Acta Adriatica 66 (2025):
https://doi.org/10.32582/aa.66.1.2

www.izor.hr

ORIGINAL ARTICLE

Morphological and molecular (*cox*1, 28S rRNA) data of *Microcotyle erythrini* Van Beneden & Hesse, 1863 *sensu stricto* (Polyopisthocotyla, Microcotylidae) infecting the gill lamellae of a marine sparid fish in the Western Mediterranean

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Abstract: The polyopisthocotylan *Microcotyle erythrini* was first described from the common pandora (*Pagellus erythrinus*) collected off Brest, Northeast Atlantic, and subsequently recorded from various sparid hosts across the Mediterranean. Recent studies combining morphometric and molecular data resolved this complex into distinct species, including *M. isyebi* from *Boops boops* and *M. whittingtoni* from *Dentex dentex*. Here, we investigate *Microcotyle* from *Pagrus pagrus* off Tunisia (Western Mediterranean), combining morphological and molecular barcodes (28S rRNA and *cox*1). The *cox*1 sequence divergence between *Microcotyle* from *P. pagrus* off Tunisia and *M. erythrini sensu stricto* from *P. erythrinus* in the Mediterranean was 0-3 %, suggesting the presence of a single species. Newly generated *cox*1 sequences clustered with high support within the *M. erythrini sensu stricto* clade reported from *P. erythrinus* in France and Spain and *P. pagrus* in Spain and Algeria. Phylogenetic analyses based on partial 28S rDNA sequences corroborated this, as the newly generated 28S sequences of *M. erythrini* from *P. pagrus* off Tunisia clustered alongside isolates from *P. pagrus* and *P. erythrinus* across the Western Mediterranean. We also provide novel morphometric and anatomical data for *M. erythrini sensu lato* based on Adriatic specimens (Montenegro) and discuss host specificity patterns in *Microcotyle* spp. from the Mediterranean. These findings emphasize the importance of comprehensive taxonomic revisions and highlight the complexity of *Microcotyle* species associated with sparid hosts in the Mediterranean.

Keywords: Monogenea; Polyopisthocotylea; *Microcotyle*; DNA barcoding; Sparidae; Tunisia; Adriatic; Mediterranean

Sažetak: MORFOLOŠKI I MOLEKULARNI (*COX*1, 28S RRNA) PODACI O JEDNORODNOM METILJU *MICROCOTYLE ERYTHRINI* VAN BENEDEN & HESSE, 1863 *SENSU STRICTO* (POLYOPISTHOCOTYLA, MICROCOTYLIDAE) KOJI INFICIRA LAMELE ŠKRGA SPARIDA U ZAPADNOM SREDOZEMNOM MORU. Jednorodni metilj *Microcotyle erythrini* prvi je put pronađen i opisan na sparidnom domaćinu, arbunu *Pagellus erythrinus*, ulovljenom uz obalu Bresta u sjeveroistočnom Atlantiku, a zatim je zabilježen na drugim sparidnim domaćinima širom Sredozemnog mora. Nedavne studije koje kombiniraju morfometrijske i molekularne podatke razdvojile su ovaj kompleks u različite vrste, uključujući *M. isyebi* na bukvi *Boops boops* i *M. whittingtoni* na zubacu *Dentex dentex*. Ovdje istražujemo metilje *Microcotyle* prikupljene s pagra *Pagrus pagrus* uz obalu Tunisa (zapadno Sredozemno more), a za identifikaciju je kombinirana morfologija i molekularni bar kodovi (28S rRNA i *cox*1). Divergencija *cox*1 sekvenci između roda *Microcotyle* s vrste *P. pagrus* ulovljene u Tunisu i *M. erythrini sensu stricto* s *P. erythrinus* iz Sredozemnog mora bila je 0-3 %, što ukazuje na prisutnost samo jedne vrste. Nova *cox*1 sekvenca *Microcotyle* s *P. pagrus* iz Tunisa grupirana je s visokom podrškom unutar skupine *M. erythrini sensu stricto* s *P. erythrinus* iz Francuske i Španjolske i izolatima s vrste *P. pagrus* iz Španjolske i Alžira. Filogenija temeljena na djelomičnim sekvencama 28S rDNA poduprla je ovu hipotezu, jer su se nove sekvence *M. erythrini* s *P. pagrus* iz Tunisa grupirale zajedno s izolatima s *P. pagrus* i *P. erythrinus* iz zapadnog Sredozemnog mora. Nadalje, donosimo nove morfometrijske i anatomske podatke za *M. erythrini sensu lato* na temelju uzoraka iz Jadranskog mora (Crna Gora) i raspravljamo o specifičnosti domaćina *Microcotyle* spp. iz Sredozemnog mora. Ovi nalazi ističu važnost sveobuhvatnih taksonomskih revizija i naglašavaju složenost vrsta metilja iz roda *Microcotyle* povezanih sa sparidnim domaćinima u Sredozemnom moru.

Ključne riječi: Monogenea; Polyopisthocotyla; *Microcotyle*; DNA barkodiranje; Sparidae; Tunis; Jadran; Sredozemno more

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Received: 13 September 2024, accepted: 5 December 2024
ISSN: 0001-5113, eISSN: 1846-0453

INTRODUCTION

The polyopisthocotylan *Microcotyle erythrini* Van Beneden and Hesse, 1863 has an unusual host specificity pattern, as it has a relatively large host spectrum and has been recorded from several fish host species in the Mediterranean Sea and the Atlantic Ocean. In contrast to many species that are considered strictly host specific, *M. erythrini* parasitizes closely related hosts (Sparidae) (Bouguerche *et al*., 2019a) and thus has a stenoxenic specificity. This microcotylid, listed to date as a valid species (WoRMS, 2024), was first described by Van Beneden and Hesse (1863) from the common pandora *Pagellus erythrinus* (Sparidae) collected off Brest (Brittany, France), Northeast Atlantic Ocean.

Similar to many early descriptions of *Microcotyle* spp., *M. erythrini* was succinctly characterised and its original description was brief (Víllora-Montero *et al*., 2020), with distinction from the congeners primarily based on the number of clamps and testes, as well as the features of the genital atrium (Van Beneden and Hesse, 1863). In several studies, *M. erythrini* was recorded from three other sparid hosts, the axillary seabream *Pagellus acarne*, the bogue *Boops boops,* and the common dentex *Dentex dentex* in several localities in the Mediterranean (Bouguerche *et al.,* 2019a). Most importantly, another problem in the systematics and records of *M. erythrini*, as highlighted by Víllora-Montero *et al.* (2020) is that several subsequent records assigned microcotylids from various sparid species to *M. erythrini*, based on a combination of measurements of specimens from different host species, enlarging unreasonably the host range and the morphometric range of *M. erythrini*. In addition, the original description by Van Beneden and Hesse (1863) provided only the body length, and thus the subsequent records of *M. erythrini* from the Mediterranean could not be verified (Bouguerche *et al.,* 2019a).

In recent years, one of the earliest efforts in tackling the *M. erythrini*-like species complex used integrative taxonomy combining morphometry and DNA sequences (using a partial fragment of *cox*1) and has led to the differentiation of *M. erythrini* into two distinct species, each associated with a different sparid host off the Algerian coast: *M. erythrini* from *P. erythrinus* and a cryptic species, *M. isyebi* Bouguerche, Gey, Justine & Tazerouti, 2019, from *B. boops* (Bouguerche *et al*., 2019a). A more comprehensive and extensive effort is that of Víllora-Montero *et al*. (2020), who depicted another species previously identified as *M. erythrini*, *M. whittingtoni* Víllora-Montero, Pérez-del-Olmo, Georgieva, Raga & Montero, 2020 from *D. dentex* and provided a revision of the taxonomy of *Microcotyle* spp. in sparids from the Western Mediterranean off Spain, and provided novel morphological and molecular data valuable for the taxonomy of *Microcotyle* spp.

Among sparids reported as hosts for *M. erythrini* is the red porgy *Pagrus pagrus* (Víllora-Montero *et al*., 2020; Lablack *et al*., 2022a, 2022b). Furthermore, this sparid hosts different parasitic groups: Acanthocephala, Nematoda, Copepoda, Isopoda, Cestoda, Digenea (Paraguassú *et al*., 2002a; Soares and Luque, 2015; Soares *et al*., 2018; Hamdi, 2022; Lablack *et al*., 2022a) and both monopisthocotylan and polyopisthocotylan “Monogenea” (see Table 1). During a collaborative parasitological survey of helminth parasites of marine fishes from the western Mediterranean (off Tunisia), aiming at describing and barcoding microcotylid Polyopisthocotyla, several representatives of “*M. erythrini*” were collected from the gills of *P. pagrus*. We used integrative taxonomy combining molecular markers (nuclear and mitochondrial markers; 28S rRNA and *cox*1) and morphology to characterize and provide a formal description of the newly collected specimens. We also re-examine specimens of *M. erythrini sensu lato* from the collection of Louis Euzet at the Muséum National d’Histoire Naturelle (MNHN) collections and provide a detailed description.

