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Gyrodactylus orecchiae sp. n. (Monogenea: Gyrodactylidae) from farmed populations of gilthead seabream (*Sparus aurata*) in the Adriatic Sea

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Abstract: *Gyrodactylus orecchiae* sp. n. (Monogenea, Gyrodactylidae) is described from the skin, fins, eyes and gills of juvenile *Sparus aurata* L. (gilthead seabream) following two outbreaks of gyrodactylosis amongst stocks held in inshore floating cages on the Adriatic coast of Albania and Croatia. Fish were heavily infected (1000+ gyrodactylids/fish) with *G. orecchiae* which reportedly resulted in ~2–10% mortality amongst the infected stock. Morphologically, the haptoral hooks of *G. orecchiae* most closely resemble those of *Gyrodactylus arcuatus* Bychowsky, 1933 in the approximate shape of the ventral bar with its pronounced ventral bar processes and marginal hook sickles which possess a square line to the inner edge of the sickle blade and large rounded heels. The marginal hooks are also morphologically similar to those of *Gyrodactylus quadratidigitus* Longshaw, Pursglove et Shinn, 2003 and *Gyrodactylus colemanensis* Mizelle et Kritsky, 1967, but *G. orecchiae* can be readily discriminated from all three species by the characteristic infolding of the hamuli roots and the shape of the marginal hook sickle. Molecular sequencing of the ITS1, 5.8S, ITS2 regions (513+157+404 bp, respectively) of *G. orecchiae* and alignment with other gyrodactylids for which these same genomic regions have been determined, suggests that this is a new species. No similarities were found when the ITS1 region of *G. orecchiae* was compared with 84 species of *Gyrodactylus* available on GenBank.

Key words: Monogenea, *Gyrodactylus orecchiae*, gilthead seabream, *Sparus aurata*, Croatia, Albania

Monogeneans, notably infections with the microcotylid *Sparicotyle chrysophrii* (van Beneden et Hesse, 1863) and the diplectanid *Furnestinia echeneis* (Wagener, 1857), are commonly encountered in both cultured and wild populations of gilthead seabream *Sparus aurata* L. (Sparidae) within the Mediterranean (Euzet 1984, Radujkovic and Euzet 1989, Di Cave et al. 1998, Varriale and Baroncelli 1998, De Liberato et al. 2000). Of these, the report by De Liberato et al. (2000) and a chemotherapy study by Santamarina et al. (1991) also refer to the presence of an unidentified *Gyrodactylus* von Nordmann, 1832 on *S. aurata*. During 2005–2006, routine diagnostic sampling of inshore floating gilthead seabream cages at Orikum, Albania and Ugljan Island, Croatia revealed heavy infections with gyrodactylids on the skin and gills of juvenile stock. Infected fish were observed to be hypermelanotic, lethargic, anorexic and displayed a progressive loss of weight. Stock mortality was determined to be 2–5% within the inland-based farm at Orikum, rising to 10% in the floating cages at the same location. Looking at the on-line database "GyroDb" (www.gyrodb.net, Harris et al. 2008), only one other gyrodactylid, *Gyrodactylus alviga* Dmitrieva et Gerasev, 2000, is known to parasitize the sparids *Diplodus annularis* (L.) and *Sarpa salpa* (L.) from the Black Sea (Dmitrieva and Gerasev 2000). Given the increasing importance of *S. aurata* in the Mediterranean as a species for aquaculture (86,700 tonnes in 2006; FAO/GLOBEFISH 2007), this study was undertaken to describe a new species of *Gyrodactylus* using molecular, light and scanning electron microscopy techniques.

MATERIALS AND METHODS

Collection of material and morphological determination. At each of the two farm sites (Fig. 1), approximately 20 juvenile *S. aurata* (weight ca. 5–10 g) were randomly sampled from several cages, killed by pithing and then fixed immediately in 70%

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Fig. 1. *Gyrodactylus orecchiae* sp. n. sample sites within the Adriatic. 1 – Ugljan Island, Croatia (44°7'45.87"N, 15°6'9.77"E); 2 – Orikum, Albania (40°18'50.92"N, 19°28'32.93"E).

ethanol. On return to the laboratory at the University of Bologna, the fish were screened using an Olympus SZ40 stereomicroscope at ×4 magnification and specimens of *Gyrodactylus* were removed using mounted triangular surgical needles (size 16, Barber of Sheffield, UK). All fish (*i.e.* 2 sites; n = 40 fish screened) were found to be infected with mean intensities in excess of 1,000 gyrodactylids per fish; no other metazoan parasites were detected. A further five fish from each site were processed for histology following standard procedures.

Parasite specimens were washed in distilled water and representatives prepared as whole mounts by clearing them in ammonium picrate glycerine following the procedure detailed by Malmberg (1970). A further 40 worms were removed, washed in distilled water and then digested on glass slides using a modification of the proteolytic method given in Harris and Cable (2000) and then mounted in ammonium picrate. The haptoral hooks of ten specimens were digested on 11 mm round glass coverslips, sputter-coated with gold and then examined using a JEOL JSM 5200 scanning electron microscope operating at an accelerating voltage of 25 kV. Five specimens were removed from their hosts, their haptors excised and prepared for proteolytic digestion and morphological study while the bodies were fixed in 95% ethanol for molecular characterisation.

