Development of Multiplex Microsatellite PCR Panels for the Seagrass Thalassia hemprichii (Hydrocharitaceae)

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Primer Note

Development of multiplex microsatellite PCR panels for the seagrass *Thalassia hemprichii* (Hydrocharitaceae)[1]

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- *Premise of the study:* New microsatellites were developed for the seagrass *Thalassia hemprichii* (Hydrocharitaceae), a long-lived seagrass species that is found throughout the shallow waters of tropical and subtropical Indo-West Pacific. Three multiplex PCR panels were designed utilizing new and previously developed markers, resulting in a toolkit for generating a 16-locus genotype.
- *Methods and Results:* Through the use of microsatellite enrichment and next-generation sequencing, 16 new, validated, polymorphic microsatellite markers were isolated. Diversity was between two and four alleles per locus totaling 36 alleles. These markers, plus previously developed microsatellite markers for *T. hemprichii* and *T. testudinum*, were tested for suitability in multiplex PCR panels.
- *Conclusions:* The generation of an easily replicated suite of multiplex panels of codominant molecular markers will allow for high-resolution and detailed genetic structure analysis and clonality assessment with minimal genotyping costs. We suggest the establishment of a *T. hemprichii* primer convention for the unification of future data sets.

Key words: clonality; Hydrocharitaceae; microsatellites; population genetics; seagrass; *Thalassia hemprichii*.

Thalassia hemprichii (Ehrenb.) Asch. is a widely distributed seagrass of the family Hydrocharitaceae, and one of four marine angiosperm lineages (Waycott et al., 2004). Seagrasses provide substantial and valuable habitat that has been significantly declining (Waycott et al., 2009). An important trait of all seagrasses is clonality through vegetative growth leading to some very large and old individual plants (Arnaud-Haond et al., 2012). Identification of genets (plants originating from one seed) with a high degree of confidence is impossible in the field and only possible through the use of high-resolution molecular markers capable of generating individual specific genotypes such as microsatellites. Despite the potential of new techniques to generate genetic data for individuals and populations, microsatellites are still the marker of choice to study the clonal biology of seagrasses. Microsatellites have been developed in the recent past (Matsuki et al., 2012; Wainwright et al., 2013), but were not available at the time when this study was initiated. Due to the substantial number of loci available, a selection following criteria to optimize outcomes was made to design multiplex panels that are easily amplified and give high-resolution power. In addition, many of the previously published loci, in particular those published by Matsuki et al. (2012), were based on compound microsatellites and primers anchored in the repeats themselves. Compound loci have been used in our laboratory for previous studies (e.g., van Dijk et al., 2007, 2009), and alleles of perfect repeats were usually scored with less difficulty. For this reason we propose to use loci with a higher likelihood of providing unambiguous genotypes, which are particularly useful for determining clonality based on probability estimates.

METHODS AND RESULTS

Novel microsatellite development—Genomic DNA was extracted from *T. hemprichii* shoot tissue from 13 samples originating from seven populations along the northeastern Australian coast (Magnetic Island: 19°110.772′S, 146°50.447′E; Thursday Island: 10°58.598′S, 142°21.623′E). Voucher material from each population was deposited at the State Herbarium of South Australia (Appendix 1). DNA was extracted using the DNeasy Plant Mini Kit (QIAGEN, Valencia, California, USA) following the manufacturer's protocol. DNA was sent to GenoScreen (Lille, France) for microsatellite enrichment and high-throughput 454 pyrosequencing (GS-FLX Titanium; Roche Diagnostics, Basel, Switzerland) following methodology described in Malausa et al. (2011). Microsatellite enrichment was done for motifs TG$_{10}$, TC$_{10}$, AAC$_8$, AAG$_8$, AGG$_8$, ACG$_8$, ACAT$_6$, and ACTC$_6$.

