Genetic Diversity of *Symbiodinium* in Selected Corals in the Western Indian Ocean

by

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Abstract

Coastal communities along the east African coastline rely on coral reefs and their associated resources for food security and income. However, increases in the frequency and severity of episodes of coral bleaching have resulted in mass coral moralities in many locations around the world including the western Indian Ocean (WIO). Reef corals obligately host dinoflagellate algal symbionts of the genus *Symbiodinium*. Coral bleaching is caused by the loss of these symbionts from the host, resulting from a variety of stresses, the major ones being increased seawater temperature and irradiance. The *Symbiodinum* genus is diverse and the distribution of symbionts is influenced by the host biology, external light environment and geographic location. Ten distinct clades of *Symbiodinium* have been identified. Although the Caribbean and Great Barrier Reef have been studied intensively with respect to *Symbiodinium* diversity in many locations in the WIO *Symbiodinium* diversity is unknown. The aim of this study was to determine diversity, distribution and prevalence of *Symbiodinium* types in corals along the east African coastline. The *Symbiodinium* ssrDNA region was analysed using restriction fragment length polymorphism (RFLP) in order to assess the cladal diversity of *Symbiodinium*. The results showed all samples analysed to belong to clade C. To gain more insight into *Symbiodinium* genetic diversity, the ITS region was employed to assess *Symbiodinium* diversity at the subcladal level. Twenty ITS types were identified. The most prevalent type was found to be subclade C1. No phylogeographic structuring was found amongst the symbiont types, however, specificity of symbiont types to coral hosts was demonstrated indicating potential susceptibility to perturbations such as increased seawater temperature.

Preface

The research described in this thesis was carried out through the School of Biological and Conservation Sciences, University of KwaZulu-Natal, Durban, from February 2006 to December 2007, under the supervision of Professor Michael Schleyer and Dr Jennifer Lamb.

These studies present original work by the author and have not otherwise been submitted in any form for any degree or diploma to any other university. Where the use has been made of work of others, it has been duly acknowledged in the text.

Dorota E. Starzak

As the candidate's supervisor I have approved this dissertation for submission.

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Chapter 1

1. Introduction

1.1 General Introduction

Coral reefs are of great importance, both in the biological and economic sense. They maintain a rich biodiversity in shallow tropical waters and, as a result, are vital in sustaining the coastal communities in these regions. Coral reefs are essential to the fishing sector, as a large portion of the local fish harvest may be attributed to coral reefs (Linden *et al*., 2002). Local communities also benefit from income generated from the non-extractive use of coral reefs and other tourism-related activities (Bellwood *et al*., 2004). The maintenance of coral reefs is thus necessary to ensure food security for coastal communities. At present, however, coral reefs are under threat as a result of anthropogenic perturbation. In many regions of the world, man-made stresses to coral reefs have exceeded their regenerative capacity, causing dramatic shifts in species composition and resulting in severe economic loss (Bellwood *et al*., 2004). These stresses include direct damage resulting from detrimental fishing practices as well as eutrophication caused by poor agricultural procedures, deforestation and poor land-resource management (Bellwood *et al*., 2004; Linden *et al*., 2002). Climate change also poses a major threat to coral reefs. The 1998 El Niño Southern Oscillation (ENSO) event resulted in the destruction of approximately one-sixth of the world's coral colonies due to mass coral bleaching, caused by mild increases in ocean temperature (Glynn, 1991; Brown, 1997, Dennis, 2002). In the past two decades there has been an increase in the frequency and severity of episodes of mass coral bleaching (Wilkinson, 2002; Baker, 2003). The WIO underwent the most significant coral bleaching ever recorded, resulting in 90–95% mortality of corals at the most affected sites, with 30% mortality on a regional scale (Obura, 2005). This decline of coral reefs calls for an urgent reassessment of current management practices, which will

require a major scaling-up of management efforts based on an improved understanding of the processes that underlie reef resilience (Bellwood *et al*., 2004).

1.2 Physiology of Coral Bleaching

Coral bleaching can be defined as the whitening of corals due to the mass expulsion of symbiotic algae and/or the loss of photosynthetic pigments within individual zooxanthellae (Lesser and Ferrel, 2004). In severe cases, corals do not recover and die (Ulstrup and van Oppen, 2003). The process can be viewed as a response to a variety of environmental stresses (Celliers and Schleyer, 2002). A variety of stressors have been invoked as being responsible for causing coral bleaching, the most important being increased sea temperature, solar radiation and a combination of both factors (Brown, 1997; Lesser and Farrell, 2004). Because symbioses with zooxanthellae are commonly found in habitats at 1–2 °C below the temperature which triggers breakdown of the symbiosis, they are extremely sensitive to increases in temperature (Douglas, 2003).

In the past, attributing coral bleaching to increased sea temperatures was not straightforward. For example, the use of satellite sea temperature data presented problems. These included lack of resolution, difficulties in calibration and the fact that satellite data only provide values for the top few millimetres of surface water, thus depicting changes in solar radiation and not bulk sea temperature (Brown, 1997). However, recent work such as that of Strong *et al*., (2000) has resolved these problems. Thus, satellite measurements of changes in sea temperature can now predict with almost 95% accuracy whether or not a geographical on the planet will bleach (Strong *et al*., 2000).

Photosynthetically-active radiation (PAR) and/or ultraviolet radiation (UVR) may act in concert with elevated temperature to elicit a bleaching reponse (Brown, 1997; Werner *et al*., 1999; Lesser and Ferrel, 2004). The role that UVR may play in increasing future coral bleaching is one of the main areas of concern. The amount of UVR that submerged corals are exposed to is related to stratospheric ozone levels, changing weather patterns and the

attenuation of UVR in the water column (Brown, 1997). Therefore, increases in ozone depletion, global warming and anthropogenic influences that affect both particulate loads and dissolved organic carbon (DOS) in oceans, all contribute to coral bleaching (Brown, 1997).

A number of cellular mechanisms have been proposed for coral bleaching. These include: the *in situ* degradation of zooxanthellae, the loss of algae into the coelenteron and the release of intact endodermal cells containing intracellular zooxanthellae (Gates *et al*., 1999; Brown, 1997). It is likely that the pathway of zooxanthellar degradation may differ depending on the intensity and duration of stress and also the host species (Mise and Hidaka, 2001). Lesser and Farrel (2004) showed that corals exposed to supra-optimal solar irradiance and elevated seawater temperatures exhibit greater damage to the photosynthetic apparatus. An increase in water temperature results in a decrease in the photosynthetic activity of the symbiosis, most probably associated with a decrease in the ability of *Symbiodinium* sp. to process captured light. This causes dysfunction in photosystem II. UVR has been shown to act synergistically with increased temperature and physiological hyperoxia to produce reactive oxygen species (ROS) in host tissues. The fluxes of ROS overwhelm host and algal anti-oxidant defence systems, resulting in damage to both the light (photosystem II) and dark reactions (carbon fixation) in photosynthesis (Lesser and Farrel, 2004). Under high temperature and light conditions, components such as superoxide dismutase (SOD) and ascorbate peroxidase (APO) that quench and convert superoxides into less harmful molcules are saturated resulting in damage to cellular components (Hoegh-Guldberg and Smith 1989; Glynn and D'Croz 1990; Iglesias-Prieto *et al.,* 1992; Lesser *et al.,* 1990, Lesser 2004; Fitt and Warner, 1995).

Smith *et al*., (2005) suggested that hydrogen peroxide generated within zooxanthellae may have a signalling role in triggering the mechanisms that result in expulsion of the zooxanthellae from the coral.

1.3 *Symbiodinium* Genus

Zooxanthellae are symbiotic dinoflagellates (or any symbiotic 'brown algae') and form part of the group Dinoflagellata which is a group of single-celled protists common to aquatic environments (Trench, 1993; Rowan, 1998; Coffroth and Santos et al., 2005). Freudenthal (1962) formally described the taxonomy, life cycle and morphology of these dinoflagellates and erected the genus *Symbiodinium* to encompass the symbiotic dinoflagellates associated with a phylogenetically diverse range of invertebrate hosts.

1.3.1 Description, Identification and Phylogeny of Symbiodinium

An understanding of algal symbionts found in reef corals is essential to elucidate the past evolution, present distribution and future of coral reefs and the ecosystems they support (Baker, 2003). Dinoflagellates in the genus *Symbiodinium* are the most common endosymbionts and their presence contributes substantially to the productivity, survival and success of their hosts (LaJeunesse, 2002). A true mutualistic relationship exists between the host and symbiont, in that the symbiont receives inorganic nutrients from the host and provides the host with translocated photosynthetic products (Muscatine, 1990). *Symbiodinium* species possess chlorophyll *a* and *c*, plus various carotenoids, particularly the peridinins which confer the brown colour. In Cnidaria, the *Symbiodinium* cells are generally intracellular, located in cells of the endoderm (Douglas, 2003; Schoenberg and Trench, 1980).

Symbiotic dinoflagellates were first considered to be members of a single pandemic species, *Symbiodinium microadriaticum* (Freudenthal, 1962, Taylor, 1971, 1974). In the mid-1970s, however, evidence began to accumulate that these dinoflagellates were characterised by a high degree of genetic diversity and, at present, eleven species have been identified in the genus *Symbiodinium* (Baker, 1999). Initially, species were distinguished using the morphological species concept (Taylor, 1971, Schoenberg and Trench, 1980b); however, this was problematic as difficulties arose when culturing these microalgae for morphological description (Baker, 1999). An observed lack of sexual reproduction in this

algal group also prevented the use of the biological species concept to define species boundaries. Other studies aimed at assessing symbiont diversity included methods based on behavioural as well as biochemical and physiological symbiont traits (Schoenberg and Trench, 1980c; Chang and Trench, 1982; Withers *et al*., 1982; Chang *et al*., 1983; Fitt and Trench, 1983; Colley and Trench, 1983; Kokke *et al*., 1984; Iglesias-Prieto *et al*., 1991; Iglesias-Prieto and Trench, 1997; Iglesias-Prieto and Trench, 1994; Rodriguez-Lanetty, 2001). Withers *et al*., (1982) for example, used sterol analysis of cultured zooxanthellae to reveal marked variation between sterol patterns among zooxanthellae from different invertebrate hosts.

In recent years molecular methods are being used to define diversity with increasing success and, as a result the phylogenetic species concept is now being employed to distinguish taxa (Baker, 2003). Molecular studies have yielded between four and ten distinct clades of *Symbiodinium*. Five of these clades (A-D, F) are known to occur in corals (Rowan and Powers, 1991; Carlos *et al*., 1999; Baker, 1999; Toller, 2001a,b; LaJeunesse, 2001; Ulstrup and van Oppen, 2003). Host-symbiont relationships were initially believed to be highly specific. This is because, up to the early 1990s, *Symbiodinium* taxonomy relied on the morphological study of cultured material, and culturing *Symbiodinium* reduces the diversity of heterogeneous isolates, favouring non-representative members over previously dominant types (Baker, 2003). Since then studies have revealed that multiple strains of zooxanthellae may occur within hosts and that diverse ecological factors drive ecological zonation of different strains among coral colonies, and even over the surface of individual coral colonies (Rowan and Knowlton, 1995; Rowan *et al*., 1997; Toller *et al*., 2001a,b; Chen *et al*., 2005).

1.3.2 Specificity of Hosts and Symbionts

Recent studies have shown that some coral hosts are able to associate with a variety of distantly-related symbiont types, while others are restricted to a single symbiont or sub-set of closely-related types. The same pattern has been observed amongst algal symbionts,

with some being widely-distributed and found in many hosts ("generalists") and others being endemic to certain regions and restricted to a particular host taxon ("specialists"). A single association can be both flexible and highly-specific, meaning that the host may have high symbiont specificity but the symbiont may be a generalist or *vice versa.* Strict specificity may reflect a stable equilibrium between the symbiont and host, and disturbances such as environmental change, bleaching, or disease may trigger the formation of opportunistic partnerships. Zooxanthellar diversity thus provides a mechanism for corals to adapt to changing environmental conditions (Baker, 2003). Variation in environmental factors with time and from one location to another make regional differences in zooxanthellar diversity within a single host or among multiple host species important (Chen *et al*., 2005). The presence of physiological differences among zooxanthellar types has led to the suggestion that these types may affect their hosts' sensitivity to environmental conditions and/or that changes in environmental conditions may affect the distribution of zooxanthellar types (van Oppen *et al*., 2005). Therefore, it would appear that the ability of a coral host to live in symbiosis with several symbiont types is beneficial to the host as it permits combinations with broader physiological possibilities than those of a strict one-on-one association (Baker, 2003).

The adaptive bleaching hypothesis (ABH) has been proposed to account for the loss of photosymbionts which allows some representatives of a host species to re-establish a symbiosis with a different alga, resulting in a new holobiont that is better suited to the altered environmental circumstances. A change in dominant symbionts potentially enhances the long-term survival of the host, which may explain why coral reefs are sensitive to environmental perturbation in the short term, but are robust over geological time (Fautin and Buddemeier, 2004). From previous studies, it has been demonstrated that recovery of coral-algal symbioses after a bleaching event is not solely dependent on the *Symbiodinium* complement initially acquired in the host's ontogeny, but may also occur through the establishment of new symbiont associations from an environmental pool (Lewis and Coffroth, 2004). There are three ways in which a change in symbiont community structure following mass bleaching events can arise: Differential mortality of

bleaching-susceptible combinations (natural selection), quantitative changes in the relative abundance of existing symbiont communities within colonies (symbiont "shuffling") and qualitative changes by recombination with symbionts acquired from the environment (symbiont "switching"). The latter two modes form part of the ABH (Baker, 2003).

Bleached corals may serve as nutrient rich habitats for zooxanthellae as the waste products of coral metabolism go largely unutilized. As a result, competition for nutrients amongst different zooxanthellar strains may be minimal. However, as the coral recovers and the zooxanthellar biomass increases, so will the competition for nutrients. The zooxanthellar genotype that competes for the nutrients best should ultimately become the dominant symbiont (Toller *et al*., 2001a). However, a high diversity of symbiont types has been described in the literature (LaJeunesse *et al*., 2003; LaJeunesse, 2004a; Coffroth and Santos, 2005) which suggests that symbionts may not be selected against as in any given area there is diversity of *Symbiodinium* types and not a single dominant type.

A key assumption of symbiont change is that corals are able to host multiple symbiont genotypes either concurrently or sequentially. By combining data from 1991 to 2006, however, Goulet (2006) showed that the majority of corals (77%) host only one symbiont clade. She also demonstrated that the coral species that host a single zooxanthellar clade have not been shown to switch to another zooxanthellar clade, even at the sub-cladal level, when coral colonies were monitored over time, after transplantation to novel environments as well as after exposure to diseases or elevated temperatures. Goulet (2006) also indicated that no study has thus-far demonstrated that coral species hosting a single symbiont clade form new combinations with "cryptic" zooxanthellae. These findings indicate that the majority of symbionts may not be able to adapt to changing environmental conditions as has been hypothesized and, in the context of global change, may be at greater risk than previous studies have implied.

The reproductive mode of the coral host affects how its offspring become symbiotic. Symbionts must be acquired from the surrounding environment (horizontal acquisition) in

corals that broadcast spawn aposymbiotic gametes into the water column. This allows for new host-symbiont combinations in each new generation. Corals that brood larvae internally must, on the other hand, acquire symbionts from the parent (vertical acquisition), resulting in the maintenance of specific combinations among various coral species and various *Symbiodinium* species (Thornhill *et al*., 2006).

Coffroth *et al*., (2001) demonstrated that the primary polyp stage, in coral species that host only a single zooxanthellar clade as adults, is more flexible in hosting multiple zooxanthellar genotypes, even from different symbiont clades. It has been suggested that the zooxanthellar complement of the adult colonies may arise by two processes: 1) Newly settled polyps that acquire a zooxanthellar clade not found in the adults may die, resulting in the survival of only the polyps hosting the clade found in the adults or 2) the zooxanthellae belonging to the clade characteristic of the adult may out-compete all other zooxanthellae (Goulet and Coffroth, 2003a; Goulet, 2006).

1.3.3 Ecology and Biogeography of Symbiodinium

Past studies on Caribbean scleractinian corals have yielded *Symbiodinium A-, B-,* and *D*-types in shallow water (0-6 m), while *Symbiodinium C*-types are found in deeper water (3-14 m) (Rowan and Knowlton 1995; Rowan *et al*., 1997; Toller *et al*., 2001b). Mixed symbiont populations have been found in single colonies in intermediate or shallow reef habitats. This indicates that different symbiont types are suited to different light conditions (Baker, 1999). These depth patterns of symbiont distribution are, however, not found in the tropical Pacific. Instead, different symbionts within clade *C* exhibit patterns of depth-zonation (Baker, 1999, LaJeunesse, 2003; van Oppen *et al*., 2001). High-light environments, both in the Caribbean and Pacific, are characterised by higher symbiont diversity than low-light environments, which is probably a result of greater variability in high-light environments (LaJeunesse, 2002; Baker, 2003). Symbiont diversity has also been demonstrated within individual coral colonies. Cases of intracolony diversity generally occur at intermediate depths where both shallow- and deep-water symbiont taxa

co-occur (Baker, 1999). The distribution of symbionts within a colony correlates with irradiance levels and may thus relate to differences in photophysiology among symbionts (Thornhill *et al*., 2006).

Symbiont distributions in scleractinian corals have also been shown to vary in different regions of the world. Members of *Symbiodinium A, B* and/or *F* are more common at higher latitudes worldwide, while *Symbiodinium C* is more abundant in tropical latitudes (Rodriguez-Lanetty *et al*., 2002; Savage et al., 2002). Host assemblages in the Western Atlantic (Baker and Rowan, 1997) and the Red Sea (Karako-Lampert *et al*., 2004) are commonly associated with members of clade *A*. Clade *B* has been frequently found to be associated with gorgonian corals in the Western Atlantic (Baker and Rowan, 1997; Goulet and Coffroth, 1997). Clade *C* has been found to be particularly abundant in the Indo-Pacific (LaJeunesse, 2005) and seems to be more sensitive to increases in sea water temperature than the other *Symbiodinium* clades (Rowan, 2004). However, clade C has been shown to consist of a large number of ecologically and physiologically distinct 'types' which have individual physiological sensitivities and tolerances (LaJeunesse 2001). Scleractinian corals in the tropical western Atlantic have been found to be associated with clades *A*, *B*, *C* and *D* but clades *A* and *B* are absent from tropical Pacific scleractinians despite them being present in other invertebrates in that region (Baker and Rowan, 1997, Baker 1999). This conclusion, however, may not be true for temperate Pacific Scleractinia as clade *B* has been identified in *Plesiastrea versipora* (Baker, 1999). The observation that multiple symbioses within scleractinian corals always involve clade *C* and the wide biogeographic distribution of *Symbiodinium C* suggest that this may have been the ancestral symbiotic state of Scleractinia (Baker and Rowan 1997; Baker, 1999). More recent work by LaJeunesse (2002) and LaJeunesse *et al*., (2002, 2003, 2004a, 2004b and 2005) has shown subclades C1 and C3 to be ancestral varieties in both the Pacific and Caribbean realms. The latitudinal and bathymetric variation in symbiont types further suggest that both temperature and light play important roles in determining *Symbiodinium* distribution (Baker, 2003).

Symbiodinium D has been inferred, from phylogenetic analysis, to be ancestral to clades *B* and *C* (Baker, 1999). It has a relatively low diversity, is haphazardly distributed throughout the tropics and does not appear to be the dominant symbiont in any particular species (LaJeunesse, 2002). It has been shown to be favoured under conditions to which other symbionts are poorly suited, such as the transition depth between the shallow *Symbiodinium A*-types and the deep *Symbiodinium C*-types in the scleractinian coral *Stephanocoenia intersepta* (Baker, 1999), and in extremely deep colonies of *Montastraea franksi* (Toller *et al*., 2001b). Van Oppen *et al*., (2005) noted a post-bleaching increase in the relative abundance, and possibly the absolute abundance, of *Symbiodinium D* in coral species on the Great Barrier Reef. It has also been demonstrated that far-eastern corals harbouring symbionts of clade *D* suffered less from the 1998 coral bleaching event than those associated with symbionts of a different genotype, suggesting that zooxanthellae of clade *D* increase the thermal tolerance of the coral host (Ulstrup and van Oppen, 2003).

Therefore, *Symbiodinium D* may be an opportunistic symbiont, characteristic of recently stressed or marginal habitats, in bleached corals in the process of re-establishing a steady-state equilibrium with their original symbiont communities. An abundance of *Symbiodinium D* may thus reflect coral community health by providing an indication of recent and/or recurrent stress events (Baker, 2003). The thermal tolerance attained by coral hosts harbouring the clade *D* symbiont, however, is associated with a cost as growth in juveniles of *Acropora millepora* that harbour clade *D* zooxanthellae is two to three times slower than those that harbour *C* zooxanthellae, and thus results in a reduced ability to recover from disturbances and to compete for space (van Oppen *et al*., 2005). Therefore, it appears that in the absence of stress, stress-sensitive holobionts have competitive advantages over stress-tolerant holobionts and the situation is only reversed in the presence of stress when opportunistic symbiont types become dominant (Fautin and Buddemeier, 2004).

Cladal level identity, however, does not always correlate with physiological function. Past studies have demonstrated that, when exposed to elevated temperatures, algae from

different clades respond similarly while isolates from the same clade, including most closely-related sister "types", exhibited significantly different responses. Therefore specific physiological attributes should only be assigned to particular *Symbiodinium* taxa when sufficient data are available (Coffroth and Santos, 2005). However, functional differences have been demonstrated among the clades' ability to synthesize mycosporine-like amino acids (MAAs) under the influence of ultra-violet light and photosynthetically active radiation (PAR) (Banaszak *et al*., 2000). An accumulation of UV-absorbing compounds such as MAAs is thought to increase resistance to UV inhibition (Neal *et al.*, 1998).

