FISH PARASITOLOGY - ORIGINAL PAPER

Diclidophora luscae (Monogenea: Diclidophoridae) in pouting, *Trisopterus luscus* (Linnaeus, 1758) from the northeast Atlantic; epidemiology, morphology, molecular and phylogenetic analysis

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Abstract

Diclidophora (Monogenea) species are gill parasites with a stenoxenic specificity occurring only in Gadiformes. Epidemiological, morphological, molecular and phylogenetic studies were performed on 594 *Diclidophora* specimens collected from 213 *Trisopterus luscus* captured in the northeast Atlantic off the Portuguese coast during 2012, 2013 and 2020. Prevalence, parasite abundance and infection intensity were determined. Positive correlation between fish weight and length and infection intensity was observed. The effects of preservation on the parasite morphological features were studied, highlighting that specimen's identification should be reinforced by molecular studies. A sequence of *D. luscae capelanii* from *T. capelanus* captured in the Mediterranean Sea included in the 28S rDNA molecular analysis was nested within a robust *D. luscae* clade. Data analysis suggested that this species is in fact *D. luscae*, which is compatible with *T. luscus* and *T. capelanus*. The identity of fish hosts was confirmed by barcoding. For the first time, data on the infection parameters is shown, highlighting the importance of including this parasite in the monitoring plans for a holistic approach with possible effects for the management of pouting resources aiming of attaining sustainable development and biodiversity conservation measures, according to the 14th objective of the 2030 agenda.

Keywords *Diclidophora luscae* · Pouting · *Trisopterus luscus* · Epidemiology · Morphology · Molecular and phylogenetic studies · Northeast Atlantic

Introduction

Pouting, *Trisopterus luscus* (Linnaeus 1758) (Gadiformes: Gadidae) is a common marine fish present off the Atlantic coast of Portugal, with a distribution range from Norway to Morocco including the British Isles and offshore islands, Skagerrak (Northeastern Atlantic) and the western Mediterranean (Froese and Pauly 2021). Poor cod, *Trisopterus minutus* is another pouting species that is also found off the Atlantic coast of Portugal (Carneiro et al. 2014, 2019), but it is not a target species for fishing in the studied area.

In the northwest of the Portuguese coast, pouting was the most landed species in trammel net fisheries with an average of 6 068 tons per year from 1927 to 2011 (Teixeira

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et al. 2014), which associated to its low cost in the markets highlighting the importance and interest of this species for Portuguese consumers.

Parasites are an integral part of the aquatic ecosystem, and fish parasites represent an important component of aquatic biodiversity (Palm 2011; Quiazon 2015). To avoid the commercialization of obviously parasitized fish, the detection of visible parasites in fishery products for human consumption is ruled by European Union, Commission Regulation (EC) No 2074/2005.

Studies on pouting off the Portuguese coast highlighted the presence of visible parasites on edible parts and their importance to the commercial circuit, especially *Lernaeocera lusci* (Copepoda) (Ramos, unpublished data) and the nematodes *Huffmanela lusitana* (Ramos et al. 2019) and *Anisakis simplex* s.l. (Ramos 2012). On the other hand, parasites like the gill monogenean *Diclidophora luscae* which was identified in pouting off the Portuguese coast by Kearn and Vasconcelos (1979) and Ramos et al. (2013, 2014a, b) have an unknown impact in pouting wild stocks and commercialization.

Members of the monogenean genus *Diclidophora* KrØyer, 1838 (Diclidophoridae Fuhrmann 1928) are known to exhibit a stenoxenic specificity occurring only in gadiformes and display a high degree of host attachment site specificity on the gills (Llewellyn et al. 1980). Rubec and Dronen (1994) revised the known species of *Diclidophora* and referred to *D. luscae* (van Beneden and Hesse 1863) Price, 1943, collected from *T. luscus* in the Northeast Atlantic (Plymouth, England). Other *Trisopterus* species have been associated with specific gill-infecting *Diclidophora* species (Table 1).

To date, morphologic and genetic analyses have revealed that there are only ten recognized species of the genus *Diclidophora* (Rubec and Dronen 1994; Jovelin and Justine 2001; Strona et al. 2010) (Table 1): *D. merlangi*, *D. denticulata*, *D. esmarkii*, *D. phycidis*, *D. luscae*, *D. luscae capelanii*, *D. minor*, *D. palmata*, *D. pollachii*, and *D. micromesisti*. However, recent studies have revealed the occurrence of *Diclidophora* spp. in different hosts from their normal ones: *D. merlangi*, usually a parasite of the whiting *Merlangius merlangus* (L.), in cod, *Gadus morhua* (Perdiguero-Alonso et al. 2006) and brushtooth lizardfish, *Saurida undosquamis* (Morsy et al. 2018); *D. denticulata*, usually a parasite of *Pollachius virens*, in *Trisopterus minutus* (Strona et al. 2010); and *D. phycidis*, usually a parasite of *Phycis blennoides*, in *T. minutus* (Strona et al. 2010) (Table 1).

Studies on *D. merlangi* features from *G. morhua* showed smaller morphometric values than specimens collected from *M. merlangus* (Perdiguero-Alonso et al. 2006)—its "specific" host. No data on this issue are available from the other examples.

Table 1 *Diclidophora* spp. and known host-parasite interactions based in morphological features

Species	Host	Location	References
D. merlangi	*Merlangius merlangus*	Northeastern Atlantic (off Scotland, Plymouth, England, Ireland, Sweden, Norway)	Rubec and Dronen (1994)
	Gadus morhua	Northeastern Atlantic (North Irish, Norwegian and Baltic Seas, off Iceland; farms in Scotland and Iceland	Perdiguero-Alonso et al. (2006)
	Saurida undosquamis	Red Sea, Egypt	Morsy et al. (2018)
D. denticulata	*Pollachius virens*	Northeastern (off Scotland, Norway) and Northwestern Atlantic (off Woods Hole, Massachusetts, USA, Gulf of St. Lawrence, Canada)	Rubec and Dronen (1994)
	Trisopterus minutus	Mediterranean, Italian Coast	Strona et al. (2010)
D. esmarkii	*Trisopterus esmarkii*	Northeastern Atlantic (off Scotland, Plymouth, England and Bay of Biscay, France)	Rubec and Dronen (1994)
D. luscae	*Trisopterus luscus*	River Tagus Estuary (Portugal)	Kearn and Vasconcelos (1979)
	Trisopterus luscus	Northeastern Atlantic (Plymouth, England)	Rubec and Dronen (1994)
	Trisopterus luscus	Northeastern Atlantic (Peniche, Portugal)	Ramos et al. (2013, 2014a, b)
D. luscae capelanii	*Trisopterus capelanus*	French coast of the Mediterranean Sea	Jovelin and Justine (2001)
D. minor	*Micromesistius poutassou*	Northeastern Atlantic (off Scotland, Plymouth England)	Rubec and Dronen (1994)
	Micromesistius poutassou	Mediterranean, Italian Coast	Strona et al. (2010)
D. palmata	*Molva molva*	Northeastern Atlantic (Faroe Island, North Sea off Scotland, Reyjavik, Iceland)	Rubec and Dronen (1994)
D. phycidis	*Phycis blennoides*	Northeastern Atlantic, off Scotland, Rosemary Bank	Rubec and Dronen (1994)
	T. minutus *Phycis blennoides*	Mediterranean, Italian Coast	Strona et al. (2010)
D. pollachii	*Pollachius pollachius*	Northeastern Atlantic (off Scotland, Ireland)	Rubec and Dronen (1994)
D. micromesisti	*Micromesistius australis*	Southwestern Atlantic, off Argentina	Rubec and Dronen (1994)

Additionally, based on the observation of haptor and copulatory organ morphology and on molecular markers applied to the study of polyopisthocotylean monogeneans phylogenetic relationships, *D. luscae capelanii* was identified on *T. capelanus* inhabiting the French coast of the Mediterranean Sea (Jovelin and Justine 2001).

In Portugal, there are no molecular data available either on *D. luscae* in *T. luscus* or on *T. luscus* itself. Furthermore, data on this hematophagous *Diclidophora* infection on natural stocks is scarce.

The present study was designed to investigate the relationship between *D. luscae* and *T. luscus* based on data from samples collected in 3 different years, aiming to:

1. Determine *Diclidophora* infection parameters in pouting, *T. luscus*, from the Northeast Atlantic coast of Portugal and their interannual variation to evaluate the parasite's importance in pouting ecology and to propose the inclusion of their further study in the National Plan for Biological Samples (PNAB).
2. Characterize *Diclidophora* specimens in pouting, *T. luscus* from continental coast of Portugal based on epidemiological, morphological, molecular and phylogenetic studies.

Knowledge of this fish parasite interaction will be considered as a future biodiversity indicator for stock sustainability, according to the 14th objective of the 2030 agenda for sustainable development.

Material and methods

Collection of material

Pouting were captured in the northeast Atlantic off the Portuguese coast in three periods, May 2012 ($n = 58$) (38° 51′ 500″N; 09° 30′ 300″W), June 2013 ($n = 124$) (39° 09′ 000″N; 09° 25′ 300″W) and January 2020 ($n = 31$) (41° 12.800′ N; 8° 58.800′ W). Fish specimens were preserved deep-frozen until they reached the laboratory. A fresh pouting ($n = 15$) sample was obtained from market for parasite morphometric comparative purposes. In the laboratory, each fish was measured, weighed and sexed for basic biological information and examined for the presence of *Diclidophora*. The sampling procedure and the flow and codification of the parasites were designed to enable all the techniques developed in this study (morphometric and morphological studies, comparative analysis of different preservation techniques, epidemiological analysis and genetic and phylogenetic approaches).

