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Difficulties in obtaining microsatellites from acroporid corals

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ABSTRACT

Microsatellites are currently the molecular marker of choice in population genetics but their recovery from acroporid corals has been largely unsuccessful. We report the results of six attempts to develop microsatellites, representing five distinct methodological approaches, undertaken within three independent labs. Conventional screening of 50,000 clones from two *Acropora formosa* genomic libraries produced five single- copy, polymorphic microsatellites, of which only one may be useful for population genetics. Other projects tried to improve on this low recovery using different microsatellite enrichment techniques, such as affinity chromatography, strand extension using biotin-labelled probes, PCR with synthetic microsatellites and isolation from RAPD fragments. However, all of them failed to yield any additional loci. Apart from technical problems involved in microsatellite recovery, the small genome of acroporid corals and the correlation between genome size and microsatellite abundance suggests that biological constraints may limit the number of microsatellite loci in these corals.

Keywords *Acropora*, Scleractinia, Molecular markers, Microsatellite abundance, Genome size.

Introduction

Microsatellites are DNA sequences consisting of short, randomly repeated motifs (1-6 bp). Because of their high variability in repeat size, driven by slipped-strand mispairing errors during DNA eplication (Eisen 1999), microsatellites have become the molecular marker of choice for population genetic studies, distinguishing closely related individuals and/or taxa (Sunnucks 2000a). Microsatellites have been used to estimate a wide range of population attributes, such as dispersal and migration, relatedness and parentage, inbreeding, effective population sizes and bottlenecks (Luikart and England 1999, and references therein).

Several studies, including some presented in this symposium, demonstrated the limitations of the current repertoire of molecular markers in scleractinian corals and the need to develop additional techniques (Romano and Palumbi 1996, van Oppen et al. 2001, Romano and Richmond 2001). Multi-locus PCR based techniques, such as RAPDs (randomly amplified polymorphic DNA) and AFLPs (amplified fragment length polymorphism), have proved informative in some occasions (Coffroth and Mulawka 1995, Coffroth and Lasker 1998, Brazeau et al. 1998, Lopez et al. 1999). However, both techniques often contend with repeatability between amplifications and sampling constraints due to the requirement of zooxanthellae free tissues (i.e. sperm) to either avoid or control for contamination from endosymbiont DNA (Lopez et al. 1999, Romano and Richmond 2001). Single copy nuclear introns and intergenic regions may be informative with screening techniques such as RFLP (restriction fragment length polymorphism) (Smith et al. 1997) or SSCP (single stranded conformation polymorphism) (Sunnucks et al. 2000), but interpretation of data can often be ambiguous, and loci must be analysed individually.

Mitochondrial DNA in corals shows extremely slow rates of evolution and thus low level of variation that is only suitable for phylogenetic studies (Pont-Kingdon et al. 1995, Romano and Palumbi 1996, van Oppen et al. 2001, Fukami et al. 2000, Snell et al. 2001). Allozymes are variable enough to differentiate between populations and estimate gene flow (e.g. Ayre and Hughes 2000), however the logistics of freezing samples in remote areas make their use impractical. Allozyme levels of variability are sometimes too low for studies of clonality (McFadden 1997) or paternity (Ayre and Willis unpublished data of sperm competition in *Acropora millepora*), requiring scoring of many loci to reliably estimate these parameters.

Until now, the only study published documenting the finding and use of microsatellites in corals is that of Lopez et al. (1999). These authors accidentally found a single microsatellite locus, Mfra-gttt1, in a genomic clone from *Montastrea franksi*. This locus demonstrated the usefulness of microsatellite markers by successfully distinguishing *Montastrea faveolata* from *M. franksi* and *M. annularis*. These three *Montastrea* species are very closely related and had been previously grouped as a single species (Knowlton et al. 1992).

