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The first report of *Gyrodactylus salaris* Malmberg, 1957 (Platyhelminthes, Monogenea) on Italian cultured stocks of rainbow trout (*Oncorhynchus mykiss* Walbaum)

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ABSTRACT

The monogenean *Gyrodactylus salaris* Malmberg, 1957 is considered one of the most important parasites of wild salmonids in the European Community due to the heavy ecological and economical damage it has inflicted on Atlantic salmon (*Salmo salar*) parr populations. Rainbow trout (*Oncorhynchus mykiss*) is susceptible to *G. salaris* and can act as a suitable carrier host and, consequently, its trade in EU territory is restricted in relation to the status of "recognized free" zones. Despite the economic importance of rainbow trout farming in Italy, information on the Italian gyrodactylid fauna is lacking and prior to this study, *G. salaris* had not been officially reported. During a routine health examination of farmed rainbow trout stock throughout Central and Northern Italy in 2004–2005, five fish farms were found to be infected with *G. salaris* alongside three other gyrodactylids. Morphological and molecular characterisation confirmed the presence of *G. salaris*, *Gyrodactylus teuchis* Lautraite, Blanc, Thiery, Daniel et Vigneulle, 1999 and *Gyrodactylus derjavinoides* Malmberg, Collins, Cunningham et Jalali, 2007, while *Gyrodactylus truttae* Gläser, 1974 was identified by morphological analysis only. The findings from this study extend the distribution of *G. salaris* within Europe and highlight the importance of the rainbow trout trade in its dissemination.

1. Introduction

The importance of *Gyrodactylus salaris* Malmberg, 1957 is demonstrated by the heavy losses that this monogenean has caused over the last thirty years in parr and smolt stages of wild Atlantic salmon (*Salmo salar* L.), principally throughout Norway (Johnsen et al., 1999; Bakke et al., 2007). Apart from being a parasite of Atlantic salmon, *G. salaris* can colonise and reproduce on a wide number of salmonid species without clinical signs of disease and these hosts represent important carriers of the parasite (Bakke et al., 2002). In particular, rainbow trout (*Oncorhynchus mykiss* Walbaum) is considered as an ideal reservoir for *G. salaris*, being a very receptive and generally asymptomatic host (Bakke et al., 2002). For this reason, the movement of rainbow trout within the European Community is strictly regulated and is permitted only between regions of equivalent health status (Peeler et al., 2006).

Most species of *Gyrodactylus* can be differentiated by the morphological features of their haptoral hard parts (Malmberg, 1970) and/or by differences in their ribosomal internal transcribed spacer (ITS1 and ITS2) regions

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(Matejusová et al., 2001; Ziętara and Lumme, 2002). The discrimination of *G. salaris* from other related species is, however, not always straightforward. *Gyrodactylus salaris* and the purported non-pathogenic *Gyrodactylus thymalli* Žitňan, 1960 from grayling, *Thymallus thymallus* L., are morphologically similar, their ITS sequences are practically identical, and recent studies indicate that the two species might be conspecific (Ziętara and Lumme, 2002; Hansen et al., 2003, 2006, 2007; Meinilä et al., 2004). Analyses of the mitochondrial cytochrome oxidase I gene (COI), however, show that *G. salaris* and *G. thymalli* can be grouped in different clades and that all the gyrodactylids from rainbow trout appear to belong to the same COI haplotype (Hansen et al., 2003; Meinilä et al., 2004). Although characterisation by COI does not seem to be linked to the virulence of *G. salaris* (Hansen et al., 2007), its determination, however, does provide hints to its possible origin and relationship to other populations within the species. Similarly *Gyrodactylus teuchis* Lautraite, Blanc, Thiery, Daniel et Vigneulle, 1999 was first considered to be morphologically similar to *G. salaris* but it was later shown that it could be characterised as a separate species based on its ITS sequences (Lautraite et al., 1999; Cunningham et al., 2001).

