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NOTA

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Isolation and characterization of new microsatellite markers for the Pacific geoduck (*Panopea generosa*) using next generation sequencing

Aislamiento y caracterización de nuevos marcadores microsatélites para la almeja generosa (*Panopea generosa*) por medio de secuenciación de nueva generación

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ABSTRACT

Background. *Panopea generosa* is a large and long-lived infaunal clam with a considerable commercial value in Canada, United States and Mexico, in need of population genetic studies across its range of distribution. **Goals**. We set to develop new genetic markers (microsatellites) specific for *P. generosa*. **Methods**. We tested 30 microsatellite loci generated using next-generation genome sequencing (Illumina Hi-Seq 2500). **Results**. We identified eight as suitable polymorphic genetic markers. The number of alleles per locus ranged from 5 to 22 and heterozygosity from 0.429 to 0.818 (observed) and from 0.548 to 0.962 (expected). Deviation from Hardy-Weinberg equilibrium was found in three loci, after Dunn-Šidák correction, as a result of heterozygote deficiencies suggesting the presence of null alleles and linkage disequilibrium was found between two loci. **Conclusions**. These markers are highly informative and useful for population genetic studies aimed at informing management and conservation measures of this valuable resource.

Keywords: Genetic markers, next-generation sequencing, microsatellite, *Panopea generosa*

RESUMEN

Antecedentes. *Panopea generosa* es una almeja de gran tamaño, infáunica y longeva con un valor comercial considerable en Canadá, Estados Unidos y México, y de la que se requiere conocer su estructura genética poblacional a lo largo de su rango de distribución. **Objetivos**. Desarrollar nuevos marcadores microsatélites específicos para *P. generosa*. **Métodos.** En este reporte evaluamos 30 loci microsatélites generados mediante secuenciación genómica de siguiente generación (Illumina Hi-Seq 2500). **Resultados**. Se identificaron a ocho como marcadores genéticos polimórficos adecuados. El número de alelos por locus varió entre 5 y 22, la heterocigosidad observada entre 0.429 y 0.818 y la esperada entre 0.548 y 0.962. Tres marcadores se desviaron del equilibrio de Hardy-Weinberg, después de la corrección de Dunn-Šidák, como resultado de un déficit de heterocigosidad, sugiriendo la presencia de alelos nulos y se encontró desequilibrio de ligamiento entre dos microsatélites. **Conclusiones.** Estos marcadores son altamente informativos y útiles para estudios de genética poblacional encaminados a la implementación de medidas de administración y conservación de este valioso recurso.

Palabras clave: marcadores genéticos, microsatélites, *Panopea generosa*, secuenciación de siguiente generación

The Pacific geoduck, *Panopea generosa* Gould, 1850, is a large and long-lived infaunal clam, inhabiting sediments from the low intertidal to subtidal waters from Alaska, USA, to Baja California, México, and is the most important commercial geoduck (Bivalvia: Hiatellidae) in the northeast Pacific (Aragón-Noriega *et al*., 2012). Its commercial harvest started in 1970's in the U.S. and Canada, and in early 2000 in Mexico (Aragón-Noriega *et al*., 2012). Commercial aquaculture operations started in the late 1990's in the U.S. The species is sensible to over-exploitation because of its extended longevity, low recruitment, late sexual maturity, and slow growth (Bureau *et al*., 2002; Calderon-Aguilera *et al*., 2010; Orensanz *et al*., 2004; Sloan & Robinson, 1984). Therefore, assessing the structure and dynamics of wild populations is a prerequisite to address their potential for sustainable exploitation and to manage risk reduction in aquaculture (Straus *et al*., 2008). Ge-

netic analyses using polymorphic loci are a powerful tool to investigate population genetic structure, and to assess levels of genetic variability, effective population size and extinction risk (Evans & Sheldon, 2008). Among molecular genetic markers, microsatellites show several advantages (Jarne & Lagoda, 1996) however, their isolation in a new species requires significant effort (time- and money-wise) and expertise (Zane *et al*., 2002). In addition, the genomic abundance of microsatellites is variable among taxa; and in some genomes, such as those of bivalves, they appear in low frequency (Peñarrubia *et al*., 2015). On the other hand, next generation DNA sequencing (NGS) technologies provide a cost-time-efficient means to isolate large number of microsatellites (Abdelkrim *et al*., 2009; Csencsics *et al*., 2010; Inoue *et al*., 2013; Lance *et al*., 2013).

