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Review article.

Why are there so few genetic markers available for coral population analyses?

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Abstract
Coral reefs are in serious decline, and research in support of reef management objectives is urgently needed. Reef connectivity analyses have been highlighted as one of the major future research avenues necessary for implementing effective management initiatives for coral reefs. Despite the number of new molecular genetic tools and the wealth of information that is now available for population-level processes in many marine disciplines, scleractinian coral population genetic information remains surprisingly limited. Here we examine the technical problems and approaches used, address the reasons contributing to this delay in understanding, and discuss the future of coral population marker development. Considerable resources are needed to target the immediate development of an array of relevant genetic markers coupled with the rapid production of management focused data in order to help conserve our globally threatened coral reef resources.

Keywords: Population genetics, molecular methods, connectivity, symbiosis

1. Introduction

The health of coral reef ecosystems continues to be challenged by a suite of anthropogenically derived environmental stressors that are predicted to increase in magnitude and frequency over the next several decades (Hoegh-Guldberg, 1999). While we have considerable insight into the types of disturbances that are deleterious to coral health, it is clear that corals, and the reefs systems they support, show enormous variation in their response to any given stressor and in their ability to recover from the disturbance. Recovery from disturbance appears contingent upon the availability of genetically diverse (physiologically flexible) new recruits, which is driven by the relative connectivity of reef systems within any geographic region. Thus, a detailed understanding of the genetic diversity of individual coral populations within a given geographic region is a critical part of the information base that will allow us to manage coral reef resources in the face of future increasing environmental disturbances. Not surprisingly then, connectivity analyses have been highlighted as one of the major future research avenues for coral reefs (Hughes et al., 2003).

Population genetics is the study of variation in genes among a group of individuals, and offers a useful advance in understanding the relationship between the dispersive ability of organisms and the genetic differentiation of populations. A measure of genetic relatedness also represents a proxy to the extent of recruitment that is occurring between habitats and the connectivity between different areas within an ecosystem; information that is critical to the development of ecosystem management plans. Despite new molecular genetic tools and the plethora of information that is now available for population-level processes in many marine disciplines, our knowledge of coral population genetics is surprisingly poor. Here we examine the reasons behind this delay in understanding and explore whether the data that are currently available are sufficient to focus management efforts aimed at conserving our globally threatened coral reef resources.

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2. Coral Specific Technical Problems

Despite the rapid expansion of molecular biology, DNA studies on corals have trailed other disciplines, probably due to technical difficulties specific to working with the scleractinians. Prior to the Polymerase Chain Reaction (PCR), early studies were hampered by the difficulty in preparing high molecular weight DNA necessary for DNA hybridization analysis, as stripping coral tissue off the skeleton rendered it unsuitable for digestion with restriction enzymes (McMillan et al., 1988). More recently there have been problems with specificity associated with the contamination of the coral genomic DNA with symbiotic dinoflagellates genomic DNA. Thus, pursuing work with scleractinian DNAs involves using isolated symbiont DNA as back controls in all experiments and adds a level of complexity and cost to an already expensive and time consuming endeavor. This can be overcome by the use of symbiont-free coral tissue for genomic DNA extractions, however, obtaining gametes from corals is not straightforward as most spawning corals release their gametes only once a year and many brooders incorporate symbionts into their gametes during development in the mesenteries, which makes it extremely difficult to obtain symbiont free material. A new protocol involving centrifugation and flow cytometry has recently been developed to separate adult coral and symbiont tissues prior to extracting the coral genomic DNA (T. Lewis, pers. comm.), but it remains to be demonstrated whether all traces of the symbionts have been removed.

The development of 'coral-specific' PCR primers is the ideal way to circumvent problems associated with mixed DNAs, however, this approach has not been broadly applied to corals because the experimental design requires prior knowledge of the genetic sequence of the target molecule. Prior to 1999 there was very little sequence information available for corals, but over the last five years a diverse array of coral-specific sequences have been reported, due largely to phylogenetic studies and more recently as a result of high throughput sequencing endeavors. Given the amount of coral sequence data now publicly available, it is somewhat surprising that the application of 'coral-specific' DNA-based approaches to coral population genetics still remains largely unexplored.

