTCGCTTGAAGTCCGCCGTGTACGGTGCTCGCTCTCAAAGTCACGCTCCTCGGAATTGGAGCGTAAATCAGGT : 263
K12 : GTGAGAATCCTGTGTTTCGCTTGAAGTCCGCCGTGTACGGTGCTCGCTCTCAAAGTCACGCTCCTCGGAATTGGAGCGTAAATCAGGT : 263
K13 : GTGAGAATCCTGTGTTTCGCTTGAAGTCCGCCGTGTACGGTGCTCGCTCTCAAAGTCACGCTCCTCGGAATTGGAGCGTAAATCAGGT : 263
K14 : GTGAGAATCCTGTGTTTCGCTTGAAGTCCGCCGTGTACGGTGCTCGCTCTCAAAGTCACGCTCCTCGGAATTGGAGCGTAAATCAGGT : 263
K15 : GTGAGAATCCTGTGTTTCGCTTGATGTCCGCTGTCCACAGCACTTGTTCTCAGAGTCACGCTCCTCGGAATTGGAGCGTAAATAAGGT : 263
K16 : GTGAGAATCCTGTGTTTCGCTTGATGTCCGCTGTCCACAGCACTTGTTCTCAGAGTCACGCTCCTCGGAATTGGAGCGTAAATAAGGT : 263
K17 : GTGAGAATCCTGTGTTTCGCTTGATGTCCGCTGTCCACAGCACTTGTTCTCAGAGTCACGCTCCTCGGAATTGGAGCGTAAATAAGGT : 263
K18 : GTGAGAATCCTGTGTTTCGCTTGATGTCCGCTGTCCACAGCACTTGTTCTCAGAGTCACGCTCCTCGGAATTGGAGCGTAAATAAGGT : 254
K19 : GTGAGAATCCTGTGTTTCGCTTGATGTCCGCTGTCCACAGCACTTGTTCTCAGAGTCACGCTCCTCGGAATTGGAGCGTAAATAAGGT : 254
K20 : GTGAGAATCCTGTGTTTCGCTTGATGTCCGCTGTCCACAGCACTTGTTCTCAGAGTCACGCTCCTCGGAATTGGAGCGTAAATAAGGT : 252
K21 : GTGAGAATCCTGTGTTTCGCTTGATGTCCGCTGTCCACAGCACTTGTTCTCAGAGTCACGCTCCTCGGAATTGGAGCGTAAATAAGGT : 263
K22 : GTGAGAATCCTGTGTTTCGCTTGATGTCCGCTGTCCACAGCACTTGTTCTCAGAGTCACGCTCCTCGGAATTGGAGCGTAAATAAGGT : 263
K23 : GTGAGAATCCTGTGTTTCGCTTGATGTCCGCTGTCCACAGCACTTGTTCTCAGAGTCACGCTCCTCGGAATTGGAGCGTAAATAAGGT : 263
K24 : GTGAGAATCCTGTGTTTTGCTTGATGTCCGCTGTCCACAGCACTTGTTCTCAGAGTCACGCTCCTCGGAATTGGAGCGTAAATAAGGT : 251
K25 : GTGAGAATCCTGTGTTTCGCTTGATGTCCGCTGTCCACAGCACTTGTTCTCAGAGTCACGCTCCTCGGAATTGGAGCGTAAATAAGGT : 263
K26 : GTGAGAATCCTGTGTTTCGCTTGATGTCCGCTGTCCACAGCACTTGTTCTCAGAGTCACGCTCCTCGGAATTGGAGCGTAAATAAGGT : 254
K27 : GTGAGAATCCTGTGTTTCGCTTGATGTCCGCTGTCTACAGCACTTGTTCTCAGAGTCATGCTCCTCGGAATTGGAGCGTAAATAAGGT : 263
K28 : GTGAAAATCCTGTGTTTCGCTTGATGTCCGCTGTCTACAGCACTTGTTCTCAGAGTCATGCTCCTCGGAATTGGAGCGTAAATAAGGT : 263
K29 : GTGAGAATCCTGTGTTTCGCTTGATGTCCGCTGTCTACAGCACTTGTTCTCAGAGTCATGCTCCTCGGAATTGGAGCGTAAATAAGGT : 263
K30 : GTGAGAATCCTGTGTTTCGCTTGATGTCCGCTGTCCACAGCACTTGTTCTCAGAGTCACGCTCCTCGGAATTGGAGCGTAAATAAGGT : 263
K31 : GTGAGAATCCTGTGTTTCGCTTGATGTCCGCTGTCTACAGCACTTGTTCTCAGAGTCATGCTCCTCGGAATTGGAGCGTAAATAAGGT : 263
K32 : GTGAGAATCCTGTGTTTCGCTTGATGTCCGCTGTCTACAGCACTTGTTCTCAGAGTCATGCTCCTCGGAATTGGAGCGTAAATAAGGT : 263
K33 : GTGAGAATCCTGTGTTTCGCTTGATGTCCGCTGTCTACAGCACTTGTTCTCAGAGTCATGCTCCTCGGAATTGGAGCGTAAATAAGGT : 263
K34 : GTGAGAATCCTGTGTTTCGCTTGATGTCCGCTGTCTACAGCACTTGTTCTCAGAGTCCTGCTCCTCGGAATTGGAGCGTAAATAAGGT : 263
K35 : GTGAGAATCCTGTGTTTCGCTTGATGTCCGCTGTCCACAGCACTTGTTCTCAGAGTCACGCTCCTCGGAATTGGAGCGTAAATAAGGT : 263
K36 : GTGAGAATCCTGTGTTTCGCTTGATGTCCGCTGTCCACAGCACTTGTTCTCAGAGTCACGCTCCTCGGAATTGGAGCATAAATAAGGT : 263

              *         280         *         300         *         320         *         340         *
M1  : GGTAAATTTCATCTAAAGCTAAATACAGGCTCGAGACCGATAGCAAACAAGTACCATGAGGGAAAGATGAAAAGGACTTTGAAAAGAG : 315
M2  : GGTAAATTTCATCTAAAGCTAAATACAGGCTCGAGACCGATAGCAAACAAGTACCATGAGGGAAAGATGAAAAGGACTTTGAAAAGAG : 315
M3  : GGTAAATTTCATCTAAAGCTAAATACAGGCTCGAGACCGATAGCAAACAAGTACCATGAGGGAAAGATGAAAAGGACTTTGAAAAGAG : 297
M4  : GGTAAATTTCATCTAAAGCTAAATACAGGCTCGAGACCGATAGCAAACAAGTACCATGAGGGAAAGATGAAAAGGACTTTGAAAAGAG : 342
M5  : GGTAAATTTCATCTAAAGCTAAATACAGGCTCGAGACCGATAGCAAACAAGTACCATGAGGGAAAGATGAAAAGGACTTTGAAAAGAG : 315
M6  : GGTAAATTTCATCTAAAGCTAAATACAGGCTCGAGACCGATAGCAAACAAGTACCATGAGGGAAAGATGAAAAGGACTTTGAAAAGAG : 315
M7  : GGTAAATTTCATCTAAAGCTAAATACAGGCTCGAGACCGATAGCAAACAAGTACCATGAGGGAAAGATGAAAAGGACTTTGAAAAGAG : 315
M8  : GGTAAATTTCATCTAAAGCTAAATACAGGCTCGAGACCGATAGCAAACAAGTACCATGAGGGAAAGATGAAAAGGACTTTGAAAAGAG : 315
M9  : GGTAAATTTCATCTAAAGCTAAATACAGGCTCGAGACCGATAGCAAACAAGTACCATGAGGGAAAGATGAAAAGGACTTTGAAAAGAG : 315
M10 : GGTAAATTTCATCTAAAGCTAAATACAGGCTCGAGACCGATAGCAAACAAGTACCATGAGGGAAAGATGAAAAGGACTTTGAAAAGAG : 351
M11 : GGTAAATTTCATCTAAAGCTAAATACAGGCTCGAGACCGATAGCAAACAAGTACCATGAGGGAAAGATGAAAAGGACTTTGAAAAGAG : 351
M12 : GGTAAATTTCATCTAAAGCTAAATACAGGCTCGAGACCGATAGCAAACAAGTACCATGAGGGAAAGATGAAAAGGACTTTGAAAAGAG : 351
M13 : GGTAAATTTCATCTAAAGCTAAATACAGGCTCGAGACCGATAGCAAACAAGTACCATGAGGGAAAGATGAAAAGGACTTTGAAAAGAG : 351
M14 : GGTAAATTTCATCTAAAGCTAAATACAGGCTCGAGACCGATAGCAAACAAGTACCATGAGGGAAAGATGAAAAGGACTTTGAAAAGAG : 351
M15 : GGTAAGTTTCATCCAAAGCTAAATAGAAGCTCGAGACCAATAGCAAACAAGTACCATGAGGGAAAGATGAAAAGGACTTTGGAAAGAG : 341
K1  : GGTAAATTTCATCTAAAGCTAAATACAGGCTCGAGACCGATAGCAAACAAGTACCATGAGGGAAAGATGAAAAGGACTTTGAAAAGAG : 351
K2  : GGTAGATTTCATCTAAAGCCAAATACAGACCTGAGACCGATAGTGAACAAGTACCATGAGGGAAAGATGAAAAGGACTTTGGAAAGAG : 343
K3  : GGTAGATTTCATCTAAAGCCAAATACAGACCTGAGACCGATAGTGAACAAGTACCATGAGGGAAAGATGAAAAGGACTTTGGAAAGAG : 342
K4  : GGTAGATTTCATCTAAAGCCAAATACAGACCTGAGACCGATAGTGAACAAGTACCATGAGGGAAAGATGAAAAGGACTTTGGAAAGAG : 351
K5  : GGTAGATTTCATCTAAAGCCAAATACAGACCTGAGACCGATAGTGAACAAGTACCATGAGGGAAAGATGAAAAGGACTTTGGAAAGAG : 351
K6  : GGTAGATTTCATCTAAAGCCAAATACAGACCTGAGACCGATAGTGAACAAGTACCATGAGGGAAAGATGAAAAGGACTTTGGAAAGAG : 345
K7  : GGTAGATTTCATCTAAAGCCAAATACAGACCTGAGACCGATAGTGAACAAGTACCATGAGGGAAAGATGAAAAGGACTTTGGAAAGAG : 342
K8  : GGTAGATTTCATCTAAAGCCAAATACAGACCTGAGACCGATAGTGAACAAGTACCATGAGGGAAAGATGAAAAGGACTTTGGAAAGAG : 345
K9  : GGTAGATTTCATCTAAAGCCAAATACAGACCTGAGACCGATAGTGAACAAGTACCATGAGGGAAAGATGAAAAGGACTTTGGAAAGAG : 352
K10 : GGTAGATTTCATCTAAAGCCAAATACAGACCTGAGACCGATAGTGAACAAGTACCATGAGGGAAAGATGAAAAGGACTTTGGAAAGAG : 351
K11 : GGTAGATTTCATCTAAAGCCAAATACAGACCTGAGACCGATAGTGAACAAGTACCATGAGGGAAAGATGAAAAGGACTTTGGAAAGAG : 351
K12 : GGTAGATTTCATCTAAAGCCAAATACAGACCTGAGACCGATAGTGAACAAGTACCATGAGGGAAAGATGAAAAGGACTTTGGAAAGAG : 351
K13 : GGTAGATTTCATCTAAAGCCAAATACAGACCTGAGACCGATAGTGAACAAGTACCATGAGGGAAAGATGAAAAGGACTTTGGAAAGAG : 351
K14 : GGTAGATTTCATCTAAAGCCAAATACAGACCTGAGACCGATAGTGAACAAGTACCATGAGGGAAAGATGAAAAGGACTTTGGAAAGAG : 351
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K16 : GGTAAATTTCATCTAAAGCTAAATACGGGCTCGAGACCGATAGTGAACAAGTACCATGAGGGAAAGATGAAAAGGACTTTGGAAAGAG : 351
K17 : GGTAAATTTCATCTAAAGCTAAATACGGGCTCGAGACCGATAGTGAACAAGTACCATGAGGGAAAGATGAAAAGGACTTTGGAAAGAG : 351
K18 : GGTAAATTTCATCTAAAGCTAAATACGGGCTCGAGACCGATAGTGAACAAGTACCATGAGGGAAAGATGAAAAGGACTTTGGAAAGAG : 342
K19 : GGTAAATTTCATCTAAAGCTAAATACGGGCTCGAGACCGATAGTGAACAAGTACCATGAGGGAAAGATGAAAAGGACTTTGGAAAGAG : 342
K20 : GGTAAATTTCATCTAAAGCTAAATACGGGCTCGAGACCGATAGTGAACAAGTACCATGAGGGAAAGATGAAAAGGACTTTGGAAAGAG : 340
K21 : GGTAAATTTCATCTAAAGCTAAATACGGGCTCGAGACCGATAGTGAACAAGTACCATGAGGGAAAGATGAAAAGGACTTTGGAAAGAG : 351
K22 : GGTAAATTTCATCTAAAGCTAAATACGGGCTCGAGACCGATAGTGAACAAGTACCATGAGGGAAAGATGAAAAGGACTTTGGAAAGAG : 351
K23 : GGTAAATTTCATCTAAAGCTAAATACGGGCTCGAGACCGATAGTGAACAAGTACCATGAGGGAAAGATGAAAAGGACTTTGGAAAGAG : 351
K24 : GGTAAATTTCATCTAAAGCTAAATACGGGCTCGAGACCGATAGTGAACAAGTACCATGAGGGAAAGATGAAAAGGACTTTGGAAAGAG : 339
K25 : GGTAAATTTCATCTAAAGCTAAATACAGGCTCGAGACCGATAGTGAACAAGTACCATGAGGGAAAGATGAAAAGGACTTTGGAAAGAG : 351
K26 : GGTAAATTTCATCTAAAGCTAAATACGGGCTCGAGACCGATAGTGAACAAGTACCATGAGGGAAAGATGAAAAGGACTTTGGAAAGAG : 342
K27 : GGTAAATTTCATCTAAAGGTAAATACGGGCTCGAGACCGATAGTGAACAAGTACCATGAGGGAAAGATGAAAAGGACTTTGGAAAGAG : 351
K28 : GGTAAATTTCATCTAAAGGTAAATACGGGCTCGAGACCGATAGTGAACAAGTACCATGAGGGAAAGATGAAAAGGACTTTGGAAAGAG : 351
K29 : GGTAAATTTCATCTAAAGGTAAATACGGGCTCGAGACCGATAGTGAACAAGTACCATGAGGGAAAGATGAAAAGGACTTTGGAAAGAG : 351
K30 : GGTAAATTTCATCTAAAGCTAAATACGGGCTCGAGACCGATAGTGAACAAGTACCATGAGGGAAAGATGAAAAGGACTTCGGAAAGAG : 351
K31 : GGTAAATTTCATCTAAAGGTAAATACGGGCTCGAGACCGATAGTGAACAAGTACCATGAGGGAAAGATGGAAAGGACTTTGGAAAGAG : 351
K32 : GGTAAATTTCATCTAAAGGTAAATACGGGCTCGAGACCGATAGTGAACAAGTACCATGAGGGAAAGATGAAAAGGACTTTGGAAAGAG : 351
K33 : GGTAAATTTCATCTAAAGGTAAATACGGGCTCGAGACCGATAGTGAACAAGTACCATGAGGGAAAGATGAAAAGGACTTTGGAAAGAG : 351
K34 : GGTAAATTTCATCTAAAGGTAAATACGGGCTCGAGACCGATAGTGAACAAGTACCATGAGGGAAAGATGAAAAGGACTTTGGAAAGAG : 351
K35 : GGTAAACTTCATCTAAAGCTAAATACGGGCTCGAGACCGATAGTGAACAAGTACCATGAGGGAAAGATGAAAAGGACTTTGGAAAGAG : 351
K36 : GGTAAATTTCATCTAAAGCTAAATACGGGCTCGAGACCGATAGTGAACAAGTACCATGAGGGAAAGATGAAAAGGACTTTGGAAAGAG : 351
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Figure 3.7: continued on next page

↓ D2 Start

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             360         *         380         *         400         *         420         *         440
M1  : AGTTAAAAGTGTTTGAAATCGCTGAACTGGAAGCGAATGGAACTAGTGCTCTGGCTGAGATTGCTGTAAGTGGCAGCCAAATTTGGG- : 402
M2  : AGTTAAAAGTGTTTGAAATCGCTGAACTGGAAGCGAATGGAACTAGTGCTCTGGCTGAGATTGCTGTAAGTGGCAGCCAAATTTGGG- : 402
M3  : AGTTAAAAGTGTTTGAAATCGCTGAACTGGAAGCGAATGGAACTAGTGCTCTGGCTGAGATTGCTGTAAGTGGCAGCCAAATTTGGG- : 384
M4  : AGTTAAAAGTGTTTGAAATCGCTGAACTGGAAGCGAATGGAACTAGTGCTCTGGCTGAGATTGCTGTAAGTGGCAGCCAAATTTGGG- : 429
M5  : AGTTAAAAGTGTTTGAAATCGCTGAACTGGAAGCGAATGGAACTAGTGCTCTGGCTGAGATTGCTGTAAGTGGCAGCCAAATTTGGG- : 402
M6  : AGTTAAAAGTGTTTGAAATCGCTGAACTGGAAGCGAATGGAACTAGTGCTCTGGCTGAGATTGCTGTAAGTGGCAGCCAAATTTGGG- : 402
M7  : AGTTAAAAGTGTTTGAAATCGCTGAACTGGAAGCGAATGGAACTAGTGCTCTGGCTGAGATTGCTGTAAGTGGCAGCCAAATTTGGG- : 402
M8  : AGTTAAAAGTGTTTGAAATCGCTGAACTGGAAGCGAATGGAACTAGTGCTCTGGCTGAGATTGCTGTAAGTGGCAGCCAAATTTGGG- : 402
M9  : AGTTAAAAGTGTTTGAAATCGCTGAACTGGAAGCGAATGGAACTAGTGCTCTGGCTGAGATTGCTGTAAGTGGCAGCCAAATTTGGG- : 402
M10 : AGTTAAAAGTGTTTGAAATCGCTGAACTGGAAGCGAATGGAACTAGTGCTCTGGCTGAGATTGCTGTAAGTGGCAGCCAAATTTGGG- : 439
M11 : AGTTAAAAGTGTTTGAAATCGCTGAACTGGAAGCGAATGGAACTAGTGCTCTGGCTGAGATTGCTGTAAGTGGCAGCCAAATTTGGG- : 438
M12 : AGTTAAAAGTGTTTGAAATCGCTGAACTGGAAGCGAATGGAACTAGTGCTCTGGCTGAGATTGCTGTAAGTGGCAGCCAAATTTGGG- : 438
M13 : AGTTAAAAGTGTTTGAAATCGCTGAACTGGAAGCGAATGGAACTAGTGCTCTGGCTGAGATTGCTGTAAGTGGCAGCCAAATTTGGG- : 438
M14 : AGTTAAAAGTGTTTGAAATCGCTGAACTGGAAGCGAATGGAACTAGTGCTCTGGCTGAGATTGCTGTAAGTGGCAGCCAAATTTGGG- : 438
M15 : ACTTAAAAGTGTTTGAAATTGCTGAGAGCGAAGCGAACGGAGCCACATGCTCGTGCTGAGATTGCTGTGGGTCTTGTGAGCCTTGAG- : 428
K1  : AGTTAAAAGTGCTTGAAATCGCTGAACTGGAAGCGAATGGAACTAGTGCTCTGGTTGAGATTGCTGTACATGGCTGCGACATCTGGG- : 438
K2  : AGTTAAAAGTGCTTGAAATCGCTGAAAGGGAAGCGAATGGAACCACATG█CTTGCTGAGATTGCTGCCAATGCTTGTGAGCCCTGGT- : 429
K3  : AGTTAAAAGTGCTTGAAATCGCTGAAAGGGAAGCGAATGGAACCACATG█CTTGCTGAGATTGCTGCCAATGCTTGTGAGCCCTGGT- : 428
K4  : AGTTAAAAGTGCTTGAAATCGCTGAAAGGGAAGCGAATGGAACCACATG█CTTGCTGAGATTGCTGCCAATGCTTGTGAGCCCTGGT- : 437
K5  : AGTTAAAAGTGCTTGAAATCGCTGAAAGGGAAGCGAATGGAACCACATG█CTTGCTGAGATTGCTGCCAATGCTTGTGAGCCCTGGT- : 437
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K7  : AGTTAAAAGTGCTTGAAATCGCTGAAAGGGAAGCGAATGGAACCACATG█CTTGCTGAGATTGCTGCCAATGCTTGTGAGCCCTGGT- : 428
K8  : AGTTAAAAGTGCTTGAAATCGCTGAAAGGGAAGCGAATGGAACCACATG█CTTGCTGAGATTGCTGCCAATGCTTGTGAGCCCTGGT- : 431
K9  : AGTTAAAAGTGCTTGAAATCGCTGAAAGGGAAGCGAATGGAACCACATG█CTTGCTGAGATTGCTGCCAATGCTTGTGAGCCCTGGT- : 438
K10 : AGTTAAAAGTGCTTGAAATCGCTGAAAGGGAAGCGAATGGAACCACATG█CTTGCTGAGATTGCTGCCAATGCTTGTGAGCCCTGGT- : 437
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K12 : AGTTAAAAGTGCTTGAAATCGCTGAAAGGGAAGCGAATGGAACCACATG█CTTGCTGAGATTGCTGCCAATGCTTGTGAGCCCTGGT- : 437
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K22 : AGTTAAAAGTGCTTGAAATCGCTGAAAGGGAAGCGAAGAGAACCACACGTCTTGCTGAGATTGCTGTAGGCTGCTGTGAGCCTTGGG- : 438
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K33 : AGTTAAAAGTGCTTGAAATCGCTGAAAGGGGAGCGAAGAGAACCACACGTCTTGCTGAGATTGCTGTAGGCTGCTGTGAGTCTTGGG- : 438
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K35 : AGTTAAAAGTGCTTGAAATCTTTGAAAGGGAAGCGAAGAGAACCACACGTCTTGCTGAGATTGCTGTAGGCTGCTGTGAGCCTTGG█ : 439
K36 : AGTTAAAAGTGCTTGAAATCGCTGAAAGGGAAGCAAAGAGAACCACACGTCTTGCTGAGATTGCTGTAGGCTGCTGTGAGCCTTGGG- : 436

               *         460         *         480         *         500         *         520
M1  : TGTCTGAACG-CAAGTCTCTCACCTGGCTGTAAGCTGTTGCTTGCAATGTGTCTCAGCTTGAATGTCAATGTCTATGCGCGCATGAGG : 489
M2  : TGTCTGAACG-CAAGTCTCTCACCTGGCTGTAAGCTGTTGCTTGCAATGTGTCTCAGCTTGAATGTCAATGTCTATGCGCGCATGAGG : 489
M3  : TGTCTGAACG CAAGTCTCTCACCTGGCTGTAAGCTGTTGCTTGCAATGTGTCTCAGCTTGAATGTCAATGTCTATGCGCGCATGAGG : 471
M4  : TGTCTGAACG-CAAGTCTCTCACCTGGCTGTAAGCTGTTGCTTGCAATGTGTCTCAGCTTGAATGTCAATGTCTATGCGCGCATGAGG : 516
M5  : TGTCTGAACG-CAAGTCTCTCACCTGGCTGTAAGCTGTTGCTTGCAATGTGTCTCAGCTTGAATGTCAATGTCTATGCGCGCATGAGG : 469
M6  : TGTCTGAACG-CAAGTCTCTCACCTGGCTGTAAGCTGTTGCTTGCAATGTGTCTCAGCTTGAATGTCAATGTCTATGCGCGCATGAGG : 489
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M11 : TGTCTGAACG-CAAGTCTCTCACCTGGCTGTAAGCTGTTGCCTGCAATGTGTCTCAGCTTGAATGTCAATGTCTATGCGCGCATGAGG : 525
M12 : TGTCTGAACG-CAAGTCTCTCACCTGGCTGTAAGCTGTTGCTCGCAATGTGTCTCAGCTTGAATGTCAATGTCTATGCGCGCATGAGG : 525
M13 : TGTCTGAACG-CAAGTCTCTCACCTGGCTGTAAGCTGTTGCTTGCAATGTGTCTCAGCTTGAATGTCAATGTCTATGCGCGCATGAGG : 525
M14 : TGTCTGAACG-CAAGTCTCTCACCTGGCTGTAAGCTGTTGCTTGCAATGTGTCTCAGCTTGAATGTCAATGTCTATGCGCGCATGAGG : 525
M15 : CATCTAAGCG-CAAGCTGACTGCTTATGCGTGAGCATTTGCCCGCAGTGTTTCTCAGCATGCGAGTCATTAGCCACATTTGTTTGAGG : 515
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K2  : CATTAAAGCG-CAAGCTTCTTGTCTAGGAGTGAGTTGGCATTTGTAGTGCTTCTTAGCTTGCGCGTCACCTGCCACATACGCATGAGG : 516
K3  : CATTAAAGCG-CAAGCTTCTTGTCTAGGAGTGAGTTGGCATTTGTAGTGCTTCTTAGCTTGCGCGTCACCTGCCACATACGCATGAGG : 525
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K7  : CATTAAAGCG-CAAGCTTCTTGTCTAGGAGTGAGTTGGCATTTGTAGTGCTTCTTAGCTTGCGCGTCACCTGCCACATACGCATGAGG : 515
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K12 : CATTAAAGCG-CAAGCTTCTTGTCTAGGAGTGAGTTGGCATTTGTAGTGCTTCTTAGCTTGCGCGTCACCTGCCACATACGCATGAGG : 524
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K27 : CACATCAGCG-CAAGCTGTGTGCTTAAGCGTGAATAGTTGTCTGCTGTGTTTCTTAGCTTGCGCGTCACTTGCCACACCTGTTTGAGG : 525
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K36 : CACATCAGCG-CAAGCTGTGTGCTTAAGCGTGAATAGTTGTCTGCTGTGTTTCTTAGCTTGCGCGTCACTTGCCACACCTGTTTGAGG : 525
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Figure 3.7: continued on next page

D2 End

Key
? = missing data
M = A or C
R = A or G
Y = C or T
- = gap

Figure 3.7: Multiple alignments of *Symbiodinium* 24S rRNA gene sequences in samples from Kenya and the Mediterranean. Sequence codes are as outlined in Table 3.2. Residue differences are highlighted in green. Variable regions D1 and D2 are shaded grey in sequential order. Probable indel mutational sites are shaded in black.

