Bio – 3910

Master's Thesis in Biology

GYRODACTYLUS MARINUS INFECTING THE GILL FILAMENTS OF FARMED AND WILD ATLANTIC COD IN NORWAY

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Acknowledgements

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"If your future is not in science, remember parasites anyway - they make extraordinary dinner conversation!"
Albert. O. Bush

Tromsø, May 2009

Pål Haugen

To my dear parents Sidsel Bangtvedt and Tarjei Haugen

Abstract

Cod aquaculture is a rapidly expanding industry in Norway and with an increase of biomass in mariculture the concern for diseases spread to and from farmed populations of cod is increasing. The genus *Gyrodactylus* Nordmann, 1832 are parasitic monogeneans infecting teleost fish in marine and fresh water habitats worldwide. To investigate a possible problematic species of this genus the gill filaments of Atlantic cod, *Gadus morhua* L. were examined for parasites. Both farmed and wild caught cod were sampled from Ålesund, Kvarøy, Brønnøysund and Øksfjord Norway. From these samples a total of 48 specimens of *Gyrodactylus* sp. were investigated through morphological and molecular techniques. The opisthaptoral hard parts from each individual were compared through morphology and measurements and all specimens were found to have a similar morphology. The ITS1 and ITS2 together with the 5.8S subunits from rDNA were compared between the specimens investigated and confirmed the observed morphological homogeneity All species were found to be *Gyrodactylus marinus* Bychowsky and Poljansky 1953. Furthermore untreated farmed cod have a higher prevalence of *G.marinus* than what is found in wild cod populations.

Key words Monogenea, *Gyrodactylus marinus*, Atlantic cod, *Gadus morhua*, mariculture

Table of content

Introduction

The Atlantic cod, its fisheries, aquaculture and diseases

The Atlantic cod (*Gadus morhua* L.) is one of the most important commercial fish species in the marine environment (Kurlansky, 1999). It has been an attractive commercial fish for almost 500 years but in several areas the Atlantic cod populations are now heavily overfished. Thus there has been a concern for how well sustainable fisheries management is working (Cook et al., 1997). The importance of the cod is demonstrated historically by the fact that cod wars have been fought over access to cod fisheries (Kurlansky, 1999). Although stocks have been declining and fishing quotas have been reduced, the cod fishery is still an important industry in Norway. In 2007 Norwegian fisheries caught 217 401 metric tonnes of cod in the Northeast Atlantic and the value of these fisheries was in the range of 3.6 billion NOK (Norwegian Directorate of Fisheries, 2008a). In recent years the cod farming industry in Norway has been expanding. In 2008 the production of farmed cod reached 13 500 tonnes, an increase of 25% compared to 2007. In the same year 20 million cod were placed in production. In 2008 this industry exported 6 200 tons of farmed cod to a value of approximately 219 million NOK. Mainly the export is going to the EU, with Denmark and France as the largest importers (Boxaspen et al., 2009).

In 2007 approximately 1.5 million dead fish were reported from cod aquaculture, but there are no statistics on what causes the death of these fish (Norwegian Directorate of Fisheries, 2008b). Although there are no statistics available on causes of mortality in cod farms, several diseases have been reported from the cod farming industry in Norway. Among the diseases reported are viral encephalopathy and retinopathy (VER) and bacterial infections with *Photobacterium* sp., *Vibrio* sp., *Aeromonas* sp., *Francisella* sp. and *Vibrio* sp. Not surprisingly several parasite species, such as *Gyrodactylus* sp. and *Ichthyobodo necator* are frequently reported from farmed fish (Norwegian Veterinary Institute, 2008).

Diseases of fish in mariculture are one of the main limiting factors for production (MacKenzie and Hemmingsen, 2003) and the most important environmental challenges posed by cod farming are dispersal of farmed fish and spreading of diseases from cod farms (Boxaspen et al., 2009).

The Atlantic cod harbours an especially rich parasitic fauna and several potentially problematic species for aquaculture are present in wild cod populations. One hundred and seven species of both metazoan and protozoan parasites have been recorded from Atlantic cod and there are numerous recordings of parasites only described to the genus level (Hemmingsen and MacKenzie, 2001). This rich parasitic fauna and the importance of cod fisheries makes the Atlantic cod an interesting subject for studying parasites, and the ecological impact of parasite faunas on host populations. With the increase of cod farming in Norway the diseases of cod becomes important not only in an ecological context, but could also have an economic impact.

The CODPAR project

The CODPAR project is a 3-year survey of the parasitic fauna of both farmed and wild cod. The aim of the project was to establish a baseline of parasites present in areas of intensive cod farming, and to record the intensities of the infections. In the project, all organs from a total of 343 cod were examined for the presence of parasites. Altogether 50 different species from all main parasitic groups were found and the preliminary results from the project show some clear patterns. Gastrointestinal helminths, such as *Anisakis simplex*, *Cucullanus cirratus* and *Echinorhynchus gadi,* were almost absent from farmed cod, while some of the ectoparasites showed a higher prevalence in farmed cod than in wild caught cod. Several of the parasitic species found in the project, e.g. ectoparasites such as *Ichthyobodo* sp., *Trichodina* sp. and *Gyrodactylus* sp., are potential pests if introduced to fish farms. One of the most interesting findings during the project was that the *Gyrodactylus* species on the gill filaments occurred in higher prevalence and intensity on farmed cod than on the wild caught cod (Heuch et al., 2007).

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The genus Gyrodactylus: taxonomy and systematics

The genus *Gyrodactylus* von Nordmann 1832 (Plathyhelminthes; Monogenea) consists of viviparous ectoparasitic species. In addition to viviparity they are both hermaphroditic and progenetic (Lester and Adams, 1974; Harris, 1983; for a summary of the reproduction of the gyrodactylids see also Bakke et al., 2007). The opisthaptor of *Gyrodactylus* consists of 16 marginal hooks and a pair of hamuli, or anchor bars, which are attached with both a ventral and a dorsal bar (Bakke et al., 2007).

There are over 400 valid species descriptions in the genus (Harris et al., 2004), but it has been suggested that the number of species could be as high as 20 000 based on the assumption of strict host specificity and the number of potential teleost hosts (Bakke et al., 2002). Malmberg (1970) divided the genus into six subgenera: *Gyrodactylus*, *Mesonephrotus*, *Paranephrotus*, *Metanephrotus*, *Neonephrotus* and *Limnonephrotus*. Traditionally the morphology of the excretory systems and that of the opisthaptor have been used as diagnostic features for species, species group and subgenus (Malmberg, 1964; Malmberg, 1970). Recent molecular studies have shown that there is a similarity between the taxonomy based on morphology of the opisthaptor and the sequences of the ITS segments from rDNA in the genus *Gyrodactylus*. However the excretory system is not recommended for use in phylogenetic research and the subgenera proposed by Malmberg have thus been challenged (Matejusová et al., 2003). The morphology of the opisthaptoral hard parts has been used in several studies, often combined with both molecular and statistical methods, to discriminate between species of *Gyrodactylus* (Cunningham et al., 2001; Shinn et al., 2001; Shinn et al., 2004; Garcia-Vasquez et al., 2007).

Molecular systematics

Molecular methods are widely used in taxonomy and phylogenetic research. Although there are several different methods, Schlötterer (2004) proposes that there are only three different classes of molecular markers: allozymes (variation in proteins), DNA sequence polymorphism and DNA repeat variation. For phylogenetic research within the genus *Gyrodactylus* several authors have applied rDNA analysis (Cunningham et al., 1995; Ziętara et al., 2000; Matejusová et al., 2001; Huyse and Volckaert, 2002; Ziętara et al.,

2002; Matejusová et al., 2003; Ziętara and Lumme, 2003). The rDNA consists of rRNA coding regions and spacer regions and is repeated tandemly. In eukaryotes the rDNA array is arranged with a non-transcribed spacer (NTS) between the copies, and each rDNA starts with an external transcribed spacer before the 18S coding region begins. Downstream of the 18S is the 5.8S and 28S coding regions where the internal transcribed spacers (ITS) are placed between 18S and 5.8S (ITS1) and 5.8S and 28S (ITS2) (Hillis and Dixon, 1991 and references therein). Due to differences in evolutionary mutation rate between the subunits within the rDNA, the regions are used for examining phylogenetic hypotheses on different levels of the taxonomic hierarchy. The 18S and 28S are utilized in studies with emphasis on ancient evolutionary events, whereas the more rapid evolving ITS segments are applied in studies focusing on closely related taxa. To distinguish between species or populations the variation between the spacer regions (ITS1 and ITS2) can be analyzed (Hillis and Dixon, 1991).

The ITS 1 and ITS 2 segments of rDNA have been established as molecular markers discriminating between species of *Gyrodactylus* (Ziętara and Lumme, 2003).

For species determination, redescriptions and phylogenic studies of gyrodactylids, the most common molecular method is to sequence ribosomal DNA (rDNA) (Cunningham et al., 1995; Cunningham et al., 2001; Cable et al., 2005; Garcia-Vasquez et al., 2007), although mitochondrial DNA has also been applied in some studies (Meinilä et al., 2004)

Gyrodactylus sp. infections on Atlantic cod

Six different species of *Gyrodactylus* have been reported from cod, and the different species are suggested to be organ specific (Malmberg, 1970). Infections of *Gyrodactylus* sp. have been found on the skin (especially fins), gill filaments, gill arches, pharynx and the preopercular sensory canal on cod (Malmberg, 1970, CODPAR unpublished results). The gill filaments of gadoid fish in the Barents Sea, the Norwegian Sea near Lofoten and from the Sea of Japan are known to be infected with *Gyrodactylus marinus* Bychowsky and Poljansky 1953. This species is reported from the hosts Atlantic cod, *Gadus morhua*, Pacific cod, *Gadus macrocephalus* Tilesius 1810 and Alaska pollock, *Theragra chalcogramma* (Pallas 1814) (Bychowsky and Poljansky, 1953).

