Development and characterization of microsatellite loci for the haploid-diploid red seaweed Gracilaria vermiculophylla

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Microsatellite loci are popular molecular markers due to their resolution in distinguishing individual genotypes. However, they have rarely been used to explore the population dynamics in species with biphasic life cycles in which both haploid and diploid stages develop into independent, functional organisms. We developed microsatellite loci for the haploid-diploid red seaweed Gracilaria vermiculophylla, a widespread non-native species in coastal estuaries of the Northern hemisphere. Forty-two loci were screened for amplification and polymorphism. Nine of these loci were polymorphic across four populations of the extant range with two to eleven alleles observed. Mean observed and expected heterozygosities ranged from 0.265 to 0.527 and 0.317 to 0.387, respectively. Overall, these markers will aid in the study the invasive history of this seaweed and further studies on the population dynamics of this important haploid-diploid primary producer.
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INTRODUCTION

In the last decade, genetic approaches to answering evolutionary and ecological questions have become less expensive and more easily applied to non-model species (Allendorf et al. 2010; Guichoux et al. 2011). Microsatellites, or tandem repeats of two to six nucleotides, are popular molecular markers due to their resolution in distinguishing individual genotypes (Selkoe & Toonen 2006) and their ability to describe patterns of population connectivity across landscapes (Manel et al. 2003) and seascapes (Galindo et al. 2006). Much of the literature focuses on organisms with single free-living diploid stages (i.e., animals and higher plants). Yet, there are many species with both haploid and diploid stages in the same life cycle in which both ploidies undergo somatic development and live as independent, functional organisms.

While theory predicts that selection should favor either diploidy or haploidy (Mable & Otto 1998), Hughes and Otto (1999) demonstrated the maintenance of both haploid and diploid stages when the two stages occupy different ecological niches. However, there are relatively few empirical tests of these alternative hypotheses (but see Destombe et al. 1992; Thornber & Gaines 2004; Guillemin et al. 2013), and for isomorphic species in which ploidy is not easily identified through morphological traits, molecular markers will be essential to advance research in this field. These same markers can additionally be used to understand connectivity and demographic history in haploid-diploid populations. Among marine haploid-diploid macroalgae, relatively few microsatellites have been developed to address any of these issues (but see Table 1).

Understanding the consequences of biphasic life cycles and land- or seascape features on population structure is particularly relevant in light of the increasing frequency of biological introductions. There are numerous examples of widespread, and putatively invasive species, that have free-living haploid and diploid stages, including macroalgae (e.g., Asparagopsis spp.;
Andreakis et al. 2007), ferns (e.g., Lygodium spp.; Lott et al. 2003) and mosses (e.g., Campylopus introflexus; Schirmel et al. 2010). Macroalgae, or seaweeds, account for approximately 20% of the world’s introduced marine species (Andreakis & Schaffelke 2012) and a subset of these invasions are by species that are exploited in their native range, either for the phycocolloid industry or as food products (Williams & Smith 2007).

The red seaweed Gracilaria vermiculophylla (Omhi) Papenfuss is native to the northwest Pacific and, in the last 30-40 years, has spread throughout high to medium salinity estuaries of the eastern North Pacific (Saunders 2009), the western North Atlantic (Byers et al. 2012) and the eastern North Atlantic (Weinberger et al. 2008; Guillemin et al. 2008a). G. vermiculophylla transforms the ecosystems into which it is introduced through negative impacts on native species (e.g., direct competition, Hammann et al. 2013), the addition of structural complexity to soft-bottom systems (e.g., Nyberg et al. 2009, Wright et al. 2014) and the alteration of community structure, species interactions and detrital pathways (e.g., Byers et al. 2012). Previous studies of the population genetics of G. vermiculophylla focused on the mitochondrial gene cytochrome b oxidase I (Kim et al. 2010, Gulbransen et al. 2012), but mitochondrial genetics do not necessarily predict the population genetics of the nuclear genome and cannot assess patterns of ploidy and mating system. Thus, we developed nine polymorphic microsatellite loci for G. vermiculophylla.

MATERIALS AND METHODS

A library of contigs for G. vermiculophylla was generated using the 454 next-generation sequencing platform (Cornell University Life Sciences Core Laboratory Center) from a single individual collected from Charleston, SC, USA. For library preparation, DNA was extracted
using CTAB (Eichenberger et al. 2000) and library construction followed Hamilton et al. (1999).