The conserved regions of 24S rRNA genes were predicted to have fewer variable sites than the D1 and D2 domains. The percent of each domain comprising variable and constant sites is shown in Table 3.3. Approximately 40% and 63% of the D1 and D2 domains respectively, comprised variable nucleotides. This contrasts with a composition of variable nucleotides of approximately 19%, 22% and 29% in the 5', conserved core and 3' domains of 24S rDNA. The observed pattern confirms a non-random distribution.

Table 3.3: The distribution of variable and constant sites in zooxanthellal 24S rRNA gene sequences from this study.

Domain	Length of domain (bp)	Percent of nucleotides that are variable	Percent of nucleotides that are constant
5' conserved	80	19	81
D1 variable	145	40	60
Conserved core	159	22	78
D2 variable	235	63	37
3' conserved	28	29	71
Total Length (bp)	647		

ClustalX alignments, shown in Figure 3.7, include gaps that indicate probable insertion/deletion (indel) mutational sites. There were a total of 21 probable deletions, of which 19 were in the D2 variable domain. These included a single-bp deletion that was present in haplotype 11 (sequences K2-K14; the algae from *Galaxea fascicularis* and *Acropora palifera*), and a two-bp deletion that was present in four sequences of algae from *Pocillopora damicornis* (sequences K27, K29, K32 and K34). In addition, there were three probable single-bp insertions, each of which fell within the D2 domain of sequences of algae housed by *Pocillopora damicornis* (sequences K28 and K35), and two of which were from the same sample (sequence K28).

The level of variation between sequences corresponded with the algal phylotype, as identified by restriction analysis of PCR-amplified rRNA genes. Sequence variation within phylotype was low, and between phylotypes was high. For instance, overall divergence in sequences of zooxanthellae from the Mediterranean varied between 0 and 26.28%. However, divergence was reduced to between 0 and 1.24% (comparison of sequences M1-M14; temperate A algae) when sequence M15 (phylotype B) was excluded from the analysis. Overall divergence in sequences from Kenyan samples ranged from 0 to 23.6%. Sequences K2-K14 (phylotype D algae) were identical (haplotype 11), and phylotype C

algal divergences (sequences K15-K26, K35, K36) varied between 0 and 1.9%. Sequences with the unidentified PCR-RFLP profile (sequences K27-K34) showed divergences between 0.6 and 3.4%.

3.2.4 Phylogenetic Analysis

3.2.4.1 Diversity of Zooxanthellae from Kenya and the Mediterranean Sea

A Neighbor-Joining (NJ) tree was constructed by implementing the likelihood settings from the best-fit model (TrNef+G) (Tamura & Nei 1993) recovered by Modeltest version 3.06 (Posada & Crandall 1998). The length of alignment used for constructing trees was 603 bp. The tree topology remained unaltered when constructed with uncorrected distances (data not shown). A heuristic search was used to construct a Maximum Parsimony (MP) tree with 212 parsimony-informative characters of a total of 293 variable characters. The *Symbiodinium* group has been found to be a sister to a monophyletic lineage that includes *Gymnodinium simplex* (Genbank accession AF060901) and *G. beii* (AF060900) (Wilcox 1998). These were therefore used to outgroup trees (Saldarriaga *et al.* 2001) and have been extensively used as outgroups in phylogenetic studies on zooxanthellae (e.g. LaJeunesse & Trench 2000, Pochon *et al.* 2001, Loh *et al.* 2001, van Oppen *et al.* 2001, Baker 2003). NJ and MP trees are shown in Figures 3.8 and 3.9 respectively. Each tree provides strong (>99%) bootstrap support for 2 major clades, one comprising haplotypes from phylotype A, and another that consists of haplotypes from phylotypes B, C, D and the RFLP pattern that could not be assigned to a known phylotype. These last haplotypes (with the not-previously described 18S PCR-RFLP profile) occur as a relatively well-supported subgroup (>81%) within phylotype C. In each tree, B (consisting of a single sequence M15) and C cluster in a highly supported (>98%) grouping. Phylotype A is split further into two subgroups, namely 'standard' A (which consists of a single sequence K1) and a highly supported (100%) subgroup of 'temperate' A zooxanthellae (Savage *et al.* 2002).

The zooxanthellae in the majority (9 species of a total of 10) of sea anemone sampled from the Mediterranean were temperate A zooxanthellae (M1-M14) (Savage *et al.* 2002). The only exception to this were symbionts borne by *Bunodeopsis strumosa*, France (M15), which are phylotype B algae

Samples from Kenya comprised a single sequence of standard A algae hosted by *Acropora valida*, Mombasa (K1), sequences of phylotype D algae housed by *Galaxea fascicularis* and *Acropora palifera* (K2-K14), and sequences of phylotype C algae borne by *Acropora*

hyacinthus, Acropora valida, Cosinarea mcneilli, Porites cylindrica and *Pocillopora damicornis* (K15-K36). In both trees, the sequences of zooxanthellae from 8 samples of *Pocillopora damicornis* (K27- K34) with the previously undescribed PCR-RFLP profile (given as '?' in Tables 3.1 and 3.2) cluster in a subgroup within phylotype C.

Figure 3.8: Neighbor-Joining distance tree constructed with haplotypes of *Symbiodinium* 24S rRNA gene sequences from Kenya and the Mediterranean Sea. Bootstrap support for the tree was established with 1000 trials, and values over 50% are shown above the branches. With the exception of the outgroups, sequences are labelled by haplotype. For each haplotype, sequences are identified by a letter/number code, shown in brackets, that matches the code in the alignment shown in Figure 3.7. Phylotype designations A-D are as reviewed by Baker (2003), and temperate A zooxanthellae (A') from Savage *et al.* (2002)

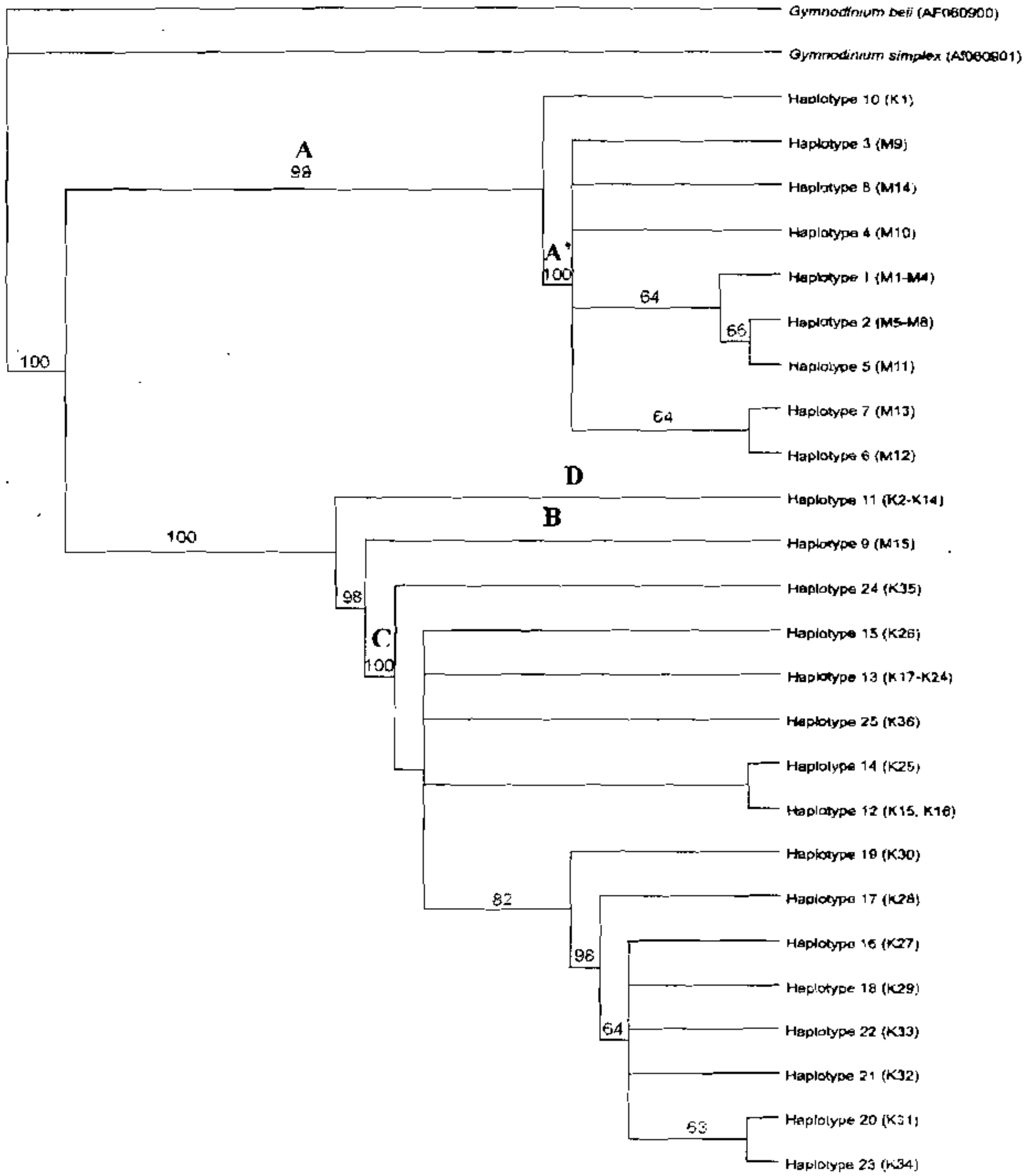


Figure 3.9: A Maximum Parsimony tree constructed with haplotypes of *Symbiodinium* 24S rRNA gene sequences from Kenya and the Mediterranean Sea. See legend to Figure 3.8 for further details.

3.2.4.2 Global Diversity of Zooxanthellae

The phylogenetic position of zooxanthellae from this study in relation to the global diversity of zooxanthellae is represented in the NJ and MP trees shown in Figures 3.10 and 3.11 respectively. The NJ tree was constructed by implementing likelihood settings from the best-fit model (TrNef+G) (Tamura & Nei 1993) selected by Likelihood Ratio Test in Modeltest version 3.06 (Posada & Crandall 1998). The alignment used to reconstruct phylogenies was 583 bp in length. When the tree was constructed with uncorrected distances, tree topology remained unaltered (data not shown). A MP tree was constructed with a heuristic search on 290 parsimony-informative characters of a total of 359 variable characters. Trees were rooted with outgroups *Gymnodinium simplex* (AF060901) and *G. beii* (AF060900) (Wilcox 1998). The broad topology of each of these trees is robust, providing strong bootstrap support (100%) for 2 major clades, one comprising phylotype A and the other that includes phylotypes B-G. All phylotypes are highly supported (100%), with the exception of phylotype F that has 88% support with NJ and 82% with MP, and phylotype E that is represented by a single sequence. Phylotype A is split further into temperate and standard A subgroups, each of which receives 100% bootstrap support. Phylotype B, C, D, F and G cluster in a group (>95%) that is monophyletic with phylotype E. Phylotypes B, C and F group together (100%) in both trees. Although B and F are monophyletic in the NJ tree, this grouping is not well supported (<50%).

The haplotypes from Kenya and the Mediterranean Sea are highlighted in bold print in Figures 3.11 and 3.12. The 24S rRNA gene sequences of zooxanthellae from Kenyan corals are closely related to phylotype standard A, C and D from distant locations in the Atlantic, Pacific and Indo-Pacific provinces. Similarly, the phylotype B sequence of symbionts housed by *Bunodeopsis strumosa*, France (M15) was closely related to phylotype B algae from the Caribbean Sea and Australia. However, the closest relation to the temperate A zooxanthellae in remaining samples of Mediterranean anemone were sequences from the study by Savage *et al.* (2002) on samples obtained from the north-east Atlantic Ocean (UK) and the Mediterranean Sea (France). Temperate A sequences are approximately 8% divergent from the globally distributed standard A zooxanthellae.

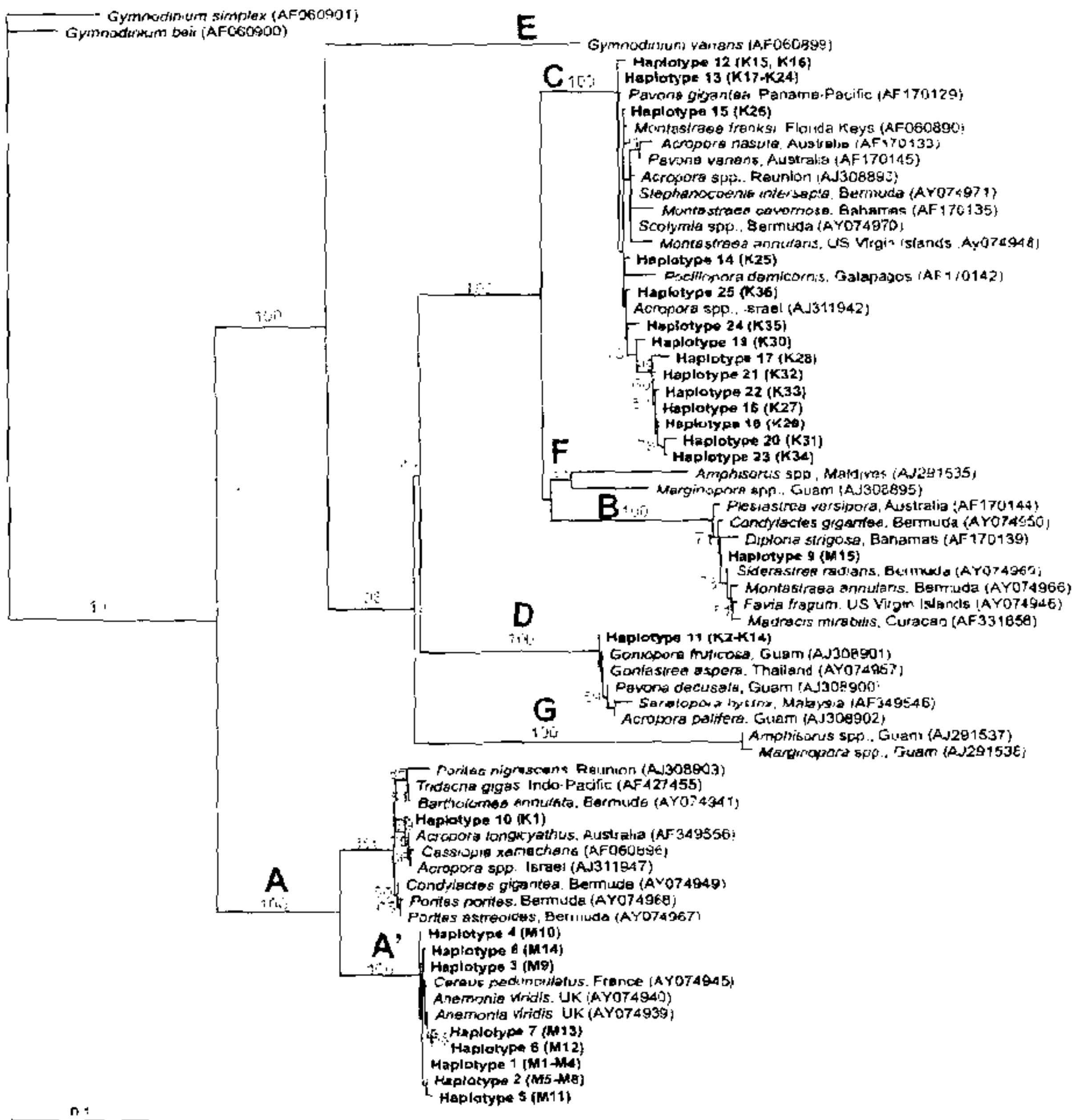


Figure 3.10: A Neighbor-Joining tree indicating the position of *Symbiodinium* 24S rRNA sequence haplotypes from Kenya and the Mediterranean in relation to the global diversity of zooxanthellae. Support for the tree was assessed by bootstrapping with 1000 replicates, and values over 50% are shown above the branches. With the exception of the outgroups and *Gymnodinium varians*, sequences are labelled by the host species, sampling location and Genbank accession number. Haplotypes from this study are highlighted in bold print. Sequences for each haplotype are identified by a letter/number code, shown in brackets, that matches the code in the sequence alignments shown in Figure 3.8. Phylotype designations A-F are as reviewed by Baker (2003), and temperate A zooxanthellae from Savage *et al.* (2002).

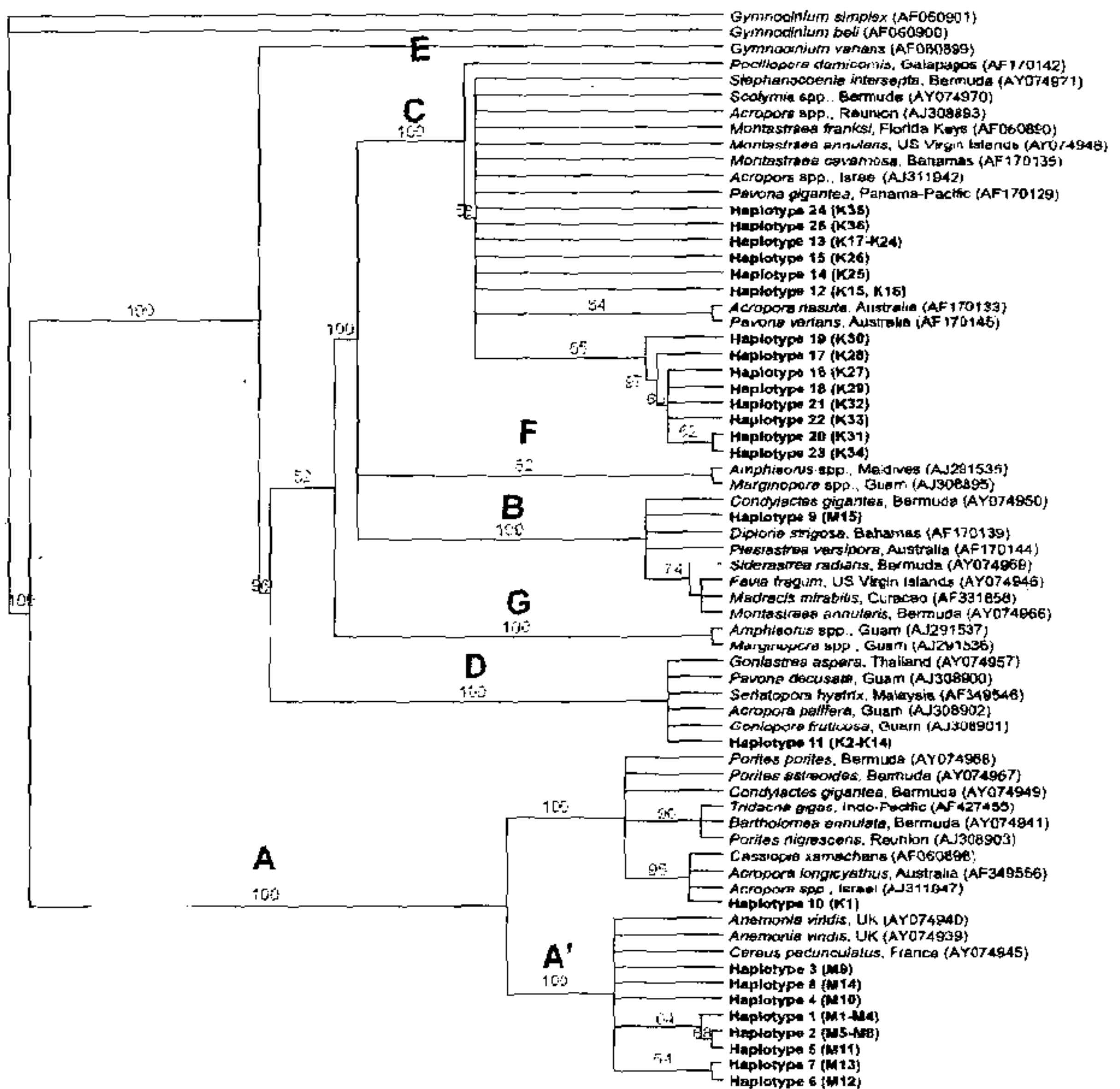


Figure 3.11: A Maximum Parsimony tree constructed with 24S rRNA gene sequences of zooxanthellae from the Genbank database. Included in the tree (shown in bold print) are algal sequence haplotypes from the Mediterranean Sea and Kenya. Bootstrap support for the tree was established with 100 bootstrap trials, and values over 50% are shown above the branches. See legend to Figure 3.11 for further details.