Apart from fortuitous findings such as the one mentioned above, microsatellite discovery conventionally involves the screening of a genomic library with specific probes. The screening is typically followed by an enrichment strategy designed to concentrate clones containing microsatellites. Here, we report six independent attempts to obtain microsatellites in acroporid corals, using 5 different approaches, each with very limited or no success. Both the methodological obstacles and possible genomic implications of our results were discussed, with the hope of simplifying future initiatives.

Methods and Results

Traditional library Screening

For the traditional screening of genomic DNA libraries,

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Acropora formosa DNA was extracted from sperm and digested with blunt end cutting restriction enzymes. Restriction fragments of ~300 – 700 bp were fractioned and ligated into commercially prepared *Sma*I cut pUC18 vectors. These plasmids were transformed into the bacterial strain DH5α and colony lifts were screened under high stringency conditions. Despite screening more than 50,000 clones for the most abundant dinucleotide microsatellite motifs (CT and GT), only five positive clones yielded microsatellites. After lengthy optimisation, only one of these microsatellite loci can be scored reliably, proving useful for population genetic studies (J.B. MacKenzie, unpublished data).

Affinity Chromatography

To construct a genomic library of *Acropora millepora* enriched with microsatellites, we used a Sepharose-DNA activated column to select restriction fragments containing microsatellites. The DNA was extracted from sperm and a rough equivalent of 5.8 x 10^6 genomes was digested using *Sau3A*I and *Mbo*I. As recovery from the affinity column is typically low, SAUL adapters were ligated to the fragments in order to provide anchors for amplifying eluted clones (Strassman et al. 1996). Preparation of the column and elution followed Brenig and Brem (1991). Initial cloning into *BamH*I digested p-Blue script showed a preference for very small inserts and so the SAUL overhangs were filled in and the inserts cloned into pGEM-T.

The clones were manually dot blotted to the nylon membranes. Probing yielded more than a hundred positive clones (see Table 1 for motifs), despite washing with very high stringency (60º C, 0.5X SSPE). Upon sequencing sixteen random positives, 13 were composed of adapter concatemers (each pair of adapters typically being interrupted by 5 to 8 bp of random sequence), while the remaining 3 were *de novo* sequences lacking microsatellites.

Strand Extension Protocol

The strand extension protocol relies upon biotin-labelled probes capturing desirable clones on streptavidin beads from random genomic starting material (Paetkau 1999). A genomic library was constructed from the insertion of size selected fragments of *Acropora millepora* sperm (200-500 bp) into *Hinc*II digested,dephosphorylated m13mp18 vector. Before beginning the enrichment for microsatellites, the library was demonstrated representative of the *Acropora* genome via repeatable amplification of several known scnDNA loci from library material. Genome size, an optimised titre (1.75 x 10^8 clones/µL), the relative ligation efficiency of the library (83% recombinant clones) and the contribution of insert to a recombinant clone (4.6%) were used to ensure 10+ genome equivalents of insert gDNA were present in the starting material of each enrichment experiment. Presented in Table 1 are eight motifs selected for screening, as well as several modifications to the published protocol. Adjustments to the reaction conditions of the 1st and 2nd strand extensions (Klenow and Long PCR, respectively), as well as attempts to enhance the elution of clones from streptavidin beads, were always unsuccessful in generating positive clones following the enrichment stage. Using a biotinylated rDNA ITS probe as a procedural control, 13 positive clones were recovered from the enrichment, however each was composed of *de novo* sequences that failed to align with any restriction fragments of the ITS consensus predicted by the enzymes used in library construction.