Dimeric to hexameric microsatellite repeats were identified with the program MSATCOMMANDER, ver 1.0.8 (Faircloth 2008) and primers were designed using PRIMER 3 (Rozen and Skalest 2000) for contigs with at least four sequences present in the library. Bioinformatics of these sequences was facilitated by the APE package (Paradis et al. 2004) in R (R Core Team 2014).

Total genomic DNA was isolated using 120 µL of a 10% Chelex solution (BioRad Laboratories, Hercules, CA, USA) in which approximately 1 cm of dried algal tissue was heated at 95°C for 30 minutes and vortexed intermittently (Walsh et al. 1991). Loci were amplified on a thermocycler (BioRad) as follows: 10 µL final volume, 2 µL of stock DNA template, 0.5 units of GoTAQ Flexi-DNA Polymerase (Promega), 1X buffer, 250 µM of each dNTP, 1.5 nM of MgCl₂, 150 nM of fluorescently-labeled forward primer, 100 nM of unlabeled forward primer and 250 nM of unlabeled reverse primer. The PCR program included 2 min at 95°C, 30 cycles of 30 sec at 95°C, 30 sec at 55°C and 30 sec at 72°C, and a final 5 min at 72°C. One µL of each PCR product was added to 10 µL of loading buffer containing 0.35 µL of size standard (GeneScan500 Liz; Applied Biosystems, Foster City, CA, USA). Samples were electrophoresed on an ABI 3130xL genetic analyzer equipped with 36 cm capillaries (Applied Biosystems). Alleles were scored manually using GENEMAPPER ver. 4 (Applied Biosystems) and allele sizes were binned with TANDEM ver. 1.08 software (Matschner & Saltzburger 2009; Krueger-Hadfield et al. 2013).

We screened a total of 42 primer pairs for amplification and polymorphism in *G. vermiculophylla* (Table 2, Supplemental Table 1). For the amplifiable loci that also showed polymorphism (nine total, see Results and Discussion), we verified single locus genetic
determinism (SGLD). Locci were in SLGD if known haploids produced a single allele and
diploids produced either one or two alleles in their homozygous or heterozygous state,
respectively. We verified SGLD in a subset of known haploid gametophytes (n = 28) and diploid
tetrasporophytes (n = 30) collected at Elkhorn Slough, CA, USA (Table 3, Supplemental Figure
1). Elkhorn Slough was the only population for which ploidy was determined by reproductive
structures and for which we had known haploids and diploids for genotyping.

The frequency of null alleles was estimated in the haploid subpopulation from Elkhorn
Slough as well as diploid tetrasporophytes for each of the four populations (Table 3). It is
possible to calculate the null allele frequency directly in the haploids based on the number of
non-amplification events, after discounting technical errors. For diploid tetrasporophytes, we
used a maximum likelihood estimator (ML-NullFreq: Kalinowski & Taper 2006).

Next, we screened loci for short allele dominance (Wattier et al. 1998). The presence of
short allele dominance is rarely tested during microsatellite development, even though it can
result in artificial heterozygote deficiencies. In contrast to null alleles, primer binding is
successful, but the larger allele is not amplified due to the preferential amplification of the
smaller allele. Wattier et al. (1998) demonstrated an analytical method to detect short allele
dominance using linear models. If a regression of allele-specific $F_{is}$ (inbreeding coefficient)
statistics on allele size reveals a significant negative slope, then short allele dominance may be
expected. We determined three to four allele size classes per locus and performed linear
regressions using the STATS package in R (R Core Team 2014).

To provide preliminary assessment of the genotypic and genetic diversity one can gain
from these loci, we genotyped diploid tetrasporophytes from one native and three non-native
populations of *G. vermiculophylla* (Table 3). Diploids were identified based either on
reproductive phenology (Elkhorn) or microsatellite genotype (after assuring SGLD) if at least one locus was heterozygous (Akkeshi, Fort Johnson and Nordstrand, Table 3).

We calculated expected allelic richness using rarefaction in order to account for differences in sample size (HP-Rare; Kalinowski 2005). Observed ($H_O$) and expected heterozygosities ($H_E$) were calculated using GenAlEx, ver. 6.501 (Peakall & Smouse 2006; Peakall & Smouse 2012). Tests for Hardy-Weinberg equilibrium and $F$-statistics were performed in FSTAT, ver. 2.9.3.2 (Goudet 1995). $F_{is}$ was calculated for each locus and over all loci according to (Weir & Cockerham 1984) and significance (at the adjusted nominal level of 0.001) was tested by running 1000 permutations of alleles among individuals within samples. We also tested for linkage disequilibrium in each population using GENEPOP, ver. 4.2.2 (Rousset 2008), with 1000 permutations followed by Bonferroni correction for multiple comparisons (Sokal & Rohlf 1995).

