**ONLINE RESOURCES**

# Isolation and characterization of microsatellite markers in the spiny lobster *Panulirus echinatus* Smith, 1869 (Decapoda: Palinuridae) by Illumina Miseq Sequencing

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## Introduction

Lobster fisheries is, undoubtedly, one of the most financially valuable shellfish fisheries around the world, especially for spiny and clawed lobsters. The brown spiny lobster *Panulirus echinatus* occurs on rocky reefs in the tropical western Atlantic Ocean and central Atlantic Islands (Holthuis 1991). The species is fished throughout its range, but intensively in some regions such as off Brazil, the islands of St. Helena (UK), Cape Verde and the Canary Archipelago (Spain). Although commercially important fisheries exist for the species, the activity lacks management plan to ensure that lobster populations remain at sustainable levels. Fisheries legislation and other policy tools are not established for this lobster species in any of these in countries. As biological information on *P. echinatus* is still scarce, it is of paramount importance to provide data that could be used to indicate whether an impact on the global population is existing.

Commercial fisheries in certain habitats may compromise distinct, locally adapted populations (Agardy 2000) resulting in loss of genetic diversity, which is measured by the genetic variation distributed within as well as between populations (Scribner *et al.* 2016). Thus, the knowledge of genetic variability in *P. echinatus* and its populations is essential to aid in the management of fisheries stocks (e.g. Altukhov 1981; Shaklee and Samollow 1984).

Genetic data from molecular markers can be used to measure genetic differentiation and variability among marine populations and, therefore, address questions of fishery management relevance (e.g. identification of population and conservation units).

So far, only very few papers can be found in the literature containing more than distribution records on this species (e.g. site-specific population and reproductive biology of *Panulirus echinatus*, Pinheiro *et al.* 2003; 2006; molecular identification of phyllosoma larva, Konishi *et al.* 2006; measurements of biometric relationships, Barreto *et al.* 2009), therefore, very little is known about the population diversity of *P. echinatus*.

Polymorphic simple sequence repeat (SSR) markers, also known as microsatellites, are useful molecular markers for fisheries population and conservation genetic studies due to their levels of intraspecific variability in many taxa, a consequence of the high mutation rate of these noncoding regions (Wright and Bentzen 1994). As no molecular markers have been identified in *P. echinatus* until now and the species has limited genomic information, it seems, therefore, an efficient alternative for microsatellite discovery and subsequent PCR primer

design to use genome assembled Illumina paired-end DNA sequences via Next Generation Sequencing (NGS) technology (Abdelkrim *et al.* 2009).

In this study we have isolated and characterized a set of 20 simple sequence repeat markers of tri- and tetranucleotide motifs from *P. echinatus* using next-generation sequencing.

## Materials and methods

An Illumina paired-end library was created using 1 ng of *Panulirus echinatus* genomic DNA, following the standard protocol of the Illumina Nextera XT Library Preparation kit (Illumina Inc., San Diego, CA). DNA was tagged and fragmented by the Nextera XT transposome, followed by limited-cycle PCR amplification, AMPure XP magnetic-bead purification (Agencourt Bioscience Corporation, Beverly, MA) and the Illumina Nextera XT bead-based normalization protocol. The DNA library was sequenced using a MiSeq Benchtop Sequencer (Illumina Inc., San Diego, CA). Contigs were created from the resulting paired-end sequence data (reads) using CLC Genomics Workbench 7.0.4 (Qiagen).

All these contigs were subsequently input into MSDB (Microsatellite Search Building Database) (https://github.com/lmdu/msdb; Du *et al.* 2013) for the detection of possible microsatellite loci with at least four repeats for tri- and tetranucleotide motifs and designing of primer pairs for each detected locus at their flanking regions. Long mononucleotide repeat stretches in sequences were ignored for marker development. Primer design was performed with the Primer3 (Rozen and Skaletsky 2000).

A total of 42 individuals were sampled from three different localities within the central Atlantic Islands of Saint Peter and Saint Paul Rocks (0° 55′ 2″ N, 29° 20′ 44″ W; 15 individuals), The Rocas Atoll (3° 51′ 50″ S, 33° 48′ 48″ W; 15 individuals), and Cape Verde (14° 55′ 0″ N, 23° 31′ 0″ W; 12 individuals). Genomic DNA was isolated from the pereiopod (walking legs) muscle using phenol–chloroform–isoamyl alcohol (25:24:1) extraction of the SDS/proteinase-K-digested tissue, followed by ethanol precipitation (Sambrook *et al.* 1989). DNA extracts from these samples were used to validate all designed primer pairs via PCR.