PCR with synthetic microsatellites

This fourth method to be discussed uses PCR as a tool for detection and amplification of clones containing microsatellites from a genomic library. As all clones of a library share common vector sequence, the universal forward vector primer may be used in combination with a synthetic microsatellite repeat to amplify only those clones containing specific microsatellite motifs. Sequences recovered from the first round of PCR target the 5' flanking region of each microsatellite locus. New primers specific to the 5' end of the microsatellite may then be used in combination with the universal reverse primer to obtain the unknown 3' flanking region of the repeat. A total of 15 motifs, composed of di-, tri- and tetra-nucleotide repeats were screened with variable success using this technique (Table 1). Eight forward primers were designed from sequences showing evidence of microsatellite repeats. In support of the method, two of the positive clones recovered aligned to a known, duplicated microsatellite locus in *Acropora*, Acyt1. This locus, which was found inadvertently within a developmental gene intron by J. Reece-Hoyes in the D.J.Miller lab at James Cook University, Australia, was selected as a positive control for the development protocol. Re-amplification, using the newly designed 5' forward primers and the universal reverse vector primer, revealed that what were thought to be new microsatellites were actually slightly repetitive sequences converted into microsatellites due to a priming artefact in the PCR process.

Isolation of microsatellites from RAPD fragments

This attempt to isolate microsatellites from *Acropora longicyathus* was unusual in that it did not involve the use of a genomic library. Instead, arbitrary RAPD (randomly amplified polymorphic DNA) primers were used in combination with the M13-40 sequence primer to PCR amplify allelic fragments from DNA of symbiont-free sperm. The resulting RAPD profiles were transferred to nylon membranes by Southern blotting and hybridized with different fluorescently labelled microsatellite probes. Hybridising fragments produced by the RAPD/M13-40 primer combination contained known flanking sequence, allowing for straightforward re-amplification and sequencing (Carter et al. 1995). Despite the strong hybridisation signals with (CA)n and (CAC)n microsatellite probes, no fragments containing either of these motifs could be successfully reamplified from extractions of either the original gel or from the Southern blot.

Discussion

Despite the relative ubiquity of microsatellites in most eukaryotes, the challenge of identifying polymorphic microsatellite loci in acroporid corals has encompassed a history of mixed success, stemming apparently from both methodological difficulties and biological complexity.

The main technical difficulty shared among the protocols discussed here is the large number of subtle yet often critical steps required for successful recovery of microsatel-

lites. Appropriate troubleshooting of these protocols requires carefully planned controls for each step, each of which may be either omitted in the original protocols or may involve more complexity than the experimental steps themselves. Therefore potential problems and/or mistakes may not become evident until completion of the protocol. For example, we thought that the affinity chromatography method might have failed at the very beginning, upon ligation of the adapters. However, we sequenced three random clones before probing and each displayed random genomic sequence of the expected size range, although none contained microsatellites. We suggest that the column preferentially selected concatemers, although not exclusively. Similarly, in the case of the strand extension protocol, probing with rDNA ITS primers as a procedural control produced 13 clones following the enrichment, however, each was composed of *de novo* sequence lacking microsatellites. Finally, all the protocols involving hybridisation of the probes showed a high incidence of false positives, which seems to be an inherent problem when the clones (or RAPDs) contain slightly repetitive sequences that do not conform microsatellites. The use of PCR probing can help to circumvent this problem (see PCR with synthetic microsatellites and Lunt et al. 1999).

Apart from the technical problems involved in the development of microsatellites, there is now clear evidence that biological constraints can affect both the abundance and motif composition of microsatellites in the genomes of different taxa (Tóth et al. 2000). For example, the abundance of microsatellites is much lower in the genomes of birds versus mammals (Primmer et al. 1997), as well as plants versus vertebrates (Lagercrantz 1993). This pattern may also extend to species within the same genus, as demonstrated by larger amount of long dinucleotide repeats in *Drosophila subobscura* and *D.pseudopscura* when compared with other *Drosophila* species (Pascual et al. 2000, Warner and Noor 2000). Genome specific rarity of microsatellites such as demonstrated in Lepidoptera (Saccheri and Bruford 1993, Nève and Meglécz 2000) and Onycophora (Sunnucks 2000b) may explain the difficulties that many researchers have had developing microsatellites, despite their use of techniques which were both familiar and successful in other invertebrates and vertebrates. It is well documented that genome size is directly correlated with microsatellite abundance (Hancock 1996). At D.J. Miller lab the genome size of *Acropora millepora* was estimated by quantifying the amount of DNA extracted from known quantities of sperm and from the frequency of single copy genes in gDNA libraries (unpublished data). According to these estimates, the amount of DNA per cell in this coral species is one order of magnitude smaller than that in mammals. Thus, if the small genome size of acroporid corals correlates with low microsatellite abundance, rarity may explain some of the difficulties the authors have faced with microsatellite development.

