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 Wildemania 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-Porcia et al. 2011). This high plasticity has made this species a biological model on oxidative stress and desiccation tolerance (Contreras-Porcia 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 (Meglécz *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 zygoteospores 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 denaturation at 95 °C for 5 min,
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, heterozygocity), inbreeding coefficient (F<sub>i</sub>) and genetic distance between the two populations were calculated using the software GenAIEx 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 microsatellite 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<sub>st</sub>) 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<sub>is</sub> 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<sub>is</sub> values were observed, such as at loci Orb7 and Orb8, suggesting a heterozygote excess; on

| Locus name | Primer sequence | Repeat motif | Size (bp) | Ta (°C) | No. of alleles | HO/HE | F<sub>is</sub> | GenBank Accession no. |
|------------|-----------------|--------------|-----------|---------|----------------|-------|-------------|----------------------|
| Orb1       | F: GCATGCTGACAGAAAGACGA R: GTATTGTGCGACCTGGGTT | (CAGC)<sup>5</sup> | 101 135 | 61 60 | 6 6 | 0.330 0.490 | 0.259 0.153 | MN449481 |
| Orb5       | F: CAGTGTATTGGAGGGCTACAG R: TTTGTCCGTAAACCAGAGC | (AGA)<sup>5</sup> | 190 303 | 59 59 | 6 6 | 0.260 0.320 | 0.153 0.320 | MN449482 |
| Orb6       | F: TCTGTGGGATATGGAGGACAT R: ACAAGTTTGGTCTGAGGGrC | (AC)<sup>3</sup> | 185 257 | 60 60 | 5 5 | 0.248 0.372 | 0.312 0.372 | MN449483 |
| Orb7       | F: CGAGGAGGTACAGAGGAGACA R: GGATAGAGGGCTACAGGaCA | (TTG)<sup>3</sup> | 252 275 | 60 60 | 3 3 | 0.940 0.550 | - 0.670 | MN449484 |
| Orb8       | F: CCTCTCCTACTGCCCTTCACAG R: ATGCATTTCAGCATTATCG | (TGAA)<sup>5</sup> | 234 286 | 60 59 | 3 3 | 0.995 0.500 | - 0.327 | MN449485 |
| Orb9       | F: TCAAGGAGCTAAAGGTGTCCTC R: CCAAGGATAGCTTGAAGGCCAAA | (TC)<sup>2</sup> | 316 338 | 59 60 | 4 6 | 0.426 0.829 | 0.380 0.839 | MN449486 |

F<sub>is</sub>: inbreeding coefficient, HO: observed heterozygosity, HE: expected heterozygosity
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 Gst 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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