RESULTS AND DISCUSSION

Of the 42 loci screened, 16 did not amplify for *G. vermiculophylla* even after several PCR modifications (Supplemental Table 1). Of the remaining 26 loci, four loci exhibited multi-peak profiles and were discarded from further use, 13 loci were considered monomorphic (Supplemental Table 1), and nine loci showed polymorphism (Table 2). The nine polymorphic loci exhibited SLGD in which known haploids always exhibited one allele. The low number of polymorphic loci revealed from this screening process is consistent with previous efforts to develop microsatellite loci for some seaweeds (e.g., Varela-Alvarez *et al.* 2011, Arnoud-Haond *et al.* 2013).
The frequency of null alleles was zero at all loci except Gverm_1803 and Gverm_2790 in which the frequencies were both 0.019 in the haploids at Elkhorn Slough (Supplemental Table 2). The only evidence of null alleles in the diploids from Elkhorn Slough was at locus Gverm_1803, with a maximum likelihood estimated frequency of 0.115. The discrepancy between the haploid and diploid estimates is likely due to assumptions underlying the maximum likelihood estimators implemented in software like HP-Rare (Kalinowski 2005), such as random mating. Krueger-Hadfield et al. (2013) demonstrated a strong bias in the estimates of null allele frequency when using these maximum likelihood estimators in macroalgal populations that have undergone non-random mating. The higher frequencies of null alleles (0.115-0.207) in the Akkeshi diploid subpopulation were most likely driven by a violation of these assumptions as well, though empirical estimates in haploid subpopulations are warranted. Nevertheless, the low frequency of null alleles and lack of evidence for short-allele dominance (all regression p-values were > 0.2, Supplemental Table 3), suggest that observed heterozygote deficiencies using these loci will be due to the mating system or spatial substructuring (Guillemin et al. 2008b; Krueger-Hadfield et al. 2011; 2013).

Previous studies have used microsatellite loci to distinguish among individual clones and to describe the genetic diversity and the mating systems of seaweed populations despite low levels of polymorphism (e.g., Guillemin et al. 2008, Arnaud-Haond et al. 2013). In the current study, the nine polymorphic markers described genetic variability in four populations sampled across the extant distribution of *G. vermiculophylla*. Overall, there was little evidence for linkage disequilibrium after Bonferroni correction (Supplemental Table 4). Additionally, allelic diversity was comparable among the one native and three non-native sites we sampled, but $F_{is}$ varied considerably (summary in Table 4; per locus statistics in Supplemental Table 5). Together, these
results suggest that unique demographic and evolutionary processes could be operating between
native and non-native ranges and within each population, but more detailed sampling is needed
to address these patterns.

In summary, we have developed and characterized microsatellite markers for the haploid-
diploid red seaweed *G. vermiculophylla*. These loci have the resolution to distinguish individual
thalli and will aid studies on the invasive history of *G. vermiculophylla*, as well as the
evolutionary ecology of rapidly spreading populations and mating system shifts in organisms that
have biphasic life cycles with free-living haploid and diploid stages (i.e., macroalgae, ferns,
mosses and some fungi).

ACKNOWLEDGMENTS

Thanks to T. M. Bell, E. Buchanan, C. E. Gerstenmaier, M. Hammann, K. Honda, B. Hughes, B.
F. Krueger, T. D. Krueger, K. Momota, M. Nakaoka, A. Pansch, T. Roth and M. Sato for field
and laboratory support.