For the morphological study, the haptoral hard parts were studied and drawn at magnifications of ×40 and ×100 oil immersion from images grabbed using a Zeiss AxioCam MRc digital camera interfacing with an Olympus BH2 compound microscope using a ×0.75 lens and MRGrab 1.0.0.4 (Carl Zeiss Vision GmbH, 2001) software. A total of 27 point-to-point morphometric measurements were made on haptoral hooks of each specimen from images grabbed using a JVC KY-F30B 3CCD video camera mounted on an Olympus BH2 microscope using a 2.5 interfacing lens at ×100 oil immersion and KS300 (ver.3.0) (Carl Zeiss Vision GmbH, 1997) image analysis software. The measurements follow those given in Shinn et al. (2004) and are expressed in micrometres as the mean ± standard deviation followed by the range in parentheses, unless otherwise stated.

Molecular characterisation. Sequencing of the ITS1, 5.8S and ITS2 regions of *G. orecchiae* was performed using primers P3b (TAGGTGAACCTGCAGAAGGATCA) and P4 (GTCCGGATCCTCCGCTTATTGAATGC) (Cable et al. 2005) which anneal to the 18S and 28S, respectively. Amplifications were carried out in a Perkin Elmer thermocycler (9700) using an initial denaturation of 95 °C, followed by 35 cycles of 94 °C 30 s, 50 °C 1 min, 72 °C 2 min and a final extension of 72 °C 10 min. PCR products were purified using Exonuclease *I* and SAP (Shrimp Alkaline Phosphatase) (Biolabs) and both strands were sequenced using BigDye (version 3.1; Applied Biosystems) on an ABI3100 sequencer. Strands were manually aligned and corrected using the program BioEdit (Hall 1999).

The consensus sequence from three individuals were aligned with EMBLALIGN: Align_000605 (Matějusová et al. 2003) using CLUSTAL X (Jeanmougin et al. 1998) following the criteria detailed by Matějusová et al. (2003) and deleting the hypervariable sections of the ITS1 and ITS2 in order to optimize the alignment without ambiguities. The following sequences from GenBank were used for the alignment analysis: *Gyrodactylus alburniensis* Prost, 1972 (AY278032); *G. alexgussevi* Ziętara et Lumme, 2003 (AY061979); *G. anguillae* Ergens, 1960 (AB063294); *G. arcuatus* Bychowsky, 1933 (AF328865); *G. branchicus* Malmberg, 1964 (AF156669); *G. bullatarudis* Turnbull, 1956 (AY692024); *G. cichlidarum* Paperna, 1968 (DQ124228); *G. elegans* von Nordmann, 1832 (AJ407870); *G. flesi* Malmberg, 1957 (AY278039); *G. lotae* Gusev, 1953 (AY061978); *G. macronychus* Malmberg, 1957 (AY061980 and AY061981); *G.* cf. *niger* Huyse, Audenaert et Volckaert, 2003 (AY338452); *G. pictae* Cable, van Oosterhout, Barson et Harris, 2005 (AY692023); *G. rarus* Wegener, 1910 (AY338445); *G. robustus* Malmberg, 1957 (AY278040); *G. rugiensis* Gläser, 1974 (AF328870); *G. rugiensoides* Huyse et Volckaert, 2002 (AJ427414); *G. salaris* Malmberg, 1957 (AF328871); and *G. turnbulli* Harris, 1986 (AJ001846). MEGA version 4.0 (Tamura et al. 2007) was used to estimate *p*-distance between species.

RESULTS

Gyrodactylus orecchiae sp. n. Figs. 2–4, Table 1

Morphological description. Coverslip-flattened specimens 275.0–455.9 (356.3) long; 62.1–92.1 (81.5) wide at level of uterus. Anterior bulb of pharynx 25.1 (22.8–28.5) long × 41.3 (36.7–46.9) wide bearing 8 processes 11.5 (8.9–15.2) long; posterior bulb 18.3 (12.8–22.6) long × 51.7 (44.8–67.2) wide. Intestinal crura, short, extend to the posterior end of uterus. Haptor, sub-ovate to spherical when attached, clearly delineated from body, 76.8 (67.6–96.1) long × 65.0 (50.0–77.7) wide (Fig. 3e, f). Male copulatory organ ventro-lateral to posterior pharyngeal bulb or posterior to it, 13.8 (11.0–15.4) long × 13.7

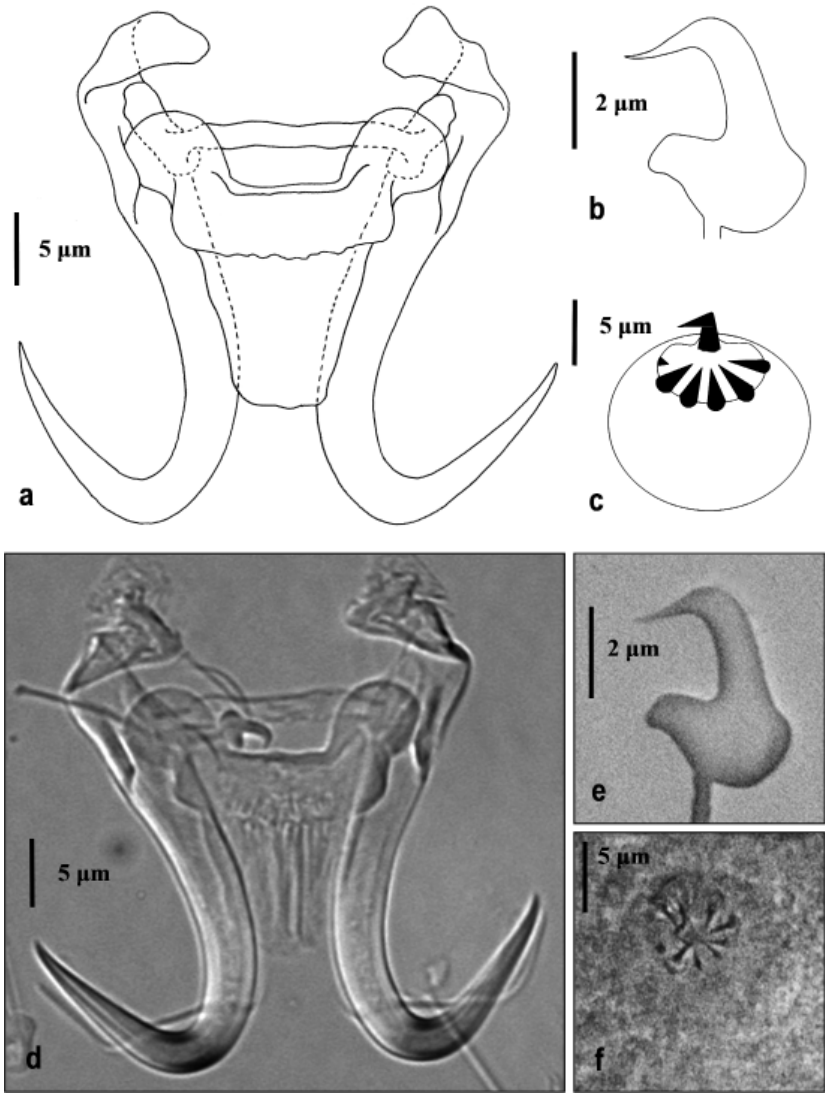

Fig. 2. *Gyrodactylus orecchiae* sp. n. **a, d –** the haptoral central hook complex of hamuli, dorsal and ventral bars; **b, e –** marginal hook sickle; **c, f –** male copulatory organ showing one large apical spine and a single row of five equal-sized small spines.

(11.1–16.6) wide, spherical, armed with large apical hook and single arch of 5 (4–6) small even-sized spines (Fig. 2c, f). Hamuli total length 34.6 (32.6–38.1); shaft length 21.1 (20.2–22.6); point 15.7 (14.6–16.2) long with a 37.3° (33.3–40.6°) aperture; inwardly directed roots 10.2 (7.7–14.2) long with central depression and thickened margins (Figs. 2a, d, 3a). Dorsal bar 16.4 (15.5–18.2) long; 2.1 (1.8–2.3) wide (Figs. 2a, d, 3a). Ventral bar 20.6 (18.6–22.0) long; 21.7 (19.0–24.0) wide; ventral bar processes prominent, rounded, 4.6 (4.0–5.6) long; ventral bar membrane lingulate, posteriorly rounded, 10.6 (9.3–11.8) long (Figs. 2a, d, 3a). Marginal hook length 18.2 (17.5–18.7); shaft length 14.7 (14.2–15.9); sickle proper length 3.3 (3.0–4.0); sickle base tangential to plane of shaft with proximal width 3.2 (2.6–3.5); rhomboid toe 1.8 (1.2–2.0) long; heel rounded; sickle shaft parallel to long axis of entire hook; sickle point perpendicular to sickle shaft, tapers to fine point with distal width 2.2 (1.9–2.5); sickle aperture 3.4 (3.1–3.8); inner curve of sickle proper approximately rhomboid (Figs. 2b, e, 3b1–b5).

Type host: *Sparus aurata* L. (gilthead seabream), Sparidae.

Site: Skin, fins, eyes and gill filaments.

