Genetics and Molecular Research

Novel genomic microsatellite markers for genetic population and diversity studies of tropical scalloped spiny lobster (*Panulirus homarus*) and their potential application in related *Panulirus* species

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Genet. Mol. Res. 15 (2): gmr.15027846
Received October 19, 2015
Accepted January 18, 2016
Published April 27, 2016
DOI http://dx.doi.org/10.4238/gmr.15027846

ABSTRACT. Fourteen polymorphic microsatellites with perfect di-, tri-, and tetra-nucleotide repeats were identified for *Panulirus homarus* using Roche 454 whole-genome sequencing method. Microsatellites were efficiently co-amplified in four multiplexes and a singleplex, providing consistent and easily interpretable genotypes. The number of alleles per locus ranged from 2 to 11 with the observed and expected

heterozygosity ranging between 0.000-0.532 and 0.031-0.836, respectively. A significant deviation from Hardy-Weinberg equilibrium was observed for majority of the loci, probably due to homozygote excess. Genetic linkage disequilibrium analysis between all the possible pairs of the loci showed significant departure from the null hypothesis in the loci pairs *Pho-G11-Pho-G33* and *Pho-G33-Pho-G57*. High success in primer cross-species amplification of these microsatellite markers indicates their utility for genetic studies of different *Panulirus* species.

Key words: Crustacea; *Panulirus homarus*; Population genetic studies; Microsatellites; Multiplexing

INTRODUCTION

Spiny lobsters are harvested from the wild in over 90 countries, with an annual catch of 80,000 tons for the year 2010 (FAO, 2012) and a continuous rise in its market price. There are major concerns about the future of spiny lobster fisheries owing to a constant decline in the catch (Rogers et al., 2010; FAO, 2012; Goddard et al., 2015), emphasizing the need for serious efforts towards sustainable fishery management and regulation of the species. Based on morphological characters, 20 different Spiny lobster species (genus *Panulirus*) have been described, five from the Atlantic and 15 from the Indo-Pacific region (Holthuis, 1991). The tropical scalloped spiny lobster, *Panulirus homarus*, is found throughout the Indo-Pacific region with centres of high concentrations in East Africa and Indonesia (Berry, 1974; Pollock, 1993) and contains three sub-species: *P.h. homarus* (Linnaeus, 1758), *P.h. megasculptus* (Pesta, 1915), and *P.h. rubellus* (Berry, 1974). Thus far, very little is understood about the genetic population structure and dynamics of the tropical scalloped spiny lobsters. Hence, genetic studies are required to obtain data on the resilience and sustainability of the exploitation of *P. homarus*, as well as for generating tools for rational conservation management plans for various *P. homarus* species. Genetic markers, such as microsatellites, have been successfully applied for the stock identification and fishery management of several marine species (Was et al., 2010; Milano et al., 2011; Cuveliers et al., 2012; Corander et al., 2013). However, for the population genetic studies of scalloped spiny lobster mainly sequencing of the control region in the mtDNA has been applied (Gopal et al., 2006; Groeneveld et al., 2007; Reddy et al., 2014). To date, only a few microsatellites markers are known in the tropical scalloped spiny lobster species (Dao et al., 2013). The development of more polymorphic microsatellite markers is significantly important for genetic population studies of the species. Here, we report the development and characterization of 14 novel microsatellite markers for use in the future studies on genetic diversity and population genetic structure of the tropical scalloped lobster *P. homarus*.

MATERIAL AND METHODS

We previously developed 30 polymorphic microsatellite markers for *P. ornatus*, based on the cost effective and rapid Roche 454 whole-genome sequencing method and demonstrated the potential of primers for cross-species amplification in closely related species (Dao et al., 2013). Considering the close taxonomic relationship between *P. ornatus* and *P. homarus*, the