3. Molecular Approaches and Current State of Knowledge

Prior to the development of PCR, coral population genetics, like most other systems, were explored using allozyme electrophoresis (e.g. Ayre and Dufty, 1994; Ayre et al., 1997; Ayre and Hughes, 2000; Benzie et al., 1995; Hellberg, 1994; Stoddart, 1984; Stobbart and Benzie, 1994). Although this technique has limitations, such as the availability of suitable material (freezing in liquid nitrogen is difficult in most locations), lack of suitable resolution, and a limited number of testable loci, allozyme analysis represents one of the most tractable ways of producing population genetic analyses in scleractinians that is applicable to reef management (Ridgway, 2005). To date, allozymes have shown that most corals show high levels of genetic subdivision, that high latitude reefs are genetically depauperate, and that levels of gene flow among corals separated by a few hundred kilometers of open water are generally low (Ayre and Hughes, 2004). Thus, the currently available allozyme data suggests that recruitment by corals on ecological time frames may well be local. Despite their proven utility, the combination of overcoming difficult sampling requirements and the lure of obtaining highly informative genetic markers for tracking individuals and/or their genes under field conditions has resulted in a number of coral population biologists switching from allozymes to DNA-based techniques.

While DNA markers provide a means for powerful analysis of individual genotypes, the challenge appears to be in finding appropriate methods that reliably reveal adequate genetic variation for a particular question with the minimum amount of effort and expense. Thus, the process of choosing a molecular genetic marker is the most critical step in any molecular analysis (Avise, 1994). Different regions of the genome experience different selective pressures depending on the genetic product and/or the tendency of the DNA to withstand changes in nucleotide sequences (Parker et al., 1998), and observed mutation rates and subsequent population divergence therefore vary accordingly. Thus, the optimal DNA segment to use for any study will depend on the degree of relationship among the individual samples, as well as on the level of selection imposed on the different regions of the genome. To date, mitochondrial and nuclear genes are the two classes of DNA markers that are the most predominant in animal population genetic studies.

In this context, the most comprehensive assessment of scleractinian genetic patterns has used the internal transcribed spacer regions of the nuclear ribosomal gene cluster (ITS). Nuclear ribosomal DNA (rDNA), spanning the ITS regions and 5.8S have been used extensively to infer phylogenetic patterns in corals (e.g. Hunter et al., 1997; Medina et al., 1999; Diekmann et al., 2001; Fukami et al., 2004), showing that coral rDNA phylogenies are typically complex and polyphyletic among closely related congeners. In terms of coral population level analyses, very few studies have been undertaken (e.g. Rodriguez-Lanetty and Hoegh-Guldberg, 2002; Ridgway, 2002; Takabayashi et al., 2003), and the general connectivity patterns observed based on the ITS data appear congruent with those previously suggested by allozyme studies of the same or similar species. According to Quijada et al. (1997), the strength of variable repetitive markers such as ITS regions lie in the fact that they are additively combined in hybrids, and whilst ITS has seemed to be the coral DNA

marker of choice, Vollmer and Palumbi (2004) have recently concluded that multiple lines of evidence demonstrate that ITS perform poorly as species- and population-level markers in scleractinian corals. The rationales for their claims are based on: 1) high intraindividual rDNA variation can overlap with the within- and between-species rDNA variation; 2) shared rDNA variation cannot be attributed to either ancestral variation or introgression; 3) discernible introgression signatures cannot be determined because the shared rDNA variation is ancient and predates the species divergence (Vollmer and Palumbi, 2004). These conclusions coupled with the multi-copy nature of the ITS region in corals, requires other markers to be assessed as alternatives to ITS.