Rainbow trout production in Italy represents a significant proportion of the nation's freshwater production with over 39,000 tons/year (API, 2008), but data on infection of *Gyrodactylus* spp. of farmed salmonids is scarce. With reference to *G. salaris*, Molnár and Ghittino (1977) reported a gyrodactylid from cultured rainbow trout and brown trout (*Salmo trutta* L.) from Italy but no further studies were carried out to confirm the identity of these or to define their distribution throughout the country. The current study, therefore, set out to establish the gyrodactylid fauna on captive held populations of rainbow trout in Northern and Central Italy.

2. Materials and methods

During the period March to May 2005, five Italian rainbow trout farms were visited throughout the Central and Northern regions of Italy and a sample of stock at each site was examined for the presence of *Gyrodactylus* spp. Ten fish, ranging in 10–40 cm total length, were sampled from each site. Fish were euthanased and a representative mucus sample was taken from the body and fins of each fish using a scalpel and then fixed immediately in 70% ethanol for analysis in the laboratory. The fish farms situated on five different water systems were positive for *Gyrodactylus*: the River Sile, Veneto (45°38′23.18″N, 12°08′14.29″E), the Avisio Torrent, Trentino Alto Adige (46°16′42.70″N, 11°26′39.52″E), the River Sérchio, Tuscany (44°02′52.00″N, 10°27′39.74″E), and two sites in the Umbria region, the Clitunno Fountain (42°44′41.95″N, 12°42′24.81″E) and the River Nera (42°51′41.38″N, 12°58′48.84″E) (Fig. 1).

Gyrodactylid parasites were isolated from the fixed mucus and prepared for morphological and molecular analyses. Individual specimens were placed on a glass slide, the haptor was removed using a scalpel and subjected to proteolytic digestion using a modification of the method given in Harris and Cable (2000), *i.e.* 3 μl of digestion solution (100 μg/ml proteinase K (Cat # 4031-1, Clontech UK Ltd., Basingstoke, UK), 75 mM Tris–HCl (Sigma–Aldrich, Poole, UK), 10 mM EDTA (Sigma–Aldrich), 5% SDS (Sigma–Aldrich)) added to each haptor. The digestion of each specimen was continuously monitored under a 4× objective on an Olympus SZ30 dissecting microscope. Tissue digestion was then arrested and mounted *in situ* by the addition of 2 μl of a 1:1 saturated ammonium picrate: 100% glycerine mix. The edges of the coverslip were then sealed with nail varnish.

The digested, ammonium picrate glycerine mounted specimens were photographed using a JVC KY-F30B 3CCD camera with an interfacing 2.5× top lens fitted to an Olympus BH2 compound fitted with phase contrast under a 100× oil immersion objective and features of the hooks measured using Zeiss KS300iC/Windows release ver 3.0 (1997) (Carl Zeiss Vision GmbH, Munchen, Germany/Imaging Associates Ltd., Thame, Oxfordshire, UK) software. For identification, a total of 25 point-to-point morphometrics (11 on the hamulus, 6 on the ventral bar and 8 on the marginal hooks) were made on each specimen (see Shinn et al., 2004) using the purpose written software PointR ver 1.0 (© Shinn and Bron, 2003, University of Stirling, UK) within KS300.

The excised body of each gyrodactylid was transferred to an individual, labelled 1.5 ml Eppendorf tube and stored in 95% ethanol until required. A limited number of specimens from each of the sampled locations were available for molecular analysis. DNA was extracted from individually isolated specimens using the DNEasyKit or Mini Kit (Qiagen) following the manufacturer's instructions. The primer pairs ITS1A (5′-GTAACAAGGTTTCCG-TAGGTG-3′) and ITS2 (5′-TCCTCCGCTTAGTGATA-3′) (Matejusová et al., 2001) were used to amplify a fragment spanning the 3′ end of the 18S subunit, ITS1-5.8S-ITS2 and the 5′ end of the 28S subunit. In instances where this full fragment did not amplify, the primers ITS4.5 and ITS2 (Matejusová et al., 2001) were used to amplify the ITS2 region separately. ITS2 alone contains less variation than ITS1, but nevertheless differs between all species studied herein.

