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FISH PARASITOLOGY - ORIGINAL PAPER

Gyrodactylus ginestrae n. sp. (Monogenea: Gyrodactylidae), a parasite of the big-scale sand smelt, *Atherina boyeri* Risso, 1810 (Actinopterygii: Atherinidae) from the Black Sea

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Abstract

We describe a new species, *Gyrodactylus ginestrae* n. sp., a parasite of the big-scale sand smelt (*Atherina boyeri*) from the Black Sea. This is the third monogenean species known from this fish host, found at 70% prevalence, but at relatively low abundance (1.9), on fish gills and fins. The new species is, both morphologically and genetically, most similar to *G. salinae*, which parasitizes the killifish *Aphanius fasciatus* (Cyprinodontidae) in the Mediterranean region. These species differ in the size of the haptoral hard parts and the number of small spines of the male copulatory organ. For molecular characterization, the internal transcribed spacer 1 (ITS1), 5.8S rRNA gene, and the internal transcribed spacer 2 (ITS2) were sequenced, completed by a fragment of the COII gene, thereby representing the first molecularly characterized gyrodactylid species from the Black Sea. Phylogenetic reconstruction based on the ITS1–5.8S–ITS2 sequence data indicated the position of *G. ginestrae* n. sp. in the marine "*rugiensis*" group of *G.* (*Paranephrotus*) and *G.* (*Neonephrotus*) subgenera which is part of the monophyletic "long ITS1" group. Taking into account the similarity of *G. ginestrae* n. sp. to several monogeneans from the Atlantic and Mediterranean regions, we suggest the Boreal-Atlantic origin of this species.

Keywords Gyrodactylidae · Brackish water · Ponto-Caspian · Phylogeny · Gulf of Odessa · Molecular study

Introduction

The order Atheriniformes Rosen, 1964 includes small inshore fish inhabiting shallow waters (Quignard and Pras 1986), forming together with the orders Cyprinodontiformes Berg, 1940 and Beloniformes Berg, 1940 the Superorder Atherinomorphae (= Atherinomorpha sensu Greenwood et al., 1966) (Betancur-R et al. 2017). Of the five species of the genus *Atherina* currently known, three inhabit European marine and brackish waters (Quignard and Pras 1986), the sand smelt (*Atherina presbyter* Cuvier, 1829), the Mediterranean sand smelt (*Atherina hepsetus* L., 1758), and the big-scale sand smelt (*Atherina boyeri* Risso, 1810). Recent molecular data from Mediterranean populations reveal the existence of three forms of *A. boyeri* (lagoon/freshwater and two exclusively marine), indicating that *A. boyeri* is a species complex, but formal description has yet to be performed (Francisco et al. 2011).

The big-scale sand smelt, *A. boyeri* sensu lato, is a small demersal fish with its range in the Eastern Atlantic and throughout the Mediterranean and Black Seas (Froese and Pauly 2019). It inhabits marine and brackish/freshwater environments, corresponding to the different forms mentioned above (Francisco et al. 2011). The brackish/freshwater forms inhabit lower reaches of rivers and some lakes, where it has established permanent freshwater populations (Kottelat and Freyhof 2007). Recently, it has actively spread into the range

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of fresh waters of the Black Sea basin (Gençoğlu et al. 2017; Kvach and Kutsokon 2017).

The Black Sea is a part of the Mediterranean region, inhabited by Boreo-Atlantic and Mediterranean fauna, as well as by relict Ponto-Caspian fauna (Zaitsev and Mamaev 1997; Zaitsev 1998). This water body, including also the Sea of Azov (just a gulf of the Black Sea from an oceanographic view), is a distinctive region because of its low salinity ranging around 18‰ (Zenkevich 1963). Part of the Black Sea fish fauna is considered as having Mediterranean origin, penetrated the Black Sea about 7000 years ago (Zaitsev and Mamaev 1997). Traditionally, Mediterranean taxa, such as *Atherina* spp., *Syngnathus* spp., and *Pomatoschistus* spp., are assigned to this group (Zaitsev and Mamaev 1997).

The monogenean fauna of Black Sea fishes consists of at least 40 species (Gaevskaya and Dmitrieva 1997; Sarabeev et al. 2013). Among them, two species have been reported from the big-scale sand smelt: *Gyrodactylus alviga* Gaevskaya & Dmitrieva, 1997 and *Gyrodactylus atherinae* Bychowsky, 1933 (Roman 1956; Gaevskaya and Dmitrieva 1997; Kvach and Drobiniak 2017). While *G. alviga* is known from a wide range of fish species across taxonomically distant orders, *G. atherinae* appears specific to the big-scale sand smelt, recorded in the offshore northern Black Sea (Gerasev and Dmitrieva 2004) and the Caspian Sea (Semenova et al. 2007). Despite extensive surveys, neither of these parasites has ever been registered in big-scale sand smelt in the other parts of its host range such as the Adriatic and Tyrrhenian Seas or the Sea of Marmara (Sasal et al. 1997; Çolak 2013; Culurgioni et al. 2014; Radujković and Šundić 2014). Moreover, all known monogenean parasites of the species of *Atherina* have been described inside the Ponto-Caspian zone, from *A. boyeri* of Black Sea origin.

