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Characterisation of a low pathogenic form of *Gyrodactylus salaris* from rainbow trout

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ABSTRACT: *Gyrodactylus salaris* was isolated from rainbow trout in a Danish freshwater trout farm, and a laboratory population of this particular parasite form was established on rainbow trout. Challenge infections were performed using different salmonid strains and species, including East Atlantic salmon *Salmo salar* (from the Danish River Skjernå), Baltic salmon *S. salar* (from the Swedish River Ume älv) and rainbow trout *Oncorhynchus mykiss* (from the Danish rainbow trout farm Fousing). These were compared to infection studies on the Norwegian Lærdalselva parasite form kept under exactly the same conditions in the laboratory. The Danish *G. salaris* form had low virulence towards both Atlantic and Baltic salmon, whereas rainbow trout proved susceptible to the parasite. The Danish *G. salaris* form was able to maintain a very low infection on East Atlantic salmon, but not on the Baltic salmon, which eliminated the infection within 2 wk. Rainbow trout developed infection intensities ranging up to several hundred parasites per host. The host colonization patterns of the parasite differed clearly from those of previous studies on microhabitats of the Norwegian form of *G. salaris*. A comparative study on morphological characters (opisthaptoral hard parts) from the Danish parasite form and Norwegian *G. salaris* showed no significant differences. Selected genes comprising internal transcribed spacers 1 and 2 (ITS), ribosomal RNA intergenic spacer (IGS) and cytochrome c oxidase subunit I (COI) regions were cloned and sequenced. Five sequenced ITS clones from 5 individuals of the Danish strain consistently revealed a single base substitution compared to ITS sequences from all other known species and strains of *Gyrodactylus*. Mitochondrial COI gene sequences demonstrated that the Danish *G. salaris* form is closely similar to the Lærdalselva parasite form found in Norway. The IGS sequences were highly variable, but very similar to those obtained from German isolates of *G. salaris.*

KEY WORDS: *Gyrodactylus salari* · Rainbow trout · Pathogenicity · Diagnostic · ITS · IGS · COI

INTRODUCTION

Monogenean platyhelminths belonging to the genus *Gyrodactylus* comprise at least 409 described species that infect a range of fish species worldwide (Malmberg 1993, Bakke et al. 2002). At least one of these parasite species has attracted considerable interest. *G. salaris* Malmberg infections on Atlantic salmon *Salmo salar* stocks have reached epidemic levels in Norwegian rivers, causing considerable financial losses on both national and local community levels, which in turn have led to calls for drastic countermeasures by the authorities (Johnsen 1978, Johnsen & Jensen 1986, 1991, Heggberget et al. 1993, Mo 1994, Appleby & Mo 1997). Several clades of the parasite exist (Hansen et al. 2003). Salmon strains with home rivers in the East Atlantic drainage area such as Norway, Scotland, Ireland, Denmark and Western Sweden are highly susceptible to at least some of these forms, whereas a number of salmon strains homing in Baltic rivers have an ability to respond to and control excessive parasite propagation (Bakke et al. 2002, Lindenstrøm et al. 2006, Heinecke & Buchmann unpubl.). Spreading of the parasite to susceptible salmon strains in European

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areas free of infection (e.g. UK and Ireland) is of great concern (Bakke & MacKenzie 1993, Dalgaard et al. 2003, Peeler 2004).

Controlled challenge infections under laboratory conditions have shown that salmon from the Danish rivers Skjernå and Storå are extremely susceptible to infections by the Norwegian Lærdalselva form of *Gyrodactylus salaris* (Dalgaard et al. 2004, Heinecke & Buchmann unpubl.), and this makes Danish salmon strains potentially vulnerable to *G. salaris* epidemics as well. The parasite species was recorded previously in Danish rainbow trout farms, but is still unknown in wild salmon in Denmark (Malmberg & Malmberg 1993, Buchmann & Bresciani 1997, Buchmann et al. 2000, Nielsen & Buchmann 2001). Whether this is due to environmental factors (affecting virulence or survival of the parasite), or to the presence of a Danish low-virulent form of the parasite is not known. This calls for controlled susceptibility studies using isolates of the parasite from Danish rainbow trout farms. A parasite with a considerable resemblance to *G. salaris*, but designated Gx (due to molecular differences in the ITS region) was isolated previously from a Danish trout farm and studied by Lindenstrøm et al. (2003). This form is of low virulence in Scottish salmon (op. cit.). The present study describes isolation of another Danish form of *G. salaris* from a rainbow trout farm, establishment of a laboratory population, performance of challenge studies using various salmonid strains and species, morphological description and finally molecular characterisation of selected genes in the parasite (cytochrome c oxidase subunit I [COI], 28S-18S ribosomal RNA intergenic spacer [IGS] and internal transcribed spacers 1 and 2 [ITS] regions) using techniques from several authors (Cunningham 1997, Matejusova et al. 2001, Meinila et al. 2002, 2004, Zietara & Lumme 2002, Cunningham et al. 2003, Hansen et al. 2003).

MATERIALS AND METHODS

Parasites. Rainbow trout infected with *Gyrodactylus salaris* were transported to the laboratory from a freshwater trout farm in Jutland (western part of Denmark), and used as source for production of laboratory micro-populations. In order to produce one micro-population, a single live parasite was removed from an anaesthetised fish and carefully placed on an anaesthetised naïve rainbow trout that was isolated subsequently in a tank containing 2 l of aerated water. A total of 30 micro-populations, each based on a single founder individual, were established. When the founder parasite had propagated to at least 10 individuals, parasites were collected for morphological (Mo 1991 a,b) and molecular species confirmation using PCR restriction fragment length polymorphism (PCR-RFLP) with restriction enzyme *Hae*III (Cunningham 1997), and the infected fish with a confirmed *G. salaris* diagnosis was transferred to a 100 l tank containing 70 l of water. A total of 50 naïve rainbow trout (body weight 2 to 6 g) were then allowed to cohabit with the infected host, which allowed further propagation of the parasite population based on one founder individual. The parasite population was monitored at weekly intervals until the population size exceeded 1000 specimens. In order to compare the morphology of the Danish form with the Norwegian Lærdalselva form of *G. salaris*, specimens of both forms were collected from our laboratory populations, kept under the same water and temperature conditions (Dalgaard et al. 2004), and used subsequently for scanning electron microscopy.

Fish. Three different salmonid strains and species (all hatched and reared in pathogen-free conditions) were used for parasite propagation and challenge infections. Fry of rainbow trout (eggs obtained from Fousing trout farm, Jutland) and Danish River Skjernå salmon (eggs obtained by stripping wild salmon caught in the river) were obtained from the Danish Centre for Wild Salmon (Randers, Jutland), where hatching and early rearing procedures were conducted. Newly hatched salmon fry from the Swedish River Ume älv were purchased from Fiskerivärket (Ume älv, Sweden). Fish were kept in the university laboratory system under the same conditions for at least 3 mo prior to experimentation. Fish were fed pelleted dry feed (Biomar, Ecostart 1.2 mm) once a week during the experimental period. The size data for the fish are in Table 1.

Water and fish tanks. Water used for all experiments was a mixture of 50% deionised water and 50% municipal water (Frederiksberg, Denmark) kept in a temperature controlled room at 12 to 14°C. This water resource has proven excellent water source for propagation of *Gyrodactylus salaris* (River Lærdalselva form from Norway) and *G. derjavini* from Denmark through 3 yr of continual parasite cultivation. A total of 2×12 tanks (total volume 15 l) were each equipped with air pumps and filled with 10 l water. Biological filtering was accomplished using of internal filters (Eheim) that kept the ammonium and nitrite concentrations at <0.1 mg l^{-1} and zero, respectively. Nitrate never exceeded 100 mg l^{-1}, due to replenishment (50%) of water twice a week.

Challenge infection. Two experiments were conducted (each in triplicate). For each challenge experiment, 10 rainbow trout, 10 River Skjernå salmon and 10 River Ume älv salmon, respectively, were introduced into separate tanks and kept for 72 h before challenge infection. Water volumes were then lowered from 10 l to 3 l, and a 'donor' rainbow trout (killed by

Table 1. *Salmo salar* and *Oncorhynchus mykiss*. Size (body weight and length, SD in parentheses) of the salmonid strains and species used for challenge studies with the Danish isolate of *Gyrodactylus salaris*

Fish strain	N (in 2 experiments)	Mean body weight (g)		Mean body length (mm)	
		Expt 1	Expt 2	Expt 1	Expt 2
Atlantic salmon from River Skjernå	80 (40 + 40)	0.73 (0.23)	1.85 (0.45)	46.13 (4.88)	62.38 (4.98)
Baltic salmon from River Ume älv	80 (40 + 40)	0.43 (0.07)	0.76 (0.20)	36.67 (1.87)	42.33 (4.10)
Rainbow trout	80 (40 + 40)	1.22 (0.46)	1.80 (0.32)	51.60 (7.09)	60.33 (2.96)

an incision into the brain) infected with approximately 50 *Gyrodactylus salaris* individuals was placed on the bottom of each tank. After 24 h the donor fish was removed and the number of parasites on the fish counted. The infection levels were monitored at weekly intervals for 6 wk. Control tanks containing River Skjernå salmon, River Ume älv salmon and rainbow trout, were treated similarly, except that no *G. salaris* parasites were introduced. Thus, a total of 240 fish (80 rainbow trout, 80 River Skjernå salmon and 80 River Ume älv salmon) were used for the experiments.

Microhabitat. To elucidate microhabitat selection, the location of all parasites on the hosts was registered once a week during counting. The fish surface was divided into 12 zones and the number of parasites occupying each zone was noted according to Buchmann & Uldal (1997).

Morphological characterisation. Specimens of the Danish *Gyrodactylus salaris* form collected from anaesthetised rainbow trout were mounted live in ammonium picrate glycerine (Malmberg 1970). A total of 10 parasites were examined and recorded under a high power light microscope (Leica DM LB X 1000). Eleven characters were measured according to (Mo 1991a,b): LMH, total length of marginal hook; LH, length of marginal hook handle; LSI, length of marginal hook sickle; LA, total length of anchor; LAS, length of anchor shaft; LAP, length of anchor point; LAR, length of anchor root; MDPVB, maximum distance between processes of ventral bar; TMWVB, total median width of ventral bar; MWVB, median width of ventral bar; LVBM, length of ventral bar membrane (TMWVB = MWVB + LVBM) (Table 2).

Scanning electron microscopy of hard parts. Ten parasites of each form (Danish and Norwegian Lærdalselva forms) were each placed on plastic discs (1 cm^2) and individually exposed to partial digestion using lysis buffer (Tween 20 (0.45%), Proteinase K (60 µl ml^{-1}), 10 mM Tris and 1 mM EDTA) at 20°C and followed under the dissection microscope. When the soft parts were partially digested, the parasites were rinsed with distilled water, partly dried and fixed in cacodylate buffered neutral 2.5% glutaraldehyde, sputtered with gold-palladium and studied in a FEI Quanta 200 scanning electron microscope (ESEM).

Molecular characterisation. Lysis of parasites and PCR were performed according to Lindenstrøm et al. (2003). In brief, parasites preserved in 96% alcohol were dried and placed in 200 µl PCR tubes containing 7.5 µl lysis buffer (Tween 20 [0.45%], Proteinase K [60 µl ml^{-1}], 10 mM Tris and 1 mM EDTA) at 65°C until complete digestion of soft parts (confirmed by microscopy). Inactivation of Proteinase K was done at 95°C for 10 min. PCR was performed with *Taq* polymerase (Bioline no. BIO21040), at an annealing temperature of 55°C, and 30 cycles. For specific primers see Table 3.

Table 2. *Gyrodactylus salaris*. Measurements of opisthaptoral hard parts of the Danish form mounted in ammonium picrate glycerine (Malmberg 1970), following Mo (1991 a,b). N_s: number of specimens; N_m: number of measurements; SD: standard deviation

Character	N_s	N_m	Mean	SD	Range
Total length of marginal hook (LMH)	9	17	42.39	1.4	40.5–44.5
Length of marginal hook handle (LH)	9	18	32.58	0.96	31.0–4.0
Length of marginal hook sickle (LSI)	9	59	8.70	0.39	8.0–9.5
Total length of anchor (LA)	9	9	74.55	2.9	71.0–80.0
Length of anchor shaft (LAS)	9	9	56.33	2.3	55.0–60.0
Length of anchor point (LAP)	10	10	35.85	1.2	34.0–37.5
Length of anchor root (LAR)	9	9	26.33	1.7	24.0–29.0
Length of ventral bar membrane (LVBM)	8	8	19.87	1.4	18.0–22.0
Median width of ventral bar (MWVB)	8	8	9.25	0.71	8.0–10.0
Total median width of ventral bar (TMWVB)	8	8	28.75	1.3	27.0–30.0
Maximum distance between processes of ventral bar (MDPVB)	8	8	29.13	1.5	27.0–31.0

Table 3. Primers used for PCR amplification. COI: cytochrome C oxidase subunit I; ITS: internal transcribed spacers; IGS: ribosomal RNA intergenic spacer

Gene	Forward primer	Reverse primer	Length (bp)
COI	COI LA: GAATCGGCGGGTTCGGTAA	COI HA: GAACCATGTATCGTGTAGCA	820
IGS	IGSV4: GATACTCATTTGACTCGGTGTG	IGSV3: CTGGCTATAATCACGTAAGACT	Approx. 820
ITS	ITS1: TTTCCGTAGGTGAACCT	ITS2: TCCTCCGCTTAGTGATA	1300

Sequencing of ITS, IGS and COI. Products for sequencing were cloned into the pCR®2.1-TOPO vector and transformed into chemically competent TOP 10 cells (Invitrogen kit no. K4500-40). Plasmids were purified with Qiaprep®Spin miniprep Kit (Qiagen Cat. no. 27106). Sequencing was performed with a BigDye 1.1 (Applied Biosystems no. 4337450) and using specific primers. Products were analysed in an ABI-310 automated sequencer. Fragments from IGS, COI and ITS were sequenced from 4 or 5 individual parasites. The sequences in the IGS region was expressed according to the terminology presented by Cunningham et al. (2003).

Data analysis. Infection parameters used were prevalence (percentage of hosts infected) and abundance (number of parasites per host—infected and uninfected) according to Bush et al. (1997). Challenge infection data were tested for normality (Kolmogorov-Smirnov test for normality, Sigma Stat, 2005). A non-parametric test (Kruskal-Wallis 1-way analysis of variance on ranks, Sigma Stat, 2005) was used to test for significant differences between replicates within the same host species. When no significant difference was found, data were pooled for further statistical analysis. To test for differences in population size of *Gyrodactylus salaris* on the 3 different hosts, the 3 previously pooled data sets were tested against each other (Kruskal-Wallis 1-way analysis of variance on ranks). The test was carried out at each sampling occasion to evaluate parasite population development over time. If a statistically significant difference among abundance values between the 3 host stocks/species was found, an All Pairwise Multiple Comparison Procedure (Dunn's Method) (Sigma Stat, 2005) was used to isolate the group or groups that differed from the others. A probability level of 0.05 was applied.

RESULTS

Morphological characterisation

The morphology of the Danish and Norwegian Lærdalselva form including hamuli, connecting bridges and marginal hooklets are shown in Fig. 1. The similarities are strong, with only minor differences. Measured values of all 11 characters from opisthaptoral hard parts of the Danish form are presented in Table 2. They are very similar to corresponding parameters in *Gyro-*

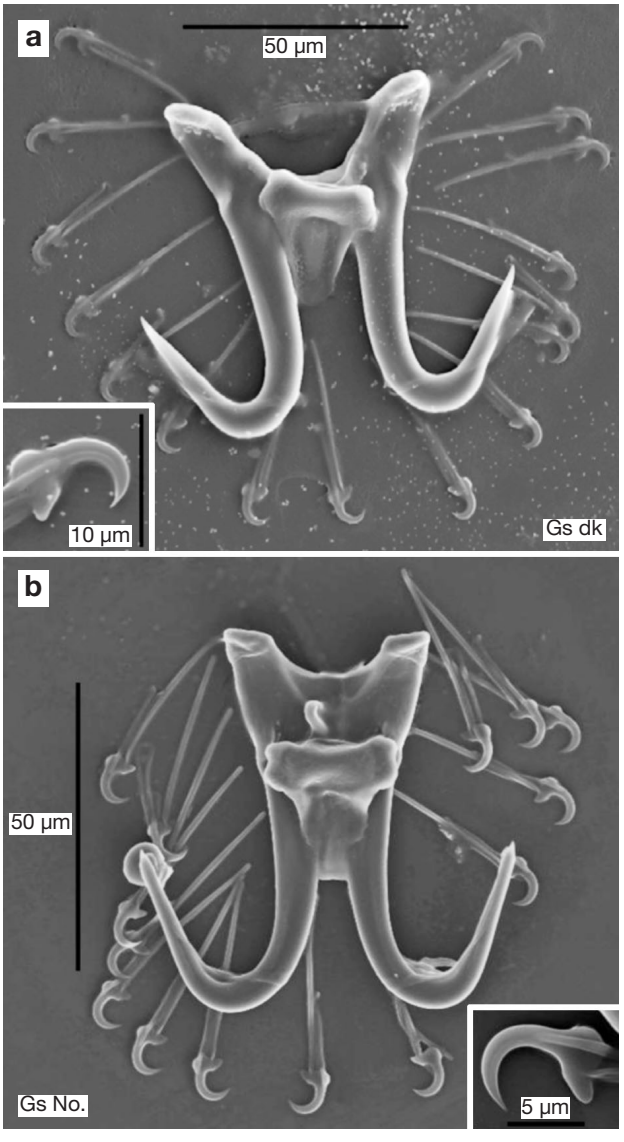

Fig. 1. *Gyrodactylus salaris*. Scanning electron micrographs of partially digested opisthaptors of (a) the Danish form (Gs dk) and (b) the Norwegian Lærdalselva form (Gs No.)

dactylus salaris (Mo 1991a,b). The main difference is the size of the ventral bridge, which differed slightly between the 2 forms.

Molecular characterisation

Five clones from 5 different individuals were sequenced for the COI region, and 100% homology was found with the COI sequence from *Gyrodactylus salaris* from the Norwegian River Lierelva (H. Hansen, Genbank accession number AY146596). Homologies with *G. salaris* from Finland (M. Meinila, GenBank accession number AY225307) and *G. thymalli* from Norway (H. Hansen, GenBank accession number AY-146612) were 98 and 97%, respectively.

Five clones from 5 individuals were sequenced for the ITS region, and general homology with *Gyrodactylus salaris* from Norway (C. Cunningham, GenBank accession number Z72477) was found, except for a consistent one base subsistution, G^{1100} to A^{1100}.

Four clones from 4 individuals were sequenced for the IGS region. They are listed and compared to published IGS sequences in Table 4. They were all different and showed high variability. Clone 1 showed high similarity to *Gyrodactylus thymalli* from Gudbrandsdalslågan in repeat region 1, and some similarity in repeat region 2 to *G. salaris* from rainbow trout in Germany. Clone 2 showed similarity (repeat region 1) to *G. salaris* rainbow trout form from Sweden and Germany. Region 2 showed highest similarity to *G. salaris* from Germany.

Clone 3 showed greatest similarities to the rainbow trout form from Sweden (region 1) and from Sweden and Germany (region 2). Clone 4 exhibited the same affinities, although based on other sequences. Thus, similarities associated the Danish form mostly with the German record. However, the variability was high.

Challenge infection

The present investigations demonstrated clearly that the Danish *Gyrodactylus salaris* form was non-pathogenic to Atlantic salmon from the River Skjernå and to Baltic salmon from the River Ume älv, but the parasite was fully able to propagate on rainbow trout. Thus, rainbow trout showed the highest parasite susceptibility, whereas the River Skjernå salmon showed a significantly lower susceptibility. Further, the River Ume älv salmon exhibited a significantly lower susceptibility compared to both River Skjernå salmon and rainbow trout (Figs. 2 & 3).

Prevalence

Prevalence data for the different hosts are shown in Fig. 2. During the experimental period, all replicates of *Oncorhynchus mykiss* showed an increase in prevalence values. Initially, the mean prevalence for all replicates was between 20 and 50% in Expt 1 and between 30 and 100% in Expt 2. In both trials, the prevalence increased to 100% in Weeks 4 to 6. For *Salmo salar* (River Skjernå), the initial mean prevalence for all replicates in Expt 1 was 80 to 90%, and 60 to 90% in Expt 2, but 6 wk post infection the mean prevalence for all replicates fell. Initially, the mean prevalence on *S. salar* (River Ume älv) for all 3 replicates was 50 to 90% (Expt 1) and 50 to 80% (Expt 2). After 2 wk post-infection, the prevalence in all replicates had declined to 0%.

Table 4. *Gyrodactylus salaris*. Repeat regions of the IGS in the Danish isolate and a comparison with published records. Four clones from 4 individuals were sequenced and all were different as indicated. The letter codes of Cunningham et al. (2003) for the different repeat regions are used. Unique sequences found in the present work are listed in the footnotes

Clone	Repeat region 1	Repeat region 2
DK1	AA[a]BBB[b]BBBDE Closest fit: *G. thymalli* BBDBBDE	$PPSU_2RVQ_5T^{1/2}$ R Exact fit: *G. salaris* from rainbow trout in Germany clone a
DK2	ABBABBBBBBBBBB Closest fit: *G. salaris* from rainbow trout in SW Sweden and Germany Clone a	$PPSU_2$[c]$RVQ_5T^{1/2}$ R Closest fit: *G. salaris* from rainbow trout in Germany Clone a
DK3	AABBBE Closest fit: *G. salaris* from rainbow trout from SW Sweden	$PPPSURVQ^{1/2}$ R Exact fit: *G. salaris* from rainbow trout from SW Sweden and Germany Clone b
DK4	ABBABBBBBB Closest fit: *G. salaris* from rainbow trout from SW Sweden and Germany Clone a	$PPPSURVQ^{1/2}$ R Exact fit: *G. salaris* from rainbow trout from SW Sweden and Germany Clone b

[a]GTCCTTCAGTGTAG**G**ACCGTACA; [b]GTCCTTC**C**GTGTAGAGCCGTACA; [c]TACTTATTACCGTAGA**A**CCGTGCG; underscore and bold letters designate nucleotide substitution

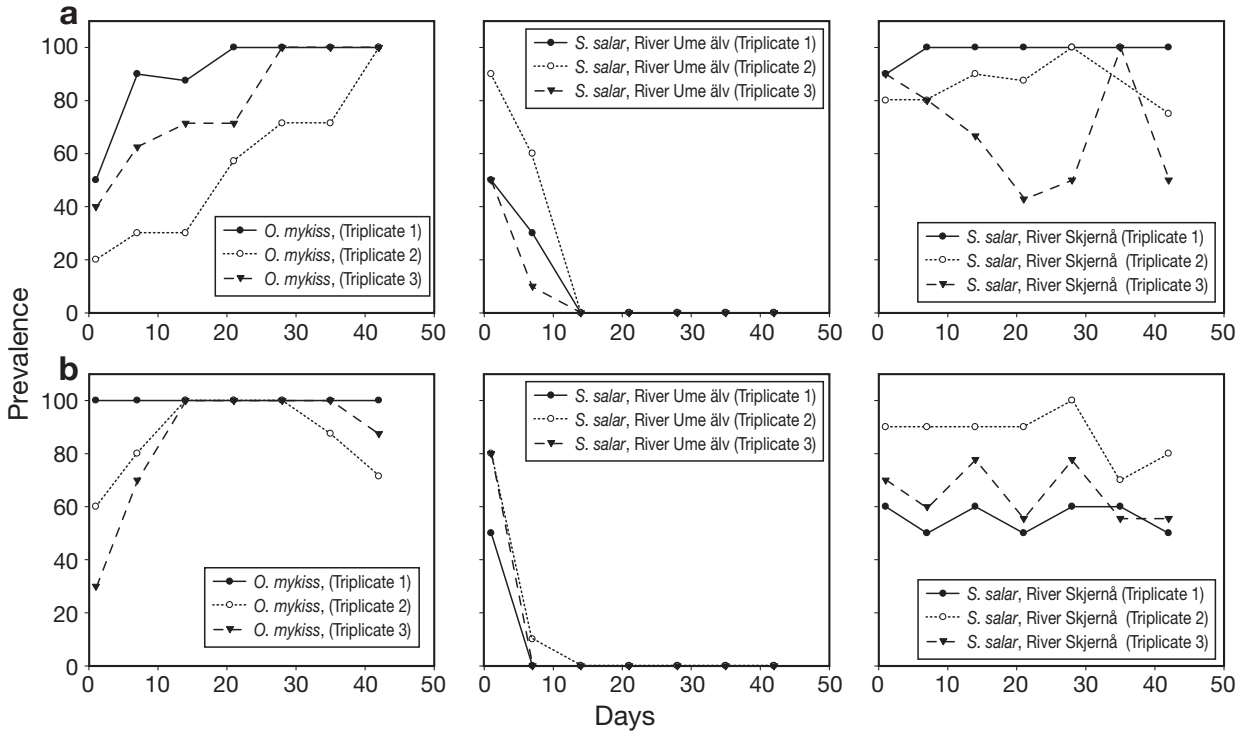

Fig. 2. Challenge infections of salmonids (*Oncorhynchus mykiss*, *Salmo salar* from River Ume älv and *S. salar* from River Skjernå) using the Danish strain of *Gyrodactylus salaris*. Prevalences in (a) Expt 1 and (b) Expt 2. Data presented as means ± SE of triplicate experiments (error bars fall within symbols)

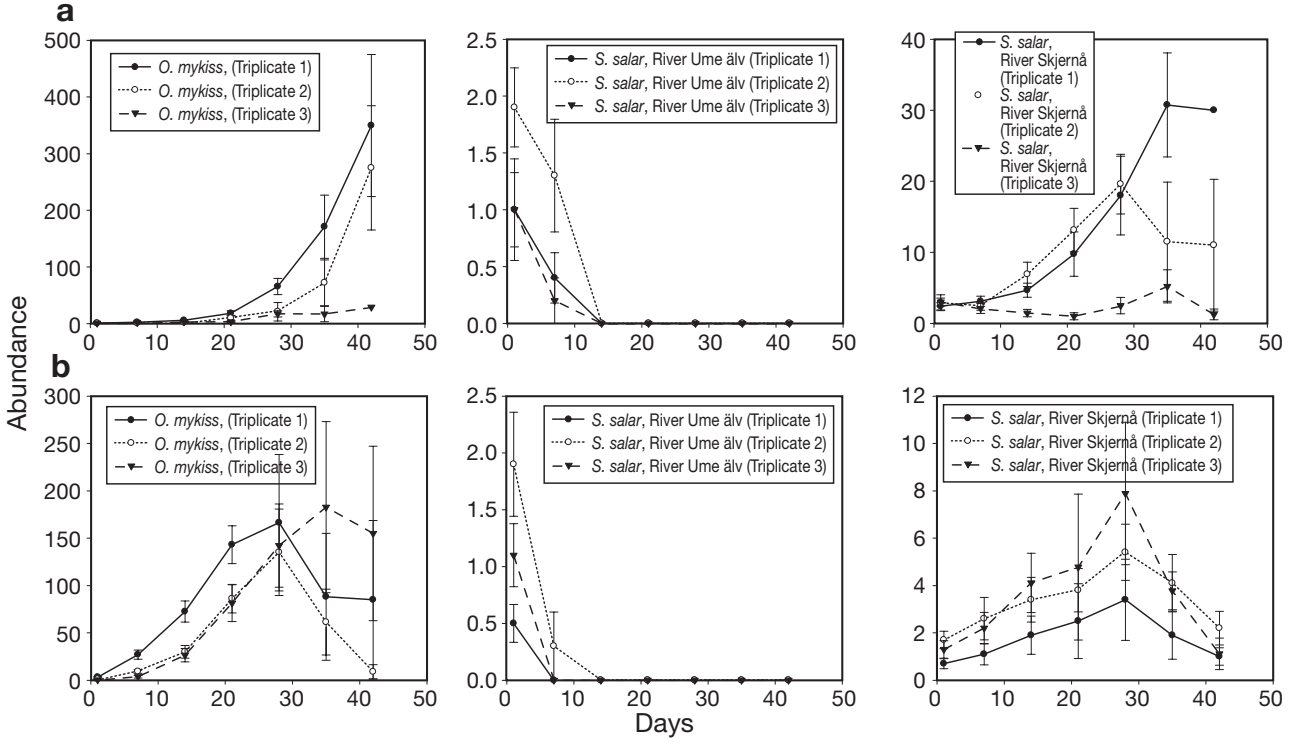

Fig. 3. Challenge infections of salmonids (*Oncorhynchus mykiss*, *Salmo salar* from River Ume älv and *S. salar* from River Skjernå) using the Danish strain of *Gyrodactylus salaris*. Abundances in (a) Expt 1 and (b) Expt 2. Data presented as means ± SE of triplicate experiments

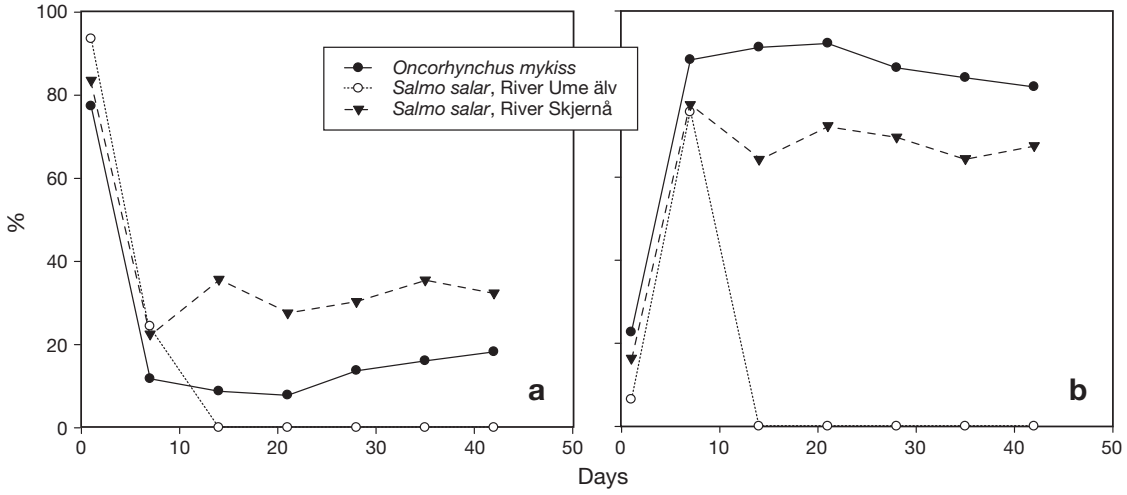

Fig. 4. *Gyrodactylus salaris*. Location (microhabitat) of the Danish strain on fins and body skin of the 3 salmonid fishes used in the challenge experiments. Values presented are percentages of all parasites in each of the triplicate experiments located on (a) fins and (b) body surfaces

Abundance

The differences in the median values among the replicate groups were not great enough to exclude the possibility that the differences were due to random variability. Accordingly, data from the 3 replicates (in each experiment) were pooled for each host strain/species respectively. Thus, abundance data for all host strains/species are presented in Fig. 3. For *Oncorhynchus mykiss,* the initial mean abundances for the 3 replicates were 0.8 and 1.6 in Expts 1 and 2, respectively. During the remaining infection, the mean abundances in all replicates increased to 217.8 and 83.0 in Expts 1 and 2, respectively. Initially, the mean abundances on *Salmo salar* (the River Skjernå) for all replicates were 2.8 and 1.2 in the 2 experiments, respectively. During the experimental period, all River Skjernå replicates showed a general increase in abundance values, and 6 wk post-infection the mean abundances for all replicates were 14.1 and 1.4 in Expts 1 and 2, respectively. Initial mean abundances on *S. salar* from the River Ume älv for Expts 1 and 2 were 1.3 and 1.2, respectively. All replicates showed a rapid decline in abundance immediately post-infection. Thus, 2 wk post infection the abundance had declined to zero in all 6 groups. From Day 7 post-infection, the parasite abundance levels for the 3 groups of hosts (rainbow trout, River Skjernå and River Ume älv salmon) were found to differ significantly.

Microhabitat

In all experiments, clear trends for initial colonisation (24 h post-infection) of the pectoral and pelvic fins on both *Oncorhynchus mykiss*, *Salmo salar* (River Skjernå) and *S. salar* (River Ume älv) were observed. However, the majority of the parasites translocated to the body regions on rainbow trout and salmon within few days post-infection (Fig. 4).

DISCUSSION

The general morphology and the morphometric data confirm that the Danish isolate fits rather well with the description of *Gyrodactylus salaris*. All character measurements fall within the range of previously measured values from Norwegian *G. salaris* on rainbow trout. The Danish parasite groups together with the *G. salaris* forms isolated from rainbow trout in various regions (G. Malmberg pers. comm.). The marginal hooklet shape does not fit that of the special Danish *G. salaris* form described by Lindenstrøm et al. (2003). In addition, the molecular work on ITS, COI, IGS leaves no doubt that the present parasite is closely related to *G. salaris* isolated in Norway, Finland, Germany and Sweden, although a single base substitution was found in the ITS region. This ITS sequence differs also from the sequences for the Danish form presented by Lindenstrøm et al. (2003). The similarity of the COI sequences suggests that this particular region is not a useful marker for pathogenicity, although it is still likely that it reflects some relatedness between the worms in question. It would be valuable to sequence further clones of IGS from additional individuals in order to obtain a more precise characterisation. However, the high variability in this region suggests that this region is less well suited for differentiation

between various forms of *G. salaris*, including the present Danish isolate.

