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Proliferative kidney disease (PKD) agent *Tetracapsuloides bryosalmonae* in brown trout populations in Estonia

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ABSTRACT: Proliferative kidney disease (PKD) caused by the myxozoan parasite *Tetracapsuloides bryosalmonae* is a serious parasitic disease threatening both farmed and wild salmonid populations, but very little is currently known about the distribution of the parasite in the Baltic Sea region. In this study we (1) report the development of a novel multiplex PCR method for fast and reliable screening of *T. bryosalmonae*; (2) use this multiplex PCR method to show that the PKD agent *T. bryosalmonae* is widespread in natural brown trout *Salmo trutta* L. populations in Estonia; (3) evaluate monthly and yearly variation of *T. bryosalmonae* prevalence in juvenile trout; (4) assess *T. bryosalmonae* prevalence in different age-classes of fish (0+ vs. 1+ and older) and report the presence of the PKD agent in the kidneys of returning sea trout spawners; and (5) suggest the freshwater bryozoan *Plumatella fungosa* as a putative invertebrate host of *T. bryosalmonae* in Estonia. Our results demonstrate a highly heterogeneous distribution of *T. bryosalmonae* at the micro-geographic scale, indicating that PKD could have an important negative effect on recruitment in Estonian brown trout populations.

KEY WORDS: *Tetracapsuloides bryosalmonae* · *Salmo trutta* · Salmonid fish · Natural population · Parasite · Host · Myxozoa · Bryozoa

INTRODUCTION

Proliferative kidney disease (PKD) is a common parasitic disease that affects both farmed and natural salmonid populations in North America and Europe (Hedrick et al. 1993, Feist et al. 2002, Wahli et al. 2002, Sterud et al. 2007). The PKD-causing agent is the myxozoan endoparasite *Tetracapsuloides bryosalmonae* (Malacosporea), previously named the PKX organism (Canning et al. 1999, 2002). To complete its lifecycle, *T. bryosalmonae* first uses an invertebrate host, belonging to a group of freshwater bryozoans commonly from the genera *Fredericella* and *Plumatella* (Anderson et al. 1999, Okamura & Wood 2002, Morris & Adams 2006), and then switches to a vertebrate host by entering salmonid fish via the gills to eventually proliferate into the kidney (Feist & Longshaw 2001). The clinical signs of *T. bryosalmonae* infection in the salmonid host are marked by a darkened body, abdominal distension, swollen kidney, and severe anemia (Hedrick et al. 1993). Both field data and experimental work have shown that increasing water temperatures enhance prevalence and severity of PKD, while recent studies suggest that eutrophication may also contribute to disease outbreaks (Hartikainen et al. 2009, Okamura et al. 2011). PKD outbreaks usually occur seasonally during the summer or early autumn when water temperatures are above 15°C (Ferguson & Ball 1979, Ferguson 1981, Clifton-Hadley et al. 1986).

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While the effects of temperature on *T. bryosalmonae* proliferation and the renal pathological responses of the salmonid host are well documented, considerably less is known about the distribution of the parasite and the severity of PKD in wild salmonid populations (Sterud et al. 2007, Peeler et al. 2008, Kristmundsson et al. 2010, Okamura et al. 2011, Skovgaard & Buchmann 2012). For example, the prevalence of PKD is spatially variable within and among river systems (Feist et al. 2002, Sterud et al. 2007, Wahli et al. 2008, Skovgaard & Buchmann 2012). However, the potential effects of temporal variation, different life history strategies (e.g. migratory vs. resident fish), and juvenile age on *T. bryosalmonae* prevalence is not known. Yet, such knowledge is necessary for a better understanding of the life cycle and dispersal of *T. bryosalmonae*, and the epidemiology of PKD.

The Baltic Sea catchment contains approximately 1000 anadromous brown trout *Salmo trutta* L. populations and numerous wild resident brown trout stocks (HELCOM 2011). Very little is currently known about the distribution of the PKD-causing parasite *T. bryosalmonae* and its bryozoan host(s) in the Baltic Sea region and its potential impact on wild trout populations (Skovgaard & Buchmann 2012). Earlier reports from Estonia documented the occurrence of *T. bryosalmonae* in Atlantic salmon and brown trout in a few rivers based on histopathological work (Kadakas et al. 2001), but no information is available on the prevalence of *T. bryosalmonae* among wild anadromous and resident brown trout populations in Estonia.

In this study, we aimed to describe the distribution and prevalence of *T. bryosalmonae* in wild anadromous and resident brown trout populations in Estonia. We evaluated the monthly and yearly variation in *T. bryosalmonae* prevalence in different age-classes (0+ versus 1+ and older) and report the development of a multiplex PCR method for fast and reliable screening of *T. bryosalmonae.* Finally, we identify the freshwater bryozoan species *Plumatella fungosa* as a putative invertebrate host of *T. bryosalmonae* in Estonia.

