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Characterisation of 30 microsatellite loci for the Tehuelche scallop, *Aequipecten tehuelchus* (d'Orbigny, 1842) and their use for estimating demographic parameters relevant to fisheries management

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ABSTRACT

Aequipecten techuelchus is an endemic scallop of the Argentine biogeographic province in the southwest Atlantic Ocean which supports a commercial fishery that has presented strong fluctuations since the 1960s. It is unclear if distinct localities constitute a single panmictic population. We used next-generation sequencing to obtain microsatellite loci that could be used to evaluate genetic diversity and differentiation among populations. We developed 30 polymorphic microsatellite loci, of which 13 meet the standard criteria required for population genetic analyses, including Hardy-Weinberg equilibrium, not being linked and with a low frequency of null alleles. The described microsatellite loci were used to estimate relatedness and effective population size, and to test for recent and historic population bottlenecks. Our results suggest that the population of the Tehuelche scallop from San Román, Gulf of San José, Patagonia shows a relatively large effective population size, high levels of genetic polymorphism, low levels of inbreeding and no signs of recent or historic drastic population reductions. These preliminary results should be confirmed with larger sample sizes and the inclusion of other nearby populations.

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Bottleneck; connectivity; effective population size; Gulf of San José; metapopulation; Patagonia

Introduction

The Tehuelche scallop *Aequipecten tehuelchus* (d'Orbigny, 1842) is an endemic species of the Argentine biogeographic province in the southwest Atlantic Ocean. It inhabits coastal areas at depths shallower than 130 m from Rio de Janeiro, Brazil (23°S) to Bahía Camarones, Argentina (45°S) (Soria et al. 2016). Historically, the Tehuelche scallop supported inshore fisheries in several gulfs of northern Patagonia, including San Matías and San José (Figure 1). The fishery started in the Gulf of San Matías at the end of the 1960s and subsequently developed in the Gulf of San José. Nowadays, the Tehuelche scallop is present at levels that sustain commercial fishing (Orensanz et al. 2007; Soria et al. 2016) only in the Gulf of San José. However, the fishery has been characterised by spasmodic recruitment over the last few decades. Overfishing and sweepstakes reproductive success can reduce genetic diversity of marine populations, limiting their viability and evolutionary potential (Hedgecock and Pudovkin 2011; Pinsky and Palumbi 2014). In addition, the fishery is currently managed as a single stock, with a global total allowable catch for the entire gulf.

As in other scallops, Tehuelche scallops display a metapopulation structure with highly dense banks connected to each other through larval flow (Soria et al. 2016). The retention-dispersal processes, in conjunction with the intensity and direction of the larval flow, may determine the patterns of population structure, either at the demographic or genetic level (Lowe and Allendorf 2010; Orensanz et al. 2016). In this sense, larval flow is constrained by the duration of the planktonic larval stage, estimated at 1 month (Narvarte and Pascual 2003). In the gulfs of northern Patagonia, the Tehuelche scallop may spawn from spring to late summer/early autumn. The timing of spawning in different grounds is mostly asynchronous, probably triggered by local conditions (Amoroso 2012; Soria et al. 2016). During the reproductive season connectivity between populations may be regulated by persistent oceanographic fronts operating at large scales (Amoroso and Gagliardini 2010; Tonini et al. 2013; Pisoni et al. 2015). Such oceanographic features may define the recurrent location of the grounds, the

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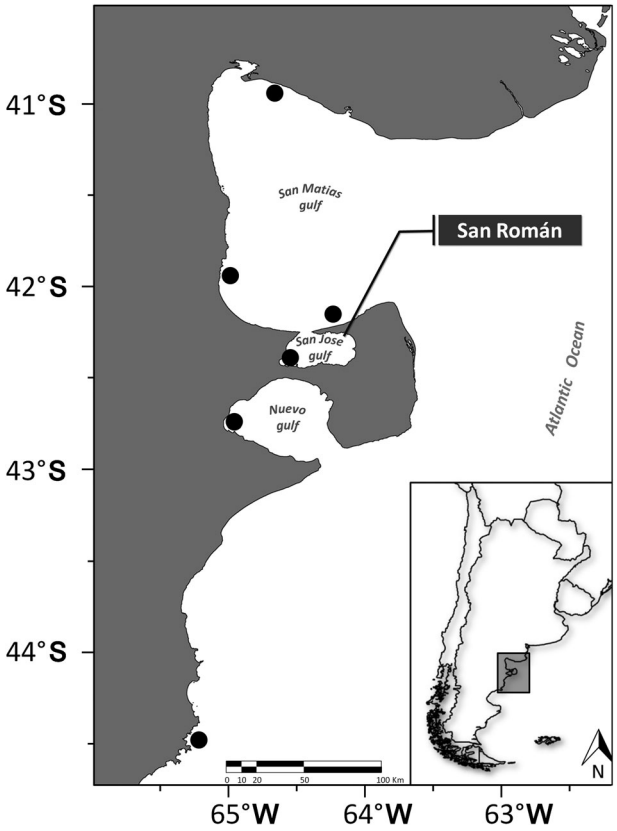