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Table 1 (on next page)

Studies in which both the haploid and diploid stages of seaweeds and mosses were investigated to reveal patterns in genetic structure and mating system.
Table 1. Studies in which both the haploid and diploid stages of seaweeds and mosses were investigated to reveal patterns in genetic structure and mating system.

| Phylum      | Species                  | Marker   | Type of study                                                                 |
|-------------|--------------------------|----------|--------------------------------------------------------------------------------|
| Rhodophyta  | *Gelidium arbuscula*     | Isozymes | Genetic structure and mating system                                           |
| Rhodophyta  | *Gelidium canariensis*   | Isozymes | Genetic structure and mating system                                           |
| Rhodophyta  | *Gracilaria gracilis*    | Microsatellites | Paternity analyses and dispersal                                           |
| Bryophyta   | *Polytrichum formosum*   | Microsatellites | Paternity analyses and dispersal                                           |
| Chlorophyta | *Cladophoropsis membranacea* | Microsatellites | Shorescape structure and mating system                                       |
| Rhodophyta  | *Gracilaria gracilis*    | Microsatellites | Shorescape structure and mating system                                       |
| Rhodophyta  | *Gracilaria chilensis*   | Microsatellites | Genetic structure, mating system and comparisons between natural and farmed populations |
| Bryophyta   | *Sphagnum lescurii*      | Microsatellites | Paternity analyses and dispersal                                           |
| Chlorophyta | *Ulva intestinalis*      | Microsatellites | Genetic structure and mating system                                           |
| Rhodophyta  | *Chondrus crispus*       | Microsatellites | Genetic structure and mating system                                           |
| Rhodophyta  | *Chondrus crispus*       | Microsatellites | Shorescape structure and mating system                                       |
| Ochrophyta  | *Ectocarpus crouanitorum*| Microsatellites | Genetic structure and mating system                                           |
| Ochrophyta  | *Ectocarpus siliculosus* | Microsatellites | Genetic structure and mating system                                           |
| Rhodophyta  | *Chondrus crispus*       | Microsatellites | Paternity analyses and dispersal                                           |
Characteristics of nine polymorphic microsatellite loci developed for *Gracilaria vermiculosphylla*

Acc. No. = genbank accession number; locus; motif; primer sequences; allele range; avg.
error: TANDEM (Matschiner & Saltzburger 2009) rounding errors for each microsatellite locus
(the authors of TANDEM suggest that good loci have an average rounding error which is
below 10% of the repeat size); $N_{\text{tall}}$ = total number of alleles. All loci showed one-locus
 genetic determinism.
Table 3. Location of the four populations used to test for polymorphism in newly characterized microsatellite loci in *Gracilaria vermiculophylla*. The region, range (native or non-native), latitude, longitude, sampling date, collector* and ploidy determination (using reproductive phenology or microsatellite genotype) is provided.

| Population          | Region         | Range     | Latitude | Longitude | Date       | Collector          | Ploidy determination |
|---------------------|----------------|-----------|----------|-----------|------------|--------------------|----------------------|
| Akkeshi, Japan      | NW Pacific     | Native    | 43.04774 | 144.9498  | 25Aug10, 31Jul12 | NMK, KH, KM, AP, MS | genotype             |
| Elkhorn Slough, CA  | NE Pacific     | Non-native| 36.50447 | -121.4513 | 3Nov13     | SAKH, BFK, TDK, BH | genotype, phenology  |
| Fort Johnson, SC    | NW Atlantic    | Non-native| 32.7513  | -79.900   | 11Dec13    | CEG                | genotype             |
| Nordstrand, Germany | North Sea      | Non-native| 54.454571| 8.874846  | 24Mar10    | MH                 | genotype             |

* Collector abbreviations: AP: A. Pansch, NMK: N. M. Kollars, KH: K. Honda, KM: K. Momota, MS: M. Sato, SAKH: S. A. Krueger-Hadfield, BFK: B. F. Krueger, TDK: T. D. Krueger, BH: B. Hughes, CEG: C. E. Gerstenmaier, MH: M. Hammann
Table 3 (on next page)

Location of the four populations used to test for polymorphism in newly characterized microsatellite loci in *Gracilaria vermiculophylla*.