In the northwestern Black Sea, the specimens identified as *G. alviga* were found on fins of *A. boyeri* (Kvach and Drobiniak 2017), but illustrations were not provided; therefore, the species identification needs confirmation. The aim of our study was to provide a morphological description of the new *Gyrodactylus* species, complemented with molecular data (ITS rDNA and mitochondrial COII sequences). Molecular characterization of *G. ginestrae* n. sp. was performed by using both rDNA and mitochondrial markers. The combination of mtDNA and rDNA markers is commonly used on platyhelminths for phylogenetic and phylogeographic studies or for species identification. First, we used the fragment of nuclear ribosomal spacers regions (ITS1–5.8S–ITS2), which represents the most often used and highly effective molecular marker for species description and inferring phylogenetic relationships in the Gyrodactylidae (Ziętara et al. 2002; Matějusová et al. 2003; Paladini et al. 2011a). According to study of Bueno-Silva and Boeger (2014), a fragment of the cytochrome oxidase II (COII) gene was chosen as an additional molecular marker for barcoding of viviparous gyrodactylids. In addition, we analyzed the phylogenetic position of *Gyrodactylus ginestrae* n. sp. within a group of selected gyrodactylids mainly from the Mediterranean region and phylogenetically related fish hosts.

Material and methods

Specimen collection

The fish were sampled in the Gulf of Odessa (46.409640, 30.762071), Black Sea, Ukraine, using a dipnet in April 2017. In total, 20 individuals of the big-scale sand smelt were transported alive in aerated cans to the laboratory of the Odessa Center of Southern Scientific Research Institute of Marine Fisheries and Oceanography, where the fish are dissected for monogenean parasites within the 2 days after capture (Kvach et al. 2016).

Each fish was measured before dissection (standard length, SL, to the nearest 1 mm), with mean ± S.D. of 7.5 ± 0.8 cm and range 6.5–9.2 cm. The fins, skin, and gills were examined for monogeneans using a stereomicroscope Crystal-45 (Konus, Italy). Collected parasites were mounted in glycerine-ammonium-picrate for morphological study (Malmberg 1957). Holotype and paratype specimens were dehydrated in ethanol and mounted in Canada Balsam for museum deposition. A subsample of collected specimens was cut into two parts: posterior and anterior. The posterior part was mounted in glycerine-ammonium-picrate as described above, and the anterior part was preserved in 96% ethanol for further molecular analysis. In total, 22 specimens were collected; out of them, 16 specimens were subjected to morphological and 11 to molecular analyses, while 8 specimens were used both for morphology (only haptor) and molecular analysis.

Parasite individuals were characterized according to the shape and size of the haptoral hard parts (hamuli, connective bars, and marginal hooks) using a light microscope (Olympus BX51) equipped with a phase contrast and differential interference contrast. Drawings of haptoral hard components were made with the aid of a drawing attachment and phase-contrast optics. Measurements were obtained using the digital image analysis package MicroImage 4.0 for Windows (Olympus Optical co., Hamburg, Germany). All measurements are presented in micrometers. Nine morphological characters of the hamuli, ventral, and dorsal bars, along with seven characters of marginal hooks (MH), were measured according to Shinn et al. (2004), supplemented by length and width of the whole body, haptor, and male copulatory organ (MCO) (Table 1).

The parasitological indices follow Bush et al. (1997): prevalence (P, %), mean intensity (MI), intensity range (IR, as minimum–maximum), mean abundance (A). Standard deviation (sd) was calculated for means.

Table 1 The list of parasites sequences from NCBI GenBank, used for comparative study

Parasite species	Host species	Locality	NCBI No.	Reference
Gyrodactylus anguillae Ergens, 1960	*Anguilla anguilla* (L., 1758)	Spain: Valencia, La Albufera	AB063294	Hayward et al. (2001)
Gyrodactylus branchialis Huyse, Malmberg & Volckaert, 2004	*Pomatoschistus marmoratus* (Risso, 1810)	France: Vaccares Lagoon	DQ821770	Huyse et al. (2006)
Gyrodactylus bubyri Osmanov, 1965	*Knipowitschia caucasica* (Berg, 1916)	Bulgaria: Lake Atanasovsko	KU355879	Stoyanov et al. (2016)
Gyrodactylus cernuae Malmberg, 1957	*Gymnocephalus cernuus* (L., 1758)	Finland: River Oulujoki, Baltic Sea basin	AF484529	Ziętara and Lumme (2002)
Gyrodactylus derjavinoides Malmberg, Collins, Cunningham & Jalali, 2007	*Salmo letnica* (Karaman, 1924)	Macedonia: River Vardar system, Aegean Sea basin	EU304810	Ziętara et al. (2010)
Gyrodactylus gracilihamatus Malmberg, 1964	*Gasterosteus aculeatus* L., 1758	Finland: Gulf of Bothnia	AF484532	Ziętara and Lumme (2002)
Gyrodactylus jusii Ziętara & Lumme, 2003	*Phoxinus phoxinus* L., 1758	Finland: River Merenoja, White Sea basin	AY061982	Ziętara and Lumme (2003)
Gyrodactylus leptorhynchi Cone, Appy, Baggett, King, Gilmore & Abbott, 2013	*Syngnathus leptorhynchus* Girard, 1854	USA: Inner Cabrillo Beach, San Pedro, California	JX110633	Cone et al. (2013)
Gyrodactylus macronychus Malmberg, 1957	*Phoxinus phoxinus* L., 1758	Finland: River Merenoja, River Kovda system, White Sea basin	AY061981	Ziętara and Lumme (2002)
Gyrodactylus notatae King, Forest & Cone, 2009	*Menidia menidia* (L., 1766)	Canada: Nova Scotia	FJ840489	King et al. (2009)
Gyrodactylus orecchiae Paladini, Cable, Fioravanti, Faria, Di Cave & Shinn, 2009	*Sparus aurata* L., 1758	Albania: Orikum, Adriatic Sea	FJ013097	Paladini et al. (2009)
Gyrodactylus ostendicus Huyse & Malmberg, 2004	*Pomatoschistus marmoratus* (Risso, 1810)	France: Vaccares lagoon	DQ821768	Huyse et al. (2006)
Gyrodactylus poeciliae Harris & Cable, 2000	*Poecilia caucana* (Steindachner, 1880)	Venezuela: La Concepción	AJ001844	Harris and Cable (2000)
Gyrodactylus pungitii Malmberg, 1964	*Pungitius pungitius* (L., 1758)	Belgium: River Dommel, River Meuse system, North Sea basin	AF328869	Ziętara and Lumme (2002)
Gyrodactylus rugiensis Glaser, 1974	*Pomatoschistus minutus* (Pallas, 1770)	France: Vaccares lagoon	DQ821761	Huyse et al. (2006)
Gyrodactylus salinae Paladini, Huyse & Shinn, 2011	*Aphanius fasciatus* (Valenciennes, 1821)	Italy: Cervia Saline, Emilia Romagna region	JF950559	Paladini et al. (2011b)
Gyrodactylus turnbulli Harris, 1986	*Poecilia reticulata* Peters, 1759	Poland: aquarium	EF445942	Lumme and Ziętara (2018)
Outgroup				
Diplogyrodactylus martini Přikrylová, Matějusová, Musilová, Gelnar & Harris, 2009	*Polypterus senegalus* Cuvier, 1829	Senegal: Niokolo Koba National Park	AM943008	Přikrylová et al. (2009)
Gyrodactyloides bychowskii Albova, 1948	*Salmo salar* L., 1758	UK: Scotland coastal waters	AJ249348	Bruno et al. (2001)
Macrogyrodactylus heterobranchi N'Douba & Lambert, 1999	*Clarias anguillaris* (L., 1758)	Senegal: Niokolo Koba National Park	GU252714	Barson et al. (2010)

