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New resource for population genetics studies on the Australasian intertidal brown alga, *Hormosira banksii*: isolation and characterization of 15 polymorphic microsatellite loci through Next Generation DNA sequencing.

Alecia Bellgrove[1*], Anthony van Rooyen[2], Andrew R. Weeks[2,3], Jennifer S. Clark[4], Martina A. Doblin[4], Adam D. Miller[1,3]

[1] Deakin University, Geelong, Australia. School of Life and Environmental Sciences, Centre for Integrative Ecology, Warrnambool Campus, P.O. Box 423, Warrnambool, Victoria 3280, Australia

[2] **cesar**, 293 Royal Parade, Parkville 3052, Australia

[3] School of BioSciences, The University of Melbourne, Parkville, Victoria, 3010 Australia

[4] C3- Climate Change Cluster, University of Technology Sydney, PO Box 123, Broadway, NSW 2007 Australia

*Corresponding author: Alecia Bellgrove (alecia.bellgrove@deakin.edu.au); Ph: +61-3-5563-3099; Fax: +61-3-5563-3462

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Abstract

The Australasian fucoid, *Hormosira banksii*, commonly known as 'Neptune's necklace' or 'bubbleweed' is regarded as an autogenic ecosystem engineer with no functional equivalents. Population declines resulting from climate change and other anthropogenic disturbances pose significant threats to intertidal biodiversity. For effective conservation strategies, patterns of gene flow and population genetic structure across the species distribution need to be clearly understood. We developed a suite of 15 polymorphic microsatellite markers using Next Generation Sequencing of 53 – 55 individuals from two sites (south-western Victoria and central New South Wales, Australia) and a replicated spatially hierarchical sampling design. We observed low to moderate genetic variation across most loci (mean number of alleles per locus = 3.26; mean expected heterozygosity = 0.38) with no evidence of individual loci deviating significantly from Hardy-Weinberg equilibrium. Marker independence was confirmed with tests for linkage disequilibrium, and analyses indicated no evidence of null alleles across loci. Independent spatial autocorrelation analyses were performed for each site using multilocus genotypes and different relatedness measures. Both analyses indicated no significant patterns between relatedness and geographic distance, complemented by non-significant Hardy-Weinberg estimates ($P < 0.05$), suggesting that individuals from each site represent a randomly-mating, outcrossing population. A preliminary investigation of population structure indicates that gene flow amongst sites is limited ($F_{ST} = 0.49$), however more comprehensive sampling is needed to determine the extent of population structure across the species range (>10,000 km). The genetic markers described provide a valuable resource for future population genetic assessments that will help guide conservation planning for *H. banksii* and the associated intertidal communities.

Introduction

25 Many perennial macroalgae function as autogenic ecosystem engineers (Jones et al. 1994,
26 1997) influencing the biodiversity of rocky coastlines around the world (Schiel and Lilley
27 2007; Jenkins et al. 1999). Aside from their habitat forming nature, macroalgae provide
28 additional ecosystem services including contributions to detrital food webs, nutrient cycling,
29 primary productivity, and carbon storage (Bennett et al. 2015; Milanese et al. 2011; Hill et al.
30 2015). Recent studies indicate that many species are likely to be vulnerable to the effects of
31 climate change and additional anthropogenic pressures. In some species, population declines
32 are occurring on local and regional scales in association with coastal urbanisation due to factors
33 such as eutrophication, herbivory and sedimentation, while ocean warming and acidification
34 are driving declines on larger spatial scales (Wernberg et al. 2016; Strain et al. 2014). As a
35 result, these environmental pressures pose significant threats to marine biodiversity and
36 ecosystem function on a global scale. This highlights the urgent need for information on the
37 biology, ecology and genetics of habitat-forming macroalgae to help identify at-risk species
38 and develop necessary management action plans.

39 *Hormosira banksii* (Turner) Decaisne is a perennial, fucoid macroalga that dominates
40 much of the rocky intertidal coastline of temperate Australasia, and is regarded as an
41 autogenic engineer with no functional equivalents (Osborn 1948; Bellgrove et al. 2004;
42 Schiel 2006). It also occurs as a free-floating form in wave protected bays, estuaries and
43 lagoons (Ralph et al. 1998). Its distribution extends from Albany in Western Australia to
44 Lennox Head in New South Wales on mainland Australia, as well as encompassing
45 Tasmania, the North and South Islands of New Zealand, and some of the smaller offshore
46 islands in southern Australasia (Osborn 1948). *H. banksii* is diplontic, dioecious and highly
47 fecund, releasing gametes during low tide (Levring 1949). Evidence suggests this species is
48 sensitive to environmental disturbance and the effects of climate change, with observed
49 population declines occurring on local and regional scales in recent decades (Schiel 2011;

50 Keough and Quinn 1998). Thus, there is a need for information on this species' adaptive
51 capacity and population connectivity across its range to help guide future conservation
52 management.

