Conserv Genet (2007) 8:987–990
DOI 10.1007/s10592-006-9208-8

TECHNICAL NOTE

Development and characterization of microsatellite markers for parentage analyses of the coral reef damselfish (*Pomacentrus amboinensis*: Pomacentridae)

Microsatellite makers for coral reef damselfish

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Received: 10 August 2006 / Accepted: 28 August 2006 / Published online: 9 November 2006

Abstract Five new polymorphic microsatellite loci were developed for the coral reef damselfish *Pomacentrus amboinensis*. Twenty-four individuals from two Great Barrier Reef populations were genotyped at the five loci, with numbers of alleles per locus ranging from 6–23 and observed heterozygosity between 0.42–0.92. In addition, the cross-species testing of six primers developed for *Stegastes partitus* revealed one primer (*Sp*GATA40) that was also polymorphic for *P. amboinensis*. Due to high levels of polymorphism (≥14 alleles) in at least four of the six loci and a high proportion of tetranucleotide repeats, these microsatellite markers should be useful for parentage assignment as well as other investigations of individual relatedness.

Keywords Damselfish · Microsatellite · Pomacentridae · Coral reef · Genetic diversity

Introduction

Many coral reef fishes are under increasing pressure from commercial fishing, aquarium collecting, and habitat destruction (Jackson et al. 2001). Whilst some fish species may be resilient due to frequent spawning, replenishment by dispersive larvae and rapid growth, others are slow-growing, long-lived, bear relatively few offspring and are thus more susceptible to exploitation (Sadovy 2001). Knowledge of mating systems should be incorporated in population assessments and is important for the effective management and conservation of marine fishes (Rowe and Hutchings 2003). Genetic analyses indicate that fish reproductive behaviour is considerably more complex than previously believed. Such studies have exposed otherwise cryptic aspects of fish mating systems and suggest that individuals may contribute disproportionately to subsequent generations (DeWoody and Avise 2001; Avise et al. 2002). Understanding individual contribution to reproductive events has implications for the development of species-specific management practices.

As part of a larger investigation exploring reproduction in reef fishes, we have developed microsatellite markers which may be useful in elucidating the mating system of the yellow damselfish, *Pomacentrus amboinensis*. This popular aquarium fish is abundant on coral reefs throughout the Indo-Pacific and is often used as a model species for ecological studies (Kerrigan 1996; Ault and Johnson 1999; Emslie and Jones 2001; McCormick 2003; McCormick and Smith 2004; McCormick 2006). *Pomacentrus amboinensis* is a polygynous, protogynous hermaphrodite, characterised by high site fidelity and limited post-settlement movement (McCormick and Makey 1997). During the breeding season (October–March), males defend benthic nest territories (e.g. overturned clam shells) that reproductively mature females visit to deposit their eggs. Due to the cryptic behavior of this species it is unclear which factors drive sexual selection, whether sneak mating by intruder males occurs, how many females may contribute to each clutch and thus which

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individuals receive the opportunity to breed. The application of molecular markers to this system may uncover valuable information about the mating system of *P. amboinensis* and other reef fishes.

Methods

Five polymorphic microsatellite loci for use in parentage analysis were developed and characterized for *P. amboinensis*. A partial genomic library was constructed for *P. amboinensis* following conventional methodologies (Sambrook et al. 1989; Glenn 1996). Genomic DNA for the library was extracted from pectoral fin clips preserved in 80% ethanol using a standard Phenol–chloroform protocol (Sambrook et al. 1989) and digested with the restriction enzymes *AluI* and *HaeIII*. DNA fragments were separated using gel electrophoresis and fragments ranging from 300 to 1,000 bp in size were excised from the gel. The fragments were purified using a QIAquick Gel Extraction Kit (Qiagen) and ligated into the pZErO-1 vector following the manufacturer's protocol (Invitrogen Life Technologies). OneShot®TOP10 chemically competent *E. coli* cells (Invitrogen Life Technologies, Mount Waverly, Australia) were transformed using the pZErO-1 plasmids containing the ligated fish genomic fragments as per supplier's instructions and plated onto LB-Kanamycin agar plates.

The pZErO-1 plasmid vector contained a lethal mutation which was inactivated in the presence of inserted fish DNA, thereby ensuring growth of only those transformants containing a vector with inserted fish DNA present. Plates were incubated overnight at 37°C. Ten clones were randomly selected and PCR-screened (primary screen) to establish the percentage of false positives prior to generating the library. Reactions were conducted using a PTC-200 Peltier Thermocycler (MJ Research, Watertown, Massachusettes, USA) with the following cycling parameters: one initial denaturing cycle at 95°C for 2 min, followed by 30 cycles at 95°C for 30 s, 30 s at annealing temperature of 55°C, 1 min extension at 72°C, with a final extension step at 72°C for 10 min, and a 25°C holding temperature. Of the ten randomly chosen clones selected for the initial PCR screening, eight contained inserts indicating the pZErO-1 lethal mutation effectively eliminates false positives. Given the low level of false positive clones, we proceeded with the construction of the library using all transformants that appeared on the LB Kanamycin plates.

