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INVITED REVIEW

Conservation genetics and the resilience of reef-building corals

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Abstract

Coral reefs have suffered long-term decline due to a range of anthropogenic disturbances and are now also under threat from climate change. For appropriate management of these vulnerable and valuable ecosystems it is important to understand the factors and processes that determine their resilience and that of the organisms inhabiting them, as well as those that have led to existing patterns of coral reef biodiversity. The scleractinian (stony) corals deposit the structural framework that supports and promotes the maintenance of biological diversity and complexity of coral reefs, and as such, are major components of these ecosystems. The success of reef-building corals is related to their obligate symbiotic association with dinoflagellates of the genus *Symbiodinium*. These one-celled algal symbionts (zooxanthellae) live in the endodermal tissues of their coral host, provide most of the host's energy budget and promote rapid calcification. Furthermore, zooxanthellae are the main primary producers on coral reefs due to the oligotrophic nature of the surrounding waters. In this review paper, we summarize and critically evaluate studies that have employed genetics and/or molecular biology in examining questions relating to the evolution and ecology of reef-building corals and their algal endosymbionts, and that bear relevance to coral reef conservation. We discuss how these studies can focus future efforts, and examine how these approaches enhance our understanding of the resilience of reef-building corals.

Keywords: connectivity, conservation, phylogeny, scleractinia, stress markers, *Symbiodinium*

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Introduction

More than two-thirds of our planet is covered by oceans, but only a tiny proportion (< 1%) of this vast marine environment harbours coral reefs. Despite this, coral reefs contribute significantly to the economies of some countries (Moberg & Folke 1999). For example, the total economic value of activities undertaken in the Great Barrier Reef Marine Park Catchment Area (i.e. tourism, commercial fishing, and cultural and recreational activity) is estimated to be over US$4.3 billion annually (Access Economics 2005). On a worldwide scale, coral reefs provide an annual net economical benefit of US$30 billion (Cesar *et al*. 2003). Unfortunately, coral reefs have not escaped human-inflicted degradation. They have suffered severe long-term decline due to overfishing and sediment/nutrient pollution from terrestrial run-off (Pandolfi *et al*. 2003) and are under threat from more recent impacts of global climate change, including coral bleaching and disease (Hoegh-Guldberg 1999; Hughes *et al*. 2003). Outbreaks of crown-of-thorns starfish have also severely affected some coral reefs, and increases in outbreak frequency have recently been linked to nutrient enrichment from terrestrial run-off (Brodie *et al*. 2005).

Twenty per cent of the world's 284 800 km^2 of coral reef (Spalding *et al*. 2001) have been effectively destroyed and show no immediate prospect of recovery (Wilkinson 2004). A further 24% are under imminent threat of collapse through anthropogenic pressures, while another 26% are under longer-term threat of collapse. In the Caribbean, coral cover in shallow waters (Bak *et al*. 2005) has been reduced from about 50% to about 10% over the last 30 years (Gardner *et al*. 2003). Although many coral reefs

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continue to recover after the 1997/1998 global bleaching event — especially those in well-managed and remote areas — this recovery may be negatively impacted by the predicted increases in sea-surface temperature. It has been projected by some that coral reefs will change rather than disappear completely (Hughes *et al*. 2003), as different species show different tolerances to climate change and coral bleaching (Marshall & Baird 2000; Hughes *et al*. 2003). However, in order to improve our predictions of what changes are most likely to occur, we require a better understanding of the resilience of corals reefs; that is, the magnitude of disturbance that can be absorbed, and recovered from, before ecosystem functions are lost and there are shifts from one stable state to another (Nyström *et al*. 2000). This review focuses on reef-building scleractinian (stony) corals (including their algal endosymbionts) and the roles that molecular biology and genetics can play in enhancing conservation efforts and our understanding of resilience of these organisms. It discusses four areas: (i) how studies of the past can be used to predict the future; (ii) genetic connectivity; (iii) molecular markers for coral stress; and (iv) the role and genetic identification of coral-inhabiting algal endosymbionts.

Coral diversity and evolution: how studying the past can be used to predict the future

An understanding of the processes that have led to existing patterns of coral diversity and the timescales over which these have occurred provides insights into the mechanisms responsible for divergence and speciation. This knowledge is valuable for reef management, as it can be projected into the future and used to make predictions about the response of reef corals to environmental changes, such as changes in sea-surface temperatures, ocean acidity, sea-level rises and circulation patterns due to climate change. The timescales over which these changes are taking place, however, are much smaller than the timescales over which natural climate change events in the past have occurred, and there may be insufficient time for some of the evolutionary processes summarized below to keep pace with current climate change. Nevertheless, the preservation of evolutionary processes that generate biodiversity has been advocated as an essential aspect of conservation efforts for complex groups of organisms (Ennos *et al*. 2005). A thorough understanding of evolutionary history is a prerequisite for successful implementation of this approach.

Molecular relationships among ancient coral lineages

Scleractinian corals are believed to have arisen in the mid-Triassic [~240 million years ago (Ma)], but the major extant families were not established until the late Cretaceous (Veron 1995). The period that followed, the Tertiary (~65–1.8 Ma), was characterized by several important palaeoclimatic and geological events that have almost certainly shaped today's reef corals, including global seawater-temperature fluctuations (Crowley & North 1991; Crame 1993) and large sea-level falls (Vail & Hardenbol 1979; Haq *et al*. 1987; Crowley & North 1991). This was followed by a series of sea-level fluctuations during the Pleistocene glacials and interglacials (Haq *et al*. 1987; Chappell *et al*. 1996). For example, the Pacific and Indian Oceans were effectively closed off from one another during each glaciation, as sea levels dropped by up to 130 m below present-day levels. These sea-level changes are likely to have led to changes in intensity and direction of sea-surface currents and, as a consequence, would have caused the repeated isolation and (re)connection of populations and species (Veron 1995; Benzie 1999). Some coral reef organisms, such as butterfly fishes (McMillan & Palumbi 1995), stomatopods (Barber *et al*. 2002) and starfish (Benzie 1999), are believed to have undergone a diversification as a result of this. As sea levels rose, new habitats became available, and a combination of founder effects and selection may have caused many new species to evolve (Potts 1983). The collision of Australia/New Guinea with the islands off the Asian continent, which began *c*. 15 Ma (Audley-Charles 1981; Hall & Holloway 1998), may have further contributed to this period of evolutionary diversification, at least in the central Indo-Pacific. Other major geological events that are likely to have affected extant reef coral lineages are the closure of the Tethys Sea and the rise of the Panama land bridge. The Tethys was a circum-tropical sea, which remained an open east–west connection between the present-day Atlantic and Pacific Oceans via the Mediterranean until approximately the mid-Miocene (*c*. 12 Ma; Rögl & Steiniger 1984; Por 1989), although it seems likely that there were several closures and re-openings in the late Oligocene or early Miocene (Rosen 1988). The final closure of the Tethys caused the fragmentation of a formerly continuously distributed benthic fauna. The rise of the Isthmus of Panama (3.0–3.5 Ma; Keigwin 1982; Duque-Caro 1990; Coates & Obando 1996) signified the final separation of the Atlantic and Pacific faunas.