53 Like other fucoid algae, the propagules of *H. banksii* are thought to typically disperse
54 short distances (< 30 m) (Bellgrove et al. 1997; Santelices 1990), while fertile floating
55 fragments have the potential to disperse greater distances through ocean current assisted
56 passive movement (McKenzie and Bellgrove 2008). Recent population genetic research
57 conducted by Coleman et al. (2011) indicated limited gene flow among populations separated
58 by less than 200 km along the central New South Wales coast. These results indicate that the
59 replenishment of depleted populations from non-local sources is likely to be minimal,
60 highlighting the vulnerability of local populations to environmental pressure and extirpation.
61 This highlights the need to sample more widely across the species distribution to test the
62 generality of these findings and to guide management efforts. At present the underlying
63 genetic structure of this alga, like many other benthic marine organisms, remains elusive.

64 The availability of genetic markers for *H. banksii* is currently limited. While Coleman
65 et al. (2011) provide valuable descriptions of population genetic structure within an area of the
66 species range, inferences were based on only 5 microsatellite loci with low allelic diversity. To
67 address this issue, we report the development and characterization of 15 polymorphic
68 microsatellite markers using next generation sequencing methods. As yet there have been
69 relatively few studies using this approach to develop genetic markers in seaweeds, but all have
70 yielded high numbers of polymorphic loci (Ayres-Ostrock et al. 2016; Candeias et al. 2015;
71 Couceiro et al. 2011). We also performed spatial autocorrelation analyses using multilocus
72 genotypes from approximately 50 individuals at two randomly chosen sites approximately
73 1500 km apart, to determine if limited propagule movement leads to high levels of relatedness

74 at fine spatial scales. These analyses are critical to guide sampling efforts for future population
75 genetic assessments of this ecologically important species.

76 **Methods**

77 *Microsatellite marker characterisation*

78 The Roche GS FLX (454) next generation sequencing platform was used to identify
79 microsatellite markers for *H. banksii*. Genomic DNA was isolated from 30 mg of freeze dried
80 *H. banksii* tissue using the Nucleospin® 96 Plant II DNA extraction kit. DNA was
81 subsequently processed by the Australian Genome Research Facility (AGRF) where it was
82 nebulized, ligated with Roche 454 sequencing primers and tagged with a unique oligo sequence
83 allowing sequences to be demultiplexed from pooled species DNA sequences using post-run
84 bioinformatic tools. The DNA sample was analyzed using high throughput DNA sequencing
85 on 1/16 of a 70 x 75 mm PicoTiterPlate using the Roche GS FLX (454) system (Margulies et
86 al. 2005). A total of 57,866 reads were obtained from the analysis, from which 1,381 unique
87 sequence contigs possessing microsatellite motifs were identified using the software QDD2
88 (Meglecz et al. 2010). Primer3 (Rozen and Skaletsky 2000) was used to design optimal primer
89 sets for each unique contig where possible, with a total of 996 contigs found to possess optimal
90 priming sites. A selection of 72 contigs was used for subsequent analysis, 55 of which
91 contained di-nucleotide repeats, 15 containing tri-nucleotide repeats, and two containing a
92 tetra-nucleotide repeat.

93 Loci were screened for polymorphism using template DNA from eight individuals,
94 consisting of four individuals from south-western Victoria (Backyards; 38°26'S, 142°35'E),
95 and four individuals from the central coast of New South Wales (Bilgola Beach; 33°38'S,
96 151°19'E). Loci were pooled into ten groups of four, labelled with unique fluorophores (FAM,
97 NED, VIC, PET) and co-amplified by multiplex PCR using a Qiagen multiplex kit (Qiagen)

98 and an Eppendorf Mastercycler *S* gradient PCR machine following the protocol described by
99 (Blacket et al. 2012). Genotyping was subsequently performed using an Applied Biosystems
100 3730 capillary analyzer (AGRF, Melbourne, Australia) and product lengths were scored
101 manually and assessed for polymorphisms using GeneMapper version 4.0 (Applied
102 Biosystems). From a total of 72 loci, 41 were found to be polymorphic (Table 1), 18 were
103 monomorphic and 13 failed to amplify.

