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Gyrodactyloides bychowskii (Monogenea: Gyrodactylidae) from sea-caged Atlantic salmon *Salmo salar* in Scotland: occurrence and ribosomal RNA sequence analysis

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ABSTRACT: *Gyrodactyloides bychowskii* has been recorded in Scottish waters for the first time. The parasite was found on the gills of Atlantic salmon reared in seawater. An integrated morphological and molecular examination of the parasite was carried out. Prevalence of the parasite was greatest in February and declined to 0 by June in 1999. During 2000, parasites were located in March and November. An overdispersion of parasites was recorded, with intensity of infection reaching over 200 parasites per gill arch in some fish. Parasitised gill tissue showed hyperplasia and hypertrophy but not always at the site of parasite attachment. The internal transcribed spacer of the ribosomal RNA gene array was amplified by PCR and sequenced. This sequence shared greatest similarity with the internal transcribed spacer of *Gyrdicotylus gallieni*, followed by *Gyrodactylus* species. This is the first molecular analysis of this parasite and provides sequence data that may be used in comparison of *G. bychowskii* from other locations or in phylogenetic analysis of this group of Monogenea.

KEY WORDS: Monogenea · *Gyrodactyloides bychowskii* · Atlantic salmon · Seawater

INTRODUCTION

Monogeneans of the family Gyrodactylidae have been reported for both wild and farmed fish, although the study of freshwater species has assumed greater significance since the emergence of *Gyrodactylus salaris* Malmberg, 1957 as a highly contagious pathogen of wild Atlantic salmon *Salmo salar*. Epidemic infections of *G. salaris* have been responsible for mass mortalities and almost total loss of salmon parr in about 40 Norwegian rivers (Mo 1994). In the UK, regular surveillance of farms culturing salmonid fish in freshwater is carried out to confirm that susceptible species are free of *G. salaris.* Other European countries also implement survey programmes designed specifically to investigate gyrodactylid infections of cultured salmonid fish. There is, however, no similar monitoring programme for fish cultured in sea water and, consequently, little is known about the impact of gyrodactylids and other monogeneans on farmed salmonids in the marine environment.

Gyrodactyloides bychowskii Albova, 1948 was originally described from the gills of wild Atlantic salmon in sea water in the White Sea and has since been reported from sea-caged Atlantic salmon in Norway (Mo & MacKenzie 1991). This present paper reports for the first time the occurrence of *G. bychowskii* in Scotland and the first molecular analysis of this species.

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The genes and associated spacers of the ribosomal RNA (rRNA) gene array have been widely used for species identification of parasitic worms (e.g., Rollinson et al. 1990, Gasser & Hoste 1995, Zhu et al. 1998). Study of the rRNA genes of *Gyrodactylus* was started in the quest for novel, objective methods to discriminate the pathogenic *G. salaris* from other non-pathogenic species such as *G. derjavini* and *G. truttae* that are commonly found on European salmonids. Nucleotide sequence analysis of variable region V4 of the small subunit (18S) rRNA gene revealed significant and consistent differences between these species (Cunningham et al. 1995). Later work on the more variable internal transcribed spacer (ITS) regions of rRNA found restriction fragment length polymorphisms (RFLP) that could also readily discriminate between species of *Gyrodactylus* (Cunningham 1997). Nucleotide sequence analysis of the ITS in *G. salaris*, *G. derjavini* and *G. truttae* has confirmed the interspecific variability, particularly noticeable in ITS1 (Cunningham et al. 2000). These new techniques and tools for the study of Monogenea were applied in this study of *Gyrodactyloides bychowskii*.

MATERIALS AND METHODS

Sample collection and examination. In Scotland an annual planned programme of visits to farms rearing Atlantic salmon in seawater is carried out. Tissues for light microscopy are selected from fish that are moribund, exhibit abnormal behaviour or show gross changes that might indicate infection. Between January 1999 and December 2000, portions of all tissues from these fish were fixed for light microscopy as described below. In 1999 additional tissue samples were collected from apparently healthy salmon at farms where the monogeneans had been located by light microscopy. In these cases both pectoral fins and a gill arch were removed from up to 10 anaesthetised salmon. These tissues were transferred into tubes containing 70% ethanol. In the laboratory, the alcohol-preserved tissues were examined using a binocular dissecting microscope. Monogenean parasites were removed with fine forceps, counted and placed in a tube containing 0.5 ml 70% ethanol. The total number of *Gyrodactyloides* specimens recovered from each fish was recorded.