1.4. Defining Conservation Units

1.4.1 Species Concepts

There are numerous definitions of species involving the use of many parameters. The Morphological Species Concept defines species as being morphological units. Species are defined on the basis of shared phenotypic characters (Mayr, 1982). However, the process of deciding which morphological characters constitute a species is subjective. Because of this, taxonomists turned to giving certain characters more priority than others. This weighting of characters, though, is also in a sense subjective. Because of this, the Phenetic Species Concept was devised. This concept defines a species as a "set of organisms that cluster at a certain distance from other such clusters" and makes use of statistics to test similarity, thus reducing the subjectivity (Sneath and Sokal, 1973). Other problems still arise when defining species based on morphology. It is often impossible to distinguish between sibling species (species that are morphologically similar but reproductively isolated), cryptic species (species that are morphologically similar but genetically distinct), sexually dimorphic species (male and females with different morphologies) and polymorphic species (different morphological forms in the same life-cycle). Also, defining species based on their morphological characters contradicts the fact that species may change over long periods of time. This concept also does not explain how differences in morphology arise amongst species (Mayr, 1996).

The Biological Species Concept states that "species are groups of actually or potentially interbreeding natural populations, which are reproductively isolated from other such populations" (Mayr, 1942). This concept is based on the logic that morphological variation in species is based on genes in the species' gene pool and that these genes are passed on from generation to generation via reproduction. Discontinuities exhibited in morphologies between species that are a result of divergence in the frequency of genes in the gene pool, are maintained because of mechanisms that restrict gene flow between diverging gene pools. This concept, which suggests that interbreeding is of prime importance in evolution, now appears to be oversimplified (Donoghue, 1985). The definition cannot be applied to autogamous (self-fertilizing) or apomicitic (bypass sexual reproduction altogether) organisms such as plants, as it would mean that many taxa would not receive recognition as separate species (Masters and Spencer, 1989; Mayr, 1996).

The Evolutionary Species Concept (Simpson, 1961) was developed because of the limitations of the Biological Species Concept. Morphological diversity is seen to be a consequence of evolution and species are viewed as dynamic entities whose morphology changes and diverges over time. This concept assumes that species that are morphologically similar have descended from a common ancestor. Simpson (1961) defined species as a lineage (an ancestral-descendent sequence of populations), evolving separately from others and with their own evolutionary role and tendencies. This concept accommodates asexual species and fossils but makes it difficult to test species empirically in order to determine if they comply with the definition. Due to this, Cracraft (1983) proposed the Phylogenetic Species Concept (PSC). This concept defines a species to be the smallest diagnosable cluster of individual organisms within which there is a parental pattern of ancestry and descent (Cracraft, 1983; Nixon and Wheeler, 1990). Species that share the same derived characters (synapomorphies) are seen to have a common ancestry. Hybridization poses theoretical and practical difficulties for the PSC, as the parent species of a hybrid are paraphyletic (Donoghue, 1985).

Methodological advances in molecular biology have led to the generation of several genetic-based data sources. These data sets have yielded cryptic species that that would probably not have been recognized based solely on classical studies of morphology (Baker and Bradley, 2006). Phylogroups experiencing stabilizing selection will evolve genetic isolation without concomitant morphological diversification (Bowen, 1999). The Genetic Species concept defines species as a group of genetically compatible interbreeding natural populations that is genetically isolated from other such groups (Baker and Bradley, 2006). This concept provides a unique level of resolution for systematists to study the number of species and their boundaries. Genetic data can quantify genetic divergence, provide an estimate of time since divergence, provide a population genetics perspective of the magnitude and types of divergence, is less subjective to convergence and parallelism, can accurately identify monophyly and sister taxa and can accurately identify hybrid individuals (Baker and Bradley, 2006). Initially karotypic and allozymic data were used to identify species, however, neither of these methods provides as much resolution as DNA sequence data (Baker and Bradley, 2006). More recently, because of methodological advances associated with automated DNA sequencing cryptic species have been identified using DNA sequence data (Bradley and Baker, 2006). These advances include the polymerase chain reaction (PCR), universal primers, GenBank, human genome project, biological informatics, computer software for analyzing molecular data and genetic resource collections associated with voucher specimens (Bradley and Baker, 2006). Because it is possible for genetic isolation to evolve without reproductive isolation, many genetically-isolated populations that are not reproductively isolated have been identified through DNA sequence data and thus the implementation of the Genetic Species Concept has resulted in a substantial number of previously unrecognized species (Baker and Bradley, 2006).

There is an overlap in the application of the Phylogenetic Species Concept and the Genetic Species Concept in use of monphyly and sister species status, however, the Genetic Species Concept places more emphasis on supporting data for isolation and on proof that there is protection of the integrity of the gene pool (Baker and Bradley, 2006). Determining the

magnitude of genetic variation required to distinguish between two putative species is still, however, an uncertain process (Bradley and Baker, 2001). Bradley and Baker (2001) concluded that a genetic distance in the cytochrome b gene of 2% corresponded to populational and intraspecific variation, whereas a genetic distance of 10% was distributed in geographically discreet phylogroups typical of different species. There is no definitive genetic distance value in a single gene that indicates two biological species. However, a single metric, when viewed against an appropriate database from other genetically defined species and subspecies with similar life histories and biological characteristics, will have an accuracy rate comparable to that of any other single character used to identify species or populations (Baker and Bradley, 2006).

Adherents of the Ecological Species Concept (EvSC) believe that ecological processes influence genetics and hence morphology (Andersson, 1990). Species are thus said to occupy ecological zones or habitats to which they are optimally adapted and differences between species are seen to be the result of their adaptations to their niche (Andersson, 1990). Van Valen (1976) defined a species as a lineage (or closely related set of lineages) which occupies an adaptive zone minimally different from that of any other lineage in its range and which evolves separately from all lineages outside its range. This concept directly connects environment and phenetic variation patterns (Andersson, 1990). However, in almost all widespread species there are local populations which differ in their niche occupation (Mayr, 1996). Also, there are numerous cases where two sympatric species occupy the same niche (Mayr, 1996; Bremer and Eriksson, 1992).

1.4.2 Ecologically Significant Unit Concepts and Management Units

Because of the differences in the definition of species encountered by the different schools of thought, problems have also arisen in deciding at what level of classification conservation efforts should be directed (Bowen, 1999). Systematists argue for the primacy of phylogenetic rankings, ecologists for the protection at the population and ecosystem level, and evolutionary biologists urge for more attention to factors that enhance adaptation

and biodiversity. However, the preservation of any single one of these objects (genes, species or ecosystems) alone is futile (Bowen, 1999). Because the disciplines of systematics, ecology and evolution each contribute uniquely and irreplaceably to conservation genetics, studies within all three domains are relevant to conservation.

There has been much debate on how to best preserve variability within threatened species, the assumption being that higher genetic variation enhances the probability of a population's survival over ecological or evolutionary time (Avise, 1989). As estimated by molecular genetic techniques, heterozygosity is known to be dramatically reduced in some threatened species and this is sometimes accompanied by lowered fitness (Avise, 1989).

Molecular genetic information can be used to detect phylogenetic discontinuities within and among species. The classification of taxa according to morphology often provides inadequate or even incorrect partitions of phylogenetic diversity; input from molecular genetics can thus provide a firmer foundation for the proper recognition and hence management of genetic diversity (Avise, 1989). If taxa are incorrectly classified, conservation efforts may focus on populations showing little evolutionary separation from other conspecifics, and neglect other populations showing considerable evolutionary genetic distinctiveness (Avise, 1989).

Given that existing taxonomy may not adequately reflect underlying genetic diversity the Evolutionary Significant Unit (ESU) concept was developed to provide a rational basis for prioritizing data for conservation efforts (Moritz, 1994). The ESU remains poorly defined, both conceptually and operationally. There is disagreement regarding the relative role that "neutral" genetic markers should play compared to other criteria in defining ESUs. Neutral genetic markers and phenotypic traits will yield different types of information, as different gauges of varying evolutionary forces may influence them (Moritz, 1994). There is also disagreement about where to focus conservation efforts along the evolutionary continuum from population segments to species (Moritz *et al.,* 1995). The definition of ESUs should

thus ensure that the evolutionary heritage is recognized and protected and that the evolutionary potential inherent across the set of ESUs is maintained (Moritz, 1994).

Initially Ryder (1986) defined an ESU as a subset of the more inclusive entity species, which possesses genetic attributes significant for the present and future generations of the species in question. Ryder (1986), however, did not offer guidelines for operational applications (Fraser and Bernatchez, 2001). Waples (1991) defines an ESU as a population segment or group of populations that is substantially reproductively isolated from other conspecific populations and represents an important component in the evolutionary legacy of the species. Waples' approach is integrative, accommodating the use of diverse biological and environmental information to discriminate ESUs, and it provides a working framework for preserving component parts of ESUs (Fraser and Bernatchez, 2001). However, although the first component of the definition (reproductive isolation) can be identified with phenotypic and/or molecular genetic methods, this is not the case with the second component (important component of evolutionary legacy), as it implies an understanding of how species arise (Fraser and Bernatchez, 2001).

When the phylogeographical approach is applied in defining ESUs, levels of genetic similarity at molecular genetic markers dictate evolutionary distinctiveness of populations (Moritz, 1994). The emphasis is on historical population structure rather than current adaptation because it can produce unique and irreplaceable combinations of genotypes (Moritz, 1994). Phenotypic divergence is not emphasized because it can happen rapidly and even recurrently through selection in natural populations, and is thus potentially replaceable (Moritz, 1994). An ESU is thus defined as a historically isolated set of populations (Moritz, 1994). According to Moritz (1994) an ESU is a population or set of populations that is reciprocally monophyletic for mtDNA alleles and shows significant divergence of allele frequencies at nuclear loci. It takes about 4N generations from the time two populations separate for there to be a high probability of their having reciprocally monophyletic alleles. Mitochondrial DNA (mtDNA) has a relatively low effective population size and high substitution rate and can thus achieve this condition more rapidly

than nuclear alleles (Moritz, 1994). This definition enables conservation biologists to apply molecular genetics but at the same time to avoid determining how much genetic variation is required to warrant the protection of a given population(s) of a species (Fraser and Bernatchez, 2001). These principles can be applied to whole communities using comparative phylogeography to define geographic areas where component species have evolutionary histories separate from their conspecifics (Moritz, 1994). Problems are, however, encountered with this approach. There is no one best method for phylogeny reconstruction, which yields the most likely phylogeny in all situations (Fraser and Bernatchez, 2001). Reciprocally monophyletic relationships may also not always infer historical isolation (Crandall *et al.,* 2000). In addition, due to reciprocal monophyly being such a stringent approach, a single individual in a new sample, if an anomaly, can overturn a population's or group of populations' reciprocally monophyletic status (Crandall *et al.,* 2000). The concept may also not put enough emphasis on the potential ability of species to maximize evolutionary success through the maintenance of adaptive diversity (Fraser and Bernatchez, 2001). Finally, because mtDNA evolves 40 – 100 times slower in plants than in animal mtDNA, this concept may be of limited use for phylogeographical studies in plants (Fraser and Bernatchez, 2001).

Because, in practice, genetic analyses often reveal differences between sampled populations, ranging from reciprocal monophyly, through substantial but incomplete phylogentic separation, to minor but statistically significant differences in allele frequency, the MU or Management Unit was recognized as fundamental to proper short-term management of the more inclusive ESUs (Moritz, 1994). MUs are recognized as populations with significant divergence of allele frequencies at nuclear or mitochondrial loci, regardless of the phylogenetic distinctiveness of the alleles (Moritz, 1994).

The Phylogenetic Species Concept (PSC) can also be used to propose conservation units by finding characters that cluster groups to the exclusion of other similar ones (Fraser and Bernatchez, 2001). It states that the smallest discernible and distinct clusters where one finds a parental pattern of decent and ancestry is considered to be it's own entity (Fraser

and Bernatchez, 2001). Population aggregation analysis can be used to identify hierarchically related groups that are the most inclusive groups of organisms united by fixed diagnostic character states (Fraser and Bernatchez, 2001). This method is stringent and thus testable; however, because of this stringency it lacks flexibility. This lack of flexibility could be problematic in small samples by chance leading to potentially erroneous management decisions (Fraser and Bernatchez, 2001). The followers of this concept believe that this approach elevates all diagnosable evolutionary lineages to species, thus removing the need to establish ESUs that are not part of formal nomenclature (Fraser and Bernatchez, 2001). The PSC is, however, sometimes not considered applicable to conservation policy. This is because, when increasingly sophisticated methods of descriptive molecular genetics are applied to natural populations, finer distinctions will result and, because most species exhibit genetic differences across their ranges, these distinctions may prove even individuals to be distinguishable from one another by some recently-derived genetic trait (Avise, 1989). Conversely, others argue that the PSC may underestimate the number of populations in need of protection and that it focuses too closely on species level. Also, the characters used to discriminate between groups can be difficult to score unambiguously and may thus be problematic (Fraser and Bernatchez, 2001).

ESUs can also be defined on the basis of molecular genetic markers alone. Population units are identified based on eight categories of population distinctiveness (Crandall *et al.,* 2000). Distinctiveness depends on the failure to reject or on the rejection of the null hypothesis of both historical and recent genetic and ecological exchangeability. Populations are assigned to the various categories depending on how distinct a particular population is (Fraser and Bernatchez, 2001). Each category has specific management recommendations. This approach recognizes the importance of adaptive distinctiveness in populations, combines ecological and genetic principles, and is testable in the form of null hypotheses (Fraser and Bernatchez, 2001). The most important advantage is, however, that this concept allows for the objective prioritization of conservation value across a broad taxonomic spectrum, thus including invertebrates and plants (Fraser and Bernatchez, 2001). There are disadvantages

to this approach. Groups of individuals, identified as belonging to the same species based on genetic and ecological nonexchangeability, have probably already been identified as such, making this approach not useful to conservation practices (Fraser and Bernatchez, 2001). Also, evidence is not given of recent genetic nonexchangeability being weighted the same as recent ecological nonexchangeability, even though this could signify an important step towards speciation (Fraser and Bernatchez, 2001).

Much of the debate over ESU concepts is a result of a similar debate over the species concepts used to define them. A unifying ESU concept should ideally operate under a unified species concept. The ESU definitions themselves are similar and thus reconciliation between ESU concepts is possible (Fraser and Bernatchez, 2001). The time scales involved under the various ESU concepts do, however, vary and the different ways in which gene flow is suggested to have been highly reduced in order to identify an ESU also varies between concepts (Fraser and Bernatchez, 2001). Thus because no ESU concept fulfils all the conservation goals across all species boundaries, differing approaches may work more effectively than others in varying circumstances and the designating of ESUs should be done on a case-by-case basis. All the ESU concepts thus guide and support the unifying conceptual framework of adaptive evolutionary conservation (AEC) goals, and, depending on the circumstances, a particular ESU concept will work more dynamically than others, and can be used alone or in combination with another concept (Fraser and Bernatchez, 2001).

There is thus agreement that the main conservation goal should be to preserve both evolutionary processes and the ecological viability of populations by maintaining genetic diversity within species, so that the process of evolution is not excessively constrained (Fraser and Bernatchez, 2001). Intraspecific ecological variation and genetic variation must be maintained in the short and long-term. Both adaptive and historical isolation should be considered, as both are functionally important and represent an extreme along the continuum of how the accumulation of genetic differences arises by the differential aspects of evolutionary forces (Fraser and Bernatchez, 2001). Reciprocally-monophyletic mtDNA

can be applied in populations of animals that have had enough evolutionary time to divide naturally into groups over the species range, thus demonstrating historical isolation. On the other hand, tests of ecological exchangeability or ecological characters may be more appropriate, in lineages experiencing rapid adaptive radiations, by the accumulation of differential genetic differences through directional selection, thus demonstrating adaptive isolation (Crandall *et al*., 2000). Thus there is consensus that entities, which we define as ESUs, may arise by accumulation of genetic differences through the various roles of evolutionary forces through time. The evolutionary continuum will vary with the organism at hand and with the criteria used. Therefore the various ESU concepts are merely tools that must operate in a complementary fashion in order to meet the goals of AEC (Fraser and Bernatchez, 2001).

1.5 Genetic Techniques Used for Identifying *Symbiodinium* Diversity

1.5.1 DNA/DNA Hybridization and Isoenzyme Analysis

The use of genetic techniques to document zooxanthellar diversity began with comparisons of isoenzyme patterns in studies by Schoenberg and Trench (1976, 1980). Isoenzyme analysis involves the isolation of enzymes which are present in all species but show heterogenicity which can be picked up by comparing migration patterns on electrophoretic gels (Steube *et al.,* 2003). Other studies that provided genetic evidence for symbiont diversity include DNA base composition analyses (Blank *et al*., 1988) and DNA/DNA hybridization analyses (Blank and Huss, 1989). DNA/DNA hybridization is a method used to measure the degree of genetic similarity between sequences in order to determine the genetic "distance" between two species (Graur and Li, 1991). DNA is heated to denaturation temperatures to form single strands and then cooled to allow the double helices to re-form (renaturation) at regions of sequence complementarity (Sibley and Ahlquist, 1984).

1.5.2 Restriction Fragment Length Polymorphism

Rowan and Powers (1991) first uncovered the cladal diversity within the *Symbiodinium* genus. They made use of restriction fragment length polymorphism (RFLP) to detect differences in the nuclear ssrDNA. RFLP is a technique in which organisms may be differentiated by analysis of patterns derived from cleavage of their DNA by particular restriction endonucleases. Restriction endonucleases are isolated from a wide variety of bacterial genera and play a part in a cell's defences against invading bacterial viruses. If two organisms differ in the distance between sites of cleavage of a particular restriction endonuclease, the length of the fragments produced will differ when the DNA is digested with a restriction enzyme. The fragments can be separated by gel electrophoresis. The similarity of the patterns generated on the gel can be used to differentiate species (and even strains) from one another (Creighton, 1999).

RFLP's can be used to study the relationships among host, symbiont and habitat diversity (Toller *et al*., 2001). They provide very conservative estimates of diversity because they only distinguish sequences that vary at restriction sites (Baker, 1999). However, despite the conservative nature of RFLP analyses, they facilitate rapid identification of taxa into closely related groups (Baker, 1999). RFLP data are also easily obtained, at a reasonable cost, from many samples of zooxanthellae and provide a good picture of the broad genetic diversity of zooxanthellae in a particular sample set (Toller *et al*., 2001).

Isolation of sufficient DNA for RFLP analysis is, however, time-consuming and labour intensive. However, the Polymerase Chain Reaction (PCR) can be used to amplify very small amounts of DNA to the levels required for RFLP analysis, allowing more samples to be analysed in a shorter amount of time.

1.5.3. Polymerase Chain Reaction

The Polymerase chain reaction is an *in vitro* technique for enzymatically replicating DNA. It allows a small amount of DNA to be amplified exponentially. PCR is used to amplify

specific regions of a DNA strand such as a single gene, a part of a gene or a non-coding sequence. The PCR technique consists of three stages: DNA denaturation, primer annealing and primer extension. During denaturation the DNA is heat-denatured such that the two strands separate (Strachan and Read, 1999). During annealing the temperature is reduced such that it is optimal for the binding of primers to specific regions of the DNA strands. Primers are complementary oligonucleotide sequences that are chemically synthesized such that they flank the region to be amplified. Next, the temperature is increased such that polymerase extension is carried out from the primer termini. The enzyme DNA polymerase controls the synthesis of the new DNA strand. For the purpose of PCR the enzyme *Taq* polymerase is utilized. This enzyme is isolated from the heat tolerant organism *Thermus aquaticus* and is thus not deactivated at DNA-denaturing temperatures. The three stage cycle can be repeated many times such that an abundance of DNA fragments can be created (Strachan and Read, 1999).

1.5.4 ssrDNA verses lsrDNA

Researchers have since used other DNA regions, such as the nuclear large ribosomal subunit DNA (lsrDNA) (Wilcox 1998, Baker 1999), to study genetic diversity amongst zooxanthellae. ssrDNA cannot be used to assess sub-cladal variation as it is relatively conservative, occurs in multiple copies and probably varies within a genome (Knowlton and Rohwer, 2003). The latter property makes it difficult to distinguish within- from between genome variation in zooxanthellae from a single clade (Knowlton and Rohwer, 2003). RFLP analysis of lsrDNA, however, allows for the elucidation of more recent divergences as lsrDNA is, in general, less conserved than ssrDNA (Hillis and Dixon, 1991). In a previous study, Wilcox (1998) showed that the mean per-nucleotide information content of the D1 – D3 region of the lsrDNA in *Symbiodinium* is approximately 3.8 times greater than in ssrDNA.

1.5.5. Internal Transcribed Spacer Region (ITS)

The internal transcribed spacer (ITS) region, consisting of ITS1, 5.8S, and ITS2 sequenced from protists, animals, plants, fungi, and macrophyte algae, typically provides phylogenetic resolution at or below the species level in each of these groups (LeJeunesse, 2001, Hunter *et al.,* 1997). The ITS regions are less-conserved than the ssrDNA and lsrDNA regions and have recently been widely used in quantifying *Symbiodinium* diversity within the various clades (Diekmann *et al.* 2003; LaJeunesse 2001, 2002; LaJeunesse *et al*., 2003, 2004a, 2004b; Santos *et al.* 2001; van Oppen *et al.* 2001). Hunter *et al*., (1997) sequenced the entire region (ITS1-5.8S-ITS2), and showed its value in distinguishing among *Symbiodinium* isolates. It exists in multiple copies of tandem repeats in the nuclear genome. However, despite concerted evolution there is a potential for intragenomic variation among these copies (Hunter *et al*., 1997). Because the ITS region is a non-coding spacer region, it is under much less selective constraint than coding regions (i.e. lsrDNA and ssrDNA). ITS sequence comparison is also very powerful due to the presence of the highly conserved 5.8S rDNA between the ITS-1 and ITS-2 regions, which allows for the anchoring of the sequence alignments (Hunter *et al*., 1997). Therefore, the ITS region allows for the detection of within-clade variation amongst *Symbidinium* isolates (Coffroth and Santos, 2005). LaJeunesse (2001) showed that sequence variation within the ITS region identified 6, 4, 2 and 2 groups within clades A, B, C and F, respectively. The sequence and length variability of ITS, however, makes it difficult to align this region between the clades, which limits its use to within-clade phylogenetics (LeJeunesse, 2002).