A total of 24,258 distinct sequences were obtained, of which 11,864 had microsatellite motifs. Primers were developed for 184 loci using the QDD pipeline (Meglécz et al., 2010). Forty-five loci were selected and sequences compared to previously published data using the online nucleotide BLAST tool of the National Center for Biotechnology Information (Altschul et al., 1990). Primers were ordered (Invitrogen, Carlsbad, California, USA) with M13-tails (5′-TGTAAAACGACGGCCAGT-3′) added to the 5′ end of the forward primer (Schuelke, 2000). A PIG-tail (5′-GTTTCT-3′) was added to

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the 5′ side of the reverse primer (Brownstein et al., 1996). Initial testing was done on eight samples from the northeastern coast of Australia (Appendix 1). Amplification under a range of temperatures and cycling conditions was conducted using MyTaq HS-DNA polymerase (Bioline, Taunton, Massachusetts, USA). Amplification was done following manufacturer instructions in a 15-µL volume using 0.5 units of polymerase. Final primer concentrations were 0.6 pmol of the M13-labeled forward primer and 2.4 pmol of the reverse primer and fluorolabeled M13 primers (NED, VIC, or FAM). The DNA template was diluted to 0.5–1.0 ng·µL^{-1}. In addition, loci previously developed for *T. testudinum* Banks & Sol. ex K. D. Koenig were also tested (Van Dijk et al., 2007) using the same amplification conditions. The only modification was that fluorolabeled (NED, VIC, or FAM) forward primers at 2.4 pmol were used instead of M13-labeled forward primers. PCR products were separated by capillary electrophoresis on a MegaBACE 1000 (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom) with an internal ET-ROX 400 size standard (GE Healthcare). A total of 16 new polymorphic microsatellite loci and one *T. testudinum* microsatellite were found to be polymorphic for *T. hemprichii* (Table 1).

Table 1. Genetic properties of polymorphic microsatellites for the seagrass *Thalassia hemprichii*.