Morphological examination. A pipette was used to transfer single parasites to a drop of 70% ethanol on a microscope slide and a coverslip was applied. Each specimen was examined by phase contrast microscopy at ×300 to ×800 magnification and measurements of 10 animals, including details of the anchors and marginal hooks of the opisthaptor, were recorded according to the criteria of Pálsson & Beverley-Burton (1982). Individual parasites were permanently mounted in Malmberg's ammonium picrate glycerin.

Light microscopy. All tissues (i.e., gills, kidney, heart, liver, spleen, brain, gut and pyloric caeca) were dissected from farmed fish for diagnostic purposes, but for this study, only gill tissues are discussed. Tissues were fixed in buffered formaldehyde and decalcified in a modified Perenyi's fluid (Bruno & Poppe 1996). After embedding, 5 µm sections were cut and stained with Harris's haematoxylin and eosin.

rRNA analysis. Following morphological examination, individual animals were placed in 7.5 µl lysis solution (proteinase K: 60 mg ml^{-1}; Tween 20: 0.45%; NP40: 0.45% in TE buffer). Tubes were incubated at 65°C for 20 min to allow proteinase K digestion, then 95°C for 10 min to denature the proteinase. Aliquots of 2.5 µl of lysate were used as the template in reactions to amplify the entire ITS1-5.8S-ITS2 region of the rRNA gene array (Cunningham 1997). The products of replicate independent amplification reactions were purified using Wizard PCR Preps (Promega, Southampton, UK) and directly sequenced in both forward and reverse directions using PCR primers and internal primers where necessary. Reactions were carried out using the dRhodamine Terminator Ready Reaction Kit and electrophoresis on an ABI PRISM 377 Sequencer (Perkin Elmer, Warrington, UK). Consensus sequences were assembled using Sequencher software (Gene Codes Corporation, Ann Arbor, MI, USA). BLAST searches of the EMBL nucleotide database were carried out using consensus sequences. CLUSTALX software (Thompson et al. 1997) was used to align the *Gyrodactyloides bychowskii* ITS sequence with similar sequences identified in the BLAST search. These pairwise alignments were used to calculate the similarity of the 5.8S gene alone and the entire ITS1-5.8S-ITS2 sequences. Regions at the termini of the alignments where 1 sequence was longer than the other were excluded from the similarity calculations. Secondary structures were predicted using MFOLD (Zuker et al. 1999) with default settings.

RESULTS

The routine examination of stained gill sections from marine-reared Atlantic salmon revealed the presence of monogeneans on fish between January and June and in December 1999 within 2 areas of Scotland. The affected fish farms were located on the west coast near Oban and in the Shetland Isles, Scotland. In the following year parasites were located histologically on fish examined in March and November on 3 farms, again in these areas. The prevalence level