104 A subset of 15 polymorphic loci were selected, pooled into four groups for multiplexing
105 based on observed locus specific allele size ranges (Table 1), and further characterized using
106 53 and 55 individuals from the Backyards and Bilgola sampling sites, respectively.
107 Microsatellite profiles were again examined using GeneMapper version 4.0 and alleles were
108 scored manually. The *Excel Microsatellite Toolkit* (Park 2001) was then used to estimate
109 expected (H_E) and observed (H_O) heterozygosities and number of alleles (N_A), while
110 examination of conformation to Hardy-Weinberg equilibrium (HWE), the inbreeding
111 coefficient (F_{IS}) and linkage disequilibrium estimates between all pairs of loci were conducted
112 using GENEPOP version 4 (Raymond and Rousset 1995). Significance values were adjusted
113 for multiple comparisons using Bonferroni corrections where necessary (Rice 1989). Finally,
114 all loci were assessed using MICRO-CHECKER to check for null alleles and scoring errors
115 (Van Oosterhout et al. 2004). The frequency of null alleles per locus was obtained using the
116 'Brookfield 1' formula as evidence of null homozygotes across loci was not observed
117 (Brookfield 1996).

118 *Population genetic analysis*

119 We compared levels of genetic diversity (H_E, N_A) and Hardy-Weinberg (HWE) estimates
120 across our two sampling sites using the methods outlined above, and determined gene flow
121 patterns using the FSTAT package (Goudet 1995) to calculate locus-by-locus and global
122 estimates of F_{ST} (Weir and Cockerham 1984) with significance determined using permutations

123 (10,000). These analyses were complemented with an analysis of molecular variation
124 (AMOVA) performed in GenAlEx version 6.501 (Peakall and Smouse 2006), based on
125 pairwise F_{ST} as the distance measure with 10,000 permutations. A factorial correspondence
126 analysis (FCA), implemented in GENETIX version 4.05 (Belkhir et al. 2004), was used to
127 summarize patterns of genetic differentiation between sample sites. The first 2 underlying
128 factors that explained the majority of variation in multi-locus genotypes across loci were
129 plotted.

130 A replicated hierarchical spatial sampling regime at each site was designed to determine
131 if individuals in close proximity on intertidal reef platforms were significantly more related
132 than expected randomly. To do this we intentionally randomly chose two different and distantly
133 located populations with varying exposures to wave activity. A total of five 10-m transects,
134 with haphazardly selected starting points and orientation, were sampled at each of two sites: 1)
135 a 25 × 57-m area of reef at Backyards, and 2) a 12 × 50-m area at Bilgola Beach. Both sites are
136 wave-exposed reefs composed of calcarenite and shale-sandstone, respectively, but wave-
137 exposure tends to be greater at Backyards due to direct exposure to the weather systems of the
138 Southern Ocean. *H. banksii* formed an almost continuous bed of densely clumped plants
139 (approx. 60 % cover J. Clark et al., unpublished data) at Bilgola Beach, but was more patchily
140 distributed at Backyards (approx. 20% cover (H. Cameron et al. unpublished data). Along each
141 transect, distal vesicles were collected from one frond from the closest distinct plant (separate
142 holdfast) at 11 set points: 0, 0.1, 0.3, 0.5, 0.7, 1.0, 1.5, 2.0, 5.0, 8.0, and 10 m. Vesicles were
143 placed in labelled individual bags, returned to the laboratory on ice and frozen at -30°C, then
144 transferred to -80°C, before being freeze-dried for DNA extraction. The GPS coordinates
145 (based on WGS 84 map datum) were recorded for the start and end points of each transect with
146 a Garmin GPSMAP 62S (Garmin Australasia, Eastern Creek, NSW, Australia
147 http://www.garmin.com/en-AU/). The closest distance between each transects' terminal points

148 (start or end depending on orientation) were also measured manually with a tape measure to
149 later calibrate distances calculated from GPS. Genomic DNA was subsequently extracted from
150 30 mg sample of tissue for individual specimens was using the NucleoSpin® 96 Plant II
151 protocol (Machery-Nagel Inc., Düren, KO, GER) by the Australian Genome Research Facility.