Figure 1. Map of Argentina showing the locations discussed in the text, including San Román within the Gulf of San José where tissue samples were collected. Black dots represent some of the known populations of the Tehuelche scallop.

source-sink connectivity pathways and the ability to recover after severe fishing depletion of local grounds.

The only existing genetic study conducted on the Tehuelche scallop (Real et al. 2004) suggested that the subpopulations of *A. tehuelchus* from the Patagonian coasts do not constitute a single panmictic population, based on paired F_{ST} values of allozymes. However, when compared to allozymes, microsatellite markers would provide a more powerful method to evaluate gene flow among populations due to a higher polymorphism, occurrence of private alleles, their utility for fine-scale assignment of individuals to populations and for estimating demographic parameters including migration rates, relatedness and effective size (Jarne and Lagoda 1996; Estoup et al. 1998). The goal of the present study was to isolate and characterise microsatellite loci for the Tehuelche scallop by means of next generation sequencing (NGS). The use of NGS methods is suitable for sequencing of non-model species, and enables the isolation of microsatellite markers in minimum time and at affordable cost (Ekblom and Galindo 2011).

Materials and methods

We obtained 32 samples of Tehuelche scallop from San Román, Gulf of San José, Patagonia, Argentina (Figure 1). We preserved tissue from abductor muscle in 96% ethanol and stored it at −20 °C. We extracted genomic DNA using the DNeasy Blood and Tissue kit (QIAGEN) following the specifications of the manufacturer. We constructed a shotgun genomic library using c. 5 µg of genomic DNA from a single individual after treating the sample with RNAse. We sequenced DNA using 454 Titanium chemistry (Roche Applied Science) at the University of Arizona Genetic Core facilities. We used the software iQDD (Meglécz et al. 2010) to search and design primers for perfect di-, tri- and tetranucleotide microsatellite loci with at least 10 repeats. We tested 30 microsatellite loci (6 tri-nucleotide and 24 di-nucleotide) with the highest number of repeats (10+). To allow fluorescent labelling, we added the universal M13 primer at the 5′ end of all forward primers (Schuelke 2000). We conducted polymerase chain reactions (PCRs) in 15 µL volumes with 20–40 ng genomic DNA, 1 × PCR buffer, 0.2 mM each dNTP, 1.5 mM $MgCl_2$, 0.2% BSA, 0.5 U *Taq* DNA polymerase (Apex, Bioresearch Products), 0.02 µM of the unlabelled M13-tailed forward primer, 0.2 µM of the fluorescently labelled 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 PCR products using an Applied Biosystems 3730XL sequencer and scored alleles using GENEMAKER v2.6.0 (SoftGenetics LLC). We assigned bins to allele sizes using FLEXIBIN (Amos et al. 2007). We calculated observed and expected heterozygosities and number of alleles using GENALEX 6.5 (Peakall and Smouse 2012). We used MICROCHECKER 2.2.3 (Van Oosterhout et al. 2004) to test for genotyping errors and presence of null alleles. Additionally, we calculated the null allele frequency by locus using the expectation maximisation (EM) algorithm proposed by Dempster et al. (1977) implemented in the software FreeNA (Chapuis and Estoup 2007). We estimated deviations from Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium (LD) using GENEPOP 4.2 (Raymond and Rousset 1995) and FSTAT 2.9.3.2 (Goudet 1995), respectively. We used a sequential Bonferroni test for multiple comparisons (Rice 1989) to adjust *P*-values ($\alpha = 0.05$).