DNA extraction, amplification, and sequencing

Anterior parts of eleven specimens of *Gyrodactylus ginestrae* n. sp. collected from *A. boyeri* were placed individually in a 1.5 ml Eppendorf tube with 95% ethanol for genomic DNA extraction. Total genomic DNA of each individual was extracted using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) following the protocol for purification of total DNA from animal

tissues. In order to make comparisons with other *Gyrodactylus* species, we amplified and sequenced widely used markers in gyrodactylids phylogenetics, comprising the 3′ end of the 18S rRNA gene, internal transcribed spacer 1 (ITS1), the 5.8S rRNA gene, the internal transcribed spacer 2 (ITS2), and the 5′end of the 28S rRNA gene. The primer pairs ITS1A (5′-GTAA CAAGGTTTCCGTAGGTG-3′) and ITS2 (5′-TCCT CCGCTTAGTGATA- 3′) (Matějusová et al. 2001) were used. The amplification reaction was performed in a final volume of 25 μl, containing of 1xPCR buffer (Fermentas), 1.5 mM MgCl2, 200 μM of each dNTP, 0.5 μM primer, 1 μl of DNA, and 1.5 UTaq Polymerase (Fermentas). The PCR was carried out in the Mastercycler ep gradient S (Eppendorf) using the following steps: an initial denaturation at 96 °C for 3 min, followed by 39 cycles of denaturation at 95 °C for 50 s, annealing at 52 °C for 50 s and extension at 72 °C for 50 s, and a final elongation at 72 °C for 7 min. A fragment of the COII gene was amplified using degenerated primers cox2F (5′-TACAYAYCGCCCGT C A A Y Y T C G - 3 ′) a n d c o x 2 R (5 ′ - AATAMWKATWGGCATRWAAGARTG-3′) following the conditions described in Bueno-Silva and Boeger (2014). All PCR products were electrophoresed on 1.5% agarose gels strained with Good View (SBS Genetech, Bratislava, Slovakia) and then were purified using ExoSAP-IT™ (Affymetrix Inc., Santa Clara, USA), following the manufacturer's protocol. The purified PCR products were sequenced directly in both directions using the same primers as in the amplification reaction. Moreover, the internal primer ITSR3A (5′-GAGC CGAGTGATCCACC-3′) (Matějusová et al. 2001) complementary to sequence at the 5′ end of 5.8S gene was used for sequencing of ITS. Sequencing was carried out using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystem by Thermo Fisher Scientific, Prague, Czech Republic) and an Applied Biosystems 3130 Genetic Analyzer (Applied Biosystems). The DNA sequences were assembled and edited using Sequencer software (Gene Codes Corp., Ann Arbor, MI, USA). The sequences were subjected to a BLAST search (Altschul et al. 1997) against GenBank for species identification. The uncorrected *p*-distances were computed in MEGA X (Kumar et al. 2018). The newly obtained sequences of *Gyrodactylus ginestrae* n. sp. were deposited in GenBank (ITS rDNA: MK550602; COII: MN061575–MN061581).

To provide phylogenetic comparison, a sample of the Ponto-Caspian monogenean, *Gyrodactylus proterorhini* Ergens, 1967, was collected from a western tubenose goby, *Proterorhinus semilunaris* (Heckel, 1837), samples inside the Ponto-Caspian zoogeographic zone, in the Danube River near Vidin, Bulgaria (ITS rDNA: MK584285). *Gyrodactylus proterorhini* was originally described from *Proterorhinus marmoratus* from the Middle Danube (Ergens 1967). That time, *P. semilunaris* was considered as a junior synonym of *P. marmoratus*. Recently, *P. semilunaris* is re-erected as a valid species with the type locality in the Middle Danube (Neilson and Stepien 2009), while *P. marmoratus* is absent in the fresh waters, but present only in the Black Sea.