152 Independent spatial autocorrelation analyses were performed for each of the two
153 sampling sites using multilocus genotypes in GenAlEx 6.501. Distance classes for each
154 analysis were based on the 'equal sample size' option, and with 9999 permutations to test for
155 levels of significance. Spatial autocorrelation analyses for each sampling site was also
156 replicated with SPAGeDi 1.2 (Hardy and Vekemans 2002). We estimated the Queller and
157 Goodnight (1989) relatedness coefficient among pairs of individuals belonging to the same *a*
158 *priori* defined distance classes. For each class, random permutations in the spatial locations of
159 individuals (10000 permutations) were then used to assess deviations of the relatedness
160 coefficient *R* from 0. Distance classes were chosen so that they contained more than 100
161 pairwise comparisons, had a participation index >50% and a coefficient of variation of
162 participation of less than 1 (Hardy and Vekemans 2002). Deviation from 0 means that
163 individuals within a given distance class are significantly more (positive values) or less
164 (negative values) related than random. To assess the reliability of the results obtained with the
165 Queller and Goodnight (1989) relatedness coefficient, we repeated the analyses using two other
166 relatedness estimators; namely, Lynch and Ritland's (1999) relatedness coefficient (*r*) and the
167 kinship coefficient of Loiselle *et al.* (1995).

168 **Results**

169 *Microsatellite marker characterisation*

170 The majority of loci were characterized by low to moderate genetic variation, with an
171 average of 3.26 alleles per locus (range = 2 – 6 alleles) and heterozygosity estimates ranging

172 between 0.02 and 0.76 (mean = 0.38). Linkage disequilibrium analyses confirmed marker
173 independence indicating no evidence of significant linkage between loci while MICRO-
174 CHECKER analyses revealed no evidence of null alleles or scoring issues across loci. After
175 significance values were adjusted for multiple comparisons all loci were found to conform to
176 Hardy-Weinberg expectations and estimates of F_{IS} indicate no significant evidence of
177 heterozygote excess or deficit (Table 2).

178 *Population genetic analysis*

179 Estimates of genetic diversity across sites were similar (Backyards mean N_A and H_E =
180 2.87 and 0.40, respectively; Bilgola mean N_A and H_E = 2.80 and 0.35, respectively), however
181 F_{ST} across all loci was significantly different from zero (global $F_{ST} = 0.27$; $P < 0.001$)
182 indicating limited gene flow and genetic structuring amongst our two sampling sites. These
183 findings were further substantiated by results from AMOVA with 27% ($P < 0.001$) of the
184 variation across microsatellite loci explained by variation between sites (variation within sites
185 = 73%, $P < 0.001$). The relationships between sites are best depicted by the 2-dimensional
186 FCA of the microsatellite variation (Figure 1). When the 2 factors explaining the highest
187 percentage of the microsatellite variation (factor 1 = 18.65%, factor 2 = 5.48%) are plotted
188 against each other, individuals from the Backyards and Bilgola sample sites are clearly
189 separated.

190 Spatial autocorrelation analyses based on different relatedness measures indicated no
191 significant patterns between relatedness and geographic distance at either sampling site
192 (Figure 2). These findings are complemented by non-significant Hardy-Weinberg estimates
193 ($P < 0.05$) across loci and all individuals for each site, suggesting that individuals from each
194 site represent randomly mating outcrossing populations.

195 **Discussion**

196 The 15 characterized microsatellite markers described in this study provide a valuable
197 resource for future population genetic assessments of *H. banksii* in Australasia. This new panel
198 of markers complement those previously developed by Coleman et al. (2011), and will result
199 in more reliable estimates of population genetic structure across the species range. Such an
200 analysis will help guide future conservation management through the characterisation of broad
201 patterns of population connectivity, and the identification of isolated, self-recruiting
202 populations that require independent management consideration. Estimates of gene flow across
203 the species range will also provide insights into the species' adaptive potential in light of
204 climate change, as gene flow is known to play a critical role in the adaptation process
205 (Frankham et al. 2011; Holt and Gomulkiewicz 1997). Furthermore, contemporary estimates
206 of genetic diversity at the population level will also provide valuable baseline data for
207 monitoring the health of *H. banksii* populations across generations.