MATERIAL AND METHODS

Host collection

A total of 60 *P. pagrus* (Sparidae) were examined for Polyopisthocotyla. Fish were obtained from local fishermen, from off the coast of Beni Khiar (36° 27’ 59.99” N, 10° 46’ 59.99” E), Tunisia, Western Mediterranean Sea (Division 37.1.1, https://www.fao.org/fishery/en/area/fao:37/en) during the period July 2018 - June 2019. Shortly after capture, fish were stored on ice and transferred to the Laboratory of Biodiversity, Parasitology, and Ecology of Aquatic Ecosystems at the Faculty of Sciences of Tunis. Fish were identified using species identification (Fischer *et al*., 1987; Kullander and Delling, 2012).

Morphological methods

Fish were dissected fresh on the day of purchase, and the gills were examined for polyopisthocotylans under a stereomicroscope. The gills were removed, placed in individual Petri dishes and examined. Newly collected polyopisthocotylans were heat-killed, fixed without pressure in near-boiling saline and preserved immediately in 70 % ethanol for parallel morphological and molecular characterisation. Two specimens were processed as hologenophores (*sensu* Pleijel *et al.* (2008)). Hologenophores consist of entire specimens showing taxonomical features (haptor, clamps, genital atrium, testes and anatomy at the level of ovarian region) and lacking only a lateral part excised and used for DNA extraction.

For morphological analyses and hologenophores, representative specimens were carefully selected following Víllora-Montero *et al.* (2023). Undamaged, contracted, stretched, wrinkled, or folded specimen were selected, stained with acetic carmine, dehydrated in a graded series of alcohol for 15 min each (70, 96,

Table 1. Monopisthocotyla and Polyopisthocotyla previously reported from *Pagrus pagrus*.

Parasites species	Family	Locality	Source
Monopisthocotyla			
Anoplodiscus longivaginatus Paraquassu, Luque & Alves, 2002	Anoplodiscidae	Brazil, SWA	(Paraguassú *et al*., 2002a, 2002b; Soares and Luque, 2015; Soares *et al*., 2018)
		Argentina, SWA	Soares *et al*., 2018
Lamellodiscus sp.	Diplectanidae	Brazil, SWA	(Paraguassú *et al*., 2002a)
Lamellodiscus baeri Oliver, 1974	Diplectanidae	Algeria, WM	(Lablack *et al*., 2022a)
		Brazil, SWA	(Soares and Luque, 2015; Soares *et al*., 2018)
		Argentina, SWA	(Soares *et al*., 2018)
Encotyllabe spari Yamaguti, 1934	Capsalidae	Brazil, SWA	(Paraguassú *et al*., 2002a; Soares and Luque, 2015; Soares *et al*., 2018)
		Argentina, SWA	(Soares *et al*., 2018)
Encotyllabe bifurcatum Taborda, Sepulveda, Luque, Escribano & Oliva, 2023	Capsalidae	Brazil, SWA	(Taborda *et al*., 2023)
Encotyllabe parvum Taborda, Sepulveda, Luque, Escribano & Oliva, 2023	Capsalidae	Brazil, SWA	(Taborda *et al*., 2023)
Encotyllabe sp.	Capsalidae	Algeria, WM	(Lablack *et al*., 2022a)
Benedenia sp.	Capsalidae	Brazil, SWA	(Paraguassú *et al*., 2002a)
Polyopisthocotyla			
Echinopelma brasiliensis Fabio, 1999	Diclidophoridae	Brazil, SWA	(Paraguassú *et al*., 2002a; Soares and Luque, 2015; Soares *et al*., 2018)
		Argentina, SWA	(Soares *et al*., 2018)
Polylabroides multispinosus Roubal, 1981	Microcotylidae	Brazil, SWA	(Paraguassú *et al*., 2002a; Soares and Luque, 2015; Soares *et al*., 2018)
		Argentina, SWA	(Soares *et al*., 2018)
M. erythrini Van Beneden & Hesse, 1863	Microcotylidae	Spain, WM	(Víllora-Montero *et al*., 2020)
		Algeria, WM	(Lablack *et al*., 2022a, 2022b)

Abbreviations: WM, Western Mediterranean. SWA, Southwest Atlantic.

and 100 %), cleared in clove oil, and mounted in Canada balsam. The hologenophores were processed according to the same methods (Fig. 1). Some specimens were mounted in Berlese's fluid to study the morphology of clamps and the genital atrium. Slides were deposited at the Invertebrates Collections of the Swedish Museum of Natural History, Stockholm, Sweden (SMNH), under the accession numbers SMNH 225213-225227.

Polyopisthocotylans were identified on stained whole mounts. Drawings were made through a Nikon Eclipse i80 microscope with DIC (differential interference contrast) (Department of Zoology, Swedish Museum of Natural History, Stockholm, Sweden) and a drawing tube. Drawings were scanned and redrawn on a computer with Adobe Illustrator 2023. Measurements of whole-mounts and hologenophores given in micrometres and indicated as a range.

For clamps nomenclature, we followed Euzet and Marc (1963). For the designation of the ventral and dorsal arms of clamps sclerites, we followed Bouguerche

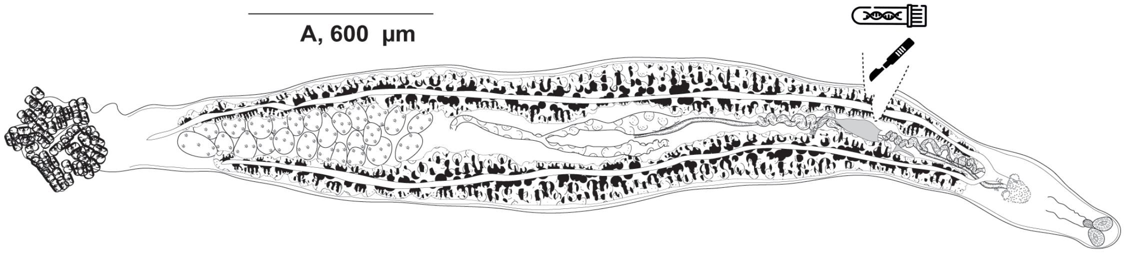

Fig. 1. *Microcotyle erythrini* Van Beneden & Hesse, 1863 *sensu stricto* ex *Pagrus pagrus* from Tunisia, Western Mediterranean, hologenophore (SMNH-225226). A small lateral part of the body was excised and used for DNA extraction.

et al. (2021). We followed the terminology as defined by Combes (2003) to describe the host specificity of a parasite in relation to the relatedness of host species: oioxenic is employed for parasites that exploit a single host species; the parasite is denoted as stenoxenic if it exploits a range of phylogenetically related species and euryxenic if it exploits a range of mutually unrelated species (Zedam *et al*., 2023). For high-level terminology of parasites, we followed the systematics of Brabec *et al*. (2023), who elevated the former subclasses of "Monogenea" to the level of classes, and we use the classes Monopisthocotyla and Polyopisthocotyla.

Molecular methods

Genomic DNA was extracted from two hologenophores using QIAmp DNA Micro Kit (Qiagen) and genetic sequence data were generated for two markers: a partial region of the *cox*1 mitochondrial region (*cox*1 mtDNA) and the large (28S) ribosomal subunit RNA coding region. The specific primers JB3 (forward 5′-TTTTTTGGGCATCCTGAGGTTTAT-3′) and JB4.5 (reverse 5′-TAAAGAAAGAACATAATGAAAATG-3′) were used to amplify a fragment of the *cox*1 gene (Bowles *et al.*, 1995; Littlewood *et al.*, 1997). PCR reaction was performed in 20 µl, containing 1 ng of DNA, 1×CoralLoad PCR buffer, 3 mM $MgCl_2$, 0.25 mM dNTP, 0.15 µM of each primer and 0.5 units of Taq DNA polymerase (Qiagen). Thermocycles consisted of an initial denaturation step at 94 °C for 2 min, followed by 37 cycles of denaturation at 94 °C for 30 s, annealing at 48 °C for 40 s and extension at 72 °C for 50 s. The final extension was conducted at 72 °C for 5 min. The sequences were edited with CodonCode Aligner software version 3.7.1, compared to the GenBank database content with BLAST and deposited in GenBank under accession number PQ520466, PQ522527.