MATERIALS AND METHODS

Sampling of fish and bryozoa

In total, 984 brown trout were collected by electrofishing at 31 sites in 20 rivers in Estonia (Table 1). Based on the location of dams that act as definite upstream migration obstacles, populations were classified as resident or anadromous life history type (Table 1). Electrofishing was done from July to October in 2009 to 2012. Age of the juvenile trout (0+, 1+ or older) was estimated based on the length distribution. Fish were euthanized in buffered 3-aminobenzoic acid ethyl ester methanesulfonate (MS-222®) and preserved in 96% ethanol. Autopsies revealed signs of proliferated, swollen, and pale kidneys in 0+ fish of some rivers (e.g. Mustoja, Vainupea, Altja), but the clinical signs of the infection were not systematically evaluated for all analyzed trout (e.g. Clifton-Hadley et al. 1986). The suspected invertebrate host, a freshwater bryozoan, was found in the River Mustoja in 2010 and 2011, and sections of the bryozoan colonies were preserved in 96% ethanol. Separate tissue samples were collected from anterior and middle kidney from 211 juveniles caught in the River Mustoja in 2009. Since the presence or absence of *Tetracapsuloides bryosalmonae* was consistent between the anterior and middle parts of the kidney using a multiplex PCR method (see below), only the middle kidney sections (below the dorsal fin) were subsequently screened in 2010 and 2011 samples. For 3 returning adult sea trout spawners caught in 2012 from River Mustoja Site 13 (Table 1), 3 separate tissue samples were taken from the anterior, middle, and posterior part of the kidney and subsequently stored in 96% ethanol. Total genomic DNA was extracted from trout kidney tissues using the salt-extraction method (Aljanabi & Martinez 1997). The salt-extraction approach was also used to isolate total genomic DNA from bryozoa.

Development of a multiplex PCR-based identification method for *Tetracapsuloides bryosalmonae*

Two sets of *T. bryosalmonae*-specific primers were used for parasite detection (Kent et al. 1998, Grabner & El-Matbouli 2009) in addition to a set of primers amplifying approximately 500 bp DNA fragments in brown trout and Atlantic salmon to verify successful amplification (Fig. 1). PKX3F (5′-CTA AGT ACA TAC TTC GGT AGA-3′) and PKX4R (5′-CCG TTA CAA CCT TGT TAG GAA-3′) (Kent et al. 1998) were used to amplify 298 bp of the 18S rRNA gene of *T. bryosalmonae*. Another short 166 bp fragment from the *T. bryosalmonae* 18S rRNA gene was amplified using the primers PKD-realF (5′-TGT CGA TTG GAC ACT GCA TG-3′) and PKD-realR (5′-ACG TCC GCA AAC TTA CAG CT-3′) (Grabner & El-Matbouli

Table 1. *Tetracapsuloides bryosalmonae* infecting *Salmo trutta*. Prevalence of *T. bryosalmonae* among anadromous and resident brown trout populations in Estonia. Total number of studied fish (n); number of noninfected (neg.) and infected fish (pos.); 95% confidence intervals (CI) are estimated separately for 0+ and older individuals. Prev.: prevalence; –: no data