208 Propagule movement in *H. banksii* is thought to be restricted to 10s of meters
209 (Bellgrove et al. 2004), and vegetative reproduction through fragmentation of the holdfast
210 (McKenzie and Bellgrove 2009) may potentially result in multiple clones of the same genotype
211 growing in close proximity. This suggests that local genetic structuring and significant
212 correlation between relatedness and geographic distance is likely in this species. The potential
213 for local genetic structuring must be accounted for when sampling individuals for population
214 genetic analysis, as the inclusion of highly related individuals can lead to biased estimates of
215 gene flow and population genetic structure (Hedrick 2011). However, our spatial
216 autocorrelation analyses indicate no significant patterns between relatedness and geographic
217 distance at either sampling site, even over very small distances. These findings are
218 complemented by Hardy-Weinberg estimates, suggesting that individuals from each site
219 represent randomly mating outcrossing populations. Our results indicate that propagule
220 movement may exceed the currently assumed dispersal distances, and that holdfast

221 fragmentation is likely to be rare in *H. banksii.* Based on these findings sampling efforts for
222 future population genetic studies should not be concerned about potential relatedness effects at
223 distances < 10 m.

224 Our preliminary population genetic analyses indicate limited gene flow among the
225 two sampling sites. This is not a surprising result given these sites are located approximately
226 1500 km apart, and that Coleman et al. (2011) found evidence of genetic structuring on much
227 finer spatial scales. These sites are also separated by a well recognised biogeographic barrier.
228 Strong genetic structuring among marine communities either side of the Wilsons Promontory
229 region in south-eastern Australia is well recognised (Ayre et al. 2009; Colton and Swearer
230 2012; Miller et al. 2013; York et al. 2008), and attributed to historical (Lambeck and
231 Chappell 2001) and contemporary physical factors, such as converging ocean currents,
232 environmental gradients (temperature and salinity), and habitat discontinuities that persist in
233 the region (Baines et al. 1983; Colton and Swearer 2012; Ridgway and Condie 2004;
234 Ridgway and Godfrey 1997; Sandery and Kaempf 2007). However these findings are
235 preliminary and more comprehensive sampling will be needed to determine the true extent of
236 gene structuring across the species range.

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388 **Table 1**. Primers sequences and characteristics of 15 microsatellite loci isolated from *H.*
389 *banksii*. Primer combinations for each multiplex reactions is provided. N_A = number of alleles.

Locus	Primer sequences (5'-3')	Repeat motif	N_A	Size range (bp)	GenBank Accession
Multiplex 1					
HB03	CGTGACTTACGGTTGCTGTG AAGTAGGTTCATTCTCCGACGA	AG	3	121-131	
HB21	CTCTTGTTTGCAGGACAGGG ATTCCAGTCCCGTTCCAGTT	AAT	3	145-151	
HB26	CTGTATCTTGTCGCCGCTCT ACACCGTTGTAAGAGCGCAT	ACC	2	158-166	
HB44	GAGCCGTATACTTTGGAAATACG ATATACGCGGTTGCCAATTC	AC	2	115-118	
Multiplex 2					
HB04	CACAGGCGGATCACAAACAT CCTTTGTCCAGCGAGATGTG	ACC	5	122-142	
HB16	TCAGGGTGAGGGACTTTCAA AACGTTTAGAAGCTACTCGCTCA	AG	3	115-119	
HB36	CCTTTCTCTGTGCTTGGTGG GTATATGGAGAGGCGAGCCC	AAT	3	199-219	
HB42	CAATATGCACGACCCACAAA CATCAGCATCACAGCACCAG	ACG	3	165-171	
Multiplex 3					
HB05	AACTCGCACCTTTGGACACT GTTACCAGGCTGTTCACCG	ACG	4	154-160	
HB10	TGAAGTTGCTGCTTCTGCTG AACAGACGGAGCGTGAAGAG	AGG	2	257-260	
HB41	TAATGGTTCGGAGATGACGG GTCCTGGATTGCGCTGTAGT	AGC	6	106-127	
Multiplex 4					
HB57	AGTTTCCCACGAAATGCAGA GCGTCCTTTGTCTTGTGACC	AG	3	255-273	
HB61	CATTTCCTGCGAAACCACAT TCATCGATGTGCACGTAACC	AG	3	129-133	
HB62	CAAGTTCACGTCGTCCATGA GTGCTTCTGGCAAGAGATGG	ACAT	4	202-214	
HB70	GACAGAGACGTGGACCACAA CGGTCGATCCGAATTGTCTA	AAT	3	114-118	