A 28S rDNA fragment was amplified using the universal primers C10 (5′-ACCCGCTGAATTTAAGCAT-3′) and D2 (3′-TCCGTGTTTCAAGACGG-5′). PCR reactions were performed in a final volume of 20 µL, containing: 1 ng of DNA, 1×CoralLoad PCR buffer, 3 mM $MgCl_2$, 66 mM of each dNTP, 0.15 mM of each primer, and 0.5 units of Taq DNA polymerase. Thermocycles consisted of an initial denaturation step at 94 °C for 1 min, followed by 40 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C, for 30 s, and extension at 72 °C for 1 min. The final extension was conducted at 72 °C for 7 min (Derouiche *et al.*, 2019). PCR products were visualised on a 1.5 % agarose gel, purified and directly sequenced in both directions on a 3730xl DNA Analyzer 96-capillary sequencers (Applied Biosystems) at Eurofins Genomics (https://eurofinsgenomics.eu). Sequences were edited and assembled using CodonCode Aligner software (CodonCode Corporation, Dedham, MA, USA), and compared to the GenBank database content with BLAST and deposited in GenBank under the accession numbers PQ522343 and PQ522345.

Phylogenetic trees and distances

Phylogenetic analyses were performed using the newly generated sequences of microcotylids from *P. pagrus* and those of closely related species available in GenBank (Tables 2, 3). Alignments for each gene region were constructed separately in AliView (Larsson, 2014). The alignments were trimmed to the shortest sequence. Analysis of phylogeny based on Bayesian inference (BI) was performed using MrBayes 3.2.7 (Huelsenbeck and Ronquist, 2001; Ronquist *et al.*, 2012). Test of best nucleotide substitution model was performed using PAUP (Swofford, 2003) implemented in MrModeltest 2.3 (Nylander, 2004). For both 28S rRNA and *cox*1 genes, BI was run with two random starting trees and four Markov chains, three heated and one "cold" for 2 x 10^6 generations under the general time reversible model (GTR) with gamma distribution and invariable sites (G+I). Tree sampling was performed at 500 generations intervals. The first 500,000 generations were discarded as burn-ins. *p*-distances were computed from the same datasets with MEGA11 (Tamura *et al.*, 2021).

RESULTS

Molecular analysis

28S analysis

The alignment of the 28S rDNA dataset for *Microcotyle* spp. (886 bp) included two newly generated sequences of *M. erythrini* ex *P. pagrus*, and 11 species retrieved from GenBank (including three unidentified species, MH700256 *Microcotyle* sp. 1, MH700266 *Microcotyle* sp. 2 , and MT890112 *Microcotyle* sp. 3 (Chou, 2018a, 2018b; Oliva et al., 2020a, 2020c). The microcotylid *Ktarius patrickbrueli* Hamdi, Benmansour, Ahmed, Gulsher & Bouguerche, 2024 (Hamdi *et al.*, 2024) was used as an outgroup. There was a total of 787 bp in the final dataset. The tree resulting from the BI analysis is shown in Fig. 2.

At the species level, seven *Microcotyle* species were recovered and resolved: 1. *M. erythrini* from various sparid hosts (see the framed clade); 2. *M. whittingtoni* ex *D. dentex*; 3. *M. isyebi* ex *B. boops*; 4. *M. arripis* Sandars, 1945 ex *Arripis georgianus*; 5. *M. bassensis* Murray, 1931 ex "flathead" (Platycephalidae); 6. *M. caudata* Goto, 1894 ex *Sebastiscus marmoratus*; and 7. *M. nemadactylus* Dillon & Hargis, 1965 ex *Cheilodactylus variegatus*. Additionally, a sequence labelled as *M. sebastis* Goto, 1894 ex *Sebastes* sp. from the North Sea, Northeast Atlantic clustered is a distinct clade, separate from those also labeled as *M. sebastis*, ex *Sebastes schlegelii* from South Korea, Northwest Pacific, suggesting that they represent different species. *Microcotyle* sp. 1 and *Microcotyle* sp. 2 clustered in different clades, suggesting that they represent also different species. The sequence of *Microcotyle* sp. 3 ex *Prolatilus jugularis* off Chile differed greatly, which calls into question the affiliation of the former species to the genus *Microcotyle*.

Table 2. Collection data for 28S sequences analysed in this study.

Polyopisthocotyla	Host	Locality	GenBank	Source
M. erythrini	*Pagrus pagrus*	Tunisia, WM	PQ520466 PQ522527	Present study
M. erythrini	*Pagellus erythrinus*	France, WM	AM157221	(Jovelin and Justine, 2001)
M. erythrini	*Pagellus erythrinus*	Spain, WM	MN814848	(Víllora-Montero *et al.*, 2020)
M. erythrini	*Pagrus pagrus*	Spain, WM	MN814849	(Víllora-Montero *et al.*, 2020)
M. erythrini	*Pagrus pagrus*	Algeria, WM	OL679677	(Lablack *et al.*, 2022b)
M. erythrini	*Pagrus pagrus*	Algeria, WM	OL679676	(Lablack *et al.*, 2022b)
M. whittingtoni	*Dentex dentex*	Spain, WM	MN814847	(Víllora-Montero *et al.*, 2020)
M. isyebi	*Boops boops*	Spain, WM	MN814850	(Víllora-Montero *et al.*, 2020)
M. arripis	*Arripis georgianus.*	Australia, EIO	GU263830	(Catalano *et al.*, 2010)
Microcotyle sp. 1	N.a.	N.a.	MH700266	(Chou, 2018b)
Microcotyle sp. 2	N.a.	N.a.	MH700256	(Chou, 2018a)
M. bassensis	“flathead”	Australia	MZ286639	(Shamsi, 2022a)
M. bassensis	“flathead”	Australia	MZ286641	(Shamsi, 2022b)
M. nemadactylus	*Cheilodactylus variegatus*	Chile, SEP	MT890110	(Oliva *et al.*, 2020b)
M. caudata	*Sebastiscus marmoratus*	Japan, NWP	MT890111	(Oliva *et al.*, 2020c)
M. sebastis	*Sebastes schlegelii*	South Korea, NWP	MT875157	(Song *et al.*, 2021)
M. sebastis	*Sebastes schlegelii*	South Korea, NWP	MT875158	(Song *et al.*, 2021)
Microcotyle sebastis	*Sebastes* sp.	North Sea, NEA	AF382051	(Olson and Littlewood, 2002)
Ktarius patrickbrueli	*Dentex gibbosus*	Tunisia, WM	PQ328200	(Hamdi *et al.*, 2024)

Abbreviations: N.a., not available; EIO, Eastern Indian Ocean; NEA, Northeast Atlantic; NWP, Northwest Pacific; SEP, Southeast Pacific; WM, Western Mediterranean.

The newly generated sequences of *M. erythrini ex P. pagrus* off Tunisia clustered in a supported clade, along with isolates from the same host off Algeria, Spain; and isolates ex *P. erythrinus* off France and Spain, supporting the presence of a single species in these sparid hosts in Mediterranean waters. *Microcotyle isyebi* ex *B. boops* from Algeria, and *M. whittingtoni* ex *D. dentex* from Spain (previously reported *as M. erythrini*) had a basal position to *M. erythrini* supporting the distinction of the three species and the hypothesis of the *M. erythrini*-like species complex.

*Cox*1 analysis

The newly generated partial *cox*1 sequences (429 bp) of *Microcotyle* ex *P. pagrus* were analyzed together with 36 published sequences for *Microcotyle* spp. The gastrocotylids *Allogastrocotyle bivaginalis* Nasir & Fuentes Zambrano, 1984 and *Pseudaxine trachuri* Parona & Perugia, 1890 were used as outgroups.

The tree resulting from the BI analysis is shown in Fig. 3. The newly generated sequences of *Microcotyle* clustered within the “*M. erythrini*” clade reported from the southern coast of the Western Mediterranean, with the isolates ex *P. erythrinus* from France and Spain and the isolates ex *P. pagrus* from Spain and Algeria, with high support. This clade was defined previously as *M. erythrini sensu stricto*.

Similarly, isolates of *M. caudata* Goto, 1894, *M. kasago* Ono, Matsumoto, Nitta & Kamio, 2020, *M. pacinkar* Kamio & Nitta, 2023, *M. algeriensis* Ayadi, Gey, Justine & Tazerouti, 2016, *M. merche* Víllora-Montero, Pérez-del-Olmo, Valmaseda-Angulo, Raga & Montero, 2023 (from scorpaenid hosts); *M. isyebi*, *M. visa* and *M whittingtoni* (from sparid hosts) clustered in highly supported clades.

The intraspecific sequence divergence between the newly generated *cox*1 sequences for *M. erythrini sensu stricto* (ex *P. erythrinus* and *P. pagrus*) was 0-2 % (Table 4). Isolates of *M. erythrini sensu stricto* differed by

Table 3. Collection data for *cox*1 sequences analysed in this study. NWP, Northwest Pacific; WM, Western Mediterranean. ^{1} from a fish farm.