River/ stream	Site no.	Coordinates (°N, °E)	Date (dd.mm.yyyy)	Life history	n	*T. bryosalmonae* infection status									
						0+ fish					1+ and older fish				
						Neg.	Pos.	Prev.	95% CI		Neg.	Pos.	Prev.	95% CI	
Pühajõgi	1	59°25′23″, 27°31′47″	23.09.2011	Anadromous	9	9	0	0	0	0.299	–	–	–	–	–
Pada	2	59°29′40″, 26°45′19″	02.07.2010	Anadromous	10	–	–	–	–	–	10	0	0	0	0.278
Pada	3	59°28′24″, 26°42′58″	02.07.2010	Anadromous	17	8	0	0	0	0.324	9	0	0	0	0.299
Pada	4	59°26′30″, 26°42′43″	02.07.2010	Resident	10	–	–	–	–	–	10	0	0	0	0.278
Pada	5	59°24′24″, 26°42′4″	02.07.2010	Resident	17	7	0	0	0	0.354	10	0	0	0	0.278
Kunda	6	59°30′42″, 26°32′16″	07.08.2010	Anadromous	33	21	0	0	0	0.155	11	1	0.083	0.015	0.354
Kunda	7	59°17′2″, 26°40′6″	07.08.2010	Resident	17	5	2	0.286	0.082	0.641	4	6	0.6	0.313	0.832
Kunda	8	59°17′26″, 26°38′33″	07.08.2010	Resident	11	9	0	0	0	0.299	1	1	0.5	0.095	0.906
Toolse	9	59°29′2″, 26°28′12″	04.08.2010	Anadromous	31	21	0	0	0	0.155	10	0	0	0	0.278
Toolse	10	59°31′29″, 26°28′13″	04.08.2010	Anadromous	8	1	0	0	0	0.794	7	0	0	0	0.354
Vainupea	11	59°34′3″, 26°15′31″	01.07.2010	Anadromous	12	6	0	0	0	0.39	1	5	0.833	0.437	0.97
Vainupea	11	59°34′3″, 26°15′31″	05.08.2010	Anadromous	36	7	15	0.682	0.473	0.836	0	14	1	0.785	1
Vainupea	11	59°34′3″, 26°15′31″	20.09.2011	Anadromous	78	0	78	1	0.953	1	–	–	–	–	–
Vainupea	12	59°33′46″, 26°14′27″	01.07.2010	Resident	2	1	0	0	0	0.794	0	1	1	0.207	1
Vainupea	12	59°33′46″, 26°14′27″	04.08.2010	Resident	8	3	0	0	0	0.562	2	3	0.6	0.231	0.882
Mustoja	13	59°34′59″, 26°10′29″	05.08.2009	Anadromous	80	0	80	1	0.954	1	–	–	–	–	–
Mustoja	13	59°34′59″, 26°10′29″	03.08.2010	Anadromous	25	0	22	1	0.851	1	0	3	1	0.439	1
Mustoja	13	59°34′59″, 26°10′29″	27.08.2011	Anadromous	17	0	17	1	0.81	1	–	–	–	–	–
Mustoja	13	59°34′59″, 26°10′29″	30.09.2012	Anadromous	3	–	–	–	–	–	0	3	1	0.439	1
Mustoja	14	59°34′9″, 26°10′44″	03.08.2010	Anadromous	51	11	39	0.78	0.648	0.873	0	1	1	0.207	1
Mustoja	15	59°33′11″, 26°10′58″	05.08.2009	Anadromous	98	2	77	0.975	0.912	0.993	0	19	1	0.832	1
Mustoja	15	59°33′11″, 26°10′58″	30.06.2010	Anadromous	15	2	6	0.75	0.409	0.929	2	5	0.714	0.359	0.918
Mustoja	16	59°32′1″, 26°10′33″	06.08.2009	Resident	33	31	2	0.061	0.017	0.196	–	–	–	–	–
Mustoja	16	59°32′1″, 26°10′33″	01.07.2010	Resident	27	24	3	0.111	0.031	0.328	–	–	–	–	–
Altja	17	59°34′46″, 26°7′14″	03.07.2010	Anadromous	14	2	0	0	0	0.658	4	8	0.667	0.386	0.797
Altja	17	59°34′46″, 26°7′14″	05.08.2010	Anadromous	30	14	1	0.067	0.012	0.298	4	11	0.733	0.481	0.891
Võsu	18	59°34′13″, 25°58′12″	03.07.2010	Anadromous	16	1	1	0.5	0.095	0.906	2	12	0.857	0.601	0.96
Võsu	18	59°34′13″, 25°58′12″	05.08.2010	Anadromous	20	8	3	0.273	0.097	0.566	1	8	0.889	0.565	0.98
Loobu	19	59°33′16″, 25°47′13″	06.08.2010	Anadromous	18	3	5	0.625	0.306	0.863	0	10	1	0.723	1
Loobu	20	59°28′1″, 25°55′51″	06.08.2010	Resident	16	12	0	0	0	0.243	3	1	0.25	0.046	0.699
Valgejõgi	21	59°20′34″, 25°49′24″	06.08.2010	Resident	21	0	8	1	0.676	1	0	13	1	0.772	1
Kuusalu	22	59°28′53″, 25°25′55″	30.07.2011	Anadromous	13	10	3	0.231	0.082	0.503	–	–	–	–	–
Valkla	23	59°29′11″, 25°19′38″	01.08.2011	Anadromous	9	0	9	1	0	0.299	–	–	–	–	–
Keila	24	59°24′2″, 24°16′58″	02.08.2010	Anadromous	43	14	26	0.65	0.495	0.779	1	2	0.667	0.208	0.939
Vodja	25	58°56′21″, 25°38′4″	31.07.2011	Resident	18	18	0	0	0	0.176	–	–	–	–	–
Preedi	26	58°56′19″, 26°6′51″	21.08.2011	Resident	16	15	0	0	0	0.204	1	0	0	0	0.794
Männiku	27	58°16′33″, 24°5′30″	04.09.2011	Anadromous	32	32	0	0	0	0.107	–	–	–	–	–
Esna	28	58°52′52″, 25°35′51″	09.08.2011	Resident	21	21	0	0	0	0.155	–	–	–	–	–
Tatra	29	58°15′6″, 26°39′35″	10.09.2011	Resident	10	10	0	0	0	0.278	–	–	–	–	–
Onga	30	58°52′25″, 26°20′43″	11.09.2011	Resident	25	25	0	0	0	0.133	–	–	–	–	–
Kaave	31	58°42′12″, 26°18′1″	11.09.2011	Resident	14	14	0	0	0	0.215	–	–	–	–	–

