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First report of *Ktariella polyorchis* (Monogenea: Calceostomatidae) infection in farmed meagre *Argyrosomus regius* (Actinopterygii: Sciaenidae), with a review of calceostomatid parasites of wild and cultured fish

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ABSTRACT

The present investigation reports a monogenean outbreak in broodstock of meagre *Argyrosomus regius* (Asso, 1801) from a Croatian farm. The parasites were visible by naked eye and were found in large numbers in the oral cavity and over the body surface. Morphological and molecular analyses allowed to identify the parasites as *Ktariella polyorchis* Vala & Euzet, 1977 (Monogenea: Calceostomatidae), here reported for the first time in farmed *A. regius*. Calceostomatids are known to parasitize different fish species of the family Sciaenidae worldwide: particularly, *K. polyorchis* had been described infecting the gills of wild *A. regius* caught off Tunisia and Egypt. This work provides the first detailed description of the parasite surface by SEM analysis, and the first molecular data (complete ITS and partial 28 S rDNA sequences), useful in future taxonomical and phylogenetic studies. Furthermore, we provide a synthesis of previous records of calceostomatid parasites of wild and cultured fish, highlighting their role as potential emerging parasites in the farming of sciaenid fish worldwide.

1. Introduction

The meagre *Argyrosomus regius* (Asso, 1801) (Actinopterygii, Sciaenidae) is a promising species in Mediterranean aquaculture, due to its fast growth rate and good adaptation to farming conditions (Duncan et al., 2013). Nevertheless, under captive conditions this species is susceptible to infections with a variety of parasites and other infectious agents (Soares et al., 2018). Among monogenean ectoparasites, the microcotylid *Sciaenocotyle pancerii* (Sonsino, 1891) Mamaev, 1989 has been reported infecting the gills of cage-reared *A. regius* from western Mediterranean areas (Merella et al., 2009; Quilichini et al., 2009; Ternengo et al., 2010), often associated with severe pathology and mortality (Merella et al., 2009; Ternengo et al., 2010). Recurrent gill infections with the diplectanid *Diplectanum sciaenae* Van Beneden & Hesse, 1863 associated with severe lesions in the branchial epithelium have been observed in *A. regius* broodstock in Spain (Andree et al., 2015). The capsalid *Benedenia sciaenae* (Van Beneden, 1852) Odhner, 1905 has been associated with hemorrhagic lesions and scale loss in the skin of *A. regius* cultured in net cages in Turkey (Toksen et al., 2007). An unidentified species of *Calceostoma* Van Beneden, 1858 (Monogenea: Calceostomatidae) has been reported from wild-caught *A. regius* subjected to adaptation experiments in captivity (Duncan et al., 2008).

Members of the family Calceostomatidae Parona and Perugia (1890) are known to parasitize different species of sciaenids worldwide. In wild *A. regius*, the species *Calceostoma calceostoma* (Wagener, 1857) has been reported from the Mediterranean and the Northeastern Atlantic (Oliver, 1993, 1980); *C. elegans* van Beneden, 1858 and *Ktariella polyorchis* Vala and Euzet, 1977, originally assigned to the genus *Calceostoma*, infects wild meagre from different Mediterranean areas (Abu Samak, 2001; Parona and Perugia, 1892; Vala and Euzet, 1977).

Other calceostomatids have been described also from non-sciaenid hosts (Gupta and Sachdeva, 1992; Mamaev, 1969; Nagibina, 1968; Pérez Ponce de León et al., 1999; Zhang et al., 2003).

In the present work we report for the first time the occurrence of *K. polyorchis* in broodstock *A. regius*, highlighting unusual sites of infection with this parasite and providing the first morphological description of its surface by SEM analysis and the first molecular data by amplification and sequencing of ITS and partial 28 S rRNA.

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Furthermore, this work provides a synthesis of previous records of calceostomatid parasites of wild and cultured fish, highlighting their role as potential emerging parasites in the farming of sciaenid fish worldwide.