Gills were examined for *Diclidophora* presence with a stereomicroscope and parasites were collected and codified

according to the pouting order number (Pn): site of infection—on the left (L) or right (R) sagittal plane of the fish and positioning on the branchial arches from anterior to posterior (1 to 4) (example P1-R2-3, meaning it is the third parasite collected from the second right branchial arch in pouting order number one). A total of 594 *Diclidophora* specimens were collected from 213 T. *luscus* samples.

Fresh fish epaxial muscle samples were taken from 5 pouting, and 15 fresh parasites were preserved in 70% ethanol for molecular purposes.

Epidemiological analysis

An excel database was produced with individual measurements (maximum fish width and height), fish weight, parasitized status, number of parasites and their location on host gills.

The measures of parasitic infection referred to mean intensity and mean abundance, according to Bush et al. (1997). Parasite intensity and abundance were estimated using quantitative parasitology (Reiczigel et al. 2019) on the web (version 1.0.15, 6 December 2020) by Jeno Reiczigel and Lajos Rozsa and web programming by Andras Reiczigel and Ibolya Fabian.

Data from samples taken in 2012 and 2013 were compared statistically with those collected in 2020. The statistical treatment was performed using the SPSS ® *software* using descriptive statistics, normality tests, Kruskal–Wallis (KW) non-parametric tests and Pearson's correlation index, ensuring the robustness of the results with the Bootstrap technique (Marôco 2018).

Morphometric and ultrastructural studies

For morphometric analysis, *Diclidophora* specimens ($n = 30$) were obtained from fresh ($n = 15$) and frozen ($n = 15$) pouting and fixed in 70% ethanol, stained with alcoholic carmine and mounted in Canada Balsam for comparative morphometric analysis. To compare morphometric measurements, *Diclidophora* sp. in pouting, samples of fresh, thawed and preserved/pressed and stained specimens were used.

The morphological variables used for component analyses were: body length (BL), body width at level of the origin of haptor (BW), distance to female genital pore (DG), first clamp length (FCL), first clamp width (FCW), pre-ovary length (POL) and post-ovary length (PTOL). These variables were associated with shape variability and homologous among all species (Llewellyn et al. 1980; Perdiguero-Alonso et al. 2006). Measurements were registered in millimetres (mm) in the form mean ± standard deviation.

The egg measurements (length without filaments and width) are reported in micrometres as average (standard deviation; minimum–maximum) and were obtained from

78 eggs from 17 specimens collected in 13 hosts. Morphometric studies were performed on a Leitz Laborlux K light microscope (LM) connected to a Leica DFC 420 camera and using the measurement software LAS (Leica Application Suite 2009).

Statistical analysis was performed using a non-parametric Kruskal–Wallis (K-W) test. Robustness of the estimates was obtained since all samples were bootstrapped, using the SPSS® statistics software.

For scanning electron microscopy (SEM), adult parasites ($n = 15$) and eggs from fresh pouting were routinely processed after post-fixation in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) at 4 °C (overnight) and then washed twice in buffer. The specimens were dehydrated through a graded ethanol series and dried using the critical point method. They were then sputter-coated with gold and mounted on metal stubs. The ultrastructural studies were performed by JSM-5410 electron microscope.

DNA extraction, amplification and sequencing

DNA extraction was performed on fish host muscle ($n = 5$) and on parasites ($n = 15$) kept in 70% ethanol. Individual parasites were rehydrated, with agitation, during 48 h in sterilized water to remove the ethanol. Fish host and parasite total DNA were extracted using Qiagen DNeasy Blood and Tissue Kit® (Qiagen, Germany) according to the manufacturer's instruction. Extracted DNA was stored at −20 °C until further use.

On fish DNA, a 464 bp region of the *cytochrome b* was amplified using the primers pair described by Calo-Mata et al. (2003): H15149AD: 5′-GCICCTCARAATGAYATT TGTCCTCA-3′ for the forward primer, and for the reverse L14735: 5′-AAAAACCACCGTTGTTATTCAACTA-3′. PCR reactions contained 12.5 μL of 2 × Red Dye Master Mix (Bioline, England), 2 μL of each primer, 6.5 μL of ultrapure water and 2 μL of template DNA for a total reaction volume of 25 μL. PCR reactions were run under the following conditions: hot start (94 °C /5 min) followed by 35 cycles of 94 °C/90 s, 50 °C/90 s and 72 °C/90 s and a final elongation at 72 °C for 7 min.

For the parasite, a portion of the 28S rDNA was amplified using the forward C1 (5′-ACC CGC TGA ATT TAA GCA T-3′) and reverse D2 (5′-TCC GTG TTT CAA GAC GG-3′) universal primers at positions 25 and 1126 of the complete *Mus musculus* 28S rDNA (Jovelin and Justine 2001; Hassouna et al. 1984). PCR reactions contained 12.5 μL of 2 × Red Dye Master Mix (Bioline, England), 1.25 μL of each primer and 10 μl of template DNA for a total reaction volume of 25 μL. PCR reactions were run under the following conditions: hot start (95 °C /3 min) followed by

29 cycles of 95 °C/30 s, 57 °C/30 s and 72 °C/1 min and a final elongation at 72 °C for 5 min. The amplification products (5 μL) from vertebrate and parasite were checked for size by gel electrophoresis with the molecular weight marker HyperLadder™ 100 bp (Bioline, England) on a 1% agarose gel.

PCR products were purified using the NucleoSpin gel and PCR clean-up kit (Macherey–Nagel, Germany), according to the manufacturer's instruction. Purified PCR products of the *cytochrome b* gene region (fish host) and 28S rDNA (gill parasite) were sequenced in both directions using its respective forward and reverse primers. Raw sequences were treated in BioEdit software. Final sequences were used for sequence similarity using BLAST analysis.

Phylogenetic analysis

MEGA-X software (Kumar et al. 2018) was used to generate phylogeny for partial *cytochrome b* gene regions with our *T. luscus* sequences and for partial 28S rDNA gene regions with our *Diclidophora* spp. Both *T. luscus* and *Diclidophora* spp. sequences were aligned with sequences of other similar fish hosts (Table 2) and parasites (Table 3), respectively, retrieved from the GenBank, NCBI. Multiple alignments were done using CLUSTAL W program, and simple trees were constructed by maximum likelihood method (Saitou and Nei 1987). Model selection (ML) implemented in MEGA X was used to find the best DNA model for evolutionary distances and phylogeny. The models with the lowest Bayesian information criterion (BIC) scores were considered to describe the substitution pattern the best. The selected DNA models were the HKY: Hasegawa-Kishino-Yano model for *Diclidophora* specimens (Online Resource 1) and HKY: Hasegawa-Kishino-Yano model for *Trisopterus* specimens (Online Resource 2).

The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbour-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum composite likelihood (MCL) approach and then selecting the topology with superior log likelihood value. A discrete Gamma distribution to model evolutionary rate differences among sites for *Diclidophora* (5 categories (+ G, parameter = 0.2631)) and *Trisopterus* (5 categories (+ G, parameter = 1.9298)) was used. In the case of *Trisopterus* analysis, the rate variation model allowed for some sites to be evolutionarily invariable ([+ I], 55.08% sites). The bootstrap value was set at 1000 to represent strong evolutionary relationships between *Trisopterus* spp. or *Diclidophora* spp. to other species.

Table 2 Sequences used in the present molecular study for *Trisopterus* spp

GenBank accession	Locality	Species	References
MW811331	Atlantic,Portugal	*Trisopterus luscus*	Present study
MW811332	Atlantic, Portugal	*Trisopterus luscus*	Present study
MW811333	Atlantic, Portugal	*Trisopterus luscus*	Present study
X76365	Norwegian coastal	*Gadus morhua*	Johansen and Johansen 1994
AF081685	Hecate Strait, NE Pacific	*Theragra chalcogramma*	Carr et al. 1999
AF081683	Hecate Strait, NE Pacific	*Gadus macrocephalus*	Carr et al. 1999
AF081684	Newfoundland Shelf, NW Atlantic	*Gadus ogac*	Carr et al. 1999
AF081686	Newfoundland Shelf, NW Atlantic	*Boreogadus saida*	Carr et al. 1999
AB091097	Japan	*Boreogadus saida*	Direct submission
AF081688	North Sea, NE Atlantic	*Merlangius merlangus*	Carr et al. 1999
AF469634	North Sea, NE Atlantic	*Pollachius virens*	Moller et al. 2002
AF081692	Gulf of St. Lawrence, NW Atlantic	*Microgadus tomcod*	Carr et al. 1999
AF081690	Barents Sea, NE Atlantic	*Eleginus navaga*	Carr et al. 1999
AF081691	Hecate Strait, NE Pacific	*Microgadus proximus*	Carr et al. 1999
DQ174068	Bay of Biscay, France	*Micromesistius poutassou*	Teletchea et al. 2006
EU492308	Baltic Sea and Skagerrak, Sweden	*Micromesistius poutassou*	Direct submission
AB571075	Chile	*Micromesistius australis*	Direct submission
AB248665	Chile	*Micromesistius australis*	Direct submission
EU224044	Bay of Biscay, France	*Trisopterus minutus*	Direct submission
AF081695	North Sea, NE Atlantic	*Trisopterus esmarkii*	Carr et al. 1999
EU492306	Baltic Sea and Skagerrak, Sweden	*Trisopterus esmarkii*	Direct submission
EU492342	Baltic Sea and Skagerrak, Sweden	*Trisopterus esmarkii*	Direct submission
EU492344	Baltic Sea and Skagerrak, Sweden	*Trisopterus esmarkii*	Direct submission
EF439620	Western Mediterranean, Spain	*Trisopterus minutus*	Direct submission
JF309490	Spain	*Trisopterus minutus*	Gonzalez et al. 2012
JF309491	Spain	*Trisopterus minutus*	Gonzalez et al. 2012
DQ174081	Bay of Biscay, France	*Trisopterus luscus*	Teletchea et al. 2006
EU224042	Bay of Biscay, France	*Trisopterus luscus*	Direct submission
KJ632964	UK	*Trisopterus luscus*	Direct submission
KJ686358	Spain	*Trisopterus luscus*	Direct submission
FR851429	Mediterranean Sea, Italy	*Sardina pilchardus*	Armani et al. 2012
FR851430	Mediterranean Sea, Italy	*S. pilchardus*	Armani et al. 2012
FR851430	Mediterranean Sea, Italy	*S. pilchardus*	Armani et al. 2012

Abbreviations: *NE*, northeast; *NW*, northwest; *UK*, United Kingdom

Results

Epidemiological data

Pouting examined in 2012 ($n = 58$) weighed (wt $\pm$ SD) 167.51 ± 62.97 g and were 24.05 ± 3.02 cm in length (lt $\pm$ SD); those examined in 2013 ($n = 124$) weighed 100.69 ± 35.67 g and were 20.66 ± 2.62 cm in length, and those examined in 2020 ($n = 31$) weighed 106.15 ± 34.19 g and were 21.10 ± 3.75 cm in length. There was a similar variation between intensity of infection and length and weight, revealing that the greater the intensity, the lower the weight and the length of the fish.