We conducted exploratory population analyses including pairwise relatedness (r) as described by Queller and Goodnight (1989) implemented in GENALEX (Peakall and Smouse 2012). We employed the software COLONY (Jones and Wang 2010) to evaluate the degree of sibship between samples and to estimate effective population size according to sibling proportions via full likelihood with both random mating and nonrandom mating (Wang 2009). Additionally, we estimated the contemporary effective population size (N_e) via the LD method with a bias

correction, a lowest allele frequency of 0.05 and 0.02 and with the Molecular Coancestry (M_C) method as implemented in the software NE-ESTIMATOR v2 (Do et al. 2014).

To test for a recent bottleneck, we compared the observed allele frequency distribution with that of a population in mutation–drift equilibrium assuming the TPM (two phase model) (Di Rienzo et al. 1994) with 95% stepwise mutation model (SMM) and 5% infinite allele model (IAM). We tested deviations between the observed and expected frequency distributions using three statistical tests: a sign test, a standardised difference test (STDV) and a Wilcoxon's signed rank test. We calculated all tests with the software BOTTLENECK and 10,000 iterations (Piry et al. 1999). We also tested for an historic bottleneck with the M-ratio test (Garza and Williamson 2001), which measures the decline in the number of alleles over the range in allele sizes that occurs during a prolonged bottleneck due to sampling effects. We tested the significance of the mean observed M-ratio value across loci by comparing it with an expected distribution generated from 10,000 simulations under equilibrium, assuming 20% of mutations of more than one step, 3.5 average size of multi-step mutations and 0.0005 mutation rate for microsatellite loci under a range of potential historical N_e values (200, 500, 1000, 2000, 10000).

Results

Once we eliminated the barcode used to identify the library and applied custom sequence quality criteria (Q ≥ 20 over a 10 bp window), we obtained 52.93 Mb of sequence distributed in 112,518 individual reads with a mean length of 470 bp. We obtained 81 loci that met our criteria and for which primers were designed. All 30 loci tested (GenBank accession numbers KX879698–KX879727; Table 1) were polymorphic (range 2–22 alleles) and a statistical test could not reject the null hypothesis of LD between pairs of loci ($P \geq 0.000115$), suggesting they are independent and unlinked. The probability of individual identity, or two individuals showing the same multilocus genotype by chance, was low (2.2^{-38}). However, 17 loci showed significant deviations from HWE due to an excess (3 loci) or a deficit (14 loci) of heterozygote individuals, even after sequential Bonferroni test ($P \leq 0.00167$) (Table 1). At the 13 remaining loci found in HWE (AETE3, AETE6, AETE11, AETE22, AETE28, AETE34, AETE35, AETE36, AETE38, AETE39, AETE40, AETE53 and AETE58), the number of alleles per locus ranged from 4 to 19 (mean 10.65) and the number of effective alleles ranged from 2.31 to 14.84 (mean 6.12). Observed heterozygosity ranged from 0.53 to 0.97 (mean 0.744) and expected heterozygosity ranged from 0.57 to 0.93 (mean 0.766). We found no evidence of large allele drop-out, but detected null alleles in 21 of the 30 loci tested according to the EM method. The frequency of null alleles (EM) among all 30 loci ranged from 0.00 to 0.40 (mean 0.123). The frequency of null alleles (EM) in the 13 loci in HWE ranged from 0.00 to 0.13 (mean 0.040). In this regard, only four loci of those in HWE showed a moderate null allele frequency (EM, range 0.075–0.133; AETE11, AETE28, AETE34 and AETE40), according to the classification of Chapuis and Estoup (2007).

The distribution of pairwise relatedness values was skewed towards unrelated individuals (i.e., values below zero) (Figure 2). We found no evidence with the software COLONY for the presence of any full-sib pairs. However, we detected seven pairs of individuals (14–16, 27–29, 2–12, 14–31, 23–30, 5–18 and 13–28) that showed a high probability ($P \geq 0.75$) of being half-sibs, representing only 1.41% of all potential individual pairs (496 total). The N_e ranged from 23.3 (M_C method, 95% C.I. 7.6–47.7) to 330.4 (LD method with lowest allele frequency 0.02, C.I. 126.8-∞). Most estimates suggested N_e approached c. 100 individuals, including the sibship methods (random mating N_e = 96 C.I. 59–186; and nonrandom mating N_e = 117, C.I. 73–239) and the LD method with lowest allele frequency 0.05 (N_e = 126.8, C.I. 65.4–761). We did not find any significant evidence for a recent bottleneck (sign test P = 0.55; STDV P = 0.39 and Wilcoxon test P = 0.21), and a lack of support in the data for the presence of a historical bottleneck (Observed M ratio = 0.8203, Critical M values ≤ 0.7493).

