Isolation and characterization of eight polymorphic microsatellites for the spotted spiny lobster, *Panulirus guttatus*

Nathan Truelove, Donald C Behringer, Mark J Butler IV, Richard F Preziosi

Microsatellite sequences were isolated from enriched genomic libraries of the spotted spiny lobster, *Panulirus guttatus* using 454 pyrosequencing. Twenty-nine previously developed polymerase chain reaction primer pairs of *Panulirus argus* microsatellite loci were also tested for cross-species amplification in *Panulirus guttatus*. In total, eight consistently amplifying, and polymorphic loci were characterized for 57 individuals collected in the Florida Keys and Bermuda. The number of alleles per locus ranged from 8 to 20 and observed heterozygosities ranged from 0.409 to 0.958. Significant deviations from Hardy-Weinberg equilibrium were found in one locus from Florida and three loci from Bermuda. Quality control testing indicated that all loci were easy to score, highly polymorphic and showed no evidence of linkage disequilibrium. Null alleles were detected in three loci with moderate frequencies ranging from (20% to 22%). These eight microsatellites provide novel molecular markers for future conservation genetics research of *P. guttatus*. 
Isolation and characterization of eight polymorphic microsatellites for the spotted spiny lobster, *Panulirus guttatus*

Nathan K. Truelove1*, Donald Behringer Jr2, Mark Butler IV3, and Richard F. Preziosi1

1Faculty of Life Sciences, The University of Manchester, M13 9PT, UK
2University of Florida, Fisheries and Aquatic Sciences, Gainesville, Florida 32653, USA
3Old Dominion University, Department of Biological Sciences, Norfolk, Virginia 23529, USA

*Corresponding author

Introduction

The spotted spiny lobster *Panulirus guttatus* is a coral reef dwelling species that occurs from Bermuda to Suriname and throughout the Caribbean Sea (Sharp, Hunt & Lyons, 1997). *P. guttatus* is believed to have a long pelagic larval duration and occupies the same coral reef habitat throughout all of its benthic stages (Sharp et al., 1997). *P. guttatus* matures at a relatively small size compared to other species of spiny lobster (females 32 mm carapace length (CL), males 36-37 mm CL). Despite its small size fishing pressure has begun to increase due to declining *Panulirus argus* fisheries in the Caribbean (Wynne & Coté, 2007; Fanning, Mahon & McConney, 2011). Fishery regulations for *P. guttatus* are either extremely limited (e.g., Bermuda and Martinique) or non-existent, and fisheries are emerging in the British West Indies and several other Caribbean nations to satisfy the demand for luxury seafood (Acosta & Robertson, 2003; Wynne & Coté, 2007). Management is hindered by a lack of basic life history, ecology, and population information – all of which would be facilitated by the development of species-specific genetic tools.

This study aims to enable future genetic studies on *P. guttatus* by characterizing new microsatellites for the species. Whilst microsatellites have already been developed for several spiny lobster species from the genus *Panulirus* (Decapoda: Palinuridae) (Ben-Horin et al., 2009; Kennington et al., 2010; Dao, Todd & Jerry, 2013; Liu, Yang & Liu, 2013), only microsatellites
for *P. argus* (Diniz et al., 2004; 2005; Tringali, Seyoum & Schmitt, 2008) were only tested for
cross-species amplification since the success rate of amplification in more distantly related
congeners (Ptacek et al., 2001) is generally low (Ben-Horin et al., 2009). These microsatellite
primers will allow researchers to identify genetically unique subpopulations, determine levels of
genetic diversity, and measure levels of genetic connectivity among subpopulations of *P.
guttatus*.