Locus[a]	Primer sequences (5′–3′)[b]	Repeat motif	Allele size range (bp)	GenBank accession no.
THH-1	F: VIC-GCATGGGAGGTAAAACCTGA R: pig-GCACTTCAGCTCGCAAAGAT	$(GA)_{11}$	153–157	KJ126795
THH-2	F: FAM-TGTTAACAAACGCACCAACG R: pig-TGGCGAAACAACTTTAACTCC	$(GA)_{10}$	123–129	KJ126796
THH-3	F: NED-GGGAATTGCCTCAACTCATT R: pig-TTTGTTAAATCGCATTTGCTTC	$(TC)_{9}$	254–258	KJ126797
THH-5	F: FAM-CACGAAATTGCAACCAACAC R: pig-TTTGTTGGATCGCTATGAGTATG	$(AC)_{14}$	167–175	KJ126798
THH-6	F: FAM-TGACACATTCGGTAAAATAAACA R: pig-TCACATGCATACACAAACTGCAT	$(CA)_{13}$	107–117	KJ126799
THH-8	F: FAM-GGACCTGAAGAGGGATGTGA R: pig-ATGCCCTGTTCTCTGCTTTC	$(GGA)_{8}$	213–222	KJ126800
THH-13	F: VIC-CCATCTCCACCCTCCATTTA R: pig-TGCAGGTTCTAGATAAGCGTGA	$(CT)_{10}(A)_{9}$	295–297	KJ126801
THH-14[c]	F: NED-TCCATCCATCCAAATAGCAA R: pig-CGTCGCCCTGAAAAGAATAC	$(TCT)_{7}$	128–131	KJ126802
THH-15	F: VIC-CTCGCTAGGGGGATTGTGT R: pig-TCAACTAAAACAAAGGCAAGGAAA	$(AC)_{12}$	160–164	KJ126803
THH-26	F: VIC-GGAGGTGGCTTAATGATAGGTG R: pig-TAGCTGCAATAGAGCGAGAGC	$(TTC)_{11}(TC)_{7}$	218–245	KJ126804
THH-29	F: FAM-TGCCCAAATATTTCTGAAAACC R: pig-TCTCACTCGAACAGTCATCCA	$(GA)_{11}$	157–159	KJ126805
THH-34	F: FAM-TGGAGATGAGGACAGGTGTG R: pig-CGCTACCCTCGGTTTATTCC	$(GT)_{8}$	291–297	KJ126806
THH-36	F: NED-TAAAGGCAGGTCTCGACGTT R: pig-TCAATGGGCATTTAGCCACTT	$(AAG)_{7}$	195–198	KJ126807
THH-39[c]	F: VIC-AAAACGTTTGACGCACTAGG R: pig-TGCATGCATGACATATTGTATTT	$(GT)_{9}$	143–145	KJ126808
THH-41	F: VIC-GAACCGCATAGAAGTACAATCAAA R: pig-TCATGGTACATGCTCAGTCTAATG	$(GGGAGA)_{5}$	234–252	KJ126809
THH-42[c]	F: NED-ATGGGTTTGGATTAAAATGGC R: pig-CATTTCTCTTATTACCTTCATTCTTCC	$(GA)_{9}$	127–129	KJ126810
TCT-58	F: NED-CCAAAACAAGGGCAACAAAG R: pig-TGGAGGAGAGACCCAAGAAGC	$(GAA)_{15}(GAT)_{4}(GAA)_{3}$	154–157	DQ512881
TH07	F: FAM- GTGGAGTTTGGGTTCTACTAAG R: pig-CAAACACAATTGCCAAGC	$(GAA)_{7}$	93–105	KC132847
TH32[d]	F: NED-ATATTCACGTCATTTGCCTT R: pig-GTACCGGTGAATTTATGTAGC	$(TC)_{15}$	97–103	KC132850
TH34	F: VIC-TTTCCATCTTCACAAACTCTT R: pig-ATAGGGAGAGGAGGAGTAGC	$(TC)_{7}$	125–135	KC132851
TH37	F: NED-GTGTGAGAGGAGAGTGAGGA R: pig-CTCACACGTAGTGACCCCT	$(GA)_{15}$	165–176	KC132853
TH43	F: FAM-ACTTGGAAAACAGCTGGTAA R: pig-CGTGTGTGACCACAATCTAC	$(AG)_{13}$	172–176	KC132855
TH52	F: NED-TCTCTAGGTTGGACGGCTAT R: pig-CCTTACCTTCGAATTAGGGT	$(GT)_{13}$	171–183	KC132863
TH66	F: VIC-GCAAGAGATCAGGTCGATAA R: pig-GACCATTGAGGGTGATTATT	$(GT)_{9}$	205–209	KC132866
TH73	F: NED-TCACTACCTTGACTTAGGGC R: pig-GATTCCAACAATCCCTAACA	$(GT)_{9}$	219–223	KC132868

[a] THH-1–THH-42 were developed for this study; TCT-58 was a cross-test of primers of the related species *Thalassia testudinum* (Van Dijk et al., 2007). TH07–TH73 are loci previously developed for *T. hemprichii* by Wainwright et al. (2013).

[b] Reverse primers all have a PIG-tail (GTTTCT) added to the 5′-end for the purposes of screening in our laboratory setup so fragment lengths will be shorter if primers are made without them.

[c] Loci that were not tested on population samples.

[d] Locus was tested but did not amplify consistently.

***Multiplex design*—**Recently Matsuki et al. (2012) and Wainwright et al. (2013) have also published microsatellites for *T. hemprichii*. A total of 49 polymorphic microsatellite loci are available if all loci are counted. To enhance genotyping outcomes for this species, we developed three sets of highly informative multiplex PCR panels. Utility of loci to be used in multiplex PCR reactions relates to amplicon size, dimer formation, stutter noise, annealing temperature, and primer complexity. Due to so many loci being available to select from, only perfect microsatellite repeats (i.e., no compound repeats) were chosen for multiplex development. As a result, loci published by Matsuki et al. (2012) were excluded from consideration, as they were all compound microsatellites. From the remaining loci, initial selection for inclusion was made based on amplicon length, number of alleles (high allelic diversity preferred), and inbreeding coefficient (loci with extreme deviation of Hardy–Weinberg equilibrium were avoided). Using Multiplex Manager version 1.2 (Holleley and Geerts, 2009), initial test multiplexes were developed and new forward primers were synthesized. Loci that were difficult to amplify were removed from the multiplexes (Table 1), resulting in three panels containing seven, six, and five loci per PCR (Appendix 2). Two loci were repeated in multiplexes to account for occasional dropouts, resulting in a 16-loci genotype in only three PCRs.