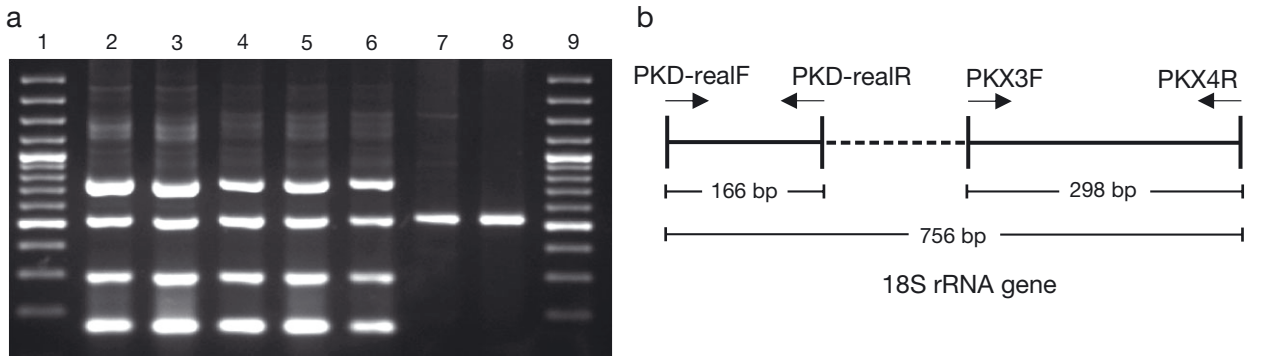

Fig. 1. *Tetracapsuloides bryosalmonae* and *Salmo trutta*. Multiplex PCR for detection of *T. bryosalmonae*. (a) Image of 2% agarose gel consisting of DNA fragments from *T. bryosalmonae* (298, 166, and 756 bp) and brown trout (approximately 500 bp); Lanes 1 and 9: 200–1500 bp DNA Ladder; Lanes 2, 3 and 4: kidney samples from 3 *T. bryosalmonae*-positive brown trout from the River Mustoja; Lanes 5 and 6: positive control; Lanes 7 and 8: negative control. (b) Amplification of 3 fragments of the 18s rRNA gene (298, 166, and 756 bp) with the primer sets PKX3F-PlusPKX4R (Kent et al. 1998), PKD-realF, PKD+realR (Grabner & El-Matbouli 2009)

2009). The combination of PKD-realF and PKX4R amplifies a longer (756 bp) fragment of the same 18S rRNA gene. The salmonid-specific forward (5′-GCC CAA AAT GTA CAG GCA AT-3′) and reverse (5′-GAT TCT CAT GTT AGC CGT CCA-3′) primers were originally designed for Atlantic salmon (GenBank EST database accession number CA064223; Vasemägi et al. 2010) but also served as a control for successful PCR reactions in brown trout (Fig. 1).

Multiplex amplification was conducted in a 6 µl total reaction volume with approximately 100 ng of total DNA, 1.8 µM of forward PKX3F and reverse PKD-realR primer, 1.2 µM of reverse PKX4R and forward PKD-realF primer in 2× Qiagen multiplex master mix. The PCR program consisted of an initial activation step at 95°C for 15 min followed by 36 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 1 min 30 s, and extension at 72°C for 1 min. The final extension was carried out at 72°C for 5 min. Amplified DNA product along with the positive and negative controls was loaded on 2% agarose gel, stained with ethidium bromide, and visualized on a UV transilluminator.

Sequencing of *Tetracapsuloides bryosalmonae*

The sequencing of the *T. bryosalmonae* 18S SSU rRNA gene fragment (756 bp) was done with the PKX3F and PKD-realR primers from Kent et al. (1998) and Grabner & El-Matbouli (2009). Single-fragment PCR was performed on the 8 *T. bryosalmonae*-infected individuals from Sites 13 and 15 in the River Mustoja (Table 1) with similar amplification reaction conditions as described above. The PCR product was then treated with 0.5 µl of exonuclease and 2 µl of shrimp alkaline phosphatase for 30 min at 37°C followed by 80°C for 15 min. The sequencing reaction was performed adding 1 µl of minimum 20 ng PCR product with 1.8 µM either PKX3F or PKD-realR primer, 2 µl of BiG Dye 5× sequencing buffer and 0.5 µl of Big Dye premix (Big Dye v. 3.1) RR-100. The sequencing reaction conditions were as follows: 96°C for 3 min, 34 cycles of 96°C for 30 s, 50°C for 30 s, and 72°C for 3 min. The reaction product was purified by using a Montage SEQ_{96} Sequencing Reaction Cleanup Kit (Millipore). An aliquot (6 µl) of each purified sequencing reaction product and 6 µl of HiDi-formamide were added for sequencing using an ABI PRISM 3130xl (Applied Biosystem) automated sequencer. Obtained 18S SSU rRNA sequences were analyzed using Geneious Pro (v. 5.6.6) software.