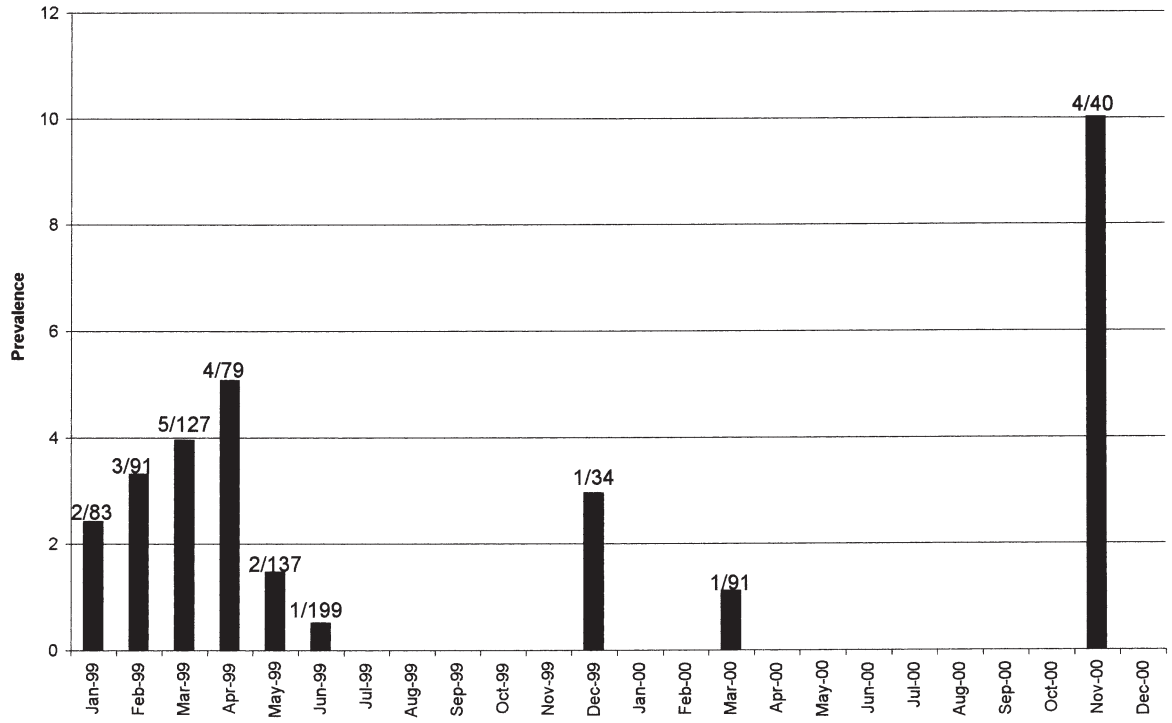

Fig. 1. Prevalence of *Gyrodactyloides bychowskii* on farmed Atlantic salmon *Salmo salar* in Scotland in 1999 and 2000. Results are from light microscopical examination. Values above columns are the numbers of infected fish/number sampled

of infection ranged between 0.5 and 10% over both years (Fig. 1). In both years parasites were not detected in stained tissue sections from the fish sampled between July and October. The total numbers of parasites recovered from 10 apparently healthy fish from 2 farms in 1999 are presented in Table 1. An overdispersed distribution of the parasites was confirmed during morphological examination, with some fish uninfected, while others collected at the same time and from the same cage or site had over 200 on a single gill arch (Table 1). No parasites were found on the pectoral fins.

Table 1. Number of *Gyrodactyloides bychowskii* on gill lamellae from marine farmed Atlantic salmon *Salmo salar*. Results are from 10 apparently normal fish sampled in 1999

Fish no.	No. of parasites per gill arch	
	Farm in Oban area (West Coast) (sampled in April)	Farm from Shetland Isles (sampled in June)
1	3	200+
2	0	0
3	100+	0
4	1	0
5	2	0
6	1	200+
7	0	0
8	3	0
9	4	0
10	1	0

Parasite morphology

The criteria of Pálsson & Beverley-Burton (1982) were used for a morphological examination of individual parasites. Initial low power examination of whole animals revealed that parasites were very similar to *Gyrodactylus* species. Each specimen was elongate with a mean length and width of 521 × 171 µm. The total length of the hamulus measured 86 to 91 µm. The opisthaptor was conspicuous with 16 marginal (lateral) hooks with long shafts arranged in 3 groups. Two groups consisting of 4 hooks each were located anterolaterally, and 8 hooks formed a posterior group. The marginal hooks were flexible with long shafts, comprising proximal and distal parts, as described by Mo & MacKenzie (1991). On the basis of morphological evidence, the parasites on the gill lamellae of farmed Atlantic salmon were identified as *Gyrodactyloides bychowskii* Albova, 1948. Morphological data obtained from 10 animals are reported in Table 2.

Table 2. Measurements of 10 *Gyrodactyloides bychowskii* from caged Atlantic salmon *Salmo salar* in the Shetland Isles, Scotland

Feature	Range (µm)	Mean (n = 10)
Total length	418–590	521
Total width	138–226	171
Width of suction disc	75–96	86
Hamulus		
Total length	86–91	89
Length of shaft	49–59	53
Deep root to curve of blade	64–71	69
Length of point	11–15	14
Length of deep root	12–17	15
Length of superficial root	34–42	39
Ratios		
Superficial root:shaft	1:1.1–1.7	1:1.4
Deep root:shaft	1:2.8–4.7	1:3.4
Length of transverse bar	23–29	25
Marginal hooks		
Total length	63–75	71
Length of sickle	7–9	8
Proximal width of sickle	5–6	5.2
Distal width of sickle	8–10	8.4
Length of distal part of shaft	26–29	28

Gross lesions and light microscopy

Tissue changes in the gills included mild to marked epidermal hyperplasia, slight oedema and hypertrophy at the site of parasite attachment; however, some parasites were associated with apparently normal tissue.

rRNA gene analysis

Amplification of the ITS resulted in a single well-defined product of approximately 950 bp. This produced a clear sequence from both strands, which was assembled to yield the full ITS sequence of 942 bp. This was submitted to the EMBL nucleotide database under accession number AJ249348.