Polyopisthocotyla	Host	Locality	GenBank	Source
M. erythrini	*Pagrus pagrus*	Tunisia, WM	PQ522343 PQ522345	Present study
M. caudata	"*Sebastes inermis* species complex"	Japan, NWP	LC472531	(Ono *et al.*, 2020)
M. caudata	"*Sebastes inermis* species complex"	Japan, NWP	LC472530	(Ono *et al.*, 2020)
M. caudata	"*Sebastes inermis* species complex"	Japan, NWP	LC472528	(Ono *et al.*, 2020)
M. caudata	*Sebastes inermis*	Japan, NWP	LC472527	(Ono *et al.*, 2020)
M. caudata	"*Sebastes inermis* species complex"	Japan, NWP	LC472529	(Ono *et al.*, 2020)
M. kasago	*Sebastiscus marmoratus*	Japan, NWP	LC472525	(Ono *et al.*, 2020)
M. kasago	*Sebastiscus marmoratus*	Japan, NWP	LC472526	(Ono *et al.*, 2020)
"*M. sebastis*"	*Sebastes schlegelii*	South Korea^1	DQ412044	(Park *et al.*, 2007)
M. pacinkar	*Sebastestaczanowskii*	Japan, NWP	LC753264	(Kamio and Nitta, 2023)
M. pacinkar	*Sebastestaczanowskii*	Japan, NWP	LC753265	(Kamio and Nitta, 2023)
M. algeriensis	*Scorpaena scrofa*	Spain, NEA	OQ243288	(Víllora-Montero *et al.*, 2023)
M. algeriensis	*Scorpaena scrofa*	Spain, NEA	OQ243289	(Víllora-Montero *et al.*, 2023)
M. algeriensis	*Scorpaena scrofa*	Spain, NEA	OQ243290	(Víllora-Montero *et al.*, 2023)
M. merche	*Helicolenus dactylopterus*	Spain, NEA	OQ243284	(Víllora-Montero *et al.*, 2023)
M. merche	*Helicolenus dactylopterus*	Spain, NEA	OQ243285	(Víllora-Montero *et al.*, 2023)
M. merche	*Helicolenus dactylopterus*	Spain, WM	OQ243286	(Víllora-Montero *et al.*, 2023)
M. merche	*Helicolenus dactylopterus*	Spain, WM	OQ243287	(Víllora-Montero *et al.*, 2023)
M. visa	*Pagrus caeruleostictus*	Algeria, WM	MK275653	(Bouguerche *et al.*, 2019b)
M. visa	*Pagrus caeruleostictus*	Algeria, WM	MK275654	(Bouguerche *et al.*, 2019b)
M. visa	*Pagrus caeruleostictus*	Algeria, WM	MK275652	(Bouguerche *et al.*, 2019b)
M. isyebi	*Boops boops*	Spain, WM	MK317922	(Bouguerche *et al.*, 2019a)
M. isyebi	*Boops boops*	Spain, WM	MN816018	(Bouguerche *et al.*, 2019a)
M. isyebi	*Boops boops*	Spain, WM	MN816019	(Víllora-Montero *et al.*, 2020)
M. isyebi	*Boops boops*	Spain, WM	MN816020	(Víllora-Montero *et al.*, 2020)
M. isyebi	*Boops boops*	Spain, WM	MN816021	(Víllora-Montero *et al.*, 2020)
M.whittingtoni	*Dentex dentex*	Spain, WM	MN816010	(Víllora-Montero *et al.*, 2020)
M.whittingtoni	*Dentex dentex*	Spain, WM	MN816011	(Víllora-Montero *et al.*, 2020)
M. erythrini	*Pagellus erythrinus*	France, WM	AY009159	(Jovelin and Justine, 2001)
	Pagellus erythrinus	Spain, WM	MN816012	(Víllora-Montero *et al.*, 2020)
	Pagellus erythrinus	Spain, WM	MN816013	(Víllora-Montero *et al.*, 2020)
	Pagrus pagrus	Spain, WM	MN816017	(Víllora-Montero *et al.*, 2020)
	Pagrus pagrus	Algeria, WM	OL675211	(Lablack *et al.*, 2022b)
	Pagrus pagrus	Spain, WM	MN816014	(Víllora-Montero *et al.*, 2020)
	Pagrus pagrus	Spain, WM	MN816016	(Víllora-Montero *et al.*, 2020)
	Pagrus pagrus	Spain, WM	MN816015	(Víllora-Montero *et al.*, 2020)
Pseudaxine trachuri	*Boops boops*	Algeria, WM	MT666075	(Bouguerche *et al.*, 2020)
Allogastrocotyle bivaginalis	*Trachurus picturatus*	Algeria, WM	MN192391	(Bouguerche *et al.*, 2019c)

Abbreviations: NEA, Northeast Atlantic; NWP, Northwest Pacific; WM, Western Mediterranean

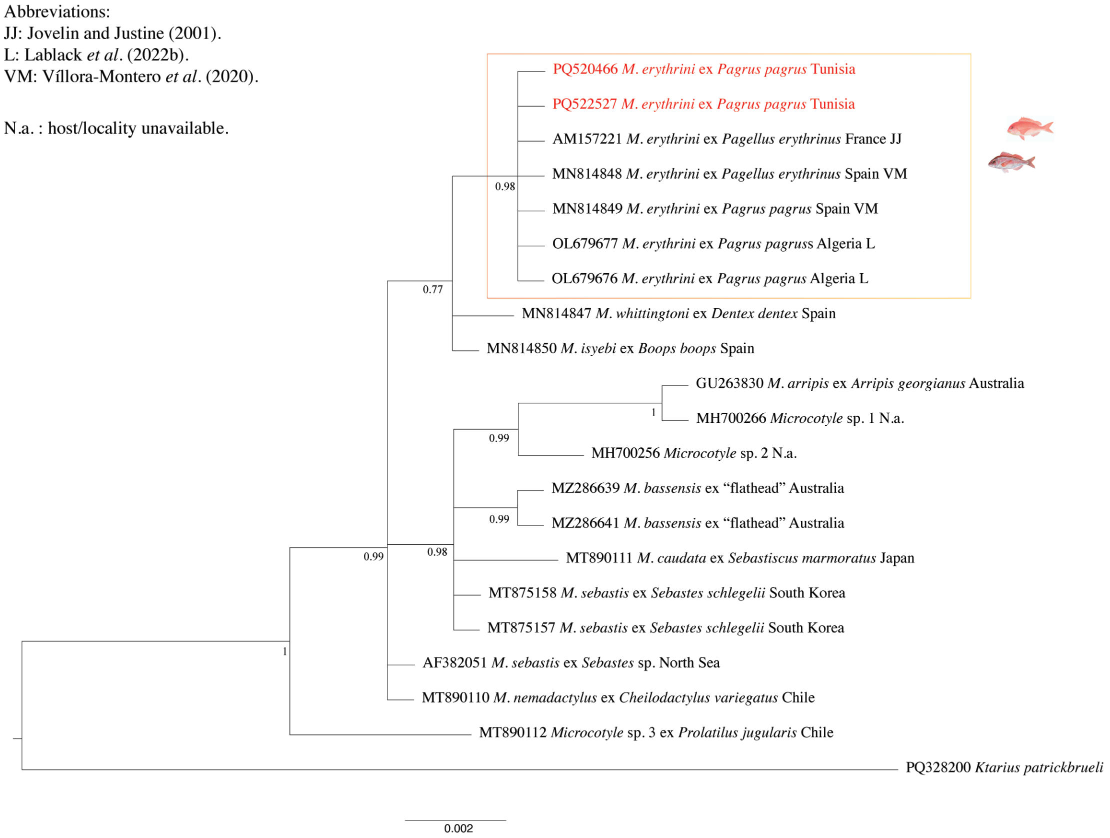

Fig. 2. Bayesian topology based on partial 28S ribosomal DNA sequences of *Microcotyle* spp. and closely related microcotylids. Outgroup: *Ktarius patrickbrueli* (Hamdi *et al.*, 2024) (Microcotylidae). GenBank accession numbers precede species names. The support values are included below the nodes as follows: posterior probabilities >0.90 are considered well-supported; posterior probabilities <0.70 are not shown. The scale bar indicates the expected number of substitutions per site. The newly generated sequences are indicated in red. For *M. erythrini*, following Lablack *et al.* (2022b), sequence identification and hosts are indicated as in GenBank, followed by a letter: JJ, Jovelin and Justine (2001), V, Víllora-Montero *et al.* (2020), L, Lablack *et al.* (2022b).

11-13 % from *M. whittingtoni* and by 13-15 % from *M. visa*, and *M. isyebi*. Isolates of *M. erythrini sensu stricto* differed from *M. pacinkar* by 13-15 % and from *M. kasago* and "*M. sebastis*" by 14-15 %. Similarly, the genetic divergence between *M. erythrini sensu stricto* and *M. caudata* and *M. merche* was 15-18 % and 17-18 %, respectively. The highest genetic divergence was to *M. algeriensis* (17-19 %).