1.5.5.1 DGGE of ITS-2

DGGE is a technique based on the electrophoretic separation of PCR-generated double-stranded DNA in an acrylamide gel containing a gradient of denaturant. As the DNA encounters an appropriate denaturant concentration, a sequence-dependent partial separation of the double strands occurs. This conformational change in DNA tertiary structure causes a reduced migration rate and results variations in band patterns (Sigler *et al.,* 2004).

LeJeunesse (2002) used DGGE to study the diversity and community structure of zooxanthellae form Caribbean coral reefs enabling the rapid assessment of variation and the examination of how it is distributed among host taxa and geographic locations. He was able to examine the disparities in the electrophoretic mobility of the ITS-2 region as a result of the differences in its primary sequence. In the same way LeJeunesse *et al.,* (2003) determined that there was low symbiont diversity in southern Great Barrier Reef corals, relative to those of the Caribbean, and that there was a high diversity and host specificity among zooxanthellae in reef communities from Hawaii (LeJeunesse *et al.,* 2004a). LeJeunesse *et al.,* (2005), again using DGGE, showed that non-Caribbean zooxanthellae persisted in Indo-Pacific Mushroom corals released in Jamaica in 1969.

1.5.5.2 SSCP of ITS-1

Van Oppen *et al.,* (2001) and Ulstrup and van Oppen (2003), used sequences of the ITS-1 region to construct the symbiont phylogeny. They used single stranded conformation polymorphism (SSCP) to detect sequence variation amongst symbiont isolates. Van Oppen *et al*., (2005) used SSCP and sequence analysis of the ITS-1 region to identify the zooxanthellar genotype on the Great Barrier Reef after the 2002 bleaching event. SSCP offers a simple, inexpensive and sensitive method for detecting whether or not DNA fragments are identical in sequence, and so can greatly reduce the amount of sequencing necessary (Sunnucks *et al.,* 2000). SSCP entails electrophoresis of single-stranded DNA fragments of suitable size through a nondenaturing polyacrylamide gel, followed by visualization (Sunnucks *et al.,* 2000). During electrophoresis the DNA strands fold into structures that migrate according to their shape. The shape of the strands is determined by their sequence and as a result different sequences have different mobilities (Sunnucks *et al.*, 2000).

1.5.6 Chloroplast lrsDNA

Santos *et al.,* (2002) constructed a *Symbiodinium* phylogeny based on chloroplast large subunit 23S rDNA sequences. They found that the chloroplast and nuclear phylogenies were congruent, which was unexpected as, in free-living dinoflagellates, phylogenies based on chloroplast genes do not always result in the same topology as nuclear genes (Santos *et al.,* 2002). This suggests that *Symbiodinium* chloroplast and nuclear rDNAs share a common evolutionary history (Santos *et al.,* 2002).

1.5.7 Microsatellites

Studies conducted by Santos *et al*., (2003, 2004) explored zooxanthellar genetic diversity within the same sub-clade or type. They employed microsatellites and their flanking regions to investigate fine-scale diversity and population structure of *Symbiodinium* type B1/B184 in symbiosis with Caribbean gorgonians. Santos *et al*., (2003) showed significant population differentiation among *Symbiodinium* spp. Clade B inhabiting the gorgonian *Pseudopterogorgia elisabethae* from different Bahamian reefs. Santos *et al*., (2004) demonstrated the existence of fine-scale specificity between Caribbean octocorals and their symbionts by comparing the genetic diversity of *Symbiodinium* belonging to the same lineage inhabiting sympatric gorgonian hosts. Magalon *et al.,* (2006) investigated the fine-scale genetic diversity and genetic structuring of the Symbiodinium inhabiting seven populations of the coral *Pocillopora meandrina* from French Polynesia and Tonga, using two polymorphic microsatellites.

1.6 Data Analysis

1.6.1 Information Required for Phylogenetic Analyses

Phylogenetic trees are used to illustrate relationships among genes and organisms. The alignment of sequences from different individuals so that the homologous residues (descended from a common ancestor) are arranged in columns is an essential prerequisite for phylogenetic analyses, as rates and patterns of change in the sequences can be analyzed

when sequences are aligned. If the sequences are evolutionarily related, they began as identical to each other and diverged over time by the accumulation of substitutions, as well as insertions and deletions. The insertion of gaps corresponding to the insertions and deletions is necessary so that the sequences can be properly aligned. Repeats in the sequences make alignment problematic, as it is difficult to determine which repeat unit in one sequence should line up with which repeat unit in another sequence. The various possible alignments are scored and the alignment with the best score is chosen. Penalties are given to gaps in the alignment (Salemi and Vandamme, 2003).

The formula used for calculating gap penalties is:

$$GP = g + hl$$

Where l is the length of the gap, g is the gap-opening penalty, and h is the gap extension penalty (Salemi and Vandamme, 2003).

Because of the variety of evolutionary forces that act on DNA sequences, sequences change in the course of time. Genetic distance is a measure of the divergence between two sequences. Genetic distance provides a measure of the dissimilarity between sequences and, if the molecular clock is assumed, then genetic distance is linearly proportional to the time elapsed. Also, when the sequences are related on an evolutionary tree, the branch lengths represent the distance between the nodes in the tree. A substitution model that provides a statistical description of the stochastic substitution of nucleotides or amino acids is an important prerequisite for computing genetic distances (Salemi and Vandamme, 2003).

The simplest of these models, the Jukes and Cantor model, assumes that the equilibrium frequencies of the four nucleotides are 25% and that during evolution, any nucleotide has the same probability to be replaced by any other (Jukes and Cantor, 1969; Salemi and Vandamme, 2003). However, in reality this is not the case, as transitions are more likely to occur than transversions. It is also very unlikely that the frequencies of the nucleotides will be equal (Salemi and Vandamme, 2003). Thus, there are more advanced models of

substitution, the most advanced of which is the general-time reversal model (GTR). This model allows for all eight parameters of a reversible nucleotide rate matrix to be specified (Lanave *et al.,* 1984; Tavare 1986; Rodriguez *et al.,* 1990). However, because often all the parameters are not known, simpler models have to be used. There are numerous models which fall in this range. The most common ones are mentioned below, with the remainder being variations of these models. The model variations may take into account the proportion of invariant sites in the data and/or the gamma shape parameter (an indication of how well the data distribution approximates gamma distribution) (Salemi and Vandamme, 2003).

The Felsenstein 1981 model (F81) assumes all base frequencies to be variable and all substitutions to be equally likely (Felsenstein, 1981). The Kimura 2-parameter model (K80) works on the principle that all base frequencies are equal but that transition and transversion frequencies are variable (Kimura, 1981). The Hasegawa-Kishino-Yano 1985 model (HKY85) proposes that purine and pyrimidine transitions have the same rate (Hasegawa *et al.,* 1985; Salemi and Vandamme, 2003). The Tamura-Nei model (TrN) assumes all base frequencies to be variable and all transition frequencies to be variable but transversion frequencies to be equal (Tamura and Nei, 1993). The Kimura 3-parameter (K3P) model assumes variable base frequencies, equal transition frequencies and variable transversion frequencies (Kimura, 1981). The substitution model used is determined by the amount of information available (Salemi and Vandamme, 2003). Computer programs such as Modeltest and Mr Modeltest can be used to select the model of nucleotide substitution that best fits the data. These programs choose among a number of models, and implement three different model selection frameworks: hierarchical likelihood ratio tests (hLRTs), Akaike information criterion (AIC), and Bayesian information criterion (BIC). The programs also implement the assessment of model uncertainty and tools for model-averaging and calculation of parameter importance, using the AIC or the BIC (Posada and Crandall, 1998).

1.6.2 Distance-Matrix Methods

The use of distance methods to infer trees from sequence data involves the fitting of a tree to a matrix of pairwise genetic distances. For each two sequences, the distance is a single value based on the fraction of positions in which the sequences differ, and is defined as the p-distance. The p-distance, however, is an underestimation of the true genetic distance as some of the aligned nucleotides may be the result of multiple substitutions at the same site. Therefore, in distance-based methods, the number of substitutions that have actually occurred is estimated by applying a specific evolutionary model (Salemi and Vandamme, 2003). Distance methods are computationally fast; however, since the original data set is not used, some information may be lost, and therefore these methods may not be as powerful as the character-state methods explained later in this chapter (Salemi and Vandamme, 2003).

The main distance-based tree-building methods are cluster analysis and minimum evolution. Both rely on a different set of assumptions, and their success or failure depends on how the data set used meets such assumptions. UPGMA (Umweighted Pair-Group Method with Arithmetic Mean) is an example of a clustering method. UPGMA involves clustering of closely distant species. At each stage of clustering, tree branches are built, and the branch lengths are calculated. UPGMA assumes a constant evolutionary rate, and so the two species in a cluster are given the same branch length from the node. It is a simple and fast method, however, it often produces incorrect topologies when the assumption of a constant evolutionary rate is not met (Nei and Kumar, 2000).

Minimum evolution (ME) is a method where the tree of minimum length, which is the sum of the lengths of the branches, is regarded as the best estimate of phylogeny (Nei and Kumar, 2000). For each tree topology it is possible to estimate the length of the branch from the estimated pairwise distances between all OTUs (operational taxonomic units). A good ME method is the Neighbour-Joining (NJ) method. The NJ method is similar to UPGMA in that it manipulates a distance matrix, reducing it in size at each step, and then

reconstructs the tree from a series of matrices; however, it does not construct clusters, but directly calculates distances to internal nodes. The advantages of the NJ method are that it does not assume clock-like behaviour, allows for unequal rates of evolution between the branches and is quick to perform. Its disadvantages are that it is dependant on the model of evolution used and, because it reduces sequence information to a single value, the estimation of ancestral states is not possible (Saitou and Nei, 1986; Salemi and Vandamme, 2003).

1.6.3 Character-State Methods

Character-state approaches include maximum parsimony, maximum likelihood and Bayesian analysis methods. These methods are said to be more powerful than distance methods because they use the raw data and thus maintain all the sequence information. However, this is usually a small fraction of the data (Saitou and Nei, 1986). Because sequence information is maintained ancestral states can be reconstructed.

Maximum Parsimony (MP) methods are based on the assumption that the most likely tree is the one that requires the fewest number of changes to explain the data in the alignment. Taxa that share a common characteristic do so because they inherited it from a common ancestor. Trees that minimize the number of evolutionary steps, required to explain the data, are selected (Salemi and Vandamme, 2003). The method only uses the relevant sites in the sequence. So when the number of informative sites is not large, this method is often less efficient than distance methods (Saitou and Nei, 1986). Invariant characters, those that have the same state in all taxa, are ignored. Characters in which the state occurs in only one taxon are also ignored. Therefore only informative sites, ones at which two different characters are represented at least twice, are used. MP is notorious for its sensitivity to codon bias and unequal rates of evolution (Saitou and Nei, 1986). It also does not make any correction for the possibility of multiple mutations occurring at the same site and can be slow to use for larger data sets (Salemi and Vandamme, 2003).

The Maximum Likelihood (ML) method evaluates the probability that the proposed evolutionary model and the hypothesized history would give rise to the observed data set. The history with a higher probability of reaching the observed state is preferred to a history with a lower probability. The method searches for the tree with the highest probability or likelihood (Salemi and Vandamme, 2003). The ML method allows for the use of an explicit model of evolution and, because it is based on a statistical algorithm, it allows for the statistical comparison of tree topologies. It often has lower variance than other methods, and as a result is frequently least affected by sampling error. It is also robust to many violations of the assumptions in the evolutionary model and, even when very short sequences are analysed, tends to out-perform alternative methods such as MP and distance methods. However, ML is very computer intensive and thus extremely slow (Salemi and Vandamme, 2003).

Bayesian inference is based on the notion of posterior probability: probabilities that are estimated based on some model, after learning something about the data. It generates a posterior distribution for a parameter based on the prior for that parameter and the likelihood of the data, generated by multiple alignment. Bayesian analysis searches for phylogenetic trees that maximize the probability of the given data and the model of evolution. The method is character based and applied to each site along the alignment (Yang and Ranala, 1997; Ronquist and Huelsenbeck, 2001 and 2003). The advantages of Bayesian inference over other methods include easy interpretation of results, the ability to incorporate prior information and faster computational time than the other character-state methods (Ronquist and Huelsenbeck, 2001).

1.6.4. Methods for Estimating Reliability of Phylogenetic Trees

Resampling procedures for testing phylogenetic reliability are popular, primarily because of the wide availability of powerful and efficient algorithms for their implementation (Hillis and Bull, 1993). The most commonly-used method for estimating the reliability of branches on phylogenetic trees is bootstrapping. Bootstrap analysis is a technique used for estimating the statistical error in situations in which the underlying sampling technique for

estimating distribution is either unknown or difficult to derive analytically. It offers a useful way to approximate the underlying distribution by resampling from the original data set. It involves the random resampling of sites in the sequence data in order to generate a number of possible phylogenetic trees. A consensus tree summarizes the results of bootstrapping in the form of a phylogenetic tree. The consensus tree contains bootstrap values on each of the branches, giving a measure of reliability of these branches (Salemi and Vandamme, 2003). Jackknifing can also be used to test phylogenetic reliability. Instead of resampling from the data, jackknifing involves the random exclusion of particular sites and the generation of phylogenetic trees from the remaining sites (Salemi and Vandamme, 2003). Bayesian inference involves the use of posterior probabilities to test the reliability of phylogenetic trees. The posterior probability of a node on a tree is the probability that the node is correct. The interpretation of posterior probabilities is direct and simple. Also, posterior probabilities can be approximated using the Markov chain Monte Carlo procedure even under complex models of substitution in a fraction of the time that would be required for maximum likelihood bootstrapping (Huelsenbeck and Ranala, 2004).

1.7 Phylogeography

Phylogeography is the field of study concerned with the principles and processes governing the geographic distributions of genealogical lineages, especially those within and among closely related species (Avise, 1998). Phylogeography serves as the link between the study of micro- and macroevolutionary processes (Bermingham and Moritz, 1998) and is an integrative discipline as the analysis and interpretation of lineage distributions usually requires input from molecular genetics, population genetics, phylogenetics, demography, ethology, and historical geography (Avise, 1998). Phylogeography was initially based on mitochondrial (mt) DNA data (Bermingham and Moritz, 1998). This is because mtDNA evolves rapidly in populations of higher animals and is usually transmitted maternally without intermolecular recombination (Avise, 1998). The inheritance of mtDNA is uniparental as mtDNA is passed on to offspring from the mother only (Parker and

Markwith, 2007). Most of the analyses using mtDNA have involved animals, as mtDNA evolves very rapidly in animals and as a result it shows appreciable variation (Parker and Markwith, 2007). In plants, however, mtDNA evolves more slowly and thus the temporal resolution of this genome is too coarse for most biogeographic questions (Parker and Markwith, 2007). mtDNA-based phylogeography did, however, succeed in an improved description of the geographical distribution, phylogenetic relationships and genetic distances among evolutionary lineages of animals, which in turn led to a better understanding of regional biogeography and areas of endemism (Bermingham and Moritz, 1998). However, the reliance of phylogeography on a single gene system such as mtDNA has many pitfalls. These include the effects of selection, inadvertent amplification of pseudogenes and interspecific hybridization, but can be avoided by conducting thorough molecular and numerical analyses and by testing for phylogenetic congruence across nuclear and mitochondrial genes (Bermingham and Moritz, 1998). Nuclear gene sequences, however, have a much greater coalescent time when compared to mitochondrial genes and as a result there is also a greater potential for reticulate evolution among nuclear alleles due to recombination. Because of this the utility of nuclear gene sequences in population-level phylogenetics may be limited (Bermingham and Moritz, 1998).

Comparative phylogeographic analyses can contribute to evolutionary and ecological studies in many ways. Phylogeographic analyses can identify historically- and evolutionarily-independent regions and can provide an evolutionary and geographical context for species comprising ecological communities (Bermingham and Moritz, 2008). This in turn allows for the determination of historical and spatial influences on patterns of species richness. An understanding of historical response to changes in the landscape and identification of evolutionarily isolated areas provides information for the creation of conservation strategies (Bermingham and Moritz, 2008).

Phylogeography is a rapidly growing field and research is expanding in many areas. More emphasis is now being placed on finding concordance among gene genealogies within a taxon, such as between mtDNA and nuclear genes (Kuchta and Meyer, 2001). It is

important to generate gene trees for multiple loci because a single genealogy may not accurately reflect the historical and demographic processes experienced by populations (Kuchta and Meyer, 2001). Additional information is provided by multilocus data such as more accurate parameter estimates and the detection of demographic differences among sexes (Kuchta and Meyer, 2001). Finding concordance among sequence characters within a gene is also important as it leads to high nodal support and increases the measure of confidence in the inferred clades (Avise 1998; Kuchta and Meyer, 2001). Another two essential aspects of genealogical concordance are; the concordance in the geography of gene-tree partitions across multiple codistributed species which will result in significant advances in understanding how organismal behaviour, and the demographic and natural histories can influence intraspecific phylogeographic patterns, and; the concordance of gene-tree partitions with other biogeographic data (Avise, 1998; Kuchta and Meyer, 2001).

1.8 Future of Coral Reefs

It is still uncertain as to the extent that symbiotic diversity and flexibility will affect the long-term future of coral reefs in response to continued climate change. Studies have indicated that diverse symbionts can significantly buffer the effects of climate change. However, it is unknown at what scales such mechanisms may occur. Field data indicate that coral bleaching on some eastern Pacific reefs was much worse during the 1982-83 El Nino than in 1997-98 despite the temperature extremes during the two events being similar. This suggests that coral communities may be able to adapt to higher temperatures over decades (Buddemeier *et al*., 2004). However, in recent years there has been a distinct increase in the frequency and magnitude of high temperature events that have resulted in widespread bleaching (Hoegh-Guldberg, 1999; Gattuso and Buddemeier, 2001). Thus, if the high frequency of sea surface temperature (SST) variation of the past 20 years continues in combination with the general warming trend, concomittant acceleration in bleaching will result in expanding degradation and near complete destruction of reefs in many areas (Hoegh-Guldberg, 1999).

It is necessary to determine what fraction of existing symbioses on coral reefs is bleaching-susceptible and to monitor the symbiont community structure on reefs over time. Also, studies aimed at determining the geographic distribution of symbiont types are needed in order to formulate better marine protected area (MPA) design. The creation of MPA networks incorporating unusual and diverse habitats that maximise symbiont diversity into their design may increase their resilience to coral bleaching (Baker, 2003).

Although many corals can survive mild bleaching events, the longer they remain bleached the more susceptible they become to diseases and other stresses. Recovery depends on the number of corals that are able to survive and reproduce, and whether the geographic placement of the reefs in relation to other currents provides a source of larvae from elsewhere (Buddemeier *et al*., 2004).

1.9 Aim and Objectives of the Study

Although the Carribbean and the Great Barrier Reef have been studied intensively with respect to *Symbiodinium* diversity, the identity of the zooxanthellae in many locations remains unknown. The only published studies of *Symbiodinium* diversity on reefs on the east coast of Africa include a survey of Kenyan corals by Baker *et al*., (2004) and study of zooxanthellae diversity in scleractinian corals of Kenya using the lsrRNA gene (Visram and Douglas, 2006). The genetic diversity of zooxanthellae in the WIO is thus still largely unknown. A broad overview of the zooxanthellar cladal diversity in the WIO is thus considered necessary. RFLP analysis of the nuclear ssrRNA gene is a conservative molecular technique that allows for the identification of zooxanthellae types at the cladal level. The use of this technique allows for the assessment of zooxanthellae genetic diversity on a broad geographical scale in the WIO, thus providing baseline information for future studies. The ITS region, on the other hand, provides insight into the sub-cladal diversity of *Symbiodinium* isolates in the sampled areas.

The aim of this study was to determine the diversity, distribution and prevalence of *Symbiodinium* types in coral reef populations along the East African coastline in an attempt to fill the knowledge gap in the region and provide baseline information for future studies. At present the MPA system along the east African coastline is inadequate, with less than 2% being protected from exploitation (Wells and Ngusaru, 2004). There is a need to establish which areas need protection. In order to do this the connectivity between locations along the east African coastline needs to be assessed so as to determine the size needed for reserve areas and the minimum distance between them to ensure sufficient gene flow. Information made available from this study on the symbiont diversity along the east African coastline will thus aid in establishing MPA networks in the region.

1.9.1 Key Objectives

1. Determine the diversity of *Symbiodinium* types in selected coral species in reef populations along the east African coastline.
2. Determine the distribution and prevalence of *Symbiodinium* types in the selected corals.
3. From the results obtained, compile baseline information for future studies.

Chapter 2

2. Materials and Methods

2.1 Sampling Sites

2.1.1 Sodwana Bay

South Africa's coral reefs lie between 26 ^{o}S and 27 ^{o}S and are the southernmost reefs in the western Indian Ocean (Obura, 2000). They are offshore reefs found at depths of > 8 m (Obura, 2000). Corals grow on submerged Pleistocene dune and beachrock sequences to near Leven Point at latitude 28^{o}, and manifest little active frame building. They are situated in the headwaters of the Agulhas Current and are not influenced by direct land run-off (Riegl *et al*., 1995). The reefs differ from true coral reefs as they lack most geomorphological traits typical of true reefs such as, lagoons, reef crests and steep reef slopes, and do not reach the surface of the water. The hard bottom area covered by corals possesses relatively homogenous topographic conditions. The major topographical features include gullies and associated small drop-offs, perpendicular to the predominant direction of the swell. Distinct communities are present on different reefs and in different zones, and differentiation was shown to occur along a depth- and sedimentation gradient (Riegl *et al*., 1995). Schleyer and Celliers (2000) reported low levels of partial bleaching on the north South African reefs post 1998 but no mortality was observed. The depth and high wave energy of the reefs were probably key elements that contributed to protecting the reefs from the impacts of the El Nino event (Obura, 2000).

