

RESEARCH NOTE

Development of six novel microsatellite markers for the Chilean red alga *Pyropia orbicularis*

Desarrollo y caracterización de seis marcadores microsatélites para el alga roja chilena *Pyropia orbicularis*

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Abstract.- Mariculture of edible *Porphyra/Pyropia* species, which are cosmopolitan red foliose algae, represent an important source of income for the pharmaceutical and food industries. In Chile, the most common alga of this complex is *Pyropia orbicularis* (Bangiales, Rhodophyta). Here we report 6 microsatellite markers obtained from *P. orbicularis* through a genomic library from 10 individuals of the gametophytic phase by next generation (Illumina) sequencing. Polymorphism analyses were done from 10 individuals of the conchocelis phase, revealing an allelic diversity ranging from three to six alleles per locus, and observed heterozygosity (HO) and expected heterozygosity (HE) ranging from 0.200 to 0.995, and 0.250 to 0.610, respectively. A large proportion of genetic variance (61%) in *P. orbicularis* was among individuals within population, and 39% genetic variance was among populations. Nonetheless, these results should be interpreted with caution; further research using larger sample sizes is required. These polymorphic markers could be useful in future studies on population genetic structure, for conservation and applied purposes.

Key words: *Pyropia orbicularis*, microsatellite markers, Rhodophyta

INTRODUCTION

A recent research on the Bangiales order (Rhodophyta) based on the mitochondrial *COI* and chloroplastal *rbcL* genes, found 18 new species along the Chilean coast (1 belonging to the *Wildemanina* genus, 1 to *Fuscifolium*, 8 to *Porphyra*, and 8 to *Pyropia*) (Guillemin *et al.* 2016). Worth highlighting among these new Chilean species is *Pyropia orbicularis*. This alga is widely distributed along the Chilean coastline, extending from 32°S to 53°S, making it the most conspicuous foliose species of the Bangiales order in Chile (Ramírez *et al.* 2014, Guillemin *et al.* 2016). *P. orbicularis* primarily inhabits the upper intertidal zone (Meynard *et al.* 2019), where this alga is able to acclimate to long periods of desiccation and rehydration during daily tide cycles (Contreras-Porcía *et al.* 2011). This high plasticity has made this species a biological model on oxidative stress and desiccation tolerance (Contreras-Porcía *et al.* 2013, López-Cristoffanini *et al.*

2015, Guajardo *et al.* 2016, Fierro *et al.* 2017). It is worth mentioning that in Asia *Pyropia/Porphyra* species are intensively cultivated for dietary ends, generating revenues of ≈US\$1.3 billion/year (Blouin *et al.* 2011).

Given the potential socio-economic benefits of *P. orbicularis* derived products to coastal communities, the food and biotechnological industries, there is a need to develop genetic markers for the evaluation of the genetic diversity of this species. The present study addresses this knowledge gap in the Southeast Pacific by developing 6 microsatellite markers for *P. orbicularis* and evaluating their polymorphism. As previously demonstrated by studies using this type of markers, these microsatellite could be useful in studies of population genetic structure (Becerra & Paredes 2000, Aranguren-Méndez *et al.* 2005), evolutionary processes (Zuo *et al.* 2007), and in studies having applied or conservation purposes.

MATERIALS AND METHODS

To construct the genomic library, 10 individuals of the gametophytic phase of *P. orbicularis* (Fig. 1) were collected from the rocky intertidal zone of Maitencillo beach, in central Chile (32°38'57"S; 71°26'34"W). Genomic DNA was extracted following methods reported by Wattier *et al.* (2000). Libraries and sequencing were performed in the core research facilities AUSTRAL-omics of the Universidad Austral de Chile (Valdivia, Chile). Five hundred ng of a DNA mix of 6 gametophytic individuals was fragmented to the desired length (200-1200 bp), and amplified by PCR to construct the genomic library using the Illumina Nextera® XT DNA Library Preparation Kit. The obtained fragments of interest were between 410 and 480 bp. The final concentration of the library was estimated through quantitative PCR (qPCR) in a LightCycler® Nano System (Roche) and using the KAPA Quantification Kit (Illumina Sequencing Platforms). Sequencing was performed using the MiSeq Reagent Kit v2 (500 cycles). Microsatellites were identified using the QDD pipeline program (Megléc *et al.* 2014). Reads having at least 95% identity were clustered as consensus sequences, while those which could not be grouped were classified as singletons, using Blast+ (Camacho *et al.* 2009). Potential primers were designed using the PRIMER v3.0 software (Rozen & Skaletsky 2000). Primers were mainly selected according to the type