Morphological analysis

Class Polyopisthocotyla Odhner, 1912
Family Microcotylidae Taschenberg, 1879
Subfamily Microcotylinae Taschenberg, 1879
Genus *Microcotyle* Van Beneden & Hesse, 1863
Microcotyle erythrini Van Beneden & Hesse, 1863 *sensu stricto* (Figs. 4-6)

Type-host: *Pagellus erythrinus* (Spariformes: Sparidae), common pandora (rödpagellen in Swedish) (Van Beneden and Hesse, 1863).

Other hosts: *Boops boops*, bogue (Perugia and Parona, 1890; López-Román and Guevara Pozo, 1973; Renaud *et al.*, 1980; Pérez-del-Olmo *et al.*, 2007a, 2007b, 2008; Fernandez-Jover *et al.*, 2010; Strona *et al.*, 2010; Marzoug *et al.*, 2012; Akmirza, 2013; Ramdane *et al.*, 2013; Benhamou *et al.*, 2017). *Pagellus acarne*, axillary seabream (Perugia and Parona, 1890; Akmirza, 2013). *Dentex dentex*, common dentex (González *et al.*, 2004). Note that the records from *B. boops* and ex *D. dentex* corresponds probably to *M. isyebi* and *M. whittingtoni* described by Bouguerche *et al.* (2019a) and Víllora-Montero *et al.* (2020), respectively.

Type-locality: off Brest, France, Northeast Atlantic (Van Beneden and Hesse, 1863).

Other localities: Central Mediterranean, Adriatic: off Montenegro (Radujkovic and Euzet, 1989). Western Mediterranean: France (Euzet, 1957; Renaud *et al.*, 1980; Jovelin and Justine, 2001); Italy (Perugia and Parona, 1890; Ulmer and James, 1981; Strona *et al.*, 2010); Spain (López-Román and Guevara Pozo, 1973; González *et al.*, 2004; Power *et al.*, 2005; Pérez-del-Olmo *et al.*,

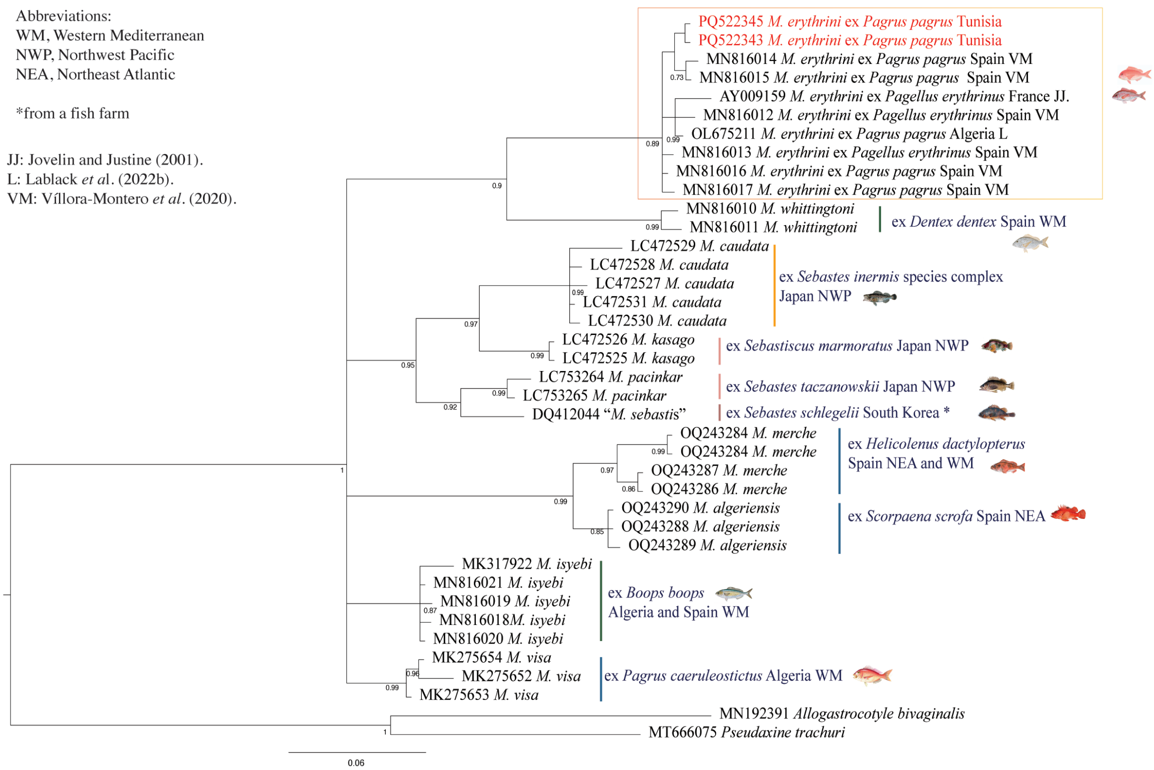

Fig. 3. Bayesian topology based on partial *cox*1 of *Microcotyle* spp. GenBank accession numbers precede species names. The support values are included below the nodes as follows: posterior probabilities >0.90 are considered well-supported; posterior probabilities <0.70 are not shown. The scale bar indicates the expected number of substitutions *per* site. The newly generated sequences are indicated in red. For *M. erythrini* Van Beneden & Hesse, 1863, following Lablack *et al.* (2022b), sequence identification and hosts are indicated as in GenBank, followed by a letter: JJ, Jovelin and Justine (2001), V, Víllora-Montero *et al.* (2020), L, Lablack *et al.* (2022b).

2007a, 2008; Fernandez-Jover *et al.*, 2010; Marzoug *et al.*, 2012); Algeria (Kouider El Ouahed-Amine, 1998; Kaouachi *et al.*, 2010; Marzoug *et al.*, 2012; Ramdane *et al.*, 2013; Benhamou *et al.*, 2017). Eastern Mediterranean, Aegean: off Turkey (Akmirza, 2013). Northeast Atlantic: off Spain (Power *et al.*, 2005; Pérez-del-Olmo *et al.*, 2007a, 2007b, 2008).

Site on host: Gills.

Infection details: Prevalence: 60 % (number of infected fish: 36, number of examined fish: 60). Mean abundance: 1.65 ± 1.93, Mean intensity: 2.8 ± 1.8.

Examined comparative material: *Microcotyle erythrini sensu lato* from *Pagellus erythrinus* from Montenegro, Adriatic, central Mediterranean, from the collection of Louis Euzet deposited in the Muséum National d'Histoire Naturelle (MNHN 306HG, Box 12, slide 10).

Specimens deposited: Specimens with molecular information: body (lacking only a small lateral part) mounted on slide as hologenophores, excised lateral part used for molecular analysis: specimens of *Microcotyle erythrini sensu stricto* from *Pagrus pagrus* from Tunisia, Western Mediterranean (SMNH 225224-225225).

Specimens examined for morphological study, wholemounts: *Microcotyle erythrini sensu stricto* from *P. pagrus* from Tunisia, Western Mediterranean (SMNH 225213-225223).

Description

Based on stained whole mounts, all measurements are in micrometers. Body fusiform, elongate, seldom slender, anterior end narrow (Fig. 4A), length of the body, length of the body proper 1610-5255; maximum width at the level of ovary 200-620; total length including the haptor 1990-6300.