2.1.2 Southern Mozambique (Inhaca Island)

Inhaca Island is the southernmost Mozambican island and is located toward the southerly latitudinal limits of coral reef growth along the seaward margins of the estuarine Maputo Bay (Perry, 2003a). It separated from the mainland peninsula, Cabo de Santa Maria, in

recent times (Salm, 1976). The island is composed of Pleistocene dune rock overlaid by Quarternary to Modern dune sands. The eastern border of the island is impacted by the surf but all the other shores are protected from heavy wave action by extensive, shallow sandbanks (Salm, 1976). The entire island is situated in an MPA (Costa *et al*., 2005). The Barreira Vermelha reef off the western shore and the Ponta Torres reef in the south are the two main coral reefs but there are several small coral patches around the island (Salm, 1976). Coral reefs are developed irregularly on the Maputo side of the island along the margins of intertidal channels (Perry, 2003b). They are subject to fluctuations in temperature and salinity, and high sedimentation and turbidity levels (Perry, 2003a). Reef development is limited to depths of approximately 6 m and framework accumulation is only present in the upper 1-2 m (Perry, 2003a). This spatial and bathymetric restriction in reef development is largely due to the high turbidity levels (Perry, 2003b). The reefs are in a state of turnover through intermittent cyclonic destruction and rejuvenation (Kalk, 1995). Massive *Porites* spp. produce a basic reef structure with other coral genera such as *Acropora* spp., *Pocillopora* spp., *Favia* spp. and *Montipora* spp. colonizing the available substrata (Perry, 2003a). Schleyer *et al*., (1999) reported extensive bleaching on the southern Mozambican reefs after the 1998 bleaching event. Coral mortality, although variable, was found to be as high as 80% in some areas (Schleyer *et al*., 1999). The Barreira Vermelha reef was found to be in relatively good condition post 1998, probably because it is situated in a protected area, and thus any bleached corals there probably had better conditions for recovery (Rodrigues *et al*., 1999).

2.1.3 Bazaruto Archipelago

The reefs in the Bazaruto Archipelago are concentrated primarily around Bazaruto Island (Schleyer and Celliers, 2005). The monthly temperature of 23 – 28 ^{o}C results in warm water conditions around the Archipelago (Schleyer and Celliers, 2005). The eastern side of the larger islands has reefs which are found in deep water whereas the western, mainland side has a shallow system of channels and seagrass beds formed as a result of sediment build up (Schleyer and Celliers, 2005). The reefs on the eastern and south-eastern shores of

the islands are patchy (Dutton and Zolho, 1989) with a high cover of *Porites* and *Acropora* thickets present on back reefs. The study site in the Bazaruto Archipelago was the Inner Two Mile Reef which consists of a partially sheltered, mixed coral community on the landward side of a fringing rock reef between Ilhas Benguerra and Bazaruto (Schleyer *et al*., 1999). The 1997-1998 El Nino event resulted in 80% coral mortality on the reef (Schleyer *et al*., 1999).

2.1.4 Northern Mozambique (Quirimbas Archipelago)

The northern Mozambican coral reefs are highly developed and have a high diversity of reef fauna (Obura, 2000a). An almost continuous fringing reef occurs along the eastern shorelines of the Quirimbas Archipelago islands and certain sections of the mainland coast, where over 50 genera of corals have been recorded (Rodrigues *et al*., 2000). Patch reefs are common on the western sides of the islands. Reefs are restricted, however, by fresh water input from numerous rivers (Whittington *et al*. 1997). High bleaching was reported on the northern Mozambican reefs post – 1998; coral mortality, however, was moderate with most areas only experiencing 20-30% mortality (Schleyer *et al*., 1999). The Sencar channel reef was severely affected by the 1997 – 1998 bleaching event, resulting in a high percentage of dead coral having being observed there by Rodrigues *et al*., (1999).

2.1.5 Southern Tanzania (Mtwara)

The entire Tanzanian coastline supports coral reefs (Obura, 2000a). Mtwara, the southernmost region of Tanzania, besides having fringing reefs, has several patch reefs and reef-fringed offshore islands (Wagner, 2004). Coral assemblages are dominated by *Acropora*, but *Galaxea* is a characteristic feature of patch reefs. Although coral bleaching and mortality have been recorded along the entire length of coastline, the degree of bleaching and mortality varied between areas (Mohammed *et al*., 2000). Wilkinson (1998) reported that 15-25% of corals bleached in Mnazi Bay in Mtwara as a result of the 1998 bleaching event. In other areas such as Matenga and Kati, live coral cover was observed to have dropped from 55 and 60% in 1997 to 28 and 42% in 1999, respectively (Guard *et al*.,

1998; Mohammed *et al*., 2000). High mortality was observed on reefs that receive direct oceanic waters and thus reefs in bays, such as Mnazi Bay, had lower levels of bleaching and mortality, possibly due to exposure to varying surface water temperatures (Mohammed *et al*., 2000). The sampled area was in Mtwara, outside the marine protected area (Mnazi Bay Marine Park).

2.1.6. The Chagos Archipelago

The Chagos Archipelago is the southern most group of atolls in the Laccadive-Chagos ridge and is located at the geographical centre of the Indian Ocean. Six major atolls are present as well as many small islands, smaller atolls and submerged shoals (Rajasuriya *et al*., 2000). The central Great Chagos Bank incorporates a large submerged reef with eight islands and constitutes the largest area of undisturbed, highly diverse reefs in the Indian Ocean (Rajasuriya *et al*., 2000). Corals around the Chagos were seriously affected by the bleaching event in 1998, which resulted in a loss of approximately 55% of the live coral cover on the outer reefs (Rajasuriya *et al*., 2000). Again, the corals in the reef lagoons fared better, possibly because they normally experience higher temperatures and acquired a measure of tolerance as a result (Rajasuriya *et al*., 2000).

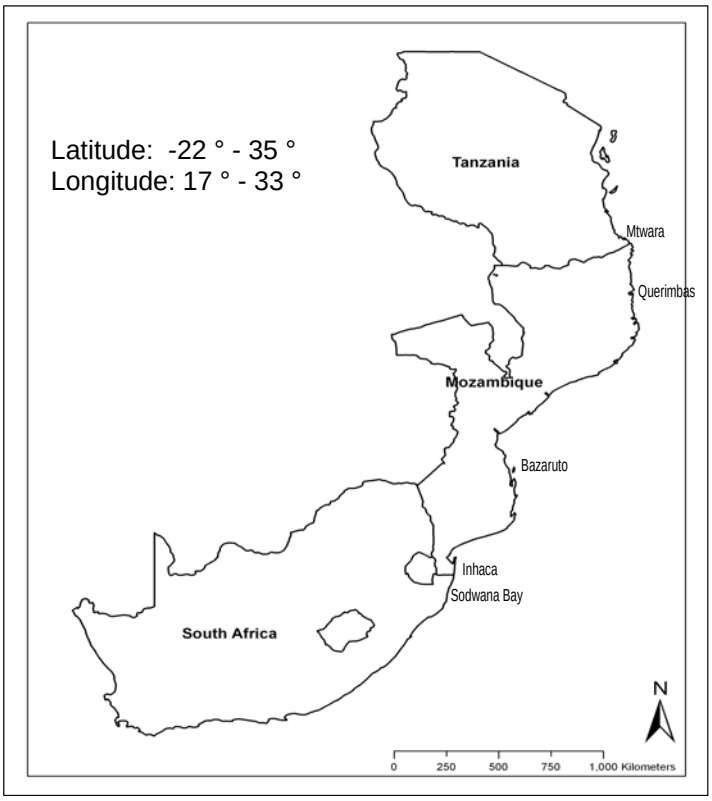

Figure 1. Map of the east coast of southern Africa showing areas the study sites namely; Sodwana Bay, Inhaca Island, Bazaruto Archipelago, Quirimbas Archipelago and Mtwara.

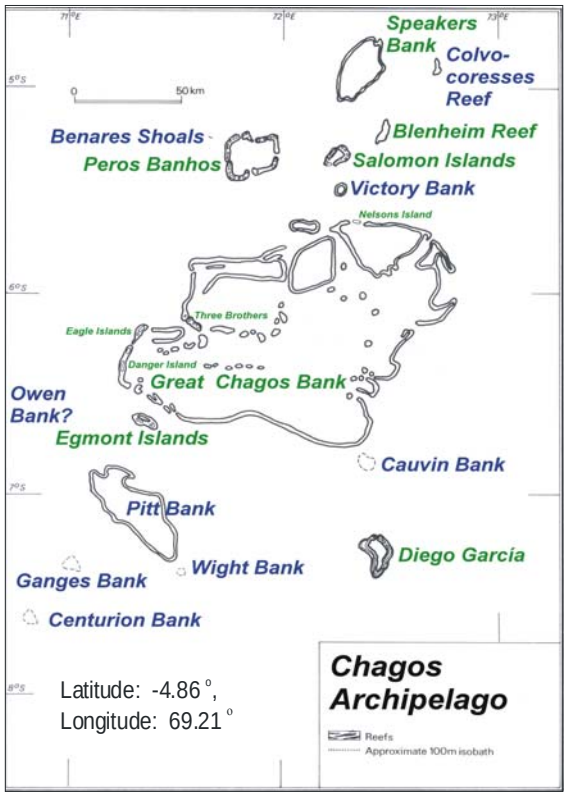

Figure 2. Map of the Chagos Archipelago. (http://www.coral.noaa.gov).

2.2 Sampling Methods

Colonies of the scleractinian corals, *Acropora austera*, *Pocillopora verrucosa*, *Pocillopora damicornis* and *Platygyra daedalea* were sampled by SCUBA at various depths from the following locations: South Africa (Sodwana Bay, KwaZulu-Natal), Southern Mozambique (Inhaca), Central Mozambique (Bazaruto Archipelago), Northern Mozambique (Quirimbas Archipelago) and the Chagos Archipelago. The coral species were selected based on their life strategies. *A. austera*, *P. verrucosa* and *P. damicornis* are fast-growing, opportunistic species whereas *P. daedalea* is a specialized, long-lived, slow-growing species (Barnes and Hughes, 1999). Corals were identified visually, based on Veron (1999), by experienced field staff. Coral fragments of around 5 cm were broken off individual colonies, preserved in 60% ethanol and refrigerated. Alternatively, in the case of the Bazaruto Archipelago samples, the tissue from coral samples was air blasted and stored in dimethyl sulphoxide

(DMSO) buffer solution (Appendix 6.1.4). All samples were stored at –20°C in the laboratory.

Table 1. Location of the study sites and number of samples of each coral species collected.

Location	**Site**	**Latitude**	**Genus**	**Species**	**No. Samples**
Sodwana Bay	Two-mile reef	27.53 S	*Acropora* *Pocillopora* *Platygyra*	*austera* *verrucosa* *daedalea*	6 2 2
Sodwana Bay	Nine-mile reef	27.42 S	*Acropora* *Pocillopora*	*austera* *verrucosa*	2 5
Southern Mozambique	Barreira Vermelho	26.02 S	*Acropora* *Pocillopora* *Platygyra*	*austera* *verrucosa* *daedalea*	3 3 1
Southern Mozambique	Baixo Danae	25.91 S	*Acropora* *Pocillopora*	*austera* *verrucosa*	7 2
Bazaruto Archipelago	Two-mile reef	21.80 S	*Acropora*	*austera*	1
Northern Mozambique: Quirimbass Archipelago	Lighthouse Point	12.33 S	*Pocillopora*	*damicornis*	3
Northern Mozambique: Quirimbass Archipelago	Sencar	12.58 S	*Pocillopora*	*damicornis*	5
Northern Mozambique: Quirimbass Archipelago	MW3	10.12 S	*Platygyra*	*daedalea*	1
Chagos Archipelago		6.15 S	*Platygyra* *Pocillopora*	*daedalea* *verrucosa*	2 3

2.3 DNA Isolation and Quantification

2.3.1 Protocol Optimisation

Qiagen® Dneasy Tissue Kits were used for the extraction of DNA from samples. The Qiagen "DNA extraction from animal tissues" protocol was first optimized for DNA

extraction from tissues of *A. austera, P. verrucosa, P. damicornis and P. daedalea*. Factors which were tested included determining the nature of the sample input (coral chip *vs* crushed coral) and the incubation time after addition of Proteinase K (3 hours *vs* overnight).

2.3.2 DNA Extraction

2.3.2.1 Extraction Using DNeasy Tissue Kit

An optimised version of the Qiagen® "DNA extraction from animal tissues" protocol was followed in order to extract total DNA from samples of *A. austera, P. verrucosa, P. damicornis and P. daedalea*.

A small piece of coral was removed from each sample, which had been stored in alcohol at -20 °C, and placed in a 1.5 ml micro-centrifuge tube. Coral was added as a chip to the tube. An aliquot of 180 µl of ATL buffer and 20 µl of Proteinase-K from the Qiagen® kit was added to the micro-centrifuge tube. Each tube was mixed thoroughly by inversion. In the case of the *P. daedalea* samples however, chips of coral were crushed in DNAB (Appendix 6.1.3) and the slurry was then decanted into a microcentrifuge tube. The tubes were incubated for a minimum of 3 hours (overnight for *P. daedalea*) in a water bath at 55 °C. Each tube was vortexed. An aliquot of 200 µl AL buffer was added and each tube vortexed again. In the case of *P. daedalea* the supernatant solution was poured into a separate tube prior to the addition of ATL buffer, and vortexing was omitted. The tubes were then incubated in a water bath at 70 °C for 10 min, after which 200 µL of 100% EtOH was added to each tube, followed by vortexing. The DNeasy mini-spin columns were placed into clear flow-through collection tubes. Samples were pipetted into the prepared spin column and centrifuged at 6000g for 1 min. The flow-through tube was removed and the liquid disposed of. The spin columns were then replaced into the tubes. An aliquot of 500 µl of AW1 buffer was added to each tube and all tubes were centrifuged at 6000g for 1 min. The flow-through was removed as before. An aliquot of 500 µl AW2 buffer was then added to each tube followed by centrifugation at 10000g for 3 min. The spin columns were removed and placed in clean micro-centrifuge tubes. The flow-through (first elution) was

stored. An aliquot of 100 µl AE buffer was pipetted directly onto the white spin column membrane. The samples were allowed to sit for 1 min, followed by centrifugation at 6000g to elute the DNA. The spin columns were placed in clean micro-centrifuge tubes and 200 µl AE buffer was pipetted directly onto the white membrane as before, followed by a 5 min standing period and 1 min centrifugation at 6000g. Both the first and second elutions were refrigerated.

2.3.2.2 Phenol: Chloroform: Isoamyl Alcohol Extraction

For certain *P. daedalea* samples from which a low yield and poor quality DNA was obtained using the Qiagen® protocol described above, alternative extraction methods were employed. These involved crushing the coral samples in DNAB (Appendix 6.1.3) solution at the beginning of the protocol and performing a phenol-chloroform-isoamyl alcohol extraction.

A small piece of coral, about 0.5 cm x 0.5 cm, was removed from each sample, which had been stored in alcohol at –20 °C, rinsed thoroughly and placed in a mortar dish containing 2 ml of DNAB (Appendix 6.1.3) solution. The sample was crushed using a pestle. The slurry was then transferred to a 1.5 ml microcentrifuge tube. An aliquot of 180 µl of ATL buffer from the Qiagen® DNeasy Tissue kit and 20 µl of Proteinase K were added to the tube and the contents mixed thoroughly by inversion. The samples were then incubated in a 55 ^{o}C water bath over night. After incubation an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added to each sample. All samples were vortexed briefly, allowed to sit for about 10 min and then centrifuged at 10000 rpm for 5 min. The DNA was then precipitated by adding an equal volume of isopropanol to each sample, followed by 15 min of centrifugation at 10000 rpm in a Eppendorf 5424® centrifuge. The supernatant was then pipetted out and the pellet rinsed with 70% ethanol. The samples were centrifuged again at 10000 rpm for 5 min and the ethanol removed. Residual ethanol was then allowed to evaporate from the samples overnight at room temperature after which

the pellets were resuspended in 500 µl of TE buffer (pH 8.0). All samples were stored at 4 ^{o}C.

2.3.3 Agarose Gel Electrophoresis

An aliquot of 10 µl from the first dilution of each sample DNA extract was combined with 2 µl of 6× loading buffer (Fermentes) and 5 µl of 100 Kbp molecular marker was combined with 2 µl of 1× loading buffer prior to loading. The samples were then separated by electrophoresis in 0.75% agarose gels (Appendix 6.2.1) at 150 V in 1× TAE buffer (Appendix 6.1.2) for approximately an hour. The gels were stained with ethidium bromide (EtBr) at a concentration of 0.5 µg/ml for 40 min and visualised under ultraviolet light with a transilluminator. Photographs of the gels were taken using a Canon PowerShot G5® digital camera. Aliquots of each extracted sample were stored in microcentrifuge tubes at 4 ^{o}C for PCR. The remainder of the extracted sample in each tube was frozen.

2.4. PCR

2.4.1. Quantification of DNA and PCR Optimisation

The DNA in each sample was quantified by spectrophotometry using a nanodrop spectrophotometer. PCR runs were set up using varying concentrations of sample DNA in the reaction mixtures, ranging from 0.01 ng/µl to 10 ng/µl, in order to determine the optimal DNA concentration for successful amplification. The PCR optimisation trials were conducted as described in 3.4.2 (ssrDNA amplification) and 3.4.3 (ITS amplification) below.

2.4.2. PCR of ssrDNA

Amplification reactions were performed in volumes of 30 µl of reaction mix containing 30 ng of DNA from *Pocillopora* or *A. austera* samples or 10 ng of DNA from *P. daedalea* samples, 1.5 µl of 0.1 mM dNTP, 1.8 µl of 25 mM $MgCl_2$, 0.75 µl of 0.2 µM primer

mixture (ss5: 5'-GGTTGATCCTGCCAGTAGTCATATGCTTG-3' and ss3z: 5'-AGCACTGCGTCAGTCCGAATAATTCACCGG-3'), 3 µl 10× buffer and 0.24 units of Supertherm *Taq* Polymerase. The thermal cycling profile included an initial denaturation step of 10 min at 94 ^{o}C followed by 40 cycles of 2.5 min at 94 ^{o}C, 1 min at 56 ^{o}C and 2 min at 72 ^{o}C alternating with 1 min at 94 ^{o}C, 1 min at 56 ^{o}C and 2.5 min at 72 ^{o}C followed by an additional 8 min at 72 ^{o}C.

For ease of preparation a master mix was initially made up and an aliquot of that mix was combined with an appropriate amount of DNA sample. ddH_2O was added to obtain a total reaction volume of 30 µl. The PCR protocol was taken from Coffroth and Goulet (2004).

2.4.3. PCR of ITS Region

Amplification reactions were performed in volumes of 30 µl of reaction mix containing 120 ng of DNA, 1.5 µl of 0.1 mM dNTP, 1.8 µl of 50 mM $MgCl_2$, 0.75 µl of 0.2 µM primer mixture (ITS FW: 5'-GTG TAT TAT TCG GAC TGA CG-3'and ITS RW: 5'-TCC TCC GCT TAT TGA TAT GC-3') and 0.5 units of Platinum *Taq* Polymerase. The thermal cycling profile included an initial denaturation step of 2 min at 95 ^{o}C followed by 40 cycles of 1 min at 94 ^{o}C, 2 min at 55 ^{o}C and 3 min at 72 ^{o}C and an additional 10 min at 72 ^{o}C. Due to low amplification success the above-mentioned primers sourced from Rodriguez-Lanetty and Hoegh-Guldberg (2003) were checked for mismatch and new primers (5' GGC TAA GCT GCT TTA GTG ACA ACT G-3'and 5'GCT TAA ATT CAG CGG GTT CAC TTG-3') were synthesized at Inqaba Biotechnology Laboratories to correct for those mismatches.

As before, a master mix was initially made up and an aliquot of that mix was combined with an appropriate amount of DNA sample. ddH_2O was added to obtain a total reaction volume of 30 µl. The PCR protocol was adapted from Rodriguez-Lanetty and Hoegh-Guldberg (2003).

2.4.4. Agarose Gel Electrophoresis

An aliquot of 3 µl of the PCR product from each sample was combined with 6 µl of 6× loading buffer (Fermentes®). The products were then run on 1% agarose gels (Appendix 6.2.2) in 1× TAE buffer (Appendix 6.1.2) at 94 V for approximately 90 min. The gels were stained with EtBr for 40 min and visualised under ultraviolet light with a transilluminator. Photographs of the gels were taken using a Canon PowerShot G5® digital camera. The remainder of the PCR product in each tube was frozen for further analysis.

2.5. Digestion of ssrDNA PCR Products (RFLP)

A standard digest mix was prepared for each sample such that each reaction contained 1 µl of 10× buffer, 0.5 µl Taq I, 0.1 µl BSA and 2.4 µl ddH_20. An aliquot of 6 µl of PCR product from each sample was added to the digest mix in the respective tubes. The samples were left to incubate at 65 ^oC overnight. An aliquot of 1.5 µl 6× loading buffer was added to the digested samples. The digest products were separated by electrophoresis in 2% agarose gels (Appendix 6.2.3) at 104 V for approximately 2 hours. An aliquot of 12 µl of 100 Kbp ladder was used as a standard. The gels were stained with EtBr at a concentration of 0.5 µg/ml for 40 min and visualised under ultraviolet light with a transilluminator. Photographs of the gels were taken using a Canon PowerShot G5 digital camera.

2.6. DNA Sequencing

PCR products were sent away to Inqaba Biotechnology Laboratories where they were sequenced directly by dideoxy sequencing (Sanger sequencing). Results were returned as electronic files. Each samples was sequenced in the forward and reverse directions using the primers used for the initial amplification.