The primer pairs ZMO1 (5′-GCGMCTAAATGCTT-TAAGGGCTTG-3′) and ZMO4 (5′-GAGGATAGCAC-TATCCCTGTCAC-3′) (Hansen et al., 2003) were used to amplify the mitochondrial COI. All PCR reactions were performed with puRe Taq Ready-to-Go PCR beads (Amersham Biosciences) in a GeneAmp PCR System 9700 (Applied Biosystems) using the following protocol: 4 min at 95 °C, followed by 35 cycles of 1 min at 95 °C, 1 min at 50 °C and 2 min at 72 °C.

All PCR-products were purified using a QIAquick PCR Purification Kit (Qiagen) or Macherey-Nagel NucleoSpin® Extract II according to the manufacturer's recommendations. Both DNA strands were sequenced on a MEGABACE 1000 (GE Healthcare) using DyeET-terminator mix (GE-Healthcare) and were carried out in 10 μl reactions. The PCR primers and the internal primers ITS1R (5′-ATTTGCGTTCGAGAGACCG-3′) and ITS2F (5′-TGGTGGAT-CACTCGGCTCA-3′) (Ziętara and Lumme, 2003) were used for sequencing of the full ITS fragment. The ITS2 fragments

Fig. 1. The location of the rainbow trout farms sampled throughout Italy and found to be positive for *Gyrodactylus* von Nordmann. (1) Avisio Torrent, Trentino Alto Adige (46°16′42.70″N, 11°26′39.52″E); (2) River Sile, Veneto (45°38′23.18″N, 12°08′14.29″E); (3) River Sérchio, Tuscany (44°02′52.00″N, 10°27′39.74″E); (4) Clitunno Fountain, Umbria (42°44′41.95″N, 12°42′24.81″E); (5) River Nera, Umbria (42°51′41.38″N, 12°58′48.84″E).

were sequenced using the PCR primers only. Amplified fragments of COI were sequenced using the PCR primers in addition to ZMO2 (5′-CCAAAGAACCAAAATAAGTGTTG-3′) and ZMO3 (5′-TGTCYCTACCAGTGCTAGCCGCTGG-3′) (Hansen et al., 2003). Sequences were proofread in VectorNTI (Invitrogen) and identity established by submitting the sequences to a GenBank BlastN search (http://www.ncbi.nlm.nih.gov/) (Altschul et al., 1990; Zhang et al., 2000). Calculation of genetic distances was performed in Mega 4.0 (Tamura et al., 2007).

In addition, one formalin fixed sample of *Gyrodactylus* was found within the fish pathology archive held by the Department of Veterinary Public Health and Animal Pathology, University of Bologna. The skin scrape, which contained five gyrodactylids, was collected in April 2000 from an unspecified rainbow trout farm within the Veneto region. The gyrodactylids were rinsed in distilled water and then prepared as whole mounts in ammonium picrate glycerine and identified by morphometry and morphology.

The sampling protocols and experimentation conducted throughout the course of the investigation complied with the laws and statutes of Italy and the diagnostic approaches required by OIE (Office International des Epizooties - World Organisation for Animal Health) for the confirmation of *G. salaris* (OIE, 2006).

3. Results

Gyrodactylids were recovered from the mucus scrapes taken from the body and the fins of the fish sampled at each of the five rainbow trout farms that were visited. All fish were found to be infected (100%) with approximately 10 (range 5–16) gyrodactylids being recovered from each site. On the basis of morphological features taken from ~10 parasites per site ($n = 53$ gyrodactylids in total), the following species were identified: *Gyrodactylus derjavinoides* Malmberg, Collins, Cunningham et Jalali, 2007, *G. salaris*, *G. teuchis* and *G. truttae* Gläser, 1974 (Table 1). Not

Table 1
A summary of the methods used to identify the specimens of *Gyrodactylus* von Nordmann, 1832 collected from the five Italian rainbow trout farms visited during the current study.

Region	*G. derjavinoides*	*G. salaris*	*G. teuchis*	*G. truttae*
Trentino Alto Adige ($n = 5$)	–	4 M; 1 ITS2[a]; 1 COI[b]	–	1 M; 1 ITS[c]; 1 COI[c]
Tuscany ($n = 16$)	–	16 M; 3 ITS1-2[a]; 3 COI[d]	–	–
Umbria (R. Nera; $n = 15$)	3 M; 2 ITS1-2[e]; 1 COI[c]	2 M; 1 ITS1-2[f]; 1 COI[g]	10 M; 3 ITS1-2[h]; 1 COI[c]	–
Umbria (Clitunno Fountain; $n = 11$)	4 M	2 M	5 M	–
Veneto (R. Sile; $n = 6$)	–	6 M; 3 ITS2[a]; 2 COI[b]	–	–
Veneto (archive sample; $n = 5$)	–	5 M	–	–

In addition, one *Gyrodactylus* positive sample collected from an unspecified rainbow trout farm dated April 2000 and deposited in the University of Bologna fish tissue archive was also examined. Each specimen was identified initially by morphology and morphometry (M) and then, where possible, confirmed by comparing the base sequence of its cytochrome oxidase 1 gene (COI) and the internal transcribed spacer 1 and 2 regions (ITS1-2) with sequences held in NCBI GenBank (http://www.ncbi.nlm.nih.gov/).

[a] 100% ITS identity with NCBI acc. nos. Z72477, DQ916137, DQ919059, AF484544 and AF328871.
[b] 100% COI identity with haplotype F from rainbow trout (Hansen et al., 2003).
[c] Not amplified.
[d] Corresponds to haplotype F from rainbow trout (Hansen et al., 2003).
[e] 100% ITS identity with NCBI acc. no. AF484530.
[f] 100% ITS identity with NCBI acc. nos. DQ919059 and AF328871.
[g] New haplotype (acc. no. GQ370816).
[h] 100% ITS identity with NCBI acc. no. AJ249350.

every species was found at all farms; *G. salaris,* however, was present on the stock at all five farms, notably Tuscany and Veneto where *G. salaris* was the only gyrodactylid species found. *Gyrodactylus teuchis* was identified from the two farm sites located in the Umbria region (Clitunno Fountain and River Nera), alongside *G. salaris* and *G. derjavinoides*. *Gyrodactylus truttae* was found in Trentino Alto Adige (Avisio Torrent) together with *G. salaris*. As the morphology of the attachment hooks of *G. salaris* and *G. teuchis* are similar and photographic images of the latter have not been formerly presented elsewhere, figure plates of these alongside *G. derjavinoides* and *G. truttae* are provided to assist in their future identification and discrimination (Figs. 2 and 3).

From Figs. 2 and 3, the four species of *Gyrodactylus* can be readily discriminated from each other based on the unique shape of the marginal hook sickle. As all specimens were collected in the same season (March–May 2005) and from water bodies of a similar temperature (~11–12.5 °C), the size of the attachment hooks of the four species can be compared. *Gyrodactylus salaris* is the largest of the four species, the total length of the hamuli and the marginal hooks were ~76 μm and ~39.5 μm respectively compared to ~68 μm and ~36 μm for *G. teuchis*, ~63 μm and ~23.5 μm for *G. truttae*, and 55 μm and ~33 μm for *G. derjavinoides*.