In cod farms the densities are high and these conditions favour transmission of parasites with direct life cycles and may lead to high intensities of infection (Rhode, 1993). Even though *Gyrodactylus* parasites are common in both wild and farmed cod, reports of *Gyrodactylus* sp. causing death in cod aquaculture are few. *G. marinus* from the gill filaments of farmed cod has been reported as pathogenic from Tromsø (Svendsen, 1991). Recently farmed cod in Canada were also reported to have up to 1000 specimens of *Gyrodactylus* on the head (David Cone, pers. comm.). The higher prevalence in the farmed fish compared to the wild fish that was found in the CODPAR project, indicates that *Gyrodactylus* might become a big parasitic problem in the cod farming industry.

Preliminary results from sequencing of ITS1 and 2 from individual *Gyrodactylus* specimens recovered from different sites on the cod (gill filaments, gill arches, skin and fins and pharynx) indicate that different species infect different sites. As the *Gyrodactylus* species infecting the gill filaments seems to have the greatest potential for causing diseases in cod, the present study will focus on this species. The aim of the study will be to determine the species of *Gyrodactylus* found on the gill filaments and to describe or redescribe them by morphological and molecular methods. Furthermore, the prevalence of infection on the gill filaments on farmed and wild caught cod will be investigated to reveal patterns of infection.

Material and methods

Sampling

Cod examined were divided into 4 categories based on origin; wild caught fish from areas with cod aquaculture, farmed cod, wild caught cod placed in cages for ongrowth (in Øksfjord only) and migratory cod (in Øksfjord only). Cod were sampled and examined for parasites at 4 different locations along the coast of Norway over a 3-year period (see Table 1 and Figure 1). Fish from Ålesund (6^o 6` N, $62^o$ 27` E) in the western part of the country, Brønnøysund (12^o 13` N, $65^o$ 28` E) and Kvarøy (12^o 52` N, $66^o$ 32` E) in Nordland to Øksfjord (22^o 21` N, $70^o$ 13` E) in Finmark were sampled from both farmed and wild populations. The farmed cod were collected directly from the pens holding the fish whereas wild cod were caught in the vicinity of the cod farms. Wild caught cod was as far as possible selcected to fit the size of farmed cod. The cod were kept in tanks with inflow of seawater for no more than 3 days and the different samples were kept separated.

All groups of cod were examined for *Gyrodactylus* sp. infections on the gills, fins, pharynx and operculum with the aid of a dissecting microscope at approximately 25 times magnification. Gill filaments were placed in Petri dishes containing seawater and live worms were counted. In order to examine as many fish as possible, only the left side (when seeing the fish from the dorsal side) of the exterior of the fish was examined. When an infection was found gyrodactylids were counted and either a subsample or the entire organ was stored in 96% ethanol for further examination in the laboratory.

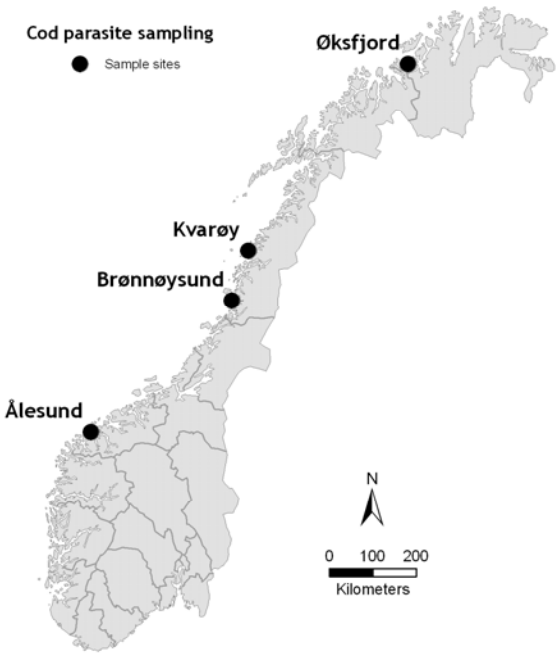

Figure 1 Map of Norway showing the four locations were cod were sampled in the CODPAR project.

Table 1 An overview of the localities and seasons for cod sampling in the CODPAR project. Note that farmed cod from Øksfjord were not hatchery reared, but wild caught cod placed in pens for ongrowth, denoted *.

Locality	Season	Farmed Cod	Wild cod	Migratory cod
Øksfjord	Spring 2006	18*	18	18
	Autumn 2006	17*	17	
Kvarøy	Spring 2007	20	20	
	Autumn 2007	20	12	
Brønnøysund	Autumn 2006	20	14	
	Spring 2007	20	15	
	Spring 2008	20	20	
Ålesund	Autumn 2007	16	18	
	Spring 2008	20	20	
Total number of cod		171	154	18

Preparation of specimens

To sample individuals of *Gyrodactylus* sp. ethanol-stored gill filaments from cod with *Gyrodactylus* sp. infections were studied under a stereo microscope (20 – 40X magnification) in the laboratory and individuals were removed from the gills for further analysis. After removing the individuals from the filaments, the opisthaptor was severed from the rest of the body with the aid of a scalpel. For subsequent PCR analysis and DNA sequencing, the body of the *Gyrodactylus* sp. was stored on 96% ethanol in individually marked Eppendorf tubes, while the opisthaptor was transferred to a coverslip for further analysis.

To remove the soft tissue surrounding the sclerites, a digestive fluid consisting of a buffer and proteinase K was added. (Harris et al., 1999). After the soft tissue was digested, glycerol was added to stop the digestive process, and a glass slide was carefully mounted on top of the coverslip. The ends were sealed using nail varnish and the specimen was studied under a microscope.

A total of 42 *Gyrodactylus* specimens were used to describe the morphological variation found between individuals from both farmed cod (28 specimens) and from wild cod (14 specimens) from one of the samples in Ålesund. In addition, 6 specimens of *Gyrodactylus* sp. from Kvarøy (n=2), Brønnøysund (n=3) and Øksfjord (n=1) were included to investigate possible similarities and differences in molecular and morphological traits.

Morphology and morphometrics

The marginal hooks, hamuli and ventral bars from the opisthaptor of the gyrodactylids were studied under an axiovert 200M Carl Zeiss microscope with a 100X (oil immersion) ocular using bright field, and pictures were taken with a mounted AxioCam MRm (Carl Zeiss) camera. Due to a high preparation depth relative to focus depth, images were taken as Z-stacs to be able to have the entire hook in focus for measuring.

Due to the addition of Canada balm on some of the type material it was recommended to use the original drawings as type material when comparing the hooks from different

gyrodactylids (Göran Malmberg pers. comm.). All hooks were individually compared to the original drawings presented by Malmberg (1970) and Bychowsky and Poljansky (1953) in Corel PHOTO-PAINT 12.0.0.458 (Corel Corporation, 2003). To determine the species of each specimen, only specimens with marginal hook, ventral bar and hamuli present were used in the analysis. Due to the lack of marginal hooks in the original description from Bychowsky and Poljansky (1953), only the marginal hooks description from Malmberg (1970) was used for comparisons.

The measuring was done with the aid of AxioVision Rel. 4.6.3.0 (Carl Zeiss Imaging Solutions, 2006-2008). The measurements on the hamuli are shown in Figure 2 and are based on those by Shinn et al. (2004), with some modifications due to the lack of the ventral bar articulation point on the hamuli on the species of *Gyrodactylus* used in this study. The remaining measurements used on the sclerites of the opisthaptor are from Shinn et al. (2004), with the exception of marginal hook instep due to lack of resolution in the obtained pictures. Table 2 shows the measurements made on *G. marinus* from cod (present study), *T. chalcogramma* (Bychowsky and Poljansky and Malmberg, taken from Malmberg (1970)) and *G. aeglefini* from haddock, *Melanogrammus aeglefinus* (L.) (Malmberg, 1970). These species were found to be the most similar gyrodactylids to the specimens used in this study and morphology and morphometrics were compared to clarify species status.

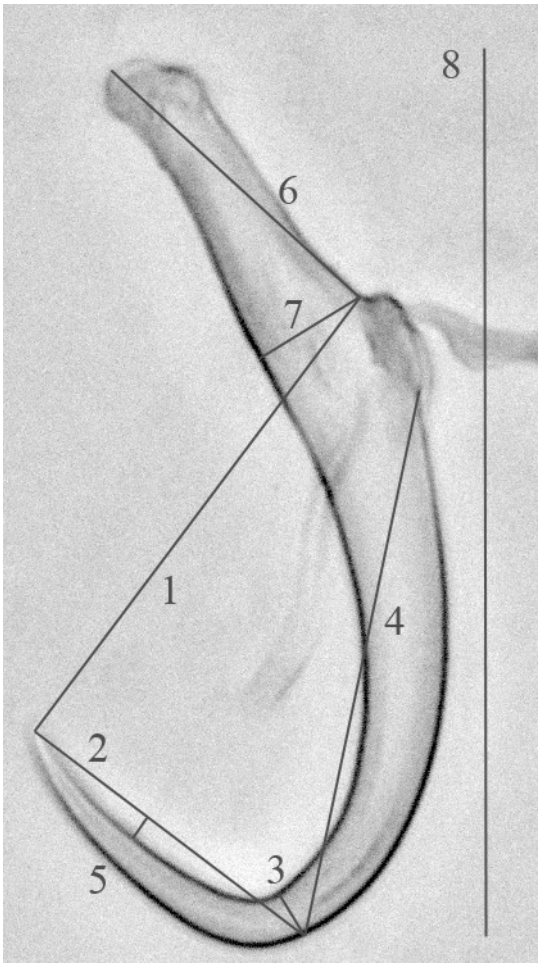

Figure 2 The measurements taken on the hamuli in this study**. 1** Hamulus Aperture Distance (HAD): the length between the hamulus point tip and the upper edge of the dorsal bar attachment point. **2** Hamulus Point Length (HPL): Measures the length of a line placed 90 degrees from measurement number 1 at the hamulus tip point to the edge of the hamulus. **3** Hamulus Distal Shaft Width (HDSW): the width of the hamulus from the point where line 2 ends at the hamuli. **4** Hamulus Shaft Length (HSL): the length between the point where line 2 ends and the lower end of the dorsal bar attachment point. **5** Hamulus Inner Curve Length (HICL): The length of a line placed 90 degrees on line 1 to the most distant part of the hamulus. **6** Hamulus root length (HRL): The length from the upper part of the dorsal bar attachment point to the most distant part of the hamulus. **7** Hamulus proximal shaft width (HPSW): the width of the hamulus measured at the upper part of the dorsal bar attachment point. **8** Hamulus total length (HTL): the length from the two most distant parts of the hamulus. Measurements taken from Shinn et al.(2004) but altered to fit the morphology of the opisthaptors measured. The angle measurements used by Shinn et al. (2004)are removed from the illustration since these measurements are recommended to be removed when measuring the hamuli from this species of *Gyrodactylus*.

Molecular analysis

Prior to extraction, the Eppendorf tubes containing the bodies were quickly centrifuged and the ethanol was removed carefully either by pipetting or by evaporation before proceeding with extraction. DNA was extracted from the excised bodies of *Gyrodactylus* sp. individuals using the QiAmp DNA® minikit (QUIAGEN) following the manufacturers' protocol for tissue extraction.

The rDNA fragment consisting of the 3`end of the 18S subunit, the internal transcribed spacer 1 (ITS1), 5.8S and the internal transcribed spacer 2 (ITS2) and the 5`end of the 28S subunit was amplified by PCR using the primers ITS1A (5`-GTAACAAGGTTTCCGTAGGTG-3`) and ITS2 (5`-TCCTCCGCTTAGTGATA-3`) (Matejusová et al., 2001). The PCR reaction was performed with PuReTaq Ready-To-Go™ PCR beads (GE Healthcare) in 0.2 ml tubes. The PCR beads contained PuReTaq polymerase (approximately 2.5 units), 200 µM dNTP and a reaction buffer (Tris-HCL pH 9.0, KCl and $MgCl_2$). In addition to the beads the solution contained 3 µl DNA template, 1 µl of each primer, 10pmol/µl and 20 µl of milli-Q-water. The samples were then placed in a GeneAmp PCR System 9700 (Applied Biosystems) and was run as follows: 4 min at 95°C, 35 cycles of 1 min at 95°C, 1 min at 55 °C and 2 min at 72°C.

PCR-products were purified using a Macherey-Nagel NucleoSpin® Extract II and subsequently sequenced 10µl reactions on a MEGABACE 1000 (GE Healthcare) using DyeET-terminator mix (GE-Healthcare). Both PCR primers and the internal primers ITS1R, ITS2F, ITS18R and in some instances the ITS28F were used for sequencing of the full ITS fragment (Ziętara and Lumme, 2003). Sequences were proof read in VectorNTI 10.3 (Invitrogen) and submitted to a GenBank BlastN search (http://www.ncbi.nlm.nih.gov/) for comparison with sequences from known species.

Statistical analysis

A total of 343 cod screened for *Gyrodactylus* sp. from the gill filaments were grouped according to infective status (infected / not infected) and capture status (farmed / wild caught). For each locality investigated a 2x2 contingency table was created and a chi-square test was performed to test if the prevalences were independent of capture status. All data processing was carried out using the R sowftware version 2.2.1 (R Development Core Team, 2008).

Results

Morphology and morphometrics

All 42 specimens investigated were found to have a very high similarity with the morphology of *Gyrodactylus marinus* as described by Bychowsky and Poljansky (1953). The material in the present study is thus used to redescribe *G. marinus* using morphology, morphometrics and molecular analyses. Although similar to the sclerites described by Malmberg, the hooks do seem to fit better to the original description. The most notable differences were in the toe of the marginal hook, which seemed broader in the investigated specimens than in the description from Malmberg, the breadth of the membrane of the ventral bar and the ventral bar total width.

Comparable measurements between *G. marinus* forms and *G. aeglefini* are presented in Table 2; from these measurements *G. marinus* measured by Malmberg are closest to the measurements done in this study. Figure 3 shows differences in morphology between the same species.

Table 2 Measurements of gyrodactylids resembling the species found on cod during fieldwork. The data are collected from Table 3 in this study and Table 2 in Malmberg (1970).

	Sample	**Present study**	***G. marinus* (Malmberg)**	***G. aeglefini* (Malmberg)**	***G. marinus* (Bychowsky and Poljansky)**	
	Measurement	**Mean**	**Mean**	**Mean**	**Min**	**Max**
Hamulus	Hamulus Point Length	28.6	24.8	24.2	24.0	29.0
	Hamulus Shaft Length	45.1	49.4	47.1	44.0	50.0
	Hamulus root length	24.1	26.7	22.8	19.0	26.0
	Hamulus total length	68.9	67.9	59.6	62.0	70.0
Ventral bar	Ventral bar total width	32.6	31.4	25.2	27.0	31.0
	Ventral bar total length	41.7	43.3	34.8		
	Ventral bar median length	9.0	5.9	6.0		
	Ventral bar process length	3.4	4.6	3.8		
	Ventral bar membrane length	26.7	26.8	22.1	26.0	32.0
Marginal hook	Marginal hook sickle length	8.5	9.0	8.0		
	Marginal hook sickle proximal width	4.9	4.5	4.5		
	Marginal hook sickle distal width	3.8	3.9	3.5		

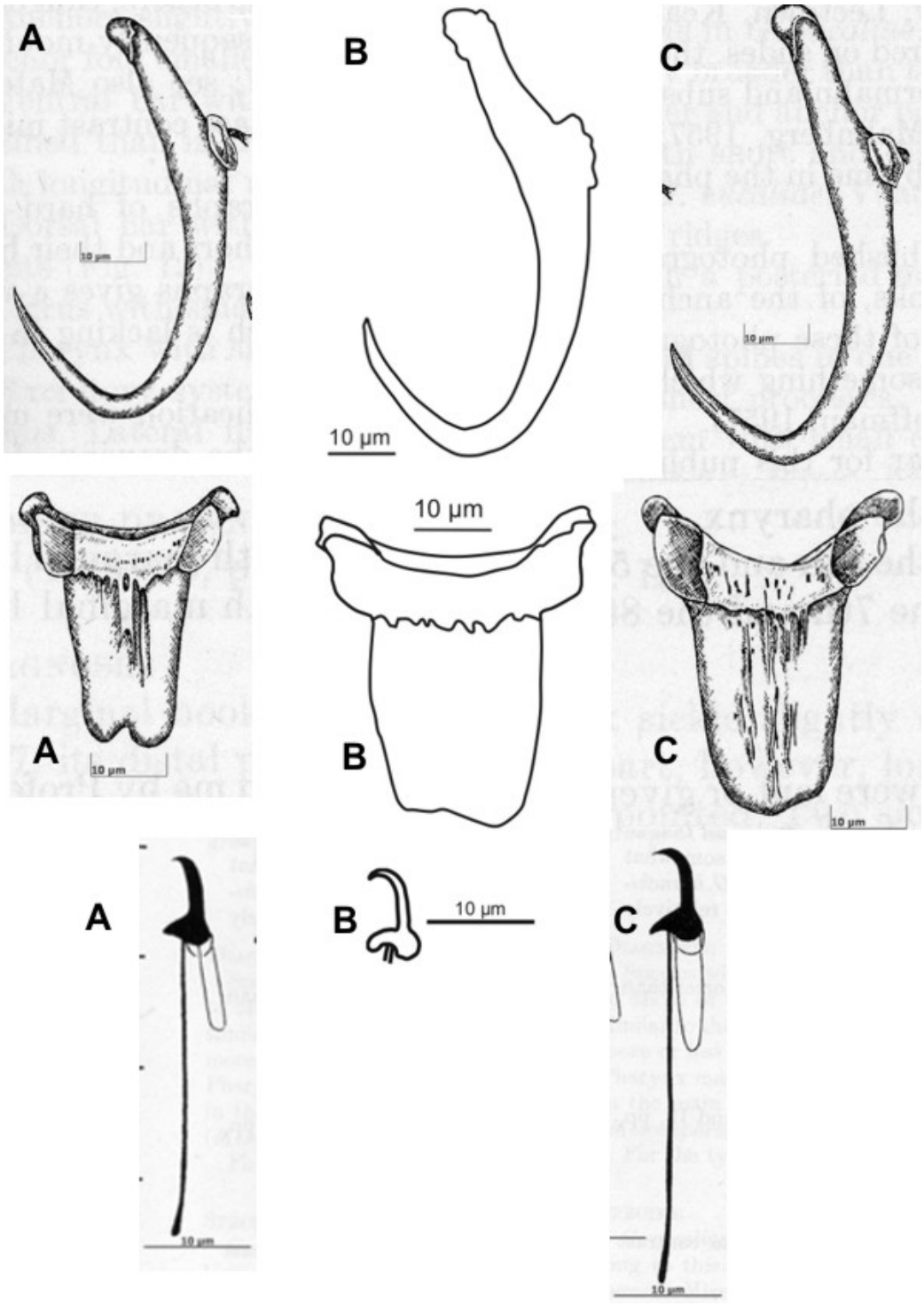

Figure 3 Drawings on the different opisthaptor hard parts from **A** *G. aeglefini*, **B** *G. marinus* from the present study and **C** *G. marinus* from *T. chalcogramma*. Figures **A** and **C** are taken from Figure 12 in Malmberg (1970) with permission from the author.

There are however some notable individual differences between the specimens investigated. Due to the small size of the sclerites, it was difficult to obtain specimens in which the sclerites were entirely flattened between the coverslip and the glass slide. Figure 4 shows two individuals with identical ITS segments showing considerable differences in the hamuli due to different spatial orientation.

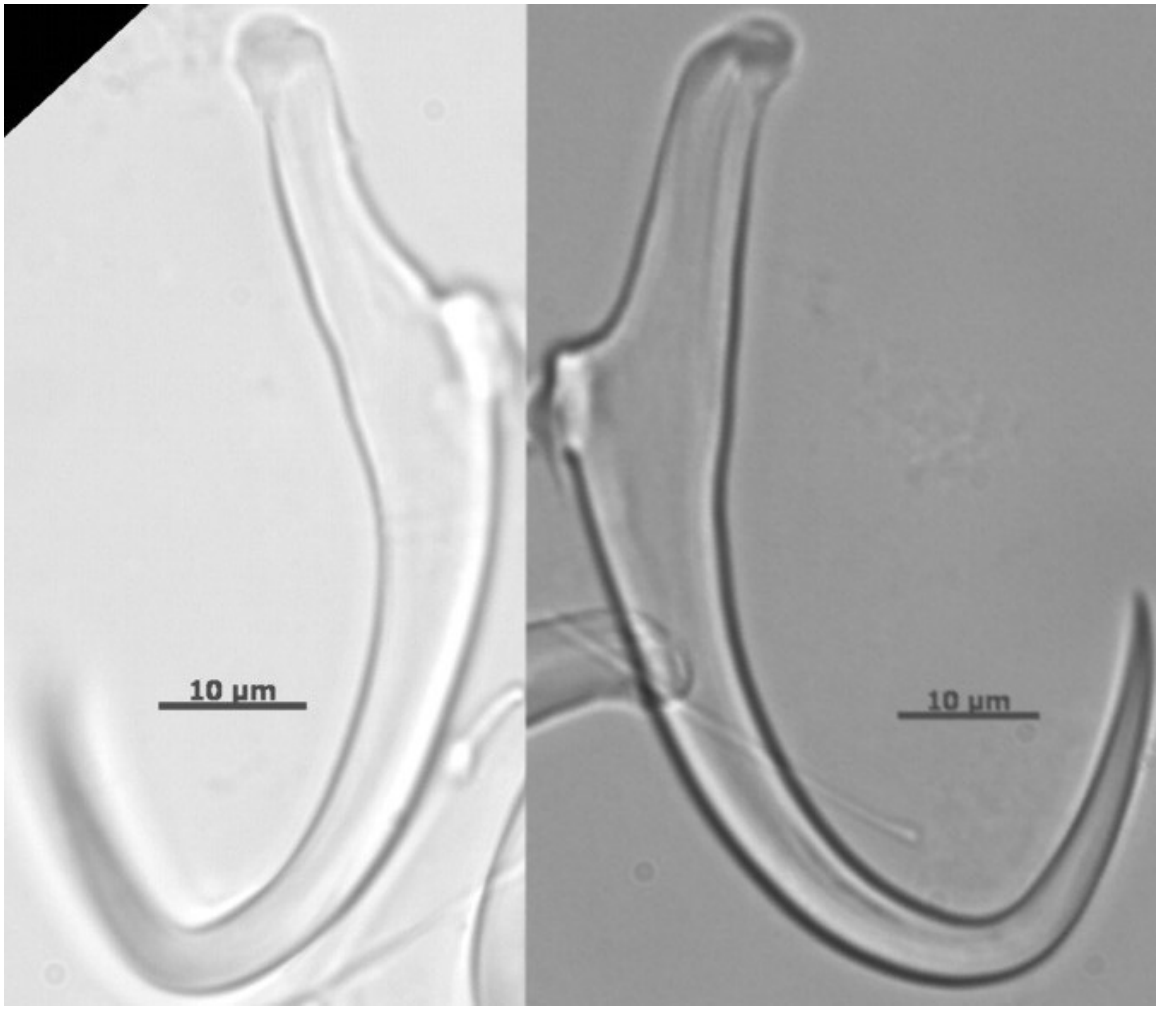

Figure 4 Pictures of to hamuli hooks taken from two different individuals of *Gyrodactylus marinus*. Left hand picture shows a flattened specimen, right hand side shows a specimen not flattened. Pictures are taken with a 100X oil immersion objective and in a single focus plane.

Redescription

Gyrodactylus marinus Bychowsky and Poljansky 1953

Class Monogenea Carus, 1863
Subclass Polyonchoinea Bychowsky, 1937
Order Gyrodactylidea Bychowsky, 1937
Family Gyrodactylidae Van Beneden et Hesse, 1863

Type host *Gadus morhua* L. This species of *Gyrodactylus* is also described from Pacific cod, *Gadus macrocephalus* and Alaska pollock *Theragra chalcogramma* (Pallas 1814) by Bychowsky and Poljansky (1953) , see Malmberg (1970) and discussion for comments on the species infecting different hosts of the family Gadidae.

Habitat Gill filaments

Material examined Forty six individuals from farmed and wild caught cod from Ålesund were compared morphologically and through rDNA. In addition, specimens from Brønnøysund (n=3), Kvarøy (n=2) and Øksfjord (n=1) were confirmed, through molecular and morphological measures, to belong to the same species.

Molecular characterization

The sequences obtained from the specimens used in this study showed no variance in the fragment of the 3' end from the 18S subunit, ITS1 (361 bp), 5.8S gene (157 bp), ITS2 (405 bp) and a short fragment of the 5' end of the 28S subunit.

Description

For details of the measurement of the haptoral hard parts see Table 3.
Total length of hamuli 68.86 ± 2.64 μm; hamulus shaft length 45.07 ± 1.90 μm; proximal shaft width 9.76 ± 0.77 μm; hamulus aperature distance 42.96 ± 2.13 μm. Inner curve of hamuli is large and gives the hook an open appearance. Shaft of hamuli slightly curved towards hamuli tip and ventral bar articulation point not present at hamuli shaft front

side. Ventral bar wide 32.59 ± 1.40 µm; ventral bar processes small, 3.37 ± 0.94 µm, membrane of ventral bar even in width, and with a notch in the lower part of the membrane.

Marginal hook sickle length 8.54 ± 0.49 µm; shaft of marginal hook straight and 30.34 ± 1.89 µm long; marginal hook toe rounded at end with end pointing downwards.

For drawings of the haptoral hard parts see Figure 5, photographs of the hooks are presented in Figure 6.

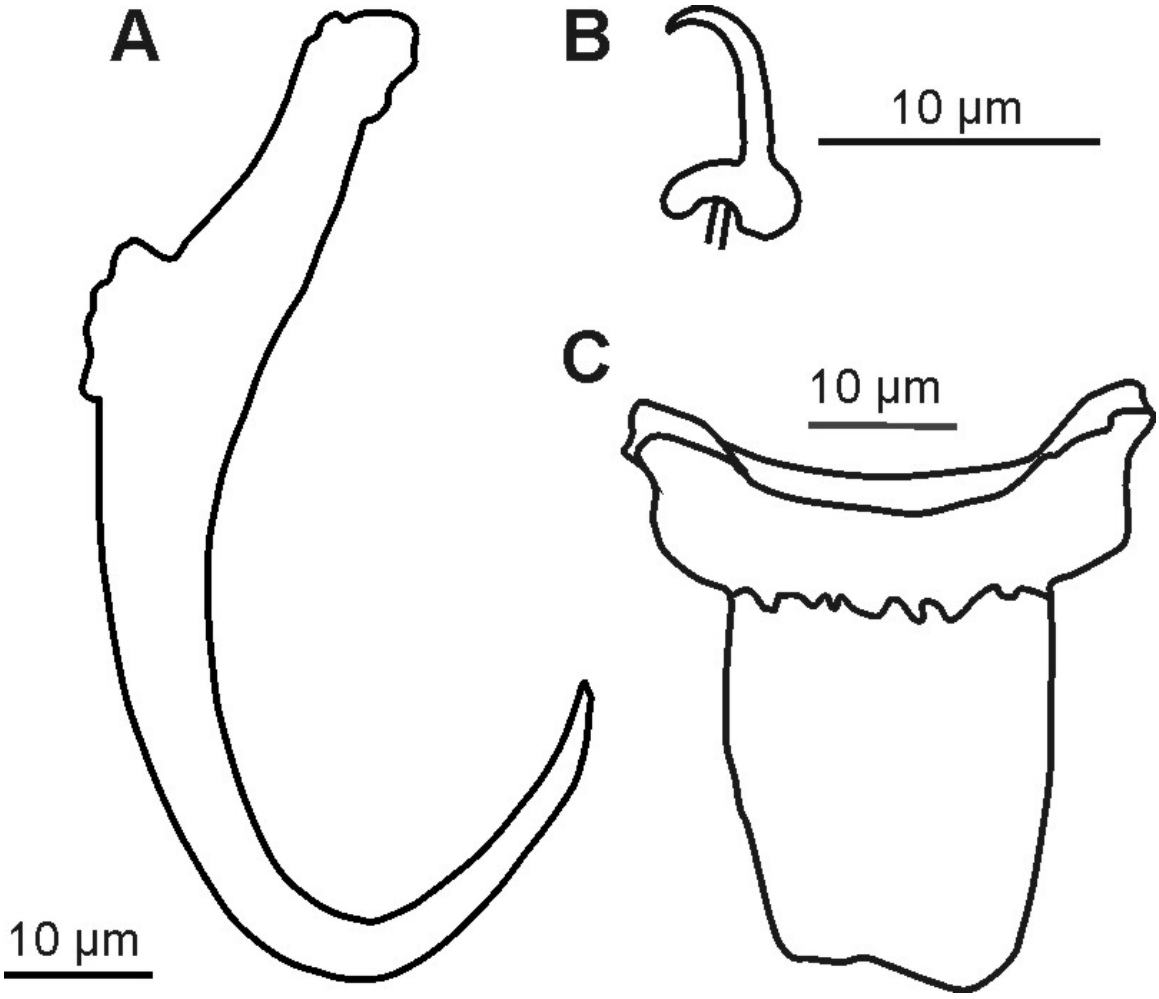

Figure 5 Drawings of the opisthaptoral hard parts: **A** hamuli, **B** marginal hook and **C** ventral bar of *Gyrodactylus marinus* from cod. The drawings were obtained through Corel draw.

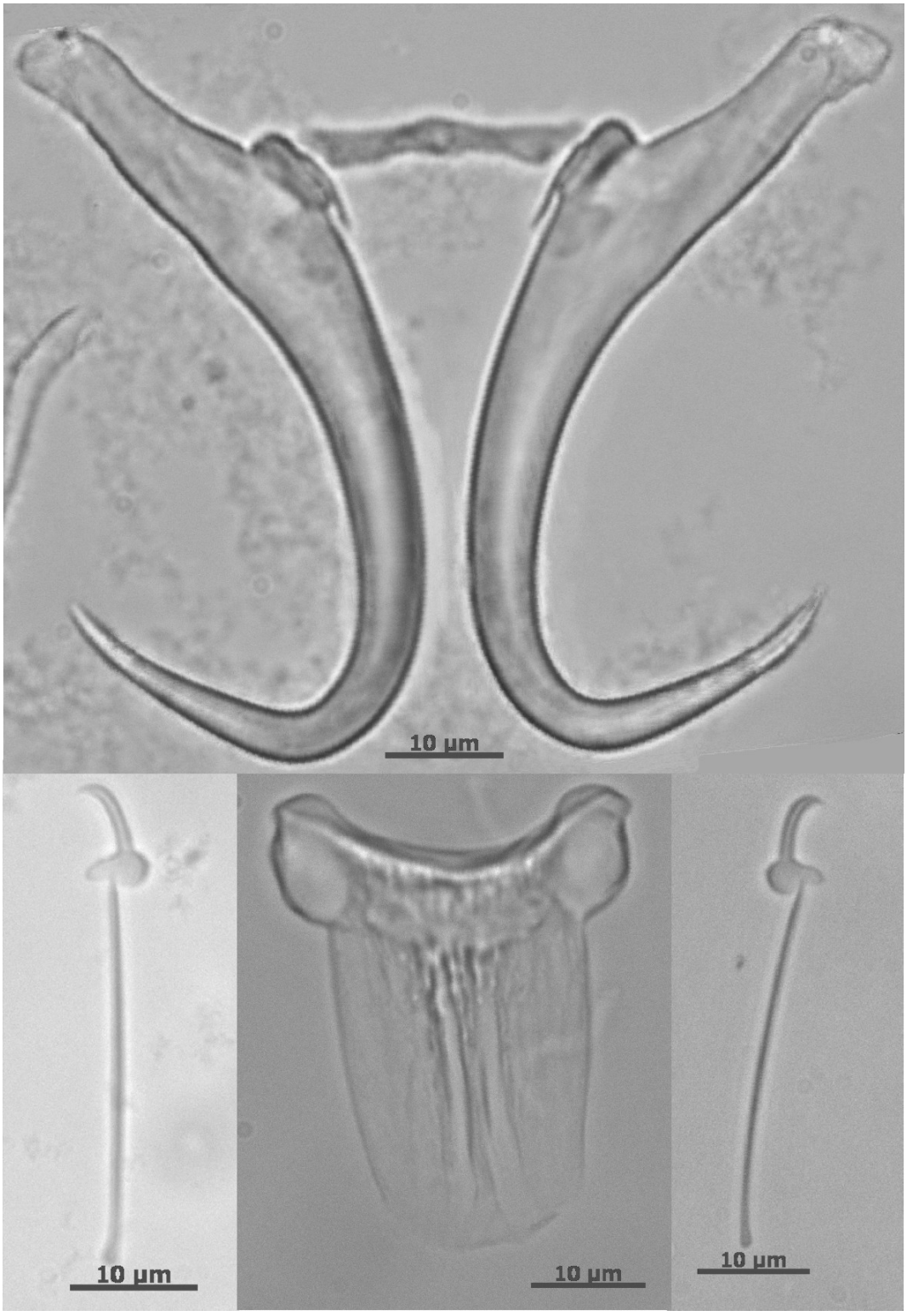

Figure 6 The opisthaptoral hard parts of *Gyrodactylus marinus* from cod (*Gadus morhua*) from a specimen from Ålesund Norway. The soft tissue of the opisthaptor is removed with a digestive fluid and the hard parts are in glycerol. All photographs were with a 100X oil immersion objective, top photograph of the hamuli is an extended focus picture computed from Z-stack (10 pictures).

Measurements

Measurements from the 42 specimens of *G. marinus* are presented in Table 3 with range, mean and the standard deviation of the mean.

Due to the relatively high variability and difficulties in correctly measuring the specimens investigated, the following measurements have been removed, and will not be used further in this study: Hamulus aperture angle, Hamulus point curve angle, Inner hamulus aperture angle, Hamulus inner curve length, Marginal hook total length and Marginal hook shaft length.

Table 3 The range and the mean with standard deviation of the measurements done on 42 specimens of *Gyrodactylus marinus* taken from farmed and wild cod in Ålesund

	Measurement	Unit	Min	Max	Mean	STD
Hamuli	Hamulus Aperture Distance	µm	36.32	48.70	42.96	2.13
	Hamulus Point Length	µm	25.83	30.96	28.61	1.19
	Hamulus Distal Shaft Width	µm	4.13	5.25	4.63	0.77
	Hamulus Shaft Length	µm	38.19	49.71	45.07	1.90
	Hamulus Inner Curve Length	µm	0.72	5.06	2.44	0.98
	Hamulus aperture angle	Deg	52.95	62.70	56.42	1.73
	Hamulus point curve angle	Deg	5.81	34.03	15.29	5.43
	Inner hamulus aperture angle	Deg	52.95	73.79	63.98	3.93
	Hamulus root length	µm	17.95	28.10	24.07	2.31
	Hamulus total length	µm	63.08	75.01	68.86	2.64
	Hamulus proximal shaft width	µm	8.15	11.22	9.76	0.77
Ventral bar	Ventral bar total width	µm	29.15	35.86	32.59	1.40
	Ventral bar total length	µm	37.35	46.57	41.71	2.28
	Ventral bar process-to-mid length	µm	2.06	8.90	6.16	1.60
	Ventral bar median length	µm	7.10	10.77	9.04	0.98
	Ventral bar process length	µm	1.67	5.21	3.37	0.94
	Ventral bar membrane length	µm	22.96	37.67	26.65	2.27
Marginal hook	Marginal hook total length	µm	33.48	42.18	37.88	2.03
	Marginal hook shaft length	µm	26.19	34.51	30.34	1.89
	Marginal hook sickle length	µm	7.29	9.68	8.54	0.49
	Marginal hook sickle proximal width	µm	3.93	6.06	4.91	0.36
	Marginal hook toe length	µm	1.29	2.52	1.89	0.24
	Marginal hook sickle distal width	µm	2.45	5.74	3.76	0.73
	Marginal hook aperture	µm	6.10	7.70	7.12	0.36

Molecular analyses

The amplified rDNA sequence was approximately 1000 bp long, and contained a short fragment of the 3' end from the 18S subunit, ITS1 (361 bp), 5.8S gene (157 bp), ITS2 (405 bp) and a short fragment of the 5' end of the 28S subunit.