In most animal groups, the mitochondrial (mt) genome has provided high-resolution genetic markers that are appropriate for population level studies. In scleractinians, mitochondrial genes such as 16S (Romano and Palumbi, 1996), cytochrome b (van Oppen et al., 1999) and cytochrome c oxidase subunit I (COI; Medina et al., 1999) have been used to explore phylogenetic relationships among coral groups. Despite the increased utilization of mitochondrial markers in studies of coral and cnidarian evolution, very few studies have focused on intraspecific mitochondrial nucleotide variation. This is not due to a lack of interest in this area of research, but rather a lack of variation in these genetic markers (Shearer et al., 2002). Mitochondrial nucleotide sequences appear invariant among conspecifics, with even potentially isolated populations showing no variability (Shearer et al., 2002). For corals, two studies have assessed intraspecific variability using the COI gene. While COI reveals population genetic structure in a number of marine invertebrates, Ridgway (2002) found no COI sequence difference among populations of *Pocillopora verrucosa* from southeast Africa, and no variation within this gene was found among 67 individuals from 18 populations of *Balanophyllia elegans* (reported in Shearer et al., 2002). These findings suggest that either the populations across the broad geographic spread of these studies are extremely closely related, which is highly unlikely, or that the scleractinian mitochondrial genome has evolved at an unexpectedly slow rate relative to other organisms. The reasons behind this apparent slow rate of mtDNA evolution in corals is extensively reviewed by Shearer et al. (2002), but include a constraint on the ability of the mitochondrial genome to accumulate mutations or from diversity within the mitochondrial genome having been severely reduced in the recent history of these species. However, Fukami et al. (2004) have reported variability in *Montastraea* in the Caribbean using non-coding regions of the mtDNA. Despite this, mitochondrial markers are generally not appropriate for population-level analyses in corals, or anthozoans in general.

Until very recently, the dearth of relevant coral specific sequence information, and the lack of resolution attained with ITS and mtDNA put random amplification techniques as the next logical avenue to explore with respect to coral population genetics. Random amplification protocols have been used extensively in plant systems because they sidestep the primer design, they require only small amounts of DNA, they are cost effective, a large number of potential markers can be generated in a single experiment, and DNA fragment patterns can be generated without prior knowledge of organism specific gene sequences. A number of these fingerprinting methods include random amplified polymorphic DNA (RAPD), arbitrarily primed PCR (AP-PCR), DNA amplification fingerprinting (DAF), intersimple sequence repeats (ISSR), and amplified fragment length polymorphism (AFLP).

Using AFLP, Lopez and Knowlton (1997) and Lopez et al. (1999) attempted to resolve the members of the *Montastrea annularis* species complex, and tested the potentially confounding problem of contaminating symbiont DNA by comparing AFLP patterns obtained using DNA from sperm, from somatic tissues containing symbionts, and from isolated symbionts. They demonstrated that their technique routinely amplified coral rather than symbiont DNA and concluded that somatic tissue might be a viable starting material for these types of studies. Likewise, Shearer et al. (2005) compared the ISSR banding patterns obtained for *Porites astreoides* and their symbionts using five primers and showed that the banding patterns of the symbiont were very different from those of the coral host. In contrast, a comparison of banding profiles amplified from DNAs extracted from *Pocillopora verrucosa* and isolated symbionts using four ISSR and 15 DAF primers, revealed that the symbiont DNA yielded more bands than the host DNA and that the banding profiles were very similar (Ridgway, unpublished data). Furthermore, sequencing of representative shared bands between the symbiont and host banding profiles showed that the shared bands were always symbiont in origin.

Whilst successful amplifications have been reported for coral DNAs using random methodologies, there are technical constraints, which make their application unreliable. Lopez et al. (1999) and Ridgway (unpublished data) both report problems with repeatability in the patterns of amplified products (a problem not exclusive to corals – see Vos et al., 1995), suggesting that either the DNA sequences contain repeats that results in secondary structure and the concomitant stochastic loss of amplified products, or that the DNA extractions contain contaminating substances that interfere with the DNA polymerases and the efficiency of the PCR reactions. Given the variable DNA concentrations that result from coral DNA extractions, the inconsistency and apparent lack of reproducibility of random amplifications, and Shearer et al.'s (2005) caution about generating molecular markers from zooxanthellate cnidarians, the use of random amplification techniques may have limited use in scleractinian population genetic studies.