Phylogenetic analyses based on the ITS1–5.8S–ITS2 rDNA

To determine the phylogenetic relationships of *Gyrodactylus ginestrae* n. sp. with other species of *Gyrodactylus*, the phylogenetic analysis based on the ITS1–5.8S–ITS2 rDNA sequences were conducted using maximum likelihood (ML) and Bayesian inference (BI) methods. A total of 19 selected *Gyrodactylus* species collected mainly from fish hosts in the Mediterranean and Atlantic regions and/or from phylogenetically related hosts (Cyprinodontiformes) (for details, see Table 1) was included into phylogenetic reconstruction. Three monogenean species, *Diplogyrodactylus martini*, *Gyrodactyloides bychowskii*, and *Macrogyrodactylus heterobranchi*, were used as outgroup. Sequences were aligned in MAFFT v. 7 (Katoh et al. 2019) and optimized manually in BioEdit (Hall 1999). The alignment was trimmed using *trimAl* v1.3. (Capella-Gutiérrez et al. 2009). The best fitting substitution model of evolution was determined using the software JModeltest v2.2.1 (Darriba et al. 2012) based on the Bayesian Information Criterion (BIC). ML analysis was conducted using the program IQ-TREE (Nguyen et al. 2015) as implemented in W-IQ-TREE (Trifinopoulos et al. 2016) under the General Time Reversible (GTR) model with gamma distribution (+ G) and invariable sites (+ I) and four gamma-rate categories. Nodal support was assessed through 10,000 ultrafast bootstrap (UFBoot) (Minh et al. 2013) and 1000 Shimodaira-Hasegava-like approximate likelihood ratio test (SH-aLRT) (Guindon et al. 2010) replicates. BI analysis was performed in MrBayes 3.2.1 (Ronquist et al. 2012) under the GTR+I+G model. Four simultaneous chains (one cold and three heated) of Markov chain Monte Carlo (MCMC) algorithm was run twice for 10 million generations. Tree topologies were sampled every 100 generations, whereby the first 25% of trees from each run were discarded as burn-in. The remaining trees were used to construct majority-rule consensus trees and determine the Bayesian posterior probability (BPP) for each clade. The trees were visualized and edited in FigTree ver. 1.4.3. (Rambaut 2017).

Results

Gyrodactylid parasites collected from 20 big-scale sand smelt were observed on fins and gills, with prevalence of 70% (14 fish infected), mean intensity of 2.7 ± 2.1, intensity range 1–8, and abundance of 1.9. All specimens represented a

morphologically similar species which did not correspond to any other *Gyrodactylus* species known from the Black Sea/Mediterranean region. Comparative measurements for the new species, other two gyrodactylids known from big-scale sand smelt, *G. atherinae* collected near Karadag (Crimea, northeastern Black Sea), reported by Gerasev and Dmitrieva (2004), and *G. alviga* collected near Sevastopol (Crimea, northwestern Black Sea) (Gaevskaya and Dmitrieva 1997), and with morphologically and genetically similar species, *Gyrodactylus salinae*, collected from *Aphanius fasciatus* (Cyprinodontidae) in Cervia Saline, Adriatic Sea (Paladini et al. 2011b), are presented in Table 2.

Family Gyrodactylidae Cobbold, 1864

Genus *Gyrodactylus* von Nordmann, 1832

Gyrodactylus ginestrae n. sp. (Figs. 1 and 2)

Type host and locality: *Atherina boyeri*, Gulf of Odessa (46.409640, 30.762071), Black Sea, Ukraine

Site on the host: fins, gills

Type specimens: Holotype and one paratype (acc. No. IPCAS M-701) are deposited in the helminthological collection at the Institute of Parasitology, Academy of Sciences of the Czech Republic, České Budějovice.

Material examined: 16 flattened specimens (morphology), 11 ethanol preserved specimens (DNA analysis)

Table 2 Morphometric parameters of *Gyrodactylus ginestrae* n. sp., *G. atherinae*, *G. alviga*, and *G. salinae*

Parameters	N	*Gyrodactylus ginestrae* n. sp. Odessa 2017 *N* = 16	*Gyrodactylus atherinae* Karadag 1947 *N* = 5	*Gyrodactylus alviga* Black Sea 1992–1995 *N* = 80	*Gyrodactylus salinae* Cervia Saline 2008 *N* = 15
Body					
Length	8	385 (259–483)	187–262	400 (363–550)	447 (375–575)
Width	7	69 (51–88)	44–56	98 (73–117)	116 (88–163)
Haptor					
Length	10	61.3 (44.1–75.9)	44–50	72 (66–80)	75 (60–88)
Width	10	60.6 (40.5–77.0)		75 (60–80)	80 (70–88)
Hamulus					
Total length	16	41.8 (39.5–44.0)	37–38	65 (63–68)	51.7 (48.7–54.6)
Shaft length	16	28.0 (25.6–30.3)		45 (43–48)	31.9 (28.2–37.3)
Root length	16	17.0 (15.4–18.8)	13–15	21 (19–22)	16.8 (14.7–18.3)
Point length	16	19.5 (17.9–21.8)	15–17	30 (30–33)	24.8 (23.9–25.9)
Aperture angle	16	35.1 (31.3–37.9)			36.6 (33.8–37.9)
Ventral bar					
Median length	15	4.8 (4.1–5.9)	4–5	5 (5–6)	6.2 (5.6–6.8)
Total width	13	19.8 (17.5–21.9)	15–16	32 (30–35)	25 (23.5–26.7)
Dorsal bar					
Total length	11	1.3 (1.0–1.6)	1	4 (4–5)	1.9 (1.6–2.2)
Total width	11	17.3 (15.08–18.7)	10–13	22 (19–24)	9.4 (8.8–9.9)
Marginal hook					
Total length	16	28.8 (26.6–30.2)	25	34 (33–34)	26.8 (25.9–27.6)
Shaft length	16	22.6 (20.7–23.8)	18	27 (27–28)	20.8 (20.2–21.5)
Sickle length	12	5.7 (5.3–6.0)	7	7	6.3 (6.1–6.6)
Aperture distance	12	5.2 (4.8–5.6)			5.6 (5.4–6.0)
Proximal width	12	3.7 (3.4–4.0)	3	4	4.0 (3.6–4.4)
Distal width	12	2.6 (2.3–2.9)	5	5	3.6 (3.3–4.0)
Sickle toe length	12	1.6 (1.4–1.8)			1.8 (1.6–2.0)
Male copulatory organ					
Length	3	16.6 (15.8–17.2)	37–50	15 (14–19)	14.4 (11.9–18.5)
Width	3	13.2 (12.3–14.7)	31–51	13 (12–16)	12.7 (10.1–17.6)