same sequence database was mined for perfect di-, tri-, and tetra-nucleotide microsatellite repeats using iQDD (Meglécz et al., 2010) and MSAT commander version 0.8.2 (Faircloth, 2008), with incorporated Primer3 software for polymerase chain reaction (PCR) primer design (parameters: product length 150-400 bps; annealing temp 50°-63°C; GC content 20-80%). From this data mining, 370 independent sequence regions with microsatellite repeats and their possible primers were identified. Potential loci along with the primers were subsequently filtered based on the distance of primers from the beginning and end of a sequence (>10 bps), distance between primers and repeat motif (>10 bps), and the PCR product length (75-400 bps). For quality check, 50 perfect di-, tri-, and tetra-nucleotide microsatellite repeats were chosen for PCR validation in *P. homarus*. Genomic DNA from 96 unrelated individuals, originating from randomly selected pueruli from a single sampling site in Vietnam, were extracted by cetyltrimethylammonium bromide (CTAB) extraction method (Sambrook and Russell, 2001). Initially, 16 DNA samples were used to check the success of cross-species amplification of the primers. The amplifications were carried out individually in 15 µL reaction volumes, containing 10-50 ng genomic DNA, 9 µL True Allele premix (Applied Biosystems (AB), Foster City, USA), 1 mM $MgCl_2$, 0.4 µM each unlabeled forward and reverse primer (Metabion; Martinsried, Germany) in a Veriti Thermal Cycler (AB). Thermal cycling conditions employed were 15 min at 95°C, followed by 32 amplification cycles at 94°C for 30 s, 56°C for 90 s, and 72°C for 60 s, and then a final extension at 72°C for 30 min. Fourteen polymorphic microsatellites were reliably amplified across the 16 individuals examined when evaluated using 2% high resolution agarose gel. For fluorescent detection and multiplexing of the scored loci, the forward primers were labeled with dyes PET, NED, VIC, or 6-FAM (AB; Table 1). A PIG-tail sequence 5'-GTTTCTT-3' (Brownstein et al., 1996) was included towards the 5'-side in the reverse primers. Genomic DNA from 96 unrelated *P. homarus* individuals were amplified in four multiplexes and a singleplex (Table 1) and screened on a 3130 capillary sequencer (AB) to evaluate the efficiency of amplification and polymorphism of the markers.

Table 1. Characteristics of the 14 microsatellite loci identified in tropical Spiny lobster (*Panulirus homarus*).

Locus (Multiplex)	Repeat motif	Primer sequence (5'- 3')	Primer concentration (µM)	*A*	Allele size range (bp)	GD	H_O	H_E	F_{IS}	GenBank accession No.
Pho-G02 (I)	ACAG	**6-FAM_F- AGACTACGCTGCAACACCTGC R- GTTTCTTCTATTGATTCATCCGTTCACCA	0.30	7	121 - 145	0.279	0.137	0.278	0.510	KT203272
Pho-G11 (II)	AGG	**6-FAM_F- AGCTGTTCGAGAGGAGG R- GTTTCTTGTGGTAAGGTTCGACCAGGA	0.13	8	107 - 131	0.455	0.115	0.453	0.748	KT203273
Pho-G19 (S)	TG	**6-FAM _F- CTGCACCAGGGTACATGAAG R- GTTTCTTCTTCAGCCTCTTCAGTGTGG	0.10	7	109 - 137	0.141	0.147	0.141	-0.044	KT203274
Pho-G24 (II)	AC	** VIC _F- CTTGTTCCCTAATTTGCGCCR- GTTTCTTCCTCACCTGCTACAGGCCT	0.20	11	87 - 119	0.475	0.156	0.473	0.671	KT203275
Pho-G26 (I)	AG	** VIC _F- ATCCTCGTTCCTTACCGTCCCTCAC R- GTTTCTTGTGCCCAGACTACTGCATCA	0.30	2	101 - 103	0.042	0.000	0.041	1.000	KT203276
Pho-G28 (IV)	AT	**PET_F- CATTATGGCATCAGTGACCG R- GTTTCTTCTTGCTGAGCGAACGGTAA	0.20	3	124 - 128	0.031	0.010	0.031	0.665	KT203277
Pho-G29 (IV)	TG	** NED_F- CTGAGCAAGTTGTCGTCCAG R- GTTTCTTGGTGGGAACCAACTTGCTTA	0.20	6	100 - 110	0.241	0.167	0.240	0.308	KT203278
Pho-G31 (I)	AT	** VIC _F- ACTTATGTATATGTTTGTATGTGGGTG R- GTTTCTTGTTTCCTGACGCTAACTCGC	0.20	3	71 - 75	0.042	0.011	0.042	0.748	KT203279
Pho-G33 (III)	CTT	**PET_F- AGGTTGTAGAGGCGAGCTG R- GTTTCTTCGGGTTTAGTTTCACGCGT	0.20	8	222 - 243	0.647	0.447	0.645	0.309	KT203280
Pho-G38 (I)	AGT	**NED_F- GCATGCAAAGGGTCTTGCC R- GTTTCTTCGTGGAAGTAGCGACGATG	0.06	11	146 - 176	0.838	0.532	0.836	0.365	KT203281
Pho-G40 (II)	CTT	**PET_F- ATTAACCCGGGCGTGGAAG R- GTTTCTTCCTCACCTGGCCCAATTCT	0.20	4	164 - 173	0.173	0.069	0.172	0.601	KT203282
Pho-G41 (IV)	GTT	**NED_F- TTCGGGCCATGGTTACGTC R- GTTTCTTGCTGAGGCCACTTGGGAT	0.20	4	246 - 261	0.341	0.046	0.339	0.865	KT203283
Pho-G47 (III)	AGAT	**6-FAM _F- AGTAAGGTCCCAAAGGGAATG R- GTTTCTTCTATTTCAAAGCTGCAGAACTCTG	0.20	8	271 - 299	0.735	0.303	0.732	0.587	KT203284
Pho-G57 (III)	CTGT	**6-FAM _F- TCAGGTGTAGGACATCCGC R- GTTTCTTGTTGTCCCGTCTCTTAACCTTG	0.20	5	314 - 330	0.595	0.084	0.592	0.858	KT203285