The different subunit was submitted to a BLASTN search and the three most similar species for each subunit was noted. The 5.8S subunit was found to be identical to the same subunit in the species *G. hrabei* and *G. harengi* (parasitizing bullhead, *Cottus gobio* (L.) and Atlantic herring, *Clupea harengus* (L.)) The ITS2 segment from *G. pterygialis* (parasitizing saithe, *Pollachius virens* (L.) and Atlantic cod) had a similarity of 399 out of 405 bp with 2 gaps to the ITS 2 of *G. marinus*. The same species *G. pterygialis* also showed a high similarity in the ITS1 segment (336/341 bp).

Even if *G. pterygialis* has sequences with similarities to the *G. marinus* specimens, they are not used for morphological comparisons. The morphology of *G. pterygialis* is too different from the investigated specimens to be considered in morphological species determination (for detailed drawings see Hodneland and Nilsen, 1994).

Comparison of Gyrodactylus marinus specimens from different localities

Gyrodactylus specimens from Øksfjord (n=1), Kvarøy (n=2) and Brønnøysund (n=3), all taken from the gill filaments, were analysed both morphologically and through rDNA analyses. These specimens showed high similarities with the *G. marinus* found on gill filaments from cod sampled in Ålesund. The rDNA analysis showed that these individuals are the same species as the individuals described from Ålesund.

The measurements of these specimens are shown relative to the measurements from Ålesund in Figures 7 and 8. The measurements are divided between the two figures according to size.

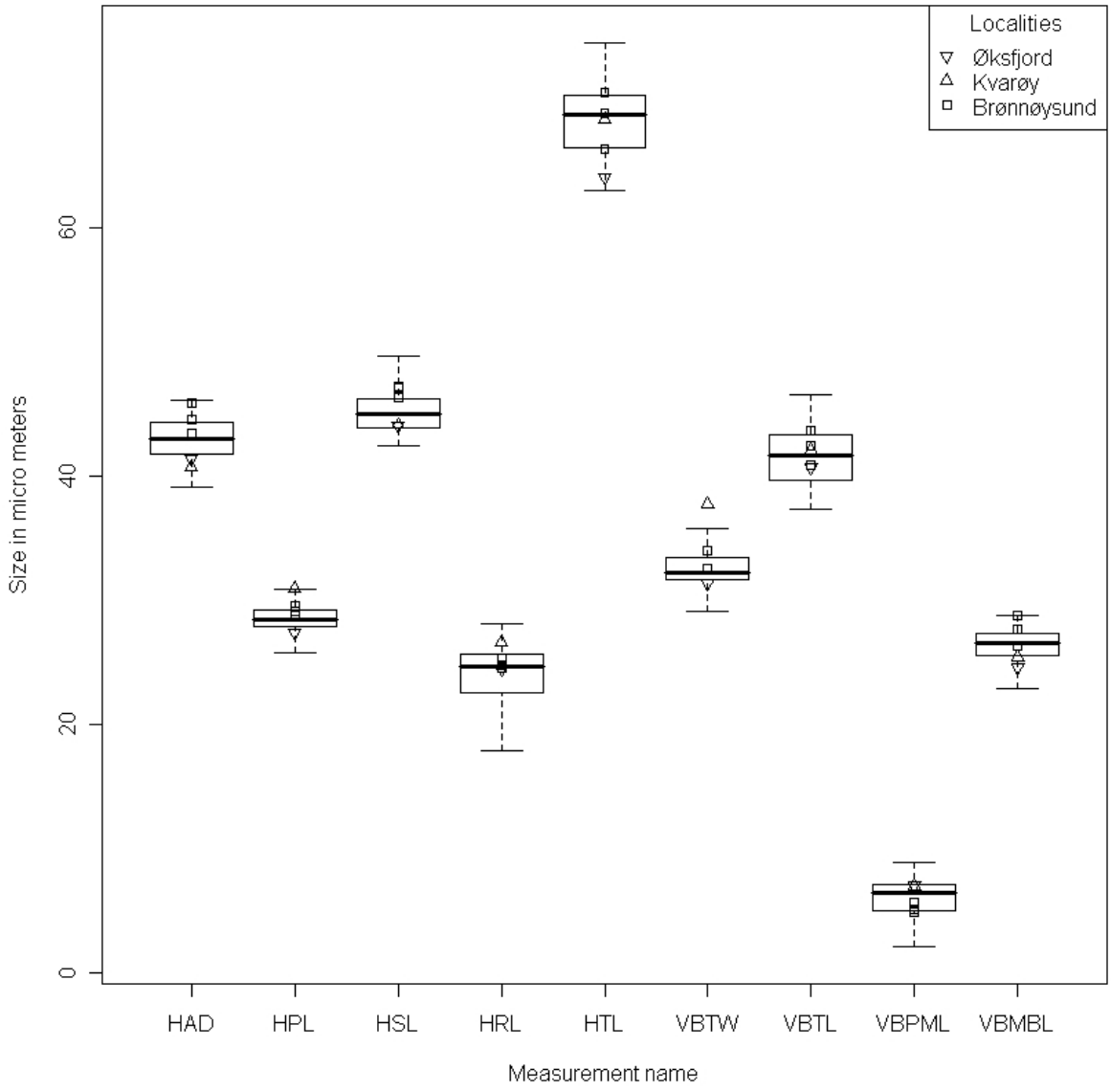

Figure 7 A boxplot showing the range of the values of measurements from the sclerites of *Gyrodactylus marinus* from Ålesund. Additional sites from the Norwegian coast are added as single points in the figure to show their values relative to the population used to redescribe *Gyrodactylus marinus*.

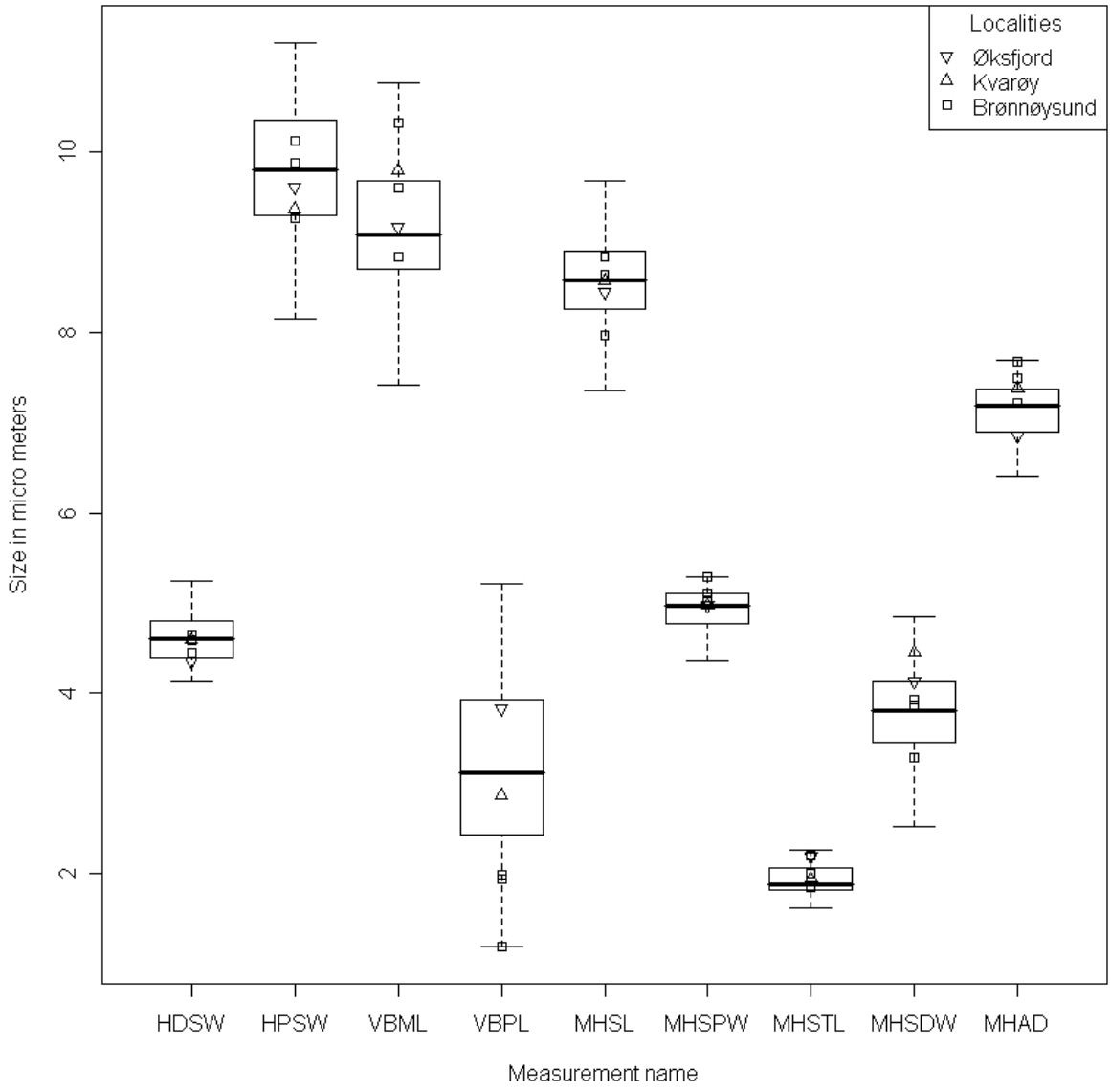

Figure 8 A boxplot showing the range of the values of measurements from the sclerites of *Gyrodactylus marinus* from Ålesund. Additional sites from the Norwegian coast are added as single points in the figure to show their values relative to the population used to redescribe *Gyrodactylus marinus*.