**Methods**

The authors collected DNA samples from *P. guttatus*, completed DNA extractions, and
tested validated microsatellite loci for polymorphism. Genoscreen, France (www.genoscreen.fr)
tested *P. guttatus* DNA for quality and quantity, developed microsatellite libraries, performed
454 pyrosequencing, used bioinformatics software to identify potentially amplifiable
microsatellite loci, and validated potentially amplifiable loci. Leg muscle tissue was collected
from 24 individuals from Long Key Florida and 33 individuals from North Rock Bermuda. Total
genomic DNA was isolated with the Wizard SV-96 Genomic DNA extraction kit (Promega).
Genomic DNA from 12 individuals from Long Key Florida was used by GenoScreen for
microsatellite development. The DNA quantity was assessed using the Picogreen assay
(Invitrogen). To improve polymorphism detection the DNA from 12 individuals were pooled
equimolarly. Microsatellite libraries were developed using 1 μg of pooled DNA and 454 GS FLX
Titanium pyrosequencing of the enriched DNA (Malausa et al., 2011). Briefly, total DNA was
enriched for microsatellite loci using 8 probes (AG, AC, AAC, AAG, AGG, ACG, ACAT and
ATCT) and subsequently amplified. The PCR products were purified, quantified, and GS FLX
libraries were developed following the manufacturer’s protocols (Roche Diagnostics) and
sequenced on a GS FLX-PTP. The level of coverage used to develop microsatellite loci was 1/32.
of a plate. This technique allowed the identification of 12676 potential microsatellite primers. The bioinformatics program QDD was used (Meglécz et al., 2010) to identify sequences that were optimal for primer design and validated 737 pairs of primers. Tri-repeats and tetra-repeats were favored in order to minimize stutter bands and increase the probability of accurate allele scoring. The following selection parameters were used to design microsatellite primers: minimum melting temperature (T_m) of 60°C; optimum T_m of 71°C; maximum difference in T_m between primer pairs of 5°C; and primer length of 20-30bp. Twenty-four validated sets of P. guttatus primers and 29 sets of previously designed microsatellite primers for P. argus (Diniz et al., 2004; 2005; Tringali et al., 2008) were tested for amplification. Primer sets were discarded if they either failed to amplify or amplified three or more distinct fragments. The 13 microsatellites developed by Genoscreen and 2 microsatellites previously developed (Tringali et al., 2008) for P. argus were tested for polymorphism in P. guttatus using the forward labeled fluorescent primers 6-FAM, HEX, NED, and PET. Twelve of the thirteen microsatellites identified by Genoscreen were partitioned into 3 multiplexes consisting of 4 primer pairs. The forth multiplex consisted of a Genoscreen primer pair and the two primer pairs developed for P. argus (Tringali et al., 2008). A unique fluorescent label was attached to the forward primers of each multiplex. Annealing temperatures for all primer pairs were calculated with Multiplex Manager (Holleley & Geerts, 2009) using 200 nanomolar primer concentration and 10°C below the primer melting temperature T_m.

Each multiplex PCR was performed with a Veriti thermal cycler (Applied Biosystems). Our protocol followed the manufacturer’s recommendations (Qiagen Microsatellite Multiplex PCR Kit), however, we initially compared reaction volumes of 25 µl, 10 µl and 5 µl for each multiplex in 24 individuals. Results were identical for each reaction volume, therefore to reduce
costs the total volume of the PCR reaction was scaled down from 25 µl to 5 µl whilst keeping the concentrations of all PCR reagents the same. The PCR reaction mix consisted of 0.5 µl of the 10X primer mix (1µM primer + 1µM fluorescent primer), 2.5 µl of Type-it Multiplex PCR Master Mix (Qiagen), 1 µl of molecular grade water and 1µl of (10-20 ng/µl) genomic DNA. The PCR conditions consisted of an initial denaturation at 95 °C for 5 min, followed by 26 cycles at 95 °C for 30 s, 59 - 65 °C for 120 s (the lowest primer annealing temperature was chosen for each multiplex; Multiplex 1 = 58 °C, Multiplex 2 = 62 °C, Multiplex 3 = 60 °C, Multiplex 4 = 65 °C), and 72 °C for 30 s. This was followed by final extension at 60 °C for 30 min. To facilitate the fragment analysis, PCR products were diluted 1:1 with 5 µl MQ water. From the diluted product, 0.5 µl was mixed with 9.5 µl of a mix consisting of Hi-Di Formamide® (Applied Biosystems) and GeneScan – 500 LIZ Size Standard (37:1) in a 96 well PCR plate. Fragment analysis was performed on an ABI 3730xl automatic DNA sequencer (Applied Biosystems, USA) at the University of Manchester DNA Sequencing Facility. Microsatellite alleles were scored using the GeneMapper® v3.7 software package (Applied Biosystems). Binning of microsatellite alleles and error checking were performed using the R package MsatAllele version 1.02 (Alberto, 2009) and R statistical software v2.15.1 (Ihaka & Gentleman, 1996). The entire data set was checked for variability and departures from Hardy-Weinberg equilibrium (HWE) and the fixation index (FIS) was calculated using the software package Genodive v2.0b23 (Meirmans & Van Tienderen, 2004; Meirmans, 2012). The Benjamini Hochberg method (i.e. the false discovery rate) was used to correct for multiple comparisons of HWE (Benjamini & Hochberg, 1995). Linkage disequilibrium between loci was tested using Genepop on the Web v4.2 (Raymond & Rousset, 1995; Rousset, 2008). Markov chain parameters for Genepop were set to the following: dememorization number 10K, number of
batches 1K, and number of iterations per batch 10K. Null allele frequencies and scoring errors
generated by stutter peaks or large allele dropout was calculated with MICROCHECKER (Van
Oosterhout et al., 2004).