3.2.5 *Symbiodinium psb*A Phylogeny

A NJ tree of *Symbiodinium psb*A was constructed using zooxanthellae isolated from *Anemonia viridis* collected from Wales, material from hosts sampled in Kenya and the Mediterranean, and samples of genomic DNA from Bermudan material provided by Anne Savage. Alignments of *psb*A sequences used for phylogenetic analysis were 357 bp in length. Sequences from the Genbank database were also used in the analysis, which is shown in Figure 3.12a. Alongside the *psb*A tree is a NJ tree (Figure 3.12b) prepared with 589 bp alignments of 24S rRNA gene sequences from corresponding samples, i.e. those used to prepare template for PCR-amplification of both genes. Both trees were constructed using uncorrected distances. A direct comparison of the trees reveals areas of congruency and dispute as follows:

3.2.5.1 Congruency Between Phylogenies

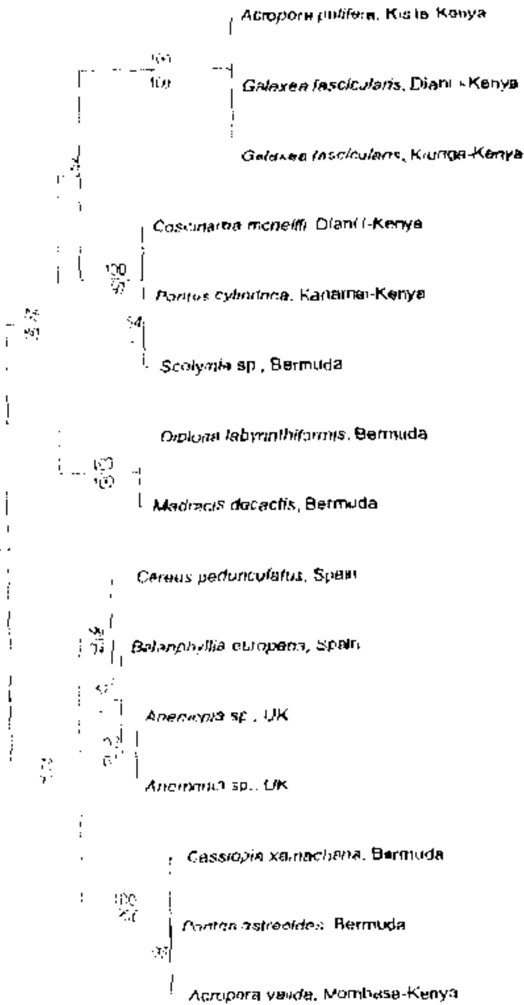
All sequences of corresponding samples clustered in corresponding lineages on both trees. Lineages were strongly supported by bootstrapping (>94%) in the *psb*A phylogeny. Therefore, *Symbiodinium* lineages defined on the basis of 24S rRNA gene sequences [A-D, including temperate A (A')] are supported by *psb*A phylogenies. Furthermore, *Symbiodinium* lineages fell within two broad clades, one comprising sequences from phylotype A (including A'), and the other consisting of sequences from phylotypes B, C and D.

3.2.5.2 Dispute Between Phylogenies

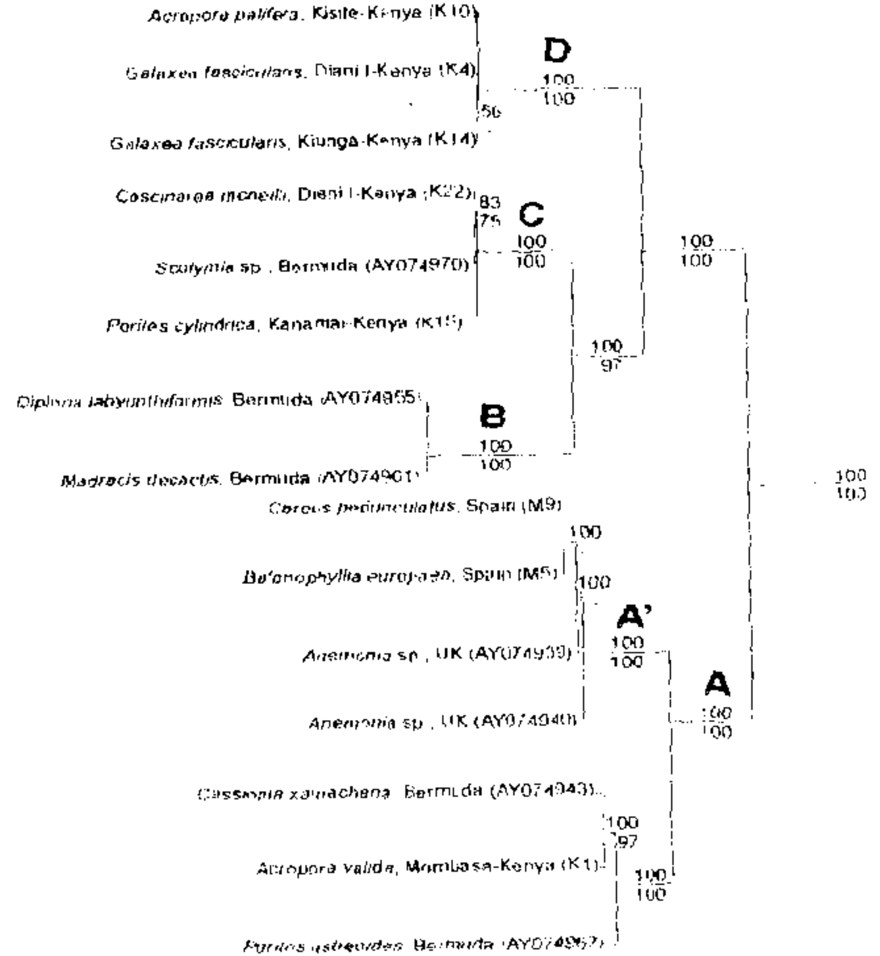
The major area of dispute between the phylogenies is that C groups with B in the 24S rRNA gene phylogeny, whereas it groups with D in the *psb*A phylogeny. However, this latter grouping is not strongly supported by bootstrapping (72% NJ, < 50% MP). In addition, there are minor points of disagreement between the trees. For example, in the 24S rRNA gene tree, *Acropora valida* is most closely related to *Cassiopia xamachana* (in A), and *Scolymia* sp. clusters with *Coscinarea mcneilli* (in C). In the *psb*A trees, however, *A. valida* and *Scolymia* sp. are more closely related to *Porites astreoides*, and *Porites cylindrica* respectively. None of these latter *psb*A groupings are strongly supported by bootstrapping

A matrix of evolutionary distances (uncorrected distances) separating pairs of *psb*A and 24S rRNA gene sequences is shown in Table 3.4. The average rate of evolution of 24S rRNA genes was just over twice (x 2.31) that undergone by *psb*A when examined over all

pairwise comparisons. *Symbiodinium* D did not vary, either in *psb*A or in 24S rRNA genes. There was greater variation in *psb*A than in 24S rRNA genes for temperate A (*psb*A: 0-1.0%, 24S rRNA genes: 0-0.5%) and phylotype B zooxanthellae (*psb*A: 0.6%, 24S rRNA genes: 0.2%), but the reverse was true for *Symbiodinium* standard A (*psb*A: 0.3-0.6%, 24S rRNA genes: 0.8-1.5%) and C (*psb*A: 0-0.3%, 24S rRNA genes: 0.2-1.1%). A comparison of sequence variation between zooxanthellae from different phylotypes indicates that 24S rRNA genes (variation between 7.9% and 25.9%) were more variable than *psb*A (variation between 4.0% and 12.9%). The distances separating the most divergent 24S rRNA gene lineages (A' and B: mean divergence of 25%) were almost twice that separating the most divergent *psb*A lineages (A and D: mean divergence of 12.8%). Zooxanthellae from phylotypes A and A' were the most closely related, both for *psb*A (mean divergence of 4.57%) and for 24S rRNA genes (mean divergence of 8.41%).



(a) Symbiodinium psbA
Gymnodinium simplex (AB096158)
Acropora palifera, Kisite-Kenya
Galaxea fascicularis, Diani I-Kenya
Galaxea fascicularis, Kiunga-Kenya
Coscinaraea mcneilli, Diani I-Kenya
Porites cylindrica, Kanamai-Kenya
Scolymia sp., Bermuda
Diploria labyrinthiformis, Bermuda
Madracis decactis, Bermuda
Cereus pedunculatus, Spain
Balanophyllia europaea, Spain
Anemonia sp., UK
Anemonia sp., UK
Cassiopia xamachana, Bermuda
Porites astreoides, Bermuda
Acropora valida, Mombasa-Kenya
100
100
0.1



(b) Symbiodinium 24S rRNA gene
Gymnodinium simplex (AF060901)
Acropora palifera, Kisite-Kenya (K10)
Galaxea fascicularis, Diani I-Kenya (K4)
Galaxea fascicularis, Kiunga-Kenya (K14)
D
Coscinaraea mcneilli, Diani I-Kenya (K22)
Scolymia sp., Bermuda (AY074970)
Porites cylindrica, Kanamai-Kenya (K15)
C
Diploria labyrinthiformis, Bermuda (AY074955)
Madracis decactis, Bermuda (AY074961)
B
Cereus pedunculatus, Spain (M9)
Balanophyllia europaea, Spain (M5)
Anemonia sp., UK (AY074939)
Anemonia sp., UK (AY074940)
A'
Cassiopia xamachana, Bermuda (AY074943)
Acropora valida, Mombasa-Kenya (K1)
Porites astreoides, Bermuda (AY074967)
A
0.1

Figure 3.12: Neighbor-Joining (NJ) trees of (a) *Symbiodinium psb*A and (b) 24S rRNA genes constructed using corresponding samples as template DNA. Trees were rooted with *Gymnodinium simplex*. Sequences are labelled by host species and sampling locations. Accession numbers identify sequences from Genbank Percent (>50%) of 1000 bootstrap replicates for NJ and Maximum Parsimony (MP) tree methods are shown above (NJ) and below (MP) the branches. Template for PCR-amplification of *psb*A was prepared from aquarium specimens of *Anemonia* sp., University of York, or obtained from Kenyan and Mediterranean hosts sampled for this study, and from Bermudan material provided by Anne Savage. Phylotypes (A-D) are as reviewed by Baker (2003), and temperate A zooxanthellae (A') from Savage *et al.* (2002).

Table 3.4: Evolutionary distance matrix of *psb*A (lower left) and nuclear 24S rRNA genes (upper right) from corresponding samples of *Symbiodinium*. The values show uncorrected distances (number of nucleotide substitutions divided by the length of alignment) between pairs of sequences. Letters A-D denote the phylotype.

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1. *Anemonia* sp.	A'	-	0.000	0.003	0.002	0.081	0.079	0.082	0.219	0.219	0.219	0.232	0.228	0.226	0.243	0.245
2. *Anemonia* sp.	A'	0.000	-	0.003	0.002	0.081	0.079	0.082	0.220	0.219	0.219	0.232	0.228	0.227	0.242	0.244
3. *B. europaea*	A'	0.003	0.003	-	0.005	0.088	0.086	0.090	0.230	0.230	0.230	0.246	0.241	0.239	0.254	0.256
4. *C. pedunculatus*	A'	0.010	0.010	0.010	-	0.087	0.085	0.089	0.235	0.235	0.235	0.243	0.241	0.239	0.258	0.259
5. *C. xamachana*	A	0.048	0.048	0.043	0.048	-	0.014	0.008	0.214	0.214	0.214	0.229	0.226	0.224	0.252	0.254
6. *P. astreoides*	A	0.045	0.045	0.040	0.045	0.003	-	0.015	0.211	0.211	0.211	0.222	0.220	0.218	0.246	0.248
7. *A. valida*	A	0.048	0.048	0.043	0.048	0.006	0.003	-	0.216	0.216	0.216	0.223	0.220	0.219	0.248	0.250
8. *G. fascicularis* (Diani)	D	0.115	0.115	0.108	0.106	0.129	0.128	0.129	-	0.000	0.000	0.178	0.174	0.172	0.197	0.199
9. *G. fascicularis* (Kiunga)	D	0.115	0.115	0.108	0.106	0.129	0.126	0.129	0.000	-	0.000	0.177	0.174	0.172	0.197	0.199
10. *A. palifera*	D	0.115	0.115	0.108	0.106	0.129	0.126	0.129	0.000	0.000	-	0.177	0.174	0.172	0.197	0.198
11. *P. cylindrica*	C	0.084	0.084	0.074	0.078	0.101	0.098	0.101	0.067	0.067	0.067	-	0.011	0.009	0.135	0.136
12. *Scolymia* sp.	C	0.087	0.087	0.077	0.081	0.098	0.101	0.104	0.070	0.070	0.070	0.003	-	0.002	0.128	0.130
13. *C. mcneilli*	C	0.080	0.080	0.074	0.077	0.095	0.092	0.095	0.071	0.071	0.071	0.000	0.003	-	0.127	0.128
14. *D. labyrinthiformis*	B	0.081	0.081	0.071	0.082	0.098	0.101	0.098	0.081	0.081	0.081	0.062	0.059	0.060	-	0.002
15. *M. decactis*	B	0.076	0.076	0.065	0.075	0.101	0.098	0.095	0.081	0.081	0.081	0.056	0.059	0.054	0.006	-

3.3 Discussion

3.3.1 PCR-RFLP and Sequence Analysis

Restriction enzyme analysis of the zooxanthellae in Kenyan corals showed that these included *Symbiodinium* A (*Acropora valida*), C (*Porites cylindrica, Coscinarea mcneilli, Acropora hyacinthus, Pocillopora damicornis, Acropora valida*) and D (*Galaxea fascicularis, Acropora palifera*). In addition, *P. damicornis* also bore zooxanthellae with a previously undescribed 18S PCR-RFLP profile. *A. hyacinthus* was polymorphic (but not mixed) for phylotypes D and C, as was *A. valida*, in which some colonies harboured mixed infections (A and C) and *P. damicornis*, in which all colonies carried mixed infections (phylotype C and zooxanthellae with an unidentified PCR-RFLP profile).

A lower diversity of symbionts was uncovered by restriction enzyme analysis of zooxanthellae from the Mediterranean than from those in Kenya. None of the phylotypes identified by PCR-RFLP of Mediterranean zooxanthellae occurred in Kenya, and conversely, zooxanthellae found in Kenya were not identified from Mediterranean samples. The vast majority of sea anemones (9 of 10 species) in the Mediterranean formed monomorphic associations with temperate A zooxanthellae throughout the range from which they were sampled. The only exception was *Bunodeopsis strumosa*, sampled only in France, which hosted *Symbiodinium* B. Polymorphic infections were not identified from Mediterranean samples.

Sequence analysis of nuclear-encoded partial 24S rRNA genes from Kenyan and Mediterranean zooxanthellae confirmed the results from PCR-RFLP above. The zooxanthella with the novel PCR-RFLP profile (labelled as '?' in Tables 3.1 and 3.2) in *P. damicornis* was found to most likely represent a subgroup within phylotype C. An unusually high number of haplotypes occurred for zooxanthellae in *P. damicornis*, with each of a total of 10 sequences processed representing a distinct haplotype. This cannot merely be attributed to a numerical bias in sequencing in favour of the zooxanthellae in *P. damicornis* (in an effort to identify the zooxanthella with the previously undescribed PCR-RFLP profile), as relatively large numbers of sequences were processed for the zooxanthellae in hosts such as *G. fascicularis* (10 sequences) and *C. mcneilli* (7 sequences), but for which all sequences from a given species had an identical haplotype. Alternative explanations must therefore be sought. These include the existence of paralogous genes (genes arising from gene duplication events) within the rRNA gene repeats (Rowan & Powers 1991a, b) and / or artefacts of PCR and cloning (Speksnijder *et al.* 2001). These explanations have previously been invoked to explain the extreme number

of sequence variants in phylotype C (Toller *et al.* 2001b, Baker 2003). These explanations however, do not adequately explain why paralogous genes and / or PCR and cloning artefacts should occur for only the zooxanthellae in *P. damicornis*. In 1997/98, an El-Niño-associated mass coral-bleaching event resulted in the complete mortality of *P. damicornis* in Kenya (Obura 2001b). Mortality rates for *G. fascicularis* were much lower and *C. mcneilli* was largely unaffected by the bleaching (Obura 2001b). The first live colony of *P. damicornis* observed in Kenya after the bleaching episode was approximately one year later, in May/June 1999 (Obura 2001b). Thus, when fieldwork was conducted in February 2001, the only colonies of *P. damicornis* available for sampling were recent recruits to the population. Juveniles are reported to be less selective for their symbionts, initially acquiring a broad spectrum of zooxanthellae before specificity is established towards adulthood (Coffroth *et al.* 2001). This then offers a further possible explanation for the high number of sequence variants in the zooxanthellae hosted by *P. damicornis*.

Kenyan corals housed zooxanthellae whose 24S rRNA gene sequences showed them to be closely related to zooxanthellae in Cnidaria from distant reef provinces in the Atlantic, Pacific and Indo-Pacific. In some cases, the sequences of zooxanthellae from Kenya were identical to the Genbank sequences of zooxanthellae elsewhere. For example, haplotype 11 (sequences K2-K14: phylotype D zooxanthellae in *G. fascicularis* and *A. palifera*) was indistinguishable from the 24S rRNA gene sequence of zooxanthellae borne by *Siderastrea siderea* in the US Virgin Islands (Genbank accession AY074951; not shown in Figures 3.11 and 3.12).

The phylotype B zooxanthellae in *B. strumosa*, France had a 24S rRNA gene sequence which showed that they were closely related to the zooxanthellae in hosts from distant locations including the Caribbean and Australia. However, temperate A zooxanthellae, the sole symbionts identified from the majority of sea anemone species sampled from the Mediterranean, had 24S rRNA gene sequences that were approximately 8% divergent from their closest relatives, the pan-tropical standard A zooxanthellae. The nearest Genbank matches for temperate A were sequences of zooxanthellae housed by *Anemonia* sp. in the UK and *Cereus pedunculatus* in France (Savage *et al.* 2002). It seems likely therefore, that temperate A zooxanthellae are regionally endemic to the North East Atlantic Ocean and the Mediterranean Sea. Their occurrence in a wide range of sea anemone speeies in waters characterised by higher seasonality and cooler temperatures than those of tropical and subtropical locations suggest that they may be specialised to these environmental

conditions. As they have not previously been reported at high latitude locations elsewhere, the divergence of temperate A may also reflect isolation from other sites by factors such as distance, land barriers and prevailing ocean currents (Palumbi 1994, Veron 1995, Cowan *et al.* 2000). The degree to which isolation and selection have shaped the distribution of temperate A zooxanthellae is far from clear, but warrants further investigation. The trend in decreasing biodiversity with increasing latitude has been well documented (Gaston 2000). Results from the current study on *Symbiodinium* lend support to this observation. A further prediction in the coral literature is that ecosystem resilience in the face of environmental change is enhanced with increased diversity (Nystrom & Folke 2001). This raises concern for the species-poor zooxanthellate communities in the Mediterranean, and especially so when considering that the dominant zooxanthella may represent an isolated and/or specialised group of *Symbiodinium*.

A research priority in the immediate future is to determine whether rRNA gene sequences vary with symbiotically important differences in zooxanthellal phenotype. Preliminary evidence suggests that *Symbiodinium* phenotypes do vary among the different genotypes. For instance, the photosynthetic responses of different genotypes maintained under uniform conditions varied with temperature (Warner *et al.* 1999) and light (Iglesias-Prieto & Trench 1994). Intensified efforts to elucidate functional differences between zooxanthellae are of paramount importance if the burgeoning information on diversity of *Symbiodinium* is to be of any practical value to reef scientists and managers alike. In the absence of specific information on functional diversity, an 'educated guess' can be made based on results from the extensive surveys undertaken to date in a wide range of hosts, geographic locations and habitats. These surveys have revealed patterns and trends in the distribution of *Symbiodinium*, which, if interpreted with due caution, can provide us with an insight on the ecology and biogeography of *Symbiodinium*.

Of particular interest are polymorphic / mixed symbioses. The study of these systems can circumvent the need to control for the potentially confounding effects of host influences on zooxanthellal phenotype. Polymorphic symbioses offer an excellent opportunity for research, and must be exploited to their full potential.

3.3.2 Ecology of Polymorphic Symbioses

It is emerging that polymorphic zooxanthellate symbioses are more common than was once perceived (Baker 2003). This challenges the conventional perspective of high fidelity

between one host and one zooxanthella. In some cases, polymorphic symbioses are uncovered with relative ease, after a period of localised or regional sampling. This was the case in Kenya, with *Acropora valida* and *Acropora hyacinthus*, respectively. However, often symbioses are only identified as being polymorphic after extensive surveys of conspecific hosts in which temporal (e.g. seasonal), spatial (e.g. depth, latitude) and environmental (e.g. near shore versus offshore reefs) scales are incorporated into the sampling regime.

Seasonal changes in the relative abundances of zooxanthellae of phylotypes C and D have been reported in *Acropora palifera* from Taiwan (Yang *et al.* 2000), with the dominance of D during summer. Similarly, seasonal changes in the relative abundances of phylotypes A and B are strongly suspected in Bermudan *Condylactis gigantea* (Alexander Venn, personal communication).

Exceptionally, the Caribbean corals *Montastraea annularis* and *Montastraea faveolata* are known to form associations with zooxanthellae of phylotypes A, B, C and D (Rowan & Knowlton 1995, Rowan *et al.* 1997, Toller *et al.* 2001a). For some colonies at certain locations, these associations are clearly non-random, being predictable by depth and/or irradiance. Whereas phylotypes A and B occur in shallow water (0-6 m), phylotype C is found in deeper water (3-14 m) or in low-irradiance microenvironments of colonies at intermediate and shallow depths, i.e. the colony sides and shaded overhangs, respectively. In an experiment designed to test the stability of these irradiance-associated patterns, Rowan *et al.* (1997) inverted shallow-water coral fragments bearing phylotypes A, B and C such that the consequent irradiance environment for A/B and C was low and high irradiance, respectively. The original pattern, with A/B and C predominant in high and low irradiance microenvironments, respectively, were re-established within six months. Partitioning of zooxanthellae by depth/irradiance in these symbioses may reflect the greater effectiveness of groups A/B at high irradiance and of C at low irradiance. Toller *et al.* (2001a) have reported *Symbiodinium* D in very deep colonies (> 35 m) of *Montastrea franksi*, as have Chen *et al.* (2003) for Taiwanese corals. In the tropical Pacific, patterns of partitioning of zooxanthellae by depth similar to those documented for Caribbean corals have generally involved different zooxanthellae within phylotype C (reviewed in Baker 2003).

Latitudinal factors also shape the distribution of *Symbiodinium*. These have been more challenging to document at the level of the individual host species than at community level. Intraspecific surveys of the wide-ranging corals *Plesiastrea versipora* (Rodriguez-Lanetty *et al.* 2001), *Seriatopora hystrix* and *Acropora longicyathus* (Loh *et al.* 2001) were conducted along the eastern Australian seaboard and the Indo-Pacific, respectively. The results indicated that phylotype C zooxanthellae were prevalent in tropical associations, but that *Symbiodinium* B (*P. versipora*) or A (*A. longicyathus*) predominated at higher latitudes (23°-35°S). At equatorial locations, D was more common in *S. hystrix*. A similar survey of the symbionts borne by the temperate sea anemone *Anthopleura elegantissima* was carried out along the North American Pacific coastline (LaJeunesse & Trench 2000). Northern populations (43.5°-48.5°N) harboured phylotype B zooxanthellae (sometimes combined with a *Chlorella*-like green alga), and southern populations (33°-36°N) housed *Symbiodinium* B and E.

Community level surveys also appear to suggest that globally, members of *Symbiodinium* A and B tend to be subtropical in their distribution, and that C leans towards a tropical distribution (Savage *et al.* 2002, Rodriguez-Lanetty *et al.* 2002, Baker 2003). In this respect, the discovery of *Symbiodinium* B in *Bunodeopsis strumosa*, France is not surprising, but the occurrence of phylotype A in *Acropora valida*, Mombasa-Kenya, is contrary to expectation. Despite latitudinal influences on the distribution of *Symbiodinium*, phylotypes A and B appear to be far more common in tropical western Atlantic corals than in their Pacific counterparts. A theory advanced to explain the current resemblance of Caribbean symbioses to those at higher latitudes in the Pacific is that the emergence of the Central American isthmus about 3.5 million years ago, which led to the evolutionary diversification of corals in the Caribbean (Collins *et al.* 1996), coincided with the onset of the Northern Hemisphere glaciation (Budd 2000). The conditions in the western Atlantic at the time may have more closely resembled those characteristic of high latitudes, selecting for scleractinian communities involving *Symbiodinum* A and B (Baker 2003). These have since diversified in Caribbean corals.