2. Materials and methods

In January 2021 a mortality outbreak, preceded by a few days of anorexia, was observed in *A. regius* broodstock (32 individuals, average weight 10.6 kg) farmed in Croatia (NE Mediterranean Sea). Broodstock was from 2011 and was selected among fish cultured at the farm, originated from a hatchery in France. The fish was cage-reared at 16 °C in a small farm, with no meagre farming in that area.

During the outbreak, small whitish parasites were noticed in the oral cavity, on the palate and over the body surface, mainly along the back and on the fins (Fig. 1). At the beginning of mortality, the fish were treated with formalin baths (250 ppm for 60–90 min, 3 baths in 3 weeks) to control the parasites. After each bath, the number of parasites on skin progressively decreased; however, during the first week, 16 fish died (before and after the first bath), while subsequently, two more baths were made and only one fish died, for a total of 17 dead specimens. No previous mortality episodes had been observed in meagre broodstock from the affected farm.

At the farm, a total of 4 fish were inspected; gills samples and skin scrapings were collected from heavily infected fish (Fig. 1, Supplementary material 1) and preserved in 70% ethanol and 10% buffered formalin. Ethanol-preserved parasites were subjected to morphological analysis following clarification in Amman's lactophenol. Before clearing the parasites, a section of the body without diagnostic characters was excised with a sterile scalpel and processed for molecular analysis.

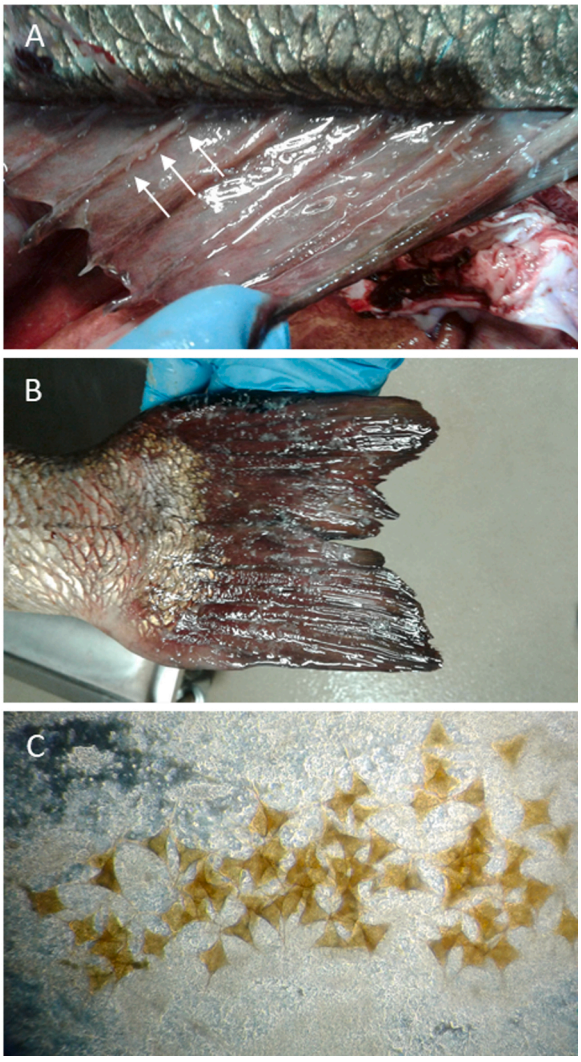

Fig. 1. Occurrence of *Ktariella polyorchis* (arrows) and abundant mucus on ventral (A) and caudal (B) fins of *Argyrosomus regius* broodstock; (C) tetrahedral egg bundles in skin scrapings.

Measurements were taken with the imaging software NIS-Elements (Nikon, Campi Bisenzio [FI], Italy), and are given in micrometers (μm; mean ± standard deviation, followed by the range in parentheses).

For Scanning Electron Microscopy (SEM), formalin-fixed specimens were washed three times with phosphate buffer, dehydrated in a graded ethanol series, critical point dried and sputter coated with gold-palladium. Observations were made using a Phenom XL G2 Desktop SEM (Thermo Fisher Scientific) operating at 5 kV.

For molecular analysis, genomic DNA was extracted from two worms using PureLink® Genomic DNA Kit (Life Technologies, Carlsbad, California) following the manufacturer's instructions.