The pathogenicity studies showed a clear difference from the River Lærdalselva form of *Gyrodactylus salaris* from Norway, which has been tested continually for several years in the same facilities and under the same experimental conditions (Dalgaard et al. 2003, 2004, Lindenstrøm et al. 2006, Heinecke & Buchmann unpubl.). Thus, populations of the Norwegian form on salmon reached abundances of more than 2000 parasites per host in this system (Lindenstrøm et al. 2006). However, the new Danish isolate achieved a low infection level of only 1 to 4 parasites per individual salmon originating from the River Skjernå, and the Swedish salmon eliminated the parasite completely within 2 wk. Furthermore, rainbow trout (which is a less susceptible host for the Lærdalselva form of *G. salaris* than Atlantic salmon, Dalgaard et al. 2004, Heinecke & Buchmann 2006) experienced infection rates of >700 Danish *G. salaris* parasites per fish (present study).

It is noteworthy that the site selection of the Danish isolate differed from previous recordings for *Gyrodactylus salaris* on various strains of Norwegian salmon (Jensen & Johnsen 1992). These authors (op. cit.) based their studies on salmon from various sources and ages, which make those data less suitable for comparison. However, site selections by the Lærdalselva form of *G. salaris* on rainbow trout and Danish salmon have also been tested under controlled and well-defined conditions corresponding to the present investigation (Heinecke & Buchmann 2006), and large differences from the Danish form were again found. The number of parasites recorded on the Swedish River Ume älv salmon in the present work was very low, due to the low susceptibility of this salmon strain, but the site selection on this host type showed a similar trend for selection of body surface location.

The genes sequenced (COI, ITS, IGS) do not encode pathogenicity traits in the parasite. However, it is noteworthy that some of the gene sequences can be used as markers for the present isolate of a non-pathogenic *Gyrodactylus salaris* form in Denmark. Thus, the consistent base substitution G^{1100} to A^{1100} in the ITS region of clones from different parasite individuals suggests that this is a valuable character of this specific form, although the presence of non-pathogenic forms without this substitution may exist. The IGS is also unique (compared to the Norwegian River Lærdalselva form), but it is highly variable and was not found consistently in the different individuals tested. Therefore, we recommend application of the sequences of the ITS region for discrimination and identification of this particular Danish form of *G. salaris*.

The present work was conducted on a clone of *Gyrodactylus salaris* derived from one founder individual propagated in the laboratory. Therefore, we cannot exclude the possibility that other forms with different morphologies, gene structures and pathogenicities will be found in future studies, and further work on such clones should be conducted.

It has been noted that the species *Gyrodactylus thymalli* occurs on grayling in Norway (Sterud et al. 2002, Hansen et al. 2003). The morphology of the hard parts of this parasite differs only slightly from that of *G. salaris*. Further, the ITS and COI regions do not differ at all, and the differences found in the IGS region are few (Cunningham et al. 2003). It would therefore be relevant to consider whether *G. thymalli* is conspecific with *G. salaris*. However, due to the fact that *G. thymalli* does not propagate to very large population size on East Atlantic salmon and does not have the same pathogenicity as *G. salaris* (Sterud et al. 2002), we consider it convenient to recognize the species *G. thymalli* in order to differentiate this low pathogenic form from the pathogenic *G. salaris* (Cunningham et al. 2003). The molecular differences between the ITS and IGS regions of the Danish and Lærdalselva forms of *G. salaris* are more extensive than the differences between *G. thymalli* and *G. salaris*. Furthermore, the pathogenicity to salmon in the Danish *G. salaris* is even lower than that in *G. thymalli*. This might suggest that the Danish form should be presented as a separate species. However, we find it is reasonable to accept that the species *G. salaris* is highly variable, exists in various forms, and that the parasite described in this work is a form with low pathogenicity towards East Atlantic salmon. Previously another morphotype, very similar to *G. salaris*, was isolated and found to have low pathogenicity towards Atlantic salmon (Lindenstrøm et al. 2003). However, molecular work on this form was limited to the ITS region, which differed from both the Norwegian form (Cunningham 1997) and the Danish form presented here, but it may indicate the existence of various forms of non-pathogenic parasites in the environment.

Restocking of the Danish rivers with conserved original salmon populations (River Skjernå and River Storå) highly susceptible to the Norwegian Lærdalselva form of *Gyrodactylus salaris* (Dalgaard et al. 2004, Heinecke & Buchmann 2006) has been carried out for more than a decade (Dalgaard et al. 2003, 2004, Heinecke & Buchmann 2006). Therefore, the benefit of such a restocking program would be doubtful if the pathogenic form of *G. salaris* were present in the rivers. However, the present work suggests that the parasites in the Danish rainbow trout farms do not pose any danger to the restocking activities. This is further

supported by the fact that no epidemics have been detected in Danish salmon populations, although the parasite was identified in a Danish rainbow trout farm in 1972 (Malmberg & Malmberg 1993). In contrast, if the Lærdalselva form were introduced, there might well be serious consequences for the Danish salmon populations.

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