Members of *Symbiodinium* D appear to have a somewhat erratic distribution. They are often documented from hosts in locations that are thought to be subject to considerable anthropogenic impacts or from marginal habitats. These include their increased abundance on inshore reefs (with higher terrestrial impacts such as freshwater runoff) relative to offshore reefs (Toller *et al.* 2001b), in intertidal and extremely deep colonies of

Montastrea franksi (Toller *et al.* 2001a), and in intertidal colonies of *Goniastrea aspera* from Thailand (Brown *et al.* 2002b). In addition, *Symbiodinium* D emerged as novel symbionts in corals following a disease-related bleaching event (Toller *et al.* 2001b). These observations have led to the speculation that *Symbiodinium* D occur transiently in recently bleached corals recovering their steady-state zooxanthellal communities, or on reefs that have undergone recent and/or recurrent stress episodes. If the suggestion that the relative abundance of phylotype D reflects coral community health is accurate (Baker 2003), then the results from the current study in Kenya are not encouraging. The phylotype D zooxanthellae identified from Kenya constituted a low-diversity group (*psb*A and 24S rRNA gene sequences were invariant) that appear to be common on Kenyan reefs. They were the sole zooxanthella in two species of corals, *Acropora palifera* and *Galaxea fascicularis*, and one of two phylotypes identified in *Acropora hyacinthus*. *A. palifera* has a narrow distribution in Kenya, found only at two southern sites, Diani II and Kisite, but it could extend further south into Tanzania. Restriction of *A. palifera* to the south of the Kenyan coastline cannot be attributed to the lack of availability of its primary symbiont north of Diani II.

From the descriptions above, it is abundantly clear that associations between hosts and their zooxanthellae can vary with environmental circumstance. How then can these observations inform us about the phenotypic traits expressed by different genotypes of zooxanthellae? Consider a hypothetical symbiosis between a particular host species that occurs at two different locations (locations 1 and 2) differing in environmental conditions (e.g. latitudinal differences). This host is known to associate with symbiont X at location 1, and with symbiont Y at location 2. Two possible explanations to account for the observed distribution are as follows:

1. Symbiont X is a better competitor than symbiont Y at location 1, whereas symbiont Y out competes symbiont X at location 2. Measures of competitive traits may include the ability to utilize available space and host-derived nutrients, and rates of proliferation. Thus, the observed specificity at either location may be the end result of *competitive exclusion*.

2. Symbiont X is a more effective symbiont at location 1, and the host derives greater benefit by associating with symbiont Y at location 2. The measure of *effectiveness* may involve the amount or the quality of photosynthate released to the host by a symbiont. An effective symbiont would promote the long-term growth and

reproduction of the host to a greater extent than a less effective one. Thus, a host may be *selective* for its symbiont. By being flexible to associate with two different symbionts, the host benefits from greater ecological and evolutionary potential.

Studies of the symbiosis between the intertidal flatworm *Convoluta roscoffensis* and algae of the genus *Tetraselmis* (Pravasoli *et al.* 1968, Douglas 1985, Douglas 1995) address key aspects of both explanations offered above. *C. roscoffensis* can establish a symbiosis with different members of *Tetraselmis*, including the subgenus *Tetraselmis*, and the subgenus *Prasinocladia*. These differ in the amount of photosynthate they release to their host, with *Tetraselmis* releasing approximately four times as much as *Prasinocladia*, thereby promoting the growth and fecundity of their hosts to a greater extent (Douglas 1995). Under laboratory conditions, transient mixed infections between juveniles of *C. roscoffensis* and both subgenus of *Tetraselmis* can be established (Pravasoli *et al.* 1968). Over a period of two weeks after infection, one is gradually expelled and the other is retained. Invariably, the alga retained is *Tetraselmis* (Douglas 1995), which confers the greatest benefit to its host, i.e. *selection* for a symbiont by a host. The transient mixed infections were shown to be costly to the host, significantly reducing the growth of the animal over 30 days (see Douglas 1998). This illustrates *competition* between multiple genotypes in a single host.

The argument by Frank (1996) that infections with multiple symbiont genotypes diminishes host fitness through the expression of competitive traits, and borne out by studies on *C. roscoffensis* / *Tetraselmis* and mycorrhizal fungi (Pearson *et al.* 1993), poses a problem for the incidence of mixed infections. However, it is believed that mixed infections with symbionts that vary in their effectiveness depending on their environmental circumstances are advantageous when shifts in environmental conditions are unpredictable or rapid relative to the lifespan of the host (Douglas 1998). In addition, specialisation for a few highly effective symbionts would not be favoured when the abundance of symbionts in the free-living state is low or unpredictable (Douglas 1998). To date, very little is known about the abundance of infective *Symbiodinium* in its free-living state.

As alluded to earlier, a cautious approach is prudent when interpreting patterns and trends in the distribution of *Symbiodinium*. These observations may have no functional basis in symbiosis. Going back to the hypothetical symbiosis described above, the observed association at location 2 may merely reflect the chance absence of symbiont X. For

instance, the composition of zooxanthellal pools has been reported to vary between different locations along the central Great Barrier Reef (van Oppen *et al.* 2001). *Symbiodinium* phylotypes should not be ascribed phenotypic characteristics. There is now persuasive evidence for substantial within-phylotype variation in symbiotically important traits (Warner *et al.* 1999, Iglesias-Prieto & Trench 1994, Savage *et al.* 2002). Such variation may arise from acclimatisation (Brown *et al.* 2000a) or from genetic variation not evident at the level of rRNA genes.

A striking illustration of the profound ecological impacts that may arise from phenotypic variation among *Symbiodinium* genotypes comes from observations of variation in bleaching susceptibilities.

3.3.3 Variation in Bleaching Susceptibility

Fresh impetus into research on diversity of zooxanthellae was provided by the discovery that members of *Symbiodinium* can vary in their susceptibility to bleaching. This was first established for the Caribbean *Montastraea* species complex, in which *Symbiodinium* C were found to be more susceptible to bleaching than phylotypes A and B (Rowan *et al.* 1997). During a natural bleaching episode, only those colonies in which the *Symbiodinium* populations comprised 35% or more of phylotype C (and 65% of phylotypes A and B) visibly bleached. *Symbiodinium* C was preferentially expelled from the upper limit of its light-dependent distribution along the sides of colonies, resulting in hitherto enigmatic 'ring' bleaching. Glynn *et al.* (2001) have made similar observations with *Pocillopora damicornis* on the west coast of Panama. Patchy bleaching in these corals was attributed to the preferential loss of *Symbiodinium* C, and the retention of phylotype D. In light of the predictions of changes in global climate, knowledge of variation in susceptibilities of different members of *Symbiodinium* is immediately relevant to our ability to understand and predict the incidence and severity of future bleaching events. This has spurred a vigorous debate (see Hoegh-Guldberg *et al.* 2002 and subsequent reply by A. Baker) on the merits of the Adaptive Bleaching Hypothesis (ABH; Buddemeier & Fautin 1993). The ABH proposed that bleaching was an adaptive mechanism that facilitated the incorporation of new zooxanthellae that were better suited (e.g. enhanced thermal tolerances) to altered environmental conditions (e.g. elevated temperatures). (See Chapter 1 for further information on the ABH).

3.3.4 *PsbA*: A New Approach

Contemporary research has seen a shift in focus from the use of nuclear-encoded rRNA genes towards the use of chloroplast-encoded rRNA genes for studies on *Symbiodinium* taxonomy (Santos *et al.* 2002, Mary Alice Coffroth, personal communication). This partly reflects the need for independent corroboration of the phylogenies established by studies using nuclear genes, but also to achieve a greater degree of phylogenetic resolution; chloroplast rRNA genes were shown to evolve faster than nuclear rRNA genes (Santos *et al.* 2002). Ribosomal RNA genes are subject to stringent purifying selection, and as such are highly conserved. In addition, although ribosomal genes are excellent molecular markers used extensively to trace the evolutionary history of genes (Hillis & Dixon 1991), they are unlikely to provide us with information on functional diversity in zooxanthellae. An accurate assessment of the level of diversity in *Symbiodinium* is only likely to be achieved through the use of multiple molecular markers. The approach taken by the current study has been to assess the suitability of *psbA* for phylogenetic studies on zooxanthellae. Takishita *et al.* (2003) set a precedent for this approach by constructing a phylogeny with *Symbiodinium psbA* with cultures for which 18S rRNA gene sequences were previously known. That study (Takishita *et al.* 2003) ascertained that *psbA* trees were congruent with trees constructed using 18S rRNA genes, and that *psbA* trees better resolved the phylogenetic relationships among the members of phylotype A than did 18S rRNA gene trees. Their results showed that *psbA* evolved faster than 18S rRNA genes. The current study appears to suggest that 24S rRNA genes evolve faster than *psbA*. However, one must bear in mind that the *psbA* tree was constructed with uncorrected distances. This was because *psbA* trees constructed with the distance settings recovered by Modeltest were not congruent with the 24S rRNA gene tree. This may have been due to a limited dataset from which the precise model of DNA evolution could not be accurately assessed. Increased confidence was therefore placed in the *psbA* tree constructed with uncorrected distances. The recovery of congruent *psbA* and 24S rRNA gene trees in the present study is encouraging. As *psbA* codes for the D1 protein, which has been implicated in bleaching susceptibility (Warner *et al.* 1999), and as concerted evolution has led to the core regions of ehloroplast minicircles being highly conserved within species (Howe *et al.* 2003), future research should be motivated by the need to sequence the entire chloroplast minicircle on which *psbA* is carried. The potential rewards from this new approach include not only the ability to distinguish the species of *Symbiodinium* with which a host forms an association, but also the ecological implications of that association.

Chapter 4

Resilience to Bleaching

4.1 Introduction

Bleaching, the paling of zooxanthellate tissues resulting from the drastic decline in zooxanthellal densities (e.g. Hoegh-Guldberg & Smith 1989) and/or the loss of photosynthetic pigments (e.g. Kleppel *et al.* 1989, Szmant & Gassman 1990), has long been recognized as a generalised response to stress (Brown 1997, Glynn 1993). As such, it is elicited by a variety of environmental and laboratory stressors. Greatest emphasis has been placed on identifying the physiological determinants of bleaching in response to elevated seawater temperatures. This is justifiable, given that elevated sea surface temperatures (SST), often combined with increased solar radiation (Brown *et al.* 2000a, Rowan *et al.* 1997, Glynn 1993), has led to the mass bleaching and mortality of reef corals after the 1980's (Hoegh-Guldberg 1999, Glynn 1993, Brown 1997), with severe impacts to tropical coastal communities (Wilkinson 1999, Hoegh-Guldberg 1999). Nonetheless, localised bleaching in the field has been reported to occur in response to a range of stressors, including sedimentation (Bak 1978), oil pollution (Guzman *et al.* 1991), reduced salinity (Goreau 1964), reduced water temperature (Kobluk & Lysenko 1994) and aerial exposure (Yamaguchi 1975). The underlying mechanisms of bleaching in response to the majority of known environmental triggers remain poorly defined (Douglas 2003). For any given zooxanthellate symbiosis, the different triggers of bleaching are predicted to have different impacts on the zooxanthella, the animal host, and symbiotic interactions between the two partners (Douglas 2003). Thus, the mechanisms and symptoms of bleaching are likely to vary with the specific trigger. Consequently, recovery processes are also likely to be influenced by the nature of the bleaching stressor.

Two bleaching-stressors that have been widely used to induce bleaching in laboratory studies are elevated seawater temperatures (e.g. Ralph *et al.* 2001, Dunn *et al.* 2002, Gates *et al.* 1992, Perez *et al.* 2001, Warner *et al.* 1996) and prolonged incubation under darkness (e.g. Titlyanov *et al.* 2002, Wang & Douglas 1998, Wang & Douglas 1999, Lewis & Coffroth 2004). Bleaching arising from exposure to elevated temperatures has most frequently been attributed to damage to the photosynthetic apparatus of the zooxanthellae (Warner *et al.* 1996, Warner *et al.* 1999, Jones *et al.* 1998, Jones *et al.* 2000, Iglesias-Prieto *et al.* 1992). Laboratory investigations have also demonstrated

damage to host tissues, particularly in the endoderm, during periods of exposure to elevated seawater temperatures (Gates *et al.* 1992, Dunn *et al.* 2002). These findings are in line with reports describing the histology of corals in the aftermath of natural temperature-mediated bleaching incidents (Hayes & Bush 1990, Glynn *et al.* 1985, Lasker *et al.* 1984). In contrast to this, prolonged exposure to darkness is not known to cause direct damage to either the photosynthetic machinery of zooxanthellae or animal tissues. Although concerns can be raised legitimately as to the relevance of using darkness as a laboratory stressor to induce bleaching, the major advantage in this is that it allows one to explore recovery of zooxanthellal populations without the confounding factor of direct damage to host tissues.

The onset of bleaching is thought to be a function of cumulative heat stress, i.e. not only is the magnitude of the stressor (e.g. positive SST anomaly) important in the incidence of bleaching, but so too is the duration for which a bleaching stressor persists (Gleeson & Strong 1995, Podestá & Glynn 1997, Winter *et al.* 1998). High values for indices assimilating duration and overall magnitude of the bleaching stressor, for example degree heating weeks (Gleeson & Strong 1995) and degree heating days (Podestá & Glynn 1997), were shown to correlate well with the incidence of bleaching. Critical values for such indices have been proposed as thresholds in excess of which bleaching may occur at the respective locations (Gleeson & Strong 1995). The duration of the bleaching stressor is also widely thought to influence recovery processes. This is reflected in statements such as "*If the stress is not too severe or prolonged, the affected corals often regain their usual complement of zooxanthellae, with normal pigmentation returning after about 1 or 2 months. If the stress continues, then the corals will die*" (Podestá & Glynn 1997). This conclusion is based largely on anecdotal reports from the field. Perhaps the logistical difficulties of appropriate experimental designs have precluded the empirical testing of this hypothesis.

East African reefs underwent mild-moderate bleaching in April 2003. Bleaching extended between Kiunga in northern Kenya (Julie Church, personal communication) and northern Tanzania in the south (David Obura, personal communication). The prevailing weather conditions in the region at the time were characterised by elevated temperatures and calm winds, and these were thought to be key factors in bringing about bleaching. *Porites cylindrica*, the species used for bleaching experiments in the laboratory, bleached to varying extents on Kanamai Reef. Coral fragments were

collected from colonies that were visually categorised as bleached, partially bleached and unbleached. These were transferred to the laboratory where the recovery of their zooxanthellal populations could be monitored. In so doing, it was hoped that the resultant recovery profiles would assist in relating the results of laboratory experiments to the incidence of natural bleaching in the field.

The major aim of the work described in this chapter was to investigate the influence of the nature of the bleaching stressor on recovery of zooxanthellal populations in bleaehed corals. Specifically, docs the nature of the bleaching stressor influence resilience, i.e. the eapacity to recover from bleaching? For the purposes of this study, resiliencc was defined as described in section 1.4.2 and illustrated in Figure 1.1 (chapter 1). Elevated seawater temperature and prolonged darkness were selected as bleaching stressors based on the different impacts they have on zooxanthellae and animal hosts, and on their widespread use in laboratory studies on bleaching. A secondary aim was to establish whether the duration over which a stressor is applied influences resilience to bleaching.

Objectives were as follows:

1. Experiments 1 and 2: To use varying durations of darkness (experiment 1: 5 -25 days; experiment 2: 7-21 days) to induce bleaching in *Porites cylindrica*. Thereafter, to monitor zooxanthellal densities and division frequencies (reeovery profiles) of treatment eorals and treatment control eorals for a period of 84 days (experiment 1) or 42 days (experiment 2) after termination of dark-treatment.
2. Experiment 3: To induee bleaehing in *P. cylindrica* by exposure to varying durations (48-96 hours) of seawater at a temperature elevated above ambient seawater temperature (treatment temperature: 32.5°C; ambient temperature: approximately 28°C). After termination of treatment, to monitor reeovery profiles of treatment corals and treatment control corals for 63 days.
3. Experiment 4: To monitor the reeovery profiles of bleached, partially bleached and unbleached *P. cylindrica* fragments for a period of 63 days after collection from the field.
4. For all experiments above, to use PCR-RFLP and sequenee analysis of *Symbiodinium* 24S rRNA genes to track changes in zooxanthellae before bleaehing, immediately after bleaching and on reeovery from bleaching.

4.2 Results

4.2.1 Experiment 1: Resilience of Zooxanthellae to Bleaching Elicited by Varying Durations of Darkness

Coral used for this experiment were bleached by treatment with darkness for varying durations (24 hr dark: 5 days, 10 days, 15 days, 20 days, 25 days). Zooxanthellal densities were monitored for a period of 84 days after treatment was terminated.

Results are displayed in Figure 4.1. The experiment ran just for under 110 days, during which the densities of zooxanthellae in treatment control corals (ambient light; 12 hr dark: 12 hr light) steadily declined from a mean of 3.14×10^6 cells cm^{-2} to 1.34×10^6 cells cm^{-2}. Densities of zooxanthellae in treatment corals, relative to densities in treatment control corals, declined with increasing duration of treatment. On termination of treatment, i.e. at the start of the recovery phase, densities of zooxanthellae in treatment corals ranged from approximately 83% of that in treatment control corals (for corals treated with darkness for 5 days) to 11% of the densities in treatment control corals (for corals subjected to 25 days of darkness). The degree to which treatment corals had undergone a decline in densities of zooxanthellal populations corresponded with the colouration of coral tissues on their return to ambient light, with corals treated for 25 days appearing visibly paler than those treated for 20 days, and so on. However, by the end of the experiment owing largely to recovery of zooxanthellal populations in bleached corals, and in part due to the decline in zooxanthellal density of treatment control corals, there were no significant differences in zooxanthellal densities between treatments (including treatment control corals; one-way ANOVA: $F_{5, 18} = 0.75$, $p = 0.595$) and corals from all treatments were of a uniform rich-brown colouration. The visual appearance and behavioural responses of all the corals used in this experiment suggested that they remained healthy throughout, extending their tentacles, presumably to feed, whenever tanks were supplied with fresh seawater from the reservoir. The mean percent of dividing zooxanthellae, shown in Figure 4.1, varied between 0.65% and 5.75% during the experiment, indicative of a healthy population.

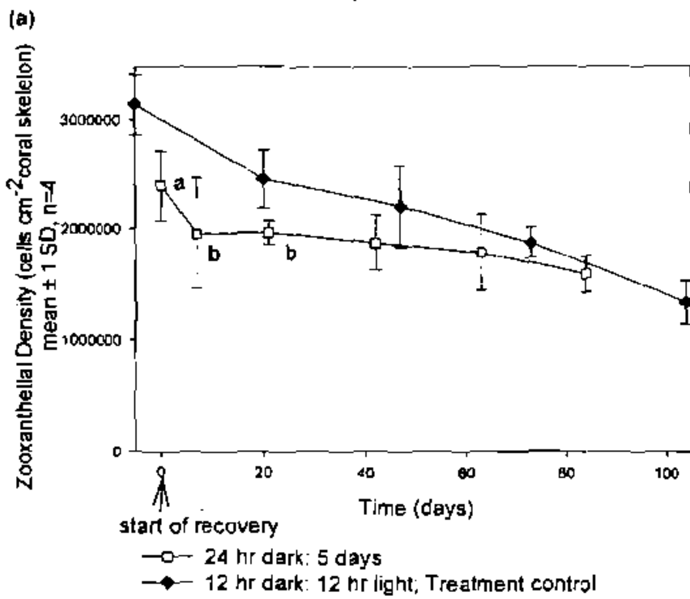
It was not known to what extent responses of corals that had been housed under artificial conditions for an extended period would provide meaningful information related to the process of recovery from bleaching. On the other hand, the initial responses of corals were considered critical, particularly when viewed in the context of recently bleached corals in a competitive reef environment once a stressor had abated.

Furthermore, treatment control corals exhibited a progressive decline in zooxanthellal density during the experiment. Data were therefore analysed for the first 21 days after successive groups of treatment corals were returned to ambient light (12 hr dark: 12 hr light). Resilience, the capacity for zooxanthellal populations to recover from bleaching, was assessed by changes in the density of zooxanthellae during this *early* phase of recovery. Corals that underwent a continued loss of zooxanthellae after return to ambient light were defined as less resilient, and those that exhibited an earlier onset of recovery of zooxanthellal densities more resilient. Data were analysed by two-way ANOVA, as shown in Figure 4.1. There were significant differences between the different durations of treatment, and the recovery responses of corals over time were significantly different for different durations of treatment, as indicated by the significant interaction term. Corals subjected to darkness for 5 days were the least resilient, undergoing a significant decline in zooxanthellal density between days 0 and 7, from a mean density of approximately 2.4 x 10^6 cells cm^{-2} to a mean of 1.96 x 10^6 cells cm^{-2}, with no significant changes thereafter. Corals treated for 20 days and 25 days had the greatest resilience, each displayed significant increases in zooxanthellal densities between days 7 and 21, from a mean of approximately 0.58 x 10^6 cells cm^{-2} to 1.36 x 10^6 cells cm^{-2}, and 0.39 x 10^6 cells cm^{-2} to 0.95 x 10^6 cells cm^{-2} respectively. Corals treated for intermediate durations, i.e. 10 days and 15 days showed no significant changes in zooxanthellal densities over the period analysed.

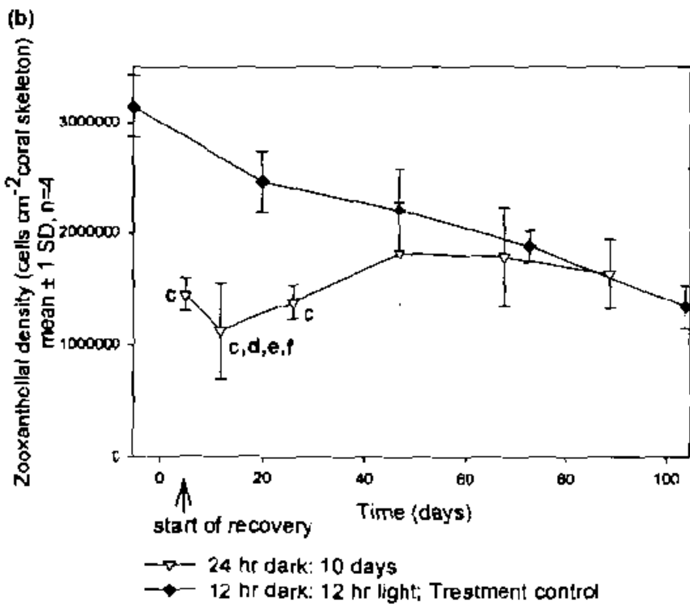
Percentages of dividing zooxanthellae, which varied between means of 0.65% and 5.75%, are also shown in Figure 4.1. A two-way ANOVA on data for the recovery interval of 21 days after treatment corals were returned to ambient light was performed. Results show that percentage division of zooxanthellae through time was dependent on the duration of treatment as indicated by the highly significant interaction term. Corals treated for 5 days underwent a significant decline in the percent of dividing zooxanthellae between days 7 and 21, from a mean of approximately 2.6% to 0.7%. There were no significant changes in the percent of dividing zooxanthellae for corals treated for 10 days over the period examined. Corals treated for 15 days and 25 days each displayed significant increases in the percent of dividing zooxanthellae between days 0 and 7 (from 1.7% to 3.4%, and 0.7% to 2.5% respectively), and no significant changes thereafter. Corals incubated under darkness for 20 days showed an increase in the percent of dividing cells between days 7 and 21 (0.9% to 4.1%).

To explore the influence of cell division on zooxanthellal densitics, the density of zooxanthellae measured at any time (T_7) relative to the preceding time (T_1), i.e. [density at T_2 / density at T_1], was plotted against pereent of dividing cells at T_1 (data not shown). No evidence for a relationship between the two variables was uneovered, either for different levels of treatment or for data combined. Neither were there any apparent correlations between densities at T_1 and the percentages of dividing cells at T_1, either for treatments in isolation or for data combined. However, the highest percentages of dividing cells observed during the experiment occurred in corals that had been subjected to relatively long durations of darkness (5.75% for 20 days darkness and 5.68% for 25 days darkness, 63 days after return to ambient light for each).