2.7. Sequence Analyses

Sequences were edited by comparison of the forward and reverse sequences and aligned in BioEdit® version 7.0.5.2 (Hall, 1999) using the ClustalW® function (Thompson *et al*.,

1994). Sequences were then trimmed to the length of the shortest sequence. The alignment was then imported into ClustalX® (Thompson *et al*., 1997) where a nexus file was created. MrModeltest® version V2 (Posada and Crandall, 1998) was used to select the model of evolution that best fit the data. Genetic distances between the various samples were calculated, and a NJ tree was produced in PAUP® V. 4.0b10 (Swofford, 1999). Bayesian analysis was performed in Mr Bayes® version 3.0b4 (Huelsenbeck and Ronquiest, 2001). TCS® version 1.21 (Clement *et al*., 2000) was used to construct a statistical parsimony haplotype network. The distribution of zooxanthellar ITS haplotypes amongst the various sampling locations was then indicated by overlaying the network with the site information. The coral species information was also overlaid on the network to show the distribution of zooxanthellar ITS haplotypes amongst the coral species sampled. Nested clade analysis was conducted using the Automated Nested Clade Analysis (ANeCA®) software which uses TCS® version 1.18 (Panchal, 2007), GeoDis® version 2.2 (Posada *et al*., 2000) and the inference key of Posada and Templeton, (2005). An analysis of molecular variance (AMOVA) was conducted in Arlequin (Excoffier *et al*., 2005) in order to determine if there is any genetic variation amongst *Symbiodinium* populations from the various sampling locations as well as amongst *Symbiodinium* populations from the various coral species.

Chapter 3

3. Results

3.1 DNA Extraction

DNA extraction from the study samples was achieved with variable success. Greater DNA yields were obtained from the *P. verrucosa*, *P. damicornis* and *A. austera* samples than from *P. daedalea* samples. The quality of the DNA obtained from the *P. verrucosa*, *P. damicornis* and *A. austera* samples (Figure 3) was also better than that of the *P. daedalea* samples which showed high levels of degradation (indicated by the smears in Figure 4).

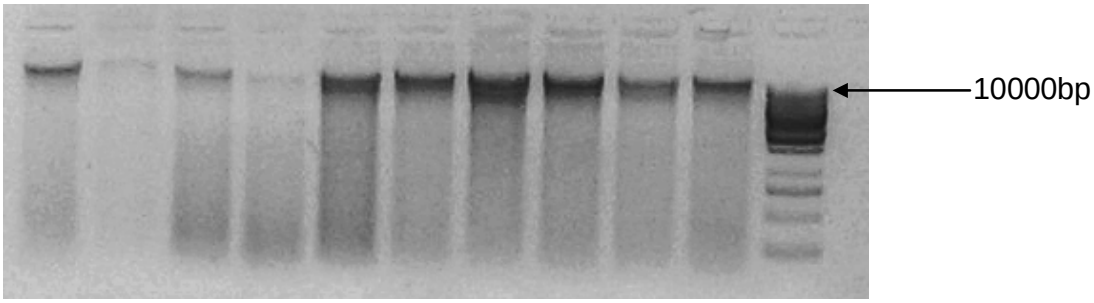

Fig. 3. Agarose gel showing total DNA extracted from selected *P. verrucosa* samples.

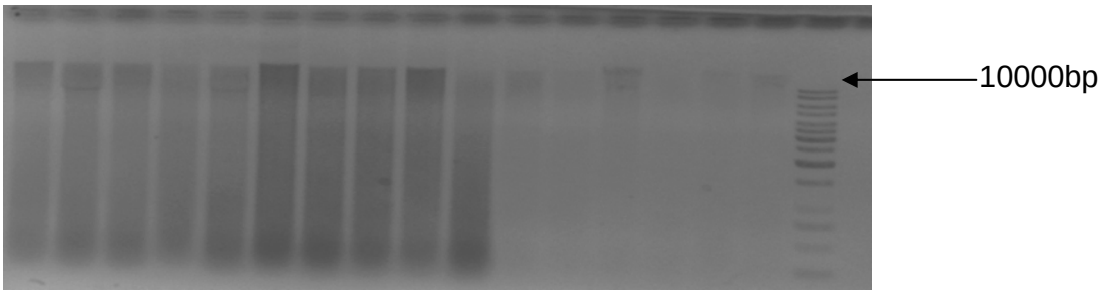

Fig. 4. Agarose gel showing total DNA extracted from selected *P. daedalea* samples.

3.2 ssrDNA Amplification

Overall, amplification using DNA extracts from *P. verrucosa*, *P. damicornis* and *A. austera* samples had good success rates. The *P. verrucosa* and *P. damicornis* samples all produced a single major amplification product of approximately 1700bp (Figure 5). The *A. austera* samples produced an amplification product of approximately 1700bp and two additional smaller amplicons of approximately 1000bp and 700bp (Figure 6). The *P. daedalea* samples failed to amplify.

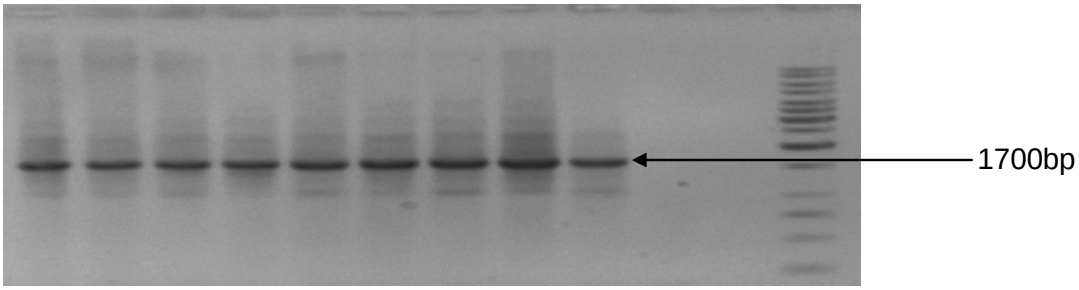

Fig. 5. PCR amplifications of zooxanthellar 18S-rDNA from selected *P. verrucosa* samples. Size of product is 1700bp.

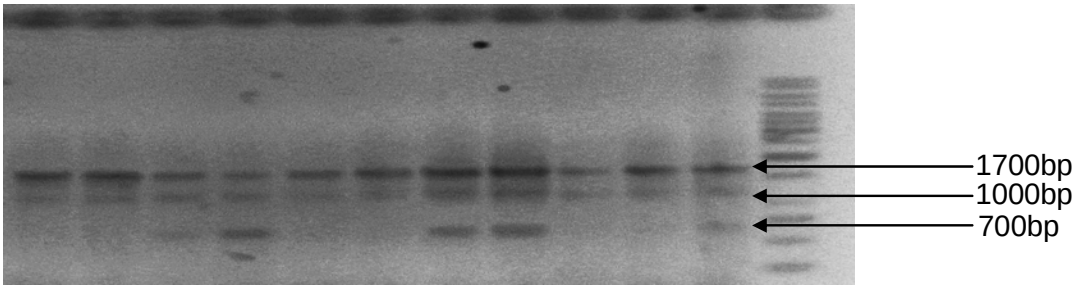

Fig. 6. PCR amplifications of zooxanthellar 18S-rDNA from selected *A. austera* samples. Sizes of products are 1700bp, 1000bp and 700bp.

3.3 ITS Amplification

DNA extracts from *P. verrucosa*, *P. damicornis* and *A. austera* samples were amplified with average success with the primers sourced from Rodriguez-Lanetty and Hoegh-Guldberg (2003). New primers (corrected for mismatch) improved the amplification

success of *P. verrucosa* (Figure 7), *P. damicornis* and *A. austera* samples. *P. daedalea* samples were difficult to amplify with both sets of primers.

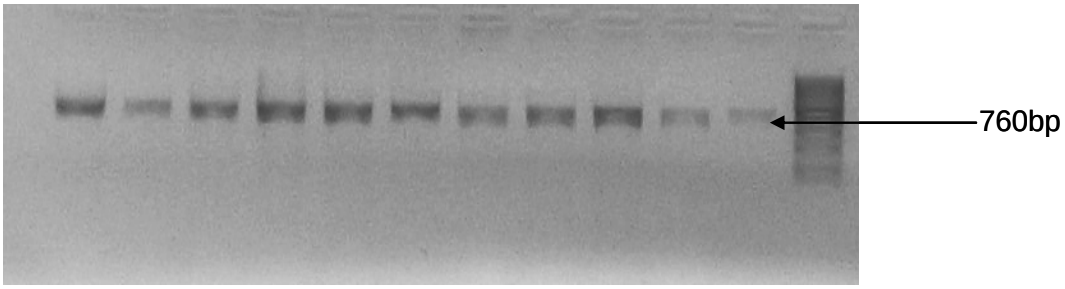

Fig. 7. ITS PCR amplifications of zooxanthellar DNA from selected *P. verrucosa* samples. Product size is 760bp.

3.4 Restriction Digests

The PCR products from all the successfully-amplified samples produced the digestion pattern corresponding to *Symbiodinium* clade C (Figure 8).

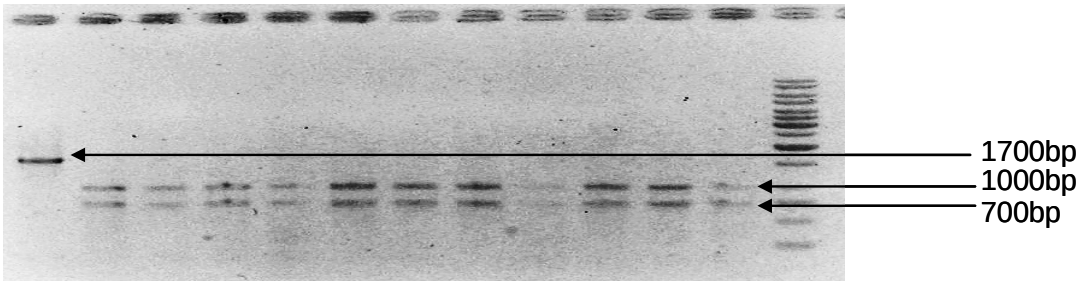

Fig. 8. RFLP gel of zooxanthellar ssrDNA from *A. austera* samples depicting a clade C digestion pattern. Sizes of digest products are 1000bp and 700bp.

3.5 ITS Sequencing

Good sequences were obtained from the *P. verrucosa*, *P. damicornis* and *A. austera* samples. The sequences obtained from the *P. daedalea* samples that successfully amplified had greater amounts of background noise when compared to the other sequences.

3.6 Haplotype Analyses

ITS sequence data (263bp) were analysed from 48 individuals of *Symbiodinium* found in *P. veruccosa*, *P. damicornis*, *A. austera* and *P. daedalea*. A total of 11 polymorphic sites and 17 haplotypes were present. The calculated haplotype diversity (Hd) was 0.878 and the variance and standard deviation were 0.0008 and 0.028 respectively. The average number of nucleotide differences (k) amongst the haplotypes was 2.646. The nucleotide diversity (Pi) was 0.0101, with a variance of 0.0000011 and a standard deviation of 0.00103.

Analysis of part of the 5.8S region (69 nucleotides) on its own as well as the sequences of eight recognized species of *Symbiodinium* revealed 9 haplotypes and 18 polymorphic sites. The calculated haplotype diversity (Hd) was 1.000 and the variance and standard deviation were 0.00274 and 0.052 respectively. The average number of nucleotide differences (k) amongst the haplotypes was 6.056. The nucleotide diversity (Pi) was 0.33642, with a variance of 0.0044089 and a standard deviation of 0.06640.

Table 2 shows a key of the abbreviated sample labels indicating the host species and location from which they were sourced. Tables 3 and 4 show the frequency, variable sites and occurrence of each *Symbiodinium* ITS and 5.8S haplotype in this study, respectively.

Table 2. Key of abbreviated sample labels, coral species and sampling locations.

Abbreviated label	Coral species	Location
PocTMR	*P. verrucosa*	Sodwana Bay, Two-mile Reef
PocNMR	*P. verrucosa*	Sodwana Bay, Nine-mile Reef
PocBD	*P. verrucosa*	Southern Mozambique, Baixo Danae
PocBV	*P. verrucosa*	Southern Mozambique, Barreira Vermelho
PocCh	*P. verrucosa*	Chagos Archipelago
PocdSen	*P. damicornis*	Northern Mozambique, Sencar
PocdLP	*P. damicornis*	Northern Mozambique, Ibo Lighthouse Point
AcTMR	*A. austera*	Sodwana Bay, Two-mile Reef
AcNMR	*A. austera*	Sodwana Bay, Nine-mile Reef
AcBD	*A. austera*	Southern Mozambique, Baixo Danae
AcBV	*A. austera*	Southern Mozambique, Barreira Vermelho
AcBazT	*A. austera*	Central Mozambique, Bazaruto Two-mile Reef
PlatTMR	*P. daedalea*	Sodwana Bay, Two-mile Reef
PlatBV	*P. daedalea*	Southern Mozambique, Barreira Vermelho
PlatCh	*P. daedalea*	Chagos Archipelago
PlatMW	*P. daedalea*	Southern Tanzania, Mtwara

Table 3. *Symbiodinium* ITS haplotypes: frequency, variable sites and occurrence. Variable nucleotides are labeled as follows: (1) nt 3, (2) nt 11, (3) nt 19, (4) nt 90, (5) nt 110, (6) nt 115, (7) nt 149, (8) nt 173, (9) nt 192, (10) nt 203, (11) nt 206, (12) nt 255.

Haplotype	N	Variable sites	Sample codes
1	1	TCCCTTTCGTTG	1PocNMR
2	1	TCCTTTTTGTTG	2PocNMR
3	2	CCCCTTTCGTTG	3PocNMR, 2PocdLP
4	11	CCCCCTTCG-TG	4PocNMR, 5PocNMR, 1PocTMR, 2PocTMR, 2PocBV, 2PocdSen, 3PocdSen, 5PocdSen, 3PocdLP, 2PlatTMR, 1PlatBV
5	1	TCCTTTTTGTTC	3PocBV
6	1	CCCTTTTCGTTG	1PocBD
7	6	CCCCCTTCA-TG	2PocBD, 2AcNMR, 1AcNMR, 2AcTMR, 7AcBD, 2AcBV
8	1	CTCCTTTCGTTG	1PocBV
9	1	CCCCTTTCGTTC	1PocdSen
10	9	CCTCCGTCA-TG	4PocdSen, 3AcTMR, 4AcTMR, 5AcTMR, 6AcTMR, 4AcBD, 6AcBD, 1AcBV, 3AcBV
11	1	TCCCTTTTGTTC	1PocdLP
12	2	CCCCCTGCGTTG	1PocCh, 3PocCh
13	1	CCCCCTTCGTTG	2PocCh
14	3	CCTCCGTCATGG	1AcNMR, 1AcBD, 2AcBD
15	1	CCTCCGTCATTG	3AcBD
16	1	TCCTTTTCG-TC	5AcBD
17	1	CCTCCTTCATTG	1AcBazT
18	1	TCCCCTTCG-TG	1PlatTMR
19	2	CCCCCTTCA-CG	1PlatCh, 2PlatCh
20	1	CCCCCTTCATTG	1PlatMW

Table 4. *Symbiodinium* 5.8S rDNA haplotypes: frequency, variable sites and occurrence. Variable nucleotides are labeled as follows: (1) nt 3, (2) nt 5, (3) nt 8, (4) nt 11, (5) nt 12, (6) nt 14, (7) nt 19, (8) nt 20, (9) nt 21, (10) nt 22, (11) nt 24, (12) nt 29, (13) nt 34, (14) nt 39, (15) nt 40, (16) nt 41, (17) nt 42, (18) nt 51, (19) nt 53.

Haplotype	N	Variable sites	Sample codes
1	28	CAGCTCCGTGGATTTT-TT	2PocCha, 3 PocCha, 2AcNMR, 1AcTMR, 2AcTMR, 1PlatBV, 5PocdSen, 2PocsLP, 3PocdLP, 1PocCha, 2PlatCh, 1PlatMW, 1PocTMR, 2PocTMR, 2PocBV, 1PocBD, 2PocBD, 1PocdSen, 2PocdSen, 3PocdSen, 7AcBD, 2AcBV, 1PlatCh, 3PocNMR, 5PocNMR, 4PocNMR, *S. goreaui* (AF333515).
2	6	TAGCTCCGTGGATTTT-TT	1PocNMR, 2PocNMR, 3PocBV, 1PocdLP, 5AcBD, 1PlatTMR.
3	14	CAGCTCTGTGGATTTT-TT	4PocdSen, 1AcNMR, 3AcTMR, 4AcTMR, 5AcTMR, 6AcTMR, 1AcBD, 2AcBD, 3AcBD, 4AcBD, 6AcBD, 1AcBV, 3AcBV, 1AcBazT.
4	1	CAGCTCCGTAGATTTT-TT	*S. kawagutti* (AF333517).
5	1	CAGTTCCGTGGATTTT-TT	1PocBV.
6	3	CAGCTTCGTGAGTTATGCT	*S. cariborum* (AF333504), *S. microadriaticum* (AF333505), *S. pilosum* (AF333506).
7	1	CGGCTCCGCGAGCTTT-CC	*S. muscatinei* (AF333510).
8	1	CGGCTCCGCGAGCTTT-CG	*S. pulchorum* (AF333511).
9	1	CAAACCCTCGAATCACGTT	*S. californium* (AF 334659).

3.7 Phylogenetic Analyses

3.7.1 Distance Methods

Genetic distances between the various ITS *Symbiodinium* haplotypes were calculated using the HKY85 model of evolution (selected using Mr Modeltest) (Table 5). Haplotype 4 included samples predominantly obtained from *P. verrucosa* and *P. damicornis* (Table 3) and haplotype 10 included samples predominantly obtained from *A. austera* (Table 3). A

genetic distance of 0.22 separates the two haplotypes. Haplotype 7, which, also appeared to be dominated by samples obtained from *A. austera* (Table 3) is separated from haplotype 4 and 10 by genetic distances of 0.07 and 0.14 respectively. Relatively large genetic distances separate the outgroup, subclade C55, from the remaining samples. For example the average number of nucleotide differences between subclade C55 and all other haplotypes was 7.350. On the other hand the average number of nucleotide differences between subclade C1 and the study haplotypes was 4.200. The average number of nucleotide differences within the study sample sequences (excluding outgroups) was 3.462.

NJ analysis (Figure 9) of the ITS dataset using the HKY85 model of evolution and 1000 bootstrap replicates yielded a 50% majority-rule consensus tree. This tree was rooted on *Symbiodinium* subclade C55 as the outgroup. All the samples formed an exclusive cluster supported by a high bootstrap value of 97%. Three weakly-supported sub-clusters were present within this cluster and were supported by bootstrap values of 62, 61 and 62%. There was a lack of resolution within the clusters indicated by the presence of polytomies. Symbiodinium included in the cluster with 61% bootstrap support were primarily associated with *Pocillopora* species, whereas those included in the cluster with 62% bootstrap support were primarily associated with *A. austera* species, ans included all the AcTMR, AcBD and AcBV samples.

Table 5. HKY85 genetic distances (substitutions per site), based on 263 nucleotides the ITS2 and 5.8S regions, between twenty *Symbiodinium* haplotypes obtained from this study.