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Table 2. Statistics for *Hormosira banksii* populations screened with 15 polymorphic microsatellite loci. Mean values per locus are presented for number of alleles (N_A), expected (H_E) and observed (H_O) heterozygosities, Hardy-Weinberg equilibrium P values, and inbreeding values (F_{IS}). Estimates of population differentiation (F_{ST}) are also provided for all loci. Bolded values indicate significance after corrections for multiple comparisons. A total of 53 and 55 individuals were genotyped for each locus from the Backyards and Bilgola sampling sites, respectively.

		HB03	HB21	HB26	HB44	HB04	HB16	HB36	HB42	HB05	HB10	HB41	HB57	HB61	HB62	HB70	Mean
Backyards	N_A	2	2	2	2	4	3	3	3	3	1	6	2	2	6	2	2.87
	H_E	0.43	0.5	0.46	0.5	0.52	0.55	0.45	0.41	0.38	0.00	0.64	0.14	0.04	0.76	0.18	0.40
	H_O	0.41	0.86	0.42	0.4	0.41	0.45	0.37	0.32	0.42	0.00	0.57	0.15	0.04	0.79	0.20	0.39
	HWE	0.70	0.00	0.54	0.14	0.63	0.20	0.49	0.01	0.02	NA	0.74	0.59	0.89	0.56	0.44	0.43
	F	0.06	-0.74	0.09	0.20	0.20	0.18	0.16	0.21	-0.10	NA	0.11	-0.08	-0.02	-0.03	-0.11	0.01
Bigola	N_A	2	3	2	2	3	3	3	3	4	2	3	3	3	4	2	2.80
	H_E	0.02	0.30	0.48	0.25	0.13	0.52	0.66	0.50	0.62	0.02	0.27	0.53	0.11	0.44	0.49	0.36
	H_O	0.02	0.27	0.48	0.21	0.13	0.42	0.67	0.50	0.73	0.02	0.27	0.52	0.12	0.50	0.50	0.36
	HWE	0.94	0.40	0.96	0.30	0.97	0.28	0.94	0.74	0.13	0.94	0.91	0.84	0.98	0.90	0.86	0.74
	F	-0.01	0.10	-0.01	0.14	-0.05	0.18	-0.02	0.01	-0.17	-0.01	-0.01	0.02	-0.05	-0.14	-0.02	0.00
F_{ST}		**0.27**	**0.18**	**0.10**	**0.29**	**0.36**	**0.30**	**0.12**	**0.20**	**0.07**	0.02	**0.45**	**0.43**	0.00	**0.16**	**0.27**	

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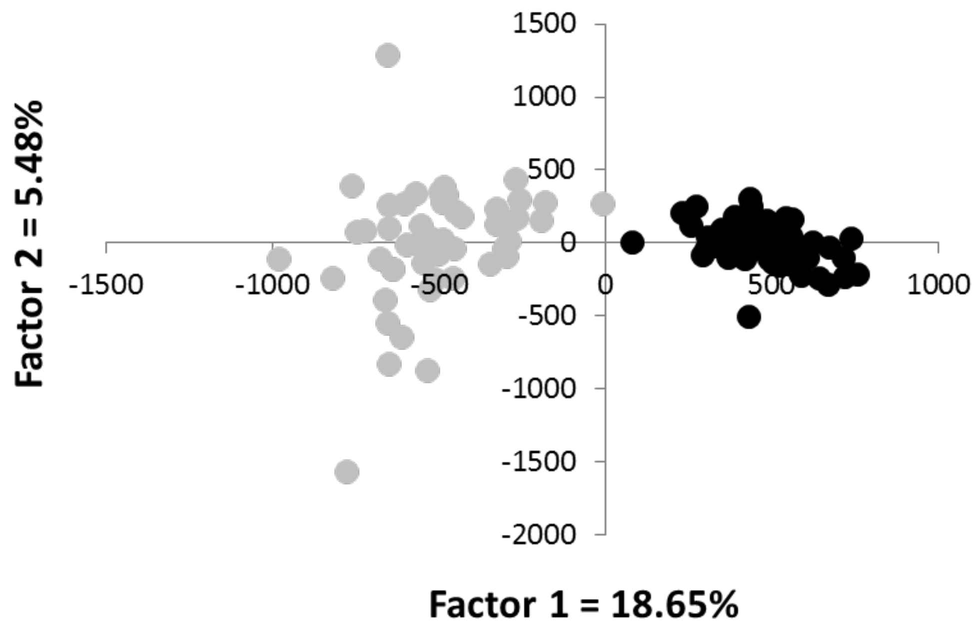