Prevalence rates obtained were slightly higher in 2020: 94% compared to 64% in 2012 and 72% in 2013 (Fig. 1a). The ratios of variance/mean were 2.76, 3.09 and 7.01 for 2012, 2013 and 2020, respectively, confirming that the distribution of samples is over-dispersed as expected. Concerning the median value of intensity (presented instead the mean, since median is a more robust estimator of intensity for over-dispersed distributions) was 2.0 ranged from 2.22 to 3.65 in 2012, 2 ranged from 2 to 3 in 2013 and 5 ranged from 5.34 to 10.4. The mean value of abundance was 1.81 ranged from 1.28 to 2.45 in 2012, 2.2 ranged from 1.81 to 2.75 in 2013 and 6.97 ranged from 4.94 to 9.71. Boxplot graphs for each year are presented (Fig. 1b) to support these results.

Table 3 Sequences used in the present molecular study for *Diclidophora* spp

GenBank accession	Country	Trematode species	Family and subfamily	Vertebrate host	References
MN860190	Portugal	*Diclidophora luscae capelanii*	Dicl; Dnae	*Trisopterus luscus*	Present study
MN860188	Portugal	*Diclidophora luscae capelanii*	Dicl; Dnae	*T. luscus*	Present study
MN860185	Portugal	*Diclidophora luscae capelanii*	Dicl; Dnae	*T. luscus*	Present study
MN860189	Portugal	*Diclidophora luscae capelanii*	Dicl; Dnae	*T. luscus*	Present study
MN860186	Portugal	*Diclidophora luscae capelanii*	Dicl; Dnae	*T. luscus*	Present study
MN860191	Portugal	*Diclidophora luscae capelanii*	Dicl; Dnae	*T. luscus*	Present study
MN860187	Portugal	*Diclidophora luscae capelanii*	Dicl; Dnae	*T. luscus*	Present study
MW287140	Portugal	*Diclidophora luscae capelanii*	Dicl; Dnae	*T. luscus*	Present study
MW287141	Portugal	*Diclidophora luscae capelanii*	Dicl; Dnae	*T. luscus*	Present study
MW287142	Portugal	*Diclidophora luscae capelanii*	Dicl; Dnae	*T. luscus*	Present study
AF311704	France	*Diclidophora luscae capelanii*	Dicl; Dnae	*T. luscus*	Jovelin and Justine (2001)
AF382047	United Kingdom (UK): North Sea	*D. denticulata*	Dicl; Dnae	*Pollachius virens*	Direct Submission
AY157169	UK: North Sea	*D. denticulata*	Dicl; Dnae	*Pollachius virens*	Direct Submission
AF382048	UK: North Sea	*D. minor*	Dicl; Dnae	*Micromesistius poutassou*	Direct Submission
FJ432588	–	*Urocotyle nibae*	Mazoc; Dicl	–	Direct Submission
KJ397730	Chile: Antofagasta	*Paraeurysorchis sarmientoi*	Dicl; Eury	*Seriolella violacea*	Oliva et al. (2014)
KJ397731	Chile: Coquimbo	*Parapedocotyle prolatili*	Dicl; Para	*Prolatilus jugularis*	Oliva et al. (2014)
KJ397726	Chile: Antofagasta	*Chalguacotyle mugiloides*	Mazoc; Dicl	*Pinguipes chilensis*	Oliva et al. (2014)
KJ397727	Chile: Antofagasta	*Choricotyle anisotremi*	Dicl; Chor	*Anisotremus scapularis*	Oliva et al. (2014)
AF382046	Australia: Coffs Harbour, NSW	*Choricotyle australiensis*	Dicl; Chor	*Rhabdosargus sarba*	Direct Submission
KJ397728	Peru: Callao	*Pedocotyle annakohni*	Dicl; Pedo	*Stellifer minor*	Oliva et al. (2014)
KJ397729	Peru: Callao	*Pedocotyle bravoi*	Dicl; Pedo	*Stellifer minor*	Oliva et al. (2014)
KU872037	India: Mumbai	*Keralina opisthopterus*	Mazoc; Dicl	*Ilisha megaloptera*	Direct Submission
KU872041	India: Mumbai	*Sauricotyle sprostoni*	Mazoc; Dicl	*Saurida tumbil*	Direct Submission
KU872040	India: Mumbai	*Sauricotyle sprostoni*	Mazoc; Dicl	*Saurida tumbil*	Direct Submission
AF382036	UK: Isle of Man	*Discocotyle sagittata*	Mazoc; Discocotylidae	*Salmo trutta*	Direct Submission
AF382042	Brazil: Paraná	*Paradewesia* sp.	Mazoc; Neo	*Scomberomorus* sp.	Direct Submission
MH011389	Brazil: Rio de Janeiro	*Pseudotagia rubri*	Macrov	*Orthopristis ruber*	Direct Submission

Abbreviations: *Dicl* Diclidophoridae, *Para* Parapedocotylinae, *Pedo* Pedocotylinae, *Eury* Eurysorchiinae, *Chor* Choricotylinae, *Dnae* Diclidophorinae, *Macrov* Macrovalvitrematidae, *Mazoc* Mazocraeidea, *MNAE* Mazocraeinae, *Neo* Neothoracocotylidae

However, when the correlations between weights, lengths and parasites found were verified, only weight and length revealed a statistically significant correlation of $R = 0.869$ (p value < 0.01).

Given that the samples failed the normality test, the non-parametric Kruskal–Wallis test was chosen to confirm the existence of differences between the analysed statistics

Fig. 1 Infective parameters. Parasitized fish, parasite numbers and prevalence (**a**); parasite abundance with standard deviation (**b**) in studied *Trisopterus luscus*
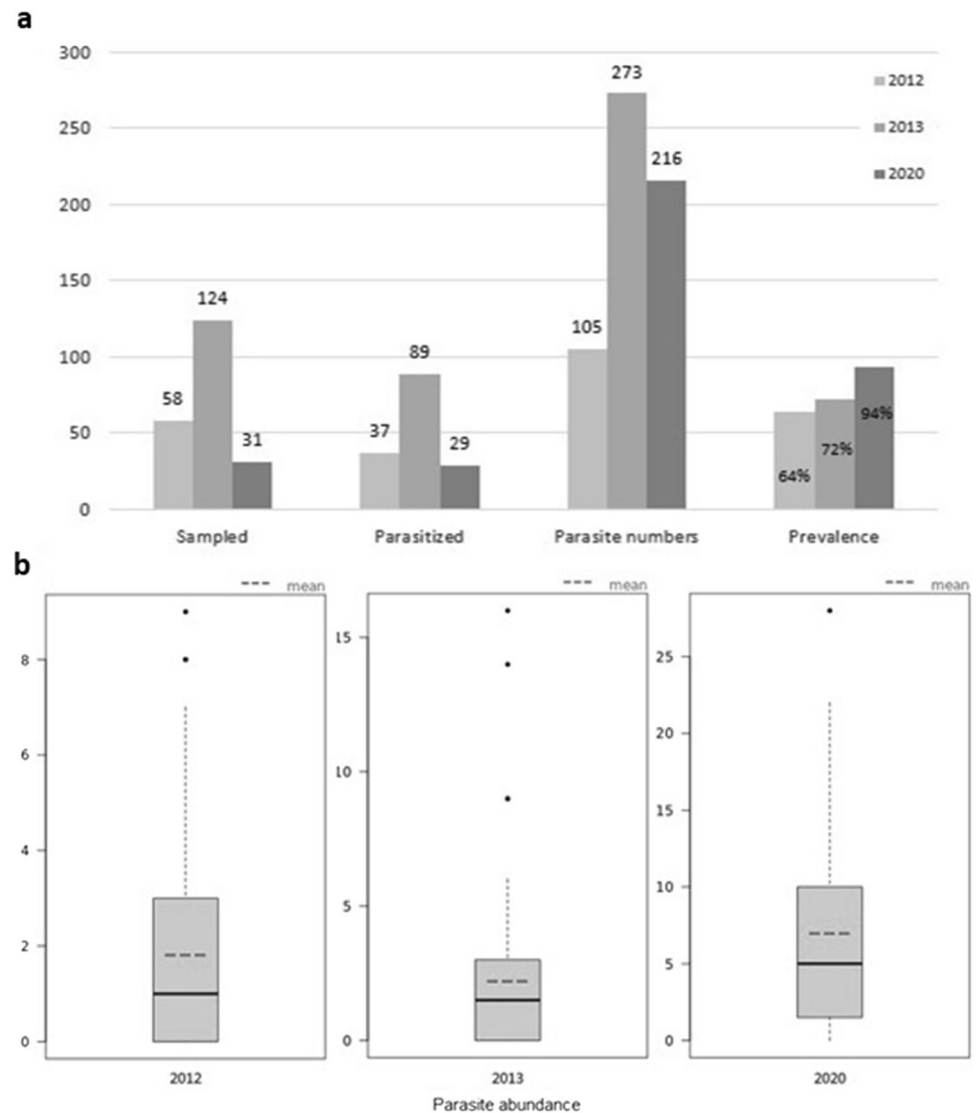
(length, weight and parasites) for the different sampled years.

The results revealed the existence of significant statistical differences for the three statistical treatments. Particularly analysing the pouting weight, it appears that there are no differences between 2013 and 2020. However, in 2012, the specimens were, on average, heavier (Table 4).

The same approach was used to analyse the length, and the results are similar. Also, in terms of length, the year 2012 stands out in relation to the years 2013 and 2020, with larger individuals (Table 5).

However, when analysing the number of parasites found, the data support the values of abundance, intensity and prevalence of 2020 (Fig. 1b). There is a clear distinction between the 2012 and 2013 results, compared to those recorded in 2020 (Table 6).

Host characterization

Hosts were identified by morphological features as *T. luscus* and confirmed by *cytochrome* b DNA marker (Fig. 2).

The tree with the highest log likelihood ($-2{,}027.73$) is shown. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 33 nucleotide sequences. Codon positions included were 1st + 2nd + 3rd + Noncoding. All positions with less than 95% site coverage were eliminated, i.e., fewer than 5% alignment gaps, missing data, and ambiguous bases, were allowed at any position (partial deletion option).

There were a total of 375 positions in the final dataset. Sequences identified in this study clustered with sequences obtained from specimens collected in the Bay of Biscay (France) (Teletchea et al. 2016), Manchester (UK) and

Table 4 Pairwise comparisons of year (average weight)

S1-S2	Test Statistic	Std. Error	Std. Test Statistic	Sig.	Adj. Sig. [a]
2013-2020	-9.601	12.376	-.776	.438	1.000
2013-2012	75.203	9.804	7.670	.000	.000
2020-2012	65.602	13.712	4.784	.000	.000

Pairwise Comparisons of Year

Each node shows the sample average rank of Year.

Each row tests the null hypothesis that the Sample 1 (S1) and Sample 2 (S2) distributions are the same

Asymptomatic significances (2-sided tests) are displayed. The significance level is .05: [a]Significance values have been adjusted by the Bonferroni correction for multiple test
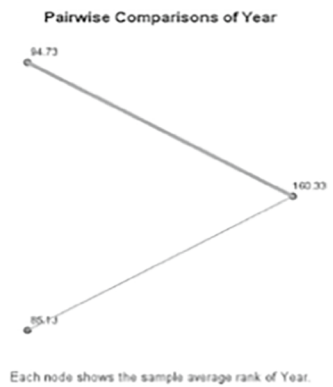
Table 5 Pairwise comparisons of year (average length)

Pairwise Comparisons of Year

Each node shows the sample average rank of Year.

S1-S2	Test Statistic	Std. Error	Std. Test Statistic	Sig.	Adj. Sig. a
2013-2020	-2.742	12.376	-.222	.825	1.000
2013-2012	68.192	9.804	6.957	.000	.000
2020-2012	65.450	13.712	4.774	.000	.000

Each row tests the null hypothesis that the Sample 1 (S1) and Sample 2 (S2) distributions are the same. Asymptomatic significances (2-sided tests) are displayed. The significance level is .05: [a]Significance values have been adjusted by the Bonferroni correction for multiple test
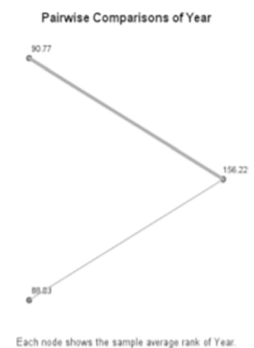
**Table 6** Pairwise comparisons of year (average of parasites)

Pairwise Comparisons of Year

Each node shows the sample average rank of Year.

S1-S2	Test Statistic	Std. Error	Std. Test Statistic	Sig.	Adj. Sig. a
2013-2020	-10.555	9.625	-1.097	.273	.818
2013-2012	-60.310	13.462	-4.480	.000	.000
2020-2012	-49.754	12.150	-4.095	.000	.000

Each row tests the null hypothesis that the Sample 1 (S1) and Sample 2 (S2) distributions are the same. Asymptomatic significances (2-sided tests) are displayed. The significance level is .05: [a]Significance values have been adjusted by the Bonferroni correction for multiple test
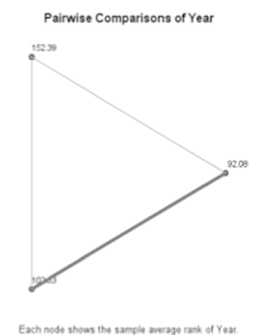
Fig. 2 *Trisopterus luscus cytochrome b* characterization. The evolutionary history was inferred by using the maximum likelihood method and Hasegawa-Kishino-Yano model (Hasegawa et al. 1985). *Present study
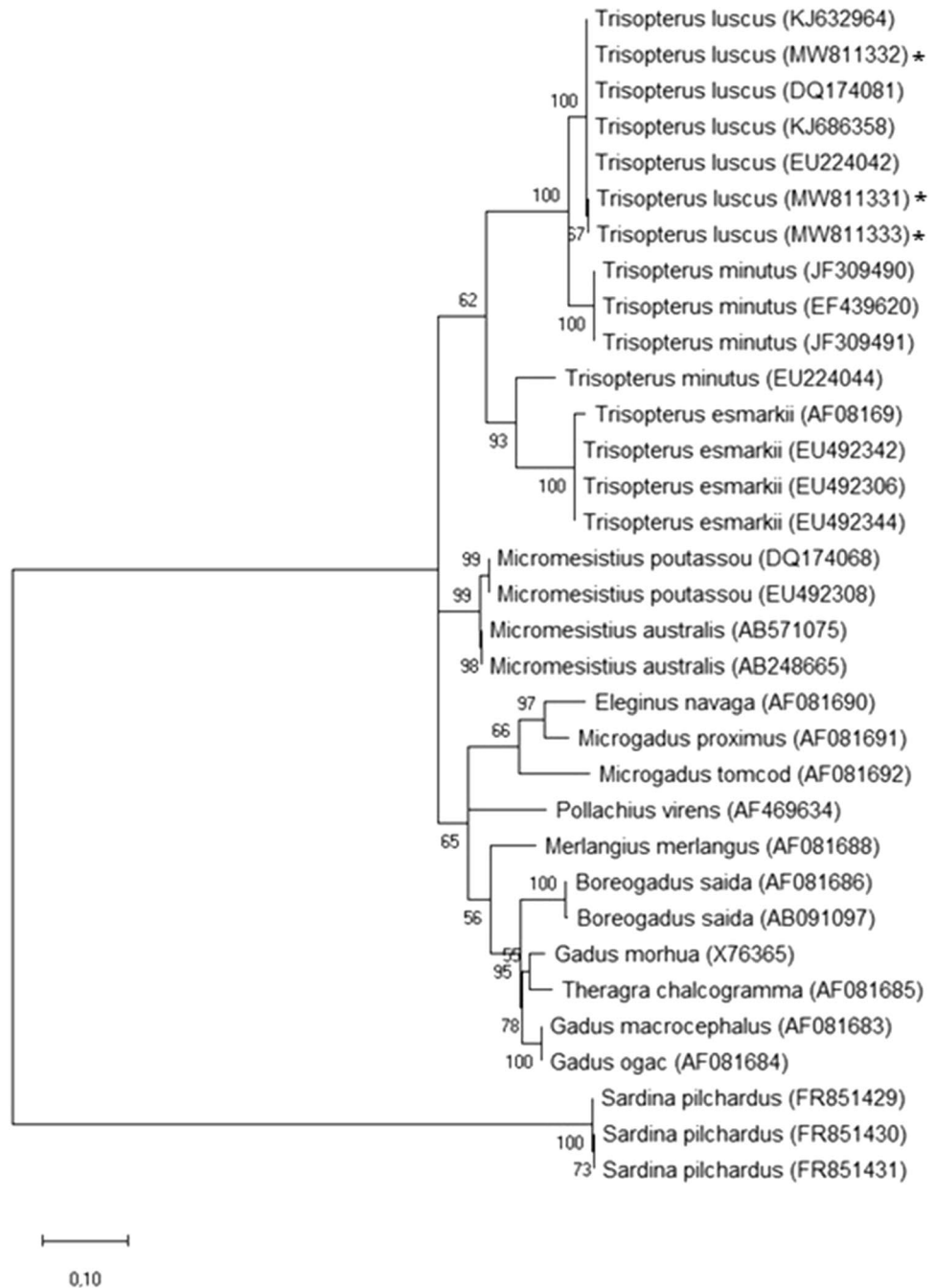
Pontevedra (Spain), and they are monophyletic with *T. minutus* from Spain (Gonzalez et al. 2012) (Fig. 2).

Site of infection

Gills. The parasites were attached with their posterior adhesive organs to a single primary lamella nearer to the gill arch of the host, between the two hemibranchs of a gill and with the anterior end nearer to the distal end of the primary lamellae (Fig. 3). A total of 594 specimens were collected from 213 hosts. The parasite was present in the outer hemibranch and more frequent in 2nd and 3rd gill arches (47.62% and 39.05% in 2012; 52.38% and 44.69% in 2013; 51.45% and 36.44% in 2020) (Table 7).

No apparent pathological effects on the gill were macroscopically observed.