Type locality: Orikum, Albania (40°18’50.92” N, 19°28’32.93” E)

Other reported localities: Ugljan Island, Croatia (44°7’45.87” N, 15°6’9.77” E)

Type material: Forty specimens were studied for light microscopy and ten digested specimens for SEM studies. Holotype (BMNH Reg. No. 2008.12.15.1) and paratype (BMNH Reg. No. 2008.12.15.2) are deposited in the parasitic worm collection at The Natural History Museum, London. Additionally, one paratype (M-475) is deposited in the gyrodactylid collection held at the Institute of Parasitology, Biology Centre of the Academy of Sciences of the Czech Republic, České Budějovice.

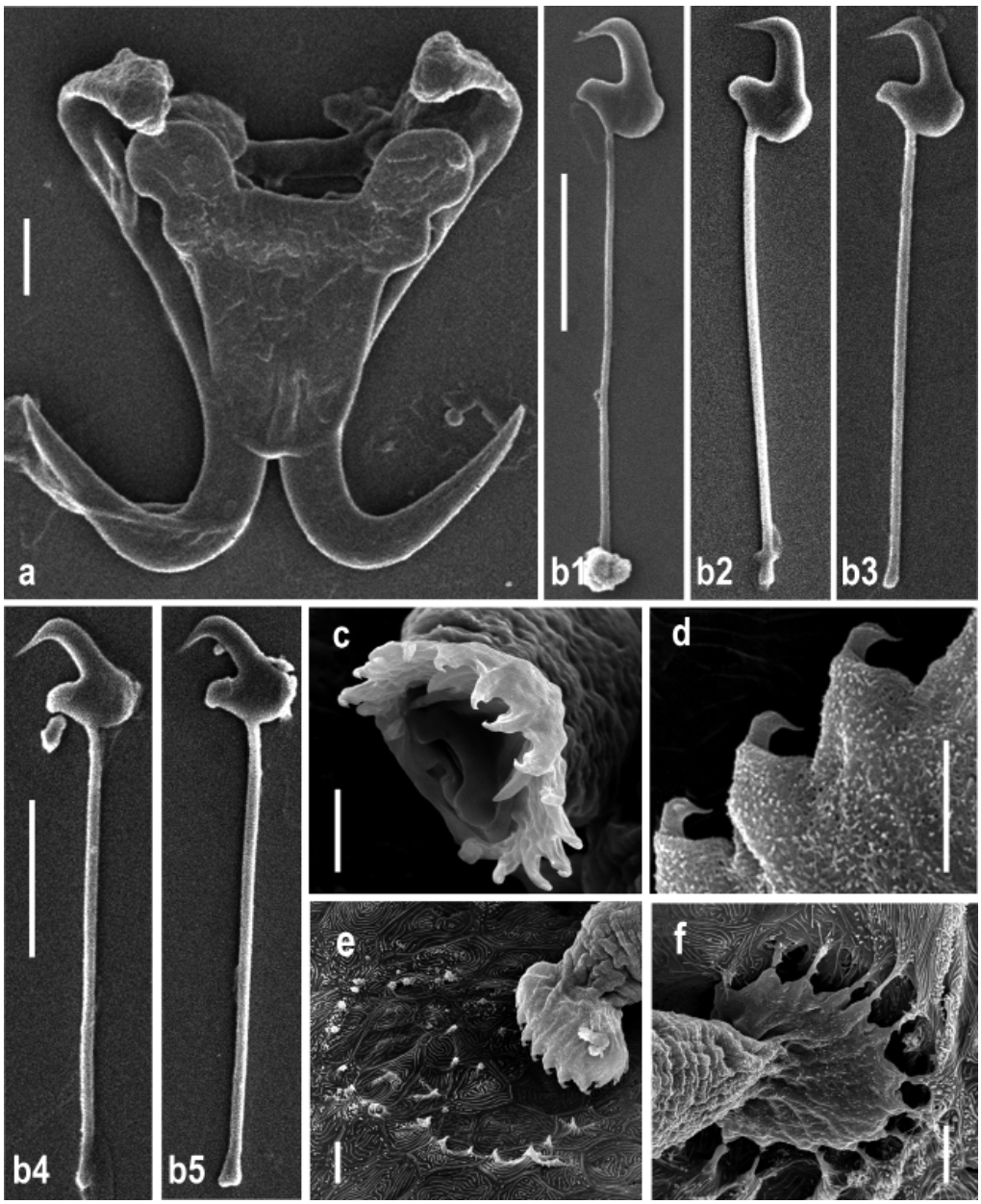

Fig. 3. Scanning electron micrographs of *Gyrodactylus orecchiae* sp. n. **a** – central hook complex showing the characteristic inwardly directed hamuli roots; **b1**–**b5** – marginal hooks; **c** – ventral surface of the haptor showing the position of the ventral and dorsal bar which may project into the epithelium of its host, preventing the haptor from slipping backwards, promoting the efficiency of attachment; **d** – characteristic marginal hook sickle points projecting from the haptoral tegument; **e** – attachment wound; **f** – sub-ovate haptor. Scale bars: a, b1–b5, d = 5 µm; c, e, f = 10 µm.

Molecular sequence data: The 1147 bp amplified fragment (18S (1–16) + ITS1 (17–529) + 5.8S (530–686) + ITS2 (687–1090) + 28S (1091–1147)) is deposited in GenBank under Accession Number FJ013097.

Etymology: Named in honour of Professor Paola Orecchia.

Histopathology. Acute dermatitis (hyperplasia and necrosis) was observed in the seabream with large numbers (1000+) of *Gyrodactylus* attached to the epidermis; epidermal spongiosis and some hydropic degeneration was also evident. Infections by *Gyrodactylus* on the gills showed secondary infection by bacteria with cellular exfoliation and mild haemorrhaging.

Molecular characterisation. The amplified nucleotide sequence of the rDNA cluster was 1147 bp and consisted of the 3' end of the 18S subunit (16 bp), the ITS1 (513 bp), the 5.8S gene (157 bp), the ITS2 (404 bp) and the 5' end of the 28S subunit (57 bp). Submitting the ITS1 to a BLASTN (Altschul 1991) search revealed no related sequences while a search using the 5.8S gave total ho-

Table 1. Morphological measurements (mean ± standard deviation followed by the range in parentheses; in micrometres) of *Gyrodactylus orecchiae* sp. n. from *Sparus aurata* collected from Orikum, Albania, which are respectively compared with those of *Gyrodactylus arcuatus* Bychowsky, 1933 from freshwater *Gasterosteus aculeatus*, *Gyrodactylus arcuatus* Bychowsky *sensu* Bychowsky et Poljansky, 1953 from *Gasterosteus aculeatus* from the Baltic Sea off Sweden, and *Gyrodactylus quadratidigitus* Longshaw, Pursglove et Shinn, 2003 from *Thorogobius ephippiatus*. Measurements taken from original descriptions in the literature are shown in a bold font, whilst those in regular font represent new measurements made in the current study. New measurements for *G. quadratidigitus* are provided from a syntype.

Measurement	***G. orecchiae* sp. n. (n = 40)**	***G. arcuatus* Bychowsky, 1933 (n = 24)**[1]	***G. arcuatus* Bychowsky *sensu* Bychowsky et Poljansky, 1953**[2]	***G. quadratidigitus* Longshaw, Pursglove et Shinn, 2003**
Total body length	356.3 ± 51.4 (275.0–455.9)	399.8 ± 36.3 (340.4–464.0)[4]	**336–460**	**430.0 ± 63.0 (334.0–486.0)**
Total body width	81.5 ± 7.6 (62.1–92.1)	102.1 ± 14.3 (82.4–122.6)[4]	**100–128**	**122.0 ± 19.0 (104.0–149.0)**
Haptor length × width	76.8 ± 12.1 (67.6–96.1) × 65.0 ± 12.0 (50.0–77.7)[3]	52.5 ± 9.7 (45.4–69.5) × 70.3 ± 8.8 (63.6–85.1)[3]	**–**	**48.9 ± 3.7 (45–54) × 81.4 ± 8.0 (70.5–88.0)**
Pharynx length × width (anterior; posterior bulb)	Ant: 25.1 ± 2.6 (22.8–28.5) × 41.3 ± 4.3 (36.7–46.9); Post: 18.3 ± 3.4 (12.8–22.6) × 51.7 ± 8.0 (44.8–67.2)[3]	Ant: 23.8 ± 5.1 (16.1–27.8) × 32.2 ± 2.8 (28.2–35.1); Post: 14.9 ± 3.8 (8.6–18.3) × 39.7 ± 2.9 (35.7–42.7)[3]	**–**	**30.0 ± 1.8 (28.5–32.5) × 30.3 ± 2.7 (27.0–33.5)**
Male copulatory organ length × width	13.8 ± 1.7 (11.0–15.4) × 13.7 ± 2.0 (11.1–16.6)[3]	13.9 ± 1.2 (12.1–14.9) × 13.9 ± 1.7 (11.9–16.4)[3]	**–**	**8.2 ± 0.5 (7.0–8.5) armed with 5–7 small spines**
Hamulus				
Ham aperture	11.5 ± 0.6 (10.5–12.4)	17.1 ± 1.0 (15.0–19.0)	**–**	12.0
Ham prox. shaft width	5.5 ± 0.3 (5.0–6.4)	6.7 ± 1.0 (5.0–9.3)	**–**	6.2
Ham point length	15.7 ± 0.4 (14.6–16.2)	18.9 ± 1.0 (17.0–20.6)	**15.2–18.5**	**14.0 ± 1.6 (12.3–15.5)**
Ham distal shaft width	3.5 ± 0.2 (3.2–3.8)	3.7 ± 0.4 (2.9–4.4)	**–**	2.2
Ham shaft length	21.1 ± 0.6 (20.2–22.6)	26.0 ± 1.2 (24.1–27.1)	**28.3–32.9**	**21.0 ± 3.8 (17.7–24.6)**
Ham inner curve length	2.3 ± 0.4 (1.5–2.9)	2.8 ± 0.7 (1.5–3.6)	**–**	1.2
Ham aperture angle (°)	37.3 ± 2.2 (33.3–40.6)	44.0 ± 4.1 (37.0–53.6)	**–**	44.0
Ham point curve angle (°)	14.0 ± 2.9 (9.8–20.7)	12.2 ± 2.1 (7.6–15.1)	**–**	20.0
Inner ham apert angle (°)	44.2 ± 3.3 (38.4–49.0)	50.9 ± 4.7 (43.1–61.7)	**–**	**53.9 ± 0.8 (53.2–54.6)**
Ham root length	10.2 ± 1.9 (7.7–14.2)	11.1 ± 1.2 (8.2–13.9)	**9.6–13.7**	**10.0 ± 0.9 (8.9–11.1)**
Ham total length	34.6 ± 1.9 (32.6–38.1)	40.4 ± 2.0 (35.8–43.5)	**35.9–43.1**	**28.0 ± 3.8 (24.4–31.8)**
Dorsal bar				
DB total length	16. 4 ± 1.0 (15.5–18.2)[3]	16.0 ± 0.9 (14.9–16.8)[3]	**17.4**	**12.3 ± 0.9 (11.4–13.4)**
DB width	2.1 ± 0.2 (1.8–2.3)[3]	2.0 ± 0.2 (1.7–2.2)[3]	**0.9**	**0.8 ± 0.1 (0.8–0.9)**
Ventral bar				
VB total width	21.7 ± 1.3 (19.0–24.0)	23.6 ± 2.0 (20.6–27.5)	**15.7–20.0**	**18.0 ± 1.5 (16.2–19.5)***
VB total length	20.6 ± 0.9 (18.6–22.0)	24.9 ± 2.1 (19.7–28.0)	**24.4–27.0**	**12.1 ± 0.7 (11.3–13.0)***
VB process to mid-length	5.2 ± 0.7 (4.0–7.5)	8.0 ± 0.9 (5.6–9.7)	**–**	3.0
VB median length	5.0 ± 0.4 (4.3–5.8)	4.9 ± 0.7 (3.6–6.0)	**3.9–5.2**	2.1
VB process length	4.6 ± 0.4 (4.0–5.6)	7.1 ± 0.9 (5.7–9.2)	**–**	**3.0 ± 0.0 (3.0–3.0)**
VB membrane length	10.6 ± 0.7 (9.3–11.8)	12.5 ± 1.7 (8.1–15.3)	**11.3–11.8**	**8.2 ± 0.6 (7.7–8.8)**
Marginal hook				
MH total length	18.2 ± 0.3 (17.5–18.7)	22.4 ± 1.3 (20.3–24.4)	**19.6–22.2**	**25.2 ± 1.1 (24.2–31.8)**
MH shaft length	14.7 ± 0.4 (14.2–15.9)	18.2 ± 1.2 (15.9–20.2)	**15.7–18.3**	**21.0 ± 0.9 (19.9–22.4)**
MH sickle length	3.3 ± 0.2 (3.0–4.0)	5.1 ± 0.3 (4.6–5.9)	**4.4**	**4.9 ± 0.4 (4.4–5.7)**
MH sick prox width	3.2 ± 0.2 (2.6–3.5)	3.9 ± 0.3 (3.4–4.3)	**3.5–3.9**	**3.0 ± 0.3 (2.8–3.7)**
MH toe length	1.8 ± 0.2 (1.2–2.0)	1.4 ± 0.1 (1.1–1.7)	**–**	1.1
MH sick dist width	2.2 ± 0.2 (1.9–2.5)	2.6 ± 0.2 (2.1–2.9)	**2.2**	**3.9 ± 0.4 (3.1–4.5)**
MH aperture	3.4 ± 0.2 (3.1–3.8)	3.9 ± 0.3 (3.4–4.3)	**–**	3.8
MH instep / arch height	0.4 ± 0.1 (0.3–0.5)	0.6 ± 0.1 (0.4–0.8)	**–**	0.2

[1]Specimens taken from a freshwater population of *Gasterosteus aculeatus* L. from Loch Airthrey, Stirlingshire, Scotland (56°8'47.6"N, 3°59'33.5"W); [2]Data taken from Malmberg (1970) represent specimens in marine environments; [3]Based on the measurement of 5 specimens; [4]Based on the measurement of 10 specimens; *The terms ventral bar length and width in this study are used in relation to longitudinal axis of the worm's body. The measurements in Longshaw et al. (2003), however, follow those of Malmberg (1970) and have been switched for direct comparison in this study.