The amplification of the partial ITS rDNA and 28 S rDNA region was performed with primers 81_f-GTAACAAGGTTTCCGTAGGTGAA (Gustinelli et al., 2010) and EDC2_r-CCTTGGTCCGTGTTTCAAGACGGG (Lockyer et al., 2003). The thermal cycler program (Tpersonal, Biometra) was 40 cycles of 30 s at 94 °C, 30 s at 52 °C and 2 m at 72 °C, preceded by a denaturation step at 94 °C for 2 min and followed by an extended elongation step at 72 °C for 10 min. The PCR products were electrophoresed on 1% agarose gel stained with SYBR® Safe DNA Gel Stain (Invitrogen, Thermo Fisher Scientific, Carlsbad, California) in 0.5X TBE. Amplicons were purified by Nucleo-Spin Gel and PCR Cleanup (Mackerey-Nagel, Düren, Germany) and sequenced with an ABI 3730 DNA analyzer (StarSEQ, Mainz, Germany). For sequencing the above-mentioned primers plus the internal primers JB9_r (Bowles and McManus, 1994) were used.

The DNA trace files were assembled with ContigExpress (VectorNTI Advance 11 software, Invitrogen, Carlsbad, California) and the consensus sequences were compared with previously published data by using BLAST tools (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Our sequences were multiple aligned with the ones retrieved from GenBank, using BioEdit 7.2.5 (Hall, 1999). Pairwise distance and maximum likelihood (ML) tree (HKY+G, bootstrap of 1000 replicates) were obtained by MEGA version X (Kumar et al., 2018). The sequences generated in this study were deposited in GenBank under the accession numbers OM985021-OM985022.

3. Results

The observation in light and scanning electron microscopy allowed to identify the collected parasites as *K. polyorchis* according to the detailed morphological description provided by Vala and Euzet (1977). The english terminology used for the morphological description follows Williams (1989).

3.1. Morphology (10 specimens)

Body elongate (Fig. 2A; 3 A), total length 3421.3 ± 803.2 (2529.2–4805.5), width at level of ovary 686.6 ± 203.3 (467.5–1035.1) with haptor 807.3 ± 151.3 (584.8–1036.2) wide. Anterior end expanded to form large lappet, with irregularly scalloped margin (Fig. 2B; 3B). Lappet with 2 conspicuous round glandular projections at opening of buccal cavity (Fig. 3B,C). Two pairs of small eye-spots close to anterior end of pharynx (Fig. 2B). Pharynx large, oval, 261.6 ± 52.2 (201.7–355.4) long by 201.1 ± 38.4 (141.4–253.5) wide (Fig. 2B). Intestine bifurcates immediately posterior to pharynx; caeca extending posteriorly to anterior margin of haptor. Haptor circular with two large hamuli associated with median sclerotized bar (Fig. 2D). Hamuli (= posterior hooks in Vala and Euzet, 1977) 110.5 ± 5.8 (102.2–117.4) long (Fig. 2D; 3E,F). A pair of hamuliform hooks, attached to central muscular area, each associated with one marginal hook (Fig. 3G,H).

