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New microsatellite markers for the seagrass *Amphibolis antarctica* reveal unprecedented genetic diversity

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ABSTRACT

The limited data available on the genetic diversity of the temperate seagrass *Amphibolis antarctica* indicate diversity may be extremely low. The available previous study was based on allozymes and restriction fragment length polymorphisms (RFLPs) as molecular markers. Numerous studies into other seagrass taxa have shown that these markers may not have the appropriate sensitivity to reveal genetic diversity. In order to determine if *A. antractica* is genuinely genetically depauperate, or if the genetic markers used were not suitable to capture the diversity, we developed novel microsatellites for this species. Forty-eight primer candidates were screened with a limited number of geographically diverse samples. Fourteen loci displayed adequate polymorphism and were arranged into three multiplex PCR panels for further testing. DNA samples of four populations were tested and statistics on locus population and genotypic diversity calculated. This is the first study that has found genetic diversity within *A. antarctica*, with allele numbers ranging between 2–10 per locus. Expected heterozygosity (H_E) for the four populations ranged between 0.355–0.507. This small-scale study has given the first insight into the genetic diversity of this species and has provided a tool to evaluate life-history strategies such as clonality, reproduction and dispersal of one of the most important southern Australian seagrass species.

1. Introduction

Amphibolis antarctica is a temperate seagrass endemic to southern Australia that forms dense, often monospecific beds (Green and Short, 2003; Waycott et al., 2014). *Amphibolis* generally grows in quite shallow waters and mainly (but not necessarily) on sandy or rocky benthic substrates in high-energy environments, where its tall, tough and wiry stems with leaf-clusters reduce water motion and stabilize the sediment (Den Hartog, 1970; Waycott et al., 2014). A member of the family Cymodoceaceae, *A. antarctica* is dioecious like many others in this family. It has a highly unusual reproductive strategy where it forms viviparous seedlings with a distinct combed grappling apparatus (Kuo and Kirkman, 1990). Very little is known about *Amphibolis*' effective dispersal strategies and the only study that has looked deeper into its population dynamics is Waycott et al. (1996). In this early population-genetic study two types of genetic markers were used: allozymes and restriction fragment length polymorphisms (RFLPs) and neither of them detected any variation among the populations studied.

In a metadata analysis conducted by Waycott et al. (2009), the rate of seagrass loss worldwide was found to be increasing, and approximately 29% of the world's seagrass areas have already been lost. Seagrasses are important as they provide a wide range of ecosystem services such as creating complex structural habitat for other organisms to live in, many of them of commercial interest. They also reduce hydrodynamic forces with their leaves and stems which results in stabilization and accretion of sediments and the settlement of suspended particles (Mtwana Nordlund et al., 2016). In southern Australia, large scale losses of seagrass have been recorded, particularly adjacent to urban areas. In the Adelaide coastal waters for example, more than 5000 Ha of seagrasses have disappeared over the last 70 years (Hart, 1997; Nayar et al., 2009). Attempts at restoration have been made over the years but the success rate has been mixed (Tanner, 2015).

Seagrass meadows may be able to stabilize and thrive if conditions are favorable, and genetic diversity has been shown to be an important factor in generating these conditions (Hughes and Stachowicz, 2009; Reynolds et al., 2012). Genetic diversity enhances seagrass ecosystem services and is an important component to meadow resilience to disturbance, and should be a consideration for conservation and restoration (Reynolds et al., 2012). Over the last two decades microsatellites have been the common genetic marker for the study of seagrass. Due to

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their high mutation rate, and thus high allelic diversity, these makers allow for accurate determination of clones and assessment of genetic diversity (Arnaud-Haond et al., 2005; Procaccini et al., 2007). Arnaud-Haond et al. (2005) made a comparison of the then available genetic markers and concluded that microsatellites had much higher genetic resolution and fewer limitation than the other markers tested. Since the first use of microsatellites in seagrass population genetics by Reusch et al. (1998) a plethora of studies have been published, seagrass geneticists now being some of the trailblazers in clonal biology.

Since the first publication on genetic diversity on *Amphibolis antarctica* (Waycott et al., 1996) no published results have progressed our understanding of diversity in this interesting species. Here we have developed novel microsatellite markers to determine if genetic diversity is present in the species. Considering that the markers used in the past might not have had the adequate mutation rate, and the fact that significant genetic diversity has been observed in most other seagrass species (see Kendrick et al., 2012) we expected to find some level of genetic diversity that is suitable for population structure analysis and clonality assessment. To test this, we genotyped a significant number of samples on a small number of populations and performed preliminary genetic structure analyses.

2. Methods

2.1. Sampling

Two separate sampling approaches were used for this project. First, broadly distributed single samples were collected for marker development and testing. Twelve samples, some with herbarium vouchers or personal collection numbers, were collected from a range of populations along the South Australian coast (Semaphore, Seaford, Rapid Bay, Ardrossan, and Stenhouse Bay, Table SP1 for additional information). Sites across a broad range of sites were selected to maximize the chances of identifying markers with polymorphisms.