Deposition of Diclidophora specimens

The parasite collection of Pathology Laboratory of Aquatic Animals at the Portuguese Institute for the Ocean and Atmosphere (Lisbon, Portugal).
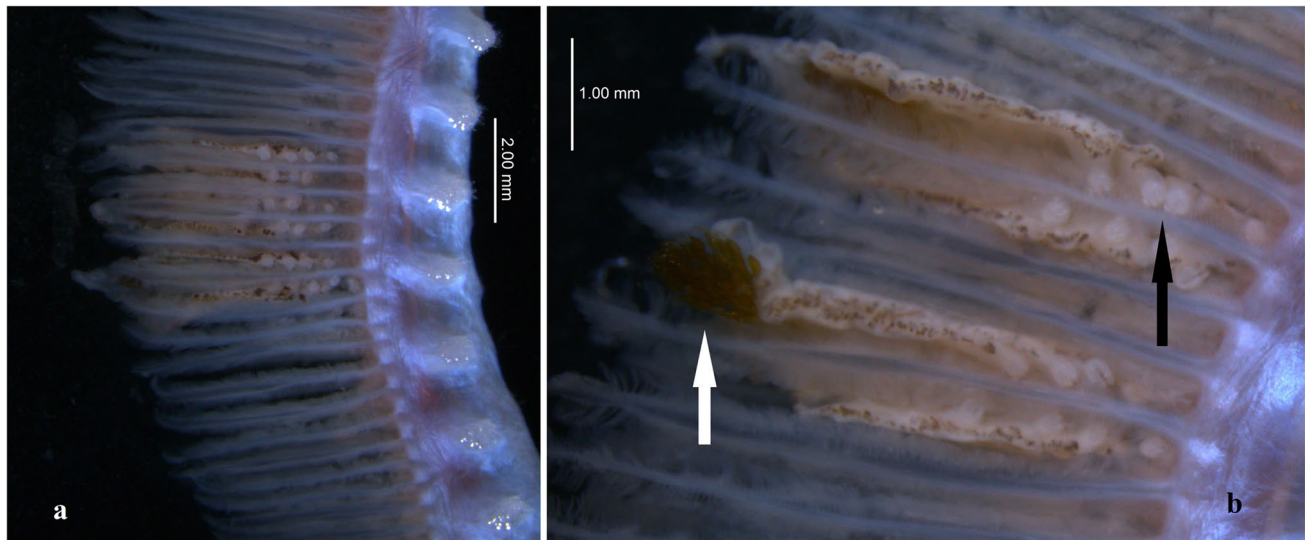
Fig. 3 *Diclidophora luscae* location on host gills (**a**) with posterior haptor (black arrow) fixed to the basis of the hemibranch and extremity anterior free from where they shed bundles of eggs (white arrow) (**b**)

Table 7 Distribution of *Diclidophora luscae* on gill arches of *Trisopterus luscus*

| RBA	Specimens (n)			LBA	Specimens (n)			Total						
	2012	2013	2020		2012	2013	2020	2012	%	2013	%	2020	%
R1	5	2	6	L1	7	2	11	12	**11.43**	4	**1.47**	17	**8.25**
R2	22	66	66	L2	28	77	50	**50**	**47.62**	143	**52.38**	116	**51.46**
R3	20	75	23	L3	21	47	50	41	**39.05**	122	**44.69**	73	**35.44**
R4	1	1	4	L4	1	3	6	2	**1.90**	4	**0.73**	10	**4.85**
Total	**48**	**144**	**99**	**Total**	**57**	**129**	117	**105**		**273**		**216**	
%	**8.08**	**24.24**	**16.67**	%	**9.6**	**21.72**	**19.70**	17.68		45.96		36.36	

RBA, right branchial arches; *LBA*, left branchial arches; *R1 to R4*, first right branchial arches to fourth; *L1 to L4*, first left branchial arches to fourth

## *Diclidophora luscae* (Van Beneden & Hesse, 1863) Price, 1943) description

Morphological identification

Diclidophoridae with bilateral symmetrical body measured 7.27 ± 1.0 in length and 2.21 ± 0.4 in width; surface topography of the parasite by SEM appears rough as microvilli-like structures are present (Fig. 4a); thin proximal end and broad posterior end; distance to female gonopore 1.09 ± 0.53; pre-ovary length 5.17 ± 0.81; paired buccal suckers aseptate; muscular pharynx larger than buccal suckers (Fig. 5a, b); the tegument around the mouth is irregular and surrounded by numerous papillae (Fig. 5c); copulatory organ consisting of muscular penis with single genital corona armed with sickle-shaped, grooved hooks (Fig. 5a). Testes follicular, numerous, pre-, para- and post-ovary, extending into haptor region (Fig. 6). Seminal receptacle roughly spherical and antero-lateral to the ovary

(Fig. 6). Inverted N-shaped ovary located in the anterior haptor region. Uterus present (Fig. 6). Separate genital aperture (male and female) (Fig. 4b). Female genital aperture opening at 1.09 ± 0.5, posteriorly to terminal male genitalia (Fig. 5a). Vitellarium extending from the anterior region into the haptor (Fig. 6). Haptor with four pairs of pedunculated clamps (Fig. 7a, b). Each clamp consists of a pair of opposable jaws supported by sclerites; first clamp measured 0.62 ± 0.08 in length and 0.35 ± 0.05 in width. In the outer ventral anterior quadrant of the clamps, small nodules variable in size were noticed in stained and SEM prepared specimens (Fig. 7c, d). The type III eggs are fusiform in shape with one appendage in each pole: one longer funnel-shaped and entangled, resembling grappling hooks held together and the other shorter and cruzier-shaped projected anteriorly in utero, remaining free (Fig. 8). Excluding polar appendages, intrauterine eggs measured 0.223 (0.014; 0.184–0.276) in length and 0.082 (0.004; 0.070–0.097) in width.
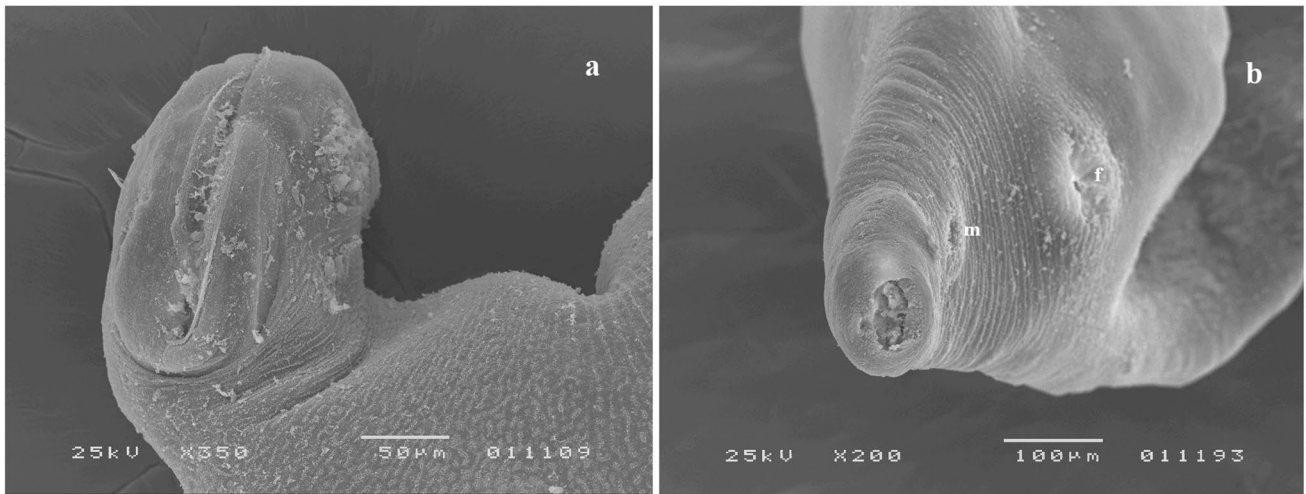
Fig. 4 *Diclidophora luscae* ventral tegument with a rough surface due to microvilli-like structures (**a**); separate female (f) and male (m) genital aperture (**b**) (SEM)
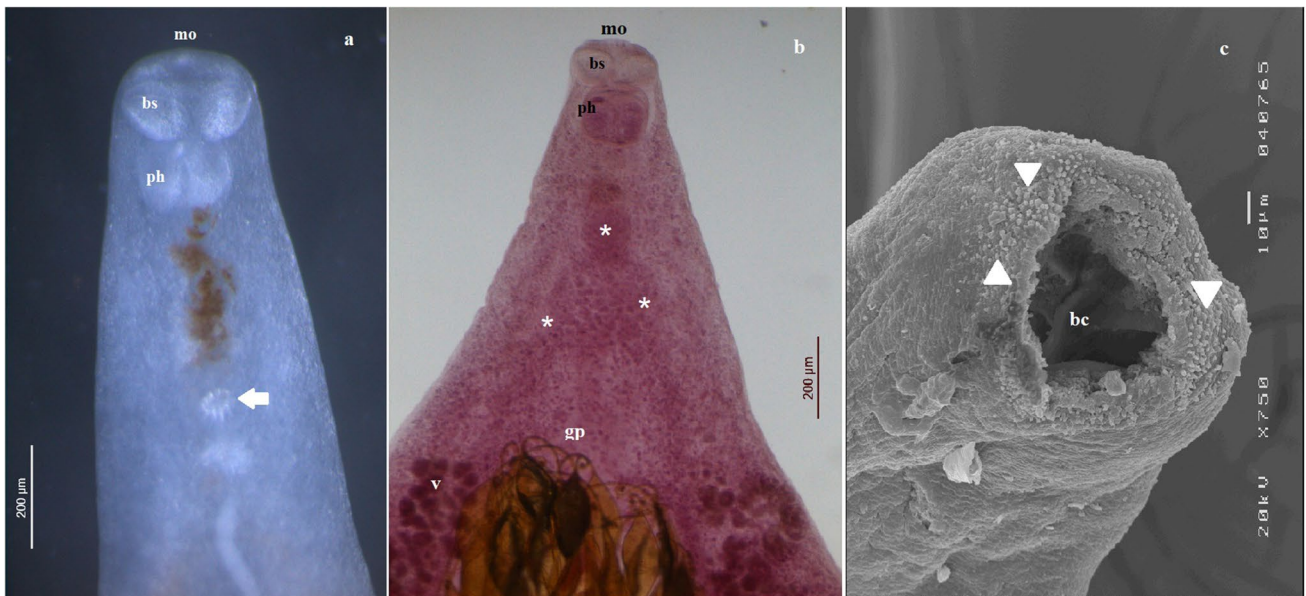
Fig. 5 Image sequences of the anterior end obtained from fresh (**a**), stained (**b**) and SEM (**c**) *Diclidophora luscae*. Mouth (mo), bucal cavity (bc), paired buccal sucker aseptate (bs), muscular pharynx (ph), muscular penis (setae), female genital pore (gp); the tegument around the mouth is irregular and characterized by numerous papillae (setae head). Vitellarium (v). Caecal bifurcation (*)

The comparative studies on morphometric *Diclidophora* sp. traits evidenced a decrease in all parameters used for component analyses from fresh to stained specimens (Table 8; Fig. 6). The analysis of data pointed out to a strong correlation (92%, 97.8%, 83.1% and 93.3%, respectively) between fixed/pressed and stained specimens in BL, BW, DG and POL ($p < 0.01$) and also (96.4%) between thawed and fixed/pressed in BL and POL ($p < 0.01$) (Table 8).

Molecular characterization and phylogenetic analysis

Molecular studies on *Diclidophora* specimens identified them as *D. luscae capelanii* by 28S rDNA marker (Fig. 9). The tree with the highest log likelihood ($-4,437.46$) is shown. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 28 nucleotide sequences. Codon positions included were 1st + 2nd + 3rd + Noncoding. All positions with less than

Fig. 6 Fresh *Diclidophora luscae* (**a**) and stained (**b**). Ventral view. Reproductive system: seminal receptacle (sr), proximal portion of uterus (u), uterus with bundle of eggs (be), follicular testes (*), ovary (ov) and ootype (setae). Vitellarium (v)
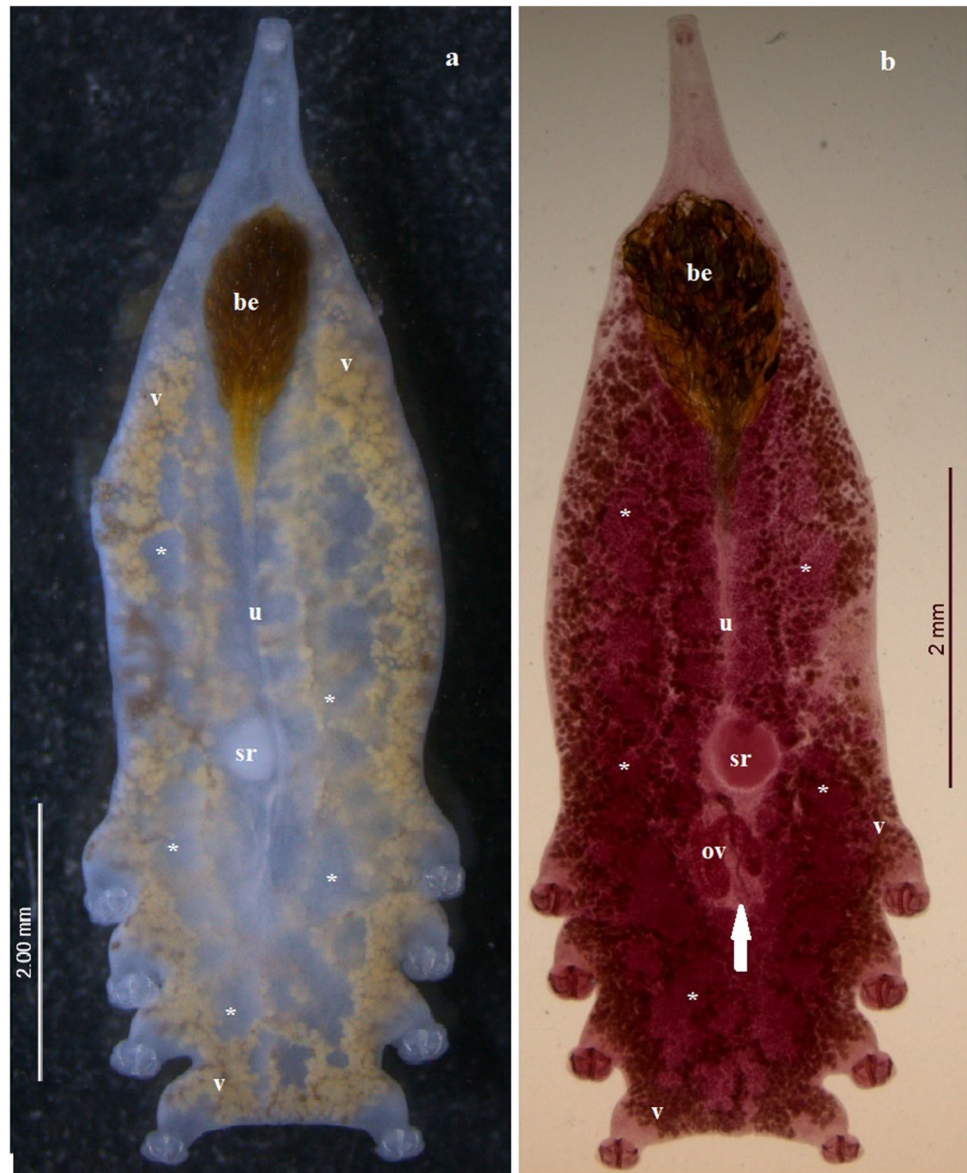

95% site coverage were eliminated, meaning that fewer than 5% alignment gaps, missing data and ambiguous bases were allowed at any position (partial deletion option). There were a total of 769 positions in the final dataset.

Phylogenetic analysis grouped the sequences obtained in this study in a cluster, which also grouped with a sequence from a gill parasite identified as *D. luscae capelanii* recovered from pouting. A similar study was performed based on Neighbour-Joining method whose results were like these showed by the maximum likelihood method (Online Resource 3) (Fig. 9).

Discussion

The importance of knowledge of community structures from shallow coastal areas and their spatial distribution over time for an adequate management of marine resources was highlighted by Felício et al. (2021). On the other hand, it has been demonstrated that fish parasites can be useful as biological indicators to illustrate the ecology of their hosts, as they are related to their distribution, migration
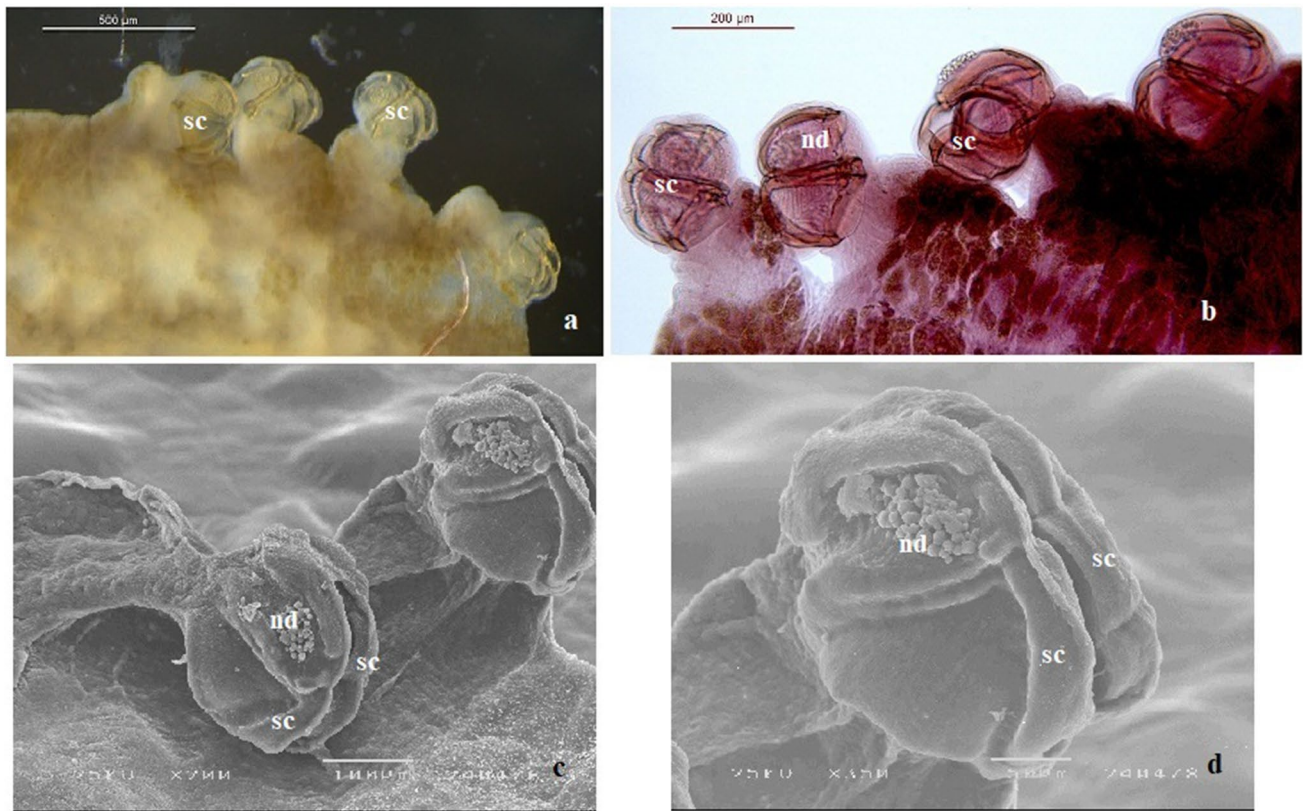
Fig. 7 Image sequence of the fresh *Diclidophora luscae* (**a**), stained (**b**) and SEM (**c**, **d**). Clamp, anterior jaw: nodules (nd) and sclerites (sc)

and biology and can reflect some environmental climatic changes over a specific population (Palm 2011). The present study can be considered as a first contribution to the natural population of pouting off the Portuguese coast and its interaction with the gill trematode *Diclidophora*, as it is the first study in which the infection parameters of *Diclidophora* infection in pouting are analysed in samples obtained over three years and inter-annually compared. The data showed variation among interannual samples concerning the host biological and parasite infective parameters considered. Despite *Diclidophora* infection seems to evidence a continuous appearance over the years, the infection levels in terms of prevalence, mean intensity and abundance pointed to an increase in the infection level. This increase could be associated with an earlier fish age of infection occurring in the catch areas where the parasite is endemic. The ratios of variance/mean (2.76, 3.09 and 7.01) confirm overdispersion of *D. luscae* in *T. luscus* samples and among them. Authors point out that several factors could determine different aggregation of parasites (Lester 2012; Lester and McVinish 2016). Heterogeneity in exposure or heterogeneity in susceptibility to infection or both, food availability and the survival of the bundles of entangled eggs sheds into water column could be contributing factors in the aggregation of *D. luscae* in pouting

samples, but at present, we have no data to support these hypotheses.