Sequencing of bryozoa

Two primer sets were used to amplify the cytochrome *c* oxidase 1 (COI) gene (forward and reverse primer: COI_LCO1_1490, HCOI_2198; Folmer et al. 1994) and the 18S small subunit ribosomal RNA (SSU rRNA) gene (forward and reverse primer: 18s rRNA1F, 18s rRNA4R; Giribet et al. 1996) in collected freshwater bryozoans. A standard PCR reaction was carried out in a 10 µl total reaction volume with 100 ng of DNA, 5 µM of each primer in 2× Qiagen multiplex master mix. The amplification program consisted of 95°C for 15 min followed by 35 cycles of 94°C for 30 s,

45°C (COI) or 53°C (18s SSU rRNA) for 1 min 30 s, and 72°C for 1 min. The final extension at 72°C lasted for 8 min. ExoSAP treatment and sequencing was conducted as described above.

Statistical analysis

Statistical tests related to *T. bryosalmonae* prevalence between the anadromous and resident fish and different age groups (0+ vs. 1+ and older) were performed using 2×2 Fisher's exact test, Mann-Whitney *U*, and Wilcoxon signed rank tests using SPSS Statistics 21. For comparison of the PKD prevalence between different age groups, a 2×2 Fisher's exact test was carried out only for 4 sites (Table 1; n = 13), with a total sample size of at least 12 individuals and a minimum sample size of 3 individuals per age group. Differences in monthly and/or yearly variation in PKD prevalence were also tested using 2×2 Fisher's exact test. The 95% confidence intervals (CI) for *T. bryosalmonae* prevalence were estimated using Wilson's score method without continuity correction according to Newcombe (1998) using a web-page calculator (www.vassarstats.net).

RESULTS

Sequence analysis of *Tetracapsuloides bryosalmonae*

We obtained sequence from a 729 bp fragment of the 18S rRNA gene for Estonian *T. bryosalmonae* (GenBank nucleotide databank accession number KF805631) that exhibited 100% homology with the 18S rRNA gene sequences of *T. bryosalmonae* from Norway (521 bp overlap; EU570235; Sterud et al. 2007) and the British Isles (317 bp overlap; AF190668; Longshaw et al. 1999). Other published 18S rRNA gene sequences from *T. bryosalmonae* also showed high similarities (97–99%) with the River Mustoja *T. bryosalmonae* sequence.

Multiplex PCR approach for detection of *Tetracapsuloides bryosalmonae*

We developed a multiplex PKD detection method that simultaneously amplifies 3 fragments from the 18S rRNA gene in *T. bryosalmonae* and an approximately 500 bp fragment in Atlantic salmon and brown trout (Fig. 1). As such, our multiplex PCR approach provides a robust and cost-effective means for screening the presence/absence of *T. bryosalmonae* in a large number of samples while simultaneously controlling for amplification failure.

Distribution and prevalence of *Tetracapsuloides bryosalmonae*

T. bryosalmonae infections were found in 524 of 984 analyzed trout (53.25%) using the developed multiplex PCR method. At individual sites, *T. bryosalmonae* prevalence varied from 0 to 100% (Fig. 2; mean prevalence: 0+, 31.55%; 1+ and older, 57.01%). As the differences in sample size were relatively large (mean: 24.00; range: 2–98), the CI estimates of *T. bryosalmonae* prevalence were relatively wide for many analyzed sites (Table 1). Altogether, *T. bryosalmonae*-positive individuals were found in half of the studied rivers (10 out of 20). The mean *T. bryosalmonae* prevalence for 0+ fish was slightly higher in rivers supporting anadromous trout compared to resident trout rivers (anadromous vs. resident: mean prevalence 33.9% vs. 10.2%). However, these differences were not significant (Mann-Whitney *U*-test, p = 0.064; 2×2 contingency table, Fisher's exact test p = 0.111). The same tendency of higher prevalence and occurrence of *T. bryosalmonae* in anadromous trout compared to rivers supporting resident trout was evident for 1+ fish (anadromous vs. resident: mean prevalence 57.5% vs. 43.3%). Similar to 0+ fish, the observed differences among 1+ fish between anadromous and resident populations were not significant (Mann-Whitney *U*-test, p = 0.539; 2×2 contingency table, Fisher's exact test, p = 1.00).

Temporal variation in *Tetracapsuloides bryosalmonae* prevalence

Because of limited temporal data, we were able to evaluate the monthly and/or yearly variation of *T. bryosalmonae* prevalence in 4 rivers: Altja, Võsu, Mustoja, and Vainupea (Table 1). Parasite prevalence for 0+ fish was lower in July compared to August at 2 studied sites (River Altja: 0% vs. 6.7%; River Vainupea, Site 11: 0% vs. 68.2%) and higher in July compared to August in the River Võsu (50.0% vs. 27.3%). However, the change in parasite prevalence was significant only in the River Vainupea (Site 11: 2×2 contingency table, Fisher's exact test, p = 0.005). For 1+ and older fish in the Rivers Altja, Võsu,

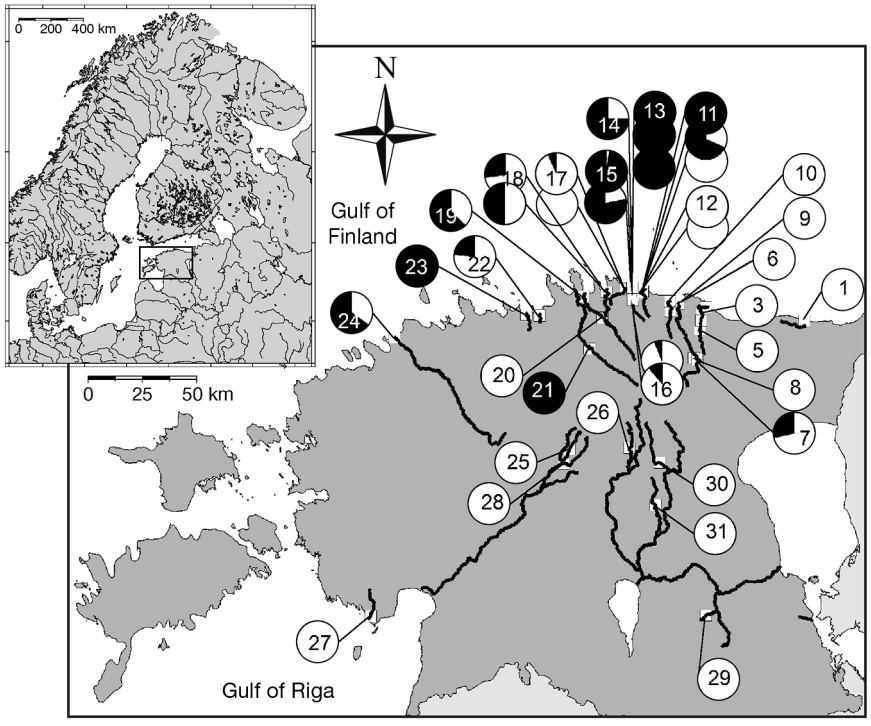

Fig. 2. *Tetracapsuloides bryosalmonae.* Geographical distribution and prevalence among 0+ anadromous and resident brown trout populations in Estonia (black box in inset; dark grey in main figure). Numbers refer to the sampling sites (see Table 1). Black and white areas in the pie chart represent the proportion of infected and uninfected individuals, respectively. Partially overlapping charts indicate temporal samples taken from the same site

and Vainupea, no significant differences in *T. bryosalmonae* prevalence was observed between July and August (Table 1; 2×2 contingency table, all Fisher's exact tests were $p > 0.05$). The *T. bryosalmonae* prevalence in 0+ fish also varied significantly between years at 2 out of the 4 studied sites (2×2 contingency table, Fisher's exact test: River Mustoja, Site 15: $p = 0.041$ and River Vainupea, Site 11: $p < 0.001$). For 1+ and older fish, *T. bryosalmonae* prevalence did not significantly differ between years (River Mustoja, Site 15: 2×2 contingency table, Fisher's exact test, $p = 0.065$).

Tetracapsuloides bryosalmonae prevalence in different age-classes

Overall, *T. bryosalmonae* prevalence was found to be significantly higher in 1+ and older fish compared to 0+ trout (Wilcoxon signed ranks test, $p = 0.005$). Significant differences were also observed in 4 out of 13 within-site comparisons (2×2 contingency table, Fisher's exact test: Rivers Võsu, Altja, and Vainupea Site 11, $p < 0.05$), where 1+ and older fish showed higher parasite prevalence compared to 0+ trout. In addition to juveniles, 3 returning adult sea trout spawners were tested and found to be carriers of *T. bryosalmonae* in the middle and posterior section of the kidney. The anterior kidneys of all examined sea trout spawners were parasite free based on the multiplex PCR method.

Molecular identification of bryozoan species

The sequenced 18S rRNA gene (accession number KF805630) and COI gene (accession number KF805632) fragments of the freshwater bryozoan samples collected in 2010 and 2011 from the River Mustoja (Site 4) were identical in both years. The sequence of the 18S rRNA gene was 100% homologous (478 bp overlap) to the published 18S rRNA gene of *Plumatella fungosa* (Pallas, 1768) (accession number DQ221748.1; Wood & Lore 2004). Since COI gene sequence data for *P. fungosa* was not available in GenBank, the closest match (86%, 576/673 bp overlap) corresponded to *Gelatinella toanensis* (accession number FJ196082.1).

DISCUSSION

By using a newly developed multiplex PCR method, we have shown for the first time that the PKD agent, the myxozoan parasite *Tetracapsuloides bryosalmonae*, is widespread in the natural brown trout populations of Estonia. We also characterized the prevalence and temporal variation of *T. bryosalmonae* in different age-classes among resident and anadromous trout.

Tetracapsuloides bryosalmonae distribution and prevalence in Estonia

The distribution of *T. bryosalmonae* varied considerably in Estonian rivers. We found *T. bryosalmonae*-positive fish in half of the studied rivers (10 out of 20) and in 55% of all sampled sites (17 out of 31). Similar to earlier studies, the prevalence of *T. bryosalmonae*-positive fish at a site was usually greater than 10%,

indicating that if *T. bryosalmonae* is present in a particular part of the river, trout are frequently infected by the parasite (e.g. Feist et al. 2002, Wahli et al. 2007, Peeler et al. 2008, Skovgaard & Buchmann 2012). The prevalence of parasites also varied markedly between geographically close rivers. For example, sea trout populations of the Rivers Toolse and Pada were found to be devoid of the parasite based on multiplex PCR while nearby rivers supporting anadromous trout (Rivers Vainupea and Mustoja) were *T. bryosalmonae* positive. In addition, we observed significant differences in *T. bryosalmonae* prevalence between sites within the same river systems. For example, in 2 cases, the upstream resident trout populations showed very low prevalence (Rivers Loobu and Mustoja, Sites 20 and 16, respectively), while a much higher parasite prevalence was observed in the downstream anadromous trout populations (Sites 13, 14, 15, and 19). As such, this study reveals a highly heterogeneous distribution of *T. bryosalmonae* at a micro-geographic scale in brown trout, consistent with earlier results from south-west England (Peeler et al. 2008) and Switzerland (Wahli et al. 2007). Our results also suggest that *T. bryosalmonae* may be more prevalent among anadromous sea trout populations compared to resident brown trout, but further analysis of more populations is necessary to confirm this. It is also possible that the anadromous populations have higher infection rates due to increased water temperature, elevated nutrient levels, and accumulation of transmissive spores.

Morris & Adams (2006) suggested possible vertical transmission and survival of *T. bryosalmonae* by the fragmentation of bryozoan colonies. Recently, Abd-Elfattah et al. (2013) demonstrated that *T. bryosalmonae* can disperse via bryozoan statoblasts that function as dormant propagules allowing bryozoans to persist during unfavorable periods. This vertical transmission capacity should enable *T. bryosalmonae* to persist indefinitely in the local bryozoan population without the vertebrate host, representing a potentially very effective dispersal strategy for the parasite. On the other hand, highly heterogeneous distributions of *T. bryosalmonae* at the micro-geographic scale shown here and by others (Wahli et al. 2007, Peeler et al. 2008) indicate that the realized dispersal ability of the parasite may be much more restricted. In order to evaluate which of the scenarios holds true for *T. bryosalmonae,* quantification of the relative roles of drift and gene flow among *T. bryosalmonae* populations using microsatellite or single nucleotide polymorphism markers would be required.

Temporal variation in *Tetracapsuloides bryosalmonae* prevalence

Although the time- and temperature-related effects of *T. bryosalmonae* proliferation have been thoroughly described in controlled experimental conditions and in farmed fish (Clifton-Hadley et al. 1986, Morris et al. 2005, Tops et al. 2006, Bettge et al. 2009 a,b, Schmidt-Posthaus et al. 2012), only a handful of studies to date have documented temporal variation in PKD prevalence in wild populations (Wahli et al. 2002, Sterud et al. 2007). Yet, several authors have stressed the importance of temporal analysis when studying the epidemiology of PKD (Feist et al. 2002, Wahli et al. 2007, Okamura et al. 2011). Similarly to earlier work showing a seasonal occurrence of PKD (Foott & Hedrick 1987, Hedrick et al. 1993), we detected a significant temporal increase in *T. bryosalmonae* prevalence in the summer 2010, during a 1 mo period (River Vainupea, Site 11) where parasite prevalence in 0+ trout increased from 0 (95% CI: 0–39%) at the beginning of July to 68% (95% CI: 47–84%) on 5 August. PKD prevalence also varied between years (Table 1), but based on current data, it is not possible to completely separate yearly and monthly effects. However, analysis of the same sites over 2 to 3 yr revealed congruent *T. bryosalmonae* presence and absence patterns. For example, all studied individuals were infected by *T. bryosalmonae* in the River Mustoja for 3 consecutive years.