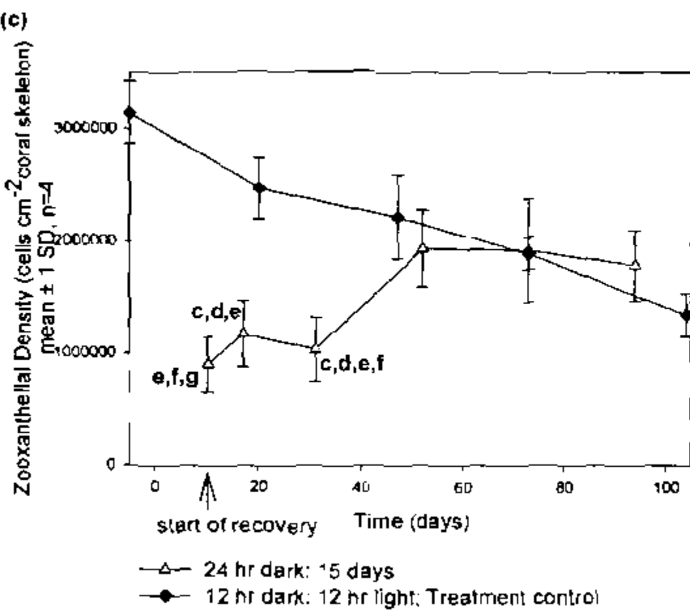
(a)



(b)



(c)



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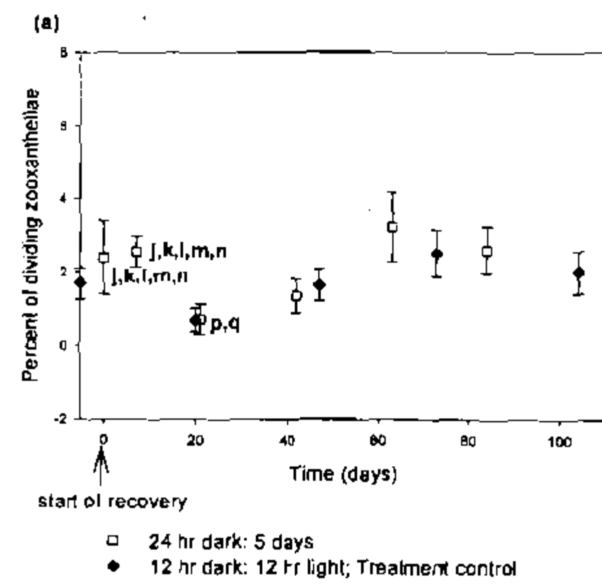

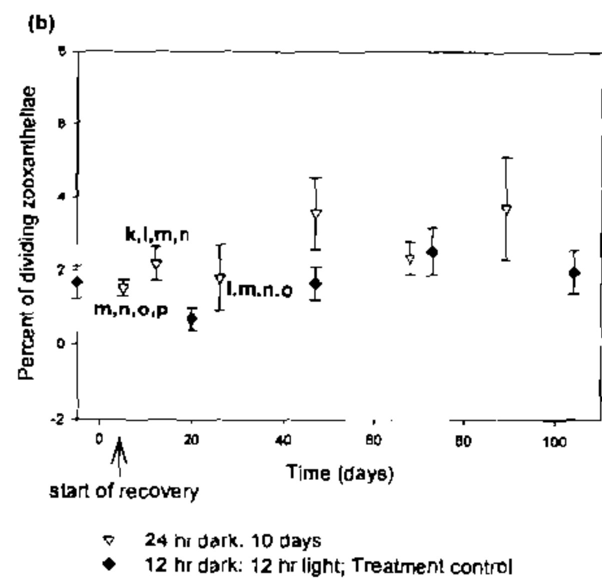

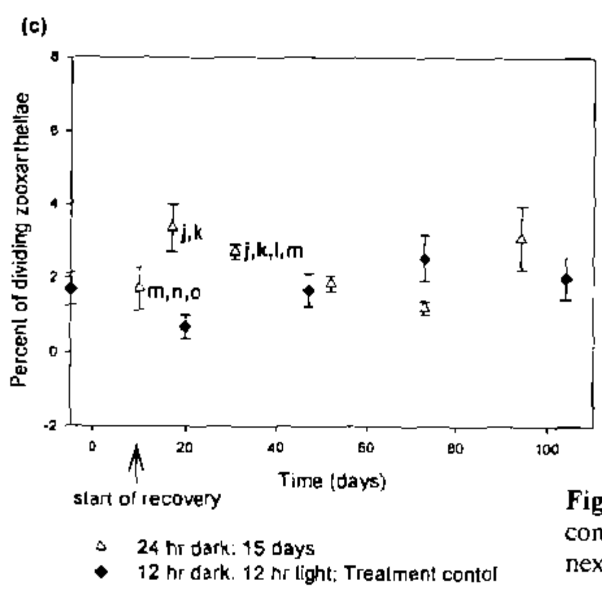

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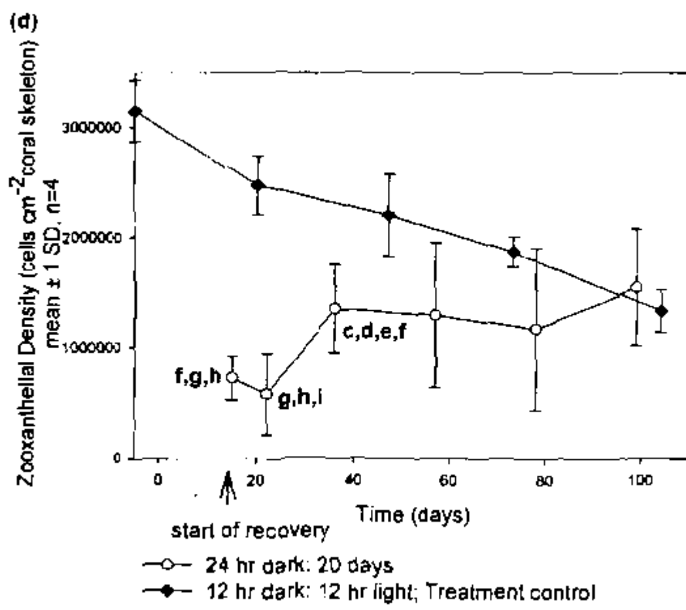

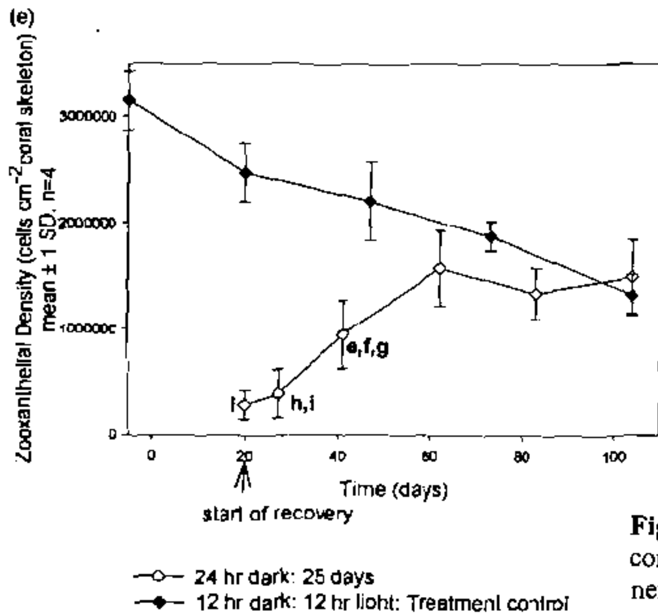

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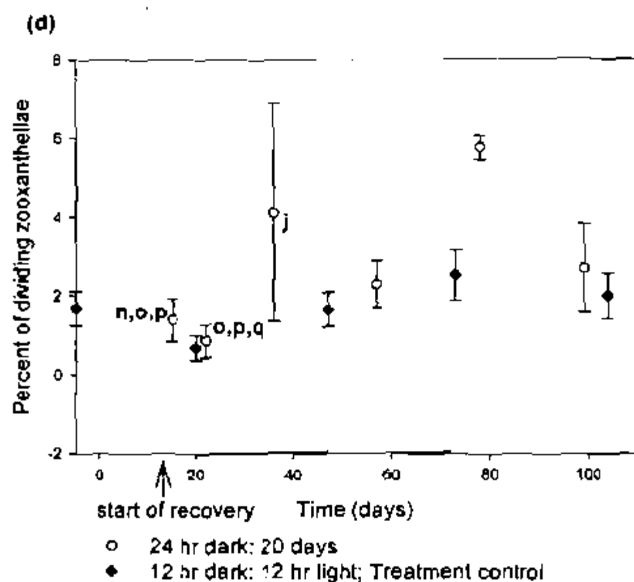
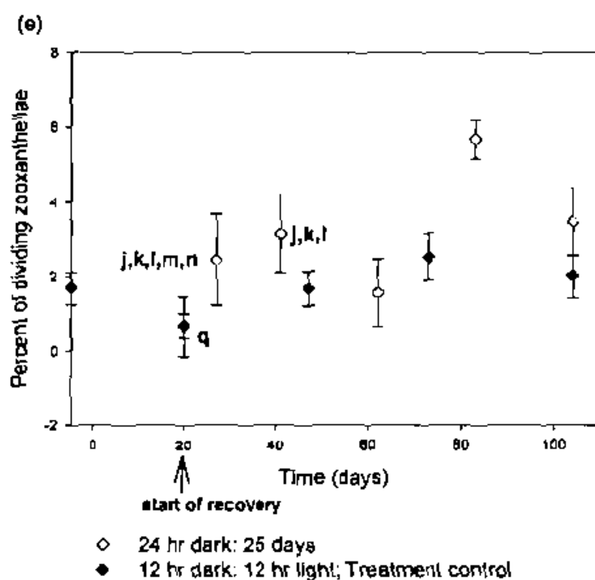
Factor	D.F.	F Ratio	P value
Treatment	4	47.37	< 0.001 ***
Time	2	4.97	< 0.05 *
Interaction	8	3.34	< 0.01 **
Error	45		





Factor	D.F.	F Ratio	P value
Treatment	4	1.62	0.186
Time	2	6.28	< 0.01 **
Interaction	8	7.26	< 0.001 ***
Error	45		

Figure 4.1: Densities (left column of graphs; mean values ± 1 SD) and division pereentages (right column of graphs) of zooxanthellae in corals recovering from bleaching elicited by treatment with darkness (open symbols) [24 hr dark: 5 days (a), 10 days (b), 15 days (c), 20 days (d), 25 days (e)]. The response of treatment control corals (closed symbols; 12 hr light: 12 hr dark) is shown on all graphs. Arrows mark times at which treatment corals were returned to ambient light (12 hr dark: 12 hr dark), i.e. recovery commenced. Results of two-way ANOVA performed on density and division data for treatment corals over the recovery interval of 21 days after return to ambient light are shown below the respective columns of graphs. Percentage data were arcsine-square root-transformed prior to analysis. Letters indicate homogeneous subsets from post-hoc analysis with Fisher's LSD test.

4.2.2 Experiment 2: Repetition of Varying Durations of a Dark Stressor

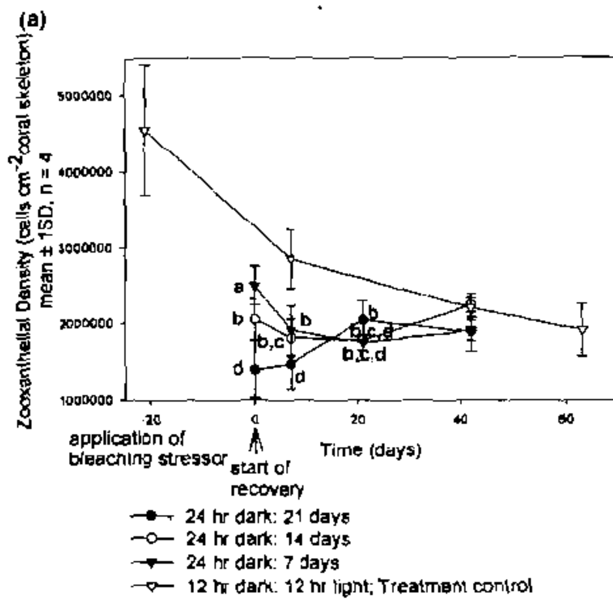
Varying durations of darkness (24 hr dark: 7 days, 14 days, 21 days) were used to bleach the corals used in this experiment. The design of this experiment differed from that of experiment 1 in that all treatment corals were returned to ambient light (12 hr light: 12 hr dark) on the same day, i.e. recovery commenced simultaneously for all levels of treatment. The densities of zooxanthellae in treatment corals were monitored for a period of 42 days after treatment was terminated. Results are shown in Figure 4.2.

Treatment corals underwent a progressive loss of zooxanthellae (and corresponding pigmentation), relative to treatment control corals, with increased duration of treatment. The greatest decline in zooxanthellal density consequently occurred in corals that had been incubated under darkness for the longest duration, i.e. 21 days. These had approximately 39% of the densities in treatment control corals on their return to ambient light. Recovery of zooxanthellal populations for the first 21 days of recovery was dependent on the duration of treatment as indicated by the highly significant interaction term in the two-way ANOVA [shown below Figure 4.2(a)]. The least resilient corals were those incubated in darkness for 7 days. These underwent a significant decline in zooxanthellal densities between days 0 (mean of approximately 2.50×10^6 cells cm^{-2}) and 7 (approximate mean of 1.91×10^6 cells cm^{-2}), with no significant changes thereafter. Corals treated for 14 days did not exhibit significant changes in zooxanthellal densities over the period analysed. The most resilient corals over the period examined were those held in darkness for 21 days, which displayed significant increases in the density of zooxanthellae between days 7 (1.47×10^6 cells cm^{-2}) and 21 (2.05×10^6 cells cm^{-2}).

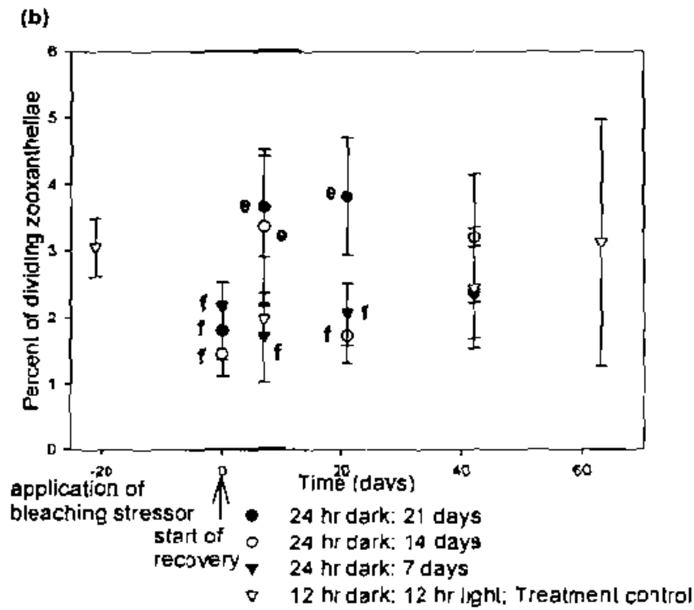
Percentages of dividing zooxanthellae, which varied between 1.5% and 3.8%, are shown in Figure 4.2 (b). A two-way ANOVA was carried out on cell division data for the first 21 days of recovery. Results show that the rate of cell division over the period analysed was dependent on duration of treatment, as indicated by the highly significant interaction term. Corals treated with 7 days of darkness did not display any significant changes in the percent of dividing zooxanthellae. Those subjected to 14 days of darkness first exhibited an increase in the percent of dividing cells between days 0 (1.5%) and 7 (3.4%), followed by a decline to 1.7% on day 21. Between days 0 and 7, there was a significant increase in dividing zooxanthellae in corals treated for 21 days, from a mean of 1.8% to 3.7%, with no significant changes in the following period.

To determine the extent to which cell division rates influenced zooxanthellal densities, [density at T_2 / density at T_1] was plotted against percent of dividing cells at T_1 (data not shown). No relationship was uncovered by this analysis. Neither were there any correlations between cell densities and percentages of dividing cells at T_1. However, the highest percentages (3.84%) of dividing cells observed during the experiment occurred after 21 days of recovery for corals subjected to the longest duration (21 days) of darkness.

Zooxanthellae in control corals and in corals assigned to 21 days dark-treatment were analysed by 24S PCR-RFLP (see chapter 3) to determine their phylotype. Samples were obtained before treatment, immediately on termination of treatment and 42 days after treatment was terminated. There were no changes in the phylotype of zooxanthellae (C) observed during the experiment, as summarized in Table 4.1.



Factor	D.F.	F Ratio	P value
Treatment	2	6.58	< 0.01 **
Time	2	2.47	0.103
Interaction	4	6.60	0.001 ***
Error	27		



Factor	D.F.	F Ratio	P value
Treatment	2	9.72	0.001 ***
Time	2	8.61	0.001 ***
Interaction	4	7.22	< 0.001 ***
Error	27		

Figure 4.2: Zooxanthellal densities (mean values ± 1 SD) **(a)** and division percentages **(b)** in corals reeovering from bleaching elicited by varying durations of darkness (24 hr dark: 21 days, 14 days, 7 days) and in treatment control corals (12 hr light: 12 hr dark, 21 days). Arrows indicate when the treatment was terminated. Results of two-way ANOVA on density and arcsine-square root-transformed division data for treatment eorals over the recovery interval of 21 days after corals were returned to ambient light are shown below the respective graphs. Letters indicate homogeneous subsets from post-hoc analysis with Fisher's LSD test.

Table 4.1: Phylotype of zooxanthellae in corals prior to the application of a bleaching stressor, immediately on termination of the stressor, and after a period of recovery of zooxanthellal populations. Phylotypes were identified by PCR-RFLP of 24S rRNA genes. Phylotypes for naturally bleached and unbleached corals, on collection from the field and 63 days after eollection (experiment 4) were also determined. No data were available for laboratory-held naturally bleached corals 63 days after collection due to unsuccessful PCR-amplification of 24S rRNA genes. Numbers indicate the number of samples that were identified.

Expt.	phylotype before treatment	treatment/duration	phylotype after treatment	recovery (days)	phylotype after recovery
2	2 C	24 hr dark: 21 days	2 C	42	2 C
3	2 C [1]	32.5°C: 96 hr	2 C	63	2 C [1]
2 & 3	2 C	Treatment controls			2 C
4		Field stressors:			
		Bleached	2 C [2]	63	1 C [2], 2 dead (field) no data for lab
		unbleached	3 C [2]	63	3 C [2] (field) 3 C (lab)

[1] 24S rRNA gene sequences of 2 samples were obtained; haplotype 12 (chapt. 3)

[2] 24S rRNA gene sequence of 1 sample was obtained; haplotype 12

4.2.3 Experiment 3: Resilience of Zooxanthellae to Bleaching Induced by Exposure to Varying Durations of Elevated Seawater Temperature

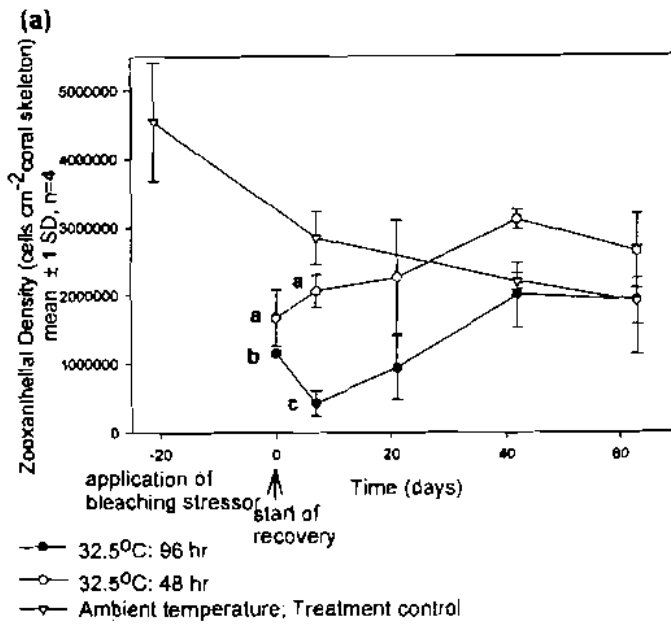
Corals used in this experiment were bleached by exposure to seawater at a temperature (32.5°C) elevated above ambient temperatures (approximately 28°C) for either 48 hours or 96 hours. The densities of zooxanthellae in treatment corals were observed over a period of 63 days after termination of treatment. Results are shown in Figure 4.3.

At the start of recovery, treatment corals had densities that varied between approximately 47% (for 48 hour treatment corals) and 32% (for 96 hour treatment corals) of that in treatment control corals. By day 42 of recovery however, the 48 hour treatment eorals had significantly higher zooxanthellal densities than either the 96 hour treatment corals or treatment control corals, both of which did not differ significantly by that stage (one-way ANOVA: $F_{2,9} = 15.86$, $p < 0.01$ **). A two-way ANOVA for the recovery interval 'days 0-21' was non-significant for the interaction between treatment and time ($F_{2,18} = 3.34$, $p = 0.058$, data not shown). However, there was a highly significant interaction term when data were analysed for an *earlier* phase of reeovery, i.e. recovery interval 'days 0-7', suggesting that changes in the density of zooxanthellae in the initial period following exposure to ambient temperature was dependent on the duration of treatment. There were no significant changes to the density of zooxanthellae in 48 hour-treated corals between days 0 and 7. However, corals treated for 96 hours were less resilient, displaying a significant decline in zooxanthellal density between days 0 (1.15×10^6 cells cm^{-2}) and 7 (0.43×10^6 cells cm^{-2}).

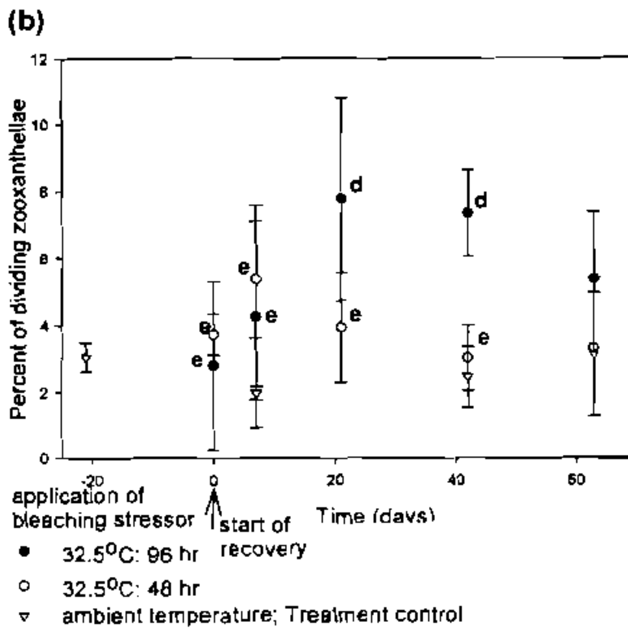
The percent of dividing zooxanthellae is shown in Figure 4.3 (b). Analysis by two-way ANOVA for the recovery interval 'days 0-21' was non-significant for the interaction between treatment and time ($F_{2,18} = 3.02$, $p = 0.074$). When data were analysed by for the recovery interval 'days 0-42' however, there was a significant interaction term. The percent of dividing zooxanthellae incrcased markedly for corals treated for 96 hours, from a mean of approximately 2.8% on day 0, to a mean of approximately 7.8% on day 21. These were more than double the maximum division rate observed for experiment 2 (maximum mean of 3.8% for 21 days dark-treated corals) in which the same coral colonies were utilised but for which darkness instead was used as a bleaching stressor. The division rates of 48 hour-treated corals did not significantly change over the period for which the analysis was performed.