Another approach that is informative in many other marine and terrestrial systems is the use of microsatellites,

because they can be used to address questions concerning degree of relatedness of individuals or groups, and using F-statistics and genetic distances they can resolve the genetic structure of subpopulations and populations and hence offer estimates of gene flow. Microsatellites were considered to be rare in scleractinian corals (Marquez et al., 2003), however, since Maier et al.'s (2001) initial note on the isolation of five polymorphic microsatellite markers (3 to 9 alleles per locus) in *Seriatopora hystrix*, several recent studies have reported microsatellites in a number of coral genera. Miller and Howard (2004) isolated five microsatellite markers (4 to 11 alleles per locus) from *Platygyra daedalea*, and five microsatellite markers (2 to 7 alleles per locus) from *Goniastrea favulus*. Magalon et al. (2004) characterized four microsatellite loci (5 to 15 alleles per locus) from *Pocillopora verrucosa* and *P. meandrina*. In addition, Severance et al. (2004) characterized seven polymorphic loci (5 to 31 alleles per locus) from *Montastraea annularis*, and five and three microsatellite markers have been isolated from *Montastraea cavernosa* (9 to 13 alleles per locus) and *Porites atreoides* (3 to 9 alleles per locus) respectively (Shearer et al., 2005).

Despite the recent flourish of microsatellite isolations, these studies are almost all short notes and it remains to be demonstrated if the polymorphic loci are due to the absence of null alleles that are valid for addressing large-scale population genetic questions or represent allelic drop-out associated with DNA quality. However, recent population genetic assessments of *Acropora palmata* (Baum et al., 2005), and *Pocillopora meandrina* (Magalon et al., 2005) provide strong support for the use of microsatellites as mainstream coral population markers, and it will be interesting to see follow up studies from other species and locations.

However, unlike mtDNA and ITS, microsatellite markers are expensive to develop and are extremely taxonomically specific. They may be useful between species in the same genus (Magalon et al., 2004; Severance et al., 2004), but they are not transferable outside of the genus boundary, even if the taxa are closely related such as *S. hystrix* and *P. damicornis* (Maier et al., 2001). Nevertheless, despite the complexity in developing and troubleshooting microsatellites, the recent demonstration of their usefulness in coral population studies definitely places them as potential mainstream tools for addressing population genetic questions in scleractinians.

4. Why is Progress in Corals so Slow?

While the principle of applying DNA-based techniques to the study of corals may seem relatively straightforward, the scleractinian coral genome has provided several surprises that remain poorly understood. As mentioned, the mitochondrial genome appears to evolve at an unusually slow rate, and individual genes show different evolutionary patterns across coral taxa. For example, ITS sequences exhibit modest to high variability between species belonging to both the genera *Acropora* and *Porites*, but vary surprisingly little between members of the *Montastrea annularis* species complex (Lopez and Knowlton, 1997; Medina et al., 1999; Odorico and Miller, 1997).

Besides these obvious irregularities in the coral genome, perhaps the nature of the symbiosis has challenged coral population genetics the most. Scleractinian coral symbioses are the intermingled sum of two phylogenetically distinct eukaryote genomes (i.e. cnidarians and dinoflagellates); an association that is generally obligate for the coral host. The symbiont cells are housed within the endodermal cells of the coral host, which is in contrast to other symbiotic systems (e.g. legume and squid symbioses), which combine eukaryote and prokaryotic genomes where the symbionts are housed within morphologically specialized structures (Edmunds and Gates, 2003). Moreover, prokaryote genomes are easier to culture and modify than eukaryote genomes, and therefore are traditionally easier to work with. Thus, the added functional and phylogenetic complexity of coral-dinoflagellate symbioses has perhaps led to the delayed progression of coral genetic studies. Nonetheless, given the advance in molecular techniques and the dramatic increase in the amount of sequence information available over the past decade, it is strange that our understanding of population genetics remains so rudimentary.