Our understanding of the evolutionary history of corals has been impeded by difficulties in interpreting variability in the skeleton and soft body parts, as well as the scant fossil record of corals (Foster 1979; Best *et al.* 1984; Veron 1995; Stanley 2003; Fukami *et al*. 2004b), as well as by the scant fossil record of soft body parts (Foster 1979; Best *et al*. 1984; Stanley 2003). Fortunately, this knowledge gap can be (partly) filled by molecular phylogenetics. Relatively few molecular phylogenies have been constructed for scleractinian corals, mainly due to the difficulties that were initially encountered in the development of suitable

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methods and markers for these organisms (reviewed in van Oppen *et al*. 2002b) and the slow rate of mitochondrial DNA (mtDNA) evolution in anthozoans (Romano & Palumbi 1997; van Oppen *et al*. 1999; Shearer *et al*. 2002); although the latter is not of major concern when deep divergences are studied. The few published studies that are available almost invariably report major inconsistencies with evolutionary relationships derived from analyses of morphological traits of extant and fossil taxa. Romano & Palumbi (1996) were the first to show that the clustering of scleractinian families in traditionally recognized suborders is not supported by mtDNA 16S sequences from 34 species of scleractinian corals, representing 29 genera in 14 of the 24 extant families. Two major mtDNA 16S clades instead of seven suborders (Veron 2000) were distinguished within the Scleractinia; one consisting of corals with relatively solid, heavily calcified skeletons ('robust' corals) and the other comprising less heavily calcified taxa ('complex' corals). Expansion of the 16S data set (partial sequences) to include representatives of 56 of the 220 extant scleractinian genera in 20 of the 24 families and all seven suborders (Romano & Cairns 2000), as well as partial mtDNA 12S sequences from 28 species including eight scleractinian families (Chen *et al*. 2002) and partial nuclear ribosomal DNA (nrDNA) 28S sequences from 40 species comprising 35 genera and 12 families (Cuif *et al*. 2003), supports the two-clade hypothesis of scleractinian coral evolution. Re-evaluation of earlier studies based on shorter sequences of the 28S ribosomal DNA (rDNA) and fewer taxa (Chen *et al*. 1995; Veron *et al*. 1996; Romano & Cairns 2000) reveals the same main dichotomy. Romano & Palumbi (1996) concluded that the separation of these two scleractinian clades took place at least 300 Ma. This separation would therefore have occurred before the first appearance of the scleractinian skeleton in the fossil record 240 Ma, which implies that the scleractinian skeleton has evolved multiple times from a soft-bodied, anemone-like ancestor.

While Romano & Palumbi (1996, 1997) as well as Chen *et al*. (2002) found that the mtDNA subordinal phylogeny differed significantly from traditional phylogenies based on skeletal morphology, traditional groupings within genera and families were supported by the mtDNA data. These results are likely to be biased due to the fact that only Indo-Pacific taxa were examined. In a more recent study using both mtDNA and nuclear DNA and including both Indo-Pacific and Atlantic corals, Fukami *et al*. (2004b) showed that most Atlantic lineages conventionally assigned to the Faviidae and Mussidae are not distributed within the more numerous Pacific lineages of these 'families', but instead represent a well-defined clade. Hence, morphological convergence at the family level has obscured the evolutionary distinctiveness of Atlantic corals. Nine of the 27 genera of reef-building Atlantic corals belong to this previously unrecognized lineage, which probably diverged over 34 Ma. The study by Fukami *et al*. (2004b) in particular highlights the importance of molecular approaches in assessing the diversity of reef corals and identifying evolutionarily distinct lineages, both of which are highly relevant to conservation efforts. A comparable example (but with the opposite outcome) is that of the endangered stony coral *Siderastrea glynni*, which is endemic to Panama. Only five colonies have ever been discovered and all were found within a few square metres of each other, downstream and close to the Pacific opening of the Panama Canal (Budd & Guzman 1994). The genus *Siderastrea* contains only five extant species. *S. glynni* is morphometrically most similar to *S. savignyana*, the only Indo-Pacific species in the genus, and it has therefore been hypothesized that *S. glynni* has originated from a rare dispersal event from the central Pacific (Budd & Guzman 1994). rDNA ITS sequence analysis, however, rejects this theory and shows that the *S. glynni* population has most likely arisen from a recent introduction from the Caribbean (Forsman *et al*. 2005). The conservation status of this taxon is therefore possibly not warranted.

More recent evolutionary processes — species boundaries and interspecific hybridization in acroporid corals

The genus *Acropora* (Acroporidae) is one of the most widespread genera of scleractinian corals, spanning the Indian and Pacific Oceans and the Caribbean Sea. It is also extremely species-rich and the largest extant reef-building coral genus with over 100 species (Wallace 1999; Veron 2000). The fossil record shows that the genus probably originated during the Palaeocene (Carbone *et al*. 1994) or Eocene (von Fritsch 1875; Latham 1929) and became widely distributed in the early Miocene (Wells 1956). It is believed that the current high diversity in the central Indo-Pacific is a result of Miocene–Pliocene and post-Pliocene speciation events (Wilson & Rosen 1998). Few species are recorded from the Miocene (Wells 1964), and most extant species have no fossil record before the Plio–Pleistocene. In contrast, most fossils of Pliocene and Pleistocene origin are identifiable with extant species (Pickett 1981; Pickett *et al*. 1985; Veron & Kelley 1988). In line with the fossil records, mitochondrial and nuclear DNA phylogenies have shown that most of the extant Indo-Pacific *Acropora* spp. have evolved relatively recently as a consequence of the Plio- and Pleistocene perturbations, suggesting that speciation of scleractinians can take place over relatively short timescales (Fukami *et al*. 2000; van Oppen *et al*. 2001a). *Acropora* therefore provides an ideal model system for examining speciation and evolution of scleractinian reef-coral species in general, on both temporal and spatial scales.

The highly synchronized spawning of many sympatric, congeneric species of corals (Harrison *et al*. 1984; Willis *et al*. 1985; Babcock *et al*. 1986) provides, undoubtedly, the

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most obvious opportunity for hybridization in the marine realm. At least 133 species of corals from 10 scleractinian families are involved in mass spawnings over four to five nights annually on the Great Barrier Reef (Willis *et al*. 1985) and multispecies spawnings, but generally involving fewer species, occur in other reef regions (Heyward *et al*. 1987; Baird *et al*. 1991; Simpson 1991; Babcock *et al*. 1994; Guest *et al*. 2002). Opportunities for interbreeding among coral species are enhanced in a variety of ways. Closely related, congeneric species spawn simultaneously or have overlapping periods of gamete release (Babcock *et al*. 1986). The positive buoyancy of gametes ensures that eggs and sperm from sympatrically occurring species, which may differ in their habitat or depth distributions, are nevertheless concentrated and mixed at the sea surface during the 4- to 8-h period of gamete viability (Heyward & Babcock 1986; Oliver & Babcock 1992; Willis *et al*. 1997). Experimental breeding trials have demonstrated that co-occurring, congeneric species are able to interbreed with up to 100% fertilization success when they do not have the option of interbreeding with conspecifics, suggesting that hybridization can occur and is likely to have contributed to the evolution of scleractinian corals (Wallace & Willis 1994; Miller & Babcock 1997; Szmant *et al*. 1997; Willis *et al*. 1997). However, during mass-spawning events, eggs are generally exposed to complex mixtures of con- and heterospecific sperm, and in these situations factors such as gamete competition are likely to modify cross-fertilization rates. Genetic approaches conducted over the past 5–10 years have fortunately been extremely valuable in unravelling the importance of natural hybridization in reef corals in the absence of data from sperm-competition experiments.

Population-genetic (Márquez *et al*. 2002c) and molecular phylogenetic (Hatta *et al*. 1999; van Oppen *et al*. 2001a, 2002a; Márquez *et al*. 2002b; Fukami *et al*. 2003; Wolstenholme *et al*. 2003; Wolstenholme 2004) analyses on Indo-Pacific acroporid corals combined with data on spawning times and cross-fertility (see above) indicate that hybridization events occur in nature, but that these are rare. Nonmonophyly at nuclear ribosomal, single-copy nuclear, as well as mitochondrial DNA markers, is a general characteristic of Indo-Pacific *Acropora* species that participate in mass-spawning events, while species that differ in the timing of release of their gametes (by several hours or weeks) tend to be monophyletic. In addition, the mtDNA and single copy nuclear DNA trees show several incongruities. These results are strongly indicative of natural hybridization and introgression in the coral genus *Acropora*, but the extent of natural hybridization is difficult to assess from phylogenetic studies. Moreover, these data cannot unambiguously refute the possibility that lineage sorting is incomplete or that some of the Indo-Pacific hybridizing species are single morphologically plastic species. Population-genetic approaches may have greater potential to distinguish between the rare hybridization vs. the 'morphologically plastic species' hypothesis than phylogenetic analyses, because comparisons are based on differences in allele frequencies, which can occur over much shorter time frames than the mutational changes on which phylogenetic analyses are based.

Genetic differences between sympatric populations of two species (*A. cytherea* and *A. hyacinthus*) have been compared with those between widely allopatric populations from eastern vs. western Australia. Eight polymorphic allozyme loci detected very low, but significant, levels of genetic differentiation between *A. cytherea* and *A. hyacinthus* in sympatry, whereas conspecific populations from eastern vs. western Australia could not be distinguished (Márquez *et al*. 2002c). These results are consistent with hybridization occurring infrequently and gene exchange between species being lower than intraspecific gene flow. The two species are clearly distinct, and the 'single morphologically polymorphic species' hypothesis can be rejected for this species pair. A second species group that has been targeted for population-genetic approaches is the *A. nasuta* group. Allele frequency differences at two nuclear introns and one microsatellite locus were highly significant between the three species, *A. nasuta, A. valida* and *A. secale* for all pairwise comparisons (MacKenzie 2005), despite the fact that the two species that were included in the nuclear intron phylogeny (van Oppen *et al*. 2001a), *A. nasuta* and *A. valida*, were para- and polyphyletic, respectively. These data are consistent with the findings for *A. cytherea* and *A. hyacinthus*, suggesting that nominal *Acropora* species constitute genetically distinct entities that can exchange genes with other such entities but do so only very rarely.

Although the coral genus *Acropora* has unsurpassed species diversity in the Indo-Pacific region, only three species are recognized in the Caribbean: *A. cervicornis*, *A. palmata* and *A. prolifera*. Two independent studies, using nuclear ribosomal DNA (nrDNA) ITS and nuclear introns (van Oppen *et al*. 2000), and nuclear introns and mtDNA data (Vollmer & Palumbi 2002), respectively, have shown that *A. prolifera* has arisen as a result of hybridization between *A. palmata* and *A. cervicornis*, and that the hybrid backcrosses with only one of the parental species (Vollmer & Palumbi 2002, 2004).

More recent evolutionary processes — species boundaries and interspecific hybridization in reef-building corals other than Acropora

With the exception of the Caribbean *Montastraea annularis* species complex (Faviidae), other reef-building corals have not been as extensively studied; hence general evolutionary patterns for scleractinian corals are not yet clear. Three putative *Montastraea* species were long thought

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to represent intraspecific variants, but later studies revealed that differences in colony morphology are matched by differences in growth rate, stable isotope chemistry, aggressive behaviour, allozymes, corallite structure, and life history (reviewed in Fukami *et al*. 2004a). Nonetheless, genetic differences are small for all markers investigated, which include allozymes (van Veghel & Bak 1993), amplified fragment length polymorphisms (Lopez *et al*. 1999), an intron in the β-tubulin gene (Lopez & Knowlton 1997), rDNA ITS (Lopez & Knowlton 1997; Medina *et al*. 1999) and mtDNA (Medina *et al*. 1999; Fukami & Knowlton 2005). These data have driven debates on the species status of these corals for over a decade, but the issue has recently been revisited in two detailed studies (Fukami *et al*. 2004a; Levitan *et al*. 2004). *Montastraea faveolata* is clearly distinct from the other two putative species based on both genetic and morphological characters, while *M. annularis* and *M. franksi* are more similar morphologically and genetically, yet are believed to represent distinct sister species (Fukami *et al*. 2004a). Levitan *et al*. (2004) showed how a variety of mechanisms are likely to maintain reproductive isolation among all three species. The occurrence of very rare introgressive hybridization cannot, however, be completely ruled out. Two of the three modern species (*M. franksi* and *M. faveolata*) first appear in the fossil record in Costa Rica in deposits dated at 2.9–3.5 Ma and are morphologically distinct throughout this period (Budd & Klaus 2001), while the third species is likely to be of more recent origin (Pandolfi *et al*. 2002). The recency of these divergences as well as the different genetic and morphological patterns observed in these two studies for northern vs. southern Caribbean populations, are likely to have contributed to the difficulty in distinguishing these taxa genetically.

Studies on two other coral genera belonging to different families have argued for the occurrence of introgressive hybridization. Allozyme divergence between three of the seven recognized *Platygyra* morphospecies (Faviidae) on the Great Barrier Reef is low (Nei's D range from 0.032 to 0.057), and preliminary electrophoretic screening of the other four species suggests that this pattern is consistent throughout the genus (Miller & Benzie 1997). All species spawn synchronously, and experimental breeding trials show that morphological species are highly cross-fertile (Miller & Babcock 1997). This has led these authors to conclude that natural hybridization occurs between these taxa. In the Caribbean coral *Madracis* (Pocilloporidae), high levels of morphological plasticity occur among the five most common sympatric species. Temporal reproductive isolation is absent in the genus, with mature gametes being present in these brooding species in autumn (August–November) (Vermeij *et al*. 2002). Three of the five species share rDNA ITS sequences (Diekmann *et al*. 2001). For one of these species, *M. decactis*, the oldest known fossils are 15–11 million years (Myr) old (Budd *et al*. 1994); *M. pharensis* is of more recent origin (1.5 Myr; Budd & Johnson 1999); while no fossils are known for *M. formosa*. Given the observed sequence divergence within the genus (*c*. 6%) and approximate age of the species, homogenization of ITS regions would normally be expected. Hence, the non-monophyly of the three species is taken as evidence for introgressive hybridization (Diekmann *et al*. 2001).

Finally, some other scleractinians appear to be good biological species that do not hybridize with closely related congeners under natural conditions. For example, the species pair *Acropora* (*Isopora*) *palifera* and *A. cuneata*, both of Pliocene origin (Wallace 1999), show fixed allozyme differences in sympatry (Ayre *et al*. 1991). *Montipora digitata* and *Montipora tortuosa* are also distinguished by fixed allelic differences in sympatry (Stobart & Benzie 1994; Stobart 2000). Five *Porites* spp. and two *Goniopora* spp. were found to be genetically distinct based on 13 allozyme loci (Garthwaite *et al*. 1994). Consistent differences in habitat preferences, tissue colouration, skeletal morphology, allozyme patterns and reproductive ecology have confirmed the separate specific status of *Pavona varians*, *Pavona frondifera* and *Pavona chiriquiensis* (Maté 2003). Finally, *Platygyra sinensis* and *Platygyra pini* from Hong Kong have been distinguished as good species based on ITS sequences, despite morphological overlap (Lam & Morton 2003).

The studies listed above are relevant in terms of understanding the resilience of reef corals, as rare hybridization events that occur in some coral species (especially in *Acropora* spp., which dominate most Indo-Pacific reefs) are important on evolutionary timescales and are likely to have resulted in increased genomic diversity and heterozygosity (Ayre & Hughes 2000; van Oppen *et al*. 2001a), which in turn may give these corals a better capacity to respond to environmental change over ecological time scales compared to species that never hybridize. Furthermore, natural selection acts directly upon genetic variation, and the presence of high levels of genetic diversity and heterozygosity at putatively neutral loci may reflect genome-wide diversity and hence a potential for adaptive evolution. Reef-building corals may diversify not only through cladogenesis, but also through hybrid speciation. These studies support the notion that preservation of evolutionary processes as a means to protect biodiversity should be an important aspect of modern management strategies (Ennos *et al*. 2005; Willis *et al*. 2006).

Genetic connectivity in scleractinian coral populations

An important factor for coral-reef resilience is the connectivity between and within coral reefs. The exchange of larvae creates and maintains high levels of genetic

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diversity, which are crucial in terms of resilience against disturbance. Migrants may carry new alleles that will be integrated into the population through reproduction, creating new gene combinations on which selection can potentially act. The spread of selectively advantageous alleles at DNA loci involved in, for instance, physiological responses such as bleaching resistance is another potential consequence of migration. Furthermore, gene flow increases local effective population sizes, thereby enhancing the ability to resist rapid random changes in allele frequencies from one generation to the next through drift. Small and isolated populations are subject to genetic erosion, with alleles being rapidly fixed or lost through drift, while selection and mutation generally play a less important role than drift in populations with small effective population size. On the other hand, high gene flow prohibits local adaptation and will slow down the process of allopatric speciation.

Larval-exporting or source reefs with diverse populations of healthy adult corals are essential to maintain the genetic diversity and resilience of larval-importing or sink reefs (Grimsditch & Salm 2005). Therefore, an assessment of larval transport in and out of reefs, i.e. the extent to which reefs are self-seeding or accumulate recruits from surrounding areas, as well as the direction of larval dispersal, will improve our ability to forecast how reef corals are likely to respond to environmental change (Warner & Cowen 2002; Sale *et al*. 2005). One of the major limitations in obtaining direct estimates of connectivity lies in tracing large numbers of small propagules with high mortality rates. Furthermore, dispersal may not result in the exchange of genes if the immigrants fail to establish themselves or are unsuccessful in interbreeding with the resident population. Estimates of connectivity are therefore generally indirect, based on inferences from sea-surface-circulation patterns or genetics. Successful migrants leave a genetic signature of their movements and allow inference of connectivity using population-genetic methods. Hence, the assessment of the genetic connectivity, which is the extent to which populations of a species range are linked by juvenile or adult exchange (Palumbi 2003), has become essential in conservation biology.

Genetic connectivity of scleractinian corals — current state of knowledge

Most studies on genetic connectivity of scleractinian coral populations are based on allozymes (Table 1), because other markers such as microsatellites and nuclear introns were initially difficult to develop (Márquez *et al*. 2002a) and have only recently become available (van Oppen *et al*. 2000; Maier *et al*. 2001; Vollmer & Palumbi 2002; MacKenzie *et al*. 2004; Magalon *et al*. 2004; Miller & Howard 2004; Severance *et al*. 2004; Shearer & Coffroth 2004; Baums *et al*. 2005a; Underwood *et al*. 2006). Mitochondrial DNA is of limited use for population-genetic application in corals due to its extremely low mutation rates in these organisms (Romano & Palumbi 1997; van Oppen *et al*. 1999; Shearer *et al*. 2002). Although wide-scale studies of the population structure of shallow-water corals have shown a range of genetic structures (e.g. Ayre & Hughes 2000), evidence for panmixia among coral populations is generally only observed at relatively small spatial scales of several tens of kilometres or less (Table 1; note that genetic structure can exist at scales smaller than tens of kilometres in some scleractinian species). For example, using allozymes, Ridgway *et al*. (2001) obtained support for panmixia among six *Pocillopora damicornis* populations in South Africa that were separated by up to 70 km. Ng & Morton (2003) found similar results for *Platygyra sinensis* in Hong Kong over a spatial scale of approximately 50 km. This is consistent with simulation studies based on population-genetic data, which have shown that the spatial scale of connectivity may be smaller than generally assumed (based on larval dispersal capabilities) in marine systems, and is in the order of several tens of kilometres per generation for species with a population size of about 1000 (Warner & Cowen 2002; Palumbi 2003). However, in some instances, extremely high levels of gene flow at large spatial scales have been inferred from genetic data. For example, Rodriguez-Lanetty & Hoegh-Guldberg (2002) and Takabayashi *et al*. (2003) argue that panmixia exists over geographical ranges of ~700 km (for *Plesiastrea versipora* in the Ryukyu Archipelago, Japan) and ~7000 km (between *Stylophora pistillata* populations from Japan and Australia), respectively. In both studies, nrDNA ITS markers were used, which are likely to lack the resolution needed for population approaches, due to their relatively slow mutation rates (e.g. Savard *et al*. 1993; Schlötterer *et al*. 1994; LaJeunesse 2005) and often high levels of intra-individual variation (Vollmer & Palumbi 2004). Caution should therefore be taken in using the lack of clear geographical structuring in ITS data as evidence for present-day high levels of gene flow. In a different study, pairwise F_{ST} values not significantly different from zero were observed for two *Acropora* species sampled from the east and west coast of Australia (~3500 km apart) based on allozyme data, while a third species sampled from the same geographical locations showed evidence for restricted gene flow (Márquez *et al*. 2002c). It remains to be confirmed with other markers whether such observations reflect true panmixia at these huge spatial scales, because allozymes are notoriously slow in reaching genetic equilibrium (Grosberg & Cunningham 2001). For example, geminate pairs of the sea urchins (Bermingham & Lessios 1993) and snapping shrimps (Knowlton *et al*. 1993) on either side of the Isthmus of Panama showed no significant allozyme differences,

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Table 1 Summary of the available literature on genetic connectivity in scleractinian corals. N.t., not tested; N.a., not applicable; GBR, Great Barrier Reef

Species	Inferred levels of gene flow	Genetic markers	Geographical location and scale, collection depth if not shallow waters	Number of populations (individuals) sampled	Isolation by distance observed	Reference
Mycedium elephantotus	High within regions (< 50 km), moderate to low between regions	Allozymes	Taiwan, ~400 km	7 (193)	N.t.	Yu *et al.* (1999)
Pocillopora damicornis	Moderate	Allozymes	Ryukyu Archipelago, Japan, ~650 km	6 (288)	No	Adjeroud & Tsuchiya (1999)
Mycedium elephantotus	Moderate to low		Taiwan, ~400 km	3 (90)	N.t.	Dai *et al.* (2000)
Plesiastrea versipora	Panmixia in Ryukyu Archipelago, restricted gene flow in East Australia	ITS	Ryukyu Archipelago, Japan, ~700 km; eastern seaboard of Australian ~4000 km	8 (40)	No in Ryukyu Archipelago; Yes along East-Australian coast	Rodriguez-Lanetty & Hoegh-Guldberg (2002)
Goniastrea aspera	Moderate to high	Allozymes	Ryukyu Archipelago, Japan, ~500 km	8 (374)	N.t.	Nishikawa & Sakai (2003)
Platygyra sinensis	Panmixia	Allozymes	Indo-West Pacific (Hong Kong), ~50 km	6 (196)	N.a.	Ng & Morton (2003)
Pocillopora meandrina	Panmixia at scales below 10 km, restricted over 2000 km	Microsatellites	South Pacific (Society and Tonga Islands), ~5–2000 km	7 (257)	No	Magalon *et al.* (2005)
Stylophora pistillata	Panmixia	ITS1	Japan, Malaysia, East Australia (GBR), 7000 km	8 (122)	N.a.	Takabayashi *et al.* (2003)
Seriatopora hystrix	Low	Allozymes	East Australia (GBR), 90 km	57 (~2280)	No	Ayre & Dufty (1994)
Pocillopora damicornis, *Seriatopora hystrix*, *Stylophora pistillata*, *Acropora cuneata*, *Acropora valida*	Moderate to high within GBR	Allozymes	East Australia (GBR), ~1200 km	9 (~450 per species)	N.t.	Ayre & Hughes (2000)
Acropora nasuta	Moderate	Microsatellite, nuclear introns	East Australia (GBR), meters to ~500 km	4 (243)	N.t.	MacKenzie *et al.* (2004)
Pocillopora damicornis, *Seriatopora hystrix*, *Stylophora pistillata*, *Acropora cuneata*, *A. palifera*, *A. valida*, *A. hyacinthus*, *A cytherea*, *A. millepora*	Little or none between Lord Howe Island and GBR	Allozymes	East Australia (GBR vs. Lord Howe Island), ~700 km	12 (~3000)	N.t.	Ayre & Hughes (2004)
Acropora millepora	Moderate to high	Allozymes	East Australia, ~1200 km	9 (249)	No	Smith-Keune & van Oppen (2006)
Acropora hyacinthus, *A. cytherea*, *A. tenuis*	Panmixia (*A. hyacinthus* and *A. cytherea*), low for *A. tenuis*	Allozymes	East vs. West Australia, ~3500 km	2 for each species (~74 per species)	N.a.	Márquez *et al.* (2002c)
Acropora palifera, *Pocillopora damicornis*	Low to moderate between crest and lagoon/microatoll	Allozymes	East Australia, < 1 km	6 (299), 9 (443)	N.t.	Benzie *et al.* (1995)
Pocillopora damicornis	Low	Allozymes	Southwest Australia, ~400 km	25 (840)	No	Stoddart (1984)
Pocillopora damicornis	Moderate to high	Allozymes	Within Rottnest Isl. (West Australia), within Kaneohe Bay (Hawaii), < 5 km	23 (25–60 per site)	N.t.	Stoddart (1988)
Fungia fungites	Moderate	Allozymes	West Australia (Dampier Archipelago), 5 km	8 (240)	N.a.	Gilmour (2002)