Reactions were performed in a 10-µL total volume containing approx. 20 ng of genomic DNA, with 1.5 µL of 10× buffer (Thermo Fisher Scientific Inc.), 2 to 2.5 mM $MgCl_2$ (Thermo Fisher Scientific Inc.), 10 mM dNTP mix (New England BioLabs Inc.), 0.25 mM of each primer, 2.0 µL de Q-solution (Qiagen), and 1 U of Taq DNA polymerase (Thermo Scientific

Inc.). All amplifications were run in a Veriti 96-well Thermal Cycler (Applied Biosystems) using the following PCR-cycle conditions: 95 °C for 10 minutes, 30× (1 minute at 95 °C, 1 minute at annealing temperature of 50 – 57 °C, depending on primer pair, 1 minute at 72 °C), and 7 minutes at 72 °C. The amplification products were screened by silver nitrate detection on denatured 6% polyacrylamide gels.

The genotyped data was initially analyzed using Micro-Checker 2.2.3 (van Oosterhout *et al.* 2004) to test for the presence of null alleles, large alleles dropout and scoring errors by stuttering. Observed and expected heterozygosities ($H_O$ and $H_E$), the number of alleles (*A*), and the polymorphic information content (*PIC*) were determined using CERVUS 3.0 (Kalinowski *et al.* 2007). Allelic richness ($A_R$) as a measure of the number of alleles per locus independent of the population size was calculated by *FSTAT* version 2.9.3.2 (Goudet 1995). Deviations from Hardy–Weinberg equilibrium (HWE) and tests for linkage disequilibrium were conducted using Genepop software (Raymond and Rousset 1995). The Bonferroni correction was applied when multiple pairwise tests were performed to assess the significance ($P < 0.05$).



## Results and discussion

The genomic library, previously loaded as 16 % of a MiSeq Reagent Kit v2 300 cycle sequencing run, produced 3,232,259 reads, which were assembled into 7,196 contigs. The software MSDB identified 2,959 microsatellite loci, being in the majority mononucleotide (1290; 43.60 %) and dinucleotide (1280; 43.26 %) repeats. For ease of imaging and scoring, we chose to examine only tri- and tetranucleotide loci, respectively showing 221 (7.47 %) and 107 (3.61 %) repeats. From these, 27 loci were chosen for primer designing and validation, of which 20 consistently amplified specific bands across individuals. Polymorphism was identified in 17 loci across the entire data set, the remaining three loci being monomorphic (*Pech*18, *Pech*19 and *Pech*20) (Table 1).

The analysis with the program Micro-Checker showed in a pool of sampled individuals the presence of low to moderate frequencies of null alleles in these loci, which is not surprising as this is commonly observed in a variety of marine invertebrate species (Kaukinen *et al.* 2004; Dailianis *et al.* 2011). Null frequencies below 0.2 are acceptable in most microsatellite data sets (Dakin and Avise 2004). Only in two loci (*Pech*11 and *Pech*15) this

estimate was higher than 0.2. The most probable reason for occurrence of this phenomenon being scoring errors due to stuttering or large allele drop out. No loci showed significant linkage disequilibrium between any pair of loci after corrections for multiple comparisons.

The genotyping of the entire dataset (N = 42), with an overall frequency of missing single locus genotypes of 0.021, has also revealed 91 alleles at all 17 polymorphic loci, ranging from 2 (*Pech*10) to 8 (*Pech*4), with an average of 5.4 ± 1.4 alleles per locus (Table 1). The number of alleles at tri- and tetranucleotide microsatellites in spiny lobsters has been reported to vary from 5.3 for Brazilian *P. argus* (Diniz *et al.* 2005) to 11.9 for *P. cygnus* (Kennington *et al.* 2010).

The size of alleles in the most polymorphic locus, *Pech*14, based on $A_R$, $H_E$ and *PIC*, varied from 160 to 178. For the less polymorphic locus, *Pech*10, allele size ranged from 78 to 86 bp; a tri- and tetranucleotide motif locus, respectively.

The level of polymorphism of each locus was also evaluated by the allelic richness ($A_R$), heterozygosities ($H$) and the polymorphic information content (*PIC*) (Table 2). The values of allelic richness varied from 1.8 to 6.2 (average of 3.9 ± 1.0), while *PIC* values ranged between 0.173 and 0.811. Mean *PIC* (0.537 ± 0.165) characterize this set of microsatellite loci as highly informative markers, as defined by Botstein *et al.* (1980). Overall mean observed and expected heterozygosity was estimated to be 0.418 ± 0.165 and 0.587 ± 0.173, respectively. These estimates were higher when compared to mean $H_O$ (0.265) and $H_E$ (0.387) of the scalloped spiny lobster (*P. homarus*) genotyped with microsatellite markers (Delghandi *et al.* 2015). However, *P. echinatus* heterozygosities were of lower magnitude when compared to other spiny lobsters, such as *P. argus* (Diniz *et al.* 2004; Diniz *et al.* 2005; Tringali *et al.* 2008), *P. cygnus* (Kennington *et al.* 2010), *P guttatus* (Truelove *et al.* 2016), *P. interruptus* (Ben-Horin *et al.* 2009), and *P. ornatus* (Liu *et al.* 2016). Except for *P. ornatus*, all these lobster species belong to the same evolutionary lineage as indicated by mtDNA phylogeny (Ptacek *et al.* 2001).

