***Parapedocotyle prolatili* gen. n. et sp. n., a representative of a new subfamily of the Diclidophoridae (Monogenea), a gill parasite of *Prolatilus jugularis* (Teleostei: Pinguipedidae) from Chile**

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Abstract: Parapedocotylinae, a new subfamily, is proposed to accommodate the gen. n. et sp. n. *Parapedocotyle prolatili* (Monogenea: Diclidophoridae), a gill parasite of the Pacific sandperch, *Prolatilus jugularis* (Valenciennes) (Pinguipedidae) from northern Chile (30°56'S; 71°20'W). Among the Diclidophoridae Cerfontaine, 1895, the species of the Pedocotylinae Yamaguti, 1963 are unique by bearing the first pair of clamps (most posteriorly) in a haptoral projection. *Pedocotyle* MacCallum, 1913, the only genus in the Pedocotylinae, is characterised by the first pair of clamps non-pedunculate, modified and non-functional, without accessory suckers, and clamps of pairs 2–4 being pedunculate and functional. In contrast, the first pair of clamps in *Parapedocotyle* is well developed and functional at the terminal end of a long haptoral appendix and having clamp pairs 2–4 pedunculated, modified and apparently not functional. Seminal receptacle is preovarian in *Parapedocotyle* in opposition to its postovarian position in *Pedocotyle*. These differences justified the erection of the new subfamily Parapedocotylinae. The new subfamily is also supported by genetic analyses (18S rDNA, 28S rDNA and *cox1* sequences) demonstrating that the Pedocotylinae and Parapedocotylinae belong to different clades in the Diclidophoridae.

Keywords: Monogenea, new subfamily, Parapedocotylinae, marine fish, Chile

Among the Diclidophoridae Cerfontaine, 1895, the Pedocotylinae Yamaguti, 1963 is unique because of the structure and arrangement of the clamps: “with three pair of pedunculate clamps just behind testicular region; last pair much smaller” Yamaguti (1963). This author did not consider that the last pair (most posterior) is smaller and deeply modified and non-functional, as described by Sproston (1946) and later by Luque-Alejos and Iannacone-Oliver (1989).

During a survey of parasites from marine fishes along the northern Chilean coast, specimens of an undescribed diclidophorid were found on the gills of the Pacific sandperch *Prolatilus jugularis* (Valenciennes) (Pinguipedidae) caught near Coquimbo, northern Chile. Characteristics of our specimens showed a slight morphological similarity with *Pedocotyle* MacCallum 1913, specifically the general arrangement of the haptor, but strongly differed in that the first pair of clamps, which is deeply modified and non-functional in *Pedocotyle*, is non-modified in our specimens. This was an indication that our specimens may belong to a new subfamily of the Diclidophoridae

In order to assess the position of the new species in the Diclidophoridae we obtained and sequenced specimens of *Pedocotyle bravo* Luque-Alejos et Iannacone-Oliver, 1989 and *P. annakohni* Luque-Alejos et Iannacone-Oliver, 1989 as well of others diclidophorids from northern Chile: *Choricotyle anisotremi* Oliva, 1987, *Paraeuryssorchis sarmientoi* (Tantalean, 1974) and the microcotylid *Paramicrocotyle* