

Host-based identification is not supported by morphometrics in natural populations of *Gyrodactylus salaris* and *G. thymalli* (Platyhelminthes, Monogenea)

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SUMMARY

Gyrodactylus salaris is a serious pest of wild pre-smolt Atlantic salmon (*Salmo salar*) in Norway. The closely related *G. thymalli*, originally described from grayling (*Thymallus thymallus*), is assumed harmless to both grayling and salmon. The 2 species are difficult to distinguish using traditional, morphometric methods or molecular approaches. The aim of this study was to explore whether there is a consistent pattern of morphometrical variation between *G. salaris* and *G. thymalli* and to analyse the morphometric variation in the context of 'diagnostic realism' (in natural populations). Specimens from the type-material for the 2 species are also included. In total, 27 point-to-point measurements from the opisthaptor hard parts were used and analysed by digital image processing and uni- and multivariate morphometry. All populations most closely resembled its respective type material, as expected from host species, with the exception of *G. thymalli* from the Norwegian river Trysiløva. We, therefore, did not find clear support in the morphometrical variation among *G. salaris* and *G. thymalli* for an *a priori* species delineation based on host. The present study also indicates an urgent need for more detailed knowledge on the influence of environmental factors on the phenotype of gyrodactylid populations.

Key words: morphology, systematics, Atlantic salmon, *Salmo salar*, grayling, *Thymallus thymallus*.

INTRODUCTION

Gyrodactylids are ubiquitous ectoparasites on the skin and gills of teleost fish both in marine and freshwater ecosystems (Bakke *et al.* 2007). The most recent species compilation lists 409 gyrodactylid species (Harris *et al.* 2004). Until the mid-1990s, most *Gyrodactylus* species were identified by comparing the morphology of the opisthaptor hard parts. Over recent years, the application of molecular markers in the taxonomy and systematics of *Gyrodactylus* species has increased, e.g., the sequencing of the internal transcribed spacers (ITS-1 and ITS-2) of nuclear ribosomal DNA (rDNA). Ziętara and Lumme (2002) showed that many *Gyrodactylus* species can be discriminated by internal transcribed spacer (ITS) sequences, and subsequent phylogenetic analyses yielded tree topologies that were basically consistent with those of classical taxonomy. However, *G. salaris* Malmberg, 1957 cannot be differentiated from its closest relative *G. thymalli* Žitňan, 1960 by means of ITS-1 and ITS-2 sequences (Cunningham, 1997), even considering parasites from a wide geographical range (Ziętara and Lumme, 2002).

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G. salaris has seriously hampered the natural juvenile production of Atlantic salmon, *Salmo salar* L., in Norway over the past 3 decades and has caused great harm both in ecological and economic terms (Johnsen *et al.* 1999; Mo *et al.* 2004), whereas *G. thymalli* is considered harmless to any of its known potential hosts. However, Jørgensen *et al.* (2007) and Olstad *et al.* (2007) have recently described 2 strains of *G. salaris* with restricted infectivity and reproduction on Atlantic salmon.

Several approaches using different markers have addressed the taxonomy and systematics of *G. salaris* and *G. thymalli*. To date, the mitochondrial cytochrome *c* oxidase subunit 1 gene (*cox1*) is the only marker allowing for a genetic discrimination of populations of *G. salaris* and *G. thymalli* (see e.g., Hansen *et al.* 2003, 2006; Meinilä *et al.* 2004). However, the 2 species differ in host preference (Soleng and Bakke, 2001; Bakke *et al.* 2002; Sterud *et al.* 2002), which is an important characteristic for considering *G. salaris* and *G. thymalli* different species. Discrimination of the 2 species based on multivariate statistics on morphometric measurements have proved possible but only a few populations (McHugh *et al.* 2000; Shinn *et al.* 2004) were included in these studies.

In the present study, we explored whether there is a consistent pattern of morphometrical variation between *G. salaris* and *G. thymalli* over a broad range

of natural populations by using a set of 'traditional' point-to-point morphometric measurements. Such morphometric measurements are known to be affected by environmental parameters, such as temperature (see Malmberg, 1970; Mo, 1991 *a, b, c*; Dmitrieva and Dimitrov, 2002; Davidova *et al.* 2005) that may impede an objective analysis of opisthaptor morphology. However, we were interested to investigate the 'diagnostic realism' in studying natural populations rather than analysing populations of gyrodactylids raised experimentally in a constant environment. To test whether *G. salaris* and *G. thymalli* are 1, 2, or more than 2 species, sample locations were chosen to comprehensively reflect the known variation in *cox1* genealogy.

MATERIALS AND METHODS

Sampling of Gyrodactylus

In total, 10 populations have been sampled and 168 specimens measured. Four *Gyrodactylus* populations were sampled from wild infected Atlantic salmon populations, namely, the rivers Skibotnelva (northern Norway), Rauma (north-western Norway), Drammenselva (southern Norway), and Göta älv (south-western Sweden). In the 3 Norwegian rivers, serious gyrodactylosis caused by *G. salaris* has been documented (Johnsen *et al.* 1999). One sample was collected from rainbow trout (*Oncorhynchus mykiss* Walbaum) (from a fish-farm in lake Bullaren, south-western Sweden), and one sample was collected from Arctic charr (*Salvelinus alpinus* L.) in lake Pålbufjord (central South-Norway). The population of *Gyrodactylus* in Lake Bullaren was identified as *G. salaris* by the OIE Reference Laboratory for *Gyrodactylus* (*G. salaris*) in Norway (Mo *et al.* 2004). The sample from Lake Pålbufjord was confirmed as *G. salaris* based on ITS rDNA and *cox1* sequences (Robertson *et al.* 2007). Four *Gyrodactylus* populations were sampled from wild infected grayling (*Thymallus thymallus* L.) populations, namely Lake Lesjaskogsvatn (central South-Norway), rivers Trysilselva (south-eastern Norway), Rena (south-eastern Norway) and Hnilec (Slovakia). The river Hnilec is one of the 2 type localities for *G. thymalli* according to Žitňan (1960) (see below). For the purpose of clarity, in this manuscript populations are referred to as *G. salaris* if recovered from Atlantic salmon and *G. thymalli* if recovered from grayling. Accordingly, the samples from Lake Bullaren and Lake Pålbufjord will be discussed independently. Wild fish were either captured by electro-fishing, gill netting or by rod-fishing. Parasite specimens were isolated from fins taken from the host and stored in 96% ethanol during transportation to the laboratory. The *cox1* sequences of the presently analysed samples are presented in Hansen *et al.* (2003, 2006). Further details are listed in Table 1.

Type-material

Gyrodactylus salaris was first described by Malmberg (1957) from Atlantic salmon in the Hölle Laboratory on the Baltic river Indalsälv, Sweden. The original description was based on 1 individual from a sample collected in 1952¹. Nevertheless, Malmberg prepared a total of 26 individuals from the same sample. The material is deposited in the Swedish Museum of Natural History, Stockholm. For the present study, 12 specimens from 'the type-sample' were photographed at the University of Stockholm using a Leica DC 300 camera mounted on a Leitz (Dialux) stereomicroscope under a $\times 63$ dry and a $\times 100$ immersion-oil objective. For scaling of the pictures, a stage micrometer from Albert Sass, Berlin (2 mm in 200 parts) was used. Measurements were performed on the Oslo Leica IM1000 v.4.0 software. (*G. salaris* ID.: 7089–7100; no. 7089 being registered as lectotype and the others as paralectotypes (Sven Boström, personal communication)).

Gyrodactylus thymalli was first described by Žitňan (1960) from grayling from the rivers Hnilec and Hron, Slovak Republic². The material is deposited in the East Slovak Museum, Košice, the Slovak Republic. A total of 12 individuals were included in the description. Of these, 6 were available for analysis in the present work (ID. no. Z-11229/1-6). The material was photographed and analysed at the Department of Zoology, Natural History Museum, University of Oslo, Norway according to the procedures described below.

Morphometry

For the morphometric analyses, individual *Gyrodactylus* specimens ($n = 12-30$) from each population were removed from fins and fixed in 96% ethanol. The opisthaptors were cut off and the opisthaptoral hard parts were subsequently prepared using a method slightly modified after Harris *et al.* (1999). The haptors were digested in 75 mM Tris, 10 mM

¹ Ergens (1983) re-described what he assumed to be the holotype of *G. salaris*, something that has turned out to be due to a misunderstanding. Malmberg did not assign any holotype-status in his original description (Malmberg, 1957). Furthermore, the individual Ergens described was from a sample taken in Hölle dating to 1957, and not from the original 1952 type-material (G. Malmberg, personal communication). The infection in Hölle Laboratory was chemically eliminated several times between 1952 and 1957 (G. Malmberg, personal communication).

² Žitňan's (1960) description was based on 12 individuals of which 2 were collected from the river Hron and 10 from the river Hnilec. Although not describing a holotype, Žitňan marked 1 (and only 1) of the slides "TYP" (ID. no.: z-11229/6 – sampled at Hnilec by Stará Voda) (V. Dudinák, personal communication). Ergens (1983) adapting the original description, highlighted the river Hron as the type locality, although this was not specifically detailed by Žitňan (1960).

Table 1. The *Gyrodactylus* populations, place of analyses and designation of the mitochondrial haplotype clades

(UoO = University of Oslo; UoS = University of Stirling.)

Natural populations (country)	Date of sampling	Temperature at sampling	Host species (Sampling method)	Place of analyses	Mitochondrial haplotype/clade according to:		
					Hansen <i>et al.</i> (2003)	Meinilä <i>et al.</i> (2004)	Hansen <i>et al.</i> (2006)
Drammenselva (Norway)	18.06.02	14.5 °C	<i>S. salar</i> (Electrofishing)	UoS	F – III	Sal F Drammen – II	F – III
Skibotnelva (Norway)	20.09.01	10.5 °C	<i>S. salar</i> (Electrofishing)	UoS; UoO	B – I	Sal B Skibotn	B – I
Rauma (Norway)	09.10.01	7.8 °C	<i>S. salar</i> (Electrofishing)	UoS	A – I	Sal A Rauma	A – I
Göta älv (Sweden)	13.11.01	3 °C	<i>S. salar</i> (Electrofishing)	UoS	E – II	Sal E Göte älv – I	E – II
Bullaren ¹ (Sweden)	13.05.02	12 °C	<i>O. mykiss</i>	UoO	F – III	Onc – II	F – II
Pålsbufjord (Norway)	Aug–Oct. 2003 ²	8–15 °C	<i>S. alpinus</i> (Gill netting)	UoS	n.a.	n.a.	n.a.
Trysilelva (Norway)	28.05.04	10 °C	<i>T. thymallus</i> (Fly-fishing)	UoO	G/H – IV	Thy G Trysilelva, Thy H Trysilelva – V	G/H/P – IV
Lesjaskogsvatn (Norway)	14.06.03	4.5 °C	<i>T. thymallus</i> (Fish trap)	UoS	n.a.	n.a.	Q – V
Rena (Norway)	24.06.06	8 °C	<i>T. thymallus</i> (Fly-fishing)	UoO	I/L – V	Thy Rena, Thy I Rena	I/L – V
Hnilec (Slovakia)	20.05.03	12 °C	<i>T. thymallus</i> (Electrofishing)	UoS	n.a.	n.a.	N – VI

¹ Samples of *O. mykiss* originated from a fish farm in Lake Bullaren.² Sampling in Pålsbufjord performed during specified period.

EDTA, 5% SDS and 100 mg/ml proteinase K, pH 8.0. After washing twice in H₂O, the released hard parts were fixed in ammonium picrate glycerine (Malmberg's fixative). The digital image analyses were performed by only 1 person (K. Olstad), but at 2 different laboratories; the Institute of Aquaculture, University of Stirling, UK, and at the Department of Zoology, Natural History Museum, University of Oslo, Norway (for details, see Table 1). At the University of Stirling, specimens were photographed using a JVC KY-F30B 3CCD camera with an interfacing ×2.5 top lens fitted to an Olympus BH2 compound microscope under a ×100 oil objective, and measured using the Zeiss KS300 iC/Windows Release v.3.0 (1997) (Carl Zeiss Vision 5 GmbH, Munich, Germany/Imaging Associates Ltd, Thame, Oxfordshire, UK) software. At the Natural History Museum, University of Oslo, specimens were photographed using a Leica DC 500 camera mounted on a Leica DM 6000B stereomicroscope under a ×100 oil objective, and measured using the Leica IM1000 v.4.0 software.

A total of 27 point-to-point measurements were taken on each specimen. Of these, 23 were identical to the measurements described by Shinn *et al.* (2004), and with the same annotations. (i) Marginal hook (MH): total length (MHTL), shaft length (MHSHL), sickle length (MHSL), sickle proximal

width (MHSPW), sickle toe length (MHSTL), sickle distal width (MHSDW), sickle aperture (MHAD), instep height (MHIH). (ii) Hamulus (H): proximal shaft width (HPSW), aperture distance (HAD), point length (HPL), distal shaft width (HDSW), hamulus inner angle (HIA), inner curve length (HICL), shaft length (HSL), root length (HRL), total length (HTL). (iii) Ventral bar (VB): total length (VBTL), process to mid length (VBPML), median length (VBML), membrane length (VBMBL), centre length (VBCL), lateral length (VBLL), total width (VBTW), width (VBW), membrane maximal width (VBMMW) and process length (VBPL).

The 2 hamulus measurements, the aperture angle (HAA) and the point curve angle (HHPCA) used by Shinn *et al.* (2004), were not considered in this study. The 4 measurements VBCL, VBLL, VBW, and VBMMW represent new morphometric variables and are depicted in Fig. 1.

Statistical analyses

All statistical analyses as well as ordination were performed using the software PAST v.1.66 (Hammer *et al.* 2001). The hamulus inner angle (HIA) was transformed to the cosine in order to obtain linear functions prior to data analyses. Since

marginal hook total length (MHTL) and shaft length (MSHSL) were not possible to obtain from the type materials, these measurements were omitted in the analyses.

Since photographing of the specimens and measuring of the sclerites were performed at 2 different localities with 2 different microscopes and software, the same specimens ($n=10$) from a randomly chosen population (Skibotnelva) first measured at the University of Stirling, were re-measured at the Natural History Museum, Oslo. The non-parametric Bray-Curtis MANOVA was used to test whether the multivariate means of the 2 data-samples were equal.

Based on the hypothesis that the sampled individuals belong to 2 distinct species, k-means clustering preset for 2 groups was run on the total data-matrix. The cluster assignments in k-means clustering are initially random which implies that the results may differ from run to run. The procedure was therefore repeated >10 times. Clustering into 3 and 4 groups was also run in order to test alternative hypotheses regarding number of groups (e.g., groupings according to host-species).

In order to test the hypothesis that any 2 populations had equal multi-dimensional means, Wilk's lambda MANOVA and Hotelling's pairwise *post-hoc* comparisons were run. The Hotelling's pairwise *post-hoc* comparisons were run both with and without Bonferroni corrections. The low number of individuals available for study in this analysis in combination with the high number of variables made a dimensionality-reduction of the data-matrix necessary. Principal Component Analysis (PCA) scores on the variance-covariance matrix were therefore obtained, and the statistical tests run on a limited number of the PCA axis-scores (but maintaining >95% of the total variation). For a numerical comparison of distances between population multivariate distributions, pair-wise Mahalanobis distances were calculated using S-PLUS 6.0, Professional Release 1.

RESULTS

The morphometric differences of 10 populations of the monogenean *G. salaris* on Atlantic salmon, rainbow trout and Arctic charr and *G. thymalli* on grayling, were studied. These particular *Gyrodactylus* populations were selected as they comprehensively represent mitochondrial haplogroups as described by Hansen *et al.* (2003, 2006) (Table 1), and are thus expected to represent the range of intra- and interspecific variation of *G. salaris* and *G. thymalli*. The assignment of mitochondrial haplotypes to parasite populations is in some instances based on the cytochrome oxidase I sequence of only 1 individual, hence intra-population mitochondrial sequence variation cannot be excluded. Individual cytochrome

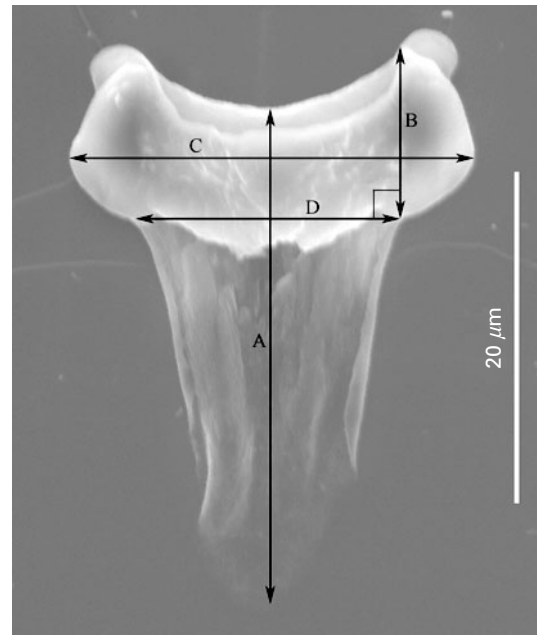


Fig. 1. Scanning electron micrograph (SEM) of the ventral bar (VB) from a *Gyrodactylus* specimen parasitizing grayling in the river Hnilec, Slovakia, illustrating the four new measurements. (A) Ventral bar centre length (VBCL): The distance between the top of the median bar and the distal part of the membrane, along a line following the centre of the structure. (B) Ventral bar lateral length (VBLL): The distance between an orthogonal line between ventral bar bases towards the membrane (measurement D) to the distal top of the ventral bar (excluding the process). (C) Ventral bar width (VBW): The maximum width of the median portion of the ventral bar, excluding the processes. (D) Ventral bar membrane maximal width (VBMMW): the distance between the ventral bar bases towards the membrane.

oxidase I sequence data were not determined and intra-population sequence variation was thus not considered in the present work.

The data obtained in the different labs were analysed together, as all measurements were performed by the same person and no significant differences were observed between any of the different instrumental set-ups used in the present investigations (non-parametric Bray-Curtis MANOVA $P=0.297$).

The type-material of *G. salaris* and *G. thymalli* constitutes whole-mounted worms that have been pre-treated with formalin. Such treatment is not favourable for light-microscope studies of the hard parts. The variance of the measurements from these individuals might thus be increased. Some individuals could not be reliably measured due to oblique positions of the hard parts and were thus discarded from the analyses (*G. salaris* measured: ID no. 7090, 7093, 7094, 7096, 7097 – *G. thymalli* measured: ID no. Z-11229/2-6). A summary of the

morphometric measurements of the hard parts of the *G. salaris* and *G. thymalli* type-material is given in Table 2. Although the averages for the measurements of the marginal hook total length (MHTL), marginal hook shaft length (MHSAL), and marginal hook sickle length (MHSL) might indicate a clear grouping in 2 species in accordance with the *a priori* species delineation, the ranges between the groups substantially overlap. MHTL and MSHAL for the *G. salaris* and *G. thymalli* type-material were unfortunately impossible to obtain. Thus, the averages and ranges did not reveal a single measurement unambiguously grouping the populations in accordance with an *a priori* species delineation.

In the k-means clustering, both for 2 and 3 groups, the *G. salaris* type-material and the *G. thymalli* type-material were categorized in separate groups (Table 3). When clustering into 2 groups, the Bullaren population (from rainbow trout) most closely resembled the '*G. salaris* group' whereas the Pålbufjord population (from Arctic charr) represented an intermediate group positioned between the *G. salaris* and *G. thymalli* groups (Table 3). The grayling parasites from Trysilelva resembled gyrodactylids collected from Atlantic salmon. The remaining populations of *Gyrodactylus* resembled type-material representing their respective species in accordance with the *a priori* species designations based on host species. When clustering into 3 groups, parasites sampled from Atlantic salmon and grayling basically followed the same pattern as when clustering into 2 groups, although some individuals from all populations but Hnilec were classified into Group 3 (Table 3). Individuals from both Bullaren (from rainbow trout) and Pålbufjorden (from Arctic charr) were mainly classified into Group 3. With few exceptions, the pattern when clustering into 4 groups was consistent with the pattern when clustering into 3 groups (Exceptions: 1, 1 and 3 individuals from *G. salaris* type-material, Drammenselva and Skibotnelva, respectively, were classified into Group 4. Data not shown in table).

In a PCA-plot (run on the variance-covariance matrix) based on the measurements from all 10 populations and the type-material of both species (Fig. 2), the 2 populations representing the name-bearing types separated well from each other. Furthermore, the populations Drammenselva, Skibotnelva, Rauma, Göta älv, Bullaren and Trysilelva apparently grouped with the *G. salaris* type-material, and the populations Hnilec, Lesjaskogsvatn and Rena with the *G. thymalli* type-material. Gyrodactylids collected from Arctic charr from Lake Pålbufjord, on the other hand, appear to represent an intermediate group positioned between the main *G. salaris* and *G. thymalli* clusters. The graphical presentation of the PCA analysis did not reveal any groupings in the material related to *cox1* phylogeny or to temperature/season of sampling. In principal

components higher than 2, the PCA plot did not reveal any clear groupings of the data.

The scores from the PCA (run on the variance-covariance matrix) were used to identify a reduced number of linear combinations explaining a sufficient amount of the total variation in the data set. The first 9 PCs explained 95.4% (PC1 – 62.70%; PC2 – 9.1%; PC3 – 6.5%) of the total variation in the data set, and were thus considered to capture a sufficient amount of the variation in the 27 variables to be the only ones included in the subsequent multivariate analyses. According to established procedures in morphometric analyses, most of the variation explained in PC1 is due to size, particularly when the first PC explains a substantial percentage of the total variation in the data set (Jolicoeur and Mosiman, 1960; Reyment *et al.* 1984). Accordingly, the following multivariate analyses were both run on a data-matrix comprised of data from the first 9 PCs (PC1–PC9) and on a data matrix in which the data for PC1 had been removed (PC2–PC9; explaining 32.7% of the total variation in the data set). The latter is expected to be an analysis in which a major part of variation due to size is removed, thereby leaving variation principally attributable to changes in shape.

The MANOVA rejected the hypothesis of equal multidimensional means (Wilks-lambda, $P < 0.05$) for all populations. This was observed both when including and excluding PC1. The Hotelling's T squared *post-hoc* tests gave significant differences ($P < 0.05$) between all populations when not Bonferroni corrected. Again, this was observed both with and without PC1. The Hotelling's T squared *post-hoc* tests when Bonferroni corrected, with few exceptions (Table 4) also gave significant results, despite very low statistical power. When analysing PC1–PC9, cases where the hypothesis of equality could not be rejected involved the salmon parasites from Drammenselva, Skibotnelva, Rauma, and Göta älv as well as the rainbow trout parasites from Bullaren. If removing the first PC, cases where the hypothesis of equality could not be rejected also involved the salmon parasites from Drammenselva, Skibotnelva, Rauma, and Göta älv. Additionally, equality could not be rejected between the grayling parasites from Lesjaskogsvatn and the salmon parasites from Drammenselva, Skibotnelva, and Rauma or between the grayling parasites from Hnilec and the salmon parasites from Skibotnelva and Rauma.

The Mahalanobis distances between the populations are listed in Table 5. For PC1–9 the magnitude of the distances indicates a grouping of the *G. salaris* populations on Atlantic salmon from Drammenselva, Skibotnelva, Rauma and Göta älv. Compared to these pairwise distances, the Mahalanobis distances between the *G. salaris* populations on rainbow trout (Bullaren) and the other

Table 2. Summary of the morphometric measurements for the 10 *Gyrodactylus* populations analysed in this study

(A detailed explanation of abbreviations is provided in the Materials and Methods section. Each measured feature is given in micrometres (μm) except HIA, for which the measurements are given in degrees, followed by the standard deviation and the range (in parentheses). n – Number of specimens measured. * – Rainbow trout. ** – Arctic charr. *** – Grayling. Others hosted by Atlantic salmon.)

	Drammen selva $n=12$	Skibotnelva $n=14$	Rauma $n=12$	Göta älv $n=17$	Bullaren* $n=14$	Pålsbu** $n=30$	Hnilec*** $n=15$	Lesjasko gsvatn*** $n=14$	Trysilelva*** $n=15$	Rena*** $n=18$	<i>G. salaris</i> , paralecto types $n=5$	<i>G. thymalli</i> , syntypes $n=4$
Marginal hook												
MHTI	37.9±0.9 (36.3–39.5)	39.5±1.1 (37.7–41.4)	39.6±0.9 (37.9–41.0)	39.9±1.0 (38.5–42.5)	38.9±1.1 (37.3–40.7)	40.2±1.1 (38.2–42.6)	41.7±0.9 (40.3–43.5)	43.0±0.7 (42.2–44.4)	41.7±1.0 (39.5–43.0)	43.3±1.1 (41.4–45.3)		
MHSHL	30.7±0.9 (29.2–32.4)	31.9±1.0 (30.3–33.3)	32.1±0.9 (30.6–33.3)	32.7±0.8 (31.4–34.6)	31.6±1.0 (30.1–33.1)	32.8±0.9 (30.9–34.4)	33.9±0.8 (32.8–35.6)	35.1±0.9 (33.8–36.7)	34.2±0.9 (32.3–35.2)	35.3±1.0 (33.4–37.1)		
MHSL	7.7±0.2 (7.5–7.9)	8.0±0.3 (7.6–8.6)	8.1±0.2 (7.8–8.3)	7.9±0.3 (7.3–8.6)	8.1±0.3 (7.7–8.5)	7.9±0.2 (7.6–8.4)	8.3±0.2 (7.9–8.7)	8.6±0.2 (8.3–8.9)	8.4±0.3 (7.8–8.8)	8.7±0.3 (8.3–9.1)	8.6±0.7 (7.5–9.3)	8.7±0.2 (8.4–9.0)
MHSPW	5.2±0.2 (4.7–5.4)	5.5±0.3 (5.1–6.1)	5.4±0.3 (5.1–6.0)	5.5±0.3 (4.7–5.9)	5.2±0.2 (4.9–5.6)	5.3±0.3 (5.0–6.2)	5.4±0.3 (4.9–6.1)	5.7±0.3 (4.9–6.3)	5.4±0.1 (5.3–5.6)	6.1±0.4 (5.4–7.0)	6.1±0.3 (5.9–6.5)	6.3±0.2 (6.2–6.7)
MHSTL	2.1±0.1 (1.9–2.3)	2.1±0.2 (1.9–2.5)	2.1±0.2 (1.8–2.3)	2.3±0.2 (1.8–2.5)	2.0±0.1 (1.8–2.3)	2.0±0.2 (1.7–2.8)	2.0±0.1 (1.9–2.2)	2.2±0.2 (1.9–2.5)	2.1±0.1 (1.9–2.3)	1.8±0.2 (1.5–2.0)	1.9±0.2 (1.7–2.1)	2.1±0.2 (1.9–2.3)
MHSD	5.8±0.7 (3.9–6.4)	6.3±0.3 (5.7–6.9)	6.2±0.3 (5.7–6.7)	6.0±0.3 (5.1–6.5)	6.0±0.2 (5.3–6.3)	6.1±0.3 (5.6–6.7)	6.4±0.3 (5.5–6.8)	6.2±0.2 (5.7–6.7)	6.1±0.2 (5.8–6.5)	5.3±0.3 (4.5–6.6)	5.3±0.2 (5.0–5.6)	5.1±0.3 (4.9–5.4)
MHAD	6.06±0.5 (4.7–6.4)	6.4±0.4 (5.8–7.0)	6.3±0.3 (5.8–6.7)	6.4±0.2 (5.7–6.9)	6.1±0.1 (5.9–6.2)	6.4±0.2 (6.1–7.2)	7.0±0.2 (6.4–7.4)	7.0±0.2 (6.3–7.3)	6.7±0.3 (6.0–7.1)	6.9±0.2 (6.5–7.3)	7.0±0.8 (6.0–7.9)	7.7±0.4 (7.2–8.2)
MHIH	0.7±0.1 (0.6–0.8)	0.7±0.1 (0.5–0.9)	0.7±0.1 (0.4–1.0)	0.8±0.1 (0.6–1.0)	0.7±0.1 (0.6–0.1)	0.6±0.1 (0.4–0.9)	1.0±0.1 (0.7–1.2)	1.0±0.1 (0.8–1.2)	0.8±0.1 (0.6–1.0)	0.9±0.1 (0.6–1.1)	0.5±0.2 (0.2–0.7)	0.7±0.2 (0.5–0.9)
Hamulus												
HPSW	11.3±0.6 (10.2–12.4)	11.7±0.7 (10.4–13.3)	11.3±0.5 (10.5–12.1)	11.8±0.5 (11.0–12.7)	11.6±0.4 (11.0–12.6)	12.1±0.8 (10.1–13.7)	14.0±0.7 (12.7–15.1)	13.9±0.7 (12.6–15.4)	11.4±0.5 (10.7–12.6)	12.8±0.6 (11.4–13.7)	9.9±0.8 (9.0–10.9)	12.6±0.7 (12.0–13.3)
HAD	24.9±1.4 (22.6–26.7)	24.1±1.2 (22.1–26.7)	25.3±1.8 (21.0–28.2)	26.4±1.2 (24.0–28.4)	24.0±1.2 (20.4–25.2)	24.3±2.9 (20.7–32.9)	28.2±2.4 (22.1–33.9)	27.3±2.0 (24.5–32.3)	21.7±1.8 (17.6–24.3)	26.8±0.8 (25.7–28.8)	22.0±3.1 (18.3–26.9)	28.1±1.5 (26.1–29.5)
HPL	35.3±1.5 (32.2–37.1)	35.6±1.1 (33.5–37.8)	34.4±0.8 (33.4–35.7)	36.1±1.1 (33.3–37.9)	37.5±1.0 (36.0–39.7)	39.3±1.0 (36.7–41.4)	40.3±0.9 (37.8–41.7)	38.4±0.8 (37.1–39.7)	35.5±1.3 (31.6–37.1)	39.5±1.2 (36.6–41.3)	35.5±1.4 (34.6–37.9)	39.2±1.3 (37.8–40.9)
HDSW	6.5±0.6 (5.5–7.9)	6.3±0.5 (5.4–7.2)	6.6±0.5 (5.8–7.3)	6.7±0.3 (5.8–7.3)	6.8±0.3 (5.9–7.2)	7.6±0.7 (6.8–9.3)	7.8±0.9 (6.8–10.7)	7.9±0.4 (7.4–8.7)	7.3±0.2 (6.9–7.7)	8.3±0.6 (7.2–9.3)	6.0±0.4 (5.5–6.5)	7.1±0.2 (6.7–7.3)
HIA	43.0±2.0 (39.8–45.8)	42.0±1.8 (39.1–44.8)	44.0±3.0 (38.7–48.2)	45.7±2.1 (41.4–49.7)	39.3±2.3 (33.3–42.5)	39.0±5.1 (33.4–55.1)	42.3±3.0 (37.1–50.9)	43.1±2.5 (39.4–47.1)	37.8±2.8 (31.0–42.1)	41.8±1.3 (39.8–43.9)	43.3±8.8 (33.1–61.5)	42.1±2.3 (40.6–45.4)
HICL	4.6±0.8 (3.8–6.6)	5.3±0.9 (3.5–7.4)	6.0±0.6 (4.7–6.8)	5.6±0.9 (3.7–7.6)	4.3±0.6 (3.4–5.8)	3.9±1.0 (1.6–6.2)	5.5±0.6 (4.4–6.5)	6.6±1.4 (4.6–9.6)	5.4±1.0 (3.6–7.9)	6.2±0.9 (4.6–7.4)	6.5±1.5 (4.8–8.1)	5.0±1.7 (3.1–6.8)
HSL	43.2±1.6 (39.40–45.3)	42.8±1.6 (40.1–46.1)	43.0±1.7 (40.62–46.1)	44.5±1.3 (42.2–48.0)	44.4±1.1 (42.0–45.9)	46.5±1.3 (44.6–49.1)	51.4±1.6 (47.2–53.7)	46.8±1.7 (44.0–50.4)	42.4±1.6 (38.7–44.4)	48.1±1.1 (46.6–50.3)	42.5±2.8 (39.9–47.0)	50.0±3.3 (46.2–54.2)
HRL	24.7±0.8 (23.6–25.8)	23.1±1.5 (20.5–26.3)	23.5±1.0 (21.7–24.6)	24.7±1.6 (20.8–27.6)	27.0±1.9 (23.4–30.7)	27.5±1.2 (24.6–29.9)	27.0±1.2 (24.9–29.2)	26.5±1.4 (24.9–30.1)	23.2±1.7 (19.5–26.4)	26.2±1.1 (24.5–28.4)	22.0±2.4 (18.0–23.7)	27.7±1.5 (25.8–29.3)
HTL	70.6±2.8 (64.0–74.2)	68.0±2.2 (63.2–72.1)	70.1±1.7 (67.7–72.9)	72.7±1.8 (68.9–75.2)	72.4±2.7 (66.3–75.2)	75.9±1.8 (72.5–79.4)	82.7±2.3 (77.3–85.6)	78.5±2.5 (74.6–82.8)	69.7±2.1 (66.6–73.1)	80.0±1.8 (75.9–82.7)	69.8±3.5 (64.0–73.1)	82.1±2.1 (79.8–84.1)

Table 3. Individual classifications based on k-means clustering into 2 and 3 groups of the morphometric measurements from the 10 populations of *Gyrodactylus* analysed as well as the *G. salaris* and *G. thymalli* type-material

(Number of specimens classified into respective groups is given. * – Rainbow trout. ** – Arctic charr. *** – Grayling. Others hosted by Atlantic salmon.)

	2 groups		3 groups		
	Group 1	Group 2	Group 1	Group 2	Group 3
<i>G. salaris</i> paralectotypes	5	0	5	0	0
<i>G. thymalli</i> syntypes	0	4	0	4	0
Drammenselva	12	0	10	0	2
Skibotnelva	14	0	13	0	1
Rauma	12	0	11	0	1
Göta älv	17	0	12	0	5
Bullaren*	14	0	3	0	11
Pålsbufjord**	15	15	0	4	26
Trysilelva***	15	0	12	0	3
Lesjaskogsvatn***	1	13	0	8	6
Rena***	0	18	0	13	5
Hnilec***	0	15	0	15	0

whereas the Pålsbufjord population represented an intermediate group positioned between the *G. salaris* and *G. thymalli* groups. The grayling parasites from Trysilelva resembled gyrodactylids collected from Atlantic salmon. The remaining populations of *Gyrodactylus* resembled the type-material representing their respective species in accordance with the *a priori* species designations. From a taxonomic viewpoint, these morphometric data confirm the species status of the *a priori* assigned populations except for the Trysilelva population.

Intra- and interspecific variation

The *Gyrodactylus* populations included in this study could be distinguished from each other by morphometry; all populations had significantly different multidimensional means. It is noteworthy that intra-specific pairwise Mahalanobis distances were lower among *G. salaris* parasitizing salmon than among the populations of *G. thymalli* parasitizing grayling, this also holds true when excluding the Trysilelva population from the comparison (Mann-Whitney U test: $P=0.03$).

When PC1, which is usually considered the size-axis (Jolicoeur and Mosiman, 1960; Reyment *et al.* 1984), was excluded from the analyses, the Mahalanobis distances indicated that *G. thymalli* specimens from Trysilelva were different from all *G. salaris* specimens, whereas the Hnilec and Lesjaskogsvatn populations resembled the *G. salaris* specimens. This pattern was, however, not fully congruent with the Hotelling's pairwise comparisons when relaxing the statistical power; the Trysilelva population was not significantly different from

the *G. salaris* populations when including size. It is noteworthy that for a size-measure such as hamulus total length (HTL) the specimens from Hnilec, with a water temperature of 12 °C at the time of sampling, were the biggest (mean 83 µm), whereas the specimens from Skibotnelva, which were collected at 3 °C, were the smallest (mean 68 µm). If one assumes that these size differences are due to ambient temperature affecting otherwise undistinguishable traits, the observed pattern would be opposite of previous publications stressing that cold conditions will lead to larger hooks due to extended development-time (suggested by Kulemina, 1977; Mo, 1991a). Whether or not the observed variation is within the range of phenotypic plasticity of size and shape or represents genetic differentiation, remains unknown. For example, it has been shown from laboratory experiments that *Gyrodactylus* exhibit differences in developmental time and fecundity on different hosts (Cable *et al.* 2000). Since the opisthaptor hard parts do not grow after birth (Kulemina, 1977), the particular parasite strain – host species relationship may be expected to constitute a significant factor for variation in size and shape given growth is allometric, or for size only if growth is isometric.

Taxonomic implications

One of the alternative taxonomic scenarios proposed by Hansen *et al.* (2003) is that *G. salaris* and *G. thymalli* may represent a complex of more than 2 sibling species. The fact that the samples from Bullaren and Pålsbufjorden grouped together separately from the Atlantic salmon and grayling

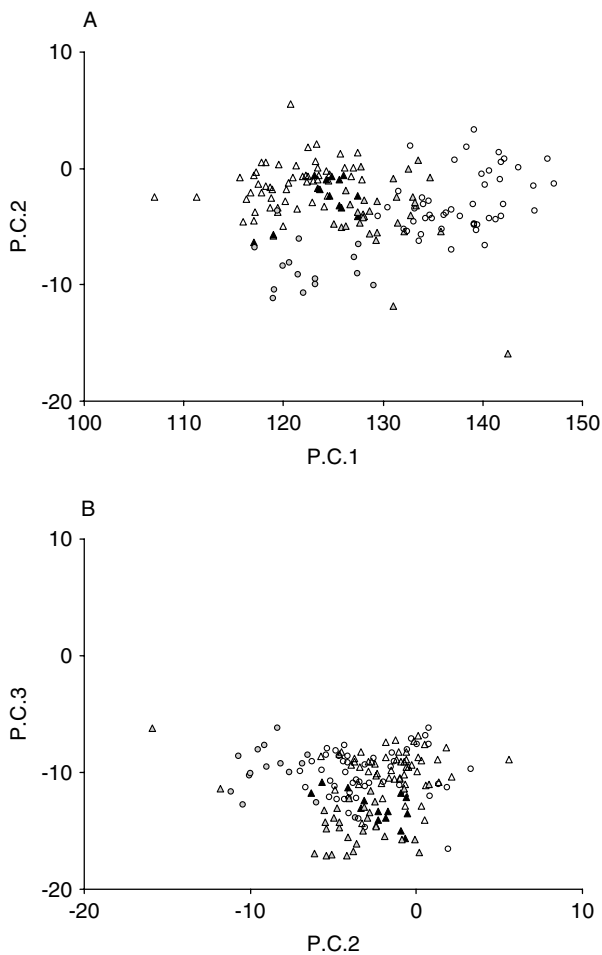


Fig. 2. Principal component analyses of 27 morphometric variables taken from 10 populations of *Gyrodactylus salaris* and *G. thymalli*. Open triangles – *G. salaris* (hosted by Atlantic salmon) from Drammenselva, Skibotnelva, Rauma, and Göta älv, as well as the *G. salaris* type-material. Black triangles – *G. salaris* from Lake Bullaren rainbow trout. Shaded triangles – *G. salaris* from Lake Pälsubufjord Arctic charr. Open circles – *G. thymalli* (hosted by grayling) from Hnilec, Lesjaskogsvatn and Rena, as well as the *G. thymalli* type-material. Shaded circles – *G. thymalli* from River Trysilelva grayling. (A) A distribution map of each *Gyrodactylus* population with the first principal component axis (PC1) plotted against the second (PC2). (B) The distribution of each *Gyrodactylus* population when the second principal component axis (PC2) is plotted against the third (PC3).

parasites might bring indications of such a scenario, particularly since they share a common *cox1* haplotype (Robertsen *et al.* 2007). However, these 2 populations also share a common *cox1* haplotype with the *G. salaris* from River Drammenselva (Hansen *et al.* 2003), a population that they did not group together with in the present analyses. The present analyses may, in this matter, be biased due to the selection of the populations, and an extended sampling including specimens from Finland and