The application of molecular techniques to coral biology has been biased towards the symbionts (Edmunds and Gates, 2003). Molecular investigations into symbiont taxonomy started in the early 1990s, and have progressed rapidly to resolve the diversity of dinoflagellate types found in coral hosts (Baker, 2003 and references therein), and recently to quantify, classify, and study the global distribution of *Symbiodinium* (e.g. LaJeunesse et al., 2004). Given how rapidly this field has progressed, it is all the more surprising how little we know regarding population level relationships in corals themselves. The field of coral biology has historically attracted coral physiologists and ecologists, whose organism and ecosystem focus have possibly delayed the application of molecular techniques to these organisms (Edmunds and Gates, 2003), but this does not explain the disparity in our understanding of coral and symbiont genetics. In our opinion, this imbalance is likely attributable to the symbiont-centric attitude that has developed over the last decade as a result of the pivotal role that the symbionts are reported to play in coral bleaching.

Coral bleaching is caused by a breakdown in the symbiosis in response to environmental disturbance and is tightly linked with global declines in coral reef health. Bleaching is often patchy, and this pattern is potentially explained by the presence of different dinoflagellate types that possess physiological tolerances that are better or less well optimized to the conditions that cause bleaching (Hoegh-Guldberg, 1999). In order to evaluate the validity of

this idea, many coral biologists have targeted their molecular research to explore the diversity of coral symbionts in relationship to the environmental resilience of the symbiosis and in doing so, resources and research effort have been diverted away from the coral host itself. Thus, besides the technical limitations of coral genetic research, the nuances of coral genome evolution, and the complex nature of the symbiosis, perhaps our slow progress in understanding coral population genetics using DNA methodologies also stems from a lack of research effort.

5. Coral Population Structure and the Future

The extent of interconnectedness of coral reef organisms has come under scrutiny in the past decade, with Roberts (1997) proposing a high correlation between ocean currents and larval dispersal, and Cowen et al. (2000) suggesting that high diffusion and mortality of larvae results in local retention of larvae. For scleractinian corals, genetic studies have indicated large-scale panmixis, however, subdivision occurs on the smaller within reef scales (e.g. Ayre and Hughes, 2000). The idea of large-scale panmixis in tropical reefs was also challenged by Jones et al. (1999) for the damselfish *Pomacentrus amboinensis* on the Great Barrier Reef (GBR), where as many as 15–60% of juveniles returned to their natal populations. Recent evidence from settlement studies (Miller and Mundy, 2003), allozymes (Ayre and Hughes, 2004), and the microsatellite studies of Baum et al. (2005) and Magalon et al. (2005) further challenge the long held views of panmixis. Miller and Mundy (2003) argue that the short competency periods and rapid settlement preferences of *Platygyra daedalea* and *Goniastrea favulus* suggest that dispersal in broadcast-spawning coral larvae may not be as great as previously assumed. Ayre and Hughes (2004) showed that the reefs of Lord Howe Island are genetically isolated from their GBR counterparts (700 km to the north). Also it appears that the western and eastern Caribbean populations of *Acropora palmata* share little or no recent genetic exchange (Baum et al., 2005), and *Pocillopora meandrina* populations show differentiation both at the regional (2,000 km) and local (5–10 km) scales (Magalon et al., 2005). Whether open or closed, coral reefs are in serious decline, and research in support of reef management objectives is thus urgently needed. This is further highlighted by the recent evidence challenging population openness, an assumption upon which many management decisions have been based. Thus as pointed out, a crucial area relevant to coral reef conservation is the genetic dissection of population structure and the modeling of reef connectivity.