To investigate the extent to which zooxanthellal densities were influenced by rates of zooxanthellal division, [density at T_2 / density at T_1] was plotted against pereent of dividing eells at T_1 (data not shown). No relationship was uncovered by this exploration of data. Corals that had been subjected to the longest duration of treatmcnt (96 hours) with elevated temperature also exhibited the highest percent of dividing zooxanthellae recorded (7.8%) during the experiment. Howcver, no correlation between the density of zooxanthellae (at T_1) and the percent of dividing cells (at T_1) was apparent.

Zooxanthellal phylotype and 24S rRNA gene sequences in corals treated for 96 hours was determined by the molecular methods discussed in chapter 3. The phylotype remained constant throughout the cxperiment, as outlined in Table 4.1. Moreover, zooxanthellae sequences at thc beginning and at the end of the experiment were identical (haplotype 12), confirming that there werc no apparent changes in thc type of zooxanthellae hosted beforc and after recovery from experimental bleaching.



Factor	D.F.	F Ratio	P value
Treatment	1	70.71	< 0.001 ***
Time	1	1.72	0.215
Interaction	1	18.85	0.001 ***
Error	12		



Factor	D.F.	F Ratio	P value
Treatment	1	2.24	0.147
Time	3	2.24	0.109
Interaction	3	4.37	< 0.05 *
Error	24		

ANOVA on zooxanthellal density data for treatment corals for the recovery interval of the first 7 days after return to ambient temperatures are shown below the respective graph. Percentage data were analysed for the first 42 days of recovery and were arcsine-square root-transformed prior to the use of ANOVA. Letters indicate homogeneous subsets from post-hoc analysis with Fisher's LSD test.

4.2.4 Experiment 4: Resilience of Zooxanthellae to Natural Bleaching

Corals used for this experiment were collected from the reef having bleached to varying extents through exposure to natural bleaching stressors, most likely a combination of elevated temperature and light. These were categorized as bleached, partially bleached and unbleached, as shown in Figure 4.4.

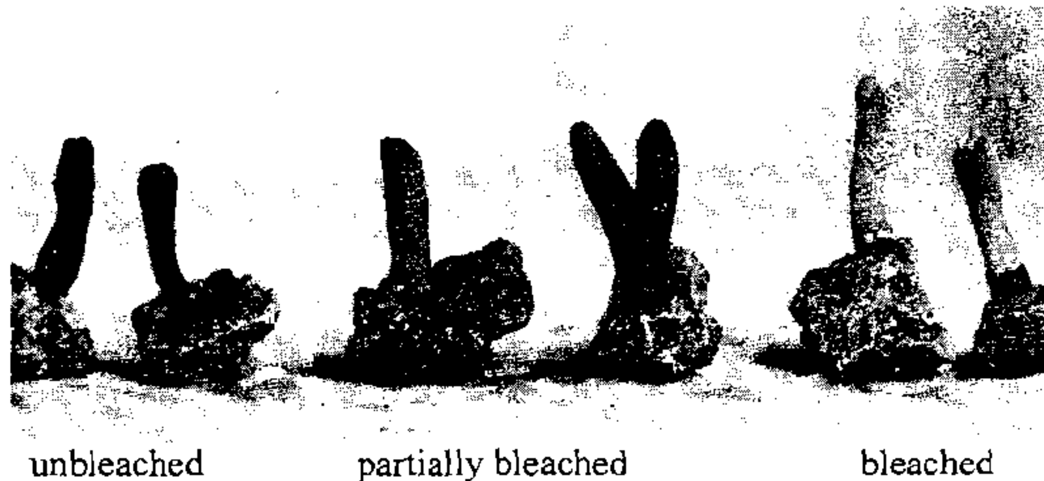
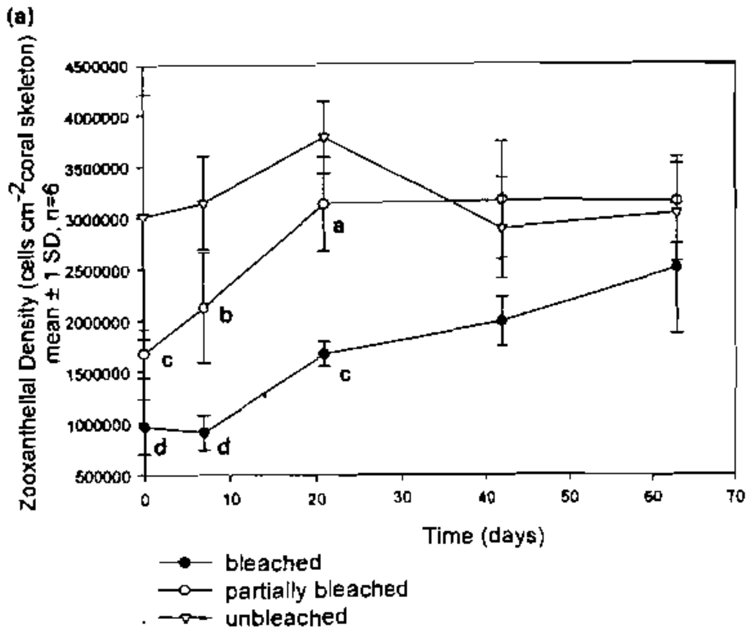


Figure 4.4: Fragments of corals that had bleached to varying extents on Kanamai Reef in April 2003. Fragments were visually categorised as being bleached, partially bleached or unbleached.

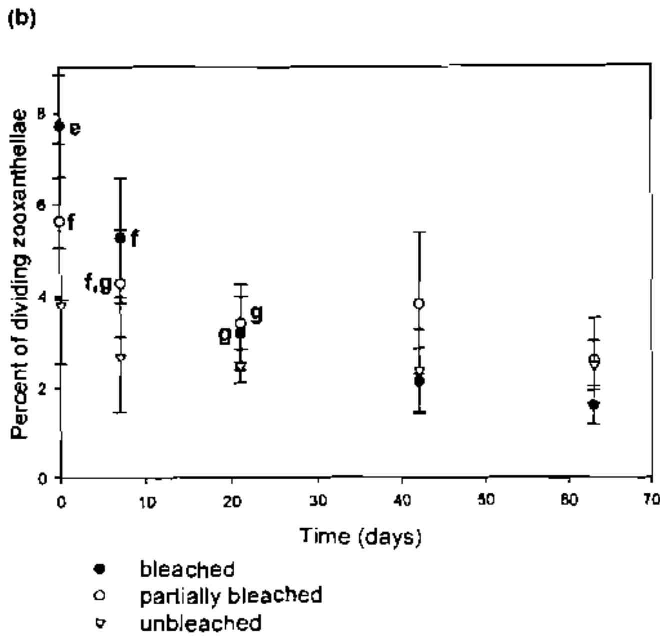
On collection from the reef, bleached and partially bleached corals had 32% and 55% respectively of the zooxanthellal population densities in unbleached corals. At the end of the experiment, however, bleached and partially bleached corals had recovered their zooxanthellal populations; there were no significant differences in the densities of zooxanthellae between bleached, partially bleached and unbleached corals (one-way ANOVA: $F_{2, 15} = 2.76$, $p = 0.095$). Changes in zooxanthellal density and the percent of dividing cells were monitored for 63 days, shown in Figure 4.5 (a) and (b) respectively. A two-way ANOVA for bleached and partially bleached corals (unbleached corals were excluded from analysis to prevent violation of the assumption for normal distribution of data) for the recovery interval 'days 0-21' was highly significant for treatment and time, but not for the interaction term. The density of zooxanthellae in partially bleached corals significantly increased from a mean of approximately 1.67×10^6 cells cm^{-2} on day 0 to 2.12×10^6 cells cm^{-2} on day 7 and further to 3.13×10^6 cells cm^{-2} on day 21. Bleached corals were less resilient, only exhibiting an increase in density of zooxanthellae after day 7.

correlation between the density of zooxanthellae and the percent of dividing zooxanthellae at any given time [bleached corals: $r = -0.906$, $F_{1,3} = 13.68$, $p < 0.05$ *; partially bleached corals $r = -0.898$, $F_{1,3} = 12.44$, $p < 0.05$ *; data combined (including unbleached corals) $r = -0.681$, $F_{1,13} = 11.25$, $p < 0.01$ **].

Molecular analysis of zooxanthellae, summarized in Table 4.1, revealed no discernible changes to the type of zooxanthellae hosted by bleached and unbleached corals in the field during the course of the experiment, although no data were available for laboratory samples of bleached corals due to unsuccessful attempts at PCR-amplification of 24S rRNA genes. It is important to note that a large percentage of colonies that had bleached naturally on the reef underwent mortality (estimated at 75%). When samples from bleached colonies were required from the field for molecular analysis of their zooxanthellae 63 days after they were first sampled, two of the three colonies originally sampled had died. However none of the partially bleached and unbleached colonies originally sampled had suffered from field-mortality. Corals originally sampled from the reef, including bleached corals, remained healthy throughout the experiment under laboratory conditions.



Factor	D.F.	F Ratio	P value
Treatment	1	112.23	< 0.001 ***
Time	2	36.52	< 0.001 ***
Interaction	2	1.95	0.160
Error	30		



Factor	D.F.	F Ratio	P value
Treatment	1	4.89	< 0.05 *
Time	2	23.64	< 0.001 ***
Interaction	2	2.44	0.105
Error	30		

Figure 4.5: Zooxanthellal densities (mean values ± 1 SD) **(a)** and division percentages **(b)** in corals collected from the reef in unbleached, partially bleached and bleached states. Results of two-way ANOVA on square root-transformed zooxanthellal density data, and arcsine-square root-transformed zooxanthellal percentage division data for bleached and partially bleached corals over the recovery interval 'days 0-21' are shown below the respective graphs. Letters indicate homogeneous subsets from post-hoc analysis with Fisher's LSD test.

4.3 Discussion

4.3.1 Processes in Recovery from Bleaching

Essential to the interpretation of results described in this chapter is an understanding of the fundamental processes by which corals that have undergone bleaching recover their zooxanthellal populations. These are briefly considered as follows:

1) An increased rate of cell division in zooxanthellae, as has previously been reported for bleached corals (Jones & Yellowlees 1997, Fitt *et al.* 1993), in combination with:
2) The division of infected host cells and distribution of their resident zooxanthellae to daughter cells (Berner *et al.* 1993), as shown in Figure 4.6a.
   and/or
3) The expulsion by exocytosis of zooxanthellae from infected endoderm cells into the gastric cavity, and their subsequent uptake by uninfected host cells (Jones & Yellowlees 1997). These could be vacant cells that had lost zooxanthellae during bleaching but that remain competent (i.e. infectible), and/or newly differentiating from stem cells replacing host cells lost during the bleaching event. This process is illustrated in Figure 4.6b.

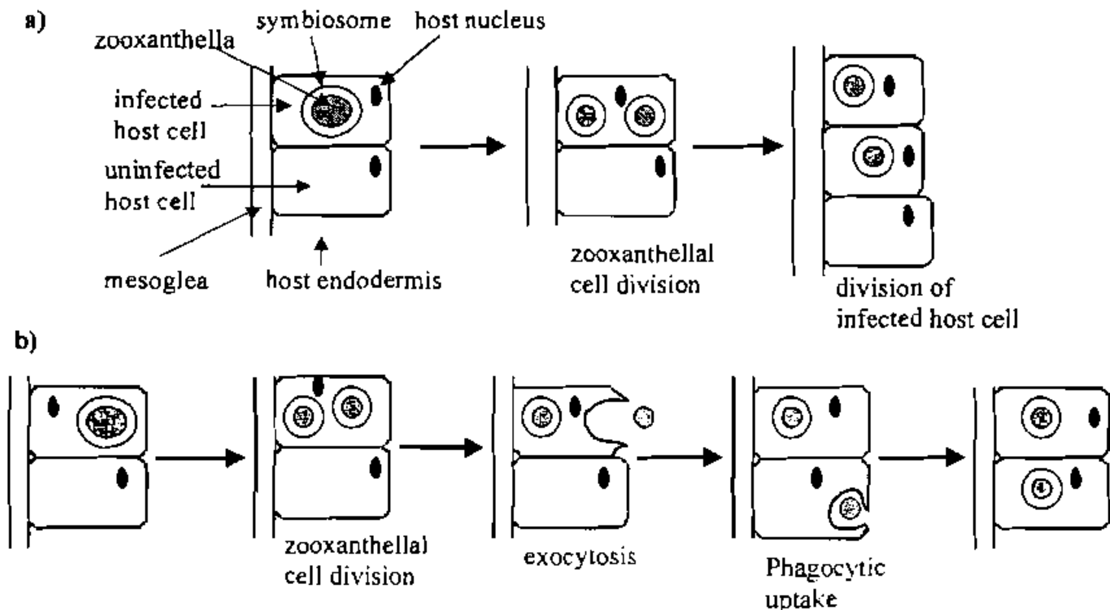


Figure 4.6: Processes occurring in the host endodermis during recovery from bleaching. **a)** The division of host cells and distribution of their resident zooxanthellae to daughter cells. b) The division, expulsion and subsequent uptake of zooxanthellae.

4.3.1.1 Recovery from Bleaching Induced by Darkness

An important consequence of prolonged exposure of corals to darkness is the reduction in cell division rate of residual zooxanthellae, without a corresponding decline in the growth and division of host eells. This is partly evidenced by the variation in the rates of zooxanthellal division in corals subjeeted to different durations of darkness, immediately on their return to ambient light. For instance, in experiment 1, the zooxanthellae subjected to relatively short durations of darkness (5 days) were dividing at a mean of 2.4% on day 0, higher than that of zooxanthellae in corals incubated under darkness for longer durations (e.g. 20 days: mean 1.4%, 25 days: mean 0.7%). Similar results were obtained for experiment 2, in which although the differences were not statistically significant, the zooxanthellae in corals treated with darkness for a relatively short duration (7 days: mean 2.2%) had a higher mean pereent of cell division on day 0 than those treated for longer durations (14 days: mean 1.5%, 21 days: mean 1.8%). The primary outcome of the eontrasting effects of darkness on the division of host cells and zooxanthellae is predicted to be a change in the ratio of uninfected host cells to residual zooxanthellae on the return of corals to ambient light conditions. The longer the exposure of corals to darkness, the larger this ratio is likely to be. This is illustrated in Figure 4.7.

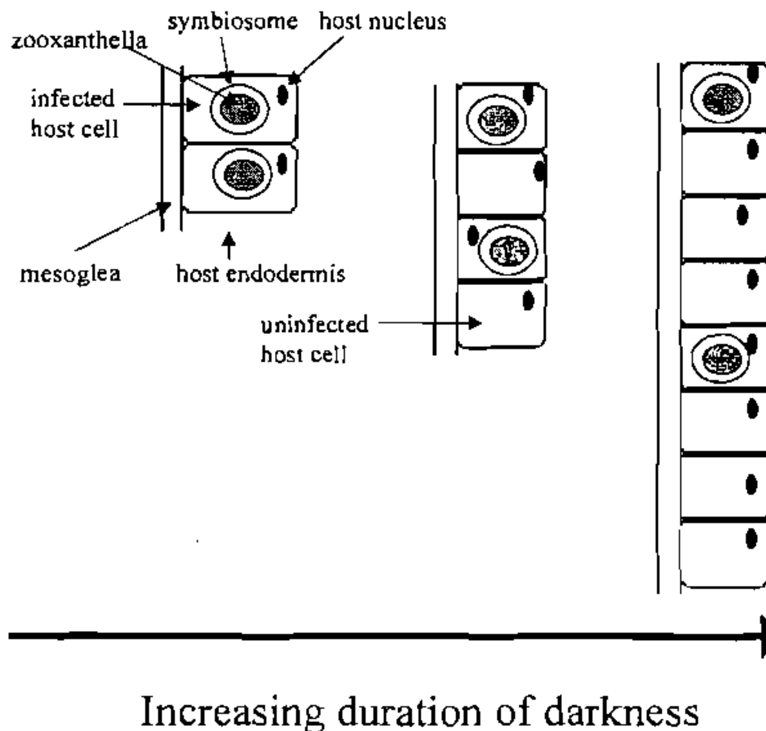


Figure 4.7: The predicted impaet of duration of darkness on the ratio of uninfected host cells to zooxanthellae.

Factors that influence the rates of division of zooxanthellae include the availability of space (Smith & Muscatine 1999), host-derived nutrients including nitrogen (Muscatine *et al.* 1998, Falkowski *et al.* 1993) and/or possibly carbon (Douglas 1994), and light; the experiments were conducted indoors, where the light levels, albeit unmeasured, were low in comparison to that which corals are likely to have experienced in their natural environment. The extent to which these factors limit the division of zooxanthellae is directly related to the density of zooxanthellae in coral tissues. In turn, zooxanthellal density, as a consequence of the inhibitory effect of darkness on zooxanthellal division, was inversely proportional to the length of exposure to darkness. It follows therefore, that the rate of zooxanthellal division in corals subjected to relatively short durations of darkness (occurring at relatively high densities) would undergo a decline, thereby bringing about a *density-dependent* reduction in zooxanthellal densities. Conversely, the above-mentioned factors, and in particular available space, i.e. the ratio of uninfected host cells to residual zooxanthellae, would not have limited the division of zooxanthellae in corals that were incubated under darkness for relatively long durations (occurring at relatively low densities). Under these conditions, zooxanthellae would proliferate. Their release into the gastric cavity, and subsequent uptake by uninfected host cells would bring about the repopulation of bleached tissues, as would an elevation in the rates of division of infected cells. The observed data on the division of zooxanthellae during the early stages of recovery are consistent with this explanation. For instance, in experiment 1, the zooxanthellae in corals treated with darkness for 5 days underwent a significant decline in percent of dividing cells, from a mean of 2.4% to 0.7% between days 0 and 21. During the same period, the percent of dividing zooxanthellae in corals incubated under darkness for 20 days and 25 days significantly increased from a mean of 1.4% to 4.1%, and from a mean of 0.7% to 3.2%, respectively. The same trend is evident in experiment 2, in which the percent of dividing zooxanthellae significantly increased between days 0 and 7 in corals subjected to treatment with darkness for 14 days (mean of 1.5% to 3.4%) and 21 days (mean of 1.8% to 3.7%), but declined, although not statistically significantly, for those in corals incubated under darkness for a period of 7 days (mean of 2.2% to 1.7%). In the context of the present study using darkness as a bleaching stressor, the observed changes in zooxanthellal density for the different levels of treatment would have been defined as elevated or diminished resilience. Resilience however, may not be an appropriate term to describe the *density dependent regulation* of zooxanthellal populations during exposure of corals to darkness, as the observed dynamics might not relate precisely to the capacity to recover from bleaching. This can be developed into a testable hypothesis.

An alternative explanation to account for the observed pattern of resilience to dark-induced bleaching may lie in the existence of a heterogeneous population of *Symbiodinium* in which dark-susceptible zooxanthellae declined and dark-resistant zooxanthellae were retained with sustained exposure to darkness. The zooxanthellae in experiment 2 were assayed by PCR-RFLP, and no changes in phylotype occurred during bleaching (see Table 4.1). However, this does not preclude the possibility of genetic variation in zooxanthellae not evident at the level of phylotype. An important connotation of zooxanthellal heterogeneity is that acclimatory changes are predicted on recovery, as corals subjected to relatively long durations of darkness would recover from bleaching by the proliferation of a residual population in which a large proportion of individuals are resistant to the stressor. Acclimation to darkness is investigated in experiment 5, which is discussed in the following chapter.

4.3.1.2 Recovery from Bleaching Induced by Elevated Temperature

The responses of zooxanthellal populations to treatment with elevated temperature displayed the opposite trend to those of zooxanthellae subjected to darkness. Not only were corals that were exposed to elevated temperature for a relatively short duration (48 hours) more resilient to bleaching than those exposed for a longer duration (96 hours), but they also exhibited an 'overshoot' of zooxanthellal populations relative to treatment controls (see Figure 4.3). In contrast, zooxanthellae in corals treated for 96 hours continued to undergo a decline in population density on their return to ambient temperatures. The zooxanthellal densities of 96-hour treatment corals did not exceed those in treatment control corals at any time during the experiment.

Damage to the photosynthetic apparatus of zooxanthellae is widely believed to be the primary determinant of bleaching during exposure to elevated seawater temperatures (Warner *et al.* 1999, Jones *et al.* 1998, Jones *et al.* 2000). Primary cellular mechanisms for the ensuing decline in zooxanthellal densities include the degradation of zooxanthellae *in situ* and the release of zooxanthellae into the gastric cavity by exocytosis (Brown *et al.* 1995). Some laboratory studies have recently challenged this perspective. Notably, the laboratory study by Dunn and colleagues (2002) using the sea anemone *Aiptasia* sp., demonstrated that the swelling and rupture of host endodermal cells caused by tissue necrosis during hyperthermal treatment was a key factor mediating the release of apparently healthy zooxanthellae into the gastric cavity. The authors pointed out that an implication of necrotic damage [as opposed to programmed cell death (PCD)] was that it

was extrinsically mediated, and not under direct host control. Necrosis and PCD of zooxanthellae, resulting in their degeneration *in situ*, did however accompany damage to host tissues after prolonged exposures to elevated temperatures. Similarly, another laboratory study (Ralph *et al.* 2001) indicated that the zooxanthellae released by the coral *Cyphastrea serailia* during temperature mediated bleaching (at 33°C) were photosynthetically competent, and only suffered from impairment to photosynthesis after the temperature was greatly elevated (to 37°C). The tissue necrosis of host endoderm indicated by laboratory studies on temperature mediated bleaching has also been observed during histological examination of corals that had undergone elevated temperature-mediated bleaching in the field (Glynn *et al.* 1985, Lasker *et al.* 1984). Zooxanthellae of normal appearance were observed in all but the most affected specimens (Glynn *et al.* 1985).

An alternative mechanism by which the structural integrity of host endodermis can be compromised is the detachment and release of intact endoderm cells with their entire complement of zooxanthellae into the gastric cavity. This has been proposed, based on laboratory experiments, as a dominant mechanism for temperature-induced bleaching (Gates *et al.* 1992, Sawyer & Muscatine 2001). A combination of epifluorescence and electron microscopy were used to detect detached viable host cells enclosing symbiosomal membrane-bound zooxanthellae (Gates *et al.* 1992). The host membranes surrounding zooxanthellae disintegrated shortly thereafter.

In the present study, neither were measurements of photosynthesis made, nor was the microscopical examination of bleached corals performed. Hence, the underlying mechanisms and symptoms of temperature mediated bleaching were not identified. However, immediately on termination of treatment, the zooxanthellae in corals subjected to 96 hours of treatment were dividing at a mean of 2.8%, not significantly different from those in corals exposed to elevated temperature for 48 hours. This rose sharply to 4.3% by day 7, and further still to a maximum mean of 7.8% on day 21 (significantly higher than that of 48-hour treatment corals; see Figure 4.3). During the same period zooxanthellal densities in these corals significantly declined between days 0 and 7, before slowly increasing. This recovery profile is not consistent with damage to the photosynthetic machinery of the zooxanthellae, but is in line with the continued disruption of host endodermis and subsequent release of zooxanthellae into the gastric cavity in the period immediately after return to ambient temperatures. The exceptionally high proliferation

rates of zooxanthellae on day 21 suggest that a large proportion of zooxanthellae counted were inside the gastric chamber and free of host suppression of their growth and division (Douglas 1994, Jones & Yellowlees 1997, Suharsono & Brown 1992), although this was not established.

