New microsatellite loci for estimating genetic diversity and structure in *Octopus hubbsorum* from Nayarit, México

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ABSTRACT

Background: *Octopus hubbsorum* Berry, 1953 is the most important species for commercial fishing in the Mexican Pacific. However, there is a lack of information regarding population structure that could have important management implications. We tested 44 microsatellite loci in *O. hubbsorum* by cross-amplification from *O. bimaculatus*. **Methods and Results**: Genetic diversity and structure was tested over 30 octopus sampled from Santa Cruz de Miramar (Nayarit, México). A total of 11 loci were successfully amplified. All loci were polymorphic with the number of effective alleles ranging from 2.13 to 23.14, while three loci significantly deviated from Hardy-Weinberg equilibrium. No significant LD was observed between pairs of loci (P ≥ 0.05). The application of the new markers in a *O. hubbsorum* population from Santa Cruz de Miramar Nayarit, México, not showed Wahlund and isolate breaking effects due to the mixing of distinct populations. **Conclusions**: The loci were useful to estimate levels of pairwise relatedness and to discard the presence of recent demographic bottlenecks in the population. We consider that eight microsatellites are adequate of the 11 amplified microsatellites.

Keywords: Octopus, Mexican Pacific, Genetic diversity, Wahlund Effect, Isolate Breaking Effect

1 Introduction

The octopus' fishery on the Mexican Pacific coast is carried out by small scale fishers, however in the last 10 years the capture has increased from ~900 to ~3500 tons per year [1]. The highest production occurs in the states of Baja California Sur, Jalisco, Guerrero, Sonora, and Nayarit [1]. *Octopus hubbsorum* is the species of greatest fishing importance [1]. It is a semelparous benthic species with a planktonic phase of approximately two to three months [2, 3]. This species inhabits rocky and sandy substrates of inter and subtidal zones down to 30 m depth [4] and its geographic range extends from the Pacific coast of the Baja California Peninsula, to Salina Cruz, Oaxaca, including the Gulf of California (GC) [5, 6]. However, in spite of its commercial relevance and wide distribution, the fishery management regulations only consider an agreement that establishes the minimum catch size and one closed season exclusively for Reserva de la Biosfera Bahía de Los Ángeles, Canal de Ballenas y Canal de Salsipuedes in the northern GC [7]. These measures are specific for *O. bimaculatus* [8] and not for *O. hubbsorum*, which could imply negative repercussions on recruitment and an overestimated minimum catch size for *O. hubbsorum* populations [2].

Although some studies have described aspects of the life history of *Octopus hubbsorum* [2], there is a lack of information about stocks or discrete populations. Using mitochondrial markers (two concatenated genes, COI+ND5) Dueñas-Romero et al. [3] did not detect any population genetic structure but Domínguez-Contreras et al. [2] using seven microsatellite loci supported the hypothesis that *O. hubbsorum* presents intermediate levels of genetic structure (AMOVA F_{ST}=0.16) among locations from Northwest Mexico, in comparison to other *Octopus* species.

A better understanding of the population structure of *O. hubbsorum* is important for proper and sustainable long-term management. Hence, having suitable molecular markers is a necessary tool to determine the existence of genetic stocks for fisheries and conservation management plans. The strategy of cross-amplifying microsatellites in other species has proven to be successful in other octopus' species (e.g., *Octopus vulgaris* and *Octopus maya [9]*). Domínguez-Contreras et al. [2] tested 15 microsatellite markers developed for *O. bimaculatus* [10] and selected seven to be applied on *O. bimaculatus*, *O. hubbsorum*, and *O. bimaculoides* collected in the Pacific coast of Mexico. Considering the economic relevance of these Octopus species in México, more microsatellite markers should be tested to increase the markers number and use them in future population genetic analysis including new sampling sites. For this study, we had access to the database of microsatellite markers obtained previously [2] and tested in *O. hubbsorum* those markers that the authors [2] did not tested. Thus, our goal was to characterize a new set of microsatellite loci to complement some markers previously published [10] and use them in future population genetic structure in *O. hubbsorum*. We show the application of the new set of loci estimating the genetic variability, effective population size, test for recent bottlenecks, and to estimating pairwise relatedness in *O. hubbsorum* individuals sampled from Nayarit, Mexico.

