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Short communication

Utility of five SSR markers for genetic diversity and paternity exclusion analysis in the Patagonian toothfish

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ABSTRACT. The Patagonian toothfish or Chilean sea bass (*Dissostichus eleginoides*), found in the Southern Ocean surrounding Antarctica, is an important fishery species for Chile. This high-value species is regarded as overfished, making it an attractive target for aquaculture. When developing a reproduction program for any aquaculture species, it is important to implement genetic tools to evaluate diversity, inbreeding, and parentage. We calculated genetic diversity and paternity/maternity exclusion probabilities based on five commonly-used microsatellite *loci* in a natural population of Patagonian toothfish from southern Chile (n = 34) in order to evaluate the potential utility of these five markers in stock management. The observed number of alleles per *locus* (N_a) and observed heterozygosities (H_O) are within range as described by studies performed in other sub-Antarctic regions. All five *loci* were strongly polymorphic, with $H_O > 0.6$ and $N_a > 7$, and paternity exclusion probabilities were high ($PE > 0.99$), indicating the potential utility of these *loci* in paternity/maternity exclusion analyses of Patagonian toothfish.

Keywords: *Dissostichus eleginoides*, Chilean sea bass, heterozygosity, microsatellites, genetic diversity.

The Patagonian toothfish (*Dissostichus eleginoides*), also marketed as "Chilean sea bass," is an important commercial fishery species found in the Southern Ocean surrounding Antarctica. It has been regarded as overfished since the 1990s (Marko *et al.,* 2011*;* Reyes *et al.,* 2012). The species is a benthopelagic fish with a long lifespan and is widely distributed along the southern Chilean coast, Patagonian shelf, and sub-Antarctic islands and seamounts at depths of 1000-2000 m (Appleyard *et al.,* 2002). Commercial fishing of the Patagonian toothfish is on the rise due to its market value (which is higher than that of salmon, for instance), and international regulations govern sustainable fishery of the species (Marko *et al.,* 2011). Therefore, the Patagonian toothfish is an attractive target for Chilean aquaculture (Reyes *et al.,* 2012). Chilean government agencies currently provide strong incentives and funding for aquaculture programs with new marine species. In Chilean Patagonia, aquaculture research projects are establishing *Dissostichus eleginoides* broodstock as a basis for developing culture technology. This research is focused on obtaining high-quality juveniles, addressing issues such as nutrition and genetics, and maximizing maturation and reproductive performance. In this context, genetic tools are needed to support reproduction programs in assessing genetic diversity, inbreeding, and parentage. Short-sequence repeats (SSR), or microsatellites, with high levels of polymorphism have been the most useful molecular markers for evaluating marine fish populations. We tested five microsatellite markers (cmr*De*2, cmr*De*4, cmr*De*9, cmr*De*13, and cmr*De*30) (Reilly & Ward, 1999) in an offshore Chilean population. These markers had been commonly used in genetic diversity studies to evaluate the distribution and population structure of the Patagonian toothfish in the

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Southern Ocean, Antarctic Islands, Patagonian shelf (Falkland/Malvinas Islands), and Atlantic, West and Western Indian Ocean sectors (Smith & McVeagh, 2000; Appleyard *et al.,* 2002, 2004; Shaw *et al.,* 2004; Rogers *et al.,* 2006). Our main objectives were to evaluate genetic diversity and estimate paternity/ maternity exclusion probabilities, in order to evaluate the potential utility of these five markers in broodstock management.

We studied 34 fishes, collected in September-October of 2011 from two sampling points in Chilean Patagonia in front of Trinidad Gulf (Magallanes and Chilean Antarctic region): n = 19 from point one (49º29'41"S, 76º26'17"S) and n = 15 from point two (50º15'05"S, 76º20'15"W). Genomic DNA was extracted using the phenol-chloroform method from fin-clip samples (Taggart *et al.,* 1992). Microsatellite analysis was performed using five markers developed for this species (cmr*De*2, cmr*De*4, cmr*De*9, cmr*De*13, and cmr*De*30) (Reilly & Ward, 1999) and the M13-tailed primer method (Schuelke, 2000) (Table 1). PCR amplifications were carried out as 15-µL reactions containing 10x PCR buffer (10 mM Tris-HCl, 50 mM KCl, 1 mM EDTA, Triton X-100 0.1%), 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 8 pmol reverse primer, 8 pmol fluorescent-labeled universal M13 primer, 2 pmol forward primer, 1 U Taq polymerase (Biotools®), and 2-µL DNA samples. The thermal profile was 94°C for 2 min, followed by 30 cycles at 94°C (30 s), specific marker Tm (Table 1) (45 s), 72°C (45 s), 8 additional cycles at 94°C (10 s), 53°C (45 s) to bind M13-labeled primer, and 72°C (45 s) with a final 10-min extension step at 72°C.