Although frequently drawn, relatively few photographic images of the male copulatory organ (MCO) exist within the literature; those of *G. derjavinoides*, *G. salaris* and *G. teuchis* are presented for the first time. Only one specimen of *G. truttae* was found in the current study but this individual was prepared for molecular analysis and therefore there was no opportunity to look at the configuration of spines on the MCO. The armature of the MCO, however, in the former three species are different from each other. The MCO of *G. salaris* which measures approximately 30 μm in diameter, bears a single arch of 6–7 spines (2 large, terminal ~6.2 μm long and 4–5 medium-sized ~5.3 μm long central spines). In addition, 4–6 small, circular studs (~1.2 μm in diameter), the precise structure of which have not been formally described, are observed scattered around the main arch of spines but are not in any set, discernible configuration (Fig. 2f–h). The MCO of *G. teuchis*, which measures ~25 μm in diameter, bears 4–5 similar, large-sized spines (~5 μm long) arranged in a single arch with 1–2 visible small, circular studs (Fig. 3f and g). The MCO of *G. derjavinoides* measures 26 μm in diameter and bears 8 spines in a single arch (2 medium-sized, terminal spines ~4.4 μm long and 6 smaller-sized ~3.2 μm long central spines). No circular studs were observed (Fig. 3j).

Only fourteen specimens were available for molecular characterisation by sequencing of the ITS1 and ITS2 or ITS2 separately (Table 1). From these samples, *G. salaris*, *G. teuchis* and *G. derjavinoides* were confirmed. Only one specimen of *G. truttae* was available for the molecular analysis and no sensible sequence reads were obtained from it. For the seven specimens identified as *G. salaris* by morphological analysis or by sequencing of ITS, the base sequence of their cytochrome oxidase I genes were also determined. Six of the sequences corresponded to the mitochondrial F haplotype that is common in rainbow trout farms across Europe (see Meinilä et al., 2004; Hansen et al., 2003, 2006, 2007) and three of these sequences contained some ambiguities that could be the result of PCR or sequencing errors. The last sequence represents a new haplotype of *G. salaris* and is submitted under GenBank accession number GQ370816. This haplotype (774 bp) differs from haplotype F with 11 nucleotide substitutions (K2-distance: 0.0147) and is not identical to any other currently known haplotypes. The most closely related sequences in GenBank are AY225307 and AY225308 (5 nucleotide substitutions).

4. Discussion

Although gyrodactylosis represents a common and economically significant parasitic disease of rainbow trout