Russia (clades III and IV according to Meinilä *et al.* 2004) may alter the results. The rejection of the hypothesis of equal multi-dimensional means and the high level of correct assignments for specimens from all studied populations in the classification tests may, on the other hand, at least not contradict the hypothesis of *G. salaris* and *G. thymalli* being a complex of more than 2 sibling species. However, the present morphometrical differentiation as presented by the PCA analysis is not suitably sufficient to allow for a clear delineation on a subspecies level and does not, therefore, provide strong support for this hypothesis. According to the sibling species complex hypothesis (Hansen *et al.* 2003), one would expect minor differentiation of the *G. salaris* populations from Skibotnelva and Rauma representing the same mitochondrial haplogroup and both parasitizing Atlantic salmon. However, the *G. salaris* populations from these 2 rivers are as differentiated as any other populations under study. When taking into account that some of the morphological characters of the opisthaptor hard parts in *Gyrodactylus* are also influenced by temperature (see Malmberg, 1970; Mo, 1991*a,b,c*; Dmitrieva and Dimitrov, 2002; Davidova *et al.* 2005), and probably also by host species (Dmitrieva and Dimitrov, 2002; Huyse and Volkaert, 2002; unpublished findings), we are tempted to conclude that the observed morphometric variation in the measured characters for *G. salaris* and *G. thymalli* does not indicate more than 2 sibling species.

In conclusion, our morphometric data did not clearly support the delineation of the *G. salaris*/*G. thymalli* species complex into 2 or more groups. Our study also illustrates that there is an urgent need for more detailed knowledge on the environmental factors influencing the phenotype of *Gyrodactylus* populations. At present, by far most of the 409 described gyrodactylid species (Harris *et al.* 2004) are ‘morphological species’, as they are described without including molecular data. More information on the environmental impact on gyrodactylid morphology is, therefore, needed for the interpretation of the taxonomic significance of the morphometrical differences observed between *Gyrodactylus* populations in general. With particular reference to *G. salaris* and *G. thymalli*, our results indicate that the species determination of gyrodactylid species taken from wild populations of fish, when based solely on morphometrics, is more complicated and problematic than initially thought.

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Table 4. Hotelling's pairwise *post-hoc* comparisons of PC1–PC9 (below the diagonal) and PC2–PC9 (above the diagonal) for 10 populations of *Gyrodactylus salaris* and *G. thymalli*, infecting four different host species (see text)(† – non-significant results ($P \geq 0.05$). * – Rainbow trout. ** – Arctic charr. *** – Grayling. Others hosted by Atlantic salmon.)

	Drammenselva	Skibotnelva	Rauma	Göta älv	Bullaren*	Pålsbufjord**	Trysilelva***	Lesjaskogsvatn***	Rena***	Hnilec***
Drammenselva	—	1.56 [†]	<0.01	0.06 [†]	0.10	<0.01	0.29 [†]	<0.01	<0.01	<0.01
Skibotnelva	2.20 [†]	—	0.47 [†]	<0.01	<0.01	<0.01	0.05 [†]	0.08 [†]	0.01	<0.01
Rauma	0.01	1.02 [†]	—	<0.01	<0.01	<0.01	1.03 [†]	0.78 [†]	<0.01	<0.01
Göta älv	0.08 [†]	<0.01	0.01	—	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Bullaren	0.06 [†]	<0.01	<0.01	<0.01	—	<0.01	<0.01	<0.01	<0.01	<0.01
Pålsbufjord	<0.01	<0.01	<0.01	<0.01	<0.01	—	<0.01	<0.01	<0.01	<0.01
Trysilelva	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	—	<0.01	<0.01	<0.01
Lesjaskogsvatn	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	—	<0.01	<0.01
Rena	<0.01	0.02	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	—	<0.01
Hnilec	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	—

Table 5. Mahalanobis distances for the principal components PC1–PC9 (above the diagonal) and PC2–PC9 (below the diagonal) for 10 populations of *Gyrodactylus salaris* and *G. thymalli*, infecting four different host species

(* – Rainbow trout. ** – Arctic charr. *** – Grayling. Others hosted by Atlantic salmon.)

	Drammenselva	Skibotnelva	Rauma	Göta älv	Bullaren*	Pålsbufjord**	Trysilelva***	Lesjaskogsvatn***	Rena***	Hnilec***
Drammenselva	—	6.84	11.81	11.24	6.34	24.68	24.21	49.42	54.76	67.28
Skibotnelva	6.84	—	6.19	8.58	14.78	28.72	11.15	39.03	45.05	68.43
Rauma	11.48	5.81	—	7.04	23.26	40.20	20.21	30.44	49.08	56.61
Göta älv	8.79	5.97	6.06	—	16.67	26.93	20.52	29.29	29.99	47.39
Bullaren	4.10	12.39	22.41	16.67	—	8.19	23.22	42.79	41.67	54.15
Pålsbufjord	11.61	15.30	30.93	22.73	3.69	—	25.49	33.00	25.46	44.14
Trysilelva	22.07	8.86	19.41	20.51	23.22	20.86	—	27.82	28.77	66.66
Lesjaskogsvatn	17.83	6.89	4.94	12.84	25.77	28.97	10.53	—	14.64	22.74
Rena	18.34	8.04	19.22	10.01	21.06	19.60	7.87	14.47	—	31.06
Hnilec	8.71	9.11	6.44	10.31	16.23	27.83	28.34	18.61	28.44	—

samples of *G. thymalli* from river Hnilec, Slovakia; H. Hansen, D. Gammelsæter, T. Haugen, L. Karlsson, I. Perä, O. Eide, P. Arnkværn, and T. Olstad for help in collecting material in Scandinavia; H.J. Berg and C. Vollev for assisting in SEM photography; and G. Robertsen for assisting in the preparation of specimens.

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