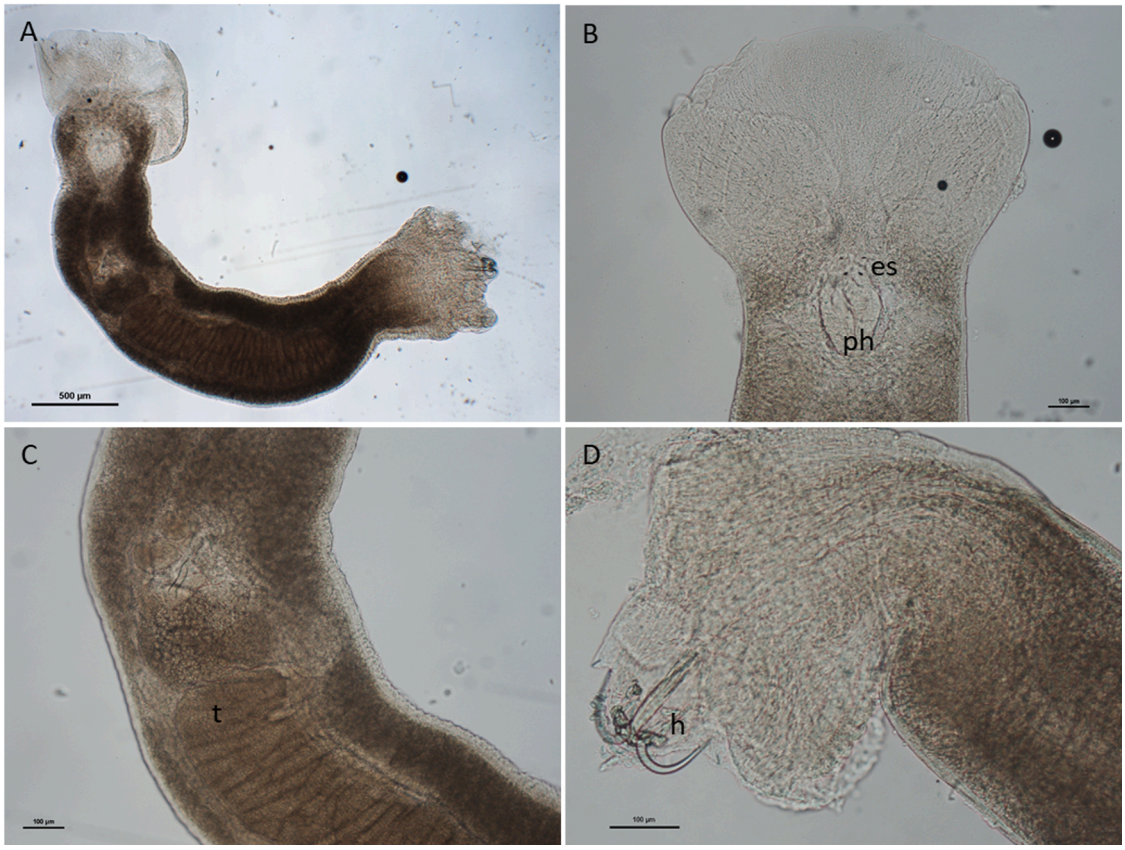

Fig. 2. *Ktariella polyorchis* observed by light microscopy A. whole specimen; B: anterior end showing the pharynx (ph) and four eyespots (es); C: middle body showing the genital complex with multiple testes (t); D: lateral view of opisthaptor with two large hamuli (h).

Testes numerous, arranged in two longitudinal rows (Fig. 2C). Anteriormost testes larger, 62.2 ± 22.3 (39.0–100.5) long by 213.7 ± 65.5 (133.6–331.9) wide. Posteriormost testes smaller, 54.2 ± 13.1 (42.3–77.7) long by 155.6 ± 55.7 (80.6–243.1) wide. Prostatic reservoir thick-walled, muscular. Ovary pretesticular, lobed 188.2 ± 58.5 (124.0–278.9) long by 327.7 ± 96.5 (231.4–554.7) wide. Ootype tetrahedral, with muscular walls. Mehlis' clustered around ootype base. Vitelline glands extending posteriorly to pharynx until haptor, surrounding intestinal caeca. Eggs tetrahedral (Fig. 1C).

3.2. Molecular analysis

The specimens were successfully amplified and sequenced producing amplicons of 1600 bp spanning the ITS-28S rDNA region. The BLAST search gave the highest identity (97,2%) with *Haliotrema* sp. (EF437158) from an unknown host, which moreover belong to a different family. No sequences of *Ktariella* were so far available in GenBank.

4. Discussion

The family Calceostomatidae currently comprises five recognized genera: the genus *Bychowskya* Nagibina (1968), the genus *Calceostoma* Van Beneden, 1858, the genus *Dicrumenia* Mamaev (1969), the genus *Ktariella* Vala and Euzet (1977) and the genus *Paracalceostoma* Caballero and Bravo-Hollis (1959). The majority of calceostomatid species have been reported only from wild fish hosts, with no details on their potential pathogenic importance.