A = number of observed alleles; GD = gene diversity; H_O = observed heterozygosity; H_E = expected heterozygosity. *Significant F_{IS} ($P < 0.001$; following sequential Bonferroni corrections). Microsatellite sequences are available on GenBank (accession Nos. KT203272 to KT203285). **Fluorescent dye labelled primer; F = forward; R = reverse.

The amplification was carried out in a 15-µL reaction volume containing 10-50 ng template, 9 µL True Allele premix, and 1 mM $MgCl_{2,}$ and the primer concentrations were adjusted to yield a consistent and relatively uniform fluorescence among the loci (Table 1 shows primer concentrations at each locus). Thermal cycling conditions were: 12 min at 95°C, followed by 32 amplification cycles at 95°C for 20 s, 58°C for 20 s, and 72°C for 30 s and a final extension at 72°C for 30 s. Genotyping was performed using a 3130 Genetic Analyser (AB). Data were collected automatically and sized with GeneMapper software (AB) using a GeneScan-500-LIZ size standard (AB).

RESULTS AND DISCUSSION

Of the 370 independent sequence regions containing microsatellites with perfect di-, tri-, and tetra nucleotide repeats, 50 were selected and their efficiency of amplification and polymorphism with genomic DNA from 16 unrelated *P. homarus* individuals was tested. On the basis of the success of amplification, pick quality, and polymorphism, 14 codominant microsatellites were selected and successfully genotyped in four multiplexes and a singleplex using a panel of 96 DNA samples. The characteristics of the 14 microsatellite loci are shown in Table 1. Statistical calculations were performed using the program Fstat (Goudet, 1995). The number of alleles per locus (A), observed heterozygosity (H_O), expected heterozygosity (H_E), gene diversity (GD), and inbreeding coefficient (F_{IS}) were calculated. The number of alleles varied from 2 to 11 per locus and the observed and expected heterozygosity ranged between 0.000-0.532 and 0.031-0.836, respectively (Table 1). The loci were further tested for conformance to both Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium. A significant deviation from HWE was observed for majority of the loci, with only *Pho-G19* conforming to the HWE. This observation was most probably due to the small sample size, genetic drift, and the presence of null alleles. Among the 91 pairwise exact tests performed, genotypic equilibrium between the loci were rejected for two tests (*Pho-G11-Pho-G33* and *Pho-G33-Pho-G57*) after sequential Bonferroni correction. Success of the cross species primer design amplification strategy indicates that the 14 microsatellite markers characterized in this study would be useful tools for future research in population genetics, reproductive ecology, and for constructing linkage maps for the two different *Panulirus* species studied here as well as for other related species.

Conflicts of interest

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

Research supported by the Research Council of Oman (TRC) (ORG/SQU/EBR/13/027) and by the Deanship of research at the Sultan Qaboos University (IG/DVC/CEMB/14/01).

REFERENCES

Berry PF (1974). A revision of the *Panulirus Homarus*-group of spiny lobsters (Decapoda, Palinuridae). *Crustaceana* 27: 31-42a. http://dx.doi.org/10.1163/156854074X00208

Brownstein MJ, Carpten JD and Smith JR (1996). Modulation of non-templated nucleotide addition by Taq DNA polymerase: primer modifications that facilitate genotyping. *Biotechniques* 20: 1004-1006, 1008-1010. PubMed

Corander J, Majander KK, Cheng L and Merilä J (2013). High degree of cryptic population differentiation in the Baltic Sea herring *Clupea harengus*. *Mol. Ecol.* 22: 2931-2940. PubMed http://dx.doi.org/10.1111/mec.12174