Distribution of Gyrodactylus sp. between the groups of Atlantic cod

An analysis of prevalence data from the CODPAR project was done to investigate possible relationships between prevalence and the status of the cod. The data were transformed to proportions and a chi-square test was performed with the null hypothesis being no difference in prevalence between the cod groups.

Due to the small number of cod in each group, the best analysis that could be performed on the data was a comparison between farmed fish and wild caught fish with respect to prevalence, the results of which are presented in Table 4. None of the expected values in the tests were below 5 and thus a chi-square distribution was assumed to fit the data.

There is however some notable differences between the sites prior to analysis. The farmed fish caught in Øksfjord were not hatchery reared fish, but wild caught fish placed in pens for further ongrowth. One could expect that these fish would have both a different parasitic fauna prior to the farming period and that their genetic variation would be higher than fish from hatcheries.

No farmed fish caught in Kvarøy were found to be infected with gyrodactylids on their gill filaments, which would indicate that prior to investigation these fish either had not been exposed to, or had been treated against, gyrodactylids. A Chi-square test between the farmed and wild caught cod found in Kvarøy would thus be of little interest since there is a clear difference between the groups.

Localities other than Kvarøy show that farmed cod had a higher prevalence of *Gyrodactylus* sp. on their gill filaments than that found on wild caught cod in the same areas.

Table 4 Summary of Atlantic cod, *Gadus morhua* L. divided into groups of wild caught and farmed cod for each locality in the CODPAR project, showing the infection status by *Gyrodactylus* sp. on the gill filaments. Chi-squared test results are presented for each group.

Locality		Infected	Uninfected	χ-squared, df=1	p-value	Prevalence	N
Øksfjord	Wild caught	7	29			0.19	36
	Farmed	32	3			0.91	35
				37.14	<0.001		
Kvarøy	Wild caught	13	19			0.41	32
	Farmed	0	40			0	40
				19.83	<0.001		
Brønnøysund	Wild caught	21	28			0.43	49
	Farmed	44	16			0.73	60
				10.41	0.0013		
Ålesund	Wild caught	21	17			0.55	38
	Farmed	34	2	14.87	<0.001	0.94	36

Discussion

All specimens of *Gyrodactylus* found on the gill filaments of cod were morphologically similar to *G. marinus* (Bychowsky and Poljansky, 1953) and showed a high similarity to the *G. marinus* specimens described by Malmberg (1970). The analysis of ITS1 and ITS2 from rDNA shows that the specimens investigated have little or no variation in these non-coding regions. This leads to the conclusion that all specimens investigated are the same species, *Gyrodactylus marinus.*

Although similar to the *G. marinus* described by Malmberg (1970), the differences in the marginal hook toe and the ventral bar membrane strongly indicate that the *G. marinus* reported from cod and Alaska pollock are not the same species. As proposed by Malmberg (1970) and discussed by Bychowsky and Poljansky (1953), these species are highly similar but show some variation in both morphology and infective patterns. These parasites utilize different hosts and thus show partly a divergent geographical distribution. Bychowsky and Poljansky (1953) reported that the different forms of *G. marinus* show different intensities of infections between localities. It might be that the three different forms of *G. marinus* described from the gill filaments of gadoid fish are in fact three different species of *Gyrodactylus*. On the other hand, studies made on *Gyrodactylus salaris* and *G. thymalli* show that differentiation between hosts and infective patterns do not necessarily mean that these parasites are two different species (Hansen, 2006).

Due to developments in microscopic equipment and computer software, a morphological study of the hard parts from the opisthaptor revealed morphological traits that, due to lower resolution, have not been observed in previous studies. Such a study would require several individuals of *Gyrodactylus* sp. of all three potential hosts as study organisms. The morphological studies should be accompanied by a comparison of the ITS1 and ITS 2 segments from rDNA. If these markers are not sufficient to explain morphological variations, mtDNA could give the additional information needed for conclusions to be drawn between the different *G. marinus* forms.

Similarity to other species

The general shape and size of the haptoral hard parts of *G. marinus* makes this species relatively simple to distinguish from other species of *Gyrodactylus* (Figure 5 and 6). The large inner curve length makes this species very different from *Gyrodactylus* sp. described from the skin of cod (*Gyrodactylus callariatis*, Malmberg 1957).
Closely related species and species infecting the same habitat could however resemble *G. marinus* in shape. *G. aeglefini*, which infects haddock, was originally described as *G. marinus aeglefini* (Bychowsky and Poljansky, 1953) but later elevated to species status by Malmberg (1970). This species resembles *G. marinus* in the shape of the hamuli, but is distinguished from *G. marinus* by the size of the hamuli and the shapes of the ventral bar and marginal hooks. Compared to other *Gyrodactylus spp.* found on cod, *G. marinus* is easily distinguished by the shape and size of the haptoral hard parts.
The present redescription of *G. marinus* is similar too, but not identical to the description of *G. marinus* from *T. chalcogramma* done by Malmberg (Malmberg, 1970).

Morphometrics

Even though genetic analyses of both rDNA and mtDNA are powerful tools for diagnostic investigations, morphological and morphometric studies of the sclerites of *Gyrodactylus* remain important tools for determining species. It is obviously necessary to analyze morphology when investigating species that are either new to science or species that have not yet been described with molecular techniques. Table 3 presents the range of the different measurements done on the opisthaptoral hard parts of *G. marinus*. The table is constructed on the basis of tables presented by Malmberg (1970) and Shinn et al. (2004). The modifications made on the measurements done by Shinn et al. (2004) are done to fit pictures of *Gyrodactylus* without the ventral bar articulation point. Although this attachment point is not found in all photographs, some specimens of *G. marinus* show a similar point of attachment, but the location of this point is different from species such as *G. salaris* and *G. callariatis,* among others. A SEM study on the sclerites of *G. marinus* is needed to reveal the exact location of this attachment point on this species.

Because an entirely flattened specimen is difficult to obtain, some of the measurements presented in Table 3 are not recommended for use in further studies. These measurements are removed partially due to the angle of the hooks, and partially due to the fixation technique. This becomes particularly apparent on the hamuli and on the marginal hooks. The hamuli could often be found with the hamuli-point facing either away or towards the objective and thus interfering with both focus depth and the measurements of hamulus aperture angles in addition to inner curve length. The marginal hooks represented a smaller problem since they are more numerous and thus the chance of finding a properly flattened hook in a specimen was large. Even so, the marginal hook shaft represents a challenge since they tend to bend easily with the fixation technique used. With respect to measurements, z-stack images can easily correct for loss of focus, but the problems with differences in angles are not solved through the measurement software used in this study. This type of variation between specimens makes comparisons between hamuli for species determination difficult and underlines Malmberg's point of using marginal hooks, ventral bar and then hamuli as the species formula (Malmberg, 1970).

When organs infected with *Gyrodactylus* sp. are stored in ethanol, the marginal hook shaft has a tendency to bend in such a manner that measurements are no longer accurate.

There are reported differences in measurements of *Gyrodactylus* spp. hard parts due to both temperature and preparation techniques (Malmberg, 1957; Malmberg, 1970; Mo, 1991; Appleby, 1996; Galli et al., 2007). When comparing measurements of *Gyrodactylus*, such as those presented in Table 2 and 3, one should always be aware of possible differences due to these factors.

In addition it is difficult to obtain high contrast pictures of the sclerites without the use of staining procedures. Phase contrast photographs increase contrast, but often result in a halo forming around the sclerites, distorting the image and thus reducing the quality of the measurements. The methods of Galli et al. (2007), where the sclerites are stained with Gomori's thrichrome and confocal microscopy techniques are applied to compensate for different spatial arrangement of the hooks, seems an improvement to the measurements of *Gyrodactylus* species. However, this technique is also more time consuming and requires costly equipment and software for measuring. Recent reviews of modern microscopic techniques point out that coherent anti-Stokes Raman scattering (CARS) is a

powerful technique for biological samples since it is not reliant on any chromophores nor does this technique destroy the sample (Muller and Zumbusch, 2007). Recent techniques like the CARS do take time to implement in a specific field of research and there are at present no available studies with the CARS technique applied to the sclerites of *Gyrodactylus* sp.

The applications of new microscopic techniques are an interesting development in biology, and could certainly increase our knowledge of the sclerites of *Gyrodactylus* spp. For diagnostic purposes however, conventional light microscopy seems more appropriate due to the need for less sophisticated equipment, and the majority of measurements on gyrodactylids that are available are done with this technique.