BLAST database searches revealed that the *Gyrodactyloides bychowskii* ITS sequence shared greatest similarity with the ITS of *Gyrdicotylus gallieni* (accession number AJ001843), followed by *Gyrodactylus turnbulli* (accession number AJ001846) and other *Gyrodactylus* species. Of the species commonly found on European salmonids in freshwater (*G. salaris*, *G. derjavini* and *G. truttae*), the ITS sequence of *G. bychowskii* shared greatest similarity with that of *G. truttae* (accession number AJ132260). Sequence similarity of the 5.8S gene alone and the full ITS1-5.8S-ITS2 sequence from these species is given in Table 3.

Five secondary structures were predicted for the ITS2 of *Gyrodactyloides bychowskii*, with free energy values of between 116.3 and –110.5. These structures were similar, with a central helix that had 2 helices radiating from each end. The 3' end of the 5.8S rRNA (20 nucleotides) is predicted to form a helix with the 5' end of ITS2. This structure corresponds to the 4-domain models of trematode and *Gyrodactylus* ITS2 structure (Morgan & Blair 1998, Cunningham et al. 2000).

DISCUSSION

Gyrodactyloides bychowskii was originally described by Albova (1948) from Atlantic salmon *Salmo salar* caught in the White Sea. The morphological examination of Atlantic salmon gills from 2 regions and distant farm sites in Scottish waters showed the presence of *G. bychowskii* between January and June 1999 and in March and November 2000. There is no previous record of this parasite from farmed salmon in Scotland. The apparent absence of the parasite from farmed salmon during the summer months of the 2 years of study may indicate a decline in numbers as the sea temperature rises, supporting the suggestion from Kulachkova (1977) that *Gyrodactyloides* is a cryophilic Arctic genus and occurs in regions with cold sea temperatures. Furthermore, this also supports the report from Mo & MacKenzie (1991), who showed that farmed salmon are a host of *G. bychowskii* in northern Norway.

The light microscopy observations of gill sections from salmon indicate that *Gyrodactyloides bychowskii* is present among farmed fish in sea water in at least 2

Table 3. Nucleotide sequence similarity for 5.8S rRNA gene (above diagonal) and entire internal transcribed spacer (ITS) (below diagonal) of *Gyrodactyloides bychowskii*, *Gyrdicotylus gallieni*, *Gyrodactylus turnbulli* and *Gyrodactylus truttae*, showing similarity value and number of aligned positions in parentheses

5.8S ITS1-5.8S-ITS2	*G. bychowskii*	*G. gallieni*	*G. turnbulli*	*G. truttae*
G. bychowskii	–	0.905 (157)	0.943 (157)	0.917 (157)
G. gallieni	0.599 (920)	–	0.943 (157)	0.917 (157)
G. turnbulli	0.593 (1008)	0.595 (965)	–	0.962 (157)
G. truttae	0.575 (1060)	0.563 (1014)	0.605 (1141)	–

distinct areas in Scotland. Further studies might help to determine its full distribution.

In some cases, *Gyrodactyloides bychowskii* infestation was associated with marked epidermal gill hyperplasia and hypertrophy and a decline in overall condition of the fish. This is consistent with the study by Mo & MacKenzie (1991), although in the current study some instances of morphological changes in the gill tissue were recorded without parasite involvement. The current study does not report the presence or significance of any systemic infections in the fish sampled during disease investigations. Mo & MacKenzie (1991) similarly noted gill damage when high numbers of *G. bychowskii* were present in fish weighing over 2.5 kg. Over 200 parasites per single gill arch were recorded from dissected tissue in the present study; however, their impact on overall fish health in Scotland is unknown. In small fish, this may have a debilitating effect, particularly in view of the gill responses to infection. Thus, a detailed study of wild and farmed salmon, similar to that already carried out by the regulatory authorities examining *Gyrodactylus* species in freshwater, would provide information on the full distribution of *G. bychowskii* and an awareness of any potentially harmful effects.

Available monogenean ITS sequences fall into 2 groups: long ITS sequences of 1200 to 1300 bp and shorter 900 to 1100 bp ITS sequences. Amplification of the ITS region employs primers located at the 3′ terminus of the small subunit rRNA gene and the 5' terminus of the large subunit rRNA gene. These sequences are highly conserved and have been used to design primers that can hybridise to an enormous variety of eukaryotes (Medlin et al. 1988). Thus, the variation in PCR product length is unlikely to be due to non-specific hybridisation of primers to regions within the ITS instead of the genes flanking the spacers.

Repetitive regions of the genome, such as the tandem array of genes encoding rRNA and the associated spacers, are most likely to experience events such as unequal crossing-over during replication, which can result in length differences. Homogenisation then spreads the variant throughout a species (Dover 1982) and, in time, clear differences can arise between species or groups of species.