Materials and methods

We tested 44 microsatellite loci previously isolated via shotgun genomic sequencing for *O. bimaculatus* [10] in 30 samples of *O. hubbsorum*. The organisms were collected by the local fishermen through diving and hook as a fishing gear in Santa Cruz de Miramar (Nayarit, Mexico). (Fig. 1). Additionally, six loci previously reported for *O. hubbsorum* (10) were included in the analysis for increase the markers number. The organisms were morphologically identified [4] and muscle tissue from each individual was preserved in 96% ethanol for genetic analysis. Total genomic DNA was obtained using the DNeasy Blood and Tissue extraction kit (QIAGEN) following the manufacturer's specifications.

We test directly the amplifiability of the markers using fluorescently labelled primers and checked the results on agarose gel. The PCRs were conducted in 15µL volumes with 20–40 ng genomic DNA, 1x PCR buffer, 0.2 mM each dNTP, 1.5 mM $MgCl_2$, 0.2 % BSA, 0.5 U *Taq* DNA polymerase (Invitrogen), 0.02 µM of the unlabeled M13-tailed forward primer, and 0.2 µM of the fluorescently labeled M13 primer, and 0.2 µM of the reverse primer. We applied a PCR touchdown protocol consisting of 94 °C for 5 min, 15 cycles of 94 °C for 30 s, 65–50 °C for 30 s (1 °C decrease each cycle), 72 °C for 30 s, followed by 40 cycles at 94 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s, and a final extension of 72 °C for 5 min.

We genotyped the PCR products using an Applied Biosystems' 3730XL sequencer and we used GeneMarker 2.4.0 for scoring the alleles. Allele sizes were assigned to bins using FLEXIBIN [11]. Genotyping errors and the presence of null alleles were detected with MICROCHECKER 2.2.3 using of Fisher's test, which calculates if the pooled probability shows a significant overall excess of homozygotes that are uniformly distributed among classes of homozygotes [12]. We tested the occurrence of linkage disequilibrium (LD) using GENEPOP 4.2 [13] and significance levels for multiple comparisons were adjusted with a sequential Bonferroni correction [14].

We use GENALEX 6.501 [15] to evaluate the genetic diversity of microsatellites through the estimation of number of alleles (Na), number of effective alleles (Ae), and observed (Ho) and expected (He) heterozygosity. An exact test for deviations from Hardy-Weinberg equilibrium (HWE) was done. GENALEX 6.501 was used also, to calculate the average number of alleles shared between pairs of individuals to estimate relatedness (r) as described by Queller and Goodnight [16].

The contemporary effective population size (Ne) via the linkage disequilibrium method (LDM) [17] was estimated using a bias correction, a lowest allele frequency of 0.05 and 0.02 and the Molecular Coancestry (MC) method [18] in the software NE-ESTIMATOR v2 [19].

We evaluate the possible occurrence of a recent bottleneck comparing the observed allele frequency distribution with that of a population in mutation–drift equilibrium assuming the two-phase model (TPM) [20]. The test was run with a 95% stepwise mutation model (SMM) and 5% infinite allele model (IAM). The deviations between the observed and expected frequency distributions was tested using a sign test, and a Wilcoxon's signed rank test with the software BOTTLENECK using 10,000 iterations [21].

Results and Discussion

We successfully amplified a total of 11 microsatellite loci, the six loci previously reported for *O. hubbsorum* (*Ocbi19, Ocbi25, Ocbi35, Ocbi39, Ocbi41* and *Ocbi47*) [10] and five new ones for this specie of the 44 tested previously isolated for *O. bimaculatus* [10] (*Ocbi17, Ocbi26, Ocbi33, Ocbi37* and *Ocbi42*, Table 1). The rest of the microsatellites loci we could not amplify it. We consider that the lack of amplification of some loci could also be due to the fact that *O. bimaculatus* is not the most closely related species to *O. hubbsorum*, such as are *O. mimus* or *O. maya* [22]. All loci were polymorphic. The number of effective alleles (Ae) ranged from 2.1 to 23.1 alleles (mean 8.8). The observed heterozygosity (Ho) varied from 0.280 to 1.00 (mean 0.797) and expected heterozygosity (He) from 0.531 to 0.957 (mean 0.800) (Table 1). No significant LD was observed between pairs of loci ($P \geq 0.05$).

