Microsatellite primer development for the seagrass *Zostera nigricaulis* (Zosteraceae)

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Abstract

Seagrasses are marine angiosperms with a worldwide distribution that form conspicous beds in nearshore habitats. Despite being universally recogised as a foundation species that performs a number of important ecosystems functions (incl. sediment stabilisation, facilitation of biodiversity, nurtient cycling and carbon sequestration), global seagrass habitats are in decline. Resilience - the ability to recover from disturbance without swithing to an alternative state - is paramount to the maintenance and persistence of seagrass habitats. Genetic diversity is a key component of seagrass resilience and contributes to an understanding of population structure, connectivity between populations, and reproductive strategies. Microsatellite primers were developed to investigate the resilience of the seagrass *Zostera nigricaulis*, which dominates subtidal habitats in the bays of south-eastern Australia. We also tested for cross-amplification of markers between *Z. nigricaulis* and previously developed markers for the sympatric species *Z. muelleri* to assess their applicability for use in assessing patterns of genetic diversity, population structure, and mating system. Using next-generation sequencing we isolated 11 novel microsatellite loci for *Z. nigricaulis*, 8 of which were polymorphic for the samples tested. Allelic diversity ranged from 1 to 8. None of the primer pairs developed for *Z. nigricaulis* cross-amplified in *Z. muelleri*; but 14 of 24 primer pairs previously developed for *Z. muelleri* amplified clearly in *Z. nigricaulis* samples with 6 of these showing polymorphism. The results demonstrate the applicability of the *Z. nigricaulis* microsatellite primers for use in the study of population genetics and limited cross-amplification with *Z. muelleri*.

Key words: *Zostera nigricaulis*; microsatellite markers; cross-amplification; genetic structure; seagrass

Seagrasses are important foundation species that occupy shallow estuarine and coastal waters and provide a variety of important ecosystem services with substantial economic value (e.g. facilitation of biodiversity; fisheries production; sediment stabilisation; nutrient cycling & carbon sequestration, Short et al. 2011). Seagrasses are under constant disturbance pressure from both natural and anthropogenic causes and have been declining globally at ever increasing rates (Waycott et al. 2009). The resilience of seagrasses depends on their ability to either withstand these external stressors, or recover from losses through both sexual and asexual reproductive strategies.

Zostera species are among the most important, widely distributed and, therefore, well-studied seagrass species globally. *Zostera nigricaulis* Kuo (Zosteracaea), has a native range extending from Western Australia around the southern coast of Australia to central New South Wales (Kuo 2005; Les et al. 2002). *Zostera nigricaulis* is distinguished from other *Zostera* species by the presence of erect, dark brown stems and is the dominant sub-tidal species found in Port Phillip Bay, Victoria, Australia (Kuo 2005). Currently there are no published genetic markers for *Z. nigricaulis*, hampering efforts to understand *Z. nigricaulis* population genetics. Here we developed a set of microsatellite markers to examine key population parameters; including: genetic structure, diversity, clonal structure, and patterns of connectivity in *Z. nigricaulis* populations from south-eastern Australia.

Approximately 10 µg of genomic DNA from fresh leaf tissue from a single individual of *Zostera nigricaulis* collected from Port Phillip Bay, Victoria, Australia using DNeasy plant kits (QIAGEN), following the manufacturers instructions. We developed microsatellites following the methodology of Sherman et al. (2012) and Gardner et al. (2011). Briefly, we sequenced one-eighth of a plate using the GS-FLX 454 platform (Roche, Germany), providing 121,156 sequenced reads. A total of 480 unique sequence reads possessing microsatellite motifs were identified using the software QDD v 1.3 (Meglecz et al. 2010). Primer pairs were designed using default settings on Primer3 for 24 of the fragments (Rozen and Skaletsky 2000). Multiplexes consisting of four loci were designed where forward primers had a fluorescent dye associated tag added (FAM-GCCTCCCTCGCGCCA; NED-GCCTTGCCAGCCCGC; VIC-CAGGACCAGGCTACCGTG; PET-CGGAGAGCCGAGAGGTG) (Blacket et al. 2012).

To test loci for levels of polymorphism, we screened six individuals collected from Port Phillip Bay, Victoria, Australia. DNA extraction followed the method reported above. Polymerase chain reactions (PCR) were