**Results**

Six out of 13 microsatellites developed by Genoscreen were found to be either
monomorphic or too difficult to score and were removed from the analysis. Twenty-seven out of
29 *P. argus* microsatellites failed to produce PCR products. One out of the two *P. argus*
microsatellites that did produce a PCR product was too difficult to score and was removed from
the analysis. Table 1 summarizes the characteristics of the eight primer pairs of polymorphic and
easy to score microsatellite loci developed for the spotted spiny lobster *P. guttatus*. No evidence
of linkage disequilibrium was found among any of these loci. We were unable to test for
Mendelian inheritance since crossbreeding of *P. guttatus* has yet to be achieved under laboratory
conditions.

Samples from Long Key Florida (*N* = 24) and Bermuda (*N* = 33) were genotyped using
the eight developed primers. The number of alleles ranged from 8 to 20 per locus. Significant
deviations from Hardy-Weinberg equilibrium were found in one locus from Florida and three
loci from Bermuda (Table 2). These deficiencies could be due to null alleles or the Wahlund
effect (Johnson & Black, 1984). The latter is possible considering the potential for extensive
geneflow in this species. However, null alleles are a common characteristic of the microsatellites
of many marine invertebrates, so could also be responsible for the deviations from HWE
(Dailianis et al., 2011). Indeed null alleles were detected by MICROCHECKER in the four loci
that deviated from HWE (Par-Fwc05, PG3, PG21, PG22). Null allele frequencies in these loci
ranged from 20% to 22% (Table 1). Although null alleles have been found to inflate levels of
population structure, they do not create population structure where it does not already exist (Chapuis & Estoup, 2007; Carlsson, 2008).

Discussion

Population genetics studies have yet to be conducted on this coral reef lobster species that is facing increasing fishing pressure. Even though 4 microsatellite primers show evidence of null alleles, the moderate null allele frequencies and number of alleles suggests these eight primers are useful for conducting future genetic studies of *P. guttatus*. This research can be used to help develop conservation and fishery management plans for this understudied species.

Acknowledgements

We thank Dr. Tammy Trott from the Bermuda Fisheries Department for providing samples for this study and Josh Anderson, Jason Spadero, and Mike Dixon for helping to collect samples in the Florida Keys. We are grateful to Antoine Destombes at Genoscreen for his help with this project.

References

Acosta CA, Robertson D 2003. Comparative spatial ecology of fished spiny lobsters *Panulirus argus* and an unfished congener *P. guttatus* in an isolated marine reserve at Glover's Reef atoll, Belize. *Coral Reefs* 22:1–9.

Alberto F 2009. MsatAllele_1.0: An R Package to Visualize the Binning of Microsatellite Alleles. *Journal of Heredity* 100:394–397.

Ben-Horin T, Iacchei M, Selkoe KA, Mai TT, Toonen RJ 2009. Characterization of eight polymorphic microsatellite loci for the California spiny lobster, *Panulirus interruptus* and cross-amplification in other achelate lobsters. *Conservation Genetics Resources* 1:193–197.

Benjamini Y, Hochberg Y 1995. Controlling the false discovery rate: a practical and powerful approach to multiple testing. *Journal of the Royal Statistical Society. Series B* (Methodological) 57:289–300.

Carlsson J 2008. Effects of microsatellite null alleles on assignment testing. *Journal of Heredity* 99:616–623.

Chapuis MP, Estoup A 2007. Microsatellite Null Alleles and Estimation of Population
Differentiation. *Molecular Biology and Evolution* 24:621–631.

Dailianis T, Tsigenopoulos CS, Dounas C, Voultsiadou E 2011. Genetic diversity of the imperilled bath sponge *Spongia officinalis* Linnaeus, 1759 across the Mediterranean Sea: patterns of population differentiation and implications for taxonomy and conservation. *Molecular Ecology* 20:3757–3772.