Prohaptoral suckers paired, septate, anteriorly placed, oval (Fig. 4B), 40-75 long × 22-48 wide; tiny papillae visible on ventral rims. Pharynx muscular, small, subspherical, immediately posterior to prohaptoral suckers, 20-45 long × 20-35 wide. Oesophagus short. Caeca predominantly pre-haptoral, with numerous medial and lateral secondary branches, extending into haptor, up to 2/3 total length of haptor; caeca not confluent posteriorly. The right caecum terminates near the beginning of the haptor, and the left branch extends into the haptor for a short distance. Testes post-ovarian, subspherical to oval, intercaecal, limited to the posterior half of body proper, 16-21 in number. Vas deferens extending along body midline to the genital atrium. Genital atrium at 220-440 from the anterior end of the body, 90-160 long, 65-210 wide. The genital atrium comprises the anterior atrium proper and two posterior 'pockets'

Table 4. Genetic distances between *cox*1 sequences of polyopisthocotylan microcotylids. *p*-distances are percentages. Distances within species are in italics; intraspecific variations are low, ranging between 0-3 %. Distances between species (interspecific variations) are higher, ranging generally between 4-5 % to 17-18 %.

p-distances	*M. caudata*	*M. kasago*	*„M. sebastis”*	*M. pacinkar*	*M. algeriensis*	*M. merche*	*M. visa*	*M. isyebi*	*M. whittingtoni*	*M. erythrinii sensu stricto*
M. caudata	*0-3*									
M. kasago	6-8	*0*								
“M. sebastis”	9-10	7	-							
M. pacinkar	9	7	4-5	*1*						
M. algeriensis	13	13	13	13	*0*					
M. merche	14	13-14	12	13-15	3-5	*0-3*				
M. visa	9-12	10-12	10-12	9-10	11-12	12-15	*0-2*			
M. isyebi	10-11	9-10	9-10	8-9	12	11-13	7-10	*0-2*		
M. whittingtoni	14-16	16	16	15-16	17	16-17	13-14	15-16	*1*	
M. erythrinii sensu stricto	15-18	14-15	14-15	13-15	17-19	17-18	13-15	13-15	11-13	*0-2*

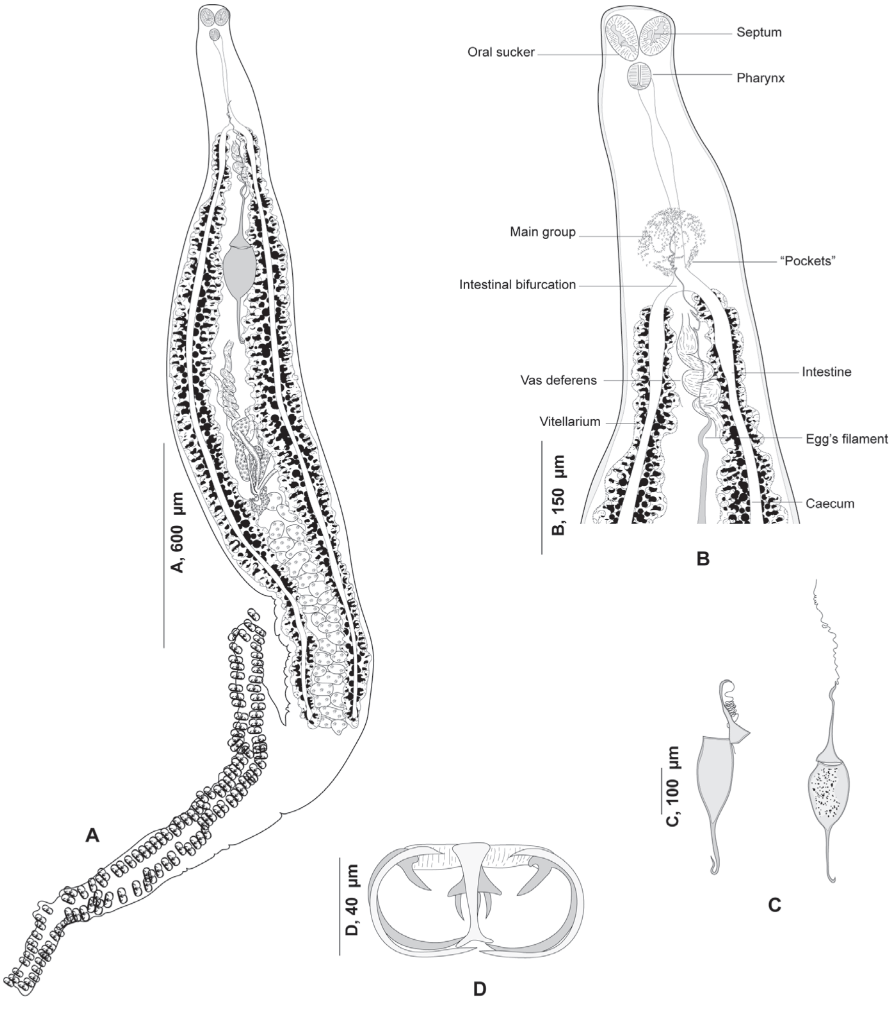

Fig. 4. *Microcotyle erythrini* Van Beneden & Hesse, 1863 *sensu stricto* ex *Pagrus pagrus* from Tunisia, Western Mediterranean. Body, ventral view (SMNH-225215) (**A**); anterior end showing male copulatory organ, ventral view (SMNH-225215) (**B**); egg ventral view (SMNH-225215, SMNH-225213) (**C**); clamp, ventral view (SMNH-225215) (**D**).

(Fig. 4B). The atrium proper inverted and heart-shaped, with numerous conical spines. Atrial spines of the atrium proper of similar sizes, denser in the center than in bilateral parts. Lumen with two lateral expansions visible only after variation in focus. Posterior pockets lined with fewer spines, similar to that of the atrium proper. Number of spines in atrium 280-355, in pockets 20-38. Vas deferens thin-walled, conspicuous in the midline, extending dorsal to the uterus and opening into the genital atrium. Egg fusiform (Fig. 4C), with a single polar filament, 148-200 long, 60-90 wide. Egg filament short and often coiled.

Haptor triangular, sub-symmetrical or symmetrical, 755-1480 long. Haptor bearing 92-120 clamps, arranged in two equal or sub-equal rows. Clamps sessile, arranged in two rows; clamps dissimilar in size: posterior and newly formed anterior clamps smallest. Length of largest clamps 22-45, width 40-65. Clamps of *Microcotyle*-type (Fig. 4D).

Clamps consist of two opposable jaws, anterior jaw **(**Fig. 5A) and posterior jaw (Fig. 5B). The ventral arm of the median spring *a* t-shaped, long, distal part with short branches of equal size. The dorsal arm of the median spring visibly shorter than its ventral arm, T-shaped, and distally broad. The ventral arm of the ventral jaw consists of two lateral sclerites *b2*, dorsal arm *b3* shorter and curved inwards; *b3* does not reach the dorsal arm of the median spring (Fig. 3B). Dorsal jaw sclerites *c* reaching the midline on the distal side. Muscles connecting *a* and *b2* are present on the proximal side (Fig. 5C).

Question mark-shaped ovary (Fig. 6). Ovary begins at the level of anterior most testes. The proximal section irregularly shaped, often overlapping the anterior most testis; the ovary continues anteriorly in the midline, reflexes at the level of confluence of vitelline ducts, reflexes again toward the anterior extremity, forms a large anterior curve and reflexes a last time posteriorly and ends as oviduct. Oviduct short and joined by a vitelline reservoir in the midline. Genito-intestinal canal (visible only in certain specimens) extending from the oviduct ventrally across the proximal end of the ovary and projecting into the right intestinal caecum. Spindle-shaped oötype, with numerous Mehlis' glands. Vitellarium follicular, co-extensive with intestinal caeca reaching up to the haptor region. Vitelline ducts paired, united anteriorly and posteriorly; anterior junction in inverted Y well visible in some specimens; posterior junction Y-shaped, conspicuous in most specimens, at the level of ovary and ventral to it.