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Table 1 *Continued*

Species	Inferred levels of gene flow	Genetic markers	Geographical location and scale, collection depth if not shallow waters	Number of populations (individuals) sampled	Isolation by distance observed	Reference
Acropora digitifera, *A. aspera*	Moderate to low	Allozymes	West Australia (Ningaloo Reef), 6.5–155 km	6 (389) *A. digitifera*, 4 (271) *A. aspera*	No	Whitaker (2004)
Balanophyllia elegans	Low	Allozymes	Northeast Pacific (California), ~3000 km	18 (966)	Yes	Hellberg (1994)
Balanophyllia elegans *Paracyathus stearnsii*	Low (*B. elegans*), high (*P. stearnsii*)	Allozymes	Northeast Pacific (California), 10–300 km	12 (?)	Yes (*B. elegans*), No (*P. stearnsii*)	Hellberg (1996)
Acropora palmata	Little or none between eastern and western Caribbean, high gene flow within eastern and western cluster	Microsatellites	Caribbean, up to ~2600 km	44 (1300)	N.t.	Baums *et al*. (2005b)
Lophelia pertusa	Low to moderate	ITS, microsatellites	Northeast Atlantic, up to ~3000 km, ~100 m depth	10 (165)	No	Le Goff-Vitry *et al*. (2004)
Balanophyllia europaea	Low	Allozymes	Mediterranean, ~35–2000 km	5 (70–131)*	No	Goffredo *et al*. (2004)
Pocillopora verrucosa	Panmixia	Allozymes	Eastern South Africa, < 200 km	6 (267)	N.a.	Ridgway *et al*. (2001)
Seriatopora hystrix	Low	Microsatellites	Red Sea, ~100 m–600 km	10 (207)	Yes	Maier *et al*. (2005)

*Sample size varied considerably among loci.

while mtDNA has revealed deep divergences. Similarly, American oysters were originally thought to show panmixia between the east American coast and the Gulf of Mexico based on allozymes (Buroker 1983), but analyses of mitochondrial (Reeb & Avise 1990) and nuclear DNA (Karl & Avise 1992) have since shown reciprocal monophyly between these populations. Nevertheless, genetic homogeneity among geographically distant reef coral populations has been documented using markers other than allozymes. Baums *et al*. (2005b) found no significant genetic structuring over large geographical scales (ranging from ~770 to ~1800 km) within the eastern or western Caribbean cluster of *Acropora palmata* populations, despite the fact that these inferences of gene flow were derived from microsatellite data.

More relevant approaches in terms of understanding resilience of reef corals

Patterns of genetic structure in marine organisms may not always reflect contemporary levels of gene flow but may show the signature of past (mainly Pleistocene) events, because populations of many marine organisms are likely not to be in equilibrium between drift and gene flow (Benzie 1999; Grosberg & Cunningham 2001). F_{ST}-based approaches are particularly sensitive to such nonequilibrium situations. Moreover, levels of gene flow estimated from *F*-statistics reflect levels of long-term gene flow, i.e. mean levels of gene flow over extensive periods of time. This can lead to situations where high levels of gene flow are inferred from *F*-statistics, while gene flow has been permanently disrupted in recent times after past periods of high gene flow. Other problems associated with F_{ST}-based estimates of gene flow ($N_e m$) are that these assume an island model of migration, while dispersal in marine organisms is often better approximated by a stepping-stone model, and that the signal:noise ratio tends to be high in high gene flow species, making it difficult to demonstrate that migration rates are sufficiently high to repopulate declined populations (Waples 1998). Examination of genetic isolation by distance has the potential to increase confidence in the biological significance of small F_{ST} values (Palumbi 2003). For management purposes, however, it is imperative to know whether a depleted population will be replenished from elsewhere and hence, whether an understanding of levels of gene

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flow over ecological rather than evolutionary time scales is required. Therefore, the more recent assignment methods to estimate population structure and migration rates on ecological time scales (in the current or over the last several generations; e.g. Paetkau *et al*. 1995; Rannala & Mountain 1997; Cornuet *et al*. 1999; Pritchard *et al*. 2000; Wilson & Rannala 2003; Paetkau *et al*. 2004) hold greater promise for management and should be employed in addition to traditional *F*-statistics (Baums *et al*. 2005b) to provide information about migration on different timescales.

Molecular stress markers in reef corals

The ability to effectively manage coral reef ecosystems is in part contingent on defining how specific stressors impact corals and on identifying which members of the community are most vulnerable to them. Human activities have dramatically modified the marine environment and, as a result, many coral reefs are now exposed to frequent disturbances in temperature and light regimes, fluctuations in salinity, high sedimentation and nutrient loading, and a diversity of organic pollutants and bacterial contaminants (Kushmaro *et al*. 1997; Hoegh-Guldberg 1999; Brown 2000). Exposure to each or any combination of these stressors manifests as changes in physiological performance and in the activation of molecular cascades that function to ameliorate and repair the biological impacts of the disturbance to maintain the organism within normal function boundaries. Ultimately, the magnitude, duration and biological complexity of the disturbance interplays with the tolerance thresholds of these molecular activities and with the energetic status of the coral, to dictate whether or not an individual survives.

The maintenance of health against a backdrop of ever changing environmental conditions is fundamental to life, and many of the genes and proteins involved in the maintenance of homeostasis and stress amelioration are evolutionarily conserved (Parsell & Lindquist 1993; Downs *et al*. 1998; Whitesell & Lindquist 2005). Such conservation has provided the scientific context to identifying key molecular players which can serve as the building blocks for designing methodological strategies aimed at examining their role in understudied taxa such as corals. For example, a polyclonal antibody raised against Hsp70 protein in mouse is cross-reactive and has been used to successfully explore Hsp70 expression in the reef coral *Goniopora djiboutiensis* (Sharp *et al*. 1997).

The advantages of targeting molecular stress markers in stress evaluation as compared to physiological and organismic level measures such as respiration rates, photophysiological performance, growth rates, reproductive outputs and bleaching status, lies in their functional diversity, the immediacy of their behaviour with respect to the onset of the stress response and, in some cases, the tight correlation between the activity of a particular molecule (or suite of molecules) and a specific stressor. As such, molecular stress markers have the potential to provide detail regarding the nature of the stressor impacting a given coral and insight into the timing of the exposure. Given the depth of information that is attainable using such tools, it is not surprising that these stress markers are the focus of increasing research activity.

Stress markers in corals—current state of the field

The best-studied molecular stress markers in corals are the heat-shock proteins (Tom *et al*. 1999) and antioxidant enzymes such as the superoxide dismutases. These proteins have predominantly been examined in corals exposed to short-term, extreme stress regimes in the laboratory (Table 2), and these studies serve to confirm that, like other taxa, corals possess temporally dynamic and responsive cellular machinery to combat the impacts of stress. More recent investigations have targeted proteins in both compartments of the symbiosis (e.g. Downs *et al*. 2002, 2005) and broadened the suite of markers under study to include proteins involved in maintaining effective function of the photosynthetic machinery of the dinoflagellates symbionts, and those indicative of DNA damage, cell cycle disturbances (Lesser & Farrell 2004) and xenobiotic responses (Downs *et al*. 2005).

