NOTE

First microsatellite loci of the myxozoan parasite *Tetracapsuloides bryosalmonae*, the causative agent of proliferative kidney disease (PKD)

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ABSTRACT: Proliferative kidney disease (PKD) caused by the myxozoan parasite *Tetracapsuloides bryosalmonae* is a severe parasitic disease of salmonid fish. Estimates of genetic variation in parasite populations across Europe are currently lacking. We developed the first polymorphic microsatellite markers for *T. bryosalmonae* using Illumina MiSeq sequence data derived from genomic DNA. Twelve polymorphic loci were identified from 24 tested loci. Allelic variation was low at most loci, ranging from 2 to 6 (average 3.0). The markers developed here are expected to be useful in future genetic studies of *T. bryosalmonae*, aimed at further understanding the dispersal of the parasite, host–parasite relationships and the epidemiology of PKD.

KEY WORDS: Fish disease · Brown trout · Parasite · Population genetics · Host–parasite relationship

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Tetracapsuloides bryosalmonae is a malacosporean (Phylum *Cnidaria*) parasite that causes proliferative kidney disease (PKD) of salmonids (Canning et al. 1999). PKD is considered as an emerging disease in the northern hemisphere, causing high mortality and significant losses of fish farm stock and impacting natural populations both in Europe and North America (Okamura et al. 2011, Skovgaard & Buchmann 2012, Dash & Vasemägi 2014). The parasite develops in the body cavity of freshwater bryozoans, and mature water-borne spores infect fish via both the gills (Morris et al. 2000, Grabner & El-Matbouli 2010) and the skin epithelium (Longshaw et al. 2002).

Despite the economic and conservation implications of PKD, only a handful of sequences from *T. bryosalmonae* have been deposited in GenBank (mostly ribosomal DNA), and there are no polymorphic microsatellite loci available to describe the population genetic structure or identify multiple infections within hosts. Recently, we described a genomic library generated from approximately 1000 *T. bryosalmonae* sacs collected by dissection from *Fredericella sultana* (Filippenko et al. 2014). We identified altogether 10653 microsatellite motifs using msatcommander v.1.0.8 software and were able to design primers for 865 microsatellite loci

using Primer3 software (repeat number 6–47, potential duplicates removed; available as supplementary material Table 1 in Filippenko et al. 2014). By testing 24 tri- and tetranucleotide microsatellite mark-

ers from this library, we identified 12 polymorphic markers for *F. sultana*, none of which successfully amplified in *T. bryosalmonae*. However, BLAST searches of small subunit rRNA gene suggested that

Table 1. Characteristics of novel microsatellite loci in *Tetracapsuloides bryosalmonae*. Forward primer sequences are presented with the M13-tail (5'-3' CACGACGTTGTAAACGAC). The GTTT 'tail' (Brownstein et al. 1996) was added to the reverse primer. Dashes indicate lack of amplification

