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TECHNICAL NOTE

Characterisation of polymorphic microsatellite loci in the freshwater bryozoan *Fredericella sultana*

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Abstract Eight polymorphic microsatellite loci were isolated from massively parallel next-generation sequencing data and tested in three populations (74 individuals) of the colonial freshwater bryozoan *Fredericella sultana*. Up to 13 alleles per locus were found and all loci were polymorphic in all populations. Minimum of three loci were sufficient to distinguish all unique multilocus genotypes. These highly variable markers are suitable for clonal identity assignment based on unique multilocus genotypes and provide tools for resolving fine scale population structure in a species characterised by clonal, vegetative growth and asexual reproduction.

Keywords Bryozoa · Myxozoa · Proliferative kidney disease · 454 · Microsatellite · Polymorphic

Freshwater bryozoans (Family Phylactolaemata, Phylum Bryozoa) are colonial suspension feeders with important ecological roles in nutrient cycling and structuring of benthic freshwater habitats (Sørensen et al. 1986). The bryozoan *Fredericella sultana* also acts as the primary host for the myxozoan parasite *Tetracapsuloides bryosalmonae*, which causes the devastating proliferative kidney disease (PKD) of salmonid fish (Anderson et al. 1999). PKD affects many endangered salmon and trout species (Hedrick et al. 1993) and *F. sultana* has a key role in the persistence and spread of the PKD parasite (Okamura et al. 2011). Thus, understanding the population dynamics of *F. sultana* is crucial for explaining the recent emergence of PKD and may contribute to salmonid conservation and management.

Fredericella sultana colonies grow and spread by budding new colony modules and can reach high densities in suitable conditions. Colonies produce asexual resting stages and may disperse via colony fragments. In contrast, sexual reproduction is short in duration and may be occasionally forgone (Wood 1973). Assignment of clonal identities within such potentially homogeneous populations can be conducted using unique multilocus genotypes (MLGs), but requires highly variable markers for confident assignment. We used newly developed (e.g. Castoe et al. 2010), rapid methods utilising massively parallel 454 sequencing technology to characterize hypervariable microsatellite loci in *F. sultana* for clone identification purposes.

About 3 μg of genomic DNA was isolated from whole colonies using a modified CTAB method and sequenced on a 454 Genome Sequencer FLX with Titanium chemistry at 1/4 plate scale. This yielded 196,025 fragments of average 388 bp in length. The program QDD2 (Meglécz et al. 2010) was used to identify microsatellite loci from the unassembled reads and primers were designed using PRIMER 3 (as implemented in QDD2 using default settings). 936 candidate loci were identified (25% dinucleotide loci, max. 26 repeats, 73% trinucleotide loci, max. 18 repeats and 0.1% tetranucleotide loci, max. repeats 6). To achieve hypervariable markers, 30 high repeat candidate loci were

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tested in 15 individuals from 5 different populations using the Qiagen Type-It microsatellite PCR kit in 8 µl reaction volumes. PCR reaction conditions were: 15 min denaturation at 95°C, 30 cycles of 95°C (30 s), 58°C (90 s), and 72°C (60 s), with final elongation step at 72°C for 30 min. 16 markers amplified consistently and showed putative polymorphisms when visualised on 3% agarose gels stained with ethidium bromide. Since the genomic DNA used for 454 sequencing originated from whole colonies (including guts with potential contaminating microorganisms), amplification specificity was tested using a pure *F. sultana* DNA sample as a template (obtained from resting stages only). All 16 markers amplified successfully and based on complementary fragment sizes, 8 loci were combined into one multiplex PCR using the same reagents and reaction conditions as above. The forward primers were labeled with four different fluorescent dyes (Table 1) and "pigtail" sequences (GTTTCTT) (Brownstein et al. 1996) were added to each reverse primer to reduce plus-A artefacts.

Fragment analysis of 74 individuals originating from three rivers in Switzerland was conducted using ABI PRISM 3730xl analyzer and allele sizes scored using GENEMAPPER 4.0. All loci were polymorphic and contained up to 13 alleles per locus. Locus Fs.14 failed to amplify consistently in the multiplex PCR and was removed from further analysis.