Results on field-collected corals (Downs *et al*. 2002, 2005) highlight some of the complexities of working with highly responsive elements of the cellular machinery in corals. Downs *et al*. (2002) show that corals belonging to the *Montastraea annularis* complex sampled in 1999 at depth (18.3 m), express less superoxide dismutases and more Hsp60, Hsp70, ubiquitin, carbonyl, lipid peroxidase and antioxidant glutathione (GHS) than their shallow counterparts (at 3 m). Furthermore, that expression is extremely dynamic on a scale of months (e.g. compare the data in Downs *et al*. 2002 Fig. 3 for March and June). However, a similar but more comprehensive suite of biomarkers examined in the same corals re-sampled in 2000 showed no significant difference among depths or across sampling intervals (e.g. see Downs *et al*. 2005 Figs 6 and 7 for March and June). While the first study clearly shows a relationship between bleached corals and the level of protein expression associated with oxidative stress, the inconsistencies between data obtained in two different years suggest that these data are highly variable in field-collected corals and are thus difficult to interpret. In fact, in the absence of a comprehensive baseline for the specific molecule under consideration collected at a relevant research site and depth over multiple years, these data suggest that it is almost impossible to derive any benefit from sampling a randomly selected coral species and measuring a suite of protein biomarkers. There is no doubt that the

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Table 2 Summary of protein stress markers examined in corals. *Abbreviations*: Hsp, heat shock protein; chlp sHsp = chloroplast small heat shock protein; sHsp, small heat shock protein; Cu/ZnSOD, copper zinc superoxidase dismutase; MnSOD, manganese superoxidase dismutase; HNE, carbonyl, 4-hydroxynonenal; GPx, glutathione peroxidase; GSH , glutathione; GST, glutathione-S-transferase; LPO, lipid peroxides; MDR, ABC family of proteins, P-glycoprotein 140 & 160; CPD, cyclobutane pyrimidine dimer; p53, expressed protein from p53 cell cycle gene; D1, D1 protein photosystem II; DTT, dithiothreitol; FKNMS, Florida Keys National Marine Sanctuary. (C), antibody specific to coral; (D), antibody specific to the dinoflagellate symbiont

Species	Molecule	Stress regime	Reference
Montastraea faveolata	Hsp 95, 90, 78, 74, 33, 28, 27	28 °C +3, +6 22 °C +5–+13 Duration variable	Black *et al*. (1995)
Montastraea annularis	Hsp 70	28 °C +2 Continuous	Hayes & King (1995)
Goniopora djiboutiensis	Hsp 70	28 °C +2, +5, +8, +10, +12 2 h shock, 4 h recovery	Sharp *et al*. (1997)
Acropora grandis	Hsp 35, 60, 70	25 °C +10 5 h	Fang *et al*. (1997)
Montastraea franksi	Hsp 70	29 °C +3 48 h	Gates & Edmunds (1999)
Montastraea faveolata	Chlp sHsp(D), sHsp(D), αB-crystallin(C), Cu/ZnSOD(C)MnSOD(C) LPO, GSH, Hsp 60, Hsp 70 ubiquitin,	27 °C +6 (over 3 h) 9 h plus light 27 °C +6 (over 3 h) 9 h in dark	Downs *et al*. (2000)
Montastraea annularis species complex	Chlp sHsp(D), Hsp 60(C), Hsp 70(C), αB-crystallin(C), Cu/ZnSOD(C) and MnSOD(C) protein carbonyl LPO, GSH, ubiquitin	FKNMS — Field 1999 Molasses Reef 3 m, 6 m, 9 m, 18 m Mar, May, Jun, July, Aug, Sept, Oct	Downs *et al*. (2002)
Goniastrea aspera	HNE, GPx (C&D), MnSOD (C&D)	DTT high light (no flow) DTT low light (no flow)	Brown *et al*. (2002)
Pocillopora damicornis	Hsp 70	500 p.p.m. of red soil 4 h	Hashimoto *et al*. (2004)
Montastraea faveolata	Cu/ZnSOD CPD p53 D1	28 °C ramp to +4 over 6 d Low light High light	Lesser & Farrell (2004)
Montastraea annularis	chlp sHsp(D) Hsp 60(D),Hsp 70(D), MnSOD(D), GPx(D), GST(D) Hsp 60(C), Hsp 70(C), sHsp(C) MnSOD(C), GPx(C), GST(C), MDR, ubiquitin	FKNMS — Field 2000 Molasses Reef 3 m, 6 m, 9 m, 18 m (same corals sampled as Downs *et al*. 2002) Alina's Reef 6 m Mar, Jun, Aug, Nov	Downs *et al*. (2005)

work that has been done on protein biomarkers in corals to date demonstrates their enormous potential as stress markers, but, as is the case with most high-resolution diagnostic tools, they will require significant development prior to their blanket use in coral biology to generate scientific data on which management decisions can be based.

In terms of gene-based stress markers, Snell and co-workers have focused their research efforts on identifying a suite of environmentally responsive genes in corals (Morgan *et al*. 2001; Morgan & Snell 2002; Snell *et al*. 2003) and this has culminated in the development of the first coral complementary DNA (cDNA) array (Edge *et al*. 2005). The 32 genes on the array were isolated from *Acropora cervicornis* and *Montastraea faveolata* challenged with a diversity of stressors in the laboratory and characterized using approaches that include Differential-Display polymerase chain reaction (PCR), Suppressive Subtractive Hybridization and Reverse-Transcriptase PCR, using primers designed to existing sequences for the target genes in GenBank (Edge *et al*. 2005). The DNA array has been shown to be effective at profiling gene expression in *Montastraea faveolata* challenged with temperature, salinity and light treatments in the laboratory (Edge *et al*. 2005) and in field-collected

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Diploria strigosa sampled at four sites at increasing distances away from Bermuda's semi-submerged municipal dump site (Morgan *et al*. 2005). These results clearly demonstrate the potential of gene-expression profiling in assessing the health status of corals and justifies future studies aimed at the simultaneous evaluation of multiple stress genes in corals.

Genomics and the implications for stress marker development in corals

Most studies utilizing stress markers in corals have focused on a small number of molecules selected for their well-defined roles in specific cellular pathways. Recent advances in genomics allow for the bulk analysis of the transcriptome and genome of an organism and the characterization and subsequent examination of large numbers of molecules whose collective activity drive fundamental aspects of organismic biology (Feder & Mitchell-Olds 2003; Hofmann *et al*. 2005).

In terms of developing such approaches to evaluate stress in corals, to our knowledge, several currently funded projects are underway and beginning to generate data. One study examines the stress responses in coral symbionts belonging to the genus *Symbiodinium* (Leggat *et al*., unpublished); a second investigates those of the coral host *Acropora millepora* (Seneca *et al*., unpublished; Klueter *et al*., unpublished); while a third evaluates symbiosis-specific gene expression in *Acropora palmata* and *Montastraea faveolata* (Schwarz *et al*. 2006). All three projects focus on high-throughput expressed sequence tag (EST) sequencing of the 5′ or 3′ ends of cloned cDNAs (complementary DNA generated for each RNA transcript in a sample) from libraries generated in organisms representing a specific state or stress exposure history. The sequences are identified by comparison to existing databases (e.g. Kortschak *et al*. 2003; Technau *et al*. 2005) and all, or a selected subset, are used for further analysis based on their putative function in other systems and relevance to the question under consideration. Using these sequences, DNA microarrays are generated, which consist of multiple individual cDNA clones arranged in a predictable way on a microscope slide. Labelled transcriptomes of individual corals or symbionts exposed to specific biological states are then hybridized against the array, and the quantitative expression of genes that are present both on the array and in the sample are measured (Thomas & Klaper 2004).

An alternative approach to that outlined above involves the generation of blind arrays (e.g. for *Porites lobata*, Matz *et al*., unpublished; for *Porites compressa* and *Montipora capitata*, Gates *et al*., unpublished). Here, cDNA is generated from animals that represent a diversity of stress-exposure histories. Clones are selected randomly and arrayed either in a micro- or a macroarray format. The smaller spot size in the former allows thousands of genes to be spotted simultaneously but the arrays are not reusable. In contrast, macroarrays have a larger spot format and thus allow for the screening of fewer clones, but they are reusable. The arrays are then hybridized to labelled samples generated for animals representing a single state. For stress evaluation in corals, this approach has the potential to rapidly characterize genes whose expression is responsive to a range of stressors, i.e. stress generalists, or those whose expression is only impacted by exposure to a single stressor, i.e. stress specialists. Once a given gene is identified as stress-responsive, it can be sequenced and potentially identified by comparison with genetic databases. This approach has the advantages of (i) reduced upfront sequencing effort and cost; (ii) selection of genes based solely on their responsiveness on the array rather than selection based on their documented role in stress amelioration; and (iii) the more rapid and comprehensive identification of stress or state specific molecular markers.