conducted in 11 µL volumes containing; 10 ng of genomic DNA; 5 µL PCR Master Mix (Qiagen, USA) and 4 µL primer multiplex (0.26 µM of each forward primer and fluorescent dye, 0.13 µM of reverse primer). PCR products were amplified using a touchdown programme; initial hot start at 94°C for 15 min; five cycles of 94°C for 45 s, 65°C for 45 s, 72°C for 45 s; five cycles of 94°C for 45 s, 60°C for 45 s, 72°C for 45 s; 10 cycles of 94°C for 45 s, 57°C for 45 s, 72°C for 45 s; 20 cycles of 94°C for 45 s, 55°C for 45 s, 72°C for 45 s; final elongation at 72°C for 15 min. PCR amplicons were electrophoresed using an ABI 3130xl Genetic Analyzer, incorporating LIZ 500 (-250) size standard (Applied Biosystems). Alleles were scored using GeneMapper, v3.7 (Applied Biosystems). Population genetic statistics were calculated with GENEPOP on the web (Raymond and Rousset 1995) and GENALEX (V6.41) (Peakall and Smouse 2006). We tested the statistical power of the marker system to identify different clones by calculating the probability of identity, P_{ID}, for increasing locus combinations (Waits et al. 2001) using the program GENALEX (V6.41) (Peakall and Smouse 2006). This identification estimator calculates the probability that two individuals drawn at random from a population will have the same genotype at multiple loci and is used to assess the statistical confidence of the marker system for individual identification. P_{ID} was calculated for each locus and then multiplied across loci to give an overall P_{ID} (Waits et al. 2001).

Eleven microsatellite primer pairs (Table 1) amplified clearly and consistently; of these 8 were polymorphic within the seagrasses sampled. Overall we detected moderate levels of polymorphism across the variable loci with the number of alleles per locus ranging from 2 to 8 and a mean of 3.50 alleles per locus. Estimates of unbiased expected heterozygosity ranged from 0.28 to 0.86, with a mean of 0.53, while observed heterozygosity varied from 0.00 to 0.80, with a mean of 0.61 (Table 2). Three loci showed significant deviations from Hardy–Weinberg equilibrium, however, none of these remained significant after sequential Bonferroni correction for multiple tests. We also report details of three monomorphic primer pairs (ZosVic 49, ZosVic 53 and ZosVic 64), as these loci may prove useful for population genetic studies of other widely geographically isolated populations despite the lack of variability in the sampled population. All individuals displayed a unique multi-locus genotype and the combined probability of identity across all variable loci was low ($P_{ID} = 5.79 \times 10^{-06}$), suggesting that these loci have a high level of power in detecting unique genotypes in this highly clonal species.

We also tested the ability of the primers developed in this study to cross-amplify in the closely related, co-occurring seagrass *Z. muelleri* Irmisch ex Asch. using samples collected from Lake Macquarie, New South Wales. Similarly, we tested the cross-amplification of recently developed *Z. muelleri* primers (Sherman et al.

2012) in *Z. nigricaulis* samples from this study. DNA was extracted and the loci genotyped for individuals and statistics generated following the protocols described above. Our results showed that none of the primer pairs developed for *Z. nigricaulis* amplified in *Z. muelleri* samples (Table 2). Of the 24 primer pairs developed for *Z. muelleri*, fourteen amplified clearly in *Z. nigricaulis* samples, but only six of these were polymorphic (Table 2). Levels of polymorphism across the six loci that cross-amplified were low ranging between 2 and 4 alleles with a mean of 2.5 alleles per locus. Low levels of cross amplification between primer pairs developed for each species supports the distinction of *Z. nigricaulis* and *Z. muelleri* as separate species (Les et al. 2002). The markers developed here will be a powerful tool for assessing patterns of population connectivity, levels of genetic diversity, and an understanding of the relative importance of asexual versus sexual reproduction to population persistence in this ecologically important species.

Acknowledgments

The Authors would like to thank A. Hirst, N. Hutchinson, C. White and D. Hatton for assistance with specimen collection. This study was financially supported by a research grant from the Victorian Department of Sustainability and Environment: 'Seagrass resilience in Port Phillip Bay: developing better predictions of how seagrasses respond to environmental change'.

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Tables

TABLE 1. Microsatellite markers developed for 11 loci in the seagrass *Zostera nigricaulis*. *N* = Number of individuals that amplified. *A* = Number of alleles. Product size range including the associated fluorescent dye tag (FAM-GCCTCCCTCGCGCCA; NED-GCCTTGCCAGCCCGC; VIC-CAGGACCAGGCTACCGTG; PET-(GGAGAGCCGAGAGGTG)(Blacket et al. 2012)