Discrete bacterial colonies (1,200) were picked from the agar plates and transferred onto replicate gridded Hybond N filters (Amersham International, Buckinghamshire, England) and one master plate using sterile toothpicks. The filters were placed onto LB-Kanamycin agar plates and grown with the master plate overnight at 37°C. The master plate was sealed and stored at 4°C. Filters were denatured and fixed as per Buluwela et al. (1989). The filters were then pre-hybridized (Glenn 1996). Pre-hybridization was conducted in a rotating oven at 60°C for 1 h. Each duplicate set of 1,200 clones was screened for the presence of microsatellites using a T4 Polynucleotide Kinase – [γ^{32}P]-dATP end-labeled cocktail of di-, tri-, and tetra-synthetic radioactive oligonucleotides. One set of filters was screened with mixture 1 containing the following oligonucleotides: $(CGCT)_7$, $(CTGT)_8$, $(GT)_{16}$ and $(GCT)_{10}$. The second duplicate set was screened with mixture 2 containing the oligos: $(CTT)_{10}$, $(GA)_{14}$, $(CAT)_{30}$ and $(CA)_{14}$. Filters were hybridized by adding the labeled oligonucleotides to the prehybridization buffer and incubating in a rotating oven at 60°C overnight. Filters were subsequently washed using decreasing concentrations of saline sodium citrate (SSC) (ranging from 3 × SSC to 1 × SSC) and 0.1% SDS until variation in the amount of radioactivity could be detected among clones using a Geiger counter. Multiple washes in the presence of reduced concentrations of SSC were used to reduce the incidence of false positive clones, which contain inserted fish DNA but lacked the microsatellite motifs. The washed filters were then exposed to Kodak Biomax film at –80°C overnight. Film was developed using Phenisol X-Ray Developer (Ilford) and fixed in Hypam X-Ray Rapid Fixer (Ilford) as per manufacturer's protocol. Colonies displaying strong radioactive signals (indicating the presence of microsatellites) appeared as dark streaks on the film.

Approximately 150 positive clones were identified by screening filters with the oligonucleotide mixtures. Radioactive signal strength, as it appeared on autoradiographs, varied greatly among clones and only those with the strongest signal (100 clones) were selected for sequencing, to identify *P. amboinensis* microsatellites. These clones were retrieved from the master plate and grown in 5 ml of Low Salt LB-Kanamycin liquid medium at 37°C overnight. Plasmid DNA was extracted for sequencing using a QIAprep Spin Miniprep Kit (Qiagen) following manufacturer's instructions. Plasmid DNA from all positive clones was PCR amplified to establish the presence of fish DNA inserts using the universal plasmid primers USP (5′-GTTTTCCCAGTCACGAC-3′) and RSP_1 (5′-AGCGGATAACAATTTCACACA-3′) prior to sequencing. Reactions were cycled on a Mastercycler Gradient

Machine (Eppendorf, New York, USA) using the cycling parameters mentioned earlier.

PCR products were used for sequencing. *Taq* polymerase error was not considered to be a confounding factor in primer design. Prior to sequencing, PCR products were purified using a QIAMinElute™ Gel Extraction Kit (Qiagen) as per manufacturer's protocol. Sequencing reactions were conducted using a MJ Research PTC-200 Peltier Thermocycler with the following cycling parameters: 35 cycles of 95°C for 20 s, 50°C for 15 s, 60°C for 2 min and a holding temperature of 11°C. Sequenced reactions were purified using a simple isopropanol clean-up protocol (as before). Sequencing was conducted on an Amersham Biosciences Megabace Capillary Sequencer using ET-Terminator Dye chemistry (Amersham Biosciences) and following the manufacturer's protocol (except that optimal template amounts ranged from 1–10 ng which was less than recommended by the supplier's protocol). Both forward and reverse sequences were generated for 96 clones containing fish DNA inserts. Eleven of the sequences contained microsatellite repeats with sufficient flanking regions appropriate for primer design.

Primers were manually designed in accordance with the principles put forth by Ehrlich (1989). Alternate primers were designed for loci which were considered particularly desirable for parentage analysis. The primer design software Oligo® version 4 (Rychlick 1992) was used to assess the chemical characteristics of each primer pair (e.g. T_m) and to check for the presence of secondary structures (e.g. potential for formation of primer-dimers or hairpin loops). Primers were synthesized by GeneWorks Pty Ltd (Adelaide, Australia) and forward primers labeled at the 5′ end with fluorochrome labels (6-FAM™ or HEX™ or TET™). Primers were optimized using the Taguchi method of primer optimization (Cobb and Clarkson 1994). Primer sequences, GenBank accession numbers and optimal annealing conditions are provided in Table 1. While only five primer pairs were optimized and characterized successfully in this study, all 11 clone sequences (and four alternate primer pairs) have been published in GenBank and may be useful in designing additional markers for this species (Table 1).