Multiplex PCRs were conducted using Type-it (QIAGEN) in 10-μL reactions using 1 μL of primer mix (Appendix 2) and ~2 ng of genomic DNA. To assess population genetic diversity, two populations of *T. hemprichii* were screened: 47 samples from Hammond Island and 47 samples from Pulau Semakau Reef, Singapore (Appendix 1). Both populations' samples were collected from an area of approximately 50 × 50 m. The distance between samples was greater than 2 m. These two populations are widely separated and were selected to reveal the potential of the developed loci. *Thalassia* has a high dispersal capacity (van Dijk et al., 2009), so very distant sites are necessary to uncover this potential. Products were analyzed as above and alleles scored using Genetic Profiler Suite version 2.2 (GE Healthcare).

Analysis of allelic data involved the initial identification of clones using GenoDive version 2.0b25 (Meirmans and Van Tierden, 2004). Clonal genotypes were removed from the data set before calculation to avoid pseudo-replicas that originate from the same plant. Genetic and marker diversity statistics were calculated using GenoDive. The 16-loci genotypes were analyzed for genotypic identity (P_{gen}) and sibling probability (P_{sex}) using GenClone version 2 (Arnaud-Haond and Belkhir, 2007). Linkage disequilibrium between loci was calculated using the log-likelihood-ratio statistic of GENEPOP version 4.2 (Raymond and Rousset, 1995; web version).

The samples screened for both populations with the multiplex panels readily amplified, providing high-probability identification of genotypes. A total of 16 genets (unique genotypes) were identified among 47 samples from Hammond Island and 42 genets among 47 samples from Singapore. Intermediate to low levels of clonality were detected in these two widely separated populations. The resolving power of the combined panels resulted in a P_{gen}(f) [the probability of obtaining a common genotype from the allele pool] of $3.3 \times 10^{-10} - 1.4 \times 10^{-8}$ for Hammond Island and $3.7 \times 10^{-5} - 1.8 \times 10^{-12}$ for Singapore and a P_{sex}(f) [probability that a shared genotype originated from a seed] of $2.0 \times 10^{-3} - 2.8 \times 10^{-3}$ for Hammond Island and 5.49×10^{-5} for Singapore. The level of expected heterozygosity was generally low for most loci (Table 2), particularly when compared to *T. testudinum* (van Dijk et al., 2009). A solid explanation is not possible at this stage, but it might be likely that the populations screened are the result of a recent radiation from a relatively small refugia. Linkage disequilibrium was detected between loci THH-5 and TH52. Near presence of these two loci on the same chromosome could lead to the association of alleles. But, random factors or linkage to genes under similar selective pressure could also lead to linkage disequilibrium.

CONCLUSIONS

The new loci developed for *T. hemprichii* in the suggested multiplex panel designs will contribute significantly to assessing population genetic structure, connectivity, kinship, and clonal diversity in an affordable and reproducible manner. We propose that at a minimum, the loci included in panels THH-a and THH-b should be used in future population genetic studies of *T. hemprichii*. This will allow for comparing and merging data sets in the future with appropriate reference comparisons.

TABLE 2. Estimates of the genetic diversity indices of *Thalassia hemprichii* samples from Hammond Island, Torres Strait, Australia, and Pulau Semakau Reef, Singapore.