Different amplicons labeled with different florescent dyes were pooled in one reaction tube and run with a GeneScan™ 500 LIZ™ dye-size standard (Applied Biosystems®) in an automatic sequencer at external laboratories. Genotypes were identified using GeneMarker® software (SoftGenetics®). Genetic diversity was assessed according to observed (H_O) and expected (H_E) heterozygosities, number of observed alleles by *locus* (N_a), polymorphism information content (*PIC*), and paternity/maternity exclusion probabilities (*PE*), with Cervus 3.0 software (Araneda *et al.,* 2004; Kalinowski *et al.,* 2007). The presence of null alleles was assessed with six different estimators (Dempster *et al.*, 1977; Chakraborty *et al.*, 1992; Brookfield, 1996; Van Oosterhout *et al.,* 2004; Kalinowski *et al.,* 2006), using Micro-Checker 2.2.3 (Van Oosterhout *et al.*, 2004), Cervus 3.0 (Kalinowski *et al.*, 2007) and FreeNA (Chapuis & Estoup, 2007). Genepop 4.2 (Rousset, 2008) was used to evaluate deviation from Hardy-Weinberg expectations along with pairwise F_{ST} to evaluate population differentiation between the two collection sites.

Genetic differentiation did not vary significantly between the two sampling points (F_{ST} = -0.002; P > 0.05), possibly due to their geographical proximity. There-fore, in subsequent analyses, all 34 samples were used together and considered to belong to the same biologic population.

N_a ranged from 8 (cmr*De*13) to 25 alleles (cmr*De*9) (Table 2). These two *loci* have also shown the lowest (cmr*De*13) and highest (cmr*De*9) allele numbers in other studies performed with the five markers used here (Reilly & Ward, 1999; Smith & McVeagh, 2000; Appleyard *et al.*, 2002, 2004; Shaw *et al.*, 2004; Rogers *et al.*, 2006).

Two *loci* (cmr*De*2 and cmr*De*9) showed significant deviations from H-W expectations, both with deficits of heterozygotes, possibly due to the presence of null alleles or the small sample size (Table 3).

Locus cmr*De*9 has also shown significant deviation from H-W expectations in previous studies (Appleyard *et al.*, 2002, 2004; Shaw *et al.*, 2004; Rogers *et al.*, 2006), suggesting that this finding may also reflect difficulties in scoring this *locus* (Rogers *et al.*, 2006). In general, all null allele frequency estimators gave similar results. Only one *locus* showed a null allele frequency above 10% (cmr*De*2) according to all estimators except the Brookfield-2 method, which indicated higher null allele frequencies for three *loci* (cmr*De*2, cmr*De*9, and cmr*De*30) (Table 3). This method assumes that all individuals that fail in amplifications are null homozygotes (Brookfield, 1996). It is widely accepted that if the null allele frequency is below 10%, relatedness/parentage estimators can be used directly, without adjustment; on the other hand, *loci* with null allele frequencies above 50% must be excluded, due to very low power (Huang *et al.*, 2016). To avoid this distortion, and to evaluate the power for paternity/maternity exclusion analysis, two datasets were used, one with and one without genotype adjustment, with the Brookfield-2 method using Micro-Checker 2.2.3.

All genetic diversity estimates were within the ranges published in previous works on Patagonian toothfish from geographic areas other than the Chilean coast, where observed heterozygosities ranged from 0.5261 for *locus* cmr*De*13 (Rogers *et al.,* 2006) to 0.9480 for *locus* cmr*De*9 (Smith & McVeagh, 2000) (Table 2). Our findings were consistent with these previous results showing high levels of genetic diversity. In our work, all *loci* were strongly polymorphic with observed heterozygosities >0.6 and PIC >0.7, indicating their potential utility in paternity exclusion analysis (Table 2).

Table 1. SSR markers and characteristics of primers used in genetic analysis of Patagonian toothfish. Underlined sequences in forward primer indicate tail to bind M13-labeled primer.