Another endeavour that will broaden our understanding of stress markers in corals will be a complete characterization of either a coral- or a *Symbiodinium* genome. An initiative to sequence the *Symbiodinium* genome is currently underway (see www.genome.gov/10001852; last accessed on 19 June 2006); some survey sequencing of the genome of *Porites lobata* has been conducted; and the genome of the anemone *Nematostella vectensis* has recently been completely sequenced (M. Martindale, personal communication). Collectively, these sequences provide a provocative glimpse of the genetic complexity within the Anthozoa and provide a plethora of information to focus the search for stress markers in corals.

The genomic approaches described here are extremely powerful in identifying, isolating and exploring the behaviour of molecular markers of stress in corals. In terms of reef management, these molecules will serve as the building blocks for developing tools that have the potential to reveal exactly which stressor(s) a coral has been exposed to, and for how long. This information is crucial in the climate of rapid coastal development and the introduction of diverse stressors into coral-reef environments. Furthermore, these approaches will allow an investigation into the potential for adaptation of corals through changes in genetically determined levels of gene expression (Oleksiak *et al*. 2002; Roberge *et al*. 2006) or by discovery of genes that are under selection (Le Quéré *et al*. 2006), and hence will improve our understanding of their resilience. While it is tempting to rush the development of these tools, their utility will be contingent upon a detailed understanding of what a given molecular response means in terms of a disturbance; knowledge that can only be achieved by examining the target molecules in corals exposed to rigorously controlled environmental disturbance regimes in the laboratory. Ultimately, this activity will allow for the development

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of a tool consisting of a suite of proteins and genes that collectively display well-characterized behaviour with respect to a diversity of stressors. This tool will allow for the generation of accurate and robust data that identify the stressor(s) and provide a measure of exposure in corals; data that will serve as scientific rationale for implementing reef management strategies and as context for legal actions aimed at halting damaging coastal development practices.

A critical assessment of the genetic tools used to identify endosymbiotic dinoflagellates in the genus *Symbiodinium*

The obligate endosymbiotic relationships between dinoflagellate algae of the genus *Symbiodinium* and scleractinian corals drive the biological success and structural integrity of modern coral reefs. These algal endosymbionts (also referred to as zooxanthellae) live inside the host's endodermal cells, where they occur at extremely high densities of > 1.10^6 cells per cm^2 of coral-surface area, are haploid and reproduce asexually (Santos & Coffroth 2003). Population-genetic data (Baillie *et al*. 2000a,b; LaJeunesse 2001; Santos *et al*. 2003a) suggest, however, that occasional sexual reproduction occurs (most likely in the free-living life phase). The zooxanthellae meet up to 95% of the energy requirement of the coral hosts and enhance their rates of calcification. *Symbiodinium* is a genetically diverse group of dinoflagellates, comprising eight phylogenetically distinct lineages, distinguished using small subunit rDNA sequences and designated clades A–H, respectively (reviewed in Baker 2003 and Coffroth & Santos 2005). The 'type' of symbiont that a coral contains may have profound ramifications for the fitness and success of a given coral host. For example, certain zooxanthellae enhance growth rates in juvenile corals (Little *et al*. 2004), while others increase the thermal tolerance of the holobiont (the coral- and symbiont unit) (Baker *et al*. 2004; Fabricius *et al*. 2004; Rowan 2004; Berkelmans & van Oppen 2006). In order to understand and predict the response of reef corals to a changing environment, it is therefore pertinent to characterize the diversity of symbiont types harboured by reef corals, and to evaluate if and how these vary over space and time. As *Symbiodinium* species have simple morphologies with few diagnostic characters, molecular methods are generally used to identify these taxa.

Over the last 15 years, a large body of research has detailed the genetic diversity of zooxanthellae found in endosymbioses with corals and other hosts (e.g. molluscs, anemones, sponges and foraminiferans), and has described the taxonomic, geographical and ecological distribution of this diversity across the globe (reviewed in Baker 2003; Coffroth & Santos 2005). The majority of these data have been generated using PCR amplifications of DNA markers located within the nuclear ribosomal array, and by analysing the amplified products using a suite of electrophoretic techniques. These markers and methods are now considered almost fool-proof and are being applied to the fields of molecular ecology and physiology without critical evaluation. In this context, we have identified a number of problems with the genetic markers and analytical methodologies being employed, and with the interpretation of the resulting sequence data that is being generated. As such, we now feel there is an urgent need to re-evaluate the strengths and limitations of these tools.

Molecular markers of the present

Rowan and co-workers (Rowan & Powers 1991a,b, 1992; Rowan 1991) were the first to assess genetic diversity of zooxanthellae using DNA-sequence analysis and focused on the small subunit (SSU) of nrDNA. Initially, three main types or clades were distinguished (A–C; Rowan & Powers 1991a, 1992), but five additional clades have since been identified based on nrDNA (D–H; Rowan & Knowlton 1995; Carlos *et al*. 1999, 2000; LaJeunesse & Trench 2000; LaJeunesse 2001; Pochon *et al*. 2001, 2004). The existence of, and relationships among, six of these clades has been confirmed by chloroplast (cp23S rDNA, Santos *et al*. 2002) and mitochondrial (*cox*1, Takabayashi *et al*. 2004) DNA. Compared to SSU/long subunit (LSU) nrDNA, levels of variation and resolution are higher in cp23S, but lower in *cox*1. Taxonomic resolution in the nrDNA internal transcribed spacer regions 1 and 2 is higher compared to the nrDNA gene regions (Hunter *et al*. 1997; Baillie *et al*. 2000a; LaJeunesse & Trench 2000; van Oppen *et al*. 2001b) and has been further increased by examining allelic variation at microsatellite repeats (Santos *et al*. 2001, 2003a; Magalon *et al*. 2006) and flanking regions (Santos *et al*. 2004). Sequence analysis of flanking regions from two microsatellite loci revealed five taxa within a single clade B lineage defined by both cp23S and ITS2 (Santos *et al*. 2004). Other genetic markers and methods that have been applied to studies of *Symbiodinium* diversity are allozymes (Schoenberg & Trench 1980; Baillie *et al*. 1998), random amplified polymorphic DNA (RAPD) (Baillie *et al*. 2000b), DNA fingerprinting (Goulet & Coffroth 2003) and an intergenic region of the chloroplast DNA (Moore *et al*. 2003). DNA fingerprinting and RAPD methods require that the host and symbiont tissues are separated before DNA analyses, or that host loci are 'subtracted' from the final results by comparing fingerprints from purified symbiont tissues with those from a mix of host and symbiont tissues. An additional concern with RAPDs is the 'noisiness' of this technique (Williams *et al*. 1993; van Oppen *et al*. 1996).

DNA fingerprinting, RAPDs and microsatellites usually provide resolution below the species level and will often

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distinguish individual genets. It is therefore important to realize that multilocus genotypes based on these markers may not equate to physiologically distinct strains or species, but that one strain/species generally comprises many of such genotypes. The plastid genomes of dinoflagellate algae are unique in having a reduced number of genes, most of which are found on unigenic minicircles of 2–3 kb (Zhang *et al*. 1999). The intergenic chloroplast region on the psbA minicircle of clade C zooxanthellae exhibits extremely high levels of sequence variation (Moore *et al*. 2003) and may provide a high-resolution marker to distinguish between *Symbiodinium* types, especially if *Symbiodinium* 'universal' PCR primers can be developed that amplify members of all clades. A potential pitfall, however, may be that recombination between chloroplast DNA (cpDNA) minicircles is believed to occur in dinoflagellates (Zhang *et al*. 2001). Further examination of patterns of sequence variation at this locus is therefore necessary to fully assess the usefulness of this marker.