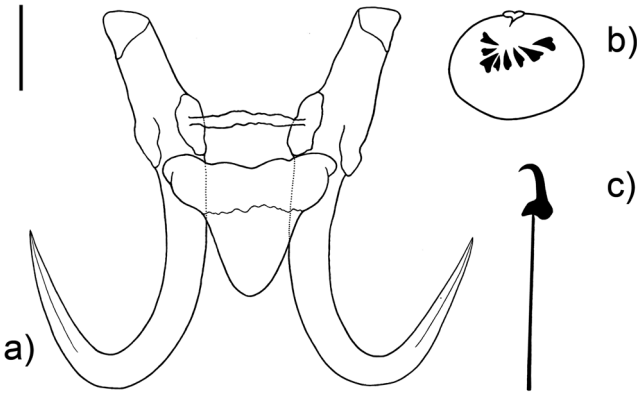

Fig. 1 Drawings of *Gyrodactylus ginestrae* n. sp. ex. *Atherina boyeri* from the Gulf of Odessa. a, opisthaptoral central hook complex; b, MCO; c, marginal hook. Scale bar: 10 μm

DNA reference sequences:

The 1204 bp sequence encoding partial 18S (17 bp), complete ITS1 (613 bp), 5.8S (157 bp), ITS2 (401 bp), and partial 28S (16 bp) is deposited in GenBank under accession No. MK550602. The partial COII sequences of 511 bp are deposited in GenBank under accession numbers (MN061575–MN061581).

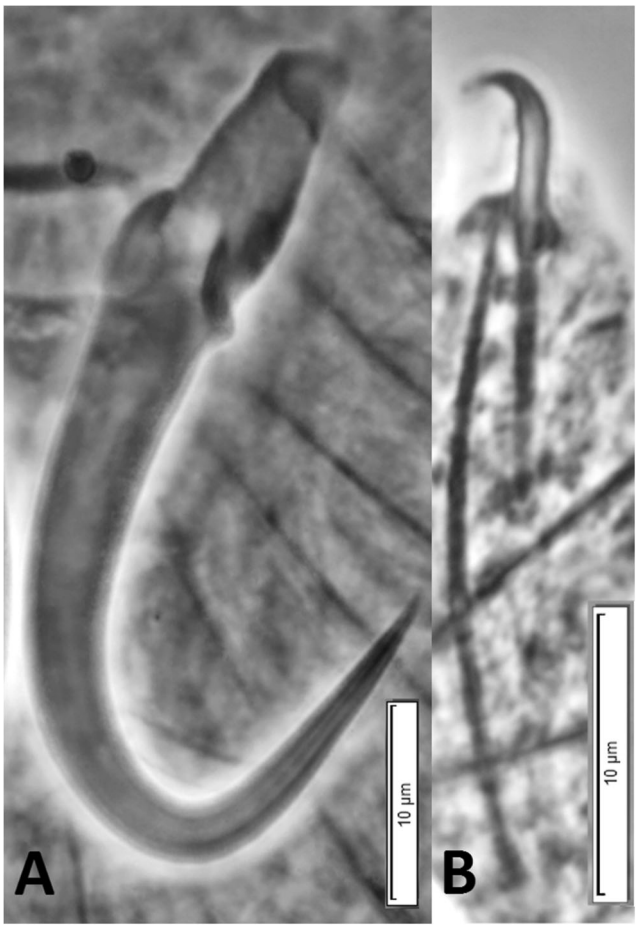

Fig. 2 *Gyrodactylus ginestrae* n. sp. ex. *Atherina boyeri* from the Gulf of Odessa. Light microphotograph of the hamulus (A) and marginal hook (B).

Etymology: The specific epithet has a root after Ginestra, the medieval Byzantine name of the region around today's Odessa, SW Ukraine.

Morphological description

General morphology based on 3–16 specimens (measurements shown in Table 2): body small, elongate, with length 385 ± 63 (259–483) comprising prohaptor and opisthaptor and width 69 ± 11 (51–88) at midbody. Haptor circular, 61 ± 10 (44–76) long, 61 ± 11 (41–77) wide. Hamuli with proportionately short straight roots, total length 42 ± 1.6 (40–44), shaft length 28 ± 1.4 (26–30), root length 17 ± 0.9 (15–19), point length 20 ± 1.1 (18–22), and hamulus aperture angle of 35 ± 2 (31–38). Dorsal bar simple 1 ± 0.2 (1–2) long, 17 ± 1 (15–19) wide. Ventral bar 4.8 ± 0.5 (4.1–5.9) long, 19.8 ± 1.3 (17.5–21.9) wide, membrane triangular. Anterolateral processes of ventral bar less prominent, projecting laterally. Marginal hook total length 29 ± 1 (27–30), shaft length 23 ± 1 (21–24). Marginal hook sickles crescent-shaped, toe pointed, short; sickle length 5.7 ± 0.2 (5.3–6.0), 2.6 ± 0.2 (2.3–2.9) wide distally, 3.7 ± 0.2 (3.4–4.0) wide proximally, aperture 5.2 ± 0.3 (4.8–5.6); base with distinct rounded heel. MCO spherical, located laterally to pharynx, 17 ± 0.6 (16–17) long and 13 ± 1.1 (12–15) wide, observed on 4 specimens. MCO armed with 1 principal spine and 8 smaller spines in a single row.