Haplotype	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	C1
1	-																				
2	0.140	-																			
3	0.063	0.230	-																		
4	0.160	0.399	0.075	-																	
5	0.211	0.063	0.307	0.492	-																
6	0.138	0.140	0.063	0.160	0.211	-															
7	0.254	0.579	0.150	0.068	0.708	0.254	-														
8	0.138	0.358	0.063	0.160	0.444	0.138	0.254	-													
9	0.130	0.307	0.063	0.142	0.228	0.130	0.222	0.130	-												
10	0.477	1.118	0.334	0.223	1.486	0.477	0.138	0.477	0.428	-											
11	0.130	0.131	0.208	0.344	0.064	0.307	0.482	0.307	0.137	0.843	-										
12	0.207	0.427	0.129	0.063	0.529	0.207	0.129	0.207	0.206	0.309	0.392	-									
13	0.137	0.340	0.063	0.000	0.427	0.137	0.063	0.137	0.130	0.222	0.303	0.063	-								
14	0.523	1.097	0.390	0.311	1.414	0.523	0.223	0.523	0.495	0.062	0.878	0.379	0.287	-							
15	0.423	0.912	0.302	0.225	1.142	0.423	0.144	0.423	0.392	0.000	0.728	0.287	0.207	0.062	-						
16	0.130	0.139	0.208	0.327	0.071	0.130	0.468	0.322	0.137	0.820	0.149	0.397	0.303	0.954	0.777	-					
17	0.338	0.778	0.225	0.150	0.962	0.338	0.075	0.338	0.303	0.074	0.608	0.207	0.137	0.133	0.063	0.643	-				
18	0.073	0.264	0.150	0.067	0.344	0.254	0.147	0.254	0.222	0.327	0.230	0.129	0.063	0.419	0.323	0.223	0.242	-			
19	0.391	0.876	0.259	0.148	1.126	0.391	0.068	0.391	0.342	0.224	0.706	0.231	0.154	0.223	0.244	0.670	0.166	0.245	-		
20	0.225	0.495	0.138	0.063	0.608	0.225	0.000	0.225	0.208	0.143	0.427	0.129	0.063	0.204	0.129	0.427	0.063	0.137	0.077	-	
C1	0.284	0.553	0.194	0.120	0.663	0.284	0.195	0.284	0.261	0.373	0.483	0.182	0.120	0.440	0.357	0.483	0.284	0.194	0.307	0.195	-
C55	0.917	1.473	0.653	0.543	1.505	0.917	0.419	0.917	5.304*	0.716	2.652*	0.599	0.485	0.574	0.614	5.304*	0.500	0.735	0.307	0.372	0.904

* undefined distances

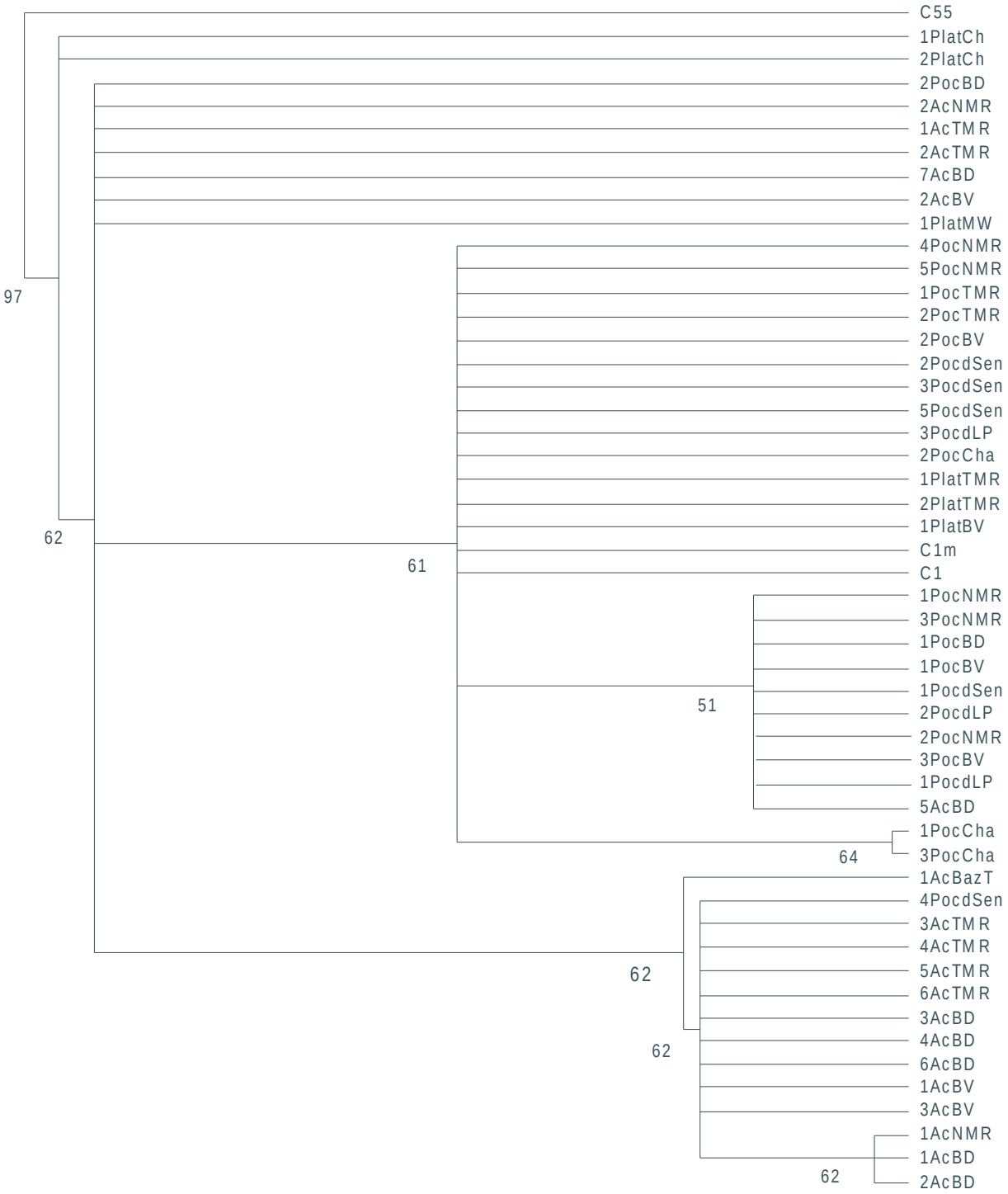

Fig. 9. NJ tree (HKY85 model of genetic distance) illustrating relationships between *Symbiodinium* samples from this study based on 263 nucleotides from the ITS2 and 5.8S regions. Numbers on the left hand side of the nodes indicate the bootstrap support for each grouping.

Genetic distances between the various 5.8S *Symbiodinium* haplotypes obtained from the study samples and Genebank sequences from morphologically identified *Symbiodinium* species, were calculated using the HKY85 model of evolution (selected using Mr Modeltest) (Table 6). Haplotypes 1, 2, and 3, which included the majority of the samples, were most genetically distant from *S. muscatinei* (0.932, 1.935 and 1.935 respectively) and least genetically distant from *S. kawagutti* (0.060, 0.130 and 0.130 respectively) and haplotype 5 (0.060, 0.132 and 0.132 respectively). The average genetic distance between the "morphological species' was 0.788. The interspecific distances ranged from 0.058 to 1.626. The average genetic distance within the study samples was 0.103, with a range of 0.060 to 0.132. The average number of nucleotide differences between *S. goreaui* and all other sample haplotypes was 0.438. The average number of nucleotide differences within the study sample sequences was 0.687.

Neighbour-joining analysis (Figure 10) of the 5.8S dataset using the HKY85 model of evolution and 1000 bootstrap replicates yielded a 50% majority-rule consensus tree. This tree was rooted on *S. microadriaticum* as the outgroup. All the samples, with the exception of *S. cariborum* and *S. pilosum*, formed an exclusive cluster supported by a bootstrap value of 82%. All the study samples formed a sub-cluster with *S. goreaui* and *S. kawagutii* supported by a bootstrap value of 71%. Within this was a moderately-supported sub-cluster containing mainly *Symbiodinium* derived from *A. austera* samples: all AcTMR, AcBD and AcBV were contained within this cluster.

Table 6. HKY85 genetic distances (substitutions per site) based on 69 nucleotides of the 5.8S RNA gene, between nine *Symbiodinium* haplotypes obtained from this study and from sequences obtained from Genebank.

Haplotype	1	2	3	4	5	6	7	8	9
1	-								
2	0.060	-							
3	0.060	0.132	-						
4	0.060	0.130	0.130	-					
5	0.060	0.132	0.132	0.130	-				
6	0.392	0.548	0.550	0.560	0.548	-			
7	0.932	1.935	1.935	1.381	1.935	0.489	-		
8	0.780	1.326	1.326	1.086	1.326	0.450	0.058	-	
9	0.891	1.295	1.295	*3.869	0.900	1.626	1.537	*3.869	-

*undefined distances

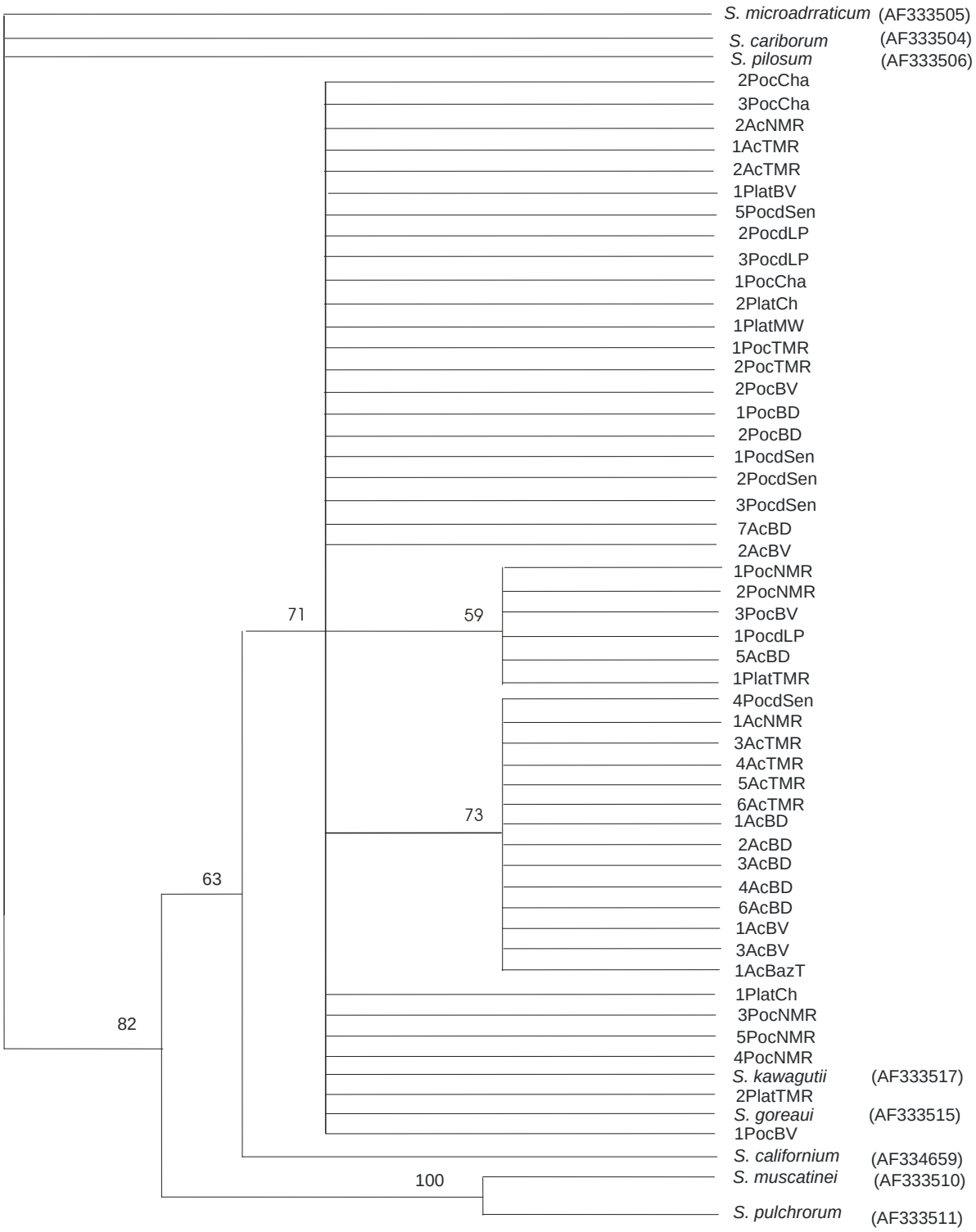

Fig. 10. NJ tree (HKY85 model of genetic distance) illustrating relationships between *Symbiodinium* samples from this study and sequences obtained from Genebank (accession numbers indicated in brackets), based on 69 nucleotides of the 5.8S gene. Numbers on the left hand side the nodes indicate the bootstrap values of each grouping.

3.7.2 Bayesian Inference

Bayesian analysis of the ITS dataset using the HKY85 + I model of evolution yielded a 99 percent credible set of 79202 trees. A fifty percent majority rule consensus tree is presented in Figure 11. A maximum parsimony analysis was also performed but was not presented as the phylogenetic tree obtained was consistent with the Bayesian tree. The reconstruction of phylogenetic relationships between *Symbiodinium* subclades was rooted on *Symbiodinium* subclade C55 as the outgroup. The tree topology was consistent with that of the NJ consensus tree based on ITS sequences (Figure 9) and included one additional poorly-supported grouping (2PocNMR, 2PocIn, 1PocdLP and 5AcBD). All the samples formed a single monophyletic clade supported by a high posterior probability value of 1.00. Three major subclades are present, weakly supported by posterior probability values of 0.78, 0.76 and 0.77 respectively. Within the groupings three are two smaller subclades supported by posterior probabilities of 0.95 and above. These include the subclade containing two samples obtained from *P. verrucosa* collected in the Chagos Archipelago as well as the subclade containing three samples obtained from *A. austera* (two from Sodwana Bay and one from southern Mozambique). The last subclade is a moderately supported subclade (posterior probability value of 0.92) comprising *Symbiodinium* mostly derived from *A. austera* samples. This contains all the ACTMR, AcBD and AcBV samples and is similar in composition to an equivelant cluster/clade found in Figures 9 and 10. However, because Bayesian analyses do not incorporate phylogenetically-informative indels, taxa that are separated by an insertion or deletion were grouped together. This is evident in the grouping of subclades C1 and C1m. A number of the samples obtained from *P. verrucosa* and *P. damicornis*, and some from *P. daedalea* cluster with subclade C1.

Bayesian analysis using the HKY85 + I model of evolution of the ITS dataset combined with the ITS sequences from a range of *Symbiodinium* belonging to clades B and C yielded 99 percent credible set of 80002 trees. A fifty percent majority rule consensus tree is presented in Figure 13 (Appendix 6.3). All the samples formed a single monophyletic clade supported by a high posterior probability value of 1.00 rooted with the outgroup *S. muscatinei*. Two strongly supported major subclades were present, both with posterior

probability values of 1.00, one consisted of *Symbiodinium* clade B sequences and the other of *Symbiodinium* clade C sequences. The majority of the study samples formed a cluster with a range of *Symbiodinium* C subclades, supported with a posterior probability value of 0.99.

Bayesian analysis of the 5.8S dataset using the HKY85 + I model of evolution yielded a 99 percent credible set of 80002 trees. A fifty percent majority rule consensus tree is presented in Figure 12. The tree was rooted on *S. microadriaticum* as the outgroup. All the study samples as well as *S. goreaui*, *S. kawagutii*, *S. californium*, *S. muscatinei* and *S. pulchrorum* formed a strongly supported monophyletic clade supported by a high posterior probability value of 0.94. Apart from a well-supported grouping of *S. muscatinei* and *S. pulchrorum* (posterior probability value of 1.00), there was little sub-structure within this clade.

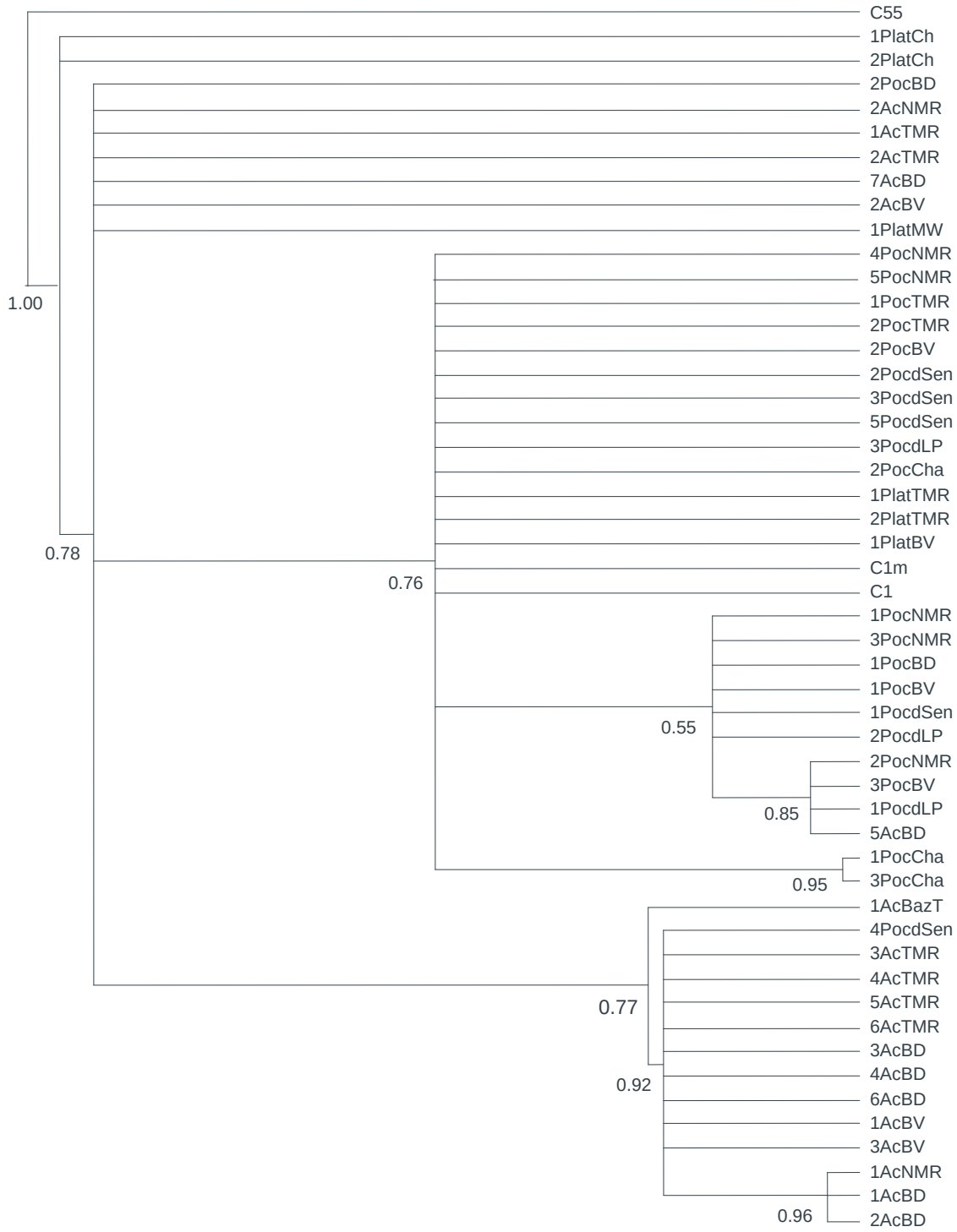

Fig. 11. Phylogenetic reconstruction of *Symbiodinium* relationships between study samples based on an alignment of 263 characters from the ITS2 and 5.8S regions using Bayesian inference. Numbers on the left hand side of the nodes indicate the posterior probabilities of each grouping.

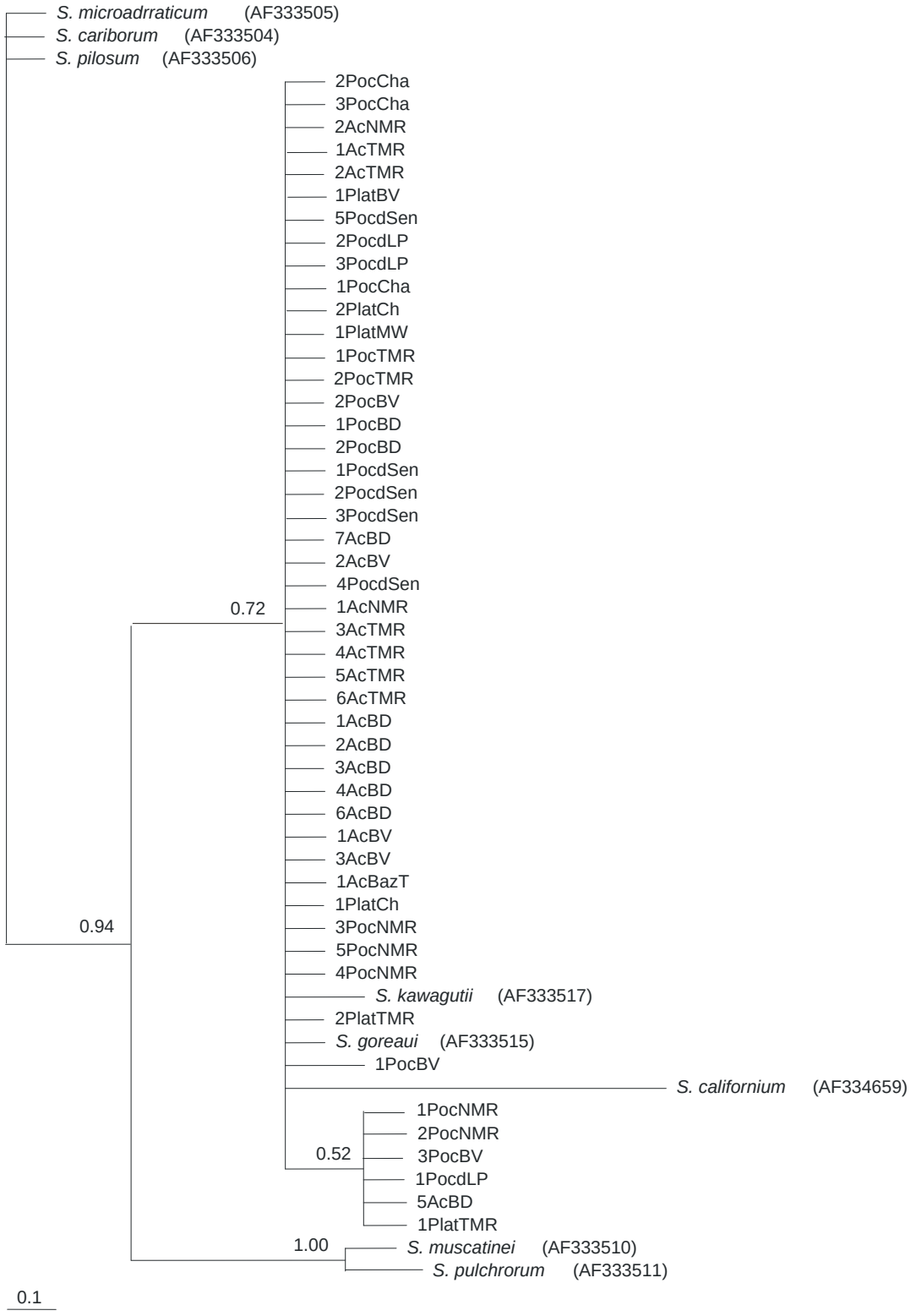

Fig. 12. Phylogenetic reconstruction of *Symbiodinium* relationships between study samples and sequences obtained from Genebank (accession numbers indicated in brackets), based on an alignment of 69 5.8S characters using Bayesian inference. Numbers on the left hand side of the nodes indicate the posterior probabilities of each grouping.

3.8 Phylogeographic Patterns

3.8.1 Haplotype Network Overlaid With Geography

Figure 14 depicts the *Symbiodinium* haplotype network showing the distribution of zooxanthellar ITS haplotypes amongst various locations in the western Indian Ocean. There was no clear association of particular haplotypes with specific geographic location, which indicated a lack of phylogeographic structuring of the zooxanthellar types in the region sampled. Haplotype 12, 13 and 19, however, were only found in the Chagos coral populations. Haplotype 7 was only found at Sodwana Bay and in southern Mozambique. The most genetically diverse area was southern Mozambique, where 9 of the 20 haplotypes were found. The Chagos Archipelago was shown to have low haplotype diversity with only 3 of the 20 haplotypes. However, sampling there was limited, therefore some of the diversity may have gone undetected.

