Chaetotaxy applied to Norwegian *Gyrodactylus salaris* Malmberg, 1957 (Monogenea) clades and related species from salmonids

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Abstract. *Gyrodactylus salaris* Malmberg, 1957 is a major pathogen of wild *Salmo salar* L. parr populations in Norway, and its delimitation from non-pathogenic species is important. The present study was undertaken to test the power of chaetotaxy to differentiate between three populations belonging to both the same and different clades (as stated by mtDNA) of *G. salaris*, in addition to three different species of gyrodactylids (*G. salaris*, *G. thymalli* and *G. caledoniensis*). The gyrodactylids were processed for chaetotaxy in situ and a maximum of 50 specimens per collection site were used to construct a generalised map over the sensilla. The sensilla were found in all populations to be symmetrically distributed around the median longitudinal axis, according to a formula of 7 dorsal (34 sensilla) and 8 ventral (44 sensilla) clusters on each side of the median line. The three Norwegian populations of *G. salaris* were found identical, as were the population of *G. thymalli*. The specimens of *G. caledoniensis* from Scotland, however, were found to differ from the Norwegian species *G. salaris* and *G. thymalli* by the position of one sensillum in two of the clusters. A comparison of the sensillum pattern of laboratory maintained *G. salaris* (River Lierelva) with results obtained ten years earlier, questions the temporal stability of the chaetotaxy pattern. The present results indicate that chaetotaxy can be used to discriminate between certain *Gyrodactylus* spp. but not generally.

Species of the monogenean genus *Gyrodactylus* Nordmann, 1832 are ubiquitous ectoparasites of marine and freshwater teleosts worldwide, and depending on the species, infect fish skin, fins or gills (Bakke and Harris 1998, Bakke et al. 2002). One species, *G. salaris* Malmberg, 1957, originally described from the Hölle hatchery, Sweden has turned out to be a major pathogen of wild anadromous Atlantic salmon (*Salmo salar* L.) parr in Norway since its introduction in the mid-seventies (Johnsen and Jensen 1991, Mo 1994, Johnsen et al. 1999). Since 1975, 44 Norwegian rivers have been found to be infected with *G. salaris*, and 28 (December 2003) of them have been treated with rotenone to exterminate the hosts and, subsequently, the parasite (Mo et al. 2004).

A recent molecular genetic study based on mtDNA mitochondrial cytochrome oxidase I gene (COI) demonstrated that at least three clades of *G. salaris* have been introduced into Norway by man, most probably from infected hatcheries located around the Baltic Sea (Hansen et al. 2003). Further distribution within Norway is believed to be through the movement of salmon parr and smolts used for restocking of rivers (Johnsen and Jensen 1986, Johnsen et al. 1999), and through natural brackish water dispersal through fiord systems (Lund and Heggerget 1992, Soleng et al. 1998, Johnsen et al. 1999).

Approximately 400 *Gyrodactylus* species have been described, 29 species of which are recorded on salmonids worldwide (Bakke et al. 2002). Traditionally, species of *Gyrodactylus* are identified on the basis of subtle differences in the shape and size of the opisthaptor sclerites (Mo 1991a, b, c, Shinn et al. 2000, 2001), the phenotype of which may be dependent on environmental factors such as temperature (Mo 1991a, c). Based on the excretory system, anchors and ventral bars, Malmberg (1970, 1993) divided the *Gyrodactylus* species into subgenera and species-groups, respectively. Malmberg also separated from the *wageneri*-group a *G. salaris*-group which he further divided into three subgroups of which *G. salaris* and *G. thymalli* Žíthán, 1960 belong to two of the subgroups. The recently described *G. caledoniensis* Shinn, Sommerville et Gibson, 1995 from salmon and rainbow trout in UK belongs to Malmberg’s *G. wageneri*-group (A. Shinn, unpub. observations). Later, however, based on molecular genetics, Cable et al. (1999) compared Malmberg’s (1970) phylogeny of the genus and found no support that *G. salaris* is a member of a separate species-group as it clustered within the *G. wageneri*-group which could be further subdivided into groups with different host preferences. In addition, Matějusová et al. (2003) suggested that the existing subgenera should be abandoned as the excretory system seems not to be conservative enough to reveal subgenera.

Several studies have used the silver nitrate staining technique of Lynch (1933) to describe the sensory sensilla in both larval (Combes and Lambert 1975, Lambert 1977a, b, 1978a, b, Tinsley 1978) and adult monogeneans (Lambert 1979, El-Naggar et al. 1993, 2001, Khidr 1977a, b, 1978a, b, Tinsley 1978) and adult monogeneans (Lambert 1979, El-Naggar et al. 1993, 2001, Khidr 1977a, b, 1978a, b, Tinsley 1978). This paper was presented at the 6th International Symposium on Fish Parasites in Bloemfontein, South Africa, 22–26 September 2003.
and El-Naggar 1996). Chaetotaxy has also been used to examine the phylogenetic position of *Gyrodactylus* within the Monogenea (Shinn et al. 1998a). Shinn et al. (1997, 1998b) applied chaetotaxy to differentiate *Gyrodactylus* species belonging to the *G. wagneri*-group parasitizing British salmonids besides two *G. salaris* populations (Swedish River Ätran and Norwegian River Lierelva), and proposed a formula for the arrangement of the natural aggregations of sensilla. They concluded that chaetotaxy had potential as a relatively simple and reliable method for distinguishing species of the genus.

In a molecular genetic study based on mtDNA (COI) sequences from *G. salaris* and *G. thymalli* populations in Norway and Sweden (Hansen et al. 2003), it was shown that these species could be separated into three and two clades, respectively. Each clade was found to be unique in having a different invasion history and pathogenesis to salmon (Bakke et al. 2002, Sterud et al. 2002). Hence, there is an urgent need to find morphological markers which can readily separate specimens into their respective species and clades. The present study aims to investigate the use of chaetotaxy to differentiate between *G. salaris* populations (on Atlantic salmon) and *G. thymalli* (on *Thymallus thymallus*) representing different clades and species-groups, and *G. caledoniensis* on *S. salar*, River Allan, representing a different subgroup.

**MATERIALS AND METHODS**

Specimens of *Gyrodactylus salaris* were collected from infected Atlantic salmon (*Salmo salar*) parr sampled by electrofishing in three different Norwegian localities: Rivers Lierelva and Drammenselva, Buskerud County, and River Rauma, More and Romsdal County. *G. salaris*-infected parr (infected fins from the River Rauma) were transported to the Zoological Aquarium Unit at the University of Stirling (IA), University of Stirling (Table 1).

The fish with the three Norwegian strains of *G. salaris*, and those with *G. thymalli*, were kept in separate tanks and boxes with a wire mesh bottom, floating within a larger tank, respectively. The Norwegian populations were supplied with charcoal-filtered, de-chlorinated water adjusted to ca. 10–12°C. The *G. caledoniensis*-infected salmon were kept in tanks supplied with water from the River Allan adjusted to ca. 10°C.

Infected fish examined for gyrodactylids after being anesthetized with 0.04% chlorobutanol for ca. 2 min were killed and fins with *Gyrodactylus* spp. were excised and washed in 2.0 M phosphate buffer (Na$_2$HPO$_4$·12H$_2$O + Na$_2$HPO$_4$·2H$_2$O) at pH 7.2 to remove excess mucus prior to staining. The gyrodactylids were processed for chaetotaxy *in situ* on the fins: the infected fins were placed directly into 2.0% silver nitrate (AgNO$_3$) at 65–70°C in the dark for 5 min (modified from Shinn et al. 1997, 1998b). Afterwards, the fins were carefully washed in 5–10 changes of distilled water. The fins were then submerged in distilled water and each side of the fin exposed to UV light (325 nm) for 5 min. After UV exposure, the fins were again washed to remove any host mucus (which takes up silver stain) before being transferred to a solution of 10% glycerine and 90% ethanol. The ethanol was allowed to evaporate, leaving the fins in glycerine. Only gyrodactylids with an empty uterus were selected and mounted in glycerine on a glass slide. Glycerine as a mountant made it possible to manipulate the parasite into a position for mapping each sensillum and to maximise the number of sensilla visible in any focal plane. Precautions were taken not to displace sensilla through excessive coverslip pressure when rolling. The slides were stored in the dark in a refrigerator at 4°C.

For scanning electron microscopy (SEM), fins were fixed for 24 h at 4°C in 2.0% glutaraldehyde buffered to pH 7.4 with 0.1 M sodium cacodylate buffer, post-fixed in 1–2% osmium tetroxide (OsO$_4$) for 1 h at 20°C. Subsequently, the fins were dehydrated through a graded ethanol series and dried in a Balzer critical-point-dryer using liquid CO$_2$, mounted on aluminium stubs and sputter coated (Polaron) with gold-palladium and examined in a JEOL JSM 6400 scanning electron microscope, operating at 10 kV. Parasites fixed for transmission electron microscopy (TEM) were individually fixed overnight in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, at 4°C. Thereafter, the specimens were rinsed in the same buffer (2 changes of 10 min each) at room temperature and post-fixed in 1% osmium tetroxide with 1.5% potassium ferricyanide (Sigma) for 1 h in the dark. The specimens were then rinsed 5 times in distilled water (10 min) before being stained in 1.5% uranyl acetate (Merck) in distilled water for 30 min in the dark. Following dehydration in ethanol, the parasites were placed in propylene oxide (Fluka) (2 changes of 10 min each) and infiltrated in Epon 812 resin (Fluka) overnight.

Table 1. A summary of the *Gyrodactylus* species, populations and numbers analysed by chaetotaxy (number examined: dorsal surface, right/left sides – ventral surface, right/left sides).

<table>
<thead>
<tr>
<th>Parasite species</th>
<th>Number examined</th>
<th>Host species</th>
<th>River localities</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>G. salaris</em> Malberg, 1957</td>
<td>45/49 – 50/50</td>
<td><em>Salmo salar</em></td>
<td>Lierelva, Buskerud County, Norway</td>
</tr>
<tr>
<td><em>G. salaris</em> Malberg, 1957</td>
<td>17/16 – 9/8</td>
<td><em>Salmo salar</em></td>
<td>Drammenselva, Buskerud County, Norway</td>
</tr>
<tr>
<td><em>G. salaris</em> Malberg, 1957</td>
<td>48/48 – 47/47</td>
<td><em>Salmo salar</em></td>
<td>Rauma, More and Romsdal County, Norway</td>
</tr>
<tr>
<td><em>G. thymalli</em> Zittel, 1960</td>
<td>18/15 – 13/8</td>
<td><em>Thymallus thymallus</em></td>
<td>Rena, Hedmark County, Norway</td>
</tr>
<tr>
<td><em>G. caledoniensis</em> Shinn, Sommerville et Gibson, 1995</td>
<td>32/31 – 31/30</td>
<td><em>Salmo salar</em></td>
<td>Allan, Perthshire, Scotland</td>
</tr>
</tbody>
</table>
Two regions with sensilla that were found to be unreliable were omitted from further analyses: (i) the area around the cephalic lobes (the rounded shape and high concentration of sensilla made a rigorous assessment of the number and position of sensilla difficult); (ii) the lateral area (the dorso-ventral compression made the precise number and position of sensilla on the lateral margins difficult to assess). In addition, the opisthaptor had a tendency to twist as the specimen was rolled into the correct orientation. This made the assessment of the number of sensilla and the definition of their position on the opisthaptor difficult to define (even if only one sensillum cluster was defined) (Fig. 5). Also, the infiltration of silver nitrate into the openings of the marginal hook could easily be mistaken for sensilla. All remaining sensilla, however, showed a consistent bi-lateral symmetrical pattern around the longitudinal median axis with the highest density of sensilla being in the circum-oral and opisthaptor peduncle regions.

The sensilla could be grouped into clusters consisting of between three to ten sensilla (Fig. 5). However, a relatively large variation was observed in occurrence of the individual sensilla within the clusters in the populations. The frequency of occurrence of each stained sensillum was determined for each population. Only sensilla that were stained with a frequency $\geq 70\%$ were used for the subsequent comparisons. Any displacement of these sensilla observed was determined. The River Rauma population had the highest level of displaced sensilla (1.6%) compared to the populations of *G. salaris* from the Rivers Lierelva and Drammenselva (1.1% and 0.9%, respectively). In *G. thymalli* specimens, the percentage displacement was 0.9%. The displacement of sensilla, in each population, was evenly dispersed throughout the clusters.

The analyses of the five populations of *Gyrodactylus* revealed that the number of sensilla within each of the defined clusters on the dorsal and ventral side for each species was the same (Table 2). There were 34 sensilla grouped into 7 clusters on the dorsal surface. The dorsal-anterior region comprised three clusters (DA1–DA3) of 5, 4 and 5 sensilla, respectively, the latter cluster of 5 sensilla (DA3) being situated close to the excretory pore (Fig. 5). The remaining dorsal-body clusters (DB1–DB3) were found to have a consistent number of 4, 4 and 8 sensilla, respectively. Ventrally, all populations had a total of 44 sensilla grouped into 8 clusters (Table 2). The ventral-anterior region harbours three clusters (VA1–VA3) of 4, 10 and 7 sensilla, respectively. The first cluster (VA1) is situated medio-laterally in close proximity to the anterior lobes, whilst the VA2 cluster which possesses the largest number of sensilla is situated towards the lateral margins. The third cluster (VA3) is composed of seven sensilla in a characteristic zigzag pattern close to the median axis (at approximately the same longitudinal position as the VA2 cluster) with 5 sensilla anterior and 2 sensilla posterior.
Fig. 1. Scanning electron micrograph (SEM) of the cephalic lobes of Gyrodactylus salaris in close proximity to the epidermis of its host, an Atlantic salmon (Salmo salar). The picture highlights the concentration of sensilla within the region. Two types of sensilla can be seen: the spike sensilla and the club-shaped sensilla. Scale bar = 10 µm.

Fig. 2. Scanning electron micrograph (SEM) of the sensilla at the anterior end of Gyrodactylus salaris demonstrating two other types of sensilla (see Fig. 1): the tapering and the common body sensilla. Scale bar = 1 µm.

Fig. 3. Transmission electron micrograph (TEM) showing a cross-section through a sensillum and its innervation within the tegument of Gyrodactylus salaris. Scale bar =1 µm.

to the oral pore (Fig. 5). The remaining four clusters (VB1–VB4) on the ventral surface have 5, 3, 8 and 4 sensilla, respectively. The second cluster (VB2) of three sensilla showed a characteristic pattern in line in the middle close to the longitudinal axis of the worm. The posterior-most cluster (VB4) is situated close to the peduncle of the body proper. The sensilla on the ventral side of the opisthaptor were difficult to map for the reasons already stated, however, when the sensilla were discernible, a set of two sensilla situated on the posterior edge of the opisthaptor and one anterior-lateral sensillum, could be observed (Fig. 5).

The general chaetotaxy map produced from stained specimens of G. salaris was used as the standard by which the other G. salaris populations were compared (Fig. 5). No significant differences in the chaetotaxy pattern were observed between the populations of G. salaris (Table 2, Fig. 5). However, a higher percentage of sensilla on G. salaris specimens from the River Rauma population were observed to be duplicated or displaced. Specimens from the Lierelva population were only occasionally observed to have a displaced sensillum, and when it was seen it was usually towards the lateral margins (e.g. sensillum 1 in DA3 and sensillum 4 in DB1; see Fig. 5).

The specimens of G. thymalli had the same number of sensilla and a similar chaetotaxy pattern to that observed on the G. salaris specimens. No significant
Fig. 4. Light micrograph (LM) displaying the distribution of sensilla around the oral aperture of *Gyrodactylus salaris* from River Lierelva, Norway. The sensilla, when stained with silver nitrate, appear as black structures with a symmetrical distribution along the longitudinal axis of the worm (long arrow). The rectangles indicate the sensilla belonging to the VA2 and VA3 clusters. The small arrows point to sensilla within the respective clusters that are out of focal range or are poorly stained as also occasionally observed. Scale bar = 5 µm.

Differences were registered between these clades representing two different species. Specimens of *G. caledoniensis* likewise had the same number and a similar arrangement of the ventral sensilla as *G. salaris* (Table 2, Fig. 5). However, two significant differences were recognized on the dorsal surface (Fig. 6, shaded regions): in cluster DB2, the two most medially positioned sensilla have a reverse configuration, and in cluster DB3 the distance between the 5th and 6th sensilla is consistently shorter than that observed in the *G. salaris* specimens.

**DISCUSSION**

The definition of sensillum clusters was assigned after visual assessment of the sensillum arrangements after staining and, therefore, may not correspond with the underlying branching structure of the nerve system. Shinn et al. (1997) suggested that the ventro-median cluster of 3 × 2 sensilla mirrored the 3 × 2 perikarya of the dorsal nerve cords that Reuter (1987) stained with the antisera to FMRF-amide in a study of neuroactive substances in *G. salaris*. The sensilla are obviously innervated as shown in the present study, but the sensillum associations with the underlying nervous system and its commissures remain to be established on gyrodyctyldis. SEM observations on *G. salaris* revealed the presence of different types of sensilla, which suggests that each type has a different function responding to either physical or chemical stimuli. It is interesting to note that the sensilla on the dorsal surface have a more even distribution than on the ventral surface. The high number of sensilla distributed ventrally around the oral pore and the region of the penis probably indicates that the sensilla serve to orientate the gyrodyctyloid during feeding and copulation. These sensilla may in all likelihood have a different function to those sensilla distributed around the cephalic lobes, which must play a crucial role when transmitting between hosts and moving over the host’s epidermis. In addition to the sensillum types mentioned here, Watson and Rohde (1994) found two other receptors which they suggested might be light sensitive, but not linked to any external structures.

A variety of approaches have been used to identify and discriminate different species of *Gyrodactylus*. Such studies have included the use of morphology using light (e.g. Malmberg 1970, Shinn et al. 1995) and scanning electron microscopy (e.g. Mo and Appleby 1990, Shinn et al. 1993), molecular-based methodologies (e.g. Cunningham et al. 1995a, b, Sterud et al. 2002, Zietara and Lumme 2002, Hansen et al. 2003), and ecological data such as host preferences (e.g. Malmberg 1970, see Bakke et al. 2002). Concerning chaetotaxy, the sensillum pattern is supposed to remain consistent throughout life because of the almost mature progeny when born. Based on this premise, Shinn et al. (1997, 1998a, b) assessed for the utility of chaetotaxy in gyrodyctyloid sys-
Fig. 5. Chaetotaxy map illustrating the distribution and approximate organisation of each coded cluster of sensilla on a generalised Gymodactylus sp. infecting Norwegian salmon and grayling. The sensilla within each cluster are linked with lines for clarity. A – ventral side; B – dorsal side. (See Table 2 for abbreviations.)