ITS diversity: Separating the wheat from the chaff

In eukaryotes, the nrDNA cistron is organized in tandem repeats at one or more loci (Gerbi 1985). Ribosomal DNA copies within a genome evolve in concert (Arnheim *et al*. 1980), usually resulting in rapid sequence homogenization within individuals and populations through gene conversion and unequal crossing over (Dover 1982). Intragenomic variation at rDNA loci, however, is not uncommon (Carranza *et al*. 1996), especially for the relatively fast evolving ITS regions (Hugall *et al*. 1999; van Herwerden *et al*. 1999; Harris & Crandall 2000; Márquez *et al*. 2003; Wörheide *et al*. 2004) and also exists in *Symbiodinium* (Baillie *et al*. 2000a; LaJeunesse 2002; LaJeunesse *et al*. 2003; Santos *et al*. 2003b). An extreme case of this is the presence of representatives of distinct rDNA clades within a single zooxanthella genome. Van Oppen *et al*. (unpublished) found that most ITS1 types within each *Symbiodinium* cell differed by 2–8 substitutions and a few small indels, but that some ITS1 types from the same cell (i.e. the same genome) isolated from the coral hosts *Acropora millepora* and *Acropora tenuis* were unalignable and classified as either clade D or C; and those isolated from the coral *Stylophora pistillata* were classified as clade A, C or D. In an independent study by Gates and co-workers (unpublished), ITS sequences representative of *Symbiodinium* clades B, C and E were observed within single symbiont cells isolated from the anemone host *Aiptasia pulchella*. The most recent common ancestor of all *Symbiodinium* clades is estimated to have existed ~50–65 Ma (Tchernov *et al*. 2004; Pochon *et al*. 2006), and the coexistence of such ancient sequence types within a single genome is difficult to explain by shared ancestral polymorphisms. A simpler hypothesis for the origin of divergent rDNA types within a single genome is that lateral gene transfer occurred between representatives of distinct *Symbiodinium* clades, possibly while these were in close proximity inside the same host.

Because rDNA copies have not been completely homogenized in *Symbiodinium*, the use of rDNA ITS sequences in genetic identification and phylogenies of *Symbiodinium* can result in comparisons of paralogues rather than orthologues. Direct sequencing of ITS PCR products often (but not always) results in good quality sequences, and only a single or a small number of SSCP (single strand conformation polymorphism) electromorphs is typically visible on SSCP gels (except for clade B from Caribbean octocorals; van Oppen *et al*. 2005), indicating that one ITS type usually dominates in copy number in the *Symbiodinium* genome. However, sequence analysis of cloned ITS PCR products tends to reveal many distinct sequence types (van Oppen *et al*. 2005), most of which are intragenomic variants that occur at relatively low copy number. These results indicate that a comparison of sequences derived from cloned PCR products can result in erroneous interpretations of the data, especially if small numbers of clones are sequenced.

The problems with denaturing gradient gel electrophoresis

Denaturing gradient gel electrophoresis (DGGE) is extremely efficient at separating mixtures of very closely related PCR-amplified DNA sequences. The method exploits the fact that DNA molecules that vary by only a few nucleotides have different melting temperatures. When separated by electrophoresis through a gradient of an increasing chemical denaturant such as formamide or urea, the mobility of the DNA sequence is retarded at the concentration at which the DNA strands dissociate. Because of the branched structure of the dissociated DNA strand, it becomes entangled in the gel matrix and stops migrating in the gel. The complete dissociation of the strand is prevented by the presence of a high-melting domain in each sequence that does not dissociate in the chemical gradient. This domain is artificially created by initially amplifying the sequences using a PCR primer with a 5′ tail that consists of approximately 40 bp of GC repeats. To characterize the sequences that are resolved at each position on a DGGE gel, the individual bands are cut, reamplified with primers that do not contain the GC clamp attached, and then sequenced. PCR products that migrate to this position in subsequent runs are assigned an identity based on the sequence type obtained from the original gel. Thus, the technique is iterative in the hands of individual researchers, allows for rapid screening of multiple samples, and it promotes the identification of novel sequences types based on new band positions on a gel, which can then be validated by sequencing.

DGGE has been used extensively in coral biology to separate mixtures of symbiont ITS2 sequences. Indeed, data

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generated using this methodology ground much of our understanding of host–symbiont specificity and the patterns and distribution of symbiont diversity across the globe (LaJeunesse 2001, 2002; LaJeunesse *et al*. 2004; Thornhill *et al*. 2006). The technique was recently subject to validation using characterized clones of ITS2 sequence obtained from multiple colonies of two coral hosts, *Porites lobata* and *Porites lutea* (Apprill & Gates, unpublished). The migration of the PCR-amplified, cloned ITS2 sequences was compared to the migration patterns of PCR-amplified ITS2 sequences from the genomic DNAs that were used to generate the clone libraries. As expected, sequences that differed by a single base pair migrated to visibly different locations on the gel. However, clone sequences differing by 14 bp migrated to almost identical positions on the gel, a position that was also the dominant band in genomic profiles. This band position has been characterized for a genomic profile by sequencing as ITS2 type C15 (LaJeunesse *et al*. 2004), and the prominence of this band in multiple genomic profiles has driven the discussion that *P. lobata* and *P. lutea* harbour a single symbiont. Data by Apprill & Gates (unpublished) do not, however, support this conclusion and indicate there is 'stacking' of sequences at this position on the DGGE gels, and that novel and potentially divergent ITS2 sequence types have been overlooked.

The inability of the currently used DGGE methodology to resolve migration difference for sequences that differ by 14 bp or more is well illustrated in the data presented in Fig. 1 of a study by LaJeunesse (2002). ITS2 types B1 and E1 belong to different clades and contain numerous changes, yet their migration positions on the gel are almost identical. Likewise, the ITS2 types C1 and F2 that belong to two less divergent clades also migrate to almost identical positions on the gel in this figure. From a methodological perspective, the inability to separate sequences that exhibit greater divergence can be addressed by modifying the chemical gradient of the gel. However, a standardized DGGE gradient of 45–80% formamide gels (8% polyacrylamide denaturing gradient gels with 100% consisting of 7 M urea and 40% deionized formamide) is consistently used in coral biology, and no such modifications are reported in the coral literature (e.g. LaJeunesse 2002; LaJeunesse *et al*. 2003, 2004; Thornhill *et al*. 2006).

Molecular markers of the future

For the identification of zooxanthellae, in particular for taxa below the clade level, we recommend that a suite of single-copy nuclear and cytoplasmic (either cpDNA or mtDNA) markers are developed and simultaneously assessed. The number and choice of loci will depend on the research questions addressed and the levels of polymorphism found in the system under study. We acknowledge that it may be a challenge to find truly single-copy markers in *Symbiodinium*, given that several DNA loci in *Symbiodinium* are known to have undergone duplications (Norris & Miller 1994; Weis *et al*. 2002). Nevertheless, results from microsatellite flanking regions (Santos *et al*. 2004) are promising. Similarly, variation at anonymous nuclear loci (Karl & Avise 1993) can be relatively easily assessed by construction of a partial genomic library or by targeting (otherwise useless) non-repeat-containing clones from a microsatellite library. In addition, variation at functional genes (e.g. identified through EST projects) or their introns may be targeted.

An important, yet unsolved, issue is what constitutes a species in *Symbiodinium*, and, perhaps even more importantly, which markers are able to delineate entities with distinct physiologies. Gene-expression studies using microarray approaches, in which thousands of genes are targeted at once, can be used to identify genes involved in certain physiological traits. Physiological differences between *Symbiodinium* strains may not only be caused by differential expression of such genes, but also by the presence of distinct alleles. Such genes may therefore represent good candidate markers for physiological identity.

Concluding remarks

The role of genetics in conservation biology, and in ecology in general, has greatly increased over the past two decades, and the available data in this field for reef-building corals are growing. We hope that the value of genetics and molecular biology is evident from the work discussed here, which, combined with physiological and ecological data, can provide a multifaceted perspective of the resilience of reef-building corals. As such, these data represent valuable components of coral-reef-conservation initiatives. Phylogenetics, phylogeography and population-genetics analyses are useful as indicators of a population's natural history and prognosis for the future. Further, the characterization of stress responses at the molecular level may lead to the development of diagnostic assays for the early detection of stress responses in corals and the rapid identification of the exact stressor(s) responsible for degradation of certain coral-reef ecosystems.

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Madeleine van Oppen's research focuses on coral bleaching and the coral-algal symbiosis, the potential for adaptation and acclimatization of reef corals to increasing seawater temperatures, connectivity of coral reefs, and evolutionary genetics of reef corals. Ruth Gates' main research interest lies in the biological mechanisms and traits that drive the ability of marine organisms to respond to changes in their environment, including the mechanisms that underlie the flexibility of coral-dinoflagellate symbioses and their sensitivity to the environment, and the evolution of animal sensory systems.

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