Three microsatellite loci (Ocbi17, Ocbi41 and Ocbi47) show significant deviations from HWE after Bonferroni correction (P=0.002). Deviations from HWE may be due to an excess of homozygotes as results of the presence of null alleles, which are common in microsatellites, particularly when cross-amplified from related species [23, 24, 25]. From these three microsatellites, only at the Ocbi17 microsatellite locus, deviation from HWE deviation was associated with an excess of homozygotes (Ho= 0.280, and He= 0.957) consistent with the presence of null alleles (frequency of null alleles 35%). The deviation from HWE caused by the excess of homozygotes can also be due to a Wahlund effect, which occurs when a sample from a mixture of populations is wrongly analyzed as if it were a single population. This effect should be more evident when the deviation occurs at all or most loci. In our case, it only occurred in one (Ocbi17). Inbreeding has a similar effect similar. In addition to, we found that the values of a relatedness (r) close to zero presented the highest frequency (Fig. 1), supporting that that the inbreeding could be discarded also as cause of deviation from HWE. Two other loci (Ocbi33 and Ocbi35) presented null alleles, however, their frequency was relatively low (<16%) and they did not show a deviation from the HWE. On the other hand, at the other microsatellite loci (Ocbi41 and Ocbi47) that showed deviation from HWE, it was due to excess of heterozygotes (Ho = 0.867, He = 0.531, and Ho = 1.000, He = 0.616; respectively) and they did not present null alleles. Because to that the deviation from HWE due to excess heterozygotes was only present in these two of the 11 loci, an isolate breaking effect, generated by population mixing, is not supported from these data. It could also be that these loci showing HWE deviations could be under selection. We used the BayeScan software and the R function "plot_bayescan" (http://cmpg.unibe.ch/software/BayeScan/download.html) to detect loci under selection. Using a False Discovery Rate of 5% (FDR=0.05) to fix the threshold value we found a deviation from neutrality only for loci Ocbi17, but when we used a Posterior Odds threshold (PO= 10), no outliers were found. Other causes of deviation could be genotyping errors/artifacts, and sex linkage [26].

Results obtained in our study did not suggest an inbreeding event (values of a relatedness, r, close to zero, Fig. 1), probably due to a large population size. We found a contemporary Ne (Ne LDM = ∞, CI. 894.1 - ∞, and Ne MC = 33.4, CI. 0.8 - 123.4) similar to previously reported for other octopuses' species, which present a dispersion by paralarvae [2]. These results are consistent with the Sign Rank Test (*P*= 0.28) and the Wilcoxon test (*P*=0.81) indicating that we did not obtain evidence that this population has gone through a recent bottleneck

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108 since. On evolutionary scale, *O. hubbsorum* populations in Mexico have experienced a demographic expansion
109 according to studies carried out with the Cytochrome Oxidase Subunit I (COI) and NADH Dehydrogenase
110 Subunit 5 (ND5) gene [3].

111 In conclusion, in this study we evaluate the potential use of 44 microsatellites for the analysis of the population
112 genetic structure in *O. hubbsorum*. Based on our results, we consider eight microsatellites to be adequate.
113 However, the Ocbi17, Ocbi41 and Ocbi47 loci should also be incorporated in subsequent analyses since a better
114 interpretation should be obtained from the analyses of more samples from other collection sites.

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127 **Conflicts of interest/Competing interests**

128 The authors declare that they have no competing interests.

129 **Ethics approval**

130 *Octopus hubbsorum* is not an endangered or a protected species in the area sampled. Sampling activities were
131 not performed at locations where specific permission is required.