and size of the repeated motifs, and the base composition of the flanking regions. Primer selection included also the following criteria: primer length close to 20 bp; 50 to 75% GC content; a distance greater than 20 bp between the primer motif and the repeated motif; and amplicon sizes between 100 bp and 500 bp.

Each amplification test for the 6 loci was conducted using 10 *in vitro* cultivated conchocelis-stage of *P. orbicularis*. Each conchocelis sample was obtained from the cultivation of zygotespores coming from 1 cm piece of a single gametophytic blade. Five gametophytic blades were collected at the rocky intertidal zones of Maitencillo and five at Salinas de Pullally (32°24'41.7"S; 71°24'57.8"W) in Central Chile. Amplification of the microsatellites loci was done by PCR using the M13-tailed primer method, including fluorophores complementary to the forward primer. The 6 microsatellite loci were evaluated through PCR in a final reaction volume of 15 µL, which contained 10-20 ng of DNA template. Each reaction contained 0.15 µM of each primer, 2 mM of MgCl₂, 0.15 mM of dNTPs, 1X of colorless GoTaq flexi buffer, and 0.35 U of GoTaq G2 flexi DNA polymerase. Ultrapure water was added to obtain the final reaction volume. PCR amplifications were conducted on a StepOnePlus™ Real-Time PCR System (Applied Biosystems) with the following thermocycler conditions: initial denaturalization at 95 °C for 5 min,

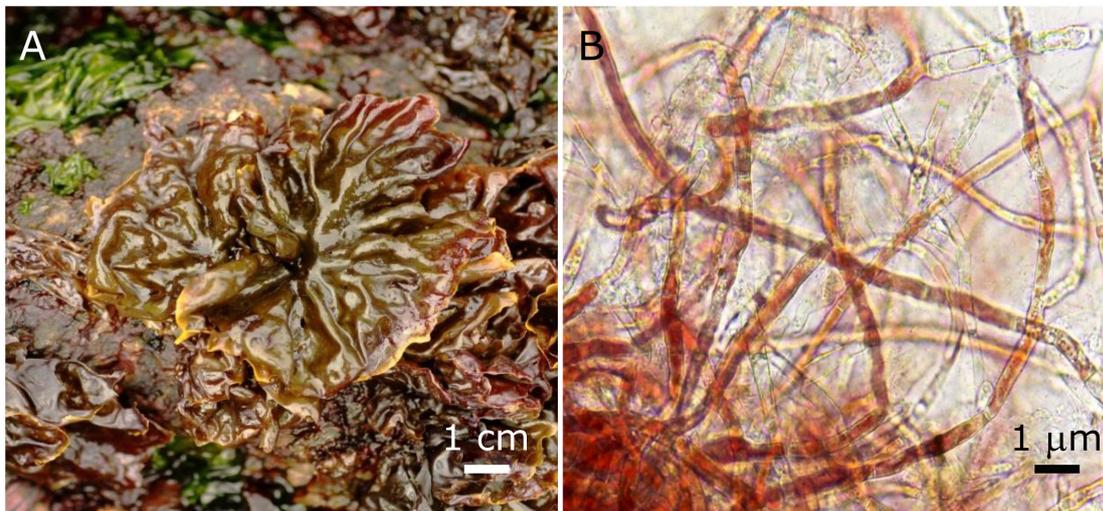


Figure 1. A) Gametophytic phase of *P. orbicularis* used for the creation of the genomic library. B) Conchocelis phase of *P. orbicularis* used for loci evaluation by PCR / A) Fase gametofítica de *P. orbicularis* usado para la creación de la librería genómica. B) Fase conchocelis de *P. orbicularis* usada para la evaluación de los loci a través de PCR