To date, only members of the genus *Calceostoma* have been reported from farmed fish (Table 1); these include *Calceostoma glandulosum* Johnston and Tiegs (1922), infecting the gills of mulloway, *A. japonicus* (Temminck and Schlegel, 1843), cultured in Australia (Hayward et al., 2007); the authors reported that treatment with praziquantel was ineffective on *C. glandulosum*, however at the intensities found in the study (0−7) no gross pathology was apparent.

Several individuals of an undetermined species of *Calceostoma* were found over the gills, the oral cavity and fins of wild-caught *A. japonicus* broodstock cultured in Japan (Nasu et al., 1987) and were associated to mortality and weakness of the parasitized fish. In the same study, different concentrations of formalin (10–1000 ppm) and different exposure times (25 min to 32 h) were tested *in vitro* against the parasite, followed by an *in vivo* trial on *A. japonicus* broodstock. These trials allowed to establish that a formalin bath at a concentration of 200 ppm for 1 h, followed by another 1-hour bath at the same concentration after 4 days were effective at eliminating the parasite and restoring the feeding activity and vitality of the fish (Nasu et al., 1987).

Calceostoma sp. was reported also from captive *A. regius*, which had been collected from the Algarve coast (Portugal) and subjected to acclimation and spawning induction experiments (Duncan et al., 2008); in the study, three consecutive daily formalin baths (100 ppm for 1 h) were given weekly for the first three weeks, followed by an oral praziquantel treatment (5 mg kg-1 BW), and allowed to effectively deparasitize the fish (Duncan et al., 2008).

Although no pathological alterations associated with the presence of calceostomatids in captive *Argyrosomus* spp. have been described during previous investigations (Hayward et al., 2007; Duncan et al., 2008), a mortality outbreak of *A. japonicus* broodstock has been associated with heavy parasite load with *Calceostoma* sp. (Nasu et al., 1987). Besides acting as primary pathogens when present in high intensities, these parasites may cause significant skin and gill damage due to their mode of attachment and feeding activity on epithelial tissue (Hayward et al., 2007), thus favoring secondary infections. In the present work we report, for the first time, the occurrence of *K. polyorchis* in *A. regius*