As noted by Cable et al. (1999), available ITS sequences from *Gyrodactylus* species occur in 2 forms based on length. The ITS of species found on salmonids, *G. salaris*, *G. derjavini*, *G. truttae* (accession numbers Z72477, AJ132259, AJ132260) and *G. gasterostei*, *G. gurleyi* and *G. pungitii* (AJ001841, AJ001842 and AJ001845), have longer ITS. Shorter ITS are found in *G. arcuatus*, *G. turnbulli*, an unidentified *Gyrodactylus* species and *Gyrdicotylus gallieni* (accession numbers AJ001839, AJ001846, AJ001844 and AJ001843). The ITS of *Gyrodactyloides bychowskii* falls into the short ITS group. The length variation in this species, as within the genus *Gyrodactylus*, is largely due to differences in the length of ITS1. Longer sequences have approximately 200 bp of additional sequence at the 5' end of ITS1.

Molecular 'characters' such as long or short ITS can add information useful in construction of morphological or molecular phylogenies. Molecular phylogenetic analysis of *Gyrodactylus* within the phylum and genus has been carried out using small subunit and ITS rRNA sequences (Cunningham et al. 1995, Cable et al. 1999). Although over 11 000 platyhelminth rRNA sequences are now available on databases, the vast majority of these are small subunit rRNA gene sequences. This gene has proved useful in determining relations within the Platyhelminthes (Blair & Barker 1993, Kralova et al. 1997, Littlewood et al. 1998) but may not be sufficiently variable to resolve phylogenetic relations at the species level. In such cases, the ITS may prove more useful and the sequence determined in this study will provide additional data for such studies.

As *Gyrodactyloides bychowskii* shares the feature of a short ITS with some *Gyrodactylus* species and *Gyrdicotylus*, it may be that this is an ancestral feature and that the longer ITS has arisen in only some groups of *Gyrodactylus*. Further ITS sequences from other members of the Monogenea will provide the evidence to show whether this is the case.

Comparison of ITS from different *Gyrodactylus* species, both RFLP and sequence analysis indicated that ITS1 was more variable than ITS2 (Cunningham 1997, Cunningham et al. 2000). The results of this study indicate that the same is also true for comparison of *Gyrdicotylus*, *Gyrodactylus* and *Gyrodactyloides* ITS. Alignment of ITS1 sequences show that the 5' end contains most of the variation, with large gaps noticeable before the start of the shorter ITS. However, not all the length variation can be attributed to the 5' terminus of ITS1, and other insertion and deletion events are evident in both ITS1 and ITS2. The 3' end of the *Gyrodactylus* ITS1 secondary structure was predicted to resemble that of Digenea (Schulenburg et al. 1999, Cunningham et al. 2000), and the relative conservation of primary nucleotide sequence in this region leads us to assume that the *Gyrodactyloides* ITS1 may also form a series of 7 helices.

The form of the *Gyrodactyloides bychowskii* ITS2 secondary structure, reminiscent of a trestle, resembles the *Gyrodactylus salaris* ITS2 structure in overall layout, but differs in detail. The 4-domain model proposed by Morgan & Blair (1998) is clearly evident. Indeed, the correlation of this structure with that of the trematodes studied by Morgan & Blair is even more clear in *Gyrodactyloides* than *G. salaris* (Cunningham

et al. 2000). It is possible that the 'short' monogenean ITS forms a clear 4-domain structure while the 'long' ITS, with additional nucleotides, may form more complex arrangements. The additional sequence in *G. salaris* is composed of a repeated element that forms 2 distinct branches on 1 of the 4 domains (Cunningham et al. 2000).

The ITS sequences demonstrate significant differences between *Gyrodactyloides bychowskii* and *Gyrodactylus* species found on salmonid fish, represented by *G. truttae* in this case. However, the 5.8S rRNA gene is highly conserved within this family. Significant conservation was found in this gene when sequences from *Gyrodactylus* and digenean Platyhelminthes were compared (Cunningham et al. 2000), so this was not unexpected.

The sequence data from *Gyrodactyloides bychowskii* has shown the clear separation between this species and *Gyrodactylus*, as does the detailed morphology of the opisthaptor. The sequence information presented here may be used to develop objective, molecular methods of identifying this parasite and distinguishing it from *Gyrodactylus* species, using probes, RFLP or sequencing. In addition, the sequence should prove useful in construction of molecular phylogenies within the Monogenea and provides a basis for comparison of *G. bychowskii* from other locations.

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