132 **Authors' contributions**

133 All authors of the paper have directly participated in the planning, execution, and analysis of this study

134 **Consent to participate**

135 All authors have approved their participation in this paper

136 **Consent for publication**

137 All authors have read and approved the final version submitted

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220   Figure caption

221   **Fig. 1** Sampling site (black dot) on the coast of Nayarit. Frequency distribution of pairwise relatedness values
222   (r) of O. hubbsorum obtained using Queller and Goodnight [18] estimator

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Table

Table 1 Characteristics of 11 microsatellites loci from *O. hubbsorum*, including: Locus name, number samples (N), GenBank accession number, repeat motif, forward (F) and reverse (R) primer sequences, Primer dye (Pd), expected size in base pairs (bp), size range of observed allelic variation, number of alleles (Na), No. effective alleles (Ae), observed (Ho) and expected (He) heterozygosities, Hardy-Weinberg equilibrium (HWE) and null alleles.

Locus name	N	GenBank accession number	Repeat motif	Primer sequence (5′–3′)	Pd	Expected size (bp)	Allele size range (bp)	Na	Ae	Ho	He	HWE	Null allele frequency (Oosterhout)
Ocbi17**	25	KF746730	tcta$_{(16)}$	F: CCTGCAGTAGCAGCAAAGGT R: CCTGAACCCACTGTGTGAGA	FAM	170	144-529	27	23.14	0.280	0.957	**0.000**	0.3509
Ocbi19*	30	KF746731	atag$_{(16)}$	F: GACGCGTCGACAAATAGTCA R: TCATTAGTCTGCCCAGCACA	FAM	122	111-178	16	8.86	0.833	0.887	0.872	0.0332
Ocbi25*	30	KF746732	cata$_{(15)}$	F: CGAGCACACAAACGTACACA R: TGCAGATTGACCCAACTCAG	NED	193	202-278	9	3.85	0.933	0.741	0.260	-0.1676
Ocbi26	30	-	gtat$_{(15)}$	F: GGCCAGTTTACCTTCGTCGATGCCTTC R: CAAAATACCCTAGTTGATGTTGAAA	FAM	290	256-299	12	8.65	0.800	0.884	0.206	0.0477
Ocbi33	30	-	taga$_{(15)}$	F: GGCCAGTGCAGGCAGGCAGGTAGATAG R: TCTGCTAGCTTTTATCCTTACTATTTT	FAM	180	103-148	12	7.86	0.667	0.873	0.022	0.1159
Ocbi35*	30	KF746734	taga$_{(15)}$	F: ATGTCCCTCAGCGTCGTTAG R: GATGTTGCGCACAAGTGTCT	VIC	156	159-216	14	11.11	0.633	0.910	0.003	0.1508
Ocbi37	30	-	taga$_{(15)}$	F: GGCCAGTCCAGACTTTGTGGACACCCT R: ATGGTTCCGGGTTAAATTCC	FAM	200	100-234	5	2.33	0.767	0.572	0.313	-0.4109
Ocbi39*	30	KF746735	tctt$_{(14)}$	F: TCTTTCCGACTGCCTTATTTG R: AAAGCAACGTCAACAACACG	FAM	186	160-245	15	9.13	0.967	0.891	0.911	-0.0435
Ocbi41*	30	KF746737	cttt$_{(14)}$	F: GGCAACAGGGACAACCTTTA R: GCCAGGCGATCAACATATTA	VIC	190	132-182	3	2.13	0.867	0.531	**0.000**	-0.4424
Ocbi42	30	-	tgta$_{(14)}$	F: GGCCAGTAAAATTATTCCTGCACCCCC R: AGTCGGACAGACAGACAGATAGA	FAM	150	102-223	24	17.30	1.000	0.942	0.014	-0.0311
Ocbi47*	30	KF746740	acat$_{(14)}$	F: CCTGTCATAAACAGTAATACCAATAAA R: GTGCCTGAAATACGGGGTAA	VIC	210	185-235	3	2.60	1.000	0.616	**0.000**	-0.5986

Microsatellite loci previously reported for *O. hubbsorum* (*), and for *O. bimaculatus* and *O. bimaculoides* (**) by Domínguez-Contreras *et al*. [12].
Values in bold showed significant deviation from HWE after Bonferroni test (p < 0.002).

Figure