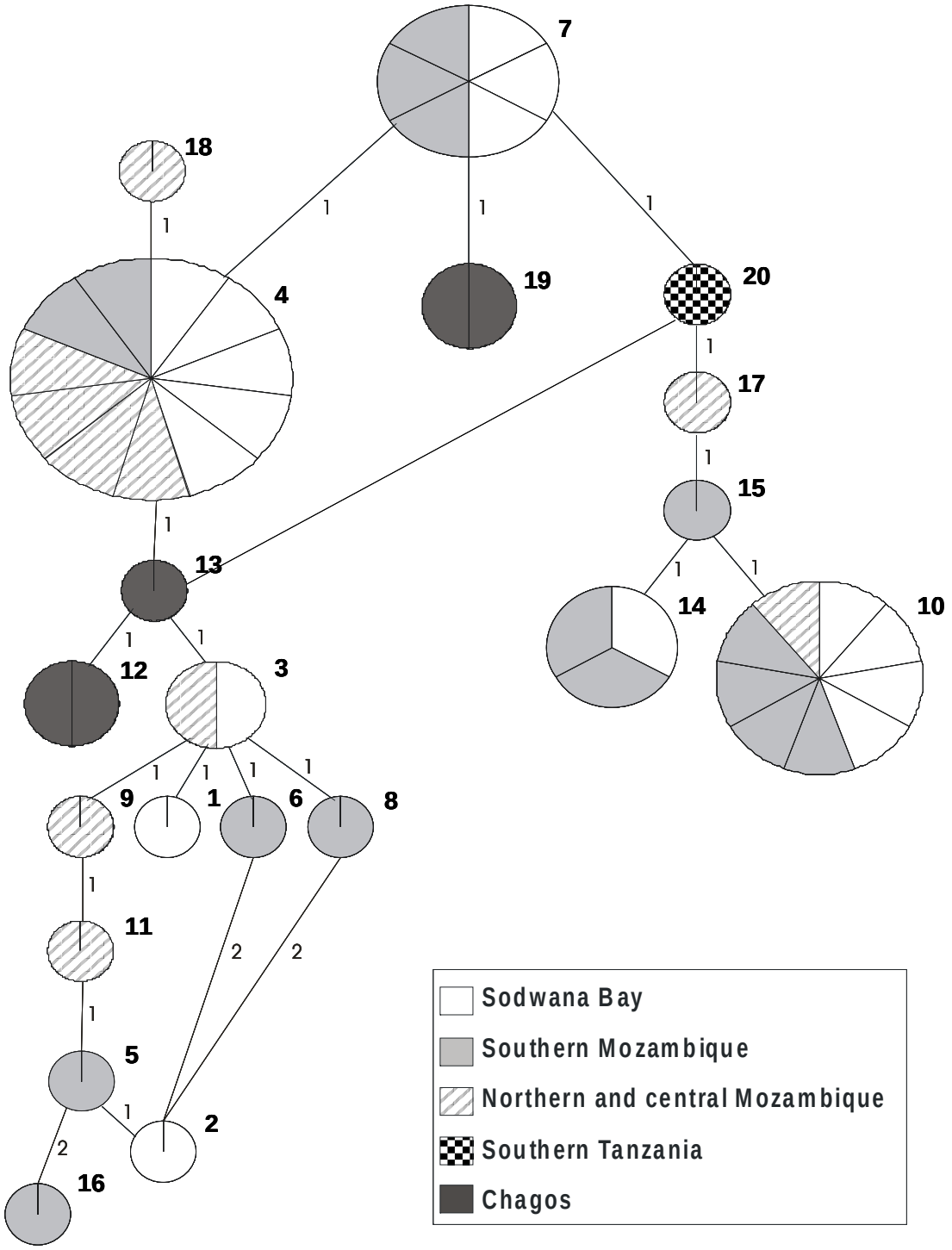

Fig. 14. Haplotype network showing distribution of *Symbiodinium* ITS haplotype amongst various locations in the western Indian Ocean. Numbers on network branches indicate informative base pair differences between haplotypes. Numbers in bold next to haplotypes indicate haplotype numbers.

3.8.2 Analysis of Molecular Variance (AMOVA)

Results of the AMOVA (Table 7 and 8) showed there to be no significant genetic variation amongst *Symbiodinium* populations in corals from all the localities sampled.

Table 7. Results of AMOVA analysis. Groups tested were: Sodwana Bay and southern Mozambique, northern and central Mozambique, southern Tanzania and the Chagos Archipelago. Fixation indices are calculated as follows: FSC = Vb/(Vb + Vc, FST = Va + Vb)/Vt and FCT = Va/Vt.

Source of variation	**d.f.**	**Sum of squares**	**Variance components**	**Percentage of variation**
Among groups	3	5.930	0.07731 Va	4.85
Among populations within groups	1	1.096	-0.02718 Vb	-1.70
Within populations	43	66.411	1.54443 Vc	96.86
Total	47	73.438	1.59456	
Fixation Indices				
FSC	-0.01791			
FST	0.03144			
FCT	0.04848			

Table 8. AMOVA significance tests based on 1023 permutations. Groups tested were: Sodwana Bay and southern Mozambique, northern and central Mozambique, southern Tanzania and the Chagos Archipelago.

	P (rand. Value < obs. Value)	**P (rand. Value = obs. Value)**	**P-value**
Vc and FST	0.29521	0.00000*	0.29521 + -0.01412
Vb and FSC	0.56500	0.00000*	0.56500 + -0.01575
Va and FCT	0.41349	0.10753	0.52102 + -0.01145

* $P < 0.05$

3.8.3 Nested Clade Analysis

Nested contingency analysis (Table 9) revealed significant association of clades and sampling locations at two cladal levels. The nested clades 1.5 and 2.1 (Figure 15) showed significant levels of phylogeographical association and the null hypothesis of no geographical association could be rejected. The phylogeographical distance analysis showed significant differences for clade (*Dc*) and nested clade (*Dn*) distances in these cases (Table 9). Contiguous range expansion was inferred for nested clade 1.5, whereas restricted gene flow with isolation-by distance was the most likely explanation for the patterns observed at nested level 2.1. The nesting design is shown in Figure 15. The inferences derived from the key in Templeton *et al*., (1995) and the geographical distribution of clades are presented in Table 10.

Table 9. Nested clade distance analysis of ITS haplotypes observed in *Symbiodinium* examined in this study. *Dc* and *Dn* are clade and nested clade distances, respectively (for details see Templeton *et al*, 1995). Interior *vs* tip contrasts for *Dc* and *Dn* are indicated with 'I–T' in the corresponding clade, with interior clades in italics bold type. Superscript S and superscript L indicate that distance measures were significantly smaller and larger, respectively, than expected under random distribution of haplotypes.

Haplotypes			**1-step clades**			**2-step clades**			**3-step clades**		
Clade	Dc	Dn	Clade	Dc	Dn	Clade	Dc	Dn	Clade	Dc	Dn
13	81.33	193.47	1.1	345.19^{S}	786.75	2.1	738.12	901.37^{S}	3.2	1275.82	1257.44
9	449.54	405.26									
14	0	128.90									
I-T	-357.49	-223.41									
16	0	691.93	**1.7**	693.19	694.39						
19	0	694.44									
			I-T	347.99^{L}	-92.36						
17	0	807.08	**1.4**	861.40	940.69	**2.3**	1281.60	1316.05			
3	869.31	863.88									
I-T	869.31	56.81	**1.5**	2095.45^{L}	1922.90^{L}						
18	0	3250.52^{L}									
6	83.24^{S}	1528.60^{S}									
I-T	83.24	-1721.92^{S}	**1.2**	0^{S}	2320.44	**2.4**	2125.82	2242.05^{L}			
11											
12											
7	0	757.95	**1.3**	881.47	1965.02						
2	785.69	946.32									
I-T	785.69	188.37									
20			1.6	0	582.76	2.2	808.67	817.96	3.1	833.91	931.98
15											
1	0	827.29	**1.11**	853.80	839.68						
10	0	1041.34									
4	0	677.62									
			I-T	853.80	839.68						
8			**1.8**	0	996.56	**2.5**	874.14	866.25			
21											
5			**1.9**	0	685.94						
22											
0			**1.10**	0	849.12						
						I-T	65.47	48.29			

Table 10. Demographic inferences from the nested clade distance analysis (Templeton *et al*, 1995; Templeton, 1998) of *Symbiodinium* examined in this study.

Clade	**Inference chain**	**Inferred pattern**
Haplotypes nested in clade 1.5	1-19-20-2-11-12 NO	Contiguous range expansion
Haplotypes nested in clade 2.1	1-2-3-4 NO	Restricted gene flow with isolation by distance (restricted dispersal by distance in non-sexual species).
Haplotypes nested in clade 2.3	1-2 IO	Inconclusive outcome
Haplotype nested in clade 2.4	1-19-20-2 IO	Inconclusive outcome
Haplotypes nested in clade 3.2	1-2 IO	Inconclusive outcome

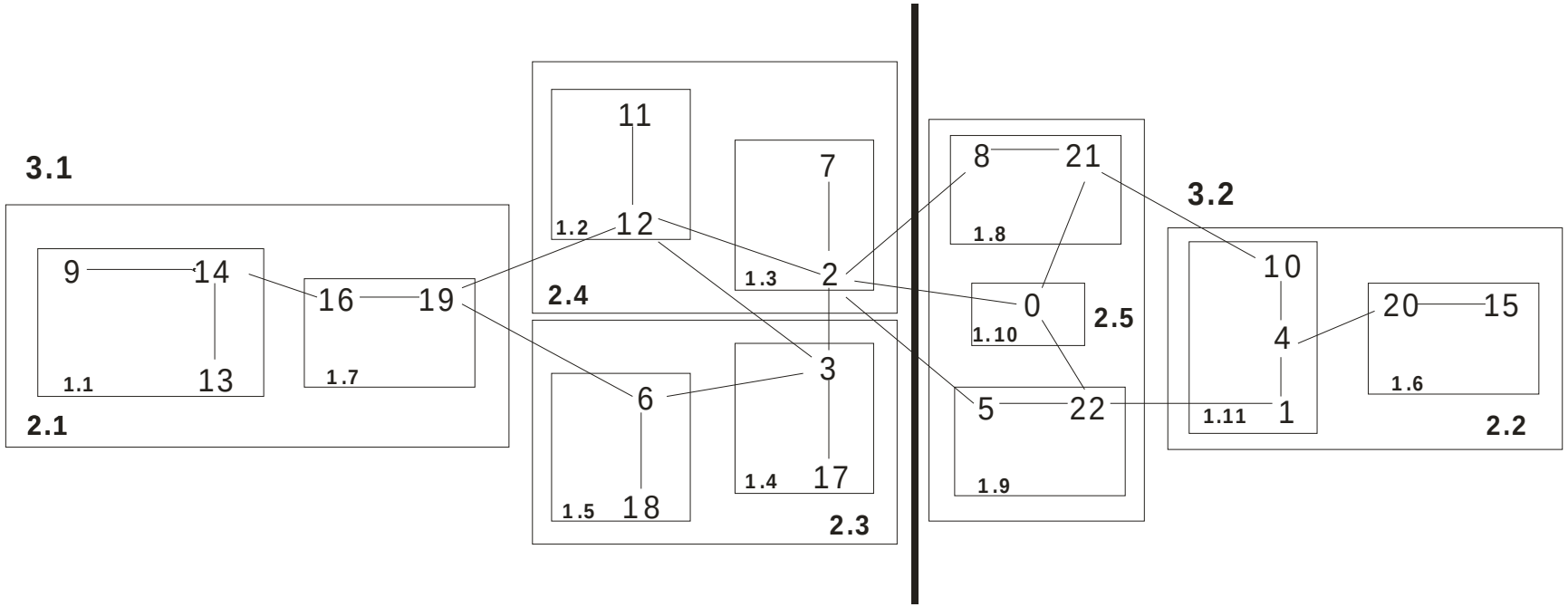

Fig 15. Nested cladogram for ITS haplotypes observed in 10 populations of *Symbiodinium* examined in this study. Rectangles designated by "1.*n*" are 1-step clades, rectangles designated by "2.*n*" are 2-step clades" and the thick line separates 3-step clades.

3.9 Patterns of Symbiodinium Distribution Amongst Coral Species

3.9.1 Haplotype Network Overlaid With Coral Species

Figure 16 depicts the *Symbiodinium* haplotype network distribution of zooxanthellar ITS haplotypes amongst corals sampled in this study. Structuring of the haplotypes amongst the different coral species is evident. Haplotypes 7, 10, 14, 15 and 17 appeared to be found primarily in *A. austera* and are located on the right hand side of the network. The *Symbiodinium* haplotypes found in *P. verrucosa* and *P. damicornis* cluster together on the left hand side of the network. Those from *P. daedalea* are mostly found in the centre of the network, haplotype 16 being the exception.

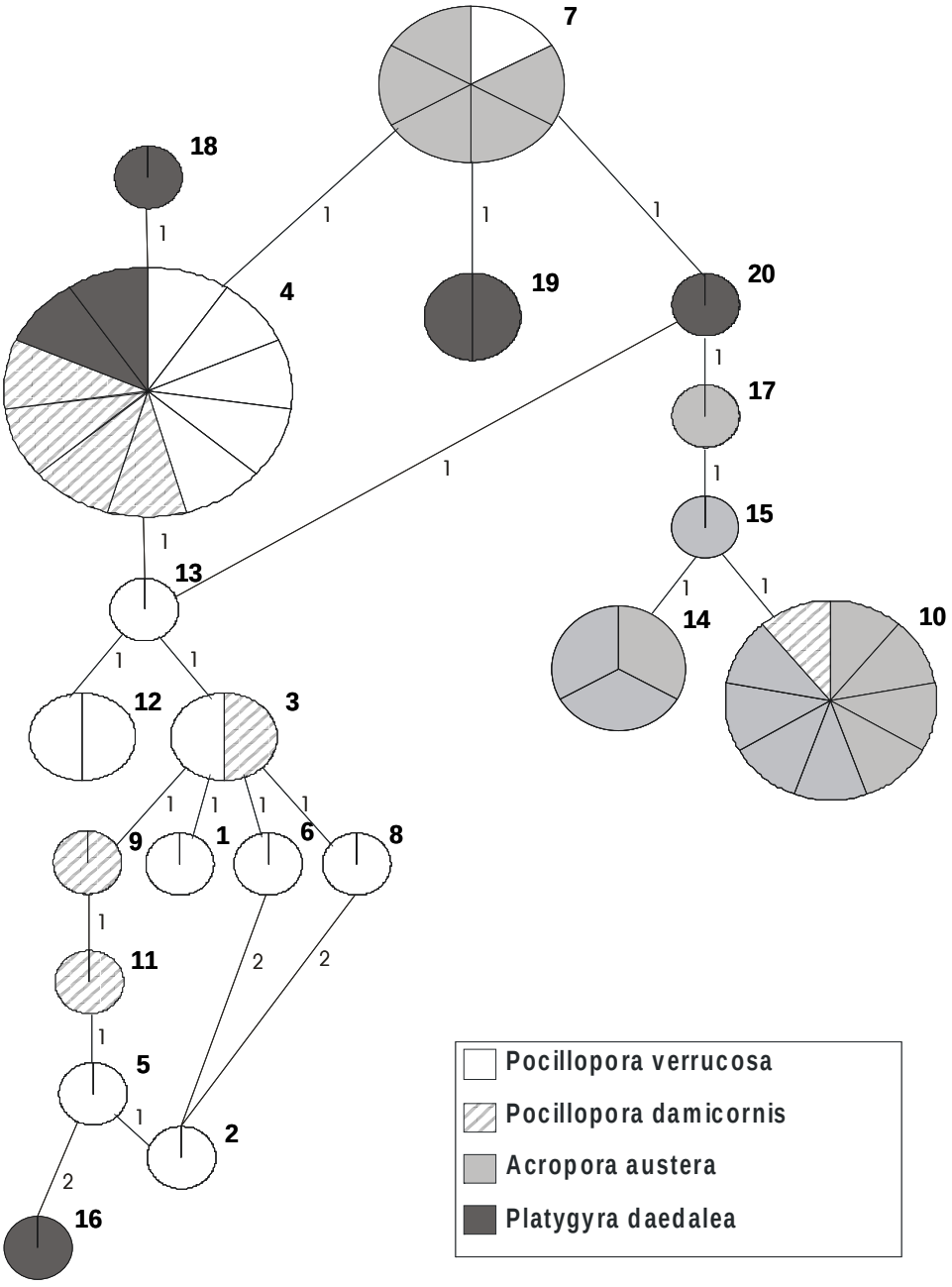

Fig. 16. Haplotype network showing distribution of *Symbiodinium* ITS haplotype in corals sampled in the western Indian Ocean. Numbers on network branches indicate informative base pair differences between haplotypes.

3.9.2 Analysis of Molecular Variance (AMOVA)

Results of the AMOVA showed there to be no significant genetic variation amongst *Symbiodinium* populations sampled from *P. verrucosa* and *P. damicornis*, *A. austera* and *P. daedalea* (Table 11). High genetic variation was present within populations. The FST probability value was found to be significant at less than the 0.05 significance level (Table 11 and 12) and there was significant variation between certain FST values calculated for *Symbiodinium* populations sampled from different corals collected in this study (Table 13).

Table 11. Results of AMOVA analysis. Groups tested were: *P. verrucosa* and *P. damicornis*, *A. austera* and *P. daedalea*. Fixation indices were calculated as follows: FSC = Vb/(Vb + Vc, FST = Va + Vb)/Vt and FCT = Va/Vt.

Source of variation	**d.f.**	**Sum of squares**	**Variance components**	**Percentage of variation**
Among groups	2	20.594	0.65085 Va	35.95
Among populations within groups	1	0.978	-0.01925 Vb	-1.06
Within populations	44	51.865	1.17876 Vc	65.11
Total	47	73.438	1.81036	
Fixation indices				
FSC	-0.01660			
FST	0.34888			
FCT	0.35951			

Table 12. AMOVA significance tests based on 1023 permutations. Groups tested were: *P. verrucosa* and *P. damicornis*, *A. austera* and *P. daedalea*.

	P (rand. Value < obs. Value)	**P (rand. Value = obs. Value)**	**P-value**
Vc and FST	0.00000	0.00000*	0.00000 + -0.00000
Vb and FSC	0.53470	0.00684*	0.54154 + -0.01720
Va and FCT	0.00000	0.16227	0.16227 + -0.01444

*P < 0.05

Table 13. Matrix of FST probability values.

	P. damicornis	*P. verrucosa*	*A. austera*
P. verrucosa	0.54054 +-0.0359		
A. austera	0.00000 +-0.0000*	0.00000 +-0.0000*	
P. daedalea	0.01802 +-0.0121*	0.18018 +-0.0332	0.00901 +-0.0091*

* $P < 0.05$

Chapter 4

4. Discussion

4.1 Phylogenetic Groupings

RFLP analysis of ssrDNA PCR products confirmed that all the samples in this study belonged to *Symbiodinium* clade C. Both the Bayesian inference and NJ ITS tree topology showed all the study samples to form a strongly supported monophyletic clade. Three major subclades were present, but were not strongly supported. Only two of the minor subclades showed strong support. The Bayesian inference tree that included a range of *Symbiodinium* sequences from the Caribbean and Indo-Pacific region (Figure 13) confirmed that all the samples in this study belonged to *Symbiodinium* clade C. This tree demonstrated specificity of *Symbiodinium* types to their coral hosts especially with regards to the symbionts in *A. austera*, *P. verrucosa* and *P. damicornis* as the majority of *A. austera* symbionts formed an independent cluster from the *Pocillopora* symbionts.

Eleven species of *Symbiodinium* namely; *S. microadriaticum*, *S. pilosum*, *S. kawagutti*, *S. goreaui*, *S. californium*, *S. corculorum*, *S. meandrinae*, *S. pulchorum*, *S. bermudense* and *S. cariborum* have been classified based on the morphological species concept (Freudenthal, 1962; Trench and Blank, 1987; Banaszak *et al*., 1993; Trench and Thinh, 1995; Rowan, 1998). Table 6 shows the 5.8S divergence amongst some of these "morphological species". The average genetic distance between them was 0.788, whilst the interspecific distances ranged from 0.000 to 1.626. In contrast, the average genetic distance within the study samples was 0.103, with a range of 0.060 to 0.132. Thus the mean genetic distance between the samples was 7.7 times lower than that between established morphological species. Use of a genetic species concept would indicate that, if the morphological species are good species, then the differences between the study samples are at sub-species level or below. However, morphological traits do not always support molecular phylogenies or correlate with the level of sequence divergence (LaJeunesse, 2001). *Gymnodium linucheae* and *S. microadriaticum*, for

example, are morphologically distinct genera and show a genetic distance of 4% for this region (Trench and Blank, 1987; Trench and Thinh, 1995; LaJeunesse, 2001).

S. cariborum, *S. microadriaticum* and *S. pilosum*, recognised morphological species, share a single 5.8S haplotype (Table 6). If three distinct species can share a single 5.8S haplotype, this would indicate that the "morphological" species referred to above should perhaps have a higher taxanomic ranking. This would be consistent with the notion that each *Symbiodinium* Clade C haplotype is a separate species (LaJeunesse, 2001).

Physiological differences in *Symbiodinium*, on the other hand, have been shown to correlate with morphological differences. *S. microadriaticum*, *S. kawagutii*, *S. pilosum*, and *S. pulchrorum* have been demonstrated to have varying photoacclimatory responses to changes in irradiance (Chang *et al*. 1983, Iglesias-Prieto and Trench 1994, 1997).

All the samples in this study clustered with *S. goreaui* and *S. kawagutii*. This is evident both in the NJ (Figure 10) and Bayesian inference trees (Figure 12) based on 5.8S data. *S. goreaui* has been found to include clade C *Symbiodinium* ITS types (LaJeunesse, 2001). *S. kawagutti*, on the other hand, usually associates with clade F symbionts (LaJeunesse, 2001). However, all the samples in this study belonged to clade C. Also, the average number of nucleotide differences between *S. goreaui* and all other sample haplotypes was 0.438, and thus smaller than the average number of nucleotide differences within the study sample sequences which was 0.687.