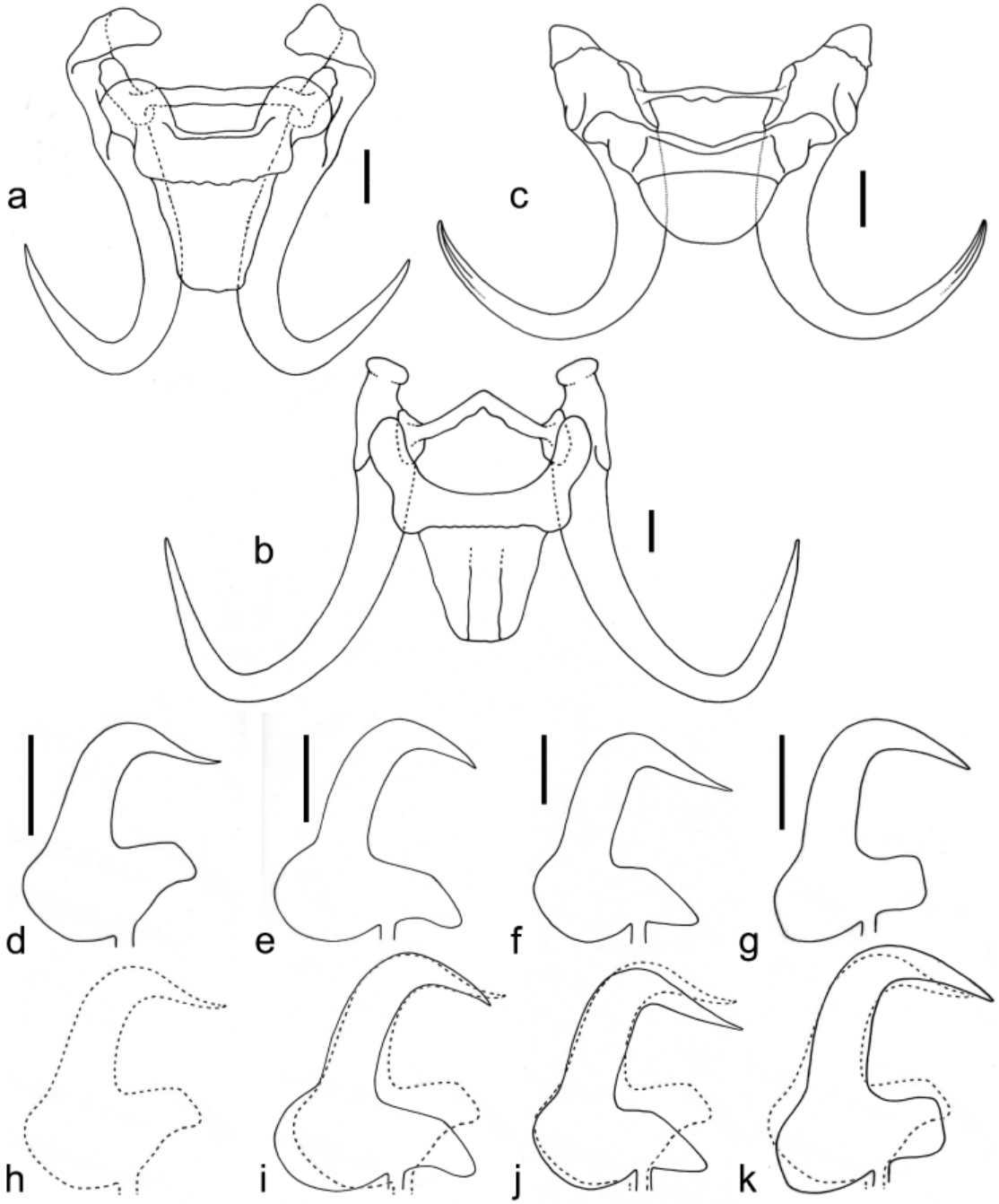

Fig. 4. A comparison of the haptoral hooks of *Gyrodactylus orecchiae* sp. n. with morphologically similar species. **a**–**c** – comparison of the central haptoral complex; **a** – *G. orecchiae*; **b** – *G. arcuatus* Bychowsky, 1933; **c** – *G. quadratidigitus* Longshaw, Pursglove et Shinn, 2003 (redrawn from original); **d**–**g** – marginal hooks of four morphologically similar species; **d** – *G. orecchiae*; **e** – *G. arcuatus*; **f** – *G. colemanensis* Mizelle et Kritsky, 1967; **g** – *G. quadratidigitus*; **h**–**k** – overlays of the marginal hook sickle for *G. orecchiae* with morphologically similar species (marginal hook sickles size invariant); **h** – *G. orecchiae* as a broken line; **i** – overlay of *G. orecchiae* with *G. arcuatus*; **j –** overlay of *G. orecchiae* with *G. colemanensis*; **k** – overlay of *G. orecchiae* with *G. quadratidigitus*. Scale bars: a–c = 5 µm; d–g = 2 µm.

mology (*p*-distance = 0) with *Gyrodactylus alexgussevi*, *G. branchicus*, *G. flesi*, *G. lotae*, *G. rarus*, *G. robustus* and *G. rugiensoides*. When the ITS2 was blasted separately, then the closest gyrodactylid with a homology of 78% (coverage of 97%) was the *Gyrodactylus* species parasitizing *Gobius niger* L. (see Huyse et al. 2003). Furthermore, homology of 86% on ITS2 (coverage of only 47%) was also obtained with *G. alexgussevi*, *G. branchicus*, *G. lotae* and *G. rarus* (*p*-distance = 0.101).