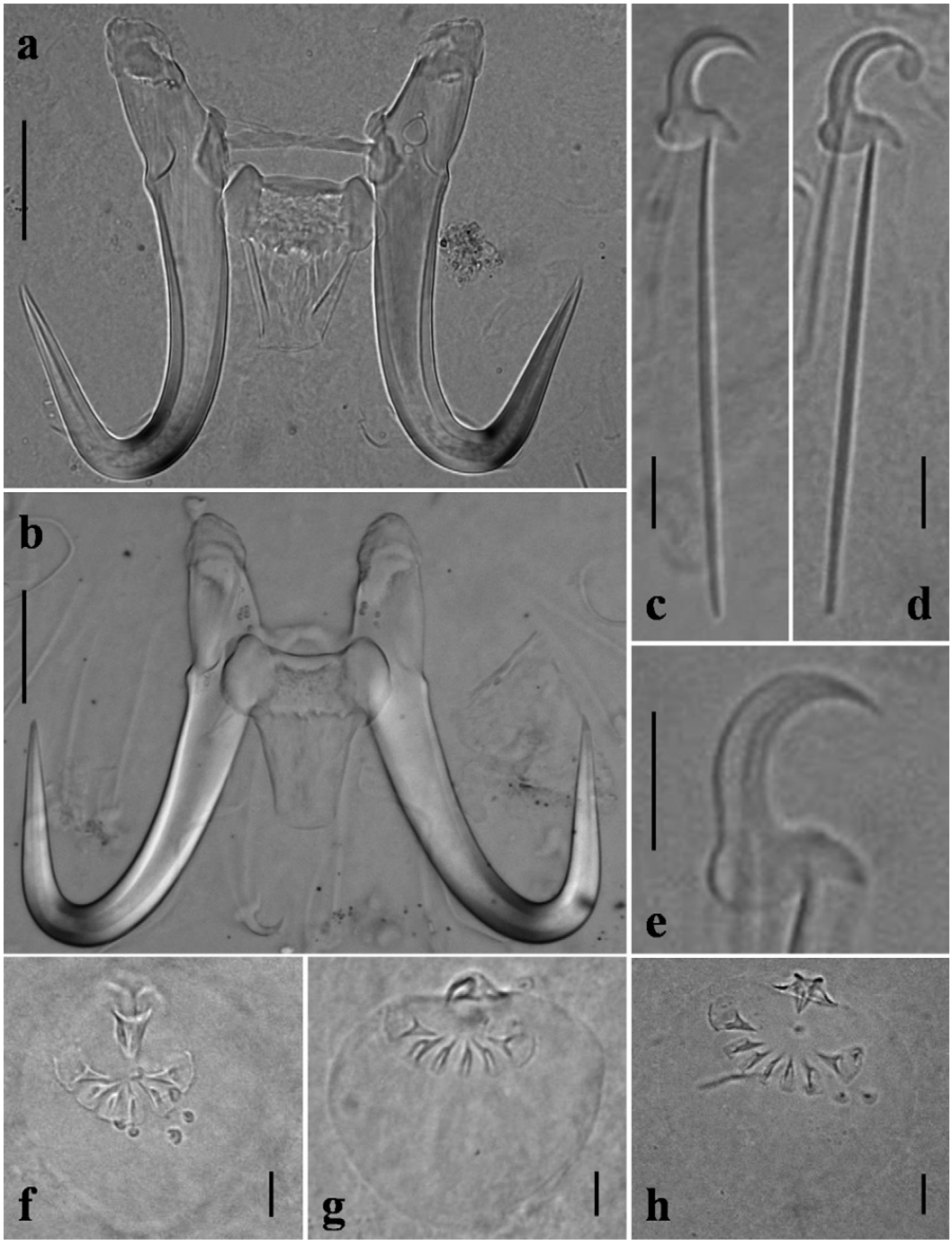

Fig. 2. Light micrographs of the haptoral hard parts and male copulatory organ of *Gyrodactylus salaris* Malmberg, 1957. (a and b) Central hook complex; (c–e) marginal hooks; (f–h) male copulatory organ. Scale bars, a and b = 20 μm; c–h = 5 μm.

farmed in Italy (Fioravanti and Caffara, 2007), studies aimed at identifying the *Gyrodactylus* species involved in its aetiology have been scarce. Molnár and Ghittino (1977) commented on the occurrence of a gyrodactylid "morphologically like *G. salaris*" on cultured rainbow trout and brown trout, but prior to the current study, this report was not confirmed and the figures that were presented in the earlier account do not permit a definitive identification. *Gyrodactylus derjavinoides* (cited as *G. derjavini* Mikhailov, 1975), however, is already known from Italy and has been reported from Italian brown and rainbow trout (Malmberg, 1993).

Malmberg and Malmberg (1993) suggested that *G. salaris* originated in the Baltic area; it is known to occur naturally at low intensities in this area including the Russian Onega and Ladoga water systems and within some Swedish and Finnish rivers that drain into the Baltic Sea (Ieshko et al., 1996; Shulman et al., 2000). Throughout Europe, *G. salaris* has also been reported from Norway (Johnsen and Jensen, 1991; Johnsen et al., 1999), from rivers on the Swedish west coast (Malmberg and Malmberg, 1993; Alenäs, 1998), Denmark (*e.g.* Buchmann and Bresciani, 1997; Buchmann et al., 2000), Finland (Rimaila-Pärnänen and Wiklund, 1987; Keränen et al., 1992; Koski