Remarks

Gyrodactylus ginestrae n. sp. is larger than *G. atherinae* in total body length, all measurements of hamuli, ventral bar width and marginal hook total length, but smaller in marginal hook sickle length (Table 2). The hamuli of *G. ginestrae* are more robust than those of *G. atherinae* and *G. alviga*, the latter two (*G. atherinae* in particular) showing narrowing of the roots towards the distal edge (Fig. 3A). The marginal hook sickles of all three species compared are morphologically distinct, being more slender in *G. atherinae* compared to other species (Fig. 3B). The newly described species is the most morphologically similar to *G. salinae*, which is larger in total length, shaft and point lengths of hamuli (Fig. 3A), ventral bar width, marginal hook sickle length and distal width, and squarer heel (Fig. 3B) and it has larger number of small spines in MCO (Paladini et al. 2011b) compared to *G. ginestrae* n. sp.

Molecular characterization

The identical sequences of the ITS fragment were obtained from 4 specimens. The results of a BLASTn search (Altschul et al. 1997) of the ITS1–5.8S–ITS2 fragment revealed no identical hits with entries in GenBank (May,

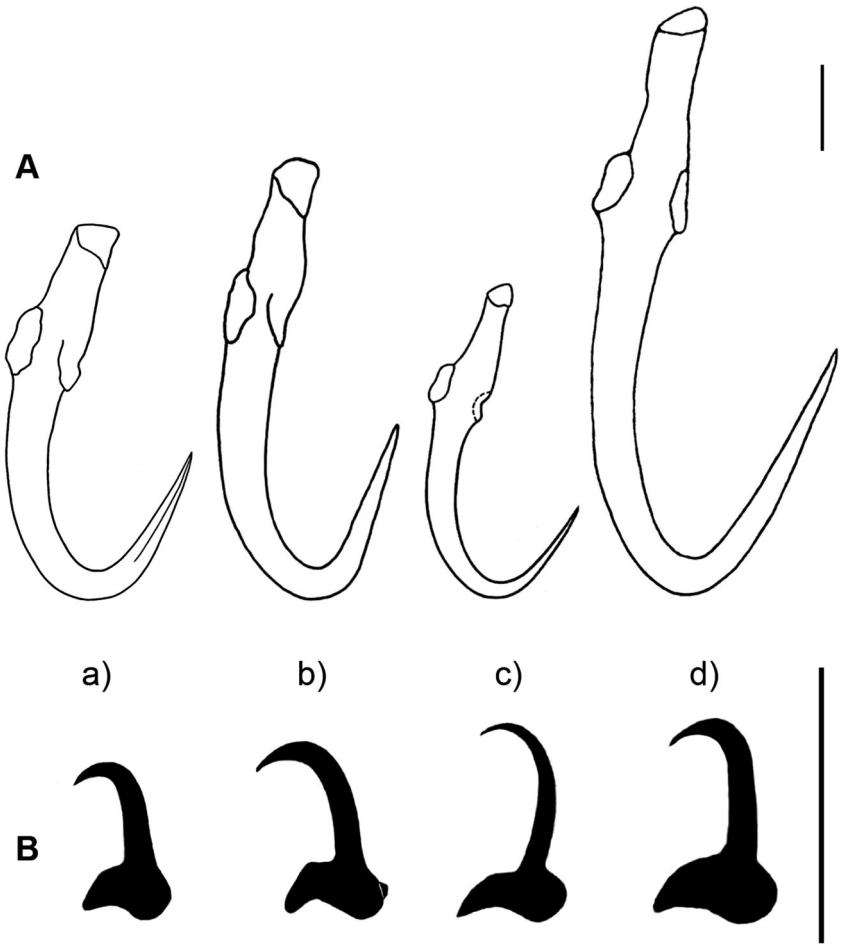

Fig. 3 Comparison of *G. ginestrae* n. sp. (a) hamuli (A) and marginal hook sickle (B) with *G. atherinae* (b), *G. alviga* (c), and *G. salinae* (d). Scale bar: 10 μm

2019). *Gyrodactylus ginestrae* n. sp. appeared most closely related to *G. anguillae* (91.81%, acc. No. AB063294) obtained from the European eel (*Anguilla anguilla*, Anguilliformes) in Spain and *Gyrodactylus salinae* (91.42%, acc. No. JF950559 from *Aphanius fasciatus* (Cyprinodontiformes) collected in Italy. When the 5.8S gene (157 bp) was submitted to a BLASTn search separately, it was found to be identical to several gyrodactylid species, namely, *G. anguillae* (AB063291), *G. bubyri* (KU355879), *G. gracilihamatus* (AF484531,32), *G. hildae* (FJ231869), *G. jussii* (AY061982), *G. micropsi* (AF328868), *G. rugiensis* (AF328870), and *G. rugiensoides* (AJ427414).