Dao HT, Todd EV, Jerry DR 2013. Characterization of polymorphic microsatellite loci for the spiny lobster *Panulirus* spp. and their utility to be applied to other *Panulirus* lobsters. *Conservation Genetics Resources* 5:43–46.

Diniz FM, Maclean N, Ogawa M, Paterson IG, Bentzen P 2005. Microsatellites in the overexploited spiny lobster, *Panulirus argus*: Isolation, characterization of loci and potential for intraspecific variability studies. *Conservation Genetics* 6:637–641.

Diniz FM, Maclean N, Paterson IG, Bentzen P 2004. Polymorphic tetranucleotide microsatellite markers in the Caribbean spiny lobster, *Panulirus argus*. *Molecular Ecology Notes* 4:327–329.

Fanning L, Mahon R, McConney P 2011. Towards marine ecosystem-based management in the wider Caribbean. In: Jentoft S, Bavinck M eds. Amsterdam University Press, 157–175.

Holleley C, Geerts P 2009. Multiplex Manager 1.0: a cross-platform computer program that plans and optimizes multiplex PCR. *Biotechniques* 46:511–517.

Ihaka R, Gentleman R 1996. R: A Language for Data Analysis and Graphics. *Journal of Computational and Graphical Statistics* 5:299–314.

Johnson MS, Black R 1984. The Wahlund effect and the geographical scale of variation in the intertidal limpet *Siphonaria* sp. *Marine Biology* 79:295–302.

Kennington WJ, Levy E, Berry O, Groth DM, Waite AM, Johnson MS, Melville-Smith R 2010. Characterization of 18 polymorphic microsatellite loci for the western rock lobster *Panulirus cygnus*. *Conservation Genetics Resources* 2:389–391.

Liu L, Yang X, Liu C 2013. Eleven novel polymorphic microsatellite loci in the ornate spiny lobster *Panulirus ornatus* (Decapoda: Palinuridae). *Journal of genetics* 92:e65–7.

Malausa T, Gilles A, Meglécz E, Blanquart H, Duthoy S, Costedoat C, Dubut V, Pech N, Castagnone-Sereno P, Délye C et al. 2011. High-throughput microsatellite isolation through 454 GS-FLX Titanium pyrosequencing of enriched DNA libraries. *Molecular Ecology Resources* 11:638–644.

Meglécz E, Costedoat C, Dubut V, Gilles A, Malausa T, Pech N, Martin J-F 2010. QDD: a user-friendly program to select microsatellite markers and design primers from large sequencing projects. *Bioinformatics* 26:403–404.

Meirmans PG 2012. AMOVA-Based Clustering of Population Genetic Data. *Journal of Heredity* 103:744–750.

Meirmans PG, Van Tienderen PH 2004. genotype and genodive: two programs for the analysis of genetic diversity of asexual organisms. *Molecular Ecology Notes* 4:792–794.

Ptacek MB, Sarver SK, Childress MJ, Herrnkind WF 2001. Molecular phylogeny of the spiny lobster genus *Panulirus* (Decapoda: Palinuridae). *Marine and Freshwater Research* 52:1037.

Raymond M, Rousset F 1995. Genepop (version 1.2): Population genetics software for exact tests and ecumenicism. *Journal of Heredity* 86:248–249.

Rousset F 2008. Genepop'007: a complete re-implementation of the Genepop software for windows and linux. *Molecular Ecology Resources* 8:103–106.

Sharp WC, Hunt JH, Lyons WG 1997. Life history of the spotted spiny lobster, *Panulirus guttatus*, an obligate reef-dweller. *Marine and Freshwater Research* 48:687–698.
Tringali MD, Seyoum S, Schmitt SL 2008. Ten di- and trinucleotide microsatellite loci in the Caribbean spiny lobster, *Panulirus argus*, for studies of regional population connectivity. *Molecular Ecology Resources* 8:650–652.

Van Oosterhout C, Hutchinson WF, Wills DPM, Shipley P 2004. MICRO-CHECKER: software for identifying and correcting genotyping errors in microsatellite data. *Molecular Ecology Notes* 4:535–538.