As part of this study, we also cross-species tested six primers (*Sp*AAC41, *Sp*AAC42, *Sp*AAC47, *Sp*GATA16, *Sp*GATA40, and *Sp*AAT40) developed for the closely related bicolor damselfish, *Stegastes partitus* (Williams et al. 2003), for their success of amplification and level of polymorphism in *P. amboinensis* (see Table 1).

DNA for genotyping was extracted from dorsal fin clips (preserved in 80% ethanol) of twenty-four

Table 1 Numbers of alleles as well as observed (H_O) and expected (H_E) heterozygosity found in *n* samples collected from two Great Barrier Reef islands are presented. All primers were 5′-end labelled with flourochrome. The *Sp*GATA40 primer set was originally designed for *Stegastes partitus* (Williams et al. 2003) and cross-amplified in *P. amboinensis*

Locus	*n*	Primers 5′ → 3′	*T*a (°C)	Repeat motif	Size (bp)	No. of alleles	H_O	H_E	GenBank Accession no.
Pamb1b	24	**F** AAGATTCCACACTGTGCCCTCTC **R** GGTGGCGAGACTTCTCAGAGTATGTAT	60	$(GT)_8$ [CT(GT)2]$_3$ $(GT)_{11}$	96–164	6	0.417	0.717	DQ841247
Pamb2b	23	**F** TCAGTTCAGTTGGTCAGTTTGGTC **R** CGTTTTCCATCTGTTCCCTGAT	60	$(GCAC)_5$ $(ACAC)_2$ $(GCAC)_3$ ACAC	188–226	14	0.870	0.820	DQ841248
Pamb3a	24	**F** TGCTCTCAGAGGATTCTACC **R** AAACCATCAGGACCCAAAGC	55	$(TGTC)_{16}$AGCT$(TGTC)_3$ACC$(TGTC)_3$	333–386	16	0.917	0.914	DQ841249
Pamb4	23	**F** ACGGTGACCCATCTGATGATCTG **R** CTGAAGCCAGAAAGTGAAGCCA	63	$(GT)_2$ $(GA)_6$ $(GT)_4$ CT $(GT)_{20}$	209–287	23	0.783	0.967	DQ841250
Pamb5	24	**F** TCAAGTAGGTGGTGATGAACTCTG **R** TTTATAAGTCAATGCACTGAAATAGC	55	$(GACA)_{22}$	154–256	21	0.708	0.946	DQ841251
*Sp*GATA40	24	**F** TTGCCTGCTGATAATTAACG **R** ATGCCTCAGAATGATGTATATTT	48	$(GATA)_6$ AATA $(GATA)_{40}$	180–230	10	0.667	0.862	AY253167

individuals from Lizard and One Tree reef populations (GBR, Australia) using a standard Phenol–chloroform protocol (Sambrook et al. 1989). PCR reactions (25 μl) contained 50 ng DNA, 0.2 μM dNTP, 2 units Taq DNA polymerase (Qiagen), 0.5 μM of each primer and 1.5 mM $MgCl_2$. Reactions were cycled using a Peltier Thermal Cycler DNA Engine TETRAD^{TM} 2 (MJ Research, Massachusettes, USA) with the following cycling parameters: one denaturing cycle at 95°C for 5 min, followed by 35 cycles of 95°C for 60 s, 30 s at the specified annealing temperature, and 72°C for 30 s, followed by a final extension at 72°C for 10 min (annealing temperatures listed in Table 1). Genotypes were screened on an Amersham Biosciences Megabace Capillary Sequencer and scored using the software Fragment Profiler© version 1.2 (Amersham Biosciences).

Results and discussion

Five of the *P. amboinensis* and one *S. partitus* (*Sp*GATA40) loci that were screened were polymorphic, with the number of alleles ranging from 6–23 (Table 1). Three of the five *P. amboinensis* loci were characterized by tetranucleotide repeats with particularly high polymorphism, characteristics which are generally associated with lower levels of null alleles and thus desirable for parentage analyses. Analysis using GENEPOP version 3.3 (Raymond and Rousset 1995) indicated that none of the six loci were out of Hardy–Weinberg equilibrium following a Bonferroni correction. Linkage analysis revealed no significant linkage among any primer pairs. Given the relatively high average number of alleles, exclusion probability models indicate this set of six loci will be useful for parentage analyses and other studies of individual relatedness in *P. amboinensis* assuming populations of appropriate sizes are screened (Bernatchez and Duchesne 2000). These makers will also be useful in population studies considering issues such as local retention, self-recruitment and dispersal. This study contributes to the small but growing collection of genetic tools available for ecological investigations of mating systems in coral reef fishes.

Acknowledgements This work was supported by a Program and RAP grant from James Cook University. We would like to thank members of the Molecular Ecology and Evolution Lab (JCU) – Dr. Brad Evans, Dr. Carolyn Smith-Keune, and Dr. Michelle Waycott for their valuable input into this project. Thanks also to Ashley Frisch and Belinda Curley who provided useful comments on the manuscript.

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