Locus	Genbank Acc. No.	Primer sequence (5'-3')	Motif	Fluorescent tag	Size range*	*N*	*A*
ZosVIC49	JQ236670	F: AAAGACATGTTCGAGAAACCC R: CGCATATGTGATACGCATAAACC	AT	FAM	157	6	1
ZosVIC55	JQ236673	F: GTGTCAAACTTGCACAACTTCC R: AAAGGTGGGCCTCAGTGTC	CT	VIC	194-206	6	3
ZosVIC50	JQ236671	F: TCAGCCAAGTATGAGGTGGTC R: GCGAGGCTTGCTTTAGTAGC	AT	VIC	163-167	6	2
ZosVIC70a	JQ236678	F: TCAGCCGGAGGAGAATTGG R: TGACGGAGATGAAGCCACC	CATT	VIC	201-208	5	2
ZosVIC70b	JQ236678	F: TCAGCCGGAGGAGAATTGG R: TGACGGAGATGAAGCCACC	CATT	VIC	215-258	5	8
ZosVIC60	JQ236675	F: ATCCTCTGTTGTGCCCGTC R: GCTGCTATCATTGGTGGCTATG	AT	FAM	167-203	6	2
ZosVIC69	JQ236677	F: TGGTTGAGCAATGAGATTCGG R: TCCACGATGAGTTGTTCTCAAG	ATCC	FAM	160-180	6	4
ZosVIC64	JQ236676	F: ACCTGAGATTTCACTTGATCCTG R: TCTGGAGGTTTGATCTTCCC	AT	PET	204	5	1
ZosVIC53	JQ236672	F: TCGAAATGTCGCTTGATGCC R: ACTAAGTTGTTGCCGACTTGAC	GT	PET	173	3	1
ZosVIC59	JQ236674	F: AGACCCTTATTGGGCTGGAG R: AACGAGGTTTGACCTTGCC	AT	NED	196-216	6	2
ZosVIC71	JQ236679	F: TTGCAGTCTTGACGAGAAAC R: TGCTAGTCTCTGCCGAATG	AGTT	VIC	343-379	5	5

TABLE 2. Results of primer screening of *Zostera nigricaulis* from Port Phillip Bay and *Z. muelleri* from Lake Macquarie. *N* = Number of individuals that amplified, *A* = Number of alleles, H_O = Observed heterozygosity, UH_E = Unbiased expected heterozygosity

	Zostera nigricaulis Port Phillip Bay (n = 6)				*Zostera muelleri* Lake Macquarie (n = 8)			
Loci	*N*	A	H_O	UH_E	*N*	A	H_O	UH_E
ZosVIC49	6	1	0.000	0.000	0	0	0.000	0.000
ZosVIC55	6	3	0.667	0.612	0	0	0.000	0.000
ZosVIC50	6	2	0.000	0.303	0	0	0.000	0.000
ZosVIC70a	5	2	1.000	0.556	0	0	0.000	0.000
ZosVIC70b	5	8	0.800	0.956	0	0	0.000	0.000
ZosVIC60	6	2	0.833	0.530	0	0	0.000	0.000
ZosVIC69	6	4	0.333	0.697	0	0	0.000	0.000
ZosVIC64	5	1	0.000	0.000	0	0	0.000	0.000
ZosVIC53	3	1	0.000	0.000	0	0	0.000	0.000
ZosVIC59	6	2	0.833	0.530	0	0	0.000	0.000
ZosVIC71	5	5	0.200	0.822	0	0	0.000	0.000
ZosNSW02	1	1	0.000	0.000	8	3	0.375	0.575
ZosNSW13	3	1	0.000	0.000	6	2	0.500	0.409
ZosNSW15	0	0	0.000	0.000	7	3	0.571	0.538
ZosNSW17	1	1	0.000	0.000	7	3	0.429	0.692
ZosNSW18	0	0	0.000	0.000	7	3	0.429	0.385
ZosNSW19	0	0	0.000	0.000	7	5	0.429	0.593
ZosNSW20	5	1	0.000	0.000	7	8	1.000	0.912
ZosNSW23	5	2	1.000	0.556	7	2	0.429	0.363
ZosNSW25	4	3	0.250	0.464	8	3	0.000	0.633
ZosNSW27	6	2	1.000	0.545	8	1	0.000	0.000
ZosNSW28	3	2	0.333	0.333	8	2	0.500	0.400
ZosNSW29	5	1	0.000	0.000	8	2	0.500	0.700
ZosNSW34	6	2	1.000	0.545	8	2	0.500	0.400
ZosNSW35	0	0	0.000	0.000	8	1	0.000	0.000
ZosNSW36	3	4	0.333	0.867	5	3	0.200	0.644
ZosNSW38	5	1	0.000	0.000	8	3	0.750	0.700
ZosNSW40	0	0	0.000	0.000	8	2	0.000	0.233
ZosNSW41	0	0	0.000	0.000	4	1	0.000	0.000
ZosNSW42	0	0	0.000	0.000	8	2	0.000	0.400
ZosNSW43	2	1	0.000	0.000	8	2	0.500	0.400
ZosNSW44	0	0	0.000	0.000	8	1	0.000	0.000
ZosNSW45	6	1	0.000	0.000	8	4	0.625	0.725
ZosNSW46	0	0	0.000	0.000	8	10	1.000	0.900
ZosNSW47	0	0	0.000	0.000	8	3	0.000	0.633

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Title:
Microsatellite primer development for the seagrass Zostera nigricaulis (Zosteraceae)

Date:
2013-09-01

Citation:
Smith, T. M., York, P. H., Stanley, A. M., Macreadie, P. I., Keough, M. J., Ross, D. J. & Sherman, C. D. H. (2013). Microsatellite primer development for the seagrass Zostera nigricaulis (Zosteraceae). CONSERVATION GENETICS RESOURCES, 5 (3), pp.607-610. https://doi.org/10.1007/s12686-013-9862-3.