Panopea generosa, formerly incorrectly named *Panopea abrupta* Conrad, 1849 (Vadopalas *et al*., 2010), has been subject to the development of seventeen specie-specific microsatellites using traditional cloning methods (Kaukinen *et al*., 2004; Vadopalas & Bentzen, 2000; Vadopalas *et al*., 2004). Some of which have been used repeatedly in population genetics studies with variable and sometimes limited success (Miller *et al*., 2006; Suárez-Moo *et al*., 2016; Vadopalas *et al*., 2004; Vadopalas *et al*., 2012). Suárez-Moo *et al.* (2016) and Vadopalas *et al.* (2012) found genetic homogeneity between samples of Baja California and Washington and among cohorts of Washington, respectively. On the other hand, Miller *et al.* (2006) and Vadopalas *et al.* (2004) revealed genetic heterogeneity in populations of Canada and USA. In this study, we aim at producing additional genetic markers and complement the molecular toolbox of the Pacific geoduck to increase the power of future genetic studies along its distribution. This information will prove valuable for managing the exploitation of wild populations and help direct aquaculture efforts along its distribution area.

DNA of gill tissue was extracted from two fresh organisms, collected near Ensenada, Baja California, using the DNeasy blood and tissue kit (QIAGEN, Hilden, Germany), obtaining in excess of 50 ng/µl of high quality ($A_{260}/A_{280} > 1.80$) genomic DNA. All processes of library construction and Illumina sequencing were done as a described in Bisbal-Pardo *et al.* (2016). Bioinformatic analyses (quality control, ends trim, *de novo* assembly, microsatellite identification and primer design) were also carried out as described in Bisbal-Pardo (2014) and Bisbal-Pardo *et al.* (2016). For marker assessment, thirty microsatellite loci (9 di-, 10 tri- and 11 tetranucleotide) were selected (primer lengths ranging 19-24 bp, matching annealing temperature (Tm) between 54-60 °C, a minimum of 5X coverage and a product size of 140-400 bp, and their amplification was tested using the same PCR conditions of Bisbal-Pardo *et al.* (2016). Genotyping was performed using ABI-3130xl automated DNA sequencer. Alleles were scored with the program Gene Marker 2.4.0 and allele sizes were assigned to bins using FLEXIBIN (Amos *et al*., 2007). We identified genotyping errors (stutters, allele dropout, typographical) and evaluated the presence of null alleles with MICRO-CHECKER (Van Oosterhout *et al*., 2004). Loci were scored in a set of 35 organisms, 7 from Hood canal (47°40'58.92" N 122°44'51.66" W) and 7 from Alden Bank (48°49'43.2" N 122°49'50.6" W), Washington, EUA; 7 from Coronado Islands (32°25'00"N 117°15'00" W), 7 from San Quintín (30°23'22.02" N 115°54'47.2" W) and 7 from Santa Rosaliíta (28°40'00" N 114°15'57" W), Baja California, Mexico. We estimated the number of alleles per locus (k), observed and expected heterozygosities (Ho and He) and polymorphic information content (PIC) using Mstools (Park, 2001). Next, we calculated the deviation from Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium (LD) with ARLEQUIN 3.1 (Excoffier *et al*., 2005). Significance was adjusted for multiple testing using Dunn-Šidák correction (Šidák, 1967). We obtained a total of 77,475,634 reads from NGS, after the trimming, the 0.76% was discarded and 7.52% were corrected resulting in reads of 99.5 bp of the average length. We identified 8,060 di-, 3,146 tri- and 2,830 tetranucleotide microsatellites in a total of 868,521 contigs of 443 bp average length, N50 was 461 bp and average coverage was 11.86 reads. Less than 26% of markers were suitable for PCR primer design. The great disparity in the number of microsatellite loci identified bioinformatically and those amenable to primer design has been reported repeatedly (Castoe *et al*., 2012; Castoe *et al*., 2010; Csencsics *et al*., 2010).

Of the 30 loci tested, only eight were consistently and accurately genotyped (Table 1). The yield obtained in this study (27%), defined as the fraction of microsatellite loci successfully genotyped from the total experimentally tested, is similar to others obtained from mollusk using NGS (43%, An & Lee, 2012; 35%, Cruz-Hernández *et al*., 2014; 22%, Greenley *et al*., 2012; 33%, O'Bryhim *et al*., 2012). In most studies, a large number of potential loci are discarded because of amplification problems, which is particularly frequent among bivalves (Selkoe & Toonen, 2006). Mollusk genomes have been found to possess a high frequency of repetitive elements that may interfere if they appear in the flanking regions of microsatellite loci, resulting in multicopy PCR products (McInerney *et al*., 2011). Also, sometimes the use of the M13 tail for fluorescently labeling the forward primer may decrease the efficiency of PCR reactions (Guichoux *et al*., 2011). We did not find evidence of genotyping errors.