Seven different COII sequences were obtained from eight specimens of *G. ginestrae* n. sp. The results from the BLASTn search revealed no identical hits with entries in GenBank (May, 2019). Average intraspecific *p*-distance of COII sequences was 1%.

Phylogenetic analysis

The phylogenetic tree based on the ML analysis is shown in Fig. 4. The final ITS1–5.8S–ITS2 alignment constituted by 22 sequences was 841 bp long and comprised 569 variable sites. Both methods of phylogenetic reconstruction recovered identical phylogenetic relationships among the species studied, with several well-supported nodes. *Gyrodactylus ginestrae* n. sp. clusters with high bootstrap support (100% of bootstrap values, 1 of pp) in a group consisting of several marine *Gyrodactylus* species collected from Mediterranean areas and *G. leptorhynchi* infecting the bay pipefish *Syngnathus leptorhynchus* (Syngnathiformes), from the Pacific coast of North America. These Mediterranean *Gyrodactylus* species were isolated from various fish hosts (Anguilliformes, Cyprinodontiformes, Gobiiformes), and, except for *G. salinae*, share identical 5.8S sequence with *G. ginestrae* n. sp.

Discussion

Gyrodactylus ginestrae n. sp. is the fifteenth described gyrodactylid species from the Black Sea, the first one molecularly characterized, and the third *Gyrodactylus* parasitizing *Atherina* (see details in Lisitsyna and Miroshnichenko 2008).

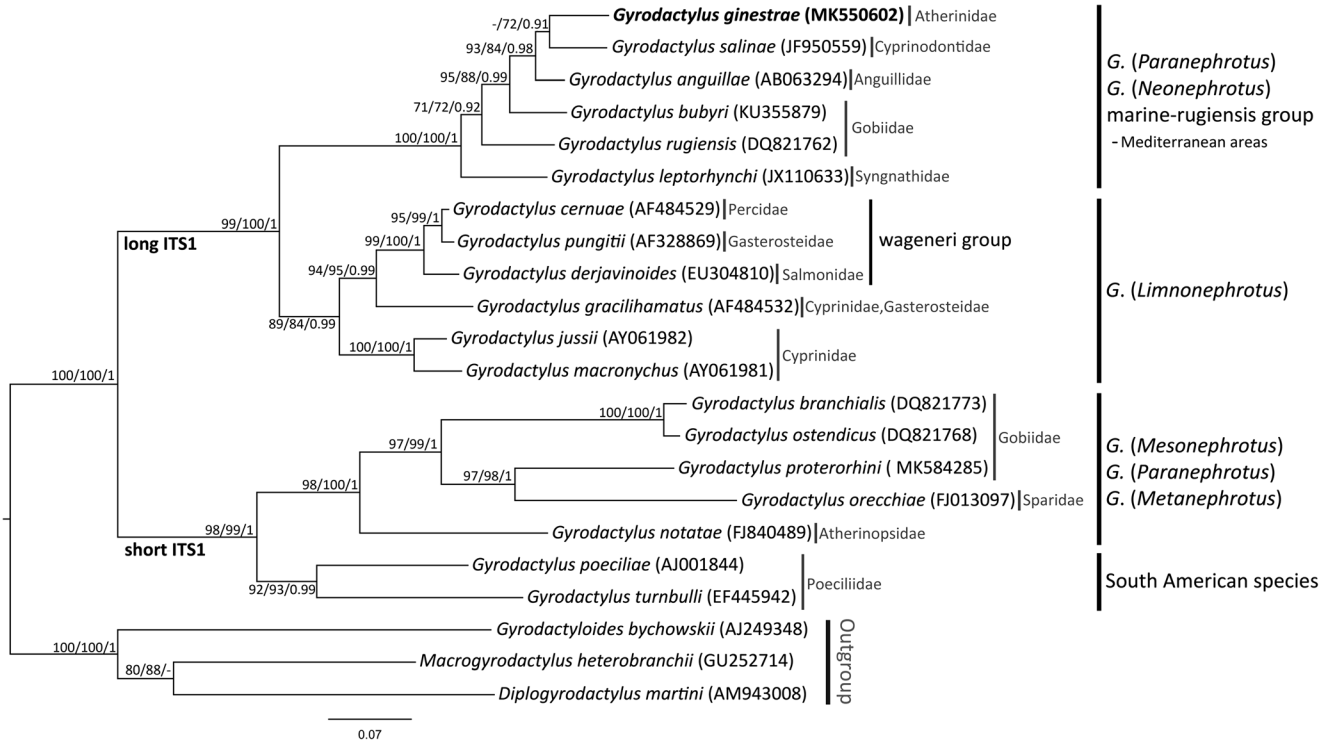

Fig. 4 The maximum likelihood tree of selected *Gyrodactylus* species based on ITS1–5.8S–ITS2 rDNA sequences. Support values beside branches represented Shimodaira-Hasegawa-like approximate likelihood ratio test (SH-aLRT)/ultrafast bootstrap (UFBoot), both implemented in IQ-TREE/ Bayesian inference (posterior probability) implemented in MrBayes. Values < 0.90 for BI and < 70% for ML are indicated by dashes (–). The phylogram is rooted with *Diplogyrodactylus martini*, *Gyrodactyloides bychowskii*, and *Macrogyrodactylus heterobranchi*. Branch lengths indicate the expected number of substitutions per site

In contrast to another specific parasite of *A. boyeri* found only on gills, *G. atherinae* (Gerasev and Dmitrieva 2004), the newly described species has been located both on fins and gills. Both morphologically and genetically, it is most similar to *G. salinae*, which parasitizes the fins and skin (occasionally on the gills) of the killifish *Aphanius fasciatus* (Cyprinodontidae) from a hyperhaline lagoon in Italy (Paladini et al. 2011b), differing from *G. ginestrae* in length of haptoral hard parts and number of small spines of the male copulatory organ. Such similarity may reflect phylogenetic proximity between the host species, i.e., representatives of Atheriniformes and Cyprinodontiformes, both belonging to the Superorder Atherimonomorphae (Betancur-R et al. 2017). On the other hand, the newly described species differs from *Gyrodactylus notatae* King et al. 2009, a parasite of the Atlantic silverside *Menidia menidia*, an atheriniform marine fish from North America (Sargent et al. 2008). This may indicate the distinguishing of the *G. ginestrae* from the Western Atlantic group. It is plausible that the geographic origin of the hosts is more important than their taxonomic relations.