followed by 40 cycles at 95 °C for 30 s, 55-60 °C for 30 s (see Table 1 for specific annealing temperatures of each locus), 72 °C for 30 s, and a final extension at 72 °C for 5 min. The amplified products were resolved on a 10% non-denaturing polyacrylamide gel, stained with GelRed and visualized under UV. PCR products were run on an ABI Prism 310 Genetic Analyzer. Alleles of *P. orbicularis* were identified using the software GeneMarker® (Hulce *et al.* 2011) (Fig. 2). Population genetic diversity indexes (allelic diversity, heterozygosity), inbreeding coefficient (F_{IS}) and genetic distance between the two populations were calculated using the software GenAlEx 6.5 (Peakall & Smouse 2006, 2012). Linkage disequilibrium (LD) and Hardy-Weinberg equilibrium (HWE) were calculated using GENEPOP 4.2 (Raymond & Rousset 1995).

RESULTS AND DISCUSSION

A total read of 8,571,507 bp was obtained, in which more than 5,000 microsatellites were found. Initially, 13 microsatellites markers were constructed that fulfilled the selection criteria previously mentioned. Nonetheless, only 6 pairs of these microsatellite primers were used to analyze

the polymorphisms of the conchocelis phase of *Pyropia orbicularis*. This was done because technical problems were experienced to obtain enough biomass of the cultivated conchocelis for DNA extraction from all samples (only 5 out of the 20 samples originally cultivated per site). Some characteristics of the 6 microsatellite loci are indicated in Table 1. Results showed that the 6 loci had 3-6 alleles per locus and observed and expected heterozygosities ranging from 0.200 to 0.995, and 0.250 to 0.610, respectively. Significant heterozygote deficiency was observed for locus Orb6 in Maitencillo ($P < 0.05$). Linkage disequilibrium calculations for each locus pair indicated that loci are not in linkage disequilibrium. According to the coefficient of gene differentiation (G_{ST}) a large proportion of genetic variance (61%) in *P. orbicularis* was among individuals within population, and 39% genetic variance was among populations, suggesting an important level of differentiation between the two sites distant about 35 km apart. The F_{IS} for each locus was significantly different from zero and ranged from -0.670 to 0.380 (Table 1). In the two sampling sites, some significant negative F_{IS} values were observed, such as at loci Orb7 and Orb8, suggesting a heterozygote excess; on

Table 1. Primers sequences and optimum annealing temperature (Ta) for six microsatellite loci from *Pyropia orbicularis* / Secuencias de los partidores utilizados y temperatura óptima de annealing (Ta) para seis loci microsatélites de *Pyropia orbicularis*

Locus name	Primer sequence	Repeat motif	Size (bp)	Ta (°C)	No. of alleles	HO/HE	F_{IS}	GenBank Accession no.
Orb1	F: GCATGCTGACAGAAAGACGA	(CAGC) ₅	101	60	6	0.330	0.259	MN449481
	R: GTATTGTGCGACCTTGCGTT		135	61		0.490		
Orb5	F: CAGTGATTGGAGTGGCTCAG	(AGA) ₉	190	59	6	0.260	0.153	MN449482
	R: TTTGTCCGTTAAACCAGACG		303	59		0.320		
Orb6	F: TCTGTGGGATATGGAGGCAT	(AC) ₃₆	185	60	5	0.248	0.312	MN449483
	R: ACAAGTTTGTGTCGAGrCGAA		257	60		0.372		
Orb7	F: CGAGGAGTGTACAGAGGGACA	(TTG) ₁₃	252	60	3	0.940	-	MN449484
	R: GGATATAGACGGCATCGAACA		275	60		0.550		
Orb8	F: CCTCTCCTACTGCCTTCACG	(TGAA) ₆	234	60	3	0.995	-	MN449485
	R: ATCGCATTTCAGCATTATCG		286	59		0.500		
Orb9	F: TCAGGGTCAACAGTGCCTC	(TC) ₂₂	316	59	4	0.426	0.380	MN449486
	R: CACAAGGATAAAGATTTAAGGCAAA		338	60		0.829		

F_{IS} : inbreeding coefficient, HO: observed heterozygosity, HE: expected heterozygosity