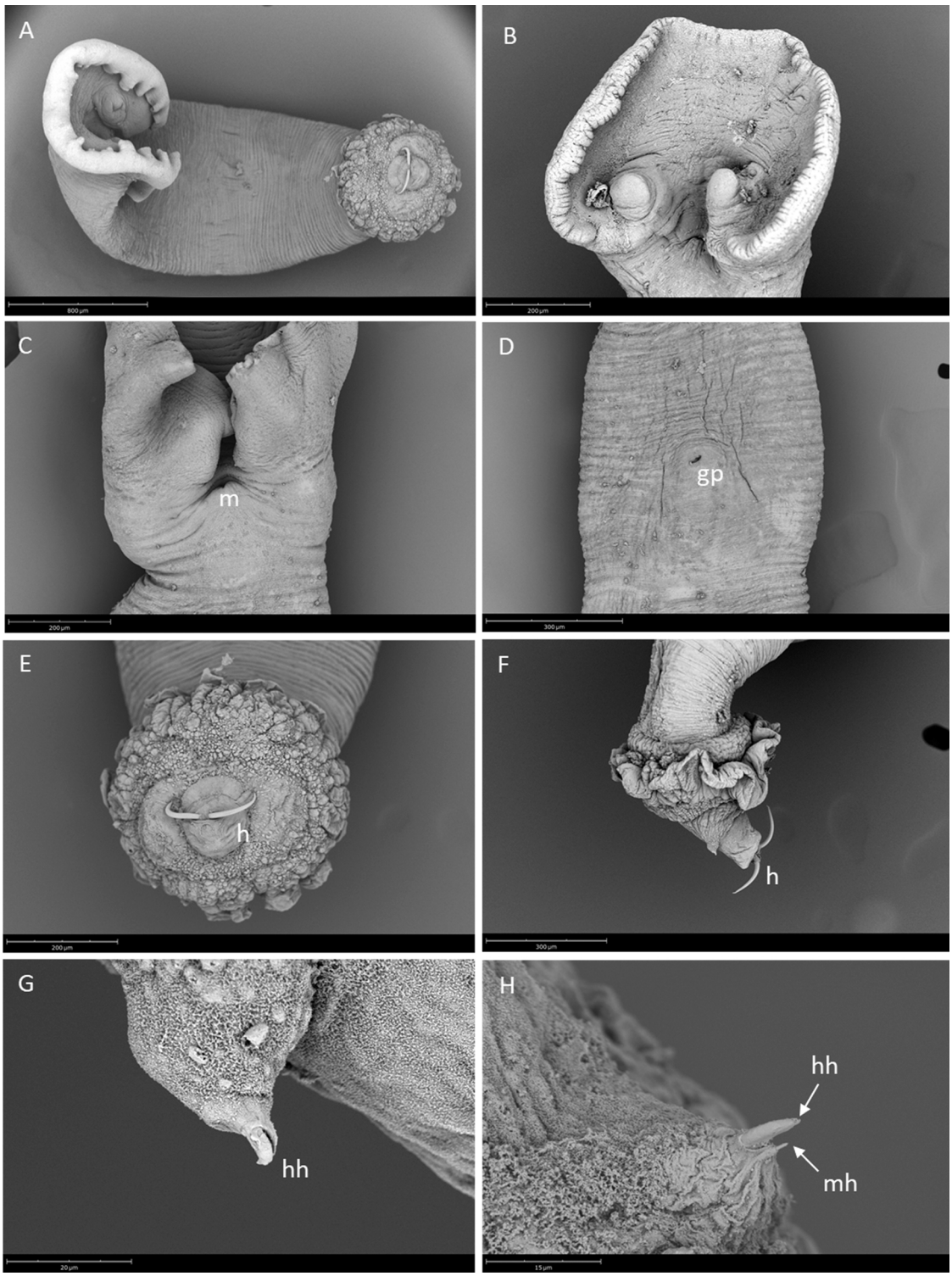

Fig. 3. *Ktariella polyorchis* observed by scanning electron microscopy A. whole specimen; B and C: anterior end with scalloped margin, two rounded projections and mouth (m); D: middle body showing the gonopore (gp); E: frontal view of opisthaptor with two large hamuli (h); F: lateral view of opisthaptor; G: detail of opisthaptor showing one hamuliform hook (hh); H: detail of opisthaptor showing hamuliform hook (hh) associated with marginal hook (mh).

broodstock from a Croatian farm. Until now, *K. polyorchis* had been reported only from wild meagre from southern Mediterranean areas, particularly from Tunisia and Egypt (Abu Samak, 2001; Vala and Euzet, 1977).

Originally assigned to the genus *Calceostoma* Van Beneden, 1958 based on the morphology of the anterior region and of the armature in the haptor, *K. polyorchis* was subsequently assigned to a separate genus based on the morphology of the male reproductive system (Vala and Euzet, 1977). Particularly, members of the genus *Calceostoma* are characterized by the presence of a single testis, while *K. polyorchis* possesses multiple testes similarly to members of the genus *Pseudocalceostoma*; nevertheless, the latter genus differs from *Calceostoma* spp. and from *K. polyorchis* with respect to haptoral armature (Vala and Euzet, 1977).

Table 1
Records of infection with calceostomatid parasites reported in the literature.