It is not disputed that damage to photosynthesis occurs when corals are subjected to elevated seawater temperature, especially during prolonged (Dunn *et al.* 2002) or extreme (Ralph *et al.* 2001) exposures. Incontrovertible too, is the fact that host tissues, particularly the endodermis, undergo damage during hyperthermic treatment. Frequently, inadequate consideration is given to repair processes in host tissues when attempting to understand factors that either promote or retard recovery of zooxanthellal populations after bleaching. These are almost certainly not instantaneous, and might take days and perhaps even weeks to occur under favourable conditions. Figure 4.8 is a diagrammatic representation of the theoretical impact of the length of exposure to elevated seawater temperature on host cells and the residual zooxanthellae in a section of the host endodermis. After a relatively short exposure to elevated temperatures, not only are there a greater number of competent zooxanthellae to proliferate but there are also a larger number of competent host cells available to acquire the dividing zooxanthellae. On the other hand, the longer the period of exposure to elevated temperature, the more vulnerable the host endodermis is to structural damage, exacerbated by photosynthetic damage to zooxanthellae, and the greater the delay in recovery of zooxanthellal populations, i.e. diminished resilience.

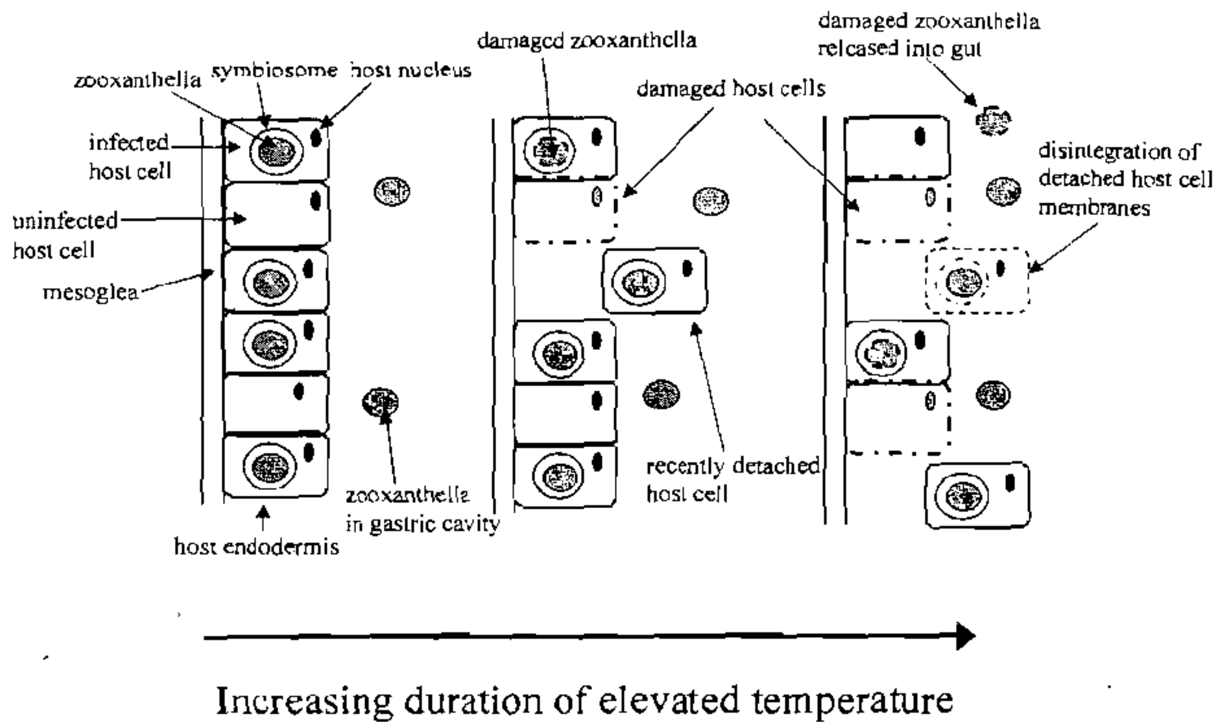


Figure 4.8: The theoretical impact of duration of elevated temperature on the availability of competent host cells and competent zooxanthellae in a section of coral endoderm tissue.

A conspicuous feature in the recovery profiles of corals recovering from temperature-induced bleaching is the 'overshoot' of zooxanthellal populations, relative to those in untreated control corals, in corals exposed to elevated temperature for 48 hours. Davy *et al.* (1997) reported a similar repopulation profile during experiments in which the aposymbiotic sea anemone *Cereus pedunculatus* was inoculated with either homologous (zooxanthellae native to *C. pedunculatus*) or heterologous (zooxanthellae native to host species different from *C. pedunculatus*) zooxanthellae. The rate at which *C. pedunculatus* was repopulated was much more rapid when homologous zooxanthellae were used to infect the aposymbiotic host. Moreover, a higher maximum zooxanthellal density was achieved by the infection with homologous zooxanthellae than with heterologous zooxanthellae. However, beyond week 8, at which time the maximum zooxanthellal density was recorded, there was a decline in the density of homologous zooxanthellae in *C. pedunculatus*, such that there were no significant differences in zooxanthellal densities of anemones infected with the different strains of zooxanthellae by week 36 post-infection. Although the authors could not explain the observed 'overshoot', they postulated that regulatory mechanisms normally exerted by the host were initially slackened, and only later imposed on their resident zooxanthellae. It is far from clear what these regulatory

mechanisms might be, but one possibility is the demand for receipt of photosynthate by the host. Support for this comes from the study of the symbiotic association between hydra and *Chlorella*. Different strains of *Chlorella* vary in the amount of photosynthetic carbon they release to their host. Strains that release low amounts of photosynthate (e.g. NC64A), retaining proportionately greater quantities of fixed carbon to support their own growth instead, exhibit significantly higher rates of cell division than do strains (e.g. 3N8/13-1) that release far less (data: Douglas & McAuley, cited in Douglas 1994). There is, therefore, the possibility that immediately after brief exposures to elevated seawater temperatures, corals do not impose as strict a requirement for their zooxanthellac to release photosynthate, thus allowing for a rapid proliferation and recovery of zooxanthellal populations. Substantial release of photosynthesis-derived products to the host may only occur after steady-state zooxanthellal densities have been restored or surpassed. In relation to this, it is worth noting that the mean percent of dividing zooxanthellae in corals exposed to elevated temperatures for 48 hours was initially 3.7%, rising to 5.4% on day 7 post-treatment, and remained above 3% thereafter. Likewise, the mean percent of dividing zooxanthellae in corals that had partially bleached on the reef only dropped below 3% on day 63 post-treatment.

4.3.1.3 Recovery from Bleaching Induced by Natural Stressors

Attention is first drawn to notable differences between experiments 3, in which zooxanthellal densitics were monitored in corals bleached in the laboratory using elevated seawater temperature, and 4, in whieh the zooxanthellal populations of corals that had bleached to varying extents on Kanamai Reef were allowed to recover in the laboratory. These include:

1. The coral fragments in experiment 4 were harvested simultaneously from colonies that were in close proximity, at the same depth and from identical habitats. It is highly unlikely that these colonies were exposed to different durations of the environmental stressors that prompted bleaching. The densities of zooxanthellae in the different colonies prior to commencement of environmental stressors were not established, and it is possible that differential bleaching of colonies arose from the application of (an) identical duration of stressor(s) in colonies with different baseline densities of zooxanthellae. The zooxanthellal densities in corals are known to show seasonal fluctuations (Fagoonee *et al.* 1999, Fitt *et al.* 2000).

2. Whereas the (two) colonies selected for treatment control corals and treatment corals in experiment 3 were identical, (three) different colonies were selected for each level of treatment (i.e. bleached, partially bleached, and unbleached) in experiment 4. The treatment controls in experiment 4 (unbleached corals) were not strictly speaking 'true' controls for bleached and partially bleached corals. There may have been genetic variation in bleaching susceptibilities between the different coral colonies selected for experiment 4. For example highly sensitive clones of a genotype of *Porites compressa* were reported to bleach in Hawaii in 1985 (C. Hunter and R.A. Kinzie cited in Jokiel & Coles 1990). As the *Symbiodinium* borne by bleached and unbleached colonies in experiment 4 were indistinguishable at the level of 24S rRNA genes (Table 4.1), genetic variation in the susceptibility of zooxanthellae (Rowan *et al.* 1997) is improbable.
3. The laboratory stressor in experiment 3 comprised exposure to elevated seawater temperature held constant for a maximum period of 4 days. The environmental stressors in experiment 4 are likewise presumed to have involved an elevation in mean seawater temperatures, but for a longer period (possibly weeks) and characterised by oscillations caused by diurnal and lunar-phase changes in tidal levels, and fluctuating with prevailing weather conditions.
4. The primary stressor, elevated temperature, was not applied in combination with high light levels in experiment 3. In experiment 4 however, elevated temperature is likely to have acted in synergy with high levels of solar radiation (Brown *et al.* 2000b, Dunne & Brown 2001) in eliciting bleaching.
5. Whereas zooxanthellal density and division measurements commenced immediately on termination of treatment in experiment 3, the precise time at which bleaching stressors abated in the field is not known. At the time, visits to Kanamai Reef were spaced approximately 7-10 days apart. The first measurements obtained during experiment 4 may have been delayed from the point at which the onset of recovery actually commenced.

Notwithstanding the above-mentioned differences, elevated temperature was very likely the key common element in both experiments, and the observed pattern of recovery after a natural bleaching incident is informative. The pattern of recovery of zooxanthellal populations in experiment 4 was similar to that observed in experiment 3; the zooxanthellae in corals that had bleached to a greater extent as a result of elevated seawater temperature were less resilient than those in corals that had only partially bleached. At the

start of experiment 4, the mean percent of dividing zooxanthellae in bleached corals was pronouncedly high, at 7.7% (see Figure 4.5), which is not dissimilar to the maximum percent of dividing zooxanthellae, 7.8%, recorded for the corals in experiment 3 that were exposed to elevated temperature for 96 hours. As the latter measurement was noted on day 21 post-treatment, this may reflect a difference between experiments in the time at which measurements were started after termination of stressor(s).

Especially noteworthy is the fact that whereas corals that had undergone bleaching on the reef survived for the entire duration of the experiment when housed in the laboratory, a large proportion of bleached colonies, estimated at 75%, suffered from mortality when left to recover on the reef. Two of the three bleached colonies selected for experiment 4 died on the reef before the end of the experiment. This is a striking illustration of the differences between the conditions under which recovery occurred in the laboratory, and those that were encountered by recently bleached corals on a typical reef in Kenya. Bleached corals are more prone to disease (Glynn 1983) and suffer from an increased susceptibility to sediments (Mascarelli & Bunkley Williams 1999). Bleached corals are also vulnerable to mechanical damage (e.g. abrasion inflicted by grazing and boring organisms, debris shifted by wave action, etc.) as they suffer from an impaired healing and regenerative capacity (Mascarelli & Bunkley Williams 1999, Fine *et al.* 2002). A visit to the reef approximately one month after the coral fragments for experiment 4 had been harvested confirmed that although all colonies were still alive, they were gradually being overgrown by filamentous and fleshy algae. Kanamai reef is a non-protected reef, where the abundance of herbivorous fish is thought to be low (McClanahan & Arthur 2001). The ecological outcome of bleaching is strongly influenced by the level of fishing pressure and nutrient inputs (Goreau *et al.* 2000). This study confirms that bleached *Porites cylindrica* had the potential to survive and recover from a natural bleaching incident, but that the competitive reef environment was not conducive to recovery. The possibility of remedial action on rare and bleaching-susceptible species of corals after a bleaching incident and allowing their recovery under laboratory conditions is possible, if not practical.

4.3.2 Molecular Analysis of Zooxanthellae

Genetic variation in the bleaching susceptibility of *Symbiodinium* has bcen described in previous studies (Rowan *et al.* 1997). Additionally, changes in the types of zooxanthellae hosted by corals on recovery from bleaching have also been reported (Toller *et al.* 2001b, Baker 2001). As initially proposed by Buddemeier & Fautin (1993) in the 'Adaptive Bleaching Hypothesis' (ABH), these changes have sometimes been interpreted as being an adaptive response to environmental perturbation, leading to bleaching-resistant combinations by the re-assortment of symbiotic partners. Potential changes in the communities of zooxanthellae in *Porites cylindrica* were therefore tracked by molecular methods (PCR-RFLP and sequenee analysis of 24S rRNA genes) during bleaching, and at the end of the designated recovery period. No genetic changes in zooxanthellae were observed in any of the experiments (Table 4.1). These results suggest that *Porites cylindrica* is highly specific for its zooxanthella at Kanamai Reef. Future recurrences in bleaching of *P. cylindrica* at Kanamai are unlikely to result in adaptive changes of the nature prescribed by the ABH.

Chapter 5

Acclimation of Corals to Bleaching Stressors

5.1 Introduction

The frequency and severity with which the principal triggers of bleaching (elevated temperature/solar light) are recurring is set to rise further still (Sheppard 2003, Hoegh-Guldberg 1999, Wilkinson 1999). The prediction is for the bleaching and mortality en-masse of corals, underlying the collapse of reef systems on a global scale (Hoegh-Guldberg 1999). A major uncertainty inherent in these predictions lies in the assumption that zooxanthellate symbioses do not undergo changes in their responses to bleaching stressors, i.e. acclimatisation. There is mounting evidence to suggest that corals can develop resistance to bleaching as a result of their recent exposure to a bleaching stressor(s). Perhaps the most persuasive evidence for acclimatisation of corals to bleaching stressors comes from field observations and studies by Brown *et al.* (2000a, 2002a, 2002b), who report that the shallow-water coral *Goniastrea aspera* from Thailand acquires host-mediated resistance to thermal/solar bleaching after having recently undergone solar bleaching.

To date, there are no studies documenting the acquisition of resistance to coral bleaching under defined laboratory conditions, i.e. acclimation of corals to bleaching stressors. Therein lies the purpose of the experiments described in this chapter. At the end of the experiments on recovery from bleaching (chapter 4), there existed three sets of corals that had recently recovered from dark-induced bleaching, elevated temperature-induced bleaching, and bleaching prompted by natural stressors. These were exploited to investigate the extent to which recent experience of a bleaching stressor had rendered the zooxanthellal populations in those corals resistant to bleaching on repetition of the stressor. Objectives of this study are best outlined in the form of a table, shown below:

Table 5.1: The objectives of the experiments described in this chapter.

Expt.	Bleaching pretreatment	Recovery (days)	Bleaching treatment
5	24 hr dark for 21 days	42	24 hr dark for 21 days
6	24 hr dark for 21 days	42	32.5°C for 72 hours
7	32.5°C for 96 hours	63	32.5°C for 24 hours [1]
8	Field stressors: bleached, partially bleached, unbleached	63	32.5°C for 72 hours

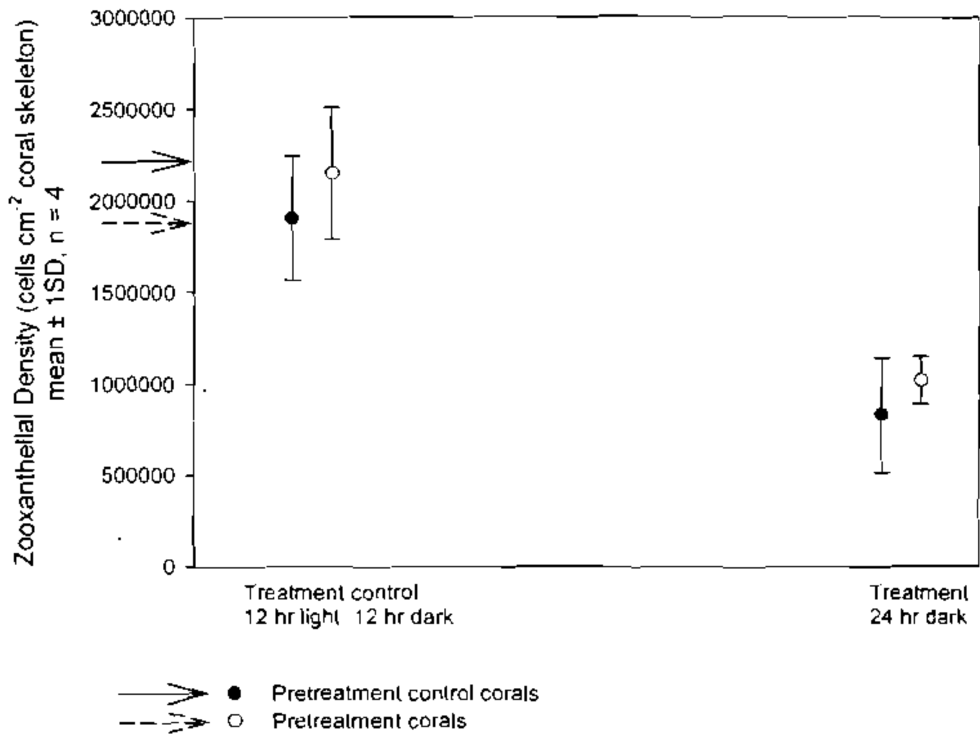
[1] The intended duration of treatment was 72 hours. Treatment was prematurely terminated owing to the onset of coral mortality.

5.2 Results

5.2.1 Experiment 5: Impact of Prior Exposure to Darkness on Response to Repetition of Darkness

The coral fragments used in this experiment had either been i) bleached by pretreatment with darkness for 21 days (24 hr dark, 21 days), then incubated for 42 days under the ambient light regime (12 hr light: 12 hr dark) or ii) maintained under ambient conditions throughout. These are referred to as *pretreatment* corals and *pretreatment control* corals, respectively. Their mean zooxanthellae densities did not differ significantly at the start of treatment [pretreatment control corals: 2.20 ± 0.13, pretreatment corals: 1.89 ± 0.26, mean density per cm^2 (x 10^6) ± 1 SD, n = 4; two-sample T-Test: T (d.f. = 4) = 2.18, $p > 0.05$].

Results are shown in Figure 5.1. Treatment with darkness resulted in a visible paling of coral tissues and a reduction of zooxanthellae density by more than 50% to levels significantly below treatment control corals. Pretreatment had no significant effect on zooxanthellae density. Coral responses to darkness were independent of levels of pretreatment, as indicated by the non-significant interaction term.



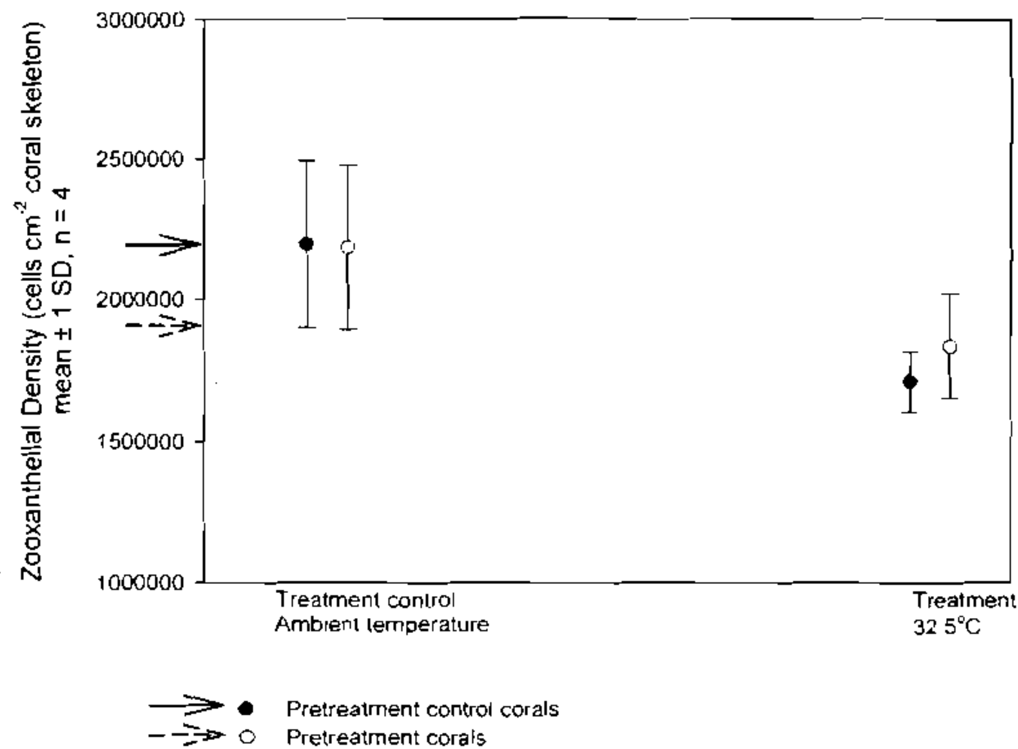
Factor	D.F.	SS (x 10^{12})	MS (x 10^{12})	F Ratio	P value
Pretreatment	1	0.192	0.192	2.15	0.168
Treatment	1	4.886	4.886	54.77	< 0.001 ***
Interaction	1	0.003	0.003	0.03	0.862
Error	12	1.071	0.089		

Figure 5.1: Zooxanthellal densities (mean values ± 1 SD) in dark-treated corals (24 hr dark, 21 days) and in treatment control corals (12 hr light: 12 hr dark, 21 days). Prior to treatment, corals were either i) pretreated with darkness (24 hr dark, 21 days; pretreatment corals), then incubated for 42 days under the ambient light regime (12 hr light: 12 hr dark) or ii) incubated under ambient conditions (12 hr light: 12 hr dark: pretreatment control corals) throughout. Arrows (dashed line: pretreatment corals, solid line: pretreatment control corals) show mean zooxanthellal densities at the start of treatment. Results of a two-way ANOVA are shown below the figure.

5.2.2 Experiment 6: Impact of Prior Exposure to Darkness on Response to Elevated Temperature

The *pretreatment* corals for this experiment comprised corals that had previously been bleached by incubation under darkness for 21 days (24 hr dark, 21 days), after which they were incubated for 42 days under the ambient light regime (12 hr light: 12 hr dark). *Pretreatment control* corals were maintained under ambient conditions throughout. Mean densities of zooxanthellae in both sets of corals did not differ significantly at the start of treatment [pretreatment controls: 2.20 ± 0.13, pretreatment corals: 1.89 ± 0.26, mean density ($\times 10^6$) ± 1 SD, n = 4; two-sample T-Test: T(d.f. = 4) = 2.18, $p > 0.05$].

Results are shown in Figure 5.2. Treatment with elevated temperature brought about a significant decline in mean zooxanthellal density. However, this was not as pronounced as the decline exhibited by corals subjected to incubation under darkness (see experiment 5). Attendant with loss of zooxanthellae was a visible paling of coral tissues. Pretreatment had no significant effect on zooxanthellal density. Coral responses to elevated temperature were independent of pretreatment, as indicated by the non-significant interaction term.



Factor	D.F.	SS (x 10^{10})	MS (x 10^{10})	F Ratio	P value
Pretreatment	1	1.297	1.297	0.24	0.635
Treatment	1	69.971	69.971	12.77	< 0.01 **
Interaction	1	1.843	1.843	0.34	0.573
Error	12	65.749	5.479		

Figure 5.2: Densities of zooxanthellae (mean values ± 1 SD) in corals treated with elevated temperature (32.5°C, 72 hours) and in treatment control corals (ambient temperature). Before being treated, corals had either been i) pretreated with darkness (24 hr dark, 21 days: pretreatment corals), then incubated for 42 days under the ambient light regime (12 hr light: 12 hr dark) or ii) incubated under ambient conditions (12 hr light: 12 hr dark; pretreatment controls) throughout. Arrows (dashed line: pretreatment corals, solid line: pretreatment control corals) show mean densities of zooxanthellae at the start of treatment. Results of a two-way ANOVA are shown below the figure.

5.2.3 Experiment 7: Impact of Prior Exposure to Elevated Temperature on Response to Elevated Temperature

The *pretreatment* corals used for this experiment had initially been bleached by exposure to elevated temperature (32.5°C) for 96 hours (*pretreatment* corals), following which they were incubated for 63 days under the ambient temperature regime. *Pretreatment control* corals were maintained under ambient conditions throughout. The mean densities of zooxanthellae in the tissues of both sets of corals did not differ significantly at the start of treatment [pretreatment controls: 1.91 ± 0.34, pretreatment corals: 1.93 ± 0.79; mean density ($\times 10^6$) ± 1 SD, n = 4; two-sample T-Test: T (d.f. = 4) = 0.05, p > 0.05].