Tetracapsuloides bryosalmonae prevalence in 0+ and older fish

Our results demonstrated higher *T. bryosalmonae* prevalence in 1+ fish compared to 0+ trout based on multiplex PCR. In fact, we observed a significant increase in *T. bryosalmonae* prevalence in 1+ and older fish for the whole data set (Wilcoxon signed ranks test) as well as for 4 individual comparisons (2×2 contingency table). These results are in line with a recent study by Schmidt-Posthaus et al. (2013), who found a similar pattern of high *T. bryosalmonae* prevalence in 1+ trout in a Swiss river system. According to Schmidt-Posthaus et al. (2013), the pathological lesions in 1+ trout, such as acute gill and kidney lesions, indicate re-infection rather than proliferation of the remaining parasites. It is therefore likely that a higher prevalence of *T. bryosalmonae* observed in this study was caused by re-infection of 1+ trout during the second summer while lack of clinical signs suggest partial immunity of 1+ fish (Schmidt-Posthaus et al. 2013).

We also detected *T. bryosalmonae* infection in the middle and posterior part of the kidney in 3 returning adult sea trout spawners. While we cannot exclude the possibility that this represents a reinfection that occurred in the river, we found it unlikely for several reasons. Firstly, all 3 spawners were caught very close to the river mouth (less than 600 m) after a sudden water level increase during the previous night because of heavy rainfall that most likely attracted spawners to enter the river. Secondly, all 3 spawners showed silvery coloration patterns indicative of recent entrance to the river. Finally, a recent study by Mo et al. (2011) in Norway documented the presence of *T. bryosalmonae* in descending Atlantic salmon and sea trout smolts, which further suggests the possibility that *T. bryosalmonae* remains in the kidney during the sea phase of the fish host. Hence, our molecular data suggest that *T. bryosalmonae* may remain in the kidney of sea trout, but further analysis of fish caught directly from the marine environment is necessary to confirm this hypothesis.

Multiplex PCR for molecular detection of *Tetracapsuloides bryosalmonae*

Several sensitive molecular genetic approaches exist for the detection of *T. bryosalmonae*, including single fragment PCR, real-time PCR (qPCR), and loop-mediated isothermal amplification (Saulnier & de Kinkelin 1997, Kent et al. 1998, Morris et al. 2002, El-Matbouli & Soliman 2005, Grabner & El-Matbouli 2009). However, compared to approaches relying on amplification of a single fragment (Kent et al. 1998, Grabner & El-Matbouli 2009), our multiplex approach is expected to be more robust as spurious amplification resulting in 3 fragments of certain sizes (298, 166, and 756 bp) is very unlikely. Co-amplification of DNA fragments from a salmonid host serves as an internal PCR control, which ensures that the failure of amplification is not misinterpreted as the absence of *T. bryosalmonae* in a sample. This is especially important when screening samples with varying levels of DNA degradation (Skovgaard & Buchmann 2012). In addition, it would be possible to add additional pathogen- or parasite-specific assays to the existing multiplex reaction. However, potential disadvantages of highly multiplexed PCR include unequal amplification of the target fragments, increased probability of primer-dimers, and misprimes that may reduce the sensitivity and increase false negatives.

Identification of the suspected bryozoan host

Since the outbreak of PKD in salmonids is dependent on the distribution of bryozoans as the invertebrate hosts, information about the distribution of freshwater bryozoans is necessary to fully understand the life cycle of *T. bryosalmonae* (Feist et al. 2002, Okamura et al. 2011). Here, we observed bryozoan colonies only in a single location in the River Mustoja (below the bridge on the concrete wall of the dam at Vihula). Based on 18S rRNA gene sequences, sampled specimens were identified as *Plumatella fungosa*, which has been listed as a possible invertebrate host species of *T. bryosalmonae* (Okamura & Wood 2002). Hence, although the collected bryozoan colony samples appeared to be *T. bryosalmonae*-negative using a multiplex PCR approach, *P. fungosa* may serve as an invertebrate host for *T. bryosalmonae* in the River Mustoja. However, further work is needed to confirm the role of *P. fungosa* or/and other bryozoans (e.g. *P. repens* found in the River Selja; H. Timm pers. comm.) as invertebrate hosts of *T. bryosalmonae* in Estonian rivers and streams.

In summary, this study represents one of the first efforts to describe the distribution and prevalence of the PKD agent *Tetracapsuloides bryosalmonae* in the Baltic Sea area. Similar to results from Denmark (Skovgaard & Buchmann 2012), we show that the parasite is widespread in natural brown trout populations showing a highly heterogeneous distribution at a micro-geographic scale. As a result, it is highly likely that PKD can have an important negative effect on recruitment in Estonian brown trout populations, especially during warm summers. However, for a more complete understanding of how environmental, ecological, and anthropogenic factors affect the host–parasite relationships, and how severe the impact of PKD is on wild salmonid populations, additional multi-disciplinary research is urgently needed.

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