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| Locus     | Acc. No. | Motif | Primer sequence                                      | Allele Range | Avg. Error | $N_{\text{tall}}$ |
|-----------|----------|-------|-----------------------------------------------------|--------------|------------|-----------------|
| Gverm_5276 | KT232089 | (AC)$_{10}$ | F: GGAGAGCAGCACGTTTTTAGG R: CTGCTTAGTTCCACGATCGAC | 282-316  | 0.14       | 11              |
| Gverm_6311 | KT232090 | (AG)$_{9}$ | F: GCGTCATTCCACTGAATGTG R: GATGAACCTCAATGCCTCGT  | 203-223  | 0.17       | 6               |
| Gverm_8036 | KT232091 | (AC)$_{12}$ | F: GCCCTTTTAAGGATGCAACA R: GGGGTAAACGACCACAGAGA | 213-251  | 0.14       | 5               |
| Gverm_3003 | KT232092 | (AG)$_{11}$ | F: CATCTTGCTTCTCTGCTCC R: TTGAAAGCCGAATTTATCG  | 198-230  | 0.11       | 4               |
| Gverm_1203 | KT232093 | (AAG)$_{8}$ | F: CTCTTGTGCAACAAGCAATA R: ACATTCTGCGCACCTTTCCT | 284-308  | 0.12       | 4               |
| Gverm_1803 | KT232094 | (AC)$_{11}$ | F: GCGTGACAGTGCTACACT R: GACAGCAACAAGTGGGTTT  | 352-356  | 0.07       | 3               |
| Gverm_804  | KT232095 | (AAG)$_{8}$ | F: TGTAGGATTGCTCTCTGGTG R: CAGGCTGGCCAAAATAACAT | 182-188  | 0.16       | 3               |
| Gverm_10367| KT232096 | (AG)$_{8}$  | F: GCTGAGAAATGAAGCGAAGG R: GCAAACCTGCCTGTGGTT | 198-200  | 0.07       | 2               |
| Gverm_2790 | KT232097 | (AATGC)$_{5}$ | F: GAAATAATGGGGAAAACATT R: GGAAGAGGCTCAAAAAGCAGA | 262-267  | 0.16       | 2               |
Table 4 (on next page)

Genetic features of four populations of *Gracilaria vermiculophylla*.

These include the sample size, $N$; the diploid genotypic richness, $N_{A_r}$ + standard error (SE); mean allelic richness, $A_E$, based on the smallest sample size of 46 alleles (23 diploid individuals) + SE; mean observed heterozygosity, $H_O$, + SE; mean expected heterozygosity, $H_E$, + SE; inbreeding coefficient, $F_{IS}$, multilocus and per locus estimates (*, $p < 0.001$, adjusted nominal value).
Table 4. Genetic features of four populations of *Gracilaria vermiculophylla*, including: the sample size, *N*; the diploid genotypic richness, *N_A*, ± standard error (SE); mean allelic richness, *A_E*, based on the smallest sample size of 46 alleles (23 diploid individuals) ± SE; mean observed heterozygosity, *H_O*, ± SE; mean expected heterozygosity, *H_E*, ± SE; inbreeding coefficient, *F_is*, multilocus and per locus estimates (*, *p* < 0.001, adjusted nominal value).

| Statistics | Akkeshi | Elkhorn Slough | Fort Johnson | Nordstrand |
|------------|---------|----------------|--------------|------------|
| *N*        | 31      | 30             | 38           | 23         |
| *N_A*      | 3.2 ± 0.5 | 2.2 ± 0.4     | 2.0 ± 0.2    | 1.9 ± 0.2  |
| *A_E*      | 3.1 ± 0.4 | 2.2 ± 0.3     | 2.0 ± 0.2    | 1.9 ± 0.2  |
| *H_O*      | 0.265 ± 0.060 | 0.311 ± 0.089 | 0.520 ± 0.110 | 0.527 ± 0.125 |
| *H_E*      | 0.374 ± 0.079 | 0.317 ± 0.084 | 0.387 ± 0.077 | 0.352 ± 0.079 |
| *F_is*     | 0.294 * | 0.017          | -0.350 *     | -0.512 *   |

*F_is* per locus

|                 | Akkeshi | Elkhorn Slough | Fort Johnson | Nordstrand |
|-----------------|---------|----------------|--------------|------------|
| Gverm_5276      | 0.484 * | 0.120          | -0.209       | -0.492     |
| Gverm_6311      | 0.435 * | 0.140          | -0.267       | -0.048     |
| Gverm_8036      | 0.334   | NA             | -0.445 *     | -0.217     |
| Gverm_3003      | 0.529   | -0.121         | -0.138       | -0.553     |
| Gverm_1203      | -0.15   | -0.206         | -0.310       | -0.508     |
| Gverm_1803      | 0.569 * | 0.460          | -0.696 *     | NA         |
| Gverm_804       | -0.278  | -0.206         | -0.310       | -0.508     |
| Gverm_10367     | -0.017  | NA             | NA           | NA         |
| Gverm_2790      | NA      | NA             | NA           | -0.913 *   |