Fig. 6. Chaetotaxy maps showing the difference in arrangement of sensilla within clusters DB2 (shaded area) and DB3 (shaded area). A – Gymodactylus salaris from the River Lierelva; B – G. caledoniensis from the River Allan, Scotland.

The present study, however, suggests that chaetotaxy is not generally applicable for the discrimination of gyrodactylids occupying different, but closely related clades.
Table 2. The number of sensilla recorded within each cluster. Only the numbers for the right side are presented as the sensilla are symmetrically distributed along the longitudinal axis of the worm (see Fig. 4–6). The three Gyrodactylus salaris populations, and G. thymalli and G. caledoniensis had the same numbers of sensilla within each cluster. Cluster acronyms: DA, dorsal anterior; DB, dorsal body; DO, dorsal opisthaptor; VA, ventral anterior; VB, ventral body; VO, ventral opisthaptor (see Fig. 5.)

<table>
<thead>
<tr>
<th>Dorsal Clusters</th>
<th>Number of sensilla</th>
<th>Ventral Clusters</th>
<th>Number of sensilla</th>
</tr>
</thead>
<tbody>
<tr>
<td>DA1</td>
<td>5</td>
<td>VA1</td>
<td>4</td>
</tr>
<tr>
<td>DA2</td>
<td>4</td>
<td>VA2</td>
<td>10</td>
</tr>
<tr>
<td>DA3</td>
<td>5</td>
<td>VA3</td>
<td>7</td>
</tr>
<tr>
<td>DB1</td>
<td>4</td>
<td>VB1</td>
<td>5</td>
</tr>
<tr>
<td>DB2</td>
<td>4</td>
<td>VB2</td>
<td>3</td>
</tr>
<tr>
<td>DB3</td>
<td>8</td>
<td>VB3</td>
<td>8</td>
</tr>
<tr>
<td>DPH1</td>
<td>4</td>
<td>VPH1</td>
<td>3</td>
</tr>
<tr>
<td>Total</td>
<td>34</td>
<td></td>
<td>44</td>
</tr>
</tbody>
</table>

Shinn et al. (1997) observed two different types of sensilla on silver nitrate-stained gyroactylid specimens, one large dark type that conformed to a pattern symmetrical along the median longitudinal axis, and a second smaller, randomly distributed type that was not included in the chaetotaxy formulae. In the present study, only the sensilla which appeared as dark rings were mapped. These sensilla were in general agreement with those mapped by Shinn et al. (1997, 1998a, b) which had an occurrence ≥70%. However, the present defined clusters and coding of sensilla differ from the previous system but attempt to increase the utility of chaetotaxy as a diagnostic method. Shinn et al. (1998b) observed a total of 154 sensilla on G. salaris from the River Lierelva (36 dorsally, 41 ventrally, on one side), the current study by comparison found a total of 156 sensilla (34 dorsally, 44 ventrally). Besides, some discrepancies in the position of certain sensilla were noted. The present study has shown that the arrangement of sensilla in the posterodorsal clusters (DB2 and DB3) is the key to separate the present species of Gyrodactylus. In contrast, Shinn et al. (1997) concluded that it was the pattern of sensilla posterior to the pharynx in the anteromedial and ventromedial clusters, that were the most informative in permitting the discrimination of the Gyrodactylus species mapped. These differences between studies, in part, arise from the designation of clusters and coding of sensilla. However, differences resulting from natural changes within the Lierelva population over time cannot be excluded (studies performed 10 years apart). In addition, the results may highlight problems when comparing laboratory isolated strains to wild-caught populations.

In the present study, the Norwegian gyroactylids of different clades and species-groups from Atlantic salmon and grayling were found to be identical, but G. caledoniensis from Scotland differed from G. salaris and G. thymalli by the position of two sensilla (in clusters DB2 and DB3). The results confirm the potential of using chaetotaxy in gyroactylids to discriminate between certain species of Gyrodactylus but not generally. The temporal stability of the chaetotaxy patterns of specific gyroactylids needs to be determined.

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