For diagnostic purposes, fluorescence microscopy opens the way for well tested techniques based on oligo-nucleotide probes with fluorophores attached. Fluorescence *in situ* hybridization (FISH) is a technique that is widely used in microbiological studies to detect different species, or even strains of bacteria (Amann et al., 1990). It is also possible to apply FISH to eukaryotic organisms (Hosoi-Tanabe and Sako, 2006) and with a combination of FISH and flow cytometry mass scannings of gyrodactylids are at least theoretically possible, although some modifications to the technique will most likely be needed to optimize the results.

rDNA sequencing

The choice of ITS1 and ITS2 as the molecular markers in the present study is made on the basis of earlier studies on gyrodactylids. The vast amount of rDNA sequences in GenBank makes the obtained results easy to compare with other closely related species. Since *G. marinus* is not available in GenBank as a sequence, the main purpose of the analysis is to confirm the results of the morphometric studies made on the specimens. The results show that both morphology and rDNA points to the conclusion of one species infecting the gill filaments of cod. And it is morphology and not rDNA that leads to the conclusion of the species in question. In this study there is the possibility of choosing molecular markers that have a higher resolution than the ITS1 and ITS2, as done by Hansen (2006), where the CO1 gene from mtDNA was used. This would however not result in a different conclusion with regards to the question of how many species are

present in this study. The higher resolution, which can be obtained from mtDNA, could be valuable in surveys concerning closely related species, and different populations of the same species of *Gyrodactylus*. If a difference in morphology, which could not be explained through rDNA, had been found one could use the CO1 marker as an additional source of information on the species in question.

The use of ITS1 and ITS2 seems to be appropriate in this context and represents a relatively easy and reliable way of diagnosing the species once described with both morphology and rDNA.

The rich parasitic fauna of cod poses a challenge for non-specialists in correctly determining the species found on the fish. Sequences of rDNA are available for several of the best known species of parasites from cod (for instance *Anisakis simplex*, *Hysterothylacium aduncum*, *Myxidium gadi* and *Pseudoterranova decipiens*), although some well known parasites from cod were not found in a search through NCBI homepage (April 2009), including species as *Holobomolochus confusus* and *Cucullanus cirratus*.

A sequencing of rDNA of all parasites recorded from cod would simplify the often challenging work of correctly identifying species of parasites. Without the aid of molecular markers, some life stages of nematodes, among other parasites, could pose a challenge even for the trained specialist (Lichtenfels et al., 1997).

Distribution of G. marinus in the study area

Sequencing of rDNA of specimens of *G. marinus* found in Øksfjord, Kvarøy and Brønnøysund show that this species infects cod along the entire study area of the CODPAR project. Although sample sizes of both cod and *Gyrodactylus sp*. were too small to draw the conclusion that *G. marinus* is the dominant species on gill filaments in all localities, the presence of the species is confirmed. Some of the morphological measurements from localities other than Ålesund are outside the range of the original measurements (Figures 7 and 8). These differences could be due to differences in temperature between the sites and underlines the importance of applying measurements of *Gyrodactylus* sp. with caution.

Infectious pattern of G. marinus

Although the dataset used to investigate the relationship of prevalence between the different groups of cod are not species specific, the *Gyrodactylus* sp. found on gill filaments will be treated as one species, *G. marinus*.

The general trend observed was that the prevalence of *Gyrodactylus* sp. on the gill filaments of cod was higher among the farmed cod than among the wild caught cod. The higher density among the farmed cod emerges as one of the main factors explaining the higher prevalence of *Gyrodactylus* sp. This pattern of infection is observed from other parasites with direct life cycles and is expected to occur in situations with high densities of the host (Anderson, 1982; MacKenzie and Hemmingsen, 2003). The cod examined in Øksfjord represented two groups with the largest differences in prevalence between wild caught and farmed fish. The farmed fish in Øksfjord were wild caught, and thus had a different infective pattern of both *Gyrodactylus* sp. and other parasites than farmed fish from the other localities. This does not however seem to influence the fact that farmed fish had higher prevalences of *G .marinus*.

For all localities except Kvarøy, the prevalence was higher in the farmed cod than in wild populations. The reproductive strategy of *Gyrodactylus* can cause mass outbreaks under farming conditions, this is however determined not only from reproductive potential, but also on immigration to new localities. It can be speculated that infections of *Gyrodactylus* under farming conditions will be of two types; one with high prevalence and intensities and the other with no outbreaks at all dependent on whether the parasite has managed to establish itself in the farming environment or not.

Pathogenic potential of G. marinus

Anderson and May (1979) argued that the interaction between parasites and host is not static, but rather a dynamic relationship. Diseases caused by parasites are shown to influence host populations, and the host population regulates the prevalence of parasites. These authors further showed that host populations with a high immigration or birth rate have a higher risk of being reduced by diseases caused by microparasites.

The effect of microparasites are however dependent on different interactions between the parasite and it host. These interactions can be divided into 4 components: the ability of the parasite to utilize the host as a habitat, host mortality caused by the parasite, the immune response from the host and the transmission between hosts.

The discussion of habitat is of minor importance in the case of *G. marinus*, as this parasite is adapted to live on the gill filaments of cod where they have the potential to reach high intensities (1100 gyrodactylids found on the gill filaments from one cod, CODPAR unpublished results). The transmission between hosts is further mediated by the high densities in aquaculture, and parasites with direct life cycles reach high intensities with a higher rate than observed in wild fish populations (Burt and MacKinnon, 1997).

The pathology of gyrodactylid infections is reported to be dependent on host immune status and the adaptation of the parasite strain to its host. The growth rate of the parasite can weaken the host immune response especially when the initial growth is rapid (Bakke et al., 2002; Bakke et al., 2007). Immune responses in fish infected with gyrodactylids have been reported in several studies. Both mechanical responses such as shedding of mucus (Lester, 1972) and host complement (Buchmann, 1998), have been demonstrated to reduce *Gyrodactylus* sp. infections.

There are however no reports on antibodies in fish infected with gyrodactylids, and specific studies on *G. salaris* and salmon (*Salmo salar* L.) have not revealed any antibodies in fish blood or mucus (Bakke et al., 2007). It is therefore reasonable to assume that although fish have an immune system that reduces the intensities of *Gyrodactylus* sp. there is no lasting immunity after infection, and fish can easily be re-infected if the initial infection is lost.

The *G. salaris* epidemic in Norwegian salmon rivers shows that gyrodactylids at least have the potential to cause severe mortality in fish populations.

With the exception of *G. salaris*, few studies are available on gyrodactylids causing disease in natural populations (Bakke et al., 2007 and references therein; van Oosterhout et al., 2007). Svendsen (1991) reported *G. marinus* causing death among medium sized cod in cages with densities lower than 5kg / M^2. These cod were however also infected with the microparasite *Trichodina* sp. and no deaths were reported among cod infected only with *G. marinus*. In addition to *Trichodina* sp., *Loma branchialis* and *Ichthyobodo necator* are found on the gill filaments of cod, and infections with a complex of these parasites are not uncommon. (Kristmundsson et al., 2006; Norwegian Veterinary Institute, 2008). If the *G. marinus* infections of cod is not directly causing a disease, secondary infections could arise due to damage caused by the attachment mechanisms of the parasite (Bakke et al., 2007).

Gyrodactylids can with respect to lifespan and transmission mechanisms "bridge the gap between micro and macroparasites" (Bakke et al., 2007), and it seems appropriate to apply the models of diseases caused by microparasites from Anderson and May (1979).

With an increase of cod mariculture along the Norwegian coast it is therefore interesting to investigate how an increase in numbers of susceptible hosts would influence the prevalence of *Gyrodactylus* sp. infections.

Even if *G. marinus* does not cause death among wild populations of cod, the addition of stress posed by mariculture could result in mortality in cod farms (Burt and MacKinnon, 1997). It is therefore possible that a benign infection with *G. marinus* in wild cod populations can cause a pathogenic infection in aquaculture.

As shown in Table 4, farmed cod have a higher prevalence of *Gyrodactylus* infection than that found in wild caught cod. Wild caught-farmed cod in Øksfjord do not seem to be more resistant to infections of gyrodactylids on the gill filaments. It is reasonable to assume that these fish had been exposed to gyrodactylids prior to capture, and the genetic variance among individuals from the group of wild caught-farmed cod would be higher than that found in the group of hatchery reared cod.

These factors seem not to play an important role in the prevalence of infection, as the wild caught-farmed fish show a similar trend in prevalence as hatchery reared cod.
The high density of farmed cod gives an ideal opportunity for gyrodactylid infections. If the parasite has established itself among the farmed cod, uninfected fish passing by the farms are exposed to the parasite. The farmed cod can in this way serve as a factor to persist the infections of gyrodactylids in local wild populations of cod. If outbreaks of *Gyrodactylus* sp. induce mortality or reduce the reproduction of cod, the impact of cod farming could pose a threat to these local populations
Experimental studies on *G. salaris* on salmon show that temperature affects the reproductive rate and the life span of the parasite. A life span of up to 5 weeks and a maximum of 4 births from a single parasite show that these species are capable of exponential growth on their host from a single individual (Jansen and Bakke, 1991).
This reproductive strategy becomes of paramount importance for the cod farming industry. Controlling the spread of infections from the surrounding environment to farmed cod seems unlikely , the only possibility being to reduce infections in farmed cod with chemical treatments or different salinity levels as for halibut (Svendsen and Haug, 1991).

MacKenzie and Hemmingsen (2003) proposed three ways by which parasites affect production of fish in mariculture: by directly affecting the health status of the fish, reduction of market value due to spoiling the appearance of the product, and threats to human health. It is apparent that *G. marinus* poses no threat to human health or appearance of the cod products. It is however possible for this species to reduce the health status of cod and cause mortalities in aquaculture.

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