Wynne SP, Coté IM 2007. Effects of habitat quality and fishing on Caribbean spotted spiny lobster populations. *Journal of Applied Ecology* 44:488–494.
### Table 1

Revised Table 1

**Table 1** Characterization of eight microsatellite loci for *Panulirus guttatus* with GenBank (GenBank Accession Number), $T_A$ (annealing temperature), $Na$ (number of alleles), $Ho$ (observed heterozygosity), $He$ (expected heterozygosity), $F_{is}$ (fixation index), $P$ ($P$-value for deviation from Hardy-Weinberg equilibrium), and $F_{NA}$ (null allele frequency). Fluorescent labels on forward primers and significant values after the false discovery rate correction for multiple comparisons (Benjamini & Hochberg, 1995) are in bold.
Table 1 Characterization of eight microsatellite loci for *Panulirus guttatus* with GenBank (GenBank Accession Number), \(T_A\) (annealing temperature), Na (number of alleles), Ho (observed heterozygosity), He (expected heterozygosity), Fis (fixation index), \(P\) (P-value for deviation from Hardy-Weinberg equilibrium), and \(F_{NA}\) (null allele frequency). Fluorescent labels on forward primers and significant values after the false discovery rate correction for multiple comparisons (Benjamini & Hochberg, 1995) are in bold.

| Locus  | Primer sequence (5' to 3') | Multiplex | GenBank | Repeat Motif | Range (bp) | \(T_A\) | Florida (N = 24) | Bermuda (N =33) | \(P\) | \(F_{NA}\) | \(P\) | \(F_{NA}\) |
|--------|-----------------------------|-----------|---------|--------------|------------|-------|----------------|----------------|------|--------|------|--------|
| Pgut-3 | F: GCTGGAGAGGGAAGAATGCT-6FAM | 1         | KC800822 | (GAG)12      | 95-131     | 66.7  | 16              | 0.696          | 0.843 | 0.175  | 0.088 | ---    |
|        | R: CCCCCATTCTTTTCTTTTCTCC    |           |         |              |            |       |                 |                 |     |        |      | 11.06  |
| Pgut-6 | F: CCAATTCTTTCCATCA-6FAM     | 1         | KC800823 | (ATC)12      | 140-165    | 58.3  | 11              | 0.75           | 0.872 | 0.139  | 0.208 | ---    |
|        | R: CCTTGATTTCAATTGCTGC       |           |         |              |            | 59.4  |                 |                 |     |        |      | 1.71   |
| Pgut-9 | F: GTGTTGTTGACGTGTGCT-VIC    | 2         | KC800824 | (TGT)17      | 78-119     | 64.6  | 8               | 0.667          | 0.834 | 0.201  | 0.06  | ---    |
|        | R: GACTGAAGACGGCAGACGTA      |           |         |              |            |       |                 |                 |     |        |      | 1.93   |
| Pgut-15| F: CACCAAGTTGAAATACCTTTGCT-PET | 2      | KC800825 | (GATA)6      | 133-178    | 63.3  | 13              | 0.958          | 0.856 | 0.146  | 0.365 | ---    |
|        | R: GTCCTAGAAAGAATAGGGA       |           |         |              |            | 62.4  |                 |                 |     |        |      | 1.84   |
| Pgut-21| F: TGCCCTG6GCAAAATCTCTA-VIC  | 3         | KC800826 | (TCTA)8      | 167-224    | 60.6  | 9               | 0.875          | 0.829 | 0.055  | 0.711 | ---    |
|        | R: GCGAACTGACGTGTGCTAA       |           |         |              |            | 62.7  |                 |                 |     |        |      | 1.69   |
| Pgut-22| F: CTTGCATCCCCAGACGTTGTA-6FAM | 3         | KC800827 | (TGA)10      | 74-115     | 64.3  | 11              | 0.5            | 0.839 | 0.404  | 0.129 | ---    |
|        | R: ACGGCAAACACATACGTCTCT     |           |         |              |            | 65.7  |                 |                 |     |        |      | 0.83   |
| Pgut-23| F: AAGGAAATAGGACCTGGCAAT-NED | 3         | KC800828 | (AGAT)11     | 133-171    | 62.5  | 10              | 0.409          | 0.844 | 0.515  | <0.001| 0.22   |
|        | R: AATGGGATACCCGTGGCAAGA     |           |         |              |            | 62.9  |                 |                 |     |        |      | 0.613  |
| Par-Fw05| F: AGAGAAAGCGCTGTTCTCTC-6FAM | 4         | EF620542 | (CA)13/(CA)10| 133-179    | 65.1  | 8               | 0.583          | 0.753 | 0.226  | 0.041 | ---    |
|        | R: AAGGGCCATCCCTGGAAGTGC     |           |         |              |            | 66.7  |                 |                 |     |        |      | 0.781  |