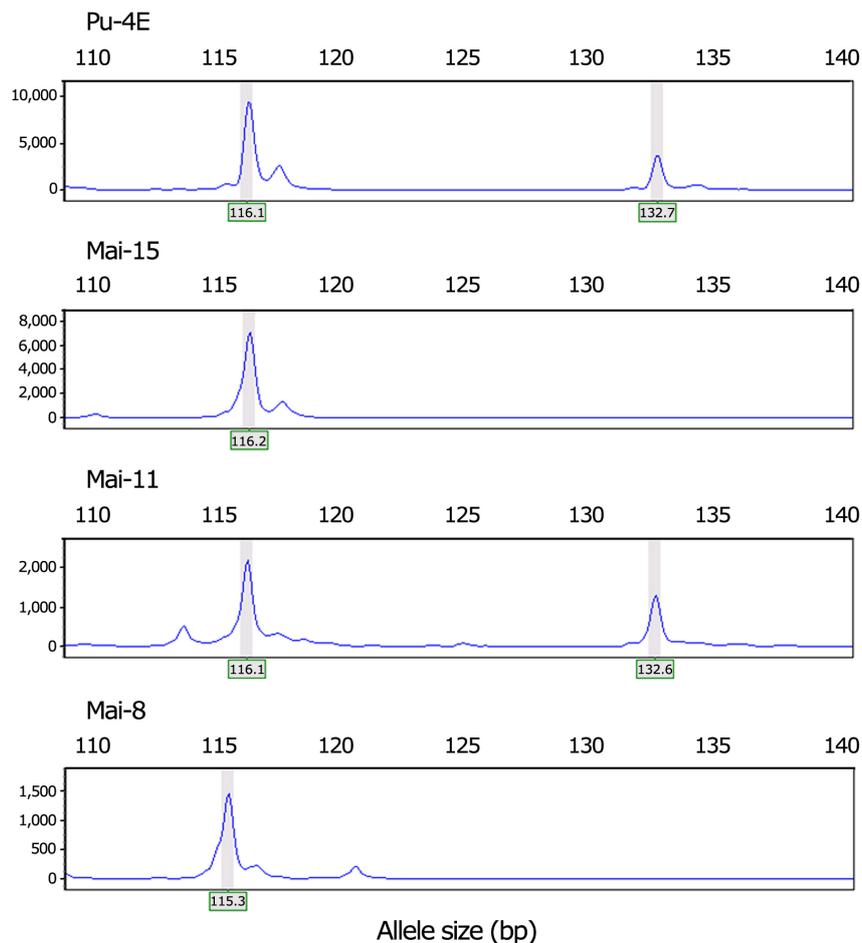


Figure 2. Analysis of genomic fragments from locus Orb1 using samples of *Pyropia orbicularis* from Maitencillo (Mai 8, 11 and 15) and Salinas de Pullally (Pu 4E) employing the software GeneMarker v 1.75 (SoftGenetics LLC, State College, PA, USA). Two homozygous and two heterozygous individuals are observed for this microsatellite / Análisis de fragmentos para el locus Orb1 en muestras de *Pyropia orbicularis* provenientes de Maitencillo (Mai 8, 11 y 15) y Salinas de Pullally (Pu 4E) empleando el programa GeneMarker v 1.75. Se observan dos individuos homocigotos y dos heterocigotos para este microsatélite

the contrary, high positive F_{IS} values were observed at the other loci, suggesting inbreeding or selection. Nonetheless, results should be interpreted with caution due the small sample sizes. Indeed, HWE, LD, F_{IS} and G_{st} should be re-calculated using larger sample sizes to confirm these results. On the other hand, taking into consideration the technical problems previously mentioned; genetic polymorphism determination at the population level would have ideally to use samples from the gametophytic phase rather than the conchocelis phase. Since the gametophytic

phase has been demonstrated to be a genetic chimera (Yan & Huang 2010); a very restricted tissue area of the gametophytic blade would have to be selected for DNA extraction; this, in order to avoid the mix of genetic material from genetically different sectors of the blade.

The 6 microsatellite primers pairs proposed herein for *Pyropia orbicularis* are the first to be published for this commercially relevant alga. These microsatellite primers are sufficiently variable for the study of genetic structure, and will facilitate population genetic studies of *P. orbicularis* for conservation and applied purposes.

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