To evaluate the power of the marker set to detect unique MLGs, a test of all locus combinations was conducted in GenClone v.2.0 (Arnaud-Haond and Belkhir 2007). Minimum of three loci were sufficient to separate all unique MLGs (Fs.1, Fs.9 and Fs.13). The probabilities (P_{sex}) that any pair of identical MLGs originated from distinct events of sexual reproduction were determined in GenClone (1,000 iterations). P_{sex} was < 0.001 in populations 1 and 2, suggesting that the marker set is suitable for identifying clonal genotypes with confidence. Population 3 consisted of two unique MLGs, with the dominant MLG representing 70% of all individuals. Comparisons within this population gave lower statistical confidence for excluding the possibility that some of the identical MLGs had arisen from independent events of sexual reproduction ($P_{sex} = 0.115$). In such highly clonal populations increasing the sampling effort is recommended to increase the number of distinct MLGs or further variable loci should be incorporated (Arnaud-Haond et al. 2007). Clonal heterogeneity (Simpson complement, D*) based on unique MLGs was calculated in GenClone. Population 1 consisted of a diverse set of unique MLGs, with only three MLGs encountered twice each (D* = 0.99). Fewer unique MLGs and thus higher proportion of clones were found in populations 2 and 3 (D* = 0.91 and D* = 0.44, respectively).

Repeated MLGs were removed, which reduced the sample sizes of populations 1, 2 and 3 to 25, 16 and 2,

Table 1 Primer characteristics, amplification conditions and basic results

Name	Primer sequence (5′–3′)	Repeat type	Primer (µM)[a]	Dye	Size range[b]	A	$N^{c}_{(1/2/3)}$	$H_{O(1/2/3)}$	$H^{d}_{e(1/2)}$
Fs.01	F:TGCACCTAATGGCATGTGTT R:*GTGTATGTGCACACGCTCG	TG_{22}	0.6	ATTO565	103–141	10/5/2	27/24/20	0.60/**0.44**/0.00	0.86/0.71
Fs.04	F:CTCGCAGCTAATGTGGAACA R:*CGCAAGAGAATGTCAGACCA	GT_{18}	0.4	YakimaYellow	168–196	8/5/3	28/23/20	0.64/0.81/1.0	0.70/0.70
Fs.09	F:CAACACATATGCGCACACAA R:*GCGAGACAGATGTGCAGGTA	CA_{18}	0.2	FAM	84–98	6/7/2	28/24/20	0.80/**0.31**/0.50	0.77/0.74
Fs.10	F:CATATCTGCATTCCAGCACG R:*GAATATGCTGCTGGCCGAT	AC_{18}	0.2	ATTO550	95–129	9/7/2	28/24/20	0.64/0.94/0.50	0.65/0.80
Fs.13	F:GAGTGATGAGAGAGTGTCGGC R:*CATCCGAACAACGCAGACTA	GT_{16}	0.4	FAM	131–157	6/6/2	28/24/20	0.80/0.75/0.50	0.78/0.84
Fs.14	F:CAGGTTCCGCTATACGTCG R:*TCTTCCCCTGAAGAACGCTA	GCA_{15}	0.4	ATTO565	na	na	na	na	na
Fs.18	F:TCTCGAAGGCTACGTTCCAC R:*GTTTGACCTTACCGCGCTC	TC_{15}	0.4	ATTO550	136–162	4/6/2	28/24/20	0.68/**0.44**/0.50	0.57/0.70
Fs.27	F:TTCAAACCATTACTAGCAGCCA R:*CTCCATGTGACATCCGCTTA	$GTAT_{13}$	0.2	YakimaYellow	89–121	4/3/2	28/24/20	0.64/**0.13**/0.50	0.69/0.41

A, number of alleles; N, number of successful amplifications when sample sizes were 28/26/20 for populations 1/2/3; H_o and H_e, observed and expected heterozygosity, respectively. Values in bold indicate significant departures from Hardy–Weinberg equilibrium at $P < 0.05$

* Indicates a pigtail (see text)

[a] Concentration in multiplex PCR, [b] including primer and flanking region, [c] 1/2/3 indicates results for each population tested, [d] only two unique MLGs were present in population 3 and no H_e was calculated

respectively. Linkage disequilibrium (LD) between all pairs of loci was estimated and observed and expected heterozygosities calculated in ARLEQUIN v.3.5.1.2 (Table 1). Populations 1 and 2 showed several loci with significant LD (up to five linked locus pairs in population 2). Significant heterozygote deficiency was detected in several loci within population 2 only (Table 1). Such results are not surprising given the widespread asexual reproduction and potential inbreeding within *F. sultana* populations, however, possibility of some null alleles in population 2 cannot be excluded. These first microsatellite markers for *F. sultana* will enable clone identity assignment at the population level, providing a high degree of confidence in distinguishing unique multilocus lineages.

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