Diclidophora species have been considered to exhibit high host specificity. Llewellyn (1958), using only one fish species, highlighted to a close host-parasite co-evolution, illustrating the evolutionary history and phylogeny of their hosts. However, recent studies provided evidence of *Diclidophora* species occurring in host species different from the original or "preferred" host (Perdiguero-Alonso et al. 2006; Strona et al. 2010; Morsy et al. 2018) (Table 1), and it was suggested that they could represent accidental infections (Perdiguero-Alonso et al. 2006). The morphology of different *Diclidophora* species is quite similar with slight differences in size, shape, haptor, ovary position and testes distribution, as summarized on Table 9. The general morphology observed in the studied specimens was similar to *D. luscae* (van Beneden and Hesse 1863) Price, 1943 (Monogenea, Diclidophoridae), as described by Dawes (1947) and added to Rubec and Dronen (1994), in brief: ovary in the anterior haptor region, testes follicular extending into haptor region, presence of uterus, separate genital aperture and fusiform eggs type III (Table 9). In this study, stained and fixed *Diclidophora* specimens were smaller than those reported from *D. luscae* in pouting by Perdiguero-Alonso et al. (2006) [($n = 2$), BL = 5.44–5.51;
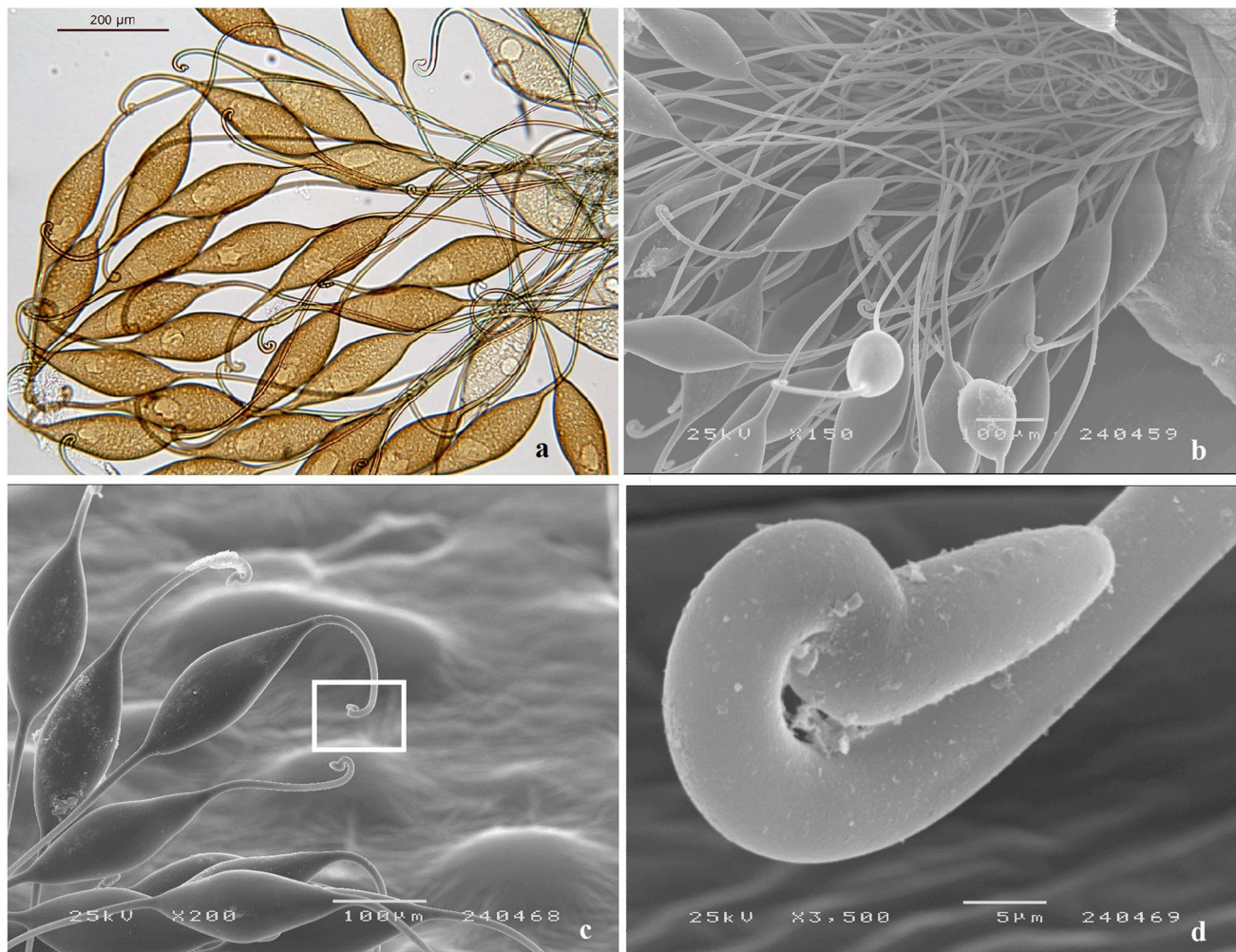
Fig. 8 Bundles of fusiform eggs with polar appendages resemble grappling hooks held together by their entangled long appendages. Wet mount (**a**) and SEM (**b** to **c**). Inset show the shorter hook-shaped opercular appendage (square) remains free from its neighbours (**c**). Hook-shaped opercular appendage detail (**d**)

Table 8 Comparative data (mean values ± SD) of the parasite variables obtained from fresh and frozen hosts and after sequential staining procedures (mm)

$n = 30$	Fresh	Defrozen	Fixed/pressed	Stained
BL	7.27 ± 1.0	4.61 ± 0.6	4.17 ± 0.5	3.85 ± 0.5
BW	2.21 ± 0.4	1.68 ± 0.2	1.53 ± 0.2	1.45 ± 0.2
DG	1.09 ± 0.5	0.67 ± 0.2	0.67 ± 0.2	0.55 ± 0.2
POL	5.17 ± 0.8	nd	2.49 ± 0.3	2.26 ± 0.4
PTOL	0.03 ± 0.08	nd	0.23 ± 0.1	0.19 ± 0.09
FCL	0.62 ± 0.08	nd	0.55 ± 0.1	0.48 ± 0.1
FCW	0.35 ± 0.05	nd	0.31 ± 0.03	0.25 ± 0.02

BL, body length; *BW*, body width at level of the origin of haptor; *DG*, distance to female genital aperture; *POL*, pre-ovary length; *PTOL*, post-ovary length; *FCL*, first clamp length; *FCW*, first clamp width; *nd*, no data available

BW = 2.31–2.4] and Llewellyn et al. (1980) from Plymouth [($n = 10$), BL = 3.4 ± 0.3 (3.1–4.1)], respectively. However, morphological and metric comparisons of *Diclidophora* are not considered as a reliable trait to characterize specimens due to their individual variability (Llewellyn et al. 1980; Perdiguero-Alonso et al. 2006), which do not allow for easy specific identification (Ramos et al. 2014b). In fact, our study provides information about the discrepancy between the morphological variables obtained from previously frozen specimens of *Diclidophora* due to the extensibility of living specimens and their varied responses to compression and histological fixatives, as already mentioned by Llewellyn et al. (1980).

In addition, the ultrastructural features revealed by SEM were like those obtained from *D. merlangi* (Halton 1979), the only data on *Diclidophora* SEM available. However, *D. luscae* has small and variable in size nodules on the outer