Locus	Primer sequence (5'-3')	Tm (ºC)	Repeat motif	Dye	GenBank accession number
cmr*De*2	**F**: TGTAAAACGACGGCCGAGACCTCTGACAGGGTAG	66	CAA	PET	AF105071
	R: TGACAGATGTTTTCTGATTAAG	59			
cmr*De*4	**F**: TGTAAAACGACGGCCAGTATCCCAACACCAAGGCTCTATCCA	67	CAA	VIC	AF105072
	R: CCGCCTATGAGAGTCATCACGTTT	56			
cmr*De*9	**F**: TGTAAAACGACGGCCTCCTAATACTGCAGCTGTGTC	65	CA	NED	AF105073
	R: GAATCCAGAGTGTTACATAGTGA	52			
cmr*De*13	**F**: TGTAAAACGACGGCCAGAAGACAGGATAAACACACTGC	65	CA	VIC	AF105074
	R: ACCCATTTTCTTGCCCCTTTG	56			
cmr*De*30	**F**: TGTAAAACGACGGCCCTGACCTTTAACCTGCGCAAT	67	CA	VIC	AF105075
	R: ACCTCACTGATAAGGAAAGGTACTG	48			

Table 2. Genetic diversity in Patagonian toothfish collected from Southern Chile. *Na*: number of observed alleles by *locus*, H_O: observed heterozygosity, H_E: expected heterozygosity, *PIC*: Polymorphism information content, PE_1: paternity/maternity exclusion probability for a single parent, PE_2: paternity/maternity exclusion probability for a single parent when one parent is known, PE_I: identity exclusion probability for a single individual.

SSR *locus*	cmr*De*2	cmr*De*4	cmr*De*9	cmr*De*13	cmr*De*30
N	26	32	28	34	31
N_a	16	12	25	8	10
Allele range size (bp)	118-180	247-283	203-267	137-161	165-185
H_O	0.615	0.844	0.786	0.706	0.645
H_E	0.912	0.869	0.966	0.788	0.804
PIC	0.885	0.840	0.947	0.746	0.766
H-W expectations (*P*-value)	<0.001	0.489	<0.001	0.6153	0.017
Non-adjusted genotypes					
PE_1	0.652	0.552	0.813	0.399	0.430
PE_2	0.789	0.713	0.897	0.578	0.608
PE_I	0.980	0.963	0.995	0.920	0.931
Adjusted genotypes					
PE_1	0.701	0.552	0.817	0.399	0.486
PE_2	0.824	0.713	0.899	0.578	0.658
PE_I	0.986	0.963	0.995	0.920	0.948

The global paternity exclusion probability for one putative parent using only the molecular information from progeny was high enough to perform a paternity/maternity exclusion analysis in a breeding population with five polymorphic SSR *loci* (PE_1 = 0.98999 and PE_1 = 0.99245 with adjusted genotypes) (Araneda *et al.,* 2004). However, in a reproduction program, even if no physical tagging is performed, it is always possible to genotype all breeders. Adding genotyping information from the other parent and progeny increases the combined paternity/maternity exclusion probability. When both datasets are available, the paternity/maternity exclusion probability for a single parent can be increased to 0.99897 and 0.99927 (adjusted genotypes) when the other parent is known (PE_2). Finally, the combined identity exclusion probability was PE_I = 0.99999998 and PE_I = 0.9999 9999 (adjusted genotypes), indicating that these five SSR markers show excellent potential for identifying an individual in a sample using its known genotype. While the presence of null alleles decreases the exclusion probability, a comparison of the statistics with and without adjustments reveals this reduction to be minor. In the context of a breeding population, the effect of a null allele is to mistakenly reject a true father, due to a lack of observed shared alleles with the offspring; that is, the number of real parents is underestimated. This scenario (type I error) is preferable to including a nonrelated individual as a parent (type II error) for breeding applications.

The potential application of these markers in paternity/maternity exclusion analysis must be evaluated in real families after Chilean reproduction aquaculture projects begin to produce families with viable offspring.

Table 3. Null allele frequencies for five SSR *loci* in Patagonian toothfish using six different estimators

	Van Oosterhout *et al.* (2004)	Chakraborty *et al.* (1992)	Brookfield (1996) Index 1	Brookfield (1996) Index 2	Kalinowski *et al.* (2006)	Dempster *et al.* (1977)
cmr*De*2	0.1586	0.1847	0.1472	0.4519	0.1904	0.1402
cmr*De*4	0.0067	0.0069	0.0063	0.1244	0.0067	0.0000
cmr*De*9	0.0863	0.0941	0.0838	0.3265	0.0947	0.0842
cmr*De*13	0.0471	0.0478	0.0399	0.0399	0.0498	0.0323
cmr*De*30	0.0905	0.1018	0.0816	0.2385	0.1010	0.0814

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