Parasite	Host	farmed/ wild	Locality	Reference
Calceostoma calceostoma (Wagener 1857)	*Argyrosomus regius*	wild	NE Atlantic; Mediterranean	Euzet et al. (1993);Oliver (1993)
	Sciaena umbra	wild	Mediterranean (Ligurian and Tyrrenian Sea)	Palombi (1949);Strona et al. (2010)
*C. durdanae*Gupta and Sachdeva (1992)	*Lutjanus yapilli*	wild	Bay of Bengal	Gupta and Sachdeva (1992)
C. elegans van Beneden 1858	*A. regius*	wild	Mediterranean	Parona and Perugia (1892)
	S. umbra	wild	Mediterranean	Parona and Perugia (1892)
*C. glandulosum*Johnston and Tiegs (1922)	*A. hololepidotus*	wild	SE Indian Ocean	Williams (1989)
	A. japonicus	farmed	Port Lincoln (Australia)	Hayward et al. (2007)
	A. japonicus	wild	Australia	Johnston and Tiegs (1922)
	A. inodorus	wild	Namibia	Amakali et al. (2022)
C. herculanea Euzet & Vala, 1976	*Umbrina canariensis*	wild	NE Atlantic; West African coast; Mediterranean	Euzet et al. (1993);Justine and Mattei (1986); Rohde et al. (1989)
*C. paronai*Gupta and Sachdeva (1992)	*S. umbra*	wild	Mediterranean	Gupta and Sachdeva (1992)
Calceostoma sp. Van Beneden, 1858	*A. japonicus*	farmed	Kadogawa (Japan)	Nasu et al. (1987)
	A. regius	wild	Algarve coast (Portugal)	Duncan et al. (2008)
	A. inodorus	wild	Namibia	Amakali et al. (2022)
	U. cirrosa	wild	Mediterranean	Parona and Perugia (1890)
*Ktariella polyorchis*Vala and Euzet (1977)	*A. regius*	wild	Mediterranean	Abu Samak (2001);Vala and Euzet (1977)
	A. argentatus	wild	East China Sea; South China Sea; Yellow Sea	Zhang et al. (2003)
K. polyorchis	***A. regius***	**farmed**	**Mediterranean (Croatia)**	**present study**
*Dicrumenia bychowskyi*Mamaev (1969)	*Pomadasys incisus*	wild	Mediterranean	Euzet et al. (1993)
	P. maculatus	wild	South China Sea	Mamaev (1969);Zhang et al. (2003);
*Paracalceostoma calceostomoides*Caballero and Bravo-Hollis (1959)	*Anisotremus interruptus*	wild	Central American/Mexican Pacific coast	Caballero and Bravo-Hollis (1959);Pérez Ponce de León et al. (1999)
	Haemulon scudderii	wild	Central American/Mexican Pacific coast	Caballero and Bravo-Hollis (1959);Pérez Ponce de León et al. (1999)
	Balistes polylepis	wild	Central American/Mexican Pacific coast	Pérez Ponce de León et al. (1999)
*Bychowskya drepanae*Nagibina (1968)	*Drepane punctata*	wild	South China Sea	Nagibina (1968)

Our study adds further morphological information, providing the first detailed description of the parasite surface by SEM analysis, and the first molecular data, useful in future taxonomical and phylogenetic studies. In fact, very few sequence data are available for calceostomatids in public online databases.

Although being reported mainly from the gills of wild *A. regius* (Vala and Euzet, 1977), in our samples *K. polyorchis* were mainly found on the fins and skin, while the gills were infected with another monogenean species, the diplectanid *D. sciaenae*, already reported in farmed *A. regius* from Spain (Andree et al., 2015). The unusual infection site observed in our study may indicate a possible displacement of *K. polyorchis* in concurrent heavy infections with *D. sciaenae*. Moreover, Bychowsky (1961) pointed out that Calceostomatidae "usually act as parasites on the fins".

In wild fish, parasitic infections often produce little or no pathological effects; nevertheless, wild fish fauna can act as reservoir of infection for farmed stocks. Under intensive farming conditions, monoxenous parasites with short generation times, such as monogeneans, can rapidly become abundant (Thoney and Hargis, 1991).

Similar to what already observed for *Calceostoma* sp. infection in *A. japonicus* broodstock during a previous investigation (Nasu et al., 1987), the mortality associated with high parasite loads observed in the present study in *A. regius* broodstock highlights the susceptibility of this species towards *K. polyorchis* and the possible importance of this parasitic infection in *A. regius* culture.

CRediT authorship contribution statement

Perla Tedesco: Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft. **Andrea Gustinelli**: Investigation, Methodology, Writing – review & editing. **Monica Caffara**: Investigation, Methodology, Writing – review & editing. **Matko Kolega**: Investigation, Review. **Slavica Čolak**: Investigation, Review. **Danijel Mejdandžić**: Investigation, Review. **Vicko Baranović**: Sampling, Investigation. **Maria Letizia Fioravanti**: Conceptualization, Supervision, Project administration, Resources, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.aqrep.2022.101105.

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