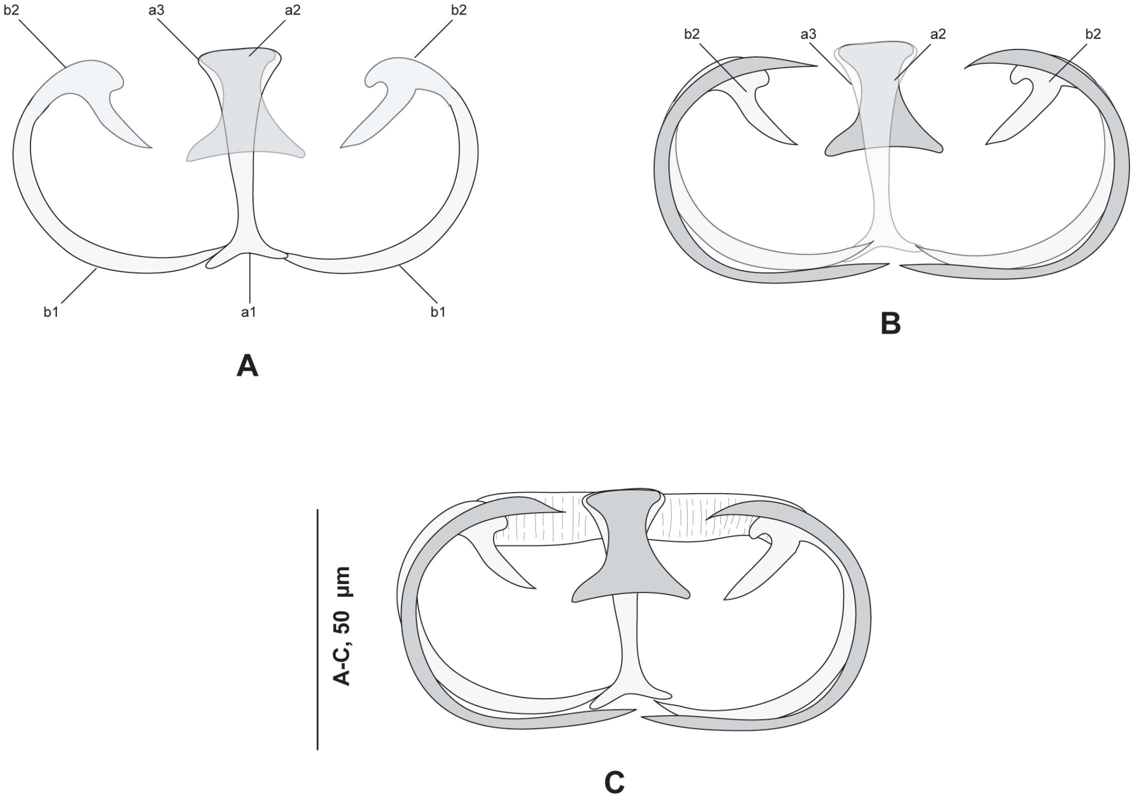

Fig. 5. *Microcotyle erythrini* Van Beneden & Hesse, 1863 *sensu stricto* ex *Pagrus pagrus* from Tunisia, Western Mediterranean, disposition of clamps sclerites (SMNH-225215). Ventral jaw (**A**); dorsal jaw (**B**); clamp, dorsal view (**C**). Abbreviations: *a1*, *a2*: ventral arm of median spring. *a3*, dorsal arm of median spring. *b1*, ventral arm of lateral sclerites. *b2*, dorsal arm of lateral sclerites.

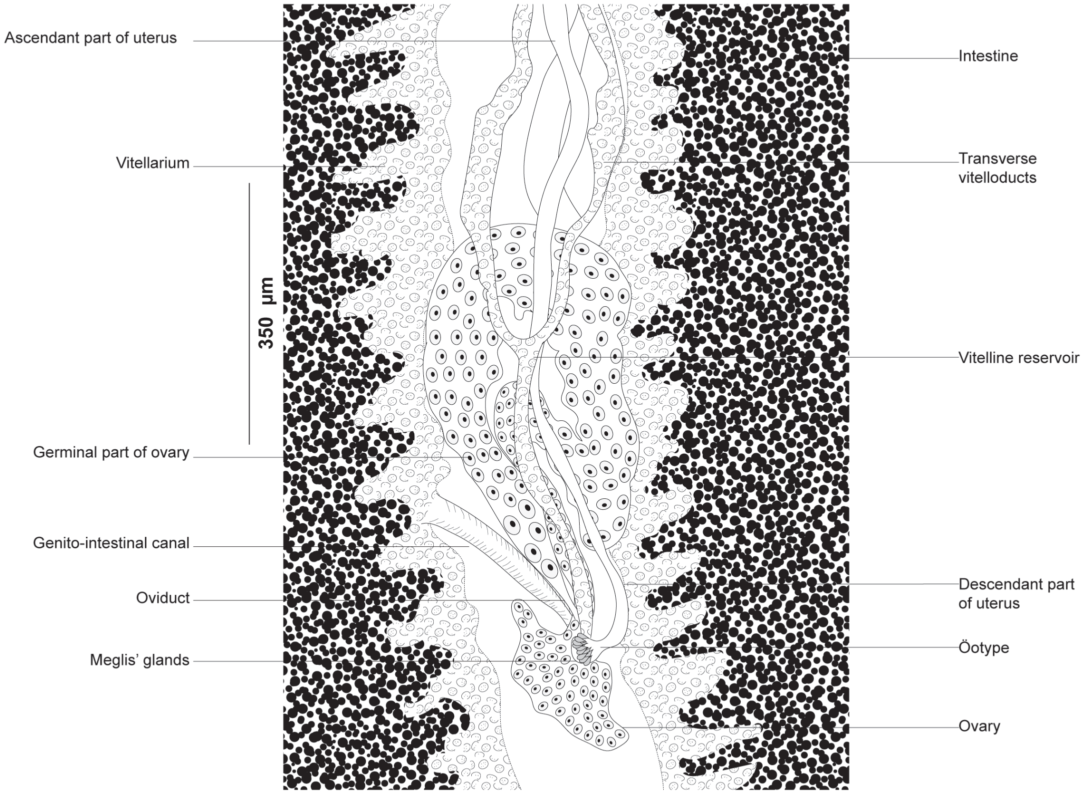

Fig. 6. *Microcotyle erythrini* Van Beneden & Hesse, 1863 *sensu stricto* ex *Pagrus pagrus* from Tunisia, Western Mediterranean, detail of the reproductive organs in the region of the ovary, ventral view (SMNH-225213).

Remark

We found one additional specimen that we refer to here as *M. erythrini sensu lato* from *P. erythrinus* from Montenegro, Adriatic, central Mediterranean, in the collection of Louis Euzet deposited at the Muséum National d'Histoire Naturelle (MNHN 306HG, Box 12, slide 10). The general morphology of this specimen is shown in Fig. 7. Since molecular barcodes are lacking for this specimen, we referred to it herein as *M. erythrini sensu lato.*

DISCUSSION

Microcotyle spp. are easily distinguished by their sub-symmetrical haptor, bearing numerous *Microcotyle*-type clamps, and their inverted heart-shaped genital atrium (Yamaguti, 1963; Mamaev, 1986). The widely reported *M. erythrini* was first described by Van Beneden and Hesse (1863) from a sparid host *P. erythrinus* collected off Brest (Brittany, Atlantic Ocean). The species has since been recorded from other sparid hosts, mainly *P. acarne*, *B. boops,* and *D. dentex* in several localities in the Mediterranean (see Bouguerche *et al*., 2019a). Previously, it has been demonstrated that "*M. erythrini*" is not a polyopisthocotylan with a stenoxenic specificity, but rather a complex of several distinct species, frequently each specific to a single host, and that could not be distinguished by morphology (Bouguerche *et al*., 2019a; Víllora-Montero *et al*., 2020). Consequently, the records of *M. erythrini* from *B. boops* was demonstrated to be *M. isyebi* (Bouguerche *et al*., 2019a), and the one from *D. dentex* (González *et al*., 2004) was subsequently described as *M. whittingtoni* (Víllora-Montero *et al.*, 2020).

In North Africa, one of the recent and extensive efforts on the barcoding and phylogeny of "Monogenea" from sparid fishes is that of Lablack *et al*. (2022b), who markedly expanded taxa representation. The authors expanded the 28S rDNA and *cox*1 sequence databases with 46 sequences for seven species of three "monogenean" families (Capsalidae Baird, 1853, Microcotylidae Taschenberg, 1879 and Diclidophoridae Cerfontaine, 1895) (Lablack *et al*., 2022b). The recent accumulation of 28S rDNA sequences for *Microcotyle* spp., especially from sparids after the latest efforts of Víllora-Montero *et al.* (2020), offers the opportunity to test the utility of this marker for the phylogenetic relationships in this group. Herein, the newly generated sequences of *M. erythrini* ex *P. pagrus* off Tunisia clustered in a supported clade, along isolates from the same host off Algeria (Lablack *et al*., 2022b) and off Spain (Víllora-Montero *et al*., 2020); and isolates ex *P. erythrinus* off France (Jovelin and Justine, 2001) and Spain (Víllora-Montero *et al.*, 2020), supporting the presence of a single species in these sparid hosts in the Mediterranean. Other *Microcotyle* species from sparids included in this analysis, *M. erythrini*, *M. whittingtoni* and *M. isyebi* were resolved in separate clades, supporting thus the distinction of three