	Hammond Island (N = 47, G = 16)				Pulau Semakau Reef (N = 47, G = 42)			
Locus	A	H_o	H_e	F_{IS}	A	H_o	H_e	F_{IS}
THH-1	2	0.33	0.29	−0.17	2	0.42	0.36	0.36
THH-2[a]	2	0.31	0.27	−0.14	2	0.24	0.29	0.29
THH-3	2	0.93	0.50	−0.86	2	0.45	0.42	0.42
THH-5	2	0.13	0.24	0.45	4	0.70	0.66	0.66
THH-6[a]	2	0.20	0.19	−0.08	1	—	—	—
THH-8	4	0.53	0.60	0.11	2	0.40	0.51	0.51
THH-13[a]	2	0.08	0.08	0.00	2	0.29	0.37	0.37
THH-15	2	0.27	0.24	−0.12	3	0.67	0.53	0.53
THH-26[a]	3	0.60	0.54	−0.11	4	0.35	0.34	0.34
THH-29	2	0.57	0.47	−0.21	2	0.14	0.13	0.13
THH-34	3	0.27	0.58	0.54	3	0.02	0.11	0.11
THH-36	2	0.13	0.13	−0.04	1	—	—	—
THH-41	2	0.07	0.07	0.00	2	0.65	0.50	0.50
TCT-58[a]	2	0.87	0.50	−0.75	1	—	—	—
TH07	3	0.60	0.63	0.05	3	0.39	0.45	0.45
TH34	4	0.14	0.21	0.32	3	0.54	0.67	0.67
TH37	4	0.71	0.58	−0.23	5	0.61	0.70	0.70
TH43	2	0.07	0.19	0.65	3	0.31	0.38	0.38
TH52	4	0.79	0.66	−0.19	7	0.41	0.42	0.42
TH66	2	0.21	0.20	−0.08	3	0.07	0.07	0.07
TH73	2	0.13	0.41	0.68	3	0.10	0.12	0.12

Note: A = number of alleles; F_{IS} = inbreeding coefficient within populations; G = number of multilocus genotypes found and used for index calculations; H_e = expected heterozygosity per population; H_o = observed heterozygosity; N = number of samples tested.

[a] Loci that were tested but were not used in the final multiplex panels.

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Appendix 1. Location and voucher information for plant material used for initial microsatellite polymorphism testing of *Thalassia hemprichii*. Vouchers were submitted to the State Herbarium of South Australia (AD).

Location	Voucher no.	Geographic coordinates
Magnetic Island, Queensland, Australia	No voucher	19°10.772′S, 146°50.447′E
Dunk Island, Queensland, Australia	No voucher	17°56.596′S, 146°8.497′E
Green Island, Queensland, Australia	AD268025	16°45.422′S, 145°58.197′E
Green Island, Queensland, Australia	AD268026	16°45.422′S, 145°58.197′E
Low Isles, Queensland, Australia	AD268036	16°23.112′S, 145°33.880′E
Horn Island, Queensland, Australia	AD268042	10°35.635′S, 142°14.706′E
Hammond Island, Queensland, Australia	AD268057	10°32.354′S, 142°13.572′E
Thursday Island, Queensland, Australia	AD268059	10°58.598′S, 142°21.623′E
Pulau Semakau Reef, Singapore[a]	No voucher	1°12.360′N, 103°45.442′E

[a] Samples not used for initial polymorphism testing.

Appendix 2. Multiplex primer mixes for the three panels designed for *Thalassia hemprichii* using fluorescent labels FAM, NED, and VIC. Primer stock concentrations were 100 μM.

Locus[a]	Label	to 500 μL[b]
Panel THH-a		
THH-34	FAM	20
TH07	FAM	10
THH-5	FAM	15
TH37	NED	20
TH73	NED	16
THH-15	VIC	12
TH66	VIC	8
Panel THH-b		
TH43	FAM	10
THH-8	FAM	10
TH52	NED	10
THH-3	NED	25
TH34	VIC	10
THH-41	VIC	10
Panel THH-c		
TH07	FAM	8
THH-29	FAM	10
THH-36	NED	10
THH-3	NED	30
THH-1	VIC	7

[a] Loci THH-3 and TH07 are duplicated.

[b] Volumes (in microliters) to be added to the panel mix are for forward and reverse primer and to be topped up to 500 μL.