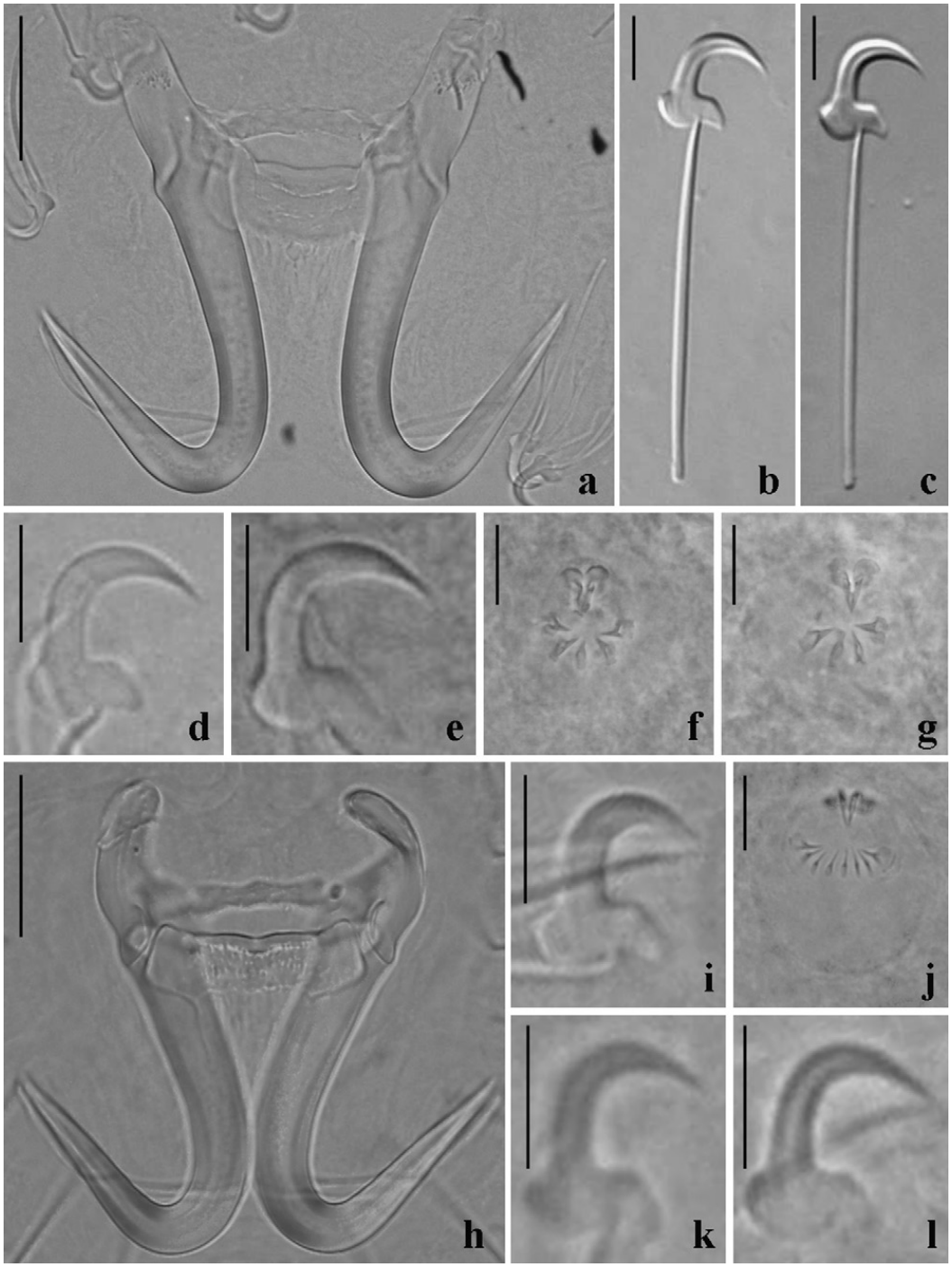

Fig. 3. Light micrographs of the haptoral hard parts of *Gyrodactylus teuchis* Lautraite, Blanc, Thiery, Daniel et Vigneulle, 1999 (a–g), *Gyrodactylus derjavinoides* Malmberg, Collins, Cunningham et Jalali, 2007 (h–j) and *Gyrodactylus truttae* Gläser, 1974 (k and l). *Gyrodactylus teuchis*: a, central hook complex; b and c, marginal hooks; d and e, marginal hook sickles; f and g, male copulatory organ; *G. derjavinoides*: h, central hook complex; i, marginal hook sickle; j, male copulatory organ; *G. truttae*: k and l, marginal hook sickles. Scale bars, a, h = 20 μm; f–g, j = 10 μm; b–e, i, k–l = 5 μm.