Locus name	Primer sequences (5'–3')	Motif/Repeat	Size range (bp)	Primer-label	No of alleles
Tb042TKU	F: CACGACGTTGTAAACGACTTGGGAATAATGAAATTTGAAAGA R: GTTTATTACGGGTTCACTTATTTAGGTATT	(AT) 5	237–241	VIC	3
Tb043TKU	F: CACGACGTTGTAAACGACTGTGCACGAAAACCTTTACAAATC R: GTTTACCCTATCGCCTCAATGAAA	(AT) 5	311–319	NED	4
Tb044TKU	F: CACGACGTTGTAAACGACTTAAACAGTCAAAATAGGCAATCA R: GTTTCCGATTTCTTATGGAAATACTTCTT	(AT) 10	220–224	PET	3
Tb045TKU	F: CACGACGTTGTAAACGACAGCCATGTAGCCCAAAACAC R: GTTTGCTTTACCTTGAATTTTCTGTTT	(AT) 5	521–523	6-FAM	2
Tb049TKU	F: CACGACGTTGTAAACGACGACGATCCAATGATGTGTT R: GTTTCGATTTTTCGGATCTCAT	(AT) 5	290–293	6-FAM	2
Tb054TKU	F: CACGACGTTGTAAACGACCCCTTTCAATGGCTGAGCAC R: GTTTCGTAGATCTCAAGTTCCAAACA	(AT) 6	325–329	VIC	2
Tb055TKU	F: CACGACGTTGTAAACGACTACCAACAGCCCAAGGTTTC R: GTTTGCTTTACCTTGAATTTTCTGTTT	(GT) 5	335–338	NED	2
Tb058TKU	F: CACGACGTTGTAAACGACTCCAGCATTATGATTGTGCAT R: GTTTAAGTCAAATAAAATAAATGGAAGAAAGA	(AT) 6	185–191	VIC	4
Tb059TKU	F: CACGACGTTGTAAACGACTGGATACTTTGAAACCATTTC R: GTTTCCTTACAGATTGCGTTACAATCA	(AT) 7	185–195	NED	2
Tb060TKU	F: CACGACGTTGTAAACGACGCAAACTTAATCTTTCTTTCCAG R: GTTTATTTTCGCACAGTTTGCTT	(AT) 5	215–244	PET	6
Tb061TKU	F: CACGACGTTGTAAACGACTGAATGAGTTTCCAATCAACG R: GTTTCCTCCACAAATAACCATTAAACC	(AT) 5	331–357	6-FAM	4
Tb063TKU	F: CACGACGTTGTAAACGACGCAAGTTCTCTTGCCCAAAT R: GTTTGATGAATCACATAGAAAATCCA	(AT) 5	181–185	NED	2
Tb046TKU	F: CACGACGTTGTAAACGACTGCGGGACTAGTTGTCAGC R: GTTTGATCCAATCTCTAACACAAA	(AT) 7	158	VIC	1
Tb047TKU	F: CACGACGTTGTAAACGACCAAGTGCAGTAGGAATGGACGA R: GTTTGGGTAAACAAATCTCAACATCG	(AT) 5	307	NED	1
Tb048TKU	F: CACGACGTTGTAAACGACTCCAATTCTCAAAGATGAAGTTTT R: GTTTCTGGTGCAATCTGATGCCTA	(AT) 5	373	PET	1
Tb050TKU	F: CACGACGTTGTAAACGACTGAGAAAGGCTGGCTATGCT R: GTTTCAGGTTCAATTCAATCAATCA	(AT) 8	172	VIC	1
Tb051TKU	F: CACGACGTTGTAAACGACCAAGTGAAACGGTAATAATTTGG R: GTTTCACCTCTCAAATTTATCCTTGTC	(AT) 7	423	NED	1
Tb053TKU	F: CACGACGTTGTAAACGACTGATGCTTCTTTGGGATATTG R: GTTTGGCTTCAAGATCATCTCGTC	(AT) 5	390	6-FAM	1
Tb056TKU	F: CACGACGTTGTAAACGACCCCTTTGTTGTTGACCATGA R: GTTTGGCAAATAAAAACAATTCAAAA	(AT) 5	394	PET	1
Tb062TKU	F: CACGACGTTGTAAACGACATCATCCGACCAAACA R: GTTTCATTTGGTAAGGAATTTGGTT	(AT) 5	537	VIC	1
Tb041TKU	F: CACGACGTTGTAAACGACTTGAACCTTTAATTACCATCTGAA R: GTTTGCCATTTCAAAAAGAACGA	(AT) 6	–	6-FAM	0
Tb052TKU	F: CACGACGTTGTAAACGACGAAGGTATTTCTCATGATTGTGGTC R: GTTTGAATGCTCAAAAACGATG	(AT) 5	–	PET	0
Tb057TKU	F: CACGACGTTGTAAACGACTGTGAGTGAATGGACGGAAG R: GTTTAATTCAAAAAGAAATCAATAGTTGAACA	(AT) 5	–	6-FAM	0
Tb064TKU	F: CACGACGTTGTAAACGACTCGAAAAATGCATGGGAAAT R: GTTTGGTTACCATAACCAACTTTTATTG	(AT) 5	–	PET	0

most of the sequences in this library were representative of *T. bryosalmonae*. To further check the proportional representation of *F. sultana* and *T. bryosalmonae* in the genomic library, BLASTn searches with 197 bp sections of putative low-copy number genes (for *T. bryosalmonae*, elongation factor 2, EF2, and for *F. sultana* elongation factor alpha-1, EF1 α) were conducted against the paired-end joined MiSeq reads. The number of fragments retrieved for *T. bryosalmonae* was 323 and 2 for *F. sultana* (in both cases all fragments overlapping the region of interest of identical length were counted). These results suggest that a substantial proportion of the library was dominated by parasite genomic DNA (gDNA) (an estimated >300 $\times$ more parasites than host gDNA). Previous work has shown that di- and trinucleotide repeats in the genome of the bryozoan host, *F. sultana*, are unusually abundant; thus, even a small amount of host contamination may result in preferential detection of host-associated microsatellite loci (Hartikainen & Jokela 2012, Schoebel et al. 2013).