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404 **Figure 1.** Two-dimensional scatter plot showing the relationships among *Hormosira*
405 *banksii* sample sites based on a factorial correspondence analysis of 15 microsatellite
406 loci. Individuals from Backyards and Bigola sample sites are depicted by grey and black
407 solid circles, respectively. The first factor (x-axis) explains 18.65% of the variance,
408 whilst the second factor (y-axis) explains 5.48%.
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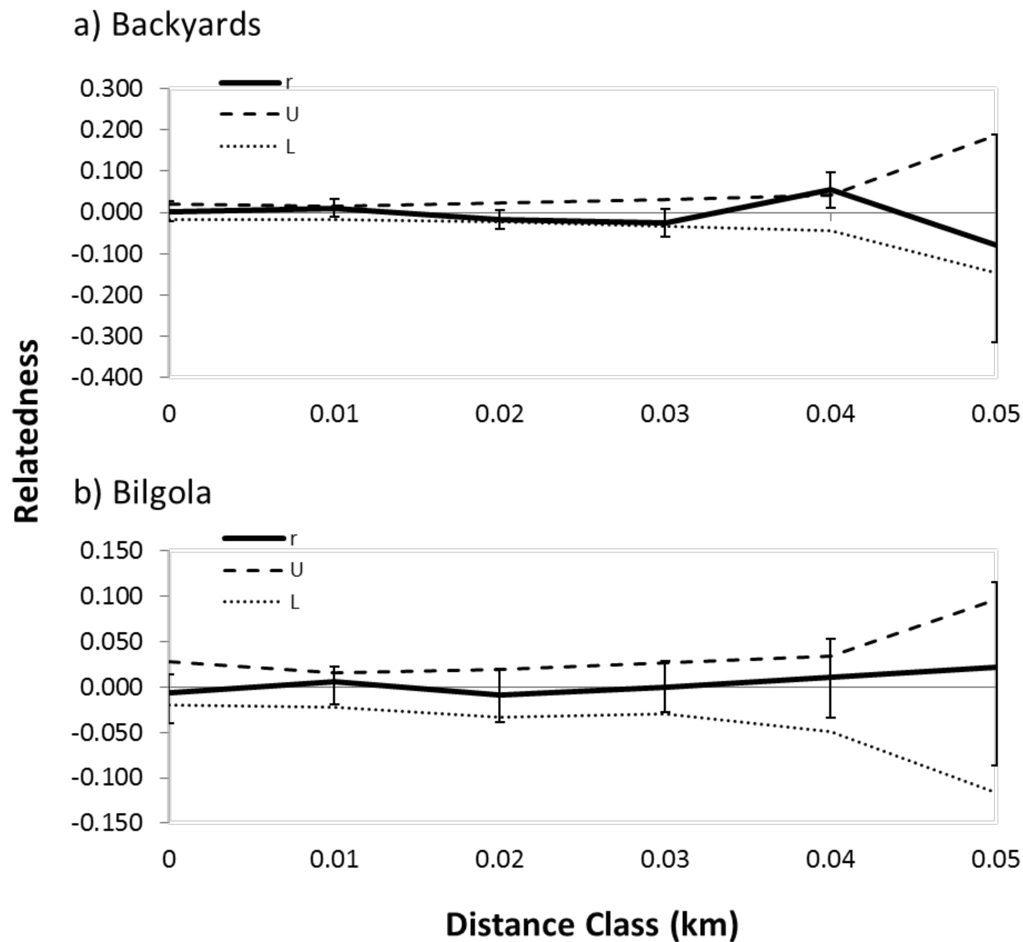

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411 **Figure 2.** Spatial auto-correlograms of average relatedness coefficient (*r*) (± SEM) and
412 upper (U) and lower (L) 95% confidence intervals in relation to distance for pairs of
413 individuals at a) Backyards and b) Bilgola. At total of 1,485 and 1,378 pairwise
414 relatedness estimates were included for the Backyards and Bilgola analyses,
415 respectively.
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