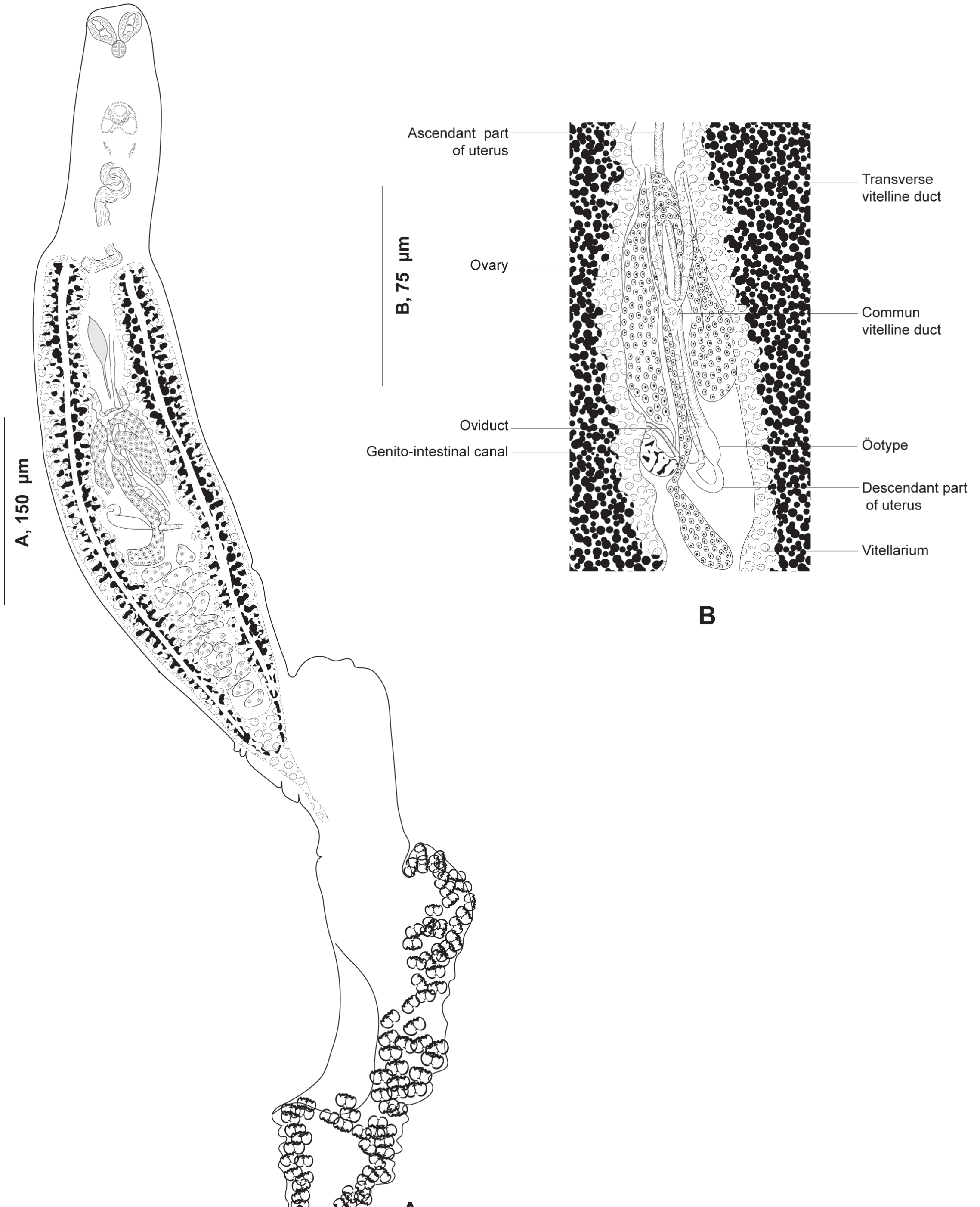

Fig. 7. *Microcotyle erythrini* Van Beneden & Hesse, 1863 *sensu lato* ex *Pagellus erythrinus* from Montenegro, Adriatic, Western Mediterranean (MNHN 306HG, Box 12, slide 10). Body, ventral view (**A**); detail of the reproductive organs in the region of ovary, ventral view (**B**).

species, as demonstrated in previous studies (Bouguerche *et al*., 2019a; Víllora-Montero *et al*., 2020; Lablack *et al*., 2022b). Unfortunately, for *M. visa*, a species also described in recent years from a sparid, the bluespotted seabream *Pagrus caeruleostictus*, 28S rDNA sequences are still lacking and only *cox*1 sequences are available for this species (Bouguerche *et al*., 2019b; Víllora-Montero *et al*., 2020).

Herein, the status of *M. sebastis* was not resolved. The sequences from Korean rockfish *S. schlegelii* from South Korea (Northwest Pacific) (Song *et al.*, 2021) and those from an unidentified rockfish *Sebastes* sp. from the North Sea (Olson and Littlewood, 2002) clustered in separate clades. This puzzling situation is hard to unravel, as *M. sebastis* was first described based on specimens obtained from the gills of also an unidentified rockfish, *Sebastes* sp. caught off Hakodate, Japan, Northwest Pacific (Goto, 1894). Hence, the only conclusion that we get in light of the available data, is that the populations from Atlantic and Pacific waters represent distinct species.

Víllora-Montero *et al.* (2020) defined morphological interspecific boundaries and generated novel morphological and molecular data for *M.* "*erythrini"* from the western Mediterranean: ex *P. pagrus* (GenBank MN816014-17) and ex *P. erythrinus* (GenBank MN816012-13), that were defined as *M. erythrini sensu stricto.* However, as the type locality is Brest, Northeast Atlantic and as *cox*1 sequences of *M. erythrini* from the type-host and type locality are still lacking, it is highly likely that future studies will reveal that the Atlantic and Mediterranean populations of *M.* "*erythrini"* represent distinct species.

The hypothesis of Víllora-Montero *et al.* (2020) suggests the presence of *M. erythrini sensu stricto* on two sparid hosts, *P. erythrinus* and *P. pagrus*, which does not eliminate the hypothesis of a host switch of *M. erythrini sensu stricto* from the Atlantic type-host *P. erythrinus* to *P. pagrus*. Previously, *M. merche* and *M. algeriensis* were demonstrated using DNA barcodes to occur in Mediterranean and Atlantic waters (Víllora-Montero *et al.*, 2023). Thus, the hypothesis of Víllora-Montero *et al.* (2020) is not to be ruled out as the two sparid hosts *P. erythrinus* and *P. pagrus* co-exist in the Eastern Atlantic. Additionally, the presence of Polyopisthocotyla in distinct localities is not unusual and has been previously challenged by *cox*1 barcodes. For instance, *Allogastrocotyle bivaginalis* occurs both in Mediterranean waters, off Algeria (Bouguerche *et al.*, 2019c), and in Australian waters of the southwest Pacific (Hossen *et al.*, 2022); also, *Kuhnia scombri* (Kuhn, 1829) Sproston, 1945 and *Pseudokuhnia minor* (Goto, 1894) Rohde & Watson, 1985 were demonstrated to occur in ten locations along the coast of China (Yan *et al.*, 2016) as well as off Australia (Hossen *et al.*, 2022). However, as mentioned above, *M. erythrini sensu stricto* is the microcotylid from the type-host and the type-locality. Unfortunately, we were unable to sample the host from localities other than Tunisia, and thus, we could not examine any Atlantic *P. erythrinus*. The only available material that we could obtain for comparison is *M. erythrini* ex *P. erythrinus* from Montenegro, Adriatic, central Mediterranean. Since DNA for these specimens is still lacking, we took a conservative position and referred to it as *M. erythrini sensu lato* pending further examinations. This is the first record of *M. erythrini* from *P. pagrus* in Tunisian waters.

Overall, for *Microcotyle* spp. both patterns of host specificity (oioxeny and stenoxeny) were demonstrated using integrative taxonomy, combining morphology and DNA barcodes. *Microcotyle isyebi*, *M. visa* and *M. whittingtoni* are oioxenic to their sparid hosts (Bouguerche *et al*., 2019a, 2019b; Víllora-Montero *et al*., 2020). Similarly, *M. merche* was demonstrated to be oioxenic to its scorpaenid host (Víllora-Montero *et al.*, 2023). Otherwise, several *Microcotyle* spp. were demonstrated to be stenoxenic: *M. algeriensis* is stenoxenic to *Scorpaena notata* and *S. scrofa* (Víllora-Montero *et al.*, 2023) and this pattern has also been demonstrated for other Polyopisthocotyla, i.e. gastrocotylids (Bouguerche *et al.*, 2020). Hence, it is premature to draw any conclusion regarding the host specificity pattern of *Microcotyle* spp. The DNA barcoding of *M. erythrini sensu stricto* from the type-host and type locality will certainly contribute to a better assessment of the host-parasite relationships for this microcotylid.

ACKNOWLEDGEMENTS

We thank the two anonymous reviewers for their insightful feedback. We extend our thanks to Jean-Lou Justine from ISYEB, Muséum National d'Histoire Naturelle (Paris, France), for making the slides collection of Louis Euzet at the Muséum National d'Histoire Naturelle available for examination. Chahinez Bouguerche was supported by the Swedish Taxonomy Initiative, Artdatabanken, Swedish University of Agricultural Sciences within the scope of the project "Systematics and integrative taxonomy of Monogenea parasitizing fishes of Sweden" (SLU.dha 2023.4.3-248).

AUTHOR CONTRIBUTIONS

IH - Data curation; Formal analysis; Funding acquisition; Investigation; Methodology; Validation; Visualization; Writing - original draft; Writing - review & editing. BB, CB - Conceptualization; Data curation; Formal analysis; Funding acquisition; Investigation; Methodology; Project administration; Resources; Software; Supervision; Validation; Visualization; Roles/Writing - original draft; Writing - review & editing. MA - Conceptualization; Data curation; Formal analysis; Methodology; Software; Validation; Visualization; Roles/Writing - original draft; Writing - review & editing. MG - DNA extraction during a traineeship at the SMNH.

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