Here, we applied an alternative strategy for identification of microsatellite markers in *T. bryosalmonae* from the same mixed genomic library (Filippenko et al. 2014) by focusing on assembled longer contigs only (500–5449 bp, $n = 9084$). The focus on longer contigs is expected to increase the likelihood of identification of contigs of *T. bryosalmonae* origin. This is because the material used for library preparation contained only trace amounts of bryozoan host, and thus sequencing coverage over the *T. bryosalmonae* genome should be higher (Filippenko et al. 2014). Due to the positively biased sequencing effort over the likely smaller *T. bryosalmonae* genome we predicted that longer contigs predominantly originate from *T. bryosalmonae*, and we used these to search for microsatellite loci.

Assembly was conducted using SOAPdenovo v.2 (Luo et al. 2012) with kmer size set to 127. A total of 1226 dinucleotide microsatellite motif (minimum number of repeats = 4) containing sequences were identified using msatcommander v.1.0.8 software. Altogether, 24 dinucleotide microsatellite loci (Table 1, Supplement; available at: www.int-res.com/articles/suppl/d113p085_supp.pdf) were tested in a panel of 28 *T. bryosalmonae*-infected brown trout (*Salmo trutta*) kidney samples and 11 *F. sultana* samples. Infected kidney samples originated from the following 7 Estonian rivers: Altja, Mustoja, Vainupea, Vösu, Valkla, Keila and Kuusalu (Dash & Vasemägi 2014). *F. sultana* samples consisted of 8 *T. bryosalmonae*-infected specimens with 3 specimens from both the

rivers Avon (Hampshire) and Dun, UK, and single specimens from the rivers Lulaine, France, and Surb, Switzerland). Three bryozoans with no apparent *T. bryosalmonae* infection from the rivers Avon, Dun and Cut, England, were used as controls.

PCR amplifications were performed in 6.1 μ l total reaction volume, which included ca. 10 to 100 ng of total gDNA, 0.6 μ M of forward and 1.2 μ M of reverse primers, 0.5 μ M of FAM, VIC, NED or PET-labeled universal M13 primer and 3.0 μ l of 2 $\times$ QIAGEN Multiplex Master Mix. Amplification conditions were as follows: initial denaturation at 95°C for 15 min, 15 cycles of 30 s at 94°C, 1 min 30 s at 58°C, and 60 s at 72°C; followed by 25 cycles of 30 s at 94°C, 1 min 30 s at 52°C, and 60 s at 72°C; and a final extension of 10 min at 60°C. Fragment analyses were carried out on ABI PRISM 3130xl (Applied Biosystems) with LIZ600 size standard. Genotyping was performed using the software GeneMarker v. 1.6 (Soft Genetics).

In contrast to our previous attempt (Filippenko et al. 2014), 20 of 24 primer pairs successfully amplified microsatellite loci in *T. bryosalmonae*-infected brown trout and bryozoan samples, while no amplification was obtained for non-infected *F. sultana* specimens. Altogether, 12 microsatellite loci were polymorphic (Table 1) with 2 to 6 alleles per locus.

As predicted, the majority of tested microsatellite primers developed from long contigs resulted in positive amplification in *T. bryosalmonae*. Our results suggest that the genomic DNA library was largely biased towards *T. bryosalmonae* and that the genome size of the parasite may be much smaller than that of its bryozoan host, *F. sultana*. The large discrepancy in microsatellite frequency in the genomes of the 2 organisms may also reflect this genome size difference. Altogether, we developed 12 polymorphic microsatellite loci, but the level of variability of these novel markers was relatively low. It remains to be seen whether the low level of polymorphism found in dinucleotide microsatellite loci is a common feature of *T. bryosalmonae*. The markers developed in the present work will be useful for population genetic studies, characterising dispersal of *T. bryosalmonae*, and examining the epidemiology of PKD.

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