DISCUSSION

Gyrodactylus orecchiae is the first species of this genus to be formally described from *Sparus aurata* although one other species, *G. alviga*, is recorded from two other sparid hosts, *Diplodus annularis* and *Sarpa salpa* (Dmitrieva and Gerasev 2000). The morphology of the attachment hooks of these two gyrodactylids, however, differs markedly. Although large ventral bar processes are a characteristic feature of many gyrodactylid species, notably among the Nearctic gyrodactylid fauna, *viz*. the freshwater species *G. colemanensis* Mizelle et Kritsky, 1967 from *Salvelinus fontinalis* (Mitchill), and the brackish/marine species *viz. G. groenlandicus* Levinsen, 1881 from *Myoxocephalus scorpius* (L.), *G. nainum* Hanek et Threlfall, 1970 from *Triglopsis* (*Myoxocephalus*) *quadricornis* (L.), *G. pleuronecti* Cone, 1981 from *Pseudopleuronectes americanus* (Walbaum) and *G. stephanus* Mueller, 1937 from *Fundulus heteroclitus* (L.), *G. orecchiae* can be discriminated from these other species based on the morphology of its marginal hook sickle. For example, when the marginal hook sickle of *G. orecchiae* is aligned to a morphologically similar species, such as *G. colemanensis*, although the shaft and point regions are proportionally alike and describe the same rhomboid inner curve to the sickle (Fig. 4j), other marginal hook features allow their differentiation from each other. For example, the toe of *G. colemanensis* is triangular whilst that of *G. orecchiae* is square to rhomboid and is upwardly oriented in the direction of the sickle point. The sickle base of *G. orecchiae* is proportionally deep with a large rounded heel (Fig. 4d, f, j). The size of the marginal hooks of these two gyrodactylids also differ markedly: (31.1 (col) *vs.* 18.2 (orec) total length; 25.8 (col) *vs.* 14.7 (orec) shaft length; 6.0 (col) *vs.* 3.3 (orec) sickle length; 4.2 (col) *vs.* 3.2 (orec) sickle proximal width; 4.1 (col) *vs.* 2.2 (orec) sickle distal width, 1.5 (col) *vs.* 1.8 (orec) toe length; 4.6 (col) *vs.* 3.4 (orec) aperture) (data for *G. colemanensis* taken from Shinn 1993).

Two other morphologically similar species are *G. arcuatus* Bychowsky, 1933 (Fig. 4b, e) and *G. quadratidigitus* Longshaw, Pursglove et Shinn, 2003 (Fig. 4c, g). The former is known from both freshwater and marine populations of three-spined sticklebacks (*Gasterosteus aculeatus* L.) and as seen in *G. orecchiae*, it also possesses large ventral bar processes, hamuli roots that are commonly observed to turn inwards over the ventral bar processes, and marginal hooks with a square line to the inner edge of the sickle blade and large rounded heels. *Gyrodactylus arcuatus*, however, can be readily discriminated from *G. orecchiae* based on the shape of the sickle proper toe, which is long and triangular in the former (Fig. 4d, e, i). *Gyrodactylus quadratidigitus* from *Thorogobius ephippiatus* (Lowe), prior to the current study, appeared to be unique in that it possesses marginal hooks with a square toe (Fig. 4g), a male copulatory organ (*sic*. cirrus) positioned in line with or anterior to the posterior pharyngeal bulb, and unusually short intestinal crura which do not extend beyond the level of the testis. The position of the male copulatory organ in mature specimens of *G. orecchiae* appears to be variable. It has been observed in positions ranging from medial or posterior to the posterior pharyngeal bulb to lateral, the centre of the male copulatory organ level with the posterior edge of the pharyngeal bulb. The intestinal crura of *G. orecchiae* also appear to be very short in that they do not extend beyond the most posterior limit of the uterus. It is the blunt-ended toe of the marginal hook sickle, however, of both *G. orecchiae* and *G. quadratidigitus* that are characteristic but the morphology of each is not so subtle as to prevent their discrimination from one another (Fig. 4d, g, k).

The angles at which the ventral bar processes and the hamuli roots project under the haptoral tegument and their alignment to one another create a series of ridges that may serve to increase the efficiency of attachment in this species (Fig. 3c). The apparently robust processes of the ventral bar, it is hypothesized, would press into the epidermal tissues of its host at an opposing angle to the principal force of action by the marginal hooks contributing to the worm's attachment and minimising the risks of its dislodgement.

Of the 409 species of *Gyrodactylus* described so far, only around 20% have been sequenced at the ITS. In the absence of molecular data for *G. colemanensis* and *G. quadratidigitus*, both of which are nominally "similar" to *G. orecchiae*, a thorough analysis of the taxonomic affinities of these species must await a more thorough molecular coverage of the group.

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