and Malmberg, 1995; Koski, 1996; Rintamäki-Kinnunen and Valtonen, 1996), Russia (Ergens, 1983; Ieshko et al., 1996, 1997; Meinilä et al., 2004), Germany (Lux, 1990; Dzika et al., 2009), Spain (Malmberg, 1993), France (Johnston et al., 1996) and, most recently, from Poland (Rokicka et al., 2007). While the presence of *G. salaris* has been confirmed by molecular methods for many of these, the reports from certain countries, *i.e.* France, Spain and Portugal, awaits verification.

In addition to Atlantic salmon, *G. salaris* has also been recorded in the wild from other salmonids such as Arctic charr (*Salvelinus alpinus* L.) (see Table 2 in Bakke et al., 1992; Robertsen et al., 2007) and Adriatic trout (*Salmo obtusirostris* Heckel) (see Žitňan and Cankovic, 1970). Although brown trout has a limited susceptibility to *G. salaris* (Mo, 1988; Jansen and Bakke, 1995), it has nevertheless been reported on this host in the wild on several occasions (*e.g.* Mo, 1988). It is also common in many

rainbow trout farms across Europe (Lux, 1990; Koski and Malmberg, 1995; Meinilä et al., 2004). In addition to these salmonid hosts, *G. salaris* has also experimentally been shown to attach and survive for a short period on some non-salmonid fish species such as the eel (*Anguilla anguilla* L.) and the flounder (*Platichthys flesus* L.) (see Mo, 1987), which may act as transport hosts (see Table 2 in Bakke et al., 1992).

The *G. salaris* findings from the current study, however, prompted a detailed study of preserved fish material from farm archives dating back to 2000. The formalin fixed *Gyrodactylus salaris* specimens were identified solely on hook morphology suggesting that this species may have been in the country for the past nine years. This latter finding has provided the impetus for a larger study of gyrodactylids on Italian salmonids which is currently underway.

Only one specimen of *G. truttae* from Trentino Alto Adige (Avisio Torrent) was found in the current study. Given the small number of fish that were sampled from each site and the sampling strategy that was used (*i.e.* skin scrapes), the likelihood of *G. truttae* occurring on stock held at the other farms cannot be ruled out. *Gyrodactylus teuchis* has previously been reported in France, sporadically in Denmark and the UK and appears to be common on rainbow trout in Polish fish farms (Lautraite et al., 1999; Cunningham et al., 2001; Rokicka et al., 2007). Lautraite et al. (1999) found *G. teuchis* to be widely distributed on both wild and farmed salmonids from Brittany to the Western Pyrénées, and the current survey now extends its distribution into Italy. In the current study, *G. teuchis* was found at two sites in Central Italy and, on both occasions, in association with *G. salaris*.

This study represents the first confirmed presence of *G. salaris* in Italy, which was the most commonly encountered gyrodactylid species on farmed rainbow trout and this extends the reported range of this parasite in Europe. Identification of most of the *G. salaris* specimens as haplotype F (Table 1), which are common in rainbow trout farms, provides supporting evidence to suggest that *G. salaris* has mainly been spread via the rainbow trout trade rather than from the local indigenous fish population. The finding of a new haplotype on rainbow trout is not surprising as several haplotypes have been recovered from salmon and grayling (Hansen et al., 2003, 2006, 2007; Meinilä et al., 2004). Further investigation, however, is needed to ascertain whether this infection originates from rainbow trout introduced to the farm or from wild fish in the River Nera. This survey together with recent studies on gyrodactylids in rainbow trout farms in Europe (*e.g.* Rokicka et al., 2007; Dzika et al., 2009) points to the importance of this industry for spreading of *G. salaris* in Europe. It seems more than likely that the examination of rainbow trout farms in other countries will extend the range further.

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