Although microsatellites have proven to be useful to study molecular variability within species or between closely related species, our experience indicates that they may be particularly difficult to obtain in acroporid corals. In a recently published discussion about the challenges of finding microsatellites in certain taxa, Sunnucks (2000b) advised that hard work, imagination and resourcefulness must be applied. We add that knowledge of previous studies will strengthen future attempts to develop microsatellites, especially with regard to probe selection, and design of experimental controls.

Note: At the time of sending this manuscript to press, Maier et al. (2001) published the development of microsatellites in *Seriatopora histrix*. Also, K. Miller group at the University of Wollongong, Australia, obtained microsatellites in Faviidae. Finally, E. Severance and S. Karl discovered microsatellites for the favid genus *Montastrea*. These findings may indicate that the difficulties experienced in their study are exclusive to the genus *Acropora*.

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Table 1. Strategis used to recover microsatellites from acroprid corals. Researchers : 1 Chen; 2 Smith ; 3 Màrquez; 4 Takabayashi; 5 MacKenzie

Strategy (researcher)	Motif screened	Method	Modification of published protocol	Results
Radio labelled probes (1)	(CT), (GT)	Standard gDNA library screening		55,000 clones yielded 5 positives: multiple sets of primers for 4 of them yield non-specific amplification, only one amplifies reliably scorable genotypes.
Radio labelled probes (2)	(GT)	"		Several false positive clones but no microsatellites identified.
Affinity chromatography (3)	(TA), (AG), (ACA)	Brenig and Brem 1991; Strassman et al. 1996	1) Column was prepared with 10 µM 30mer oligos (AGTG, TAG, ACA, AG and TA) coupled to activated Sepharose; 2) restriction fragments were incubated in the column, resuspended and PCR amplified using SAUL primers; 3) clones were probed with ^{32}P labelled oligos and washed at high stringency (60 °C, 0.5X SSPE).	Screening of 600 clones yielded >130 positives. 16 were sequenced, 13 consisted of concatemers of adaptors and 3 contained genomic sequences without microsatellites.
Isolation from RAPDs (4)	(CA), (CAC)	Carter et al. 1995	See Takabayashi 2000	RAPD fragments hybridising with (CA)n and (CAC)n probes, but no successful amplification of these fragments.
Strand Extension (5)	(ACA), (ATT), (CGA), (TAG), (AGTG), (ATGG), (CAAT), (GATT)	Paetkau 1999	1) A procedural control using a biotinylated rDNA ITS primer was attempted; 2) final primer [1.0 µM] and final dNTP [10µM] were altered for the 1st strand extension (Paetkau, pers. comm.); 3) two commercially recommended methods were compared for eluting the enrichment of biotynilated, ssDNA clones from the streptavidin beads using NaOH vs heat separation (DYNAL); and 4) annealing temperatures were adjusted to suit probes.	12 genomic equivalents (~5,000,000 recombinant clones) were used as starting material for 5 enrichment trials, twice using rDNA ITS procedural control. 13 positives were recovered from one attempt at recovering rDNA clones using the strand extension, however all contained *de novo* genomic sequences.
PCR with microsatellite oligos (5)	(ACA), (ATT), (CGA), (TAG), (AGTG), (ATGG), (CAAT), (GATT), (AT), (TA), (CA), (CT), (GA), (TC), (TG)	unpublished		Each PCR reaction screened began with 100+ genome equivalents. Of 15 motifs tested, 12 prospective primers were designed for clones that appeared to contain microsatellites. One clone contained a known microsatellite to use as a positive control, while all the other repetitive sequences recovered appear to have been artefacts of PCR.