Although treatment was initially intended to persist for 72 hours, the experiment was terminated after 24 hours. This was due to a decline in the thermal tolerance of pretreatment control corals. Fragments from this group developed a mottled appearance due to the emergence of polyps that were very dark in comparison to surrounding tissues, as shown in Figure 5.3. This was interpreted as death of individual polyps. To prevent further mortality, the experiment was immediately terminated.

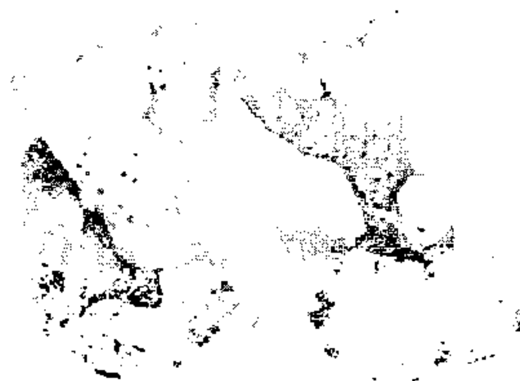
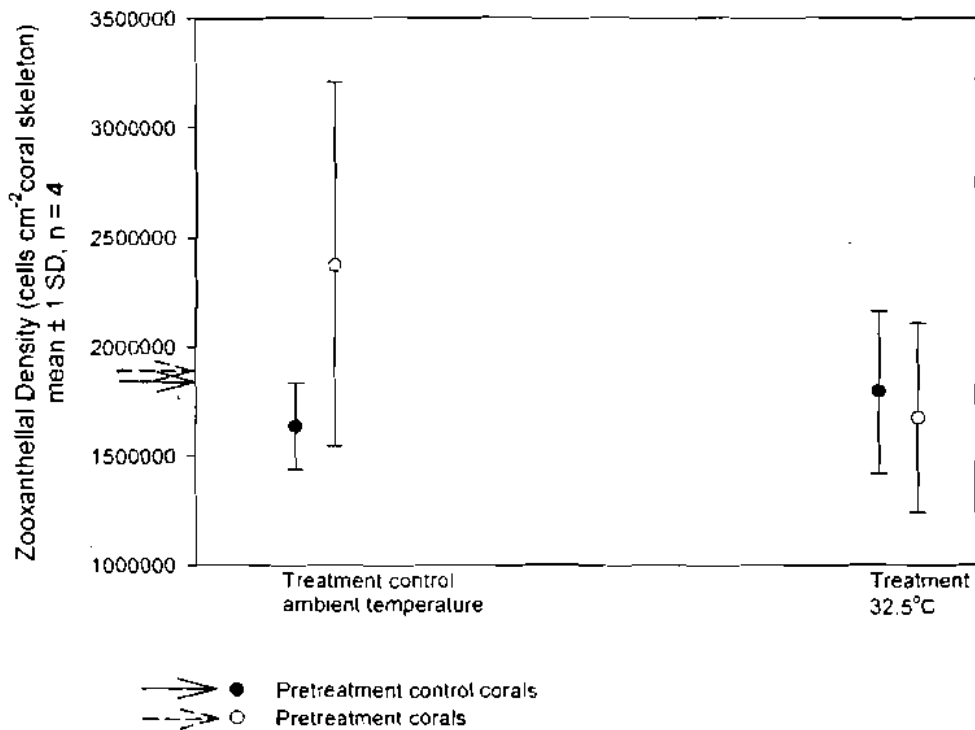


Figure 5.3: The mottled appearance of fragments from pretreatment control corals on treatment with elevated temperature (32.5°C) for 24 hours. A number of exceptionally dark polyps in comparison with surrounding tissues are visible.

Results of experiment 7 are shown in Figure 5.4. Treatment with elevated temperature did not produce a significant decline in mean density of zooxanthellae, although all fragments exhibited visible paling of tissues. Pretreatment had no significant effect on density of zooxanthellae. Coral responses to elevated temperature were independent of pretreatment, as indicated by the non-significant interaction term.



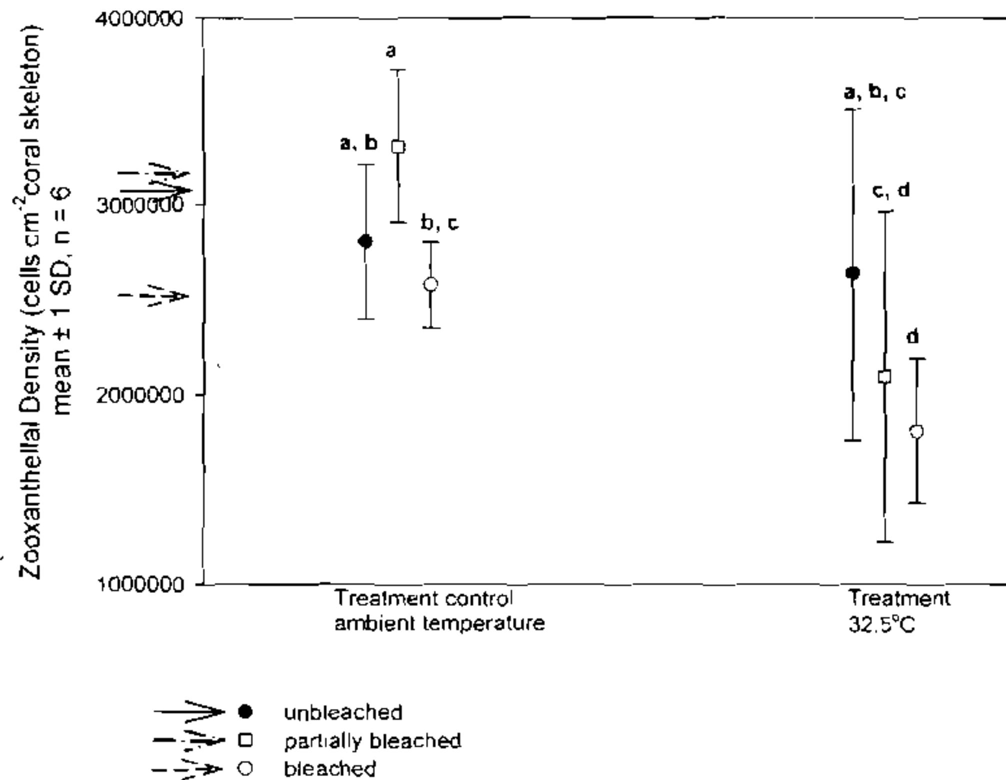
Factor	D.F.	SS (x 10^{12})	MS (x 10^{12})	F Ratio	P value
Pretreatment	1	0.384	0.384	1.45	0.251
Treatment	1	0.293	0.293	1.11	0.313
Interaction	1	0.741	0.741	2.81	0.120
Error	12	3.169	0.264		

Figure 5.4: Densities of zooxanthellae (mean values ± 1 SD) in temperature- treated corals (32.5°C, 24 hours) and in treatment control corals (ambient temperature). Prior to treatment, corals were either i) pretreated with elevated temperature (32.5°C, 96 hr dark: pretreatment corals), then incubated for 63 days under the ambient temperature regime or ii) incubated under ambient conditions (pretreatment control corals) throughout. Arrows (dashed line: pretreatment corals, solid line: pretreatment control corals) show mean zooxanthellae densities at the start of treatment. Results of a two-way ANOVA are shown below the figure.

5.2.4 Experiment 8: Impact of Prior Experience of Natural Bleaching on Responses to Elevated Temperature

The corals used for this experiment had been collected from the reef in unbleached, partially bleached and bleached states. All corals were then incubated for 63 days under the ambient temperature regime to promote recovery of zooxanthellae populations. Their mean zooxanthellal densities did not differ significantly at the start of treatment [unbleached corals: 3.05 ± 0.48, partially bleached corals: 3.17 ± 0.43, bleached corals: 2.50 ± 0.64, mean density ($\times 10^6$) $\pm$ 1 SD, $n = 6$; one-way ANOVA: $F_{2,15} = 2.76$, $p > 0.05$].

Results are shown in Figure 5.5. For each (prior) bleached state (i.e. bleached, partially bleached and unbleached), fragments were derived from separate coral colonies. Thus, a nested ANOVA (with coral colony nested within bleached state) was performed. The outcome of treatment with elevated temperature was dependent on bleached state, as indicated by the significant interaction term. There was a significant decline in mean densities of zooxanthellae in previously bleached and partially bleached corals, but not for previously unbleached corals. However, fragments from all bleached states visibly paled on treatment with elevated temperature.



Factor	D.F.	SS (x 10^{12})	MS (x 10^{12})	F Ratio	P value
Bleached State	2	2.151	1.076	5.12	< 0.05 *
Treatment	1	4.676	4.676	22.26	< 0.001 ***
Interaction: Bleached State x Treatment	2	1.656	0.828	3.94	< 0.05 *
Colony (Bleached State)	6	5.230	0.872	4.15	< 0.01 **
Error	24	5.040	0.210		

Figure 5.5: Zooxanthellal densities (mean values ± 1 SD) in temperature- treated corals (32.5°C, 72 hours) and in treatment control corals (ambient temperature). Corals had initially been collected from the reef in unbleached, partially bleached and bleached states, and then incubated for 63 days under the ambient temperature regime. Arrows (dashed line: bleached corals, solid line: unbleached corals, dotted line: partially bleached corals) show mean zooxanthellae densities at the start of treatment. Results of a two-way nested ANOVA (colony is nested within pretreatment) are shown below the figure. Letters indicate homogeneous subsets from post-hoc analysis with Fisher's LSD test.

5.3 Discussion

5.3.1 Summary of Results

The results provided no evidence for acclimation leading to the development of resistance to bleaching on repetition of the bleaching stressor; the recovered zooxanthellal populations in dark-pretreatment corals were susceptible to bleaching on treatment with darkness (experiment 5), as were those in (naturally) bleached and partially bleached corals when subjected to elevated temperature (experiment 8). Corals pretreated with elevated temperature underwent a decline in mean zooxanthellal density when re-exposed to elevated temperature (experiment 7), but the decline was not statistically significant. As the sample sizes were small, and variances within each sample large, the power of ANOVA to detect biological differences after a very brief treatment was substantially reduced (Sheppard 1999). It is possible that had treatment not been prematurely terminated, the decline in zooxanthellal density of pretreatment corals would have been statistically significant. The results from experiment 6 indicate that cross-protection between dark and elevated temperature bleaching stressors did not occur.

5.3.2 Influence of Solar radiation in Acclimatisation

The observations from the present study are in sharp contrast with those of Brown *et al.* (2000a) from the shallow-water reef flats of Thailand, in which they reported that west-facing surfaces of *Goniastrea aspera* were protected from thermal bleaching in May 1991 and May 1995 when sea surface temperatures were anomalously high. Protection resulted from exposure to high levels of photosynthetically active radiation (PAR: 400-700nm wavelength) earlier in the year. In a follow-up field experiment (Brown *et al.* 2000b), *G. aspera* colonies were rotated such that previously east-facing surfaces faced west, thereby exposing them to higher levels of solar radiation. West facing surfaces (previously protected) presented with a progressive decline in photochemical efficiency (F_v/F_m) followed by the development of a bleaching lesion, demonstrating that the physiological triggers for solar bleaching are similar to those that induce thermal bleaching. During subsequent thermal bleaching, algal cells from east and west surfaces of colonies were not different with regards to their levels of stress proteins, antioxidant enzymes and xanthophyll pigments (Brown *et al.* 2002a). Animal tissues, however, were biochemically distinctive with elevated levels of the antioxidant enzyme superoxide dismutase and stress proteins Hsp 60 and Hsp70 on the west face relative to

east. Resistance to thermal bleaching following solar bleaching in *G. aspera* therefore lies in host-mediated acclimatisation processes.

The influence of solar history in shaping the thermal bleaching response is not confined to *G. aspera* but extends over a wider range of species and genera (Dunne & Brown 2001). At their study site in Phuket, Thailand, positive temperature anomalies occurred in May of 1991, 1995, 1997 and 1998, but thermal bleaching was only observed in 1991 and 1995. In their examination of records of sea temperature and solar radiation, they come across compelling evidence that elevated PAR in the month preceding maximum temperatures (as was the case in 1997 and 1998) can protect corals from thermal bleaching. Moreover, the records indicated that sea-level anomalies exerted a strong influence on the level of light incident upon corals, and must be considered when attempting to predict thermal bleaching responses.

High levels of solar radiation preceding thermal stress appear to be a precondition to the development of resistance to thermal/solar bleaching. As all the experiments in this study were conducted at low irradiances, this may have been a contributory factor in the susceptibility to thermal bleaching of corals recently recovered from bleaching.

5.3.3 Thermal Tolerance

The responses of pretreatment control corals to elevated temperature treatment in experiment 7 were surprising in that they displayed a diminished upper tolerance limit to temperature (temperature tolerance is as defined in section 1.4.2; Chapter 1). This led to the decision to terminate treatment prematurely to prevent further polyp mortality. Corals with reduced upper temperature tolerance limits (Figure 5.3) appeared strikingly similar to the description provided by Coles & Jokiel (1978) on the condition preceding mortality - "...darkening of coral polyps...most corals having darkened polyps paled in coloration, and many colonies died." Those observations (Coles & Jokiel 1978) were made during an experiment designed to explore the temperature tolerance (assessed by survival) of corals, in which branches of *Montipora verrucosa* were pre-acclimated at varying temperatures (20°C, 24°C 26°C and 28°C) for 56 days prior to treatment with elevated temperature (32.5°C). Corals pre-acclimated at higher temperatures (28°C and 26°C) had a higher tolerance for temperature (mean survival of 74% and 61% respectively) than corals pre-acclimated at lower temperatures. Interestingly, corals pre-acclimated at 20°C (mean survival 47%) were less susceptible to high temperature

treatment than were those pre-acclimated at 24°C (mean survival 30%). The investigators noted that all the corals pre-acclimated at 20°C were already bleached and pale-bleached prior to high temperature treatment. They also noted that whereas 100% of pale-bleached branches from the 24°C pre-acclimation group survived high temperature treatment, only 29% of unbleached branches from this group survived treatment. Based on these observations they proposed that expulsion of intra-cellular algae from unbleached tissues during high temperature treatment imposed additional 'stress' on corals, partially accounting for higher mortality rates in the 24°C pre-acclimated group.

Pretreatment control corals were not subjected to any definitive trigger of bleaching on their collection from the reef. Consequently they maintained relatively large populations of zooxanthellae, albeit declining, in their tissues for approximately 10 weeks before experiment 7 was conducted. Laboratory conditions were sub optimal, with particular reference to light levels. Bearing in mind that zooxanthellae in culture (Steen 1987), and *in situ* (Steen 1986), have the capacity for heterotrophic nutrition in low light environments, pretreatment control corals may have incurred a metabolic cost during the period between pretreatment and treatment. The temperature tolerance of pretreatment control corals may thus have been greatly reduced in comparison to that of pretreatment corals, which had recovered from low initial zooxanthellal densities.

Chapter 6

General Discussion

Despite many years of intensive research on the area, we are no closer to a definitive answer on why corals bleach. There are two broad views on the matter. The first of these is that bleaching is a deleterious, maladaptive response to environmental perturbation, somewhat akin to an ailment (Hoegh-Guldberg *et al.* 2002, Douglas 2003). In stark contrast to this, the second school of thought is that bleaching is an adaptive response (Buddemeier & Fautin 1993, Baker 2001). Some advocates of this perspective have fervently argued that bleaching has evolved as a specific strategy to facilitate changes in the types of zooxanthellae hosted by corals, resulting in host-symbiont combinations better suited to altered environmental conditions. Evidence for this is limited, and not supported by the results from the present study in which detectable changes in the types of zooxanthellae hosted by *Porites cylindrica* did not occur on recovery from bleaching. More credible is that bleaching, characterised by the rapid and drastic reduction of zooxanthellae from host tissues, has evolved as a final strategy to protect from the damaging effects of a continued association with large populations of zooxanthellae under the adverse set of environmental conditions collectively termed as the triggers of bleaching. In the case of thermal/solar bleaching, these damaging effects are probably inflicted by the products of oxidative stress (Downs *et al.* 2002, Lesser 1996, 1997).

To aid in the understanding of coral bleaching, a comparison is drawn with the defensive responses of organisms to pathogens. More specifically, there are many useful parallels between coral bleaching and the immune responses of vertebrates. In much the same way as immune responses are triggered by a range of pathogens, bleaching is a generalised response to stress, being induced by varied stressors. However, some pathogens can evade immune responses with the resultant decline in health of infected individuals over time. Not all stressors trigger bleaching, and the failure to bleach under certain circumstances may lead to the gradual deterioration in the health of corals. This principle was illustrated by the outcome of experiment 7, in which the persistence of relatively high densities of zooxanthellae in corals maintained under low light levels for prolonged periods led to the decline in health of pretreatment control corals, as evidenced by their reduced thermal tolerance relative to that of pretreatment corals. In a controversial publication, Baker (2001) reported the results of experiments involving

the reciprocal transplantation of corals between shallow (high-light environment) and deep (low-light environment) water. Only corals that were transplanted upwards (i.e. deep water to shallow water) underwent bleaching, and some of these acquired new types of zooxanthellae on recovery from bleaching. None of the corals transplanted downwards (i.e. shallow water to deep water) bleached or incorporated new zooxanthellae, and some died. Baker (2001) concluded that bleaching was adaptive in that it facilitated changes in the zooxanthellal communities of corals, thereby promoting survival of transplanted corals. There is a possibility that Baker (2001) may have been correct in his belief that the shallow-water to deep-water coral transplants suffered from mortality owing to their failure to bleach, but not because they ultimately failed to acquire new types of zooxanthellae.

Immune responses sometimes have adverse side effects, for example hypersensitivity and extreme fever. The negative side effects of coral bleaching are all too evident. This explains why bleaching is a strategy of last resort rather than an early line of defence.

Why then is it that bleaching is not uniform among the corals on a reef during a period of environmental perturbation? Just as it takes varying degrees of infection with a pathogen to induce an immune response in different individuals, there is inter-specific and intra-specific genetic variation in the susceptibility of corals to bleaching stressors, i.e. variation in bleaching thresholds. It is in determining the genetic basis underlying susceptibility to bleaching where the current approach with ribosomal RNA gene markers is wanting. At best, the current molecular markers will only enable us to identify correlations between the identity of zooxanthellae and the observed susceptibility of symbioses to bleaching, after the fact. The results from the molecular survey of zooxanthellae from sea anemones in the Mediterranean Sea reveal that these comprise a distinct group of temperate A zooxanthellae that are possibly endemic to the region. With increased sampling effort, more such patterns may be discovered. Whether these populations are functionally different from other zooxanthellae, or whether they are merely atypical with respect to ribosomal RNA genes, can only be addressed by asking different molecular questions. In this respect, the work with *psb*A represents a cautious step in the right direction as *psb*A encodes the D1 protein of photosystem II (Howe *et al.* 2003), which has been implicated in coral bleaching (Warner *et al.* 1999).
Immune responses can be primed to respond rapidly and with greater intensity to a repeat-infection by a pathogen. The basis of these *secondary immune responses* lies in

the production of large clones of 'memory cells' on initial encounter with the invading organism. These memory cells reside in the spleen and lymph nodes and are extremely long-lived, sometimes conferring permanent immunity to childhood diseases such as mumps and chickenpox. The acclimatisation of corals to bleaching stressors differs from secondary immune responses on at least two important counts. Firstly, acclimatisation to bleaching stressors is short-lived/reversible, not thought to last more than three months after return of the environment to more benign conditions (Dunne & Brown 2001). Secondly, whereas secondary immunity to pathogens is highly specific, cross-protection between bleaching stressors, for instance between elevated solar radiation and elevated seawater temperature (Brown *et al.* 2000a, Dunne & Brown 2001), is known to occur. Elevated solar radiation might actually be a precondition to the development of resistance to bleaching. As all the experiments on bleaching were carried out at low irradiances, this may explain the lack of evidence in support of the acclimation of corals to bleaching stressors, described in chapter 5.

Returning a final time to the comparison between immunity and bleaching, different branches of the immune response are activated depending on the nature of the invading pathogen. The parallel with bleaching corresponds to the different mechanisms by which bleaching occurs in response to different stressors. This has important implications for resilience, i.e. the capacity to recover from bleaching, as demonstrated by the results in chapter 4, in which resilience was strongly influenced by the nature of the bleaching stressor, and the duration over which the stressor was applied. At different stages of the immune response, a characteristic profile of lymphocytes, cytokines and antibodies is produced, and this profile can inform us of the type of immune response that has been stimulated, and the nature of the infecting agent. These markers are analogous to the biomarkers produced at different stages during the network of events collectively referred to as the mechanisms of bleaching. Thus, biomarkers can be utilised to provide information on the nature of the bleaching stressor, susceptibility to bleaching and the mechanisms of bleaching. Many biomarkers produced by corals and their zooxanthellae are protective molecules, and are therefore useful as biomarkers of resistance to bleaching. Some of these that are expressed early during thermal/solar stress are identified as follows:

1. **Proteins and Enzymes:**

Heat shock proteins (hsps), sometimes referred to as heat stress proteins, are a highly conserved group of proteins whose expression is enhanced during exposure to a wide range of stressors, including elevated temperature (Downs *et al.* 1999, Brown *et al.* 2002a, Black *et al.* 1995). They act principally as molecular chaperones, regulating protein strueture, and especially during stress events when they are involved in the reconstitution of denatured proteins. Hsps protect chloroplasts from damage during heat stress (Downs *et al.* 1999), and are thought to be important in the acquisition of enhanced thermotolerance (Parsell & Lindquist 1993). Zooxanthellae and their Cnidarian hosts are both known to synthesize hsps on exposure to elevated temperatures (Brown *et al.* 2002a, Black *et al.* 1995).

Coral bleaching has been attributed to oxidative stress (Downs *et al.* 2002, Lesser 1996, 1997). The *antioxidant enzymes*, for example copper-zinc and manganese superoxide dismutases (CuZnSOD and MnSOD), produced by both zooxanthellae and their hosts (Brown *et al.* 2002a, Richier *et al.* 2003), are an important defence against oxidative stress during periods of heightened photosynthetic activity.

The host-mediated resistance to thermal bleaching acquired by *Goniastrea aspera* (Brown *et al.* 2002a) following solar bleaching was primarily due to the increased production of antioxidant enzymes and hsps.

2. **Photoprotective Pigments:**

The *xanthophylls* constitute a group of carotenoid pigments that play a vital role in the protection of photosynthesis by dissipating excess excitation energy as heat (Demmig-Adams & Adams III 1996). This is achieved by the reversible conversion of one xanthophyll to another, known as the xanthophyll cycle. In the case of zooxanthellae, this involves the interconversion between diadinoxanthin and diatoxanthin (Brown *et al.* 1999a).

The *fluorescent pigments* of corals are an important means by which the internal light environment of host tissues is regulated (Salih *et al.* 2000). In excessive sunlight, these pigments dissipate excess energy, either by fluorescence at

wavelengths of low photosynthetic activity, or by scattering and reflecting sunlight. In so doing, they serve to protect the photosynthetic apparatus of zooxanthellae.

The proteins, enzymes and pigments described above occur in both hosts and their zooxanthellae. The implication of this is that in most cases, susceptibility of symbioses to coral bleaching is ultimately governed by properties intrinsic to both symbiotic partners, and on symbiotic interactions between partners, rather than being predominantly shaped by properties of one or the other partner. This illustrates the complex nature of the problem; unravelling the network of molecular and biochemical events that define bleaching will involve the methodical and painstaking dissection of symbioses rather than efforts concentrating on one or the other symbiotic partner in isolation. This presents a major challenge for coral biologists in the coming decade. Meeting this challenge is not merely a matter of academic interest for the scientific community, but of vital concern to the millions of people globally whose lives and livelihoods are intimately entwined with the fate of the threatened coral reefs of the world.

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