Fig. 9 Evolutionary history of *Diclidophora luscae* from *Trisopterus luscus* (Northeast Atlantic) using the maximum likelihood method and Hasegawa-Kishino-Yano model (Hasegawa et al. 1985). *Present study
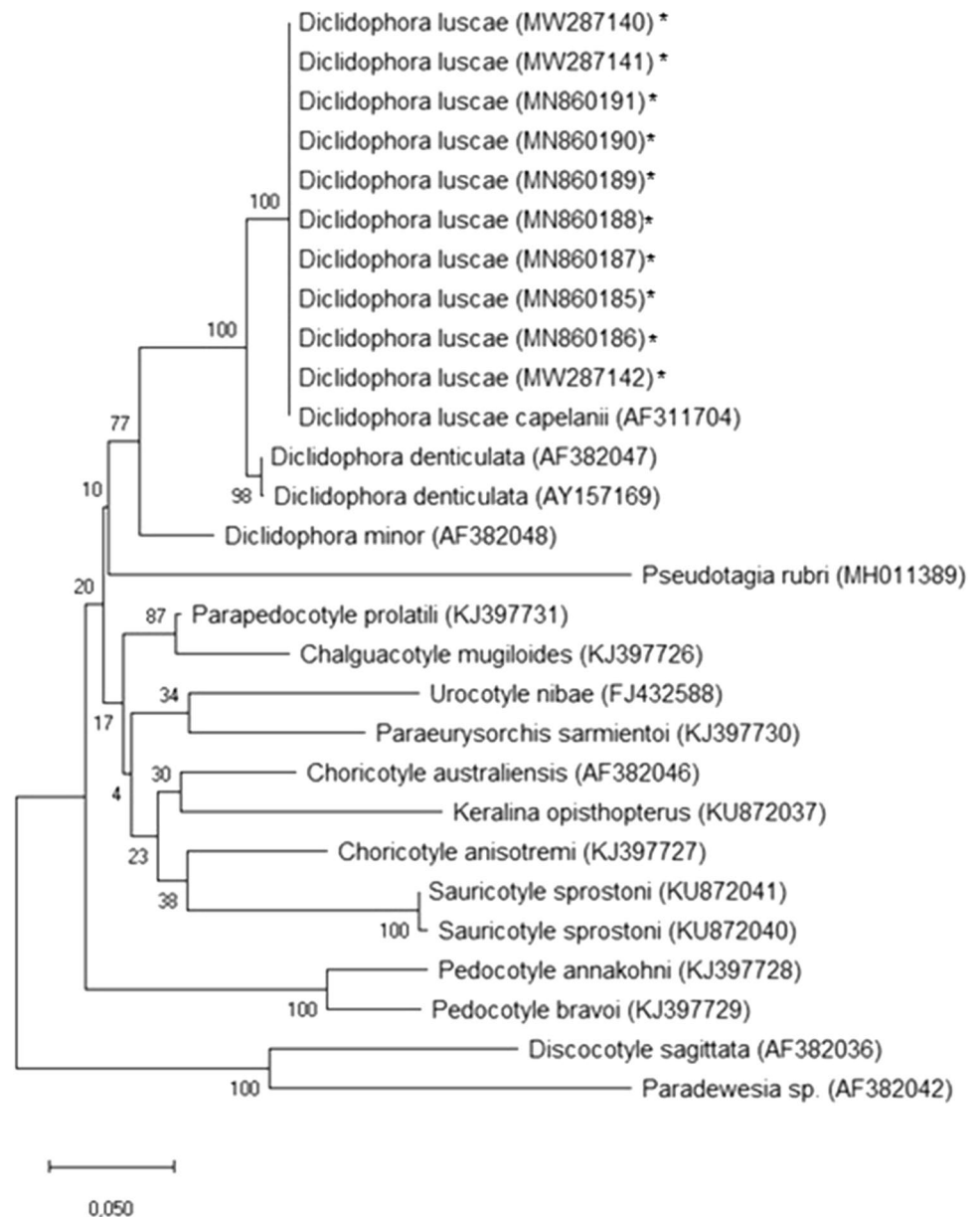
ventral anterior quadrant of the clamps, while *D. merlangi* shows a wart-like outgrowth nodule (Halton 1979).

Morphological studies performed in the present study were reinforced by genetic molecular studies on *Diclidophora* specimens, which were molecularly recognized as *D. luscae capelanii*, as inferred by phylogenetic analysis. Molecular identification of this subspecies was previously performed on *T. capelanus* captured in the Mediterranean Sea, and they were named based on their morphological similarity to *D. luscae* hosted by *T. luscus* from the Atlantic Ocean (Jovelin and Justine 2001). Based on evidence by our work, *D. luscae capelanii* found in *T. capelanus* should be renamed as *D. luscae* as this name prevails according to the international zoological code. Its host specificity involves

two different *Trisopterus* species, *T. luscus* and *T. capelanus.* The later was considered as subspecies of *T. luscus* until Delling et al. (2011) evidenced that it should be considered as a different taxonomic identity.

Fish parasites represent an important part of aquatic biodiversity (Palm 2011; Quiazon 2015), and their distributions become affected either by abiotic factors (temperature, salinity) or biotic ones (life cycle of parasite, host specificity, behaviour, availability of hosts) (Palm 2011; MacKenzie and Abaunza 2014). Thus, determining the factors which affect the distribution of parasites is complex in commercial species in the areas of its distribution (Landa and Cañás 2017). Parasite metrics such as diversity indices or species richness can reflect a possible effect of specific environmental

Table 9 *Diclidophora* species morphological main characteristics based on Rubec and Dronen (1994) and Perdiguero-Alonso et al. (2006)

Species	Size (mm)	Shape	Haptor	Ovary	Testes	Uterus	Genital aperture	Egg morphology
D. merlangi	Up to 9.0 mm and 2.6 mm in breadth posteriorly	Posterior half of body	Peduncles with equal length (0.8–0.9 length)	Posterior half of body	Numerous into haptor level of 2nd, 3rd or 4th haptoral clamp peduncle	-	Common and median	Type I, fusiform with long needle-like opercular appendages
D. denticulata	7.0 mm long	Pointed and anteriorly, but broadening abruptly in the anterior half of the body and uniformly broad in the posterior half	Sclerites with small spines in the anterior valve	Posterior third of the body	Numerous, many of them lateral to the intestine	Present	Separated	Type III, fusiform with one appendage in each pole: one longer entangled resemble grappling hooks held together and other shorter hook-shaped remaining free
D. esmarkii	Smaller size than D. luscae	Similar to D. luscae						
D. luscae	4.7 mm long	Resembling D. pollachii, but more squat, attenuated anterior end shorter, maximum breath at haptor level	Trapezoidal and broadest anteriorly, robust peduncle, each bearing a characteristic clamp with irregular nodules on the ventral valve	Anterior haptoral region between the 1st pair of peduncles; folded into an N-shape	Numerous and distributed between the components of the intestine	Present	Separated	Type III, fusiform with one appendage in each pole: one longer funnel-shaped entangled resemble grappling hooks held together and other shorter cruzier-shaped remaining free
D. minor				Second third of body	Relatively numerous (±150); post-ovary intercaecae, few lobes exceptionally postero-lateral to ovary, not extending to posterior end of body	-	-	Type I
D. palmata				Anterior half of body	Post-ovary, only intercaecae, not extended into the haptoral region	-	-	Type II, absence of polar appendages on the egg
D. phycidis				Posterior half of body	Numerous into haptor level of 2nd, 3rd or 4th haptoral clamp peduncle	-	-	nd

Table 9 (continued)

Species	Size (mm)	Shape	Haptor	Ovary	Testes	Uterus	Genital aperture	Egg morphology
D. pollachii	8.0–13 mm long	Pointed anteriorly, but broadening abruptly in the anterior half of the body, uniformly broad in the posterior half	Clamps and peduncles not as robust as *D. denticulata*, but larger than *D. pollachii*	Posterior third of body	Numerous, organized in small distinct compact groups, pre, para- and post-ovary region, some of them lateral to the intestine,	Present	Separated	Type III
D. micromesisti				Equatorial position of body, intracaecae and pre testes	intercaecae and post-ovary, numerous and compact in the posterior half of the body			Type III

conditions on the fish parasite community (Marcogliese 2008). The ecology of *Diclidophora* in the aquatic environment is unknown, but it is assumed that the acceleration trend in global warming from the early 1990s may interfere with the prevalence and average abundance of the parasite, since the thermal stress weakens the immune status of the host fish, making it more susceptible to the parasite (Marcogliese 2008). So, trying to explain the observed differences in *Diclidophora* prevalence based on environmental factors is not clear.

Parasites that are easy to collect and to identify are important factors for their selection as biological indicators (Palm 2011; MacKenzie and Abaunza 2014). *Diclidophora* is a single-host life cycle parasite whose collection involves little fish handling, and it is easily macroscopically seen, collected and identified. Despite being a hematophagous parasite, we are not aware of its impact on pouting health, nor whether it is more or less temporary in the host, which highlights the importance of monitoring *D. luscae*. On the other hand, although data on *Diclidophora* ecology and biology is scarce, the interpretation of infection parameters in terms of fish population could be more efficient and reliable (MacKenzie et al. 2008).

These features allow its use for fish assessments and should be included in regular monitoring programs, parasitological monitoring studies on PNAB, that will provide long-term datasets, important for host identification, phylogeny and its systematic position in different fish stocks. The knowledge on *Diclidophora* infection parameters in pouting as well as host distribution in the Atlantic is an important starting point that may allow us to evaluate the infection evolution in the coming years. At the current time, *Diclidophora* is under-reported, inhibiting accurate assessments of their prevalence and with their impact possibility underestimated. However, biodiversity is widely considered to correlate with ecosystem health (Mendoza-Franco et al. 2018). So concerning these, *Diclidophora* data seems to be an important contribution for the control and management of pouting stocks and adds knowledge to the total biodiversity of the northeast Atlantic off the Portuguese coast according to the 14th objective of the 2030 agenda for sustainable development.

Conclusions

Monogenea specimens in *T. luscus* gills were identified as *D. luscae* (van Beneden and Hesse 1863) Price, 1943, based on the morphometric and ultrastructural data and confirmed by 28S rDNA marker.

This study reinforced the importance of improving knowledge on the interactions between *T. luscus* and *D. luscae* and on *Diclidophora* species generally and their host

specificities, which revealed *D. luscae* compatibility with *T. luscus* and *T. capelanus*.

Measurements provided information about the effects of freezing, preservation and staining on the parasite size and attention should be drawn to methodological procedures when using morphology to study cross-infection.

For the first time, data on the infection parameters in the pouting community from the northeast Atlantic is displayed, highlighting the importance of using the parasite *Diclidophora* in the national monitoring plan (PNAB) for a holistic approach with possible implications for the management of pouting resources and its usefulness in attaining sustainable development and biodiversity conservation measures.

The variability and the increase registered in the infection parameters (prevalence and mean intensity) suggest the importance and interest of this study in understanding the host-parasite interrelationships that might result from climate and environmental change.

Finally, more research should be performed to clarify the host-parasite interactions on pouting stocks and their importance to the commercial circuit.

Declarations

Conflict of interest The authors declare no competing interests.

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