In contrast to the wide use of the ITS region, COII has been sequenced only for a few species of *Gyrodactylus* (e.g., Bueno-Silva and Boeger 2014; Huyse et al. 2017; Vanhove et al. 2018; Xavier et al. 2015). Both markers display differences mainly in intra- and interspecific sequence variation due to their different molecular evolution. While there is little or no intraspecific variation observed for the ITS region in gyrodactylids (Cable et al. 1999; Vanhove et al. 2013; García-Vásquez et al. 2015; Tu et al. 2015), the intraspecific variation of COII sequences of *Gyrodactylus* varies from 0 to 3% (Bueno-Silva and Boeger 2014; Huyse et al. 2017). Differences in intraspecific variation of these two markers have also been revealed in the present study. The ITS sequence of *G. ginestrae* n. sp. displayed no intraspecific variation, in contrast to COII, where 1% of intraspecific variation was observed.

Phylogenetic reconstruction based on the ITS1–5.8S–ITS2 sequence data indicated the position of *G. ginestrae* n. sp. in the marine "rugiensis" group of *G.* (*Paranephrotus*) and *G.* (*Neonephrotus*) sub-genera which is part of the monophyletic "long ITS1" group. Ziętara et al. (2002) observed that the genetic differences of the 5.8S locus provide objective criteria to separate *Gyrodactylus* (sub) genera (Ziętara et al. 2002), as defined by (Malmberg 1970) on the basis of the excretory system. Each *Gyrodactylus* subgenus should possess a unique sequence of the 5.8S gene. The 5.8S fragment of all *Gyrodactylus* species of "rugiensis" group observed in the present study is identical, or in case of *G. salinae*, near-identical (BLASTn searches using the 5.8S fragment of

G. salinae did not reveal any identical hits, and the highest similarity was observed with the members of this group). The uncorrected *p*-distance between species of this group varied from 0.06 to 12.62%, which corresponds to the genetic distance between *Gyrodactylus* species sharing identical 5.8S sequences (Huyse and Volckaert 2002; Paladini et al. 2011b).

Based on the length of ITS1 (613 bp), *G. ginestrae* n. sp. falls into the category of "long ITS1" group (535–688 bp) (Cable et al. 1999; Ziętara et al. 2002) (Fig. 3), similarly to species clustered based on the ITS region: a parasite of European eel, *G. anguillae* and the parasites of annual gobies of family Gobionellidae, *G. bubyri*, and *G. rugiensis*. On the other hand, the euryhaline species *G. proterorhini*, parasitizing Ponto-Caspian gobies, the only *Gyrodactylus* species of Ponto-Caspian origin analyzed, clustered with species of the "short ITS1" group.

The similarities between the Black Sea and Atlantic fauna have already been recorded, based on the phylogeny of cryptogonimid trematodes, such as *Aphalloides coelomicola* and *Timoniella imbutiforme* (Kvach et al. 2017, 2018). Both species are connected with the annual gobies in their life cycles. The gobionellid fishes of the genera *Pomatoschistus* and *Knipowitschia* appeared in the Sarmatian period (Middle Miocene) (Schwarzhans et al. 2017a). One of the species mentioned above, *T. imbutiforme*, is also a parasite of *Atherina* spp. (Maillard 1973; Kvach et al. 2018). In contrast to the cryptogonimids, gyrodactylids such as *G. atherinae* or the newly described species *G. ginestrae* n. sp. have not been recorded from the Mediterranean and Atlantic coasts of Europe, nor has, for example, the closely related *G. bubyri*. We consider both species as Boreal relict species in the Black Sea fauna, which are probably extinct in the rest of Europe, or, at least their presence needs confirmation. For example, *G. bubyri*, previously known only from the Ponto-Caspian region, has recently been registered in the Strymon River in Greece (Vanhove et al. 2014).

In the Early Miocene, the Eastern Parathetys was located where the Black Sea is now (Popov et al. 2004). This water body was inhabited by 5 extinct species of *Atherina* (Schwarzhans et al. 2017b). They probably were the source of modern *Atherina* populations in the Black Sea. The annual gobies, i.e., *Knipowitschia* and *Pomatoschistus*, inhabiting Tarkhanian Sea, appeared later, in the Middle Miocene (Schwarzhans et al. 2017a). But, the Ponto-Caspian fauna, represented by, for example, *Proterorhinus* gobies, appeared just after Carangian Crisis, during Konkian and Eastern Sarmatian Transgressions. We therefore consider that *G. ginestrae* n. sp. is related to the group of Boreal-Atlantic relict species, together with its host, *A. boyeri*. This group in the Black Sea fauna consists of the parasites of the Boreal-Atlantic relicts, such as *Pomatoschistus* and *Knipowitschia* including two digeneans, *A. coelomicola* and *T. imbutiforme* (Kvach et al. 2017, 2018). Another parasite of *Knipowitschia*, *G. bubyri*, appears to extend this group according to current data.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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