Most loci were characterized by moderate to high genetic variation, with an average of 11.4 alleles per locus (range = 5-22 alleles), heterozygosity estimates ranging between 0.429 and 0.818 (mean = 0.613) and PIC value between 0.488 and 0.939 (mean = 0.743). Three loci (Pgen3_7, Pgen4_1 and Pgen4_11) significantly deviated from HWE after the Dunn-Šidák correction ($p < 0.006$) due to heterozygote deficiencies, for which MICRO-CHECKER suggested the presence of null alleles. We found evidence of LD ($p < 0.001$) between Pgen4_1 and Pgen4_10. A similar range of alleles (4-23 per locus) were found in the congener *P. abbreviata* in microsatellite loci (n = 21) obtained from NGS (Ahanchede *et al*., 2013) and similar results have been reported in others mollusk (An & Lee, 2012; Greenley *et al*., 2012). The quality of a marker can be determined by its degree of polymorphism. In this study, the expected heterozygosity values are in the optimal range (0.6-0.8) to provide a good resolution (Taberlet & Luikart, 1999). Moreover, the PIC values of all loci are higher than 0.25, so they are informative for linkage analysis. The deviation from HWE found in some loci could be due to population phenomena such as inbreeding, Wahlund effect, and selection or genotyping errors, such as null alleles or homoplasy (Selkoe & Toonen, 2006). However, since populations of *P. generosa* have shown no genetic differentiation along the northeast Pacific it is unlikely that a Wahlund effect is playing a part in the observed disequilibria (Suárez-Moo *et al*., 2016). On the other hand, MICRO-CHECKER analysis indicated the possible presence of null alleles in some loci. Null alleles have been found to be very common in bivalves because of mutations in the flanking regions (Becquet *et al*., 2009; Hedgecock *et al*., 2004). Even though population genetic studies require the use of independent unlinked loci, linkage may be useful for mapping studies (Xiao *et al*., 2012).

Table 1. Polymerase chain reaction primers and levels of polymorphism of novel microsatellite loci developed for *Panopea generosa* Gould, 1850 using next generation sequencing.

Locus	Primer sequence (5'-3')	Tm	Motif	*n*	Na	Allelic range	Dye	H_0	H_E	P_{HWE}	PIC	GenBank accession
Pgen2_3	F: GCGTTTGATTGCRGGTGTAT R: CAGGCATCGTCGTGTAATGG	55.6	$(AT)_8$	34	5	159-167	FAM	0.559	0.673	0.311	0.6	MF668230
Pgen3_4	F: ACGGCGAAAGAACGAAATGG R: TTGGTGAGAGGTTGTTGCAG	55.6	$(ACG)_{10}$	17	6	316-337	PET	0.529	0.617	0.223	0.559	MF668231
Pgen3_7	F: GACAAACACCGCCTACACTG R: TACGAATGCAGTCACCAAGC	66	$(AAC)_{17}$	29	15	345-393	FAM	0.552	0.912	0.000*	0.888	MF668232
Pgen4_1	F: GSGTGGAATCCATTGGGGTA R: ACCACCCTGGACACTCCTTA	62	$(ACAG)_{14}$	24	22	314-474	VIC	0.667	0.962	0.000*	0.939	MF668233
Pgen4_3	F: GTTTGCCTTGTCGTCTGCAG R: GGATCCCTGGAAAGTGTGGT	62	$(AAAC)_6$	35	7	236-320	PET	0.429	0.548	0.141	0.488	MF668234
Pgen4_9	F: GTCAATCCAGCCAAGCACAG R: GCGTGTTAGCCCTCAATAGC	55.6	$(AATC)_9$	33	13	281-381	PET	0.818	0.891	0.045	0.866	MF668235
Pgen4_10	F: AACCGCAGCAGAACAAAGTC R: ATCTTCGCTTAGGAGGCGG	56.6	$(ACGC)_6$	28	13	345-409	VIC	0.724	0.823	0.039	0.786	MF668236
Pgen4_11	F: AAGTCAACCAGGATGTGCAC R: CCATTAAAGGGTCACACGGC	66	$(ATCC)_8$	32	10	242-278	NED	0.625	0.848	0.002*	0.816	MF668237

Abbreviations: Tm (°C): annealing temperature, *n*: sample size; Na: number of alleles; Dye: fluorescent dye; H_0: observed heterozygosity; H_E: expected heterozygosity; P_{HWE}: Hardy-Weinberg equilibrium test p-value (*) significant after Dunn-Sidák correction); PIC: polymorphism information content.

Nowadays, NGS is the best option to identify a high number of microsatellite loci in non-model species because it is cheaper and faster than traditional methods (Castoe *et al*., 2010; Ekblom & Galindo, 2011). These technologies are enabling more extensive and robust genetic studies in a great variety of taxa (Castoe *et al*., 2012; Huang *et al*., 2015; Mira *et al*., 2014). In this study we developed a set of new polymorphic microsatellites in *P. generosa*. These markers will useful in genetic studies applicable to conservation and management fisheries and aquaculture activities.

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