Discussion

Null alleles represent mutations in the primer regions that cause primer binding to weaken and/or fail, resulting in a failure to amplify certain alleles during PCR. Null alleles causing heterozygote deficiencies in bivalves are common and widely reported in the literature (Brownlow et al. 2008; Lemer et al. 2011), including other scallops (Pectinidae) (Kenchington et al. 2006; Silva and Gardner 2015). In Tehuelche scallops all 14 loci showing significant deviations from HWE due to a heterozygote deficiency exhibited evidence for the presence of null alleles. Apparent homozygote excess caused by the presence of null alleles could potentially bias allele frequency estimates, overestimating inbreeding and genetic differentiation among populations (Kenchington et al. 2006; Chapuis and Estoup 2007). Although loci with null alleles are generally avoided, some population genetic analyses such as population differentiation via F_{ST} values can be estimated considering the frequency of null alleles (Chapuis and Estoup 2007).

Our sampled individuals from the population of Tehuelche scallops at San Román did not show signs of significant reductions in effective population size in the present or during the recent past, and currently individuals seem to display high levels of genetic

Table 1. Characteristics of 30 microsatellite loci for the Tehuelche scallop *Aequipecten tehuelchus*, including: locus name, GenBank accession number, repeat motif/dye, forward (F) and reverse (R) primer sequences, expected size in base pairs (bp), size range of observed allelic variation, number of samples (N), number of alleles (Na), number of effective alleles (Ne), observed (Ho) and expected (He) heterozygosities, probability of Hardy-Weinberg equilibrium (HWE) (adjusted critical value after Bonferroni test $P = 0.00167$), null allele frequency according to expectation maximisation (EM) and Oosterhout methods. Italicised probability values indicate loci in HWE.