So whether it is the nature of the coral genome, a lack of research effort or a combination of the two, coral population genetics does still require a reliable and applicable population level marker. Whilst allozymes have been shown to be an appropriate technique (Ridgway, 2005), it does suffer from the problem of the need to have fresh or liquid nitrogen frozen tissue, which makes it difficult to sample remote locations. This is a real problem for corals as the vast majority of coral reefs are in remote or less developed areas. As already mentioned, the usefulness of ITS for corals has been heavily scrutinized (Vollmer and Palumbi, 2004), and the mitochondrial genome also seems to hold no possibilities, and the recent discovery of mitochondrial pseudogenes (Bensasson et al., 2001) has also greatly weakened the use of mtDNA in population genetic studies. Given the apparent inapplicability of traditional markers, what options are available to coral reefs?

Nuclear DNA is bi-parentally inherited and contains both unique single-copy and non-unique repetitive regions (Parker et al., 1998). Single-copy regions generally code for particular gene products, whereas repetitive DNA consist of core sequences that are repeated in varying degrees. Repetitive DNA may in turn be made up of coding segments (e.g. rRNA genes), or non-coding tandemly repeated units (e.g. microsatellites). In recent years, microsatellite markers have gained in popularity, as these simple repetitive sequences are present widely throughout the eukaryote genome, have high levels of polymorphism, obey Mendelian inheritance, and follow apparently simple modes of evolution (Avise, 1994). The number of published microsatellite primers over the past five years, coupled with the recent population level studies on *Acropora palmata* and *Pocillopora meandrina* place microsatellites as very good candidates to drive the much need research on discerning population structure and reef connectivity in reef building corals, and we anticipate a flourish of similar microsatellite studies to be published in the coming years.

However, given the past difficulties in scleractinian coral population genetic research, and particularly in microsatellite development, placing all hopes on a single technique should probably be done with caution. New advances in DNA data generation have yielded an exciting new avenue which may offer an alternative to microsatellites. While the slow rate of evolution of nuclear DNA has been considered a limiting factor in its use in intraspecific studies, the rate of nuclear DNA evolution is generally no lower than mtDNA (Duret, 2001). More importantly, introns no longer appear to be considered as just non-functional 'junk' sequences (Duret, 2001), and introns, because of their general high polymorphicity and ease of primer design in the flanking coding sequences, have proven useful as population makers in other organisms (e.g. Daguin and Borsa, 1999; Congdon et al., 2000; Hassan et al., 2002), and more recently in corals (MacKenzie et al., 2004). Recent and future large scale sequencing projects (e.g. Kortschak et al., 2003) will provide a wealth of information with which to screen for potential intron regions that could serve as potential suitable markers for population genetic analyses of

scleractinian corals. Current work using two newly isolated intron regions have shown them to be applicable at revealing population structuring in *Acropora millepora* populations on the GBR (Ridgway, unpublished data), and therefore present a good test case for the use of intron markers in coral population genetics. Also, as primer design can be placed in the flanking coding sequences, the potential exists where intron markers may not be hampered by the species specific nature currently experienced by microsatellites.

6. Conclusion

Population genetic markers are valuable tools to define the appropriate geographic scale for monitoring and management, to identify the origin of individuals within populations, and to detect changes in population size and connectedness (Féral, 2002). Given that coral reefs are in serious decline, with an estimated 30% already damaged, and close to 60% predicted to be lost in the next 30 years, research in support of reef management objectives is urgently needed (Hughes et al., 2003). A crucial area relevant to this mandate is the genetic dissection of population structure and the modeling of reef connectivity. While some effort has been put into technique development over the last 5 years, we are still well behind other disciplines in understanding the genetic structure of coral populations. At present, the field of coral population genetics is at a strange impasse, where some resources have been committed to technique development but little management relevant data has been produced. However, all is not lost, as the recent generation of microsatellite markers, coupled with the potential that intron markers may yield, does provide the tools and hope that has been lacking from coral population genetics in the past. Thus, an exciting time lies ahead for coral population genetics, whereby relevant genetic markers are / or are close to being available, which will enable much needed management and academic relevant data to be generated.

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