Second, four natural populations were sampled (Robe, Marino Rocks and Henley Beach in South Australia and Penguin Island in Western Australia, Table SP1) to test the selected microsatellites for genetic diversity, neutrality and intra- and inter- population variability. For Marino Rocks and Penguin Island 50 samples were taken haphazardly per site. The samples were collected by free-diving in an area of approximately 50 x 50 m with intervals of at least 3 m between samples (ramets). The Robe and Henley Beach samples were collected as wrack (i.e. drift samples) and thus do not represent a meadow, but aggregated tissue in that location.

2.2. Marker development

Genomic DNA of the 12 test samples was isolated from the meristematic leaf sheath tissue using the DNeasy Plant Kits (Qiagen, Valencia, California, USA). An equimolar mix of the DNA samples was sent to GenoScreen (Lille, France) for library preparation and followed methods described by Malausa et al. (2011). Briefly, the samples were fragmented and enriched for TG, TC, AAC, AGG, AAG, ACG, ACAT and ACTC repeats. The enriched library was then amplified and prepared for sequencing using GS-FLX Titanium chemistry (454 Life Sciences, a Roche Company, Bradford California, USA). A total of 2344 contigs containing microsatellites were isolated using the QDD software (Meglécz et al., 2010) and a shortlist of 207 validated loci with primers were provided. Forty-eight loci were selected based on repeat motif and number of repeats and ordered for testing. For cost saving purposes, the forward primers were ordered with M13 labeled tags (5′-TGTAAAAC GACGGCCAGT-3′) on the 5′-end. Additionally, universal M13 primers with fluorescent tags (6-FAM, VIC, NED, and PET) were used to allow for nested PCRs following Schuelke (2000). Reverse primers were ordered with an additional PIG-tail (5′-GTTTCT-3′) added to the 5′ end to reduce stutters (Brownstein et al., 1996).

Polymerase Chain Reactions (PCR) were done in 15 μL volumes using TypeIT® (Qiagen) chemistry (7.5 μL 2x Master Mix, 0.24 μL M13-fluorecent primer, 0.06 μL F-primer, 0.24 μL R-primer, 5.96 μL water and 1 μL DNA (diluted 1:5). PCR conditions followed manufacturer's instructions and amplifying for 36 cycles. The amplicons were separated using a capillary-based 3730xl DNA Analyzer (Applied Biosystems) at the Georgia Genomics Facility (University of Georgia, USA). Electropherograms were analyzed with Geneious R11 (Biomatters Ltd, Auckland) using the microsatellite plugin. Following initial diversity assessment with test samples, a subset of 25 loci of 34 potentially polymorphic microsatellites (Table SP2) were tested further on 48 Henley Beach samples to assess genetic diversity and linkage disequilibrium. Based on locus diversity, amplicon length and primer interactions three multiplex PCR panels were designed with Multiplex Manager v1.2 (Holleley and Geerts, 2009) to reduce laboratory expenses and labor resulting in a 14 loci genotyping panel (Table SP3). The three multiplex PCR panels were tested on samples from Robe, Marino Rocks, Henley Beach and Penguin Island (Table SP1). PCR's were done under similar conditions as testing only the fluorescent M13 and F primers were replaced with forward primers with fluorescent tags (Table SP2).

2.3. Diversity assessment

Multilocus genotypes were first determined with the R package Rclone (Arnaud-Haond and Belkhir, 2007). The clonality probability statistics p_{gen_fis} and p_{sex_fis} were calculated on complete genotypes only. The software GenoDive v2.0b27 (Meirmans and Van Tierden, 2004) was used on the full dataset (samples with some missing loci included). Multilocus lineages (referred to as genets from here) were selected based on a threshold as described by Meirmans and Van Tierden (2004) and duplicated genotypes were removed from all genetic analyses. Based on number of samples (N) and genets (G) clonal richness was calculated as $R = (G - 1)/(N - 1)$ (Dorken and Eckert, 2001). The loci were tested for Hardy-Weinberg equilibrium and linkage disequilibrium in GenePop v4.6 against a Bonferroni corrected p-value (Raymond and Rousset, 1995; Rousset, 2008). The allelic diversity, genetic diversity, AMOVA (Analysis of Molecular Variance) were also calculated in GenoDive applying 999 permutations. The package diveRsity (Keenan et al., 2013) was used to create a connectivity graph based on Alcala's $\hat{Nm}$ (Alcala et al., 2014).

3. Results and discussion

One of the main objectives of this study was to establish if *Amphibolis antarctica* had genetic diversity or not. The development of these new microsatellites clearly changed the paradigm that this species was genetically depauperate. In fact, we detected considerable genetic diversity within and among the few *A. antarctica* meadows sampled to date. Clonality varied among sites and some populations had high genotypic diversity (many genets). High genotypic diversity is generally associated with a high recruitment rate (Reusch, 2006), and this would be expected if the very large number of seedlings that are found along the southern Australian shores during the reproductive season of *Amphibolis* are derived from healthy, genetically diverse populations.

3.1. Genetic and clonal diversity

The initial development of microsatellites by GenoScreen provided a high number of potential loci. Out of the selection of 48 loci, 34 promised to be usable for population studies, which is a very high proportion (> 70%), while 25 loci made it to the second round of testing. Not all loci amplified readily or behaved as expected under Hardy-Weinberg disequilibrium. The default annealing temperature of 60 °C effectively amplified most of the primer-pairs in the multiplex PCR panels. Some loci were removed (lower part of Table SP2) from the