Locus GenBank	Repeat Motif + Dye	Primer sequence (5′–3′)	Expected size	Size range	N	Na	Ne	Ho	He	HWE	Null allele frequency	
											EM	Oosterhout
AETE2 KX879698	$AC_{(32)}$ + 6-Fam	F:TCACAAAACATCCGTAGCCTC R:GGTTGGACCTAACGCCCTAT	156	(150–204)	32	21	15.9	0.625	0.937	0.000	0.159	0.1676
AETE3 KX879699	$AC_{(29)}$ + 6-Fam	F:TCATTTAAACAATTCACGGCA R:CACTGCAGCAGGGTTTACCT	217	(169–194)	32	5	2.7	0.969	0.636	*0.025*	0.000	−0.3207
AETE4 KX879700	$AGT_{(25)}$ + 6-Fam	F:AGCCCAAACACCTGAACATC R:GGCAATGCTTTGACGGTAAT	227	(225–335)	32	22	14.4	0.500	0.931	0.000	0.223	0.231
AETE6 KX879701	$AC_{(22)}$ + 6-Fam	F:TGGTTAGGTCCGCAGAAGAT R:TCAAATGCTTTCCTTTGGAGA	177	(157–195)	32	16	10.7	0.906	0.906	*0.206*	0.007	−0.0017
AETE8 KX879702	$AT_{(19)}$ + 6-Fam	F:CATGAAATCATGACGTAACAATCA R:CAACATCAAATATGCGTTAAACA	220	(220–270)	31	15	9.2	0.581	0.891	0.000	0.182	0.1622
AETE10 KX879703	$AC_{(18)}$ + 6-Fam	F:ATGGAAATCCTTGATCGTGC R:TTGTATGTACGTGAAGAGAACCG	136	(144–160)	32	8	2.4	0.313	0.582	0.000	0.186	0.2104
AETE11 KX879704	$AC_{(17)}$ + 6-Fam	F:ATTTCTGCTTCATGCTTGGC R:TGTATGCATCGGTAGGTCTTG	103	(103–128)	30	14	7.3	0.700	0.862	*0.208*	0.075	0.0992
AETE13 KX879705	$AAT_{(17)}$ + 6-Fam	F:GGCTAACCATGCTCATCCTC R:GAACCTGGGAATCGAAGTGA	222	(197–247)	32	9	4.1	0.344	0.758	0.000	0.235	0.2669
AETE15 KX879706	$AT_{(16)}$ + 6-Fam	F:CCTCGCAATCTACATACAGCC R:CTATCCTTCGTGTGCCCAAC	260	(261–279)	24	6	3.8	0.042	0.735	0.000	0.402	0.4341
AETE17 KX879707	$AT_{(15)}$ + 6-Fam	F:TGCATTTAGAACTCACCTTTGG R:CATTGCATATGGTAGCATGG	176	(177–227)	32	14	8.3	0.500	0.88	0.000	0.203	0.2133
AETE18 KX879708	$AT_{(15)}$ + 6-Fam	F:GACACGAAGACCCTTCTTCAA R:TACACCACCTGATCACCGAA	190	(192–218)	30	20	10.8	0.700	0.908	0.000	0.105	0.1153
AETE19 KX879709	$AT_{(14)}$ + 6-Fam	F:TCGGTCGATAAATTAGGATTTGA R:CGTCACCTGTACATGGAAATC	113	(115–152)	32	17	11.8	0.594	0.915	0.000	0.167	0.1753
AETE22 KX879710	$AAT_{(14)}$ + 6-Fam	F:CACGGTGATACAGATTCCGA R:AAAAGAGGCGGAGTAAGGGA	212	(212–252)	31	9	6.3	0.839	0.842	*0.114*	0.000	0.0014
AETE23 KX879711	$AGG_{(14)}$ + 6-Fam	F:TGATGCATACAATCTCCCGA R:GTTGGCAGACACAGTGCAAC	178	(168–198)	29	10	7.2	0.414	0.86	0.000	0.240	0.256
AETE24 KX879712	$AT_{(14)}$ + 6-Fam	F:TGTGCCGAAACACAAAGAAA R:TTTGCAATCAGTTGAGCCTG	287	(280–290)	31	2	2.0	1.000	0.5	0.000	0.000	−1

AETE28 KX879713	$AT_{(13)}$ + VIC	F:GCGCACCTTAATGTGAATGA R:CGGGTGACTTTGACATGAAAT	152	(145–195)	32	19	6.7	0.656	0.851	*0.080*	0.097	0.1182
AETE34 KX879714	$AT_{(12)}$ + VIC	F:AAGCAAATCAAAATCAAAACGAA R:TCAAACCTGCCCTAGTGACC	264	(277–302)	31	14	9.7	0.645	0.897	*0.053*	0.133	0.1397
AETE35 KX879715	$AAT_{(12)}$ + VIC	F:CACGGGTAGAGATAAGATGTGTT R:TGGCTGGATATTGCTCTCATT	189	(185–277)	31	17	7.2	0.774	0.861	*0.805*	0.049	0.0477
AETE36 KX879716	$AT_{(11)}$ + VIC	F:AGCTACACGATATACAGTTGTGGT R:AGGTAGACCACACCAGACGG	131	(145–156)	32	4	2.3	0.781	0.565	*0.022*	0.000	−0.3002
AETE38 KX879717	$AT_{(11)}$ + VIC	F:AACGAATGCAGTAAACGCTG R:ACAATAAAAGGCATGCGACC	255	(270–277)	32	4	2.6	0.719	0.621	*0.302*	0.000	−0.1065
AETE39 KX879718	$ACT_{(11)}$ + VIC	F:CACAATTAGTACCGCCTTGGA R:AACAGAAGGTGCAGGAGAGC	282	(287–355)	32	18	14.8	0.875	0.933	*0.112*	0.042	0.029
AETE40 KX879719	$AT_{(11)}$ + VIC	F:AGACCAGGATGTTACCAGGG R:CTCACGCACCAACACATCTC	203	(213–228)	32	8	3.9	0.531	0.746	*0.145*	0.122	0.1411
AETE43 KX879720	$AT_{(11)}$ + VIC	F:CTTGCGTGTAACTGCATCGT R:TCCGAGATAAGAGGGTGGTG	136	(139–152)	32	6	2.9	1.000	0.651	0.000	0.000	−0.3529
AETE44 KX879721	$AT_{(11)}$ + VIC	F:CGAAACTGAACAGTTGCTGA R:ACATGTGCATCACATTGGCT	234	(246–260)	32	7	4.6	0.188	0.784	0.000	0.333	0.3668
AETE45 KX879722	$AT_{(10)}$ + VIC	F:GCTAAAATCGCAATTCCGTC R:ATCTACGGCACCAAATCGTC	141	(142–162)	32	6	2.0	0.156	0.492	0.000	0.242	0.2889
AETE47 KX879723	$AT_{(10)}$ + VIC	F:CAACCTTGAGCTCTTTTACGC R:TGCATGCAAATTTTAACAGAAAA	218	(199–247)	32	9	3.4	1.000	0.709	0.000	0.000	−0.2376
AETE48 KX879724	$AT_{(10)}$ + VIC	F:CCCCAACCCTAGCAACTTCT R:GCAAACAAGACAAGCTTCAGTC	107	(124–162)	31	18	14.7	0.323	0.932	0.000	0.316	0.3238
AETE52 KX879725	$AT_{(10)}$ + VIC	F:GCGAAGGAATACGGAAATCA R:TACCTCATGTCGCCGATGTA	183	(192–204)	31	6	3.0	0.452	0.668	0.000	0.160	0.1311
AETE53 KX879726	$AT_{(10)}$ + VIC	F:TGCATACGTGAAACTCTTTCTATCA R:CAGTACACAGTAATCAGGTGGACA	170	(184–195)	32	6	2.6	0.688	0.62	*0.853*	0.000	−0.0536
AETE58 KX879727	$AT_{(10)}$ + VIC	F:TCCGAATGTCCGTTTGTATG R:CGTCATTAATGTGGACAGCG	306	(317–324)	32	4	2.6	0.594	0.622	*0.730*	0.000	0.0307