Cuveliers EL, Larmuseau MHD, Hellemans B, Verherstraeten SLNA, et al. (2012). Multi-marker estimate of genetic connectivity of sole (*Solea solea*) in the North-East Atlantic Ocean. *Mar. Biol.* 159: 1239-1253. http://dx.doi.org/10.1007/s00227-012-1905-x

Dao HT, Todd EV and Jerry DR (2013). Characterization of polymorphic microsatellite loci for the spiny lobster *Panulirus* spp. and their utility to be applied to other *Panulirus* lobsters. *Conserv. Genet. Resour.* 5: 43-46. http://dx.doi.org/10.1007/s12686-012-9728-0

Faircloth BC (2008). msatcommander: detection of microsatellite repeat arrays and automated, locus-specific primer design. *Mol. Ecol. Resour.* 8: 92-94. PubMed http://dx.doi.org/10.1111/j.1471-8286.2007.01884.x

FAO (2012). Global Capture Production 1950-2010. Food and Agriculture Organization.

Goddard S, Delghandi M, Dobretsov S, Al-Oufi H, et al. (2015). The first GCC Marine Biotechnology Symposium: Emerging Opportunities and Future Perspectives. *Mar. Biotechnol. (NY)* 17: 285-289. PubMed http://dx.doi.org/10.1007/s10126-015-9617-0

Gopal K, Tolley KA, Groeneveld JC and Matthee CA (2006). Mitochondrial DNA variation in spiny lobster *Palinurus delagoae* suggests genetically structured populations in the southwestern Indian Ocean. *Mar. Ecol. Prog. Ser.* 319: 191-198. http://dx.doi.org/10.3354/meps319191

Goudet J (1995). FSTAT (Version 1.2): A computer program to calculate F-statistics. *J. Hered.* 86: 485-486.

Groeneveld JC, Gopal K, George RW and Matthee CA (2007). Molecular phylogeny of the spiny lobster genus *Palinurus* (Decapoda: Palinuridae) with hypotheses on speciation in the NE Atlantic/Mediterranean and SW Indian Ocean. *Mol. Phylogenet. Evol.* 45: 102-110. PubMed http://dx.doi.org/10.1016/j.ympev.2007.06.002

Holthuis LB (1991). Marine Lobsters of The world. An Annotated and Illustrated Catalogue of Species of Interest to Fisheries Known to date. *FAO Species Catalogue. FAO Fish Synop.* 13: 1-292.

Linnaeus C (1758). Systema Naturae 1. "239.Cancer". Systema Naturae 1 (10th ed.). Stockholm, Sweden: *Laurentii Salvii.* 239: 625-634.

Meglécz E, Costedoat C, Dubut V, Gilles A, et al. (2010). QDD: a user-friendly program to select microsatellite markers and design primers from large sequencing projects. *Bioinformatics* 26: 403-404. PubMed http://dx.doi.org/10.1093/bioinformatics/btp670

Milano I, Babbucci M, Panitz F, Ogden R, et al.; FishPopTrace Consortium (2011). Novel tools for conservation genomics: comparing two high-throughput approaches for SNP discovery in the transcriptome of the European hake. *PLoS One* 6: e28008. PubMed http://dx.doi.org/10.1371/journal.pone.0028008

Pesta O (1915). Bemerkungen zu einigen Langusten (Palinuridae) und ihrer Geographishen Verbreitung.S.B. *Akad. Wiss. Wein* 124: 3-12.

Pollock DE (1993). Speciation in spiny lobsters-clues to climatically-induced changes in ocean circulation patterns. *Bull. Mar. Sci.* 53: 937-944.

Reddy MM, Macdonald AHH, Groeneveld JC and Schleyer MH (2014). Phylogeography of the scalloped spiny-lobster *Panulirus homarus rubellus* in the southwest Indian Ocean. *J. Crustac. Biol.* 34: 773-781. http://dx.doi.org/10.1163/1937240X-00002284

Rogers PP, Barnard RM and Johnston MD (2010). Lobster aquaculture a commercial reality: A Review. *J. Mar. Biol. Assoc. India* 52: 327-335.

Sambrook J and Russell DW (2001). Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, New York.

Was A, Gosling E and Hoarau G (2010). Microsatellite analysis of plaice (*Pleuronectes platessa* L.) in the NE Atlantic: weak genetic structuring in a milieu of high gene flow. *Mar. Biol.* 157: 447-462. http://dx.doi.org/10.1007/s00227-009-1331-x