The haplotype analysis revealed twenty distinct *Symbiodinium* ITS types. It has been suggested that each ITS haplotype should be considered to be a separate species (LaJeunesse, 2001) based on the phylogenetic species concept. That would indicate that twenty different *Symbiodinium* species were identified in this study (Table 3). However, as will be discussed later, other factors such as ecology and physiology as well as congruence between molecular markers have to be considered when delineating species.

4.2 Prevalence

The haplotype analysis (Table 3) revealed three prominent ITS haplotypes (haplotypes 4, 7 and 10), suggesting that the reef communities in the sampling area are dominated by these *Symbiodinium* types. There are many potential explanations for this. Size, growth rates, nutrient uptake rate, photosynthetic efficiency, host species and photoacclimation ability are likely attributes that may affect distribution competition of symbiont types (LaJeunesse, 2002). This as a result, may influence the prevalence and abundance of a particular symbiont type (LaJeunesse, 2002). The most prevalent symbiont haplotype in the study area was subclade C1 (haplotype 4, Table 3), which was predominantly found in *P. verrucosa* and *P. damicornis.* Preliminary surveys of western Pacific corals indicated that C1 is the most prevalent symbiont found among hosts that must acquire symbionts from the external environment (LaJeunesse *et al.,* 2003; LaJeunesse *et al.,* 2005). This may also be the case in the southern region of the WIO as all the *P. verrucosa* samples from KwaZulu-Natal were shown to contain *Symbiodinium* belonging to subclade C1 and, although the mode of reproduction in *P. verrucosa* varies between geographically isolated regions (Ellis and Solander, 1786; Kruger and Schleyer, 1998) along the KwaZulu-Natal coast, it was found to be a simultaneous hermaphrodite and broadcast spawner (Kruger and Schleyer, 1998). Subclade C1 is found in a variety of different environments and is believed to be an ancestral, "generalist" *Symbiodinium* type (Reimer *et al*., 2007) which probably explains its high prevalence throughout the study area.

4.3 Diversity and Distribution

Although the Hd value (0.878) obtained in this study is not indicative of high levels of diversity, it is possible that more intensive sampling would reveal greater *Symbiodinium* diversity, as has been found on reefs in other parts of the world (LaJeunesse, 2004a; Coffroth and Santos, 2005). Greater haplotype diversity (12 haplotypes) was found in the southern areas (southern Mozambique and Sodwana Bay) than in the northern areas (8 haplotypes) (central and northern Mozambique and southern Tanzania) in this study. This is possibly because the corals found in the southern areas are at the margin of coral reef distribution where the more variable climatic conditions may have contributed to

greater symbiont diversity (Souter *et al.,* 2000). Also, it is possible that the southern sites have experienced less mortality associated with bleaching events (Souter *et al.,* 2000; Souter *et al.,* 2005), sustaining greater symbiont diversity.

Certain symbiont types (e.g. subclade C1) were found to be distributed over a wide geographic range (Figure 14), and no phylogeographic structuring was found amongst the symbiont types (Table 7, Table 8 and Figure 14). Similar studies on the coral host in areas with strong current regimes have demonstrated panmixia in populations (Rodriguez-Lanetty and Hoegh-Guldberg, 2002). The dominant current off the south eastern African coastline is the Agulhas Current, which runs southwards from approximately 28^{o}S, between Maputo and Durban along the south east African coastline (Lutjeharms, 2006). It is a strong, fast-flowing current and reaches a maximum velocity of around 1.5m/s at its core (Schumann *et al.*, 1988; Lutjeharms, 2006). Therefore, it is possible that the symbiont distribution in the area may have been influenced by the host distribution or *vice versa*.

4.4 Diversity and Specificity

This study provided further evidence that, at the level of ITS resolution, there is high specificity between some partners, and that these specific host-symbiont associations occur over wide geographic ranges. Specificity of *Symbiodinium* types to coral hosts was demonstrated possibly at both the species and genus level. The *Symbiodinium* ITS haplotype network overlaid with coral host information (Figure 16) clearly indicated a structuring of symbiont types amongst coral hosts. The AMOVA analyses (Table 11, 12 and 13) also demonstrated genetic differentiation between symbiont populations from different host species. Certain haplotypes (e.g. subclade C1) were obtained from different host species that were collected from distant geographic locations. This suggests that some *Symbiodinium* types must be capable of dispersing great distances and may form symbioses with a wide range of host types (LaJeunesse, 2002). *Symbiodinium* ITS types that associate selectively with a specific host and over wide biogeographic ranges must be functionally different from other ITS types due to their adaptation to that host's intracellular environment (LaJeunesse, 2003). The specificity

between certain partners is probably determined by the environmental (e.g. temperature and irradiance) and biotic (e.g. host diversity and abundance, and symbiont diversity and abundance) factors in the region where the holobiont is situated (Iglesias-Prieto *et al*., 2004).

As mentioned, the diversity of *Symbiodinium* has been found to be high in other areas such as the Caribbean and Pacific as well (LaJeunesse *et al*., 2003; LaJeunesse, 2004a; Coffroth and Santos, 2005). The utilization of genetic markers that provide greater resolution could reveal novel levels of variation below those presently recognized. This would contribute to the discovery of new *Symbiodinium* types. Questions arise regarding the processes that create such diversity as well as how such a high level of variation is maintained in natural populations (Coffroth and Santos, 2005). Host specialization and allopatric differentiation is suggested to proceed after episodic radiations of a few opportunistic "types" (Coffroth and Santos, 2005). The repetition of this process gives rise to numerous "types" with distinct host, geographic and environmental attributes (Coffroth and Santos, 2005). This diversification of *Symbiodinium* in coral reef communities leads to an increase in their genetic diversity. Genetic variation is maintained by the specialized environment that the host provides to its symbiont, structurally, biochemically and physiologically as well as by the external environment in which the host is found (Coffroth and Santos, 2005). In this study the high levels of specificity of symbiont types to their hosts probably contributes to their diversity. Both the larvae and eggs of *Pocillopora spp*., when released into the environment, possess symbionts obtained from their parents (Kinzie, 1996), which may contribute to the maintenance of specificity between the partners.

The dispersal capabilities and rate of gene flow occurring within *Symbiodinium* have not been studied, thus it is difficult to explain why these symbiont types are widely distributed (LaJeunesse, 2001). However, free-living *Symbiodinium* have been cultured and identified from environmental samples (Loeblich and Sherley, 1979; Carlos *et al*., 1999) and there is evidence that *Symbiodinium* species are capable of living and possibly remaining viable outside of the host (LaJeunesse, 2001). Some *Symbiodinium* may have a planktonic existence, this being indicated by the many isolates that have

been grown and maintained in artificial media for long periods. This suggests that they can disperse, persist and reproduce for an indefinite period of time outside the host as long as their nutrient requirements are met (LaJeunesse, 2001). Also, the coral host larvae can undergo long-distance dispersal, which may, in turn, contribute to symbiont dispersal. *P. damicornis*, for example, produces symbiotic larvae that can spend up to 100 days in the plankton before settling (Richmond, 1987). However, more insight is needed into the dispersal of *Symbiodinium* types and the possibility of gene flow among them.

LaJeunesse (2002) found host identity to be the most important factor determining the distribution of a particular symbiont. The results from this study appear to support this finding. Although it has been shown previously that there is no evidence of long-term co-evolution between partners (Rowan and Powers, 1991), it is possible that short-term co-evolutionary processes between a host and symbiont may drive speciation and help maintain symbiont diversity (LaJeunesse, 2002). However, this has not been substantiated in *Symbiodinium*-host systems. Genetic markers, such as microsatellites and their flanking regions, offer a finer genetic resolution and may be able to provide evidence for coevolution. This, in turn, would advance our understanding of *Symbiodinium* distribution amongst host taxa as well as identify one of the factors involved in the generation of *Symbiodinium* diversity over evolutionary time (Coffroth and Santos, 2005).

Buddemeier and Fautin (1993) proposed that the long-term adaptive ability of symbiotic reef invertebrates may depend largely on the degree of specificity a host species has for a particular symbiont type. Evidence of host-symbiont specificity from this study suggests that certain hosts in the sampled area may be more susceptible to perturbations than others.

The corals sampled in this study differ in their life strategies as well as bleaching susceptibilities. *A. austera* has been found to be highly bleaching-susceptible, *P. verrucosa* and *P. damicornis* have been shown to respond variably to bleaching and *P. daedalea* appears to be bleaching-resistant (Jimenez *et al*., 2001; McClanahan *et al*.,

2001). Both pocilloporan species, however, reside mainly in shallow water (Schleyer, pers. comm.) and are potentially more vulnerable to increases in temperature. *P. daedalea*, on the other hand, is found in deeper water (Schleyer, pers. comm) which may be the reason for its escape from bleaching. The finding that these coral species manifest a degree of symbiont specificity may provide insight into their ability to acclimatize to perturbations, such as increased water temperature. The bleaching susceptibility of *A. austera* in the study area is possibly explained by its specificity for particular symbiont types. In the case of *P. verrucosa* and *P. damicornis*, symbiont specificity may contribute to increased bleaching susceptibility in shallower areas. Finally, although *P. daedalea* has been found to show resistance to bleaching (Jimenez *et al*., 2001; McClanahan *et al*., 2001), this is due to its deeper habitat preference and it may be more bleaching susceptible due to its specificity for certain symbiont types.

4.5 ITS as an Ecologically Informative Marker in *Symbiodinium*

In previous studies, it has been shown that certain functional and morphological attributes correspond with phylogenetic groupings obtained from ITS sequence data (LaJeunesse, 2002). In this study, some symbiont types were distinguished by single base substitutions. These differences could thus be attributed to intraspecific variation. However, symbionts showed specificity to certain hosts and must thus inhabit different ecological niches. It has also been previously suggested that differences in the *Symbiodinium* ITS region deliniate different species (LaJeunesse, 2001). Therefore it seems likely that, in this study, these minor differences identified distinctive symbiont populations or perhaps species. Also, other studies have demonstrated that there are physiological differences (e.g. in thermal tolerance) between *Symboidinium* ITS types (Iglesias-Prieto and Trench 1997; Kinzie *et al*., 2001; LaJeunesse *et al*., 2003). Therefore, although the ITS sequence divergence between clade C symbionts is low, the lineage contains many "types" that are ecologically and physiologically distinct (LaJeunesse *et al*., 2003). Genetic divergence does not always correlate with physiological divergence, as closely-related organisms can be physiologically different (Moore *et al*., 1998). When Baillie *et al.,* (1998, 2000a and 2000b) analysed isoenzymes from *Symbiodinium* types, they found high allelic variability in isoenzymes between *Symbiodinium* isolates that had virtually identical ITS sequences, thus

demonstrating that phylogenetic similarity is not related to isoenzyme similarity (LaJeunesse, 2001). The ITS region thus serves as an ecologically important marker in *Symbiodinium* and the various ITS types may be regarded as ESUs. It provides greater systematic resolution for *Symbiodinium* than the ssrDNA and lsrDNA and allows for the correlation of morphological, physiological and biochemical traits and host-symbiont interactions (LaJeunesse, 2001). The phylogenetic groupings obtained from ITS sequence data have been demonstrated to correspond with certain functional and morphological traits (Baum 1992; Manhart and McCourt, 1992) suggesting that the phylogenetic or molecular species concept may be useful for classifying *Symbiodinium*. However, in order to better understand the genetic-physiological connection in *Symbiodinium*, studies investigating the physiological and genetic relationships need to be undertaken (LaJeunesse *et al*., 2003).

The lack of correlation between genetic markers and the high genotypic variability within and among *Symbiodinium* populations suggests that the shuffling of alleles by sexual recombination is taking place (LaJeunesse, 2001). This recombination results in greater variation, which in turn may produce populations that are more resilient to climate change (LaJeunesse, 2001).

The level of genetic resolution offered by the ITS region is not high enough to explain all morphological and physiological differences between *Symbiodinium* types. Thus new "species-specific" molecular markers for the identification and study of different ecological populations are needed for the examination of *Symbiodinium* physiology, ecological and biogeographic distribution and host-symbiont specificity (LaJeunesse, 2001).

As demonstrated by this study, the ITS region is useful for information on the diversity of *Symbiodinium* at the level of genetic resolution that it provides. However, are different *Symbiodinium* ITS types different species? Thus the question that still remains unresolved is: - what constitutes a species in *Symbiodinium*? In other words, which molecular marker differentiates a species? The same problem is experienced when dealing with the coral host and attempting to discriminate a species. In order to

compare the diversity of *Symbiodinium* with that of the host, a distinction between species needs to be made. Without this knowledge, it is difficult to compare levels of diversity and to fully address questions of host-specificity and coevolution (Coffroth and Santos, 2005).

4.6 Implications for Bleaching

Only *Symbiodinium* belonging to clade C were detected in the areas sampled in this study. *Symbiodinium* belonging to clade C are widely distributed and particularly abundant in the Indo-Pacific region (LaJeunesse *et al*., 2005). They have been reported to be more sensitive to increases in sea temperature than members of other *Symbiodinium* clades, and thus corals hosting symbionts belonging to clade C appear to undergo greater bleaching (Baker et al., 2004; Rowan, 2004). However, there appears to be variability in bleaching tolerance amongst clade C *Symbiodinium* ITS types (Fitt and Warner, 1995; Warner *et al*., 1996; Tchernov *et al*., 2004). Therefore widespread distribution of clade C, as was found in this study, does not necessarily suggest that the corals in the area sampled are all equally susceptible to bleaching. Also, recent work on stress susceptibility has concentrated on the symbiont only and has not taken into account that of the host. However, a *Symbiodinium* type that is considered to be stress-susceptible can associate with coral families that are relatively tolerant of stress (LaJeunesse *et al*., 2003). If the *Symbiodinium* type was solely responsible for the stress-tolerance of symbiotic corals, then stress-tolerant symbionts should be found in tolerant associations (Stat *et al*., 2006). Therefore, the physiology of both the symbiont and host contributes to the resilience of the holobiont.

No clade D symbionts were identified in any of the samples in this study. Subclade D2 is found in scleractinans that have survived episodes of severe bleaching and are thus considered to be thermally-tolerant (Baker *et al*., 2004; Rowan, 2004). Although the northern sites in the study area were impacted by bleaching (Souter *et al*., 2000; Souter *et al*., 2005), subclade D2 was not detected in any of the coral colonies sampled there. It is possible that more intensive sequencing may have identified symbionts belonging to subclade D2. Also, the well-documented, severe ENSO bleaching event that affected the northern reefs occurred in 1998. It is possible that bleaching may have induced a

shift in the symbiont community to subclade D2 in some of the coral hosts, but reversion to the pre-1998 symbioses may have occurred (Thornhill *et al*., 2006). Finally, coral colonies were sampled randomly in this study, and no attempt was made to record the light intensity, depth or water temperature of collection, so it is possible that colonies located in areas favourable for subclade D2 may have been under-sampled.

It has been suggested that the long term adaptive ability of corals may depend on the degree of specificity a host species has for its symbiont type and, as a result, coral hosts which are highly specific towards their symbionts may be more susceptible than others to perturbations (LaJeunesse, 2001). The specificity of *Symbiodinium* types to coral hosts that was demonstrated in this study may suggest that the corals found in the study area are susceptible to increases in sea temperature and, if their symbionts are thermally sensitive, are thus more likely to bleach as they are less likely to form associations with other more resistant symbionts. This is a concern, as it suggests that the survival of corals from bleaching events in the sampled area is potentially lower than in regions where the host-symbiont associations are more flexible. However, due to the small scale of this study, such a generalization cannot be made from its results alone. A larger sample size and a broader range of host species would provide a more realistic indication of reef-resilience in the region. Also, as mentioned before, the resilience of a particular host-symbiont association is not determined by the symbiont partner alone (LaJeunesse *et al*., 2003; Stat *et al*., 2006). So, even if a coral host possesses a high degree of specificity for a particular symbiont type, the physiology of the coral host itself also contributes to the overall resilience of the holobiont.

4.7 Future Considerations

Although no phylogeographic structuring of *Symbiodinium* types was detected in this study, this may have resulted from under-sampling. A larger sample set may have revealed greater structuring of symbiont types amongst the various study sites. However, it is possible that there is no geographic structuring in the region and that the *Symbiodinium*, like its coral hosts (Rodriguez-Lanetty and Hoegh-Guldberg, 2002), is panmicitic. A greater sample size would also have permitted individual assessment of geographic patterns amongst symbionts from the sampled coral hosts.

Problems were experienced when amplifying the ITS region from the extracted *P. daedalea* samples and, as a result, the number of ITS sequences obtained for *Symbiodinium* types from *P. daedalea* was limited. It is possible that the mucopolysaccharides present in *P. daedalea* tissues may have caused PCR inhibition (Goodacre and Wade, 2001). Although the extraction methods were modified to eliminate the mucoplysaccharides, they were not successful and, due to time constraints, only a small number of *Symbiodinium* ITS sequences could be obtained from *P. daedalea* in this study. However, it has been suggested that the inclusion of hexadecyltrimethylammonium bromide (CTAB) in the extraction mix may overcome the problems of PCR inhibition by mucopolysaccharides (Goodacre and Wade, 2001) and this will be implemented in the future.

The cost of sequencing also limited the number of samples for which sequences could be generated. A future alternative would be to employ DGGE in ITS analysis, as this technique allows for rapid screening of multiple samples and cuts down on sequencing costs, because only novel fingerprints need to be validated by sequencing (van Oppen, 2006). Unfortunately this technology was not available and thus could not be used in this study. Nevertheless, problems have been experienced with DGGE in that the technique is unable to resolve migration differences for sequences that differ by 14bp or more, thus novel and potentially divergent sequences may be overlooked (van Oppen, 2006).

The ITS region is considered to be ecologically informative for *Symbiodinium*. However, as mentioned before, it does not provide enough resolution to explain allthe variations in *Symbiodinium* morphology and physiology (LaJeunesse, 2001). It has thus been recommended that a suite of single-copy nuclear and cytoplasmic markers is developed and simultaneously assessed (van Oppen, 2006). Studies analyzing the *Symbiodinium* microsatellite flanking regions have been able to detect fine-scale diversity within the *Symbiodinium* genus (Santos *et al*., 2004). The construction of a partial *Symbiodinium* genomic library and the targeting of non-repeat-containing clones from a microsatellite library will allow for the assessment of variation in nuclear loci

(van Oppen, 2006). A molecular marker that can delineate *Symbiodinium* entities with distinct physiologies needs to be identified to determine how genetic differentiation correlates with differences in the physiology of the host-symbiont association (van Oppen, 2006). Genes which govern physiological traits thus have to be identified. This can be accomplished through gene-expression studies that utilize microarray approaches in which vast numbers of genes are targeted simultaneously. Physiological variability in *Symbiodinium* may be the result of differential expression of theses genes or the presence of distinct alleles (van Oppen, 2006).

Characterisation of the stress-response of corals at the molecular level (for both host and symbiont) will assist in the development of diagnostic assays for the early detection of stress responses in corals, as well as the rapid identification of the exact stressor(s) involved in coral bleaching (van Oppen, 2006). However, an understanding of how coral-*Symbiodinium* associations deal with stressors such as climate change is needed. This will require a greater insight into *Symbiodinium* community ecology and diversity is (LaJeunesse, 2001). The full range of symbiont partners and the extent to which a particular host-symbiont association contributes to the environmental tolerance of a coral is not yet known (LaJeunesse *et al*., 2003). Thus the genetic diversity of *Symbiodinium* has to be assessed over a broad range of host species, especially on the WIO reefs. More insight is also needed into the physiological and molecular interactions involved in the breakdown of the symbiotic association. The mechanisms that govern host-symbiont specificity need elucidation to determine this. An understanding of how the symbiosis is initiated, integrated and maintained would is also necessary. Thus, genetic and molecular work, on both the coral and symbiont, needs to be combined with physiological and ecological data to provide a multifaceted perspective on the resilience of reef-building corals (van Oppen, 2006).

The findings of this study are valuable for a number of reasons despite this being a small-scale study that utilized only one genetic marker and did not incorporate any physiological work. They provided evidence of a number of novel *Symbiodinium* ITS types in the study area and of panmixia in the *Symbiodinium* populations. The results showed that the coral species sampled possesed significant symbiont specificity,

indicative of potential susceptibility to perturbations such as increases in seawater temperature due to climate change. Finally, this was one of the first attempts at identifying the genetic diversity of *Symbiodinium* in the south West Indian Ocean and it provides a basis for future research on this subject in the area.

Chapter 5

5. References

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6. Appendices

6.1 Buffers

6.1.1 50× TAE Buffer

242 g Tris base and 18.6 g Na_2EDTA was dissolved in 800 mℓof distilled water. The pH was adjusted to 8 using glacial acetic acid. The solution was then topped up to 1 ℓ with distilled water.

6.1.2 1× TAE Buffer

A required aliquot of 50× TAE buffer was diluted 50-fold (e.g. 10 mℓ of 50× TAE buffer to 490 mℓ distilled water).

6.1.3 DNAB

A solution of 0.4 M NaCl was made up and combined with15m M NaEDTA in order to obtain pH 8.0.

6.1.4 DMSO buffer

A solution of 0.25 M EDTA was combined with 20% v/v DMSO and saturated with NaCl.

6.2 Agarose Gels

6.2.1 0.75 % Agarose Gel

An aliquot of 1× TAE buffer was combined with 1.2 g of agarose in a 250 mℓ Erlenmeyer flask in order to make a 150 mℓ solution. The solution was brought to boiling point in a microwave, poured into a horizontal gel electrophoresis tray and left to set.

6.2.2 1% Agarose Gel

An aliquot of 1× TAE buffer was combined with 1.5 g of agarose in a 250 mℓ Erlenmeyer flask in order to make a 150 mℓ solution. The solution was brought to boiling point in a microwave, poured into a horizontal gel electrophoresis tray and left to set.

6.2.3 2% Agarose Gel

An aliquot of 1× TAE buffer was combined with 3 g of agarose in a 250 mℓ Erlenmeyer flask in order to make a 150 mℓ solution. The solution was brought to boiling point in a microwave, poured into a horizontal gel electrophoresis tray and left to set.