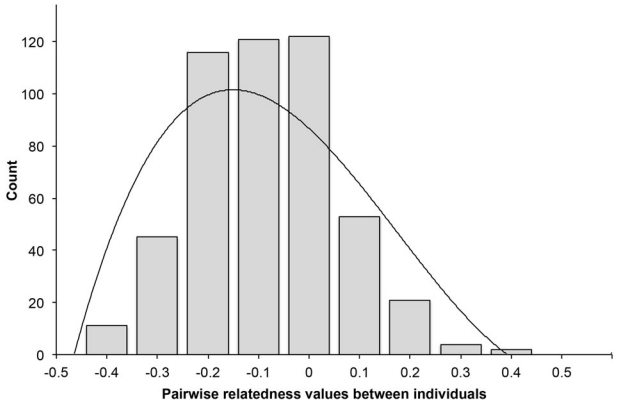

Figure 2. Frequency distribution of pairwise relatedness values obtained using Queller and Goodnight (1989) estimator between 32 individuals of Tehuelche scallops from San Román.

polymorphism and no signs of inbreeding. However, these preliminary results should be viewed with caution given our relatively small sample size from a single population that could bias many of the analyses performed. For example, the LD method could significantly underestimate N_e when sample size is less than the true N_e due to a large sampling error (Waples 2006). In addition, our results should be evaluated in the context of gene flow and divergence with other populations, since admixture can create LD due to a two-locus Wahlund effect (Waples 2006).

Although our preliminary results are in line with a scenario of a population in relatively good genetic health that is still supporting a viable fishery, it would be necessary to sample other nearby populations to verify the contribution of the population from San Roman to source-sink metapopulation dynamics, particularly regarding unexploited larval sources that may exist in deeper waters (Fiorda et al. 2013) and also outside the Gulf of San José (Picallo 1980). Contrasting the levels of genetic diversity and structure of scallops from the Gulf of San José with other overfished populations from northern Patagonia that have not yet recovered (e.g., from the Gulf of San Matías) could give important insights into patterns of larval dispersal and their respective viabilities and resilience to overexploitation. The microsatellite loci described here will be of great value for advancing research questions about genetic diversity, metapopulation structure, marine connectivity and inbreeding, which are relevant for the conservation and sustainable management of the species in Northern Patagonia.

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Disclosure statement

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