Eight microsatellite loci exhibited significant probabilities of departure from Hardy-Weinberg equilibrium expectations after sequential Bonferroni correction, adjusted critical $P < 0.0029$. In these cases the deviations occurred in the form of heterozygote deficiency, perhaps resulting from the small sample size or, considering the potential for extensive geneflow in this species, the Wahlund effect (Johnson and Black 1984). Additionally, null

alleles, even in low frequencies could have played an important part in the departures of HWE.

**Table 1.** Characteristics of 20 microsatellite loci developed in *Panulirus echinatus*[†].

| Locus | Primer sequence (5' - 3) | Core motif | Ta (°C) | *A* | Size range[§] (bp) | GenBank # |
|---|---|---|---|---|---|---|
| *Pech*1 | F: AAGCAGAACGAACTCACCGTA<br>R: TTTCTTCATTGGTCAGAAGGGT | $(CTA)_{11}$ | 55 | 5 | 119-146 | KX237634 |
| *Pech*2 | F: TCCTGGTTACGGAATAATGACC<br>R: CTGTTGTTGTTTCCCATACACG | $(CTG)_{9}$ | 55 | 6 | 109-115 | KX237635 |
| *Pech*3 | F: ACATAGATGGATGGGTAGATAGAC<br>R: CTAACTGCTTTCTTAACTGCCT | $(GATA)_{12}$ | 57 | 6 | 118-134 | KX237636 |
| *Pech*4 | F: GGGCCTTTCTTCATCTATTTCC<br>R: GTCCAGGGCAAGATTGTGATA | $(ATA)_{12}$ | 57 | 8 | 183-196 | KX237637 |
| *Pech*5 | F: TGTGTGTGTGTGTTTGTGTGTA<br>R: CACACATATACGAGTCAAAACGAA | $(TCTG)_{8}$ | 55 | 5 | 96-114 | KX237638 |
| *Pech*6 | F: CAATCTTAAAAGCAGACCAACG<br>R: GAGGAAGAATAGGTAGAAATGCAGA | $(ATA)_{15}$ | 55 | 6 | 134-158 | KX237639 |
| *Pech*7 | F: AGAGGCAAAGGGGTTCATCTA<br>R: TATACCACCATCACCAGCCATA | $(ATCT)_{6}$ | 55 | 5 | 118-150 | KX237640 |
| *Pech*8 | F: TATTCTTGTTCTTTCTTCGCCC<br>R: GAGTAGGAGGAGGAGGAAGAAGA | $(TCT)_{14}$ | 55 | 3 | 117-123 | KX237641 |
| *Pech*9 | F: TCTATCTATCTATCTTTCATTAACGC<br>R: GTTTTCGTCTCCTTTCCTGTGA | $(TATA)_{7}$ | 55 | 6 | 72-86 | KX237642 |
| *Pech*10 | F: TGTGTGGTGAGAGGAGAGTTGA<br>R: CTGGAAAGGATATGTTCAAAGGAG | $(GATA)_{8}$ | 56 | 2 | 78-86 | KX237643 |
| *Pech*11 | F: TCAGGTGGACTGTGACCAAG<br>R: CATATTATCATTATTATTATAATTATTATTAT | $(TAA)_{4}N(TAA)_{10}$ | 52 | 5 | 190-211 | KX237644 |
| *Pech*12 | F: CCTCCCACTTTGAAGCTCTG<br>R: TTATCATTAGTATCATTATGATCAT | $(ATA)_{17}$ | 52 | 6 | 99-117 | KX237645 |
| *Pech*13 | F: CATAACGTGATGATAATGATGGTG<br>R: GAGAAGAGGTACTAAACTCACTGGC | $(ATA)_{10}$ | 57 | 5 | 101-107 | KX237646 |
| *Pech*14 | F: TTTTGCTTGTGTGGCAAGATAC<br>R: ACTCCGCCCTTGTTTTAAGTTT | $(ATA)_{13}$ | 57 | 7 | 160-178 | KX237647 |
| *Pech*15 | F: GTATTGGTGCACTGGTTTATGC<br>R: TGATGATGATGATAATGATAATAATGA | $(TTA)_{15}$ | 55 | 6 | 162-194 | KX237648 |
| *Pech*16 | F: GCTTTCAGTATATGTCTATCTACCTG<br>R: GATAGATAAACAGAGAAATAGATAAA | $(TATC)_{11}$ | 55 | 6 | 87-107 | KX237649 |
| *Pech*17 | F: GCTGCCAAGCATTACGTTTT<br>R: CTGAATGAGTACCTGCGTGC | $(TATT)_{9}$ | 55 | 4 | 190-214 | KX237650 |
| *Pech*18 | F: CTCTCTCTATCTGTCTATCTATCTATC<br>R: TGGCATCAGAGGTATAGAAAGACA | $(TATC)_{11}(TCTA)_{17}$ | 56 | 1 | 175 | KX237651 |
| *Pech*19 | F: CACACACACATCATTGTATTATCATC<br>R: TGATAACAACAACAACACCAACA | $(TAA)_{14}$ | 52 | 1 | 136 | KX237652 |
| *Pech*20 | F: CGGGAAACAGCGATCATGTA<br>R: GGGGAAGATGTGTGTAGTGCAA | $(TAA)_{27}$ | 57 | 1 | 194 | KX237653 |

[†]Forty-two samples genotyped; [§]observed distribution of alleles at each locus; *T*a, annealing temperature; *A*, number of alleles.

**Table 2.** Variability of 17 microsatellite loci and frequency of null alleles *Panulirus echinatus* populations.

| Locus | All populations (*N = 42*) | | | | | | |
|---|---|---|---|---|---|---|---|
| | $A_R$ | *Ho* | $H_E$ | *PIC* | $P_{HWE}$ | $F_{IS}$ | $F_{Nulls}$ |
| *Pech*1 | 3.6 | 0.667 | 0.656 | 0.583 | 0.2629 | -0.0718 | -0.0112 |
| *Pech*2 | 4.0 | 0.595 | 0.660 | 0.589 | 0.1778 | 0.0806 | 0.0345 |
| *Pech*3 | 3.9 | 0.395 | 0.654 | 0.583 | 0.0006* | 0.3998 | 0.1522 |
| *Pech*4 | 4.5 | 0.472 | 0.698 | 0.644 | 0.0004* | 0.3155 | 0.1278 |
| *Pech*5 | 4.2 | 0.564 | 0.683 | 0.628 | 0.0616 | 0.1261 | 0.0656 |
| *Pech*6 | 4.7 | 0.415 | 0.755 | 0.704 | 0.0000* | 0.4051 | 0.1899 |
| *Pech*7 | 3.3 | 0.424 | 0.493 | 0.439 | 0.3548 | 0.0820 | 0.0414 |
| *Pech*8 | 2.4 | 0.095 | 0.293 | 0.266 | 0.0000* | 0.8609 | 0.1504 |
| *Pech*9 | 4.0 | 0.524 | 0.618 | 0.568 | 0.2030 | 0.1431 | 0.0542 |
| *Pech*10 | 1.8 | 0.214 | 0.194 | 0.173 | 1.0000 | -0.0943 | -0.0193 |
| *Pech*11 | 4.4 | 0.281 | 0.730 | 0.672 | 0.0000* | 0.5892 | 0.2543 |
| *Pech*12 | 3.8 | 0.469 | 0.515 | 0.470 | 0.1240 | 0.0639 | 0.0256 |
| *Pech*13 | 3.5 | 0.524 | 0.616 | 0.538 | 0.1258 | 0.1517 | 0.0527 |
| *Pech*14 | 6.2 | 0.595 | 0.842 | 0.811 | 0.0005* | 0.2696 | 0.1293 |
| *Pech*15 | 3.9 | 0.118 | 0.549 | 0.506 | 0.0000* | 0.7747 | 0.2746 |
| *Pech*16 | 4.8 | 0.405 | 0.696 | 0.652 | 0.0010* | 0.4045 | 0.1666 |
| *Pech*17 | 2.6 | 0.357 | 0.343 | 0.306 | 0.6560 | -0.0275 | -0.0140 |
| Mean | 3.8 | 0.418 | 0.587 | 0.537 | - | 0.2528 | - |

$A_R$, allelic richeness; $H_O$, observed heterozygosity; $H_E$, expected heterozygosity; *PIC*, polymorphic information content; $P_{HWE}$, probabilities of departure from Hardy–Weinberg Equilibrium (adjusted critical P < 0.0029); *: significant Hardy-Weinberg disequilibrium; $F_{Nulls}$: null allele frequency.



In this study, we report the isolation, using the NGS technology, and characterization of 17 polymorphic microsatellite loci for the brown spiny lobster *P. echinatus*. These microsatellites have demonstrated potential for population-level genetic studies and can provide valuable information on the genetic variation and stock structure of this lobster species. Therefore, we expect that these molecular markers will help to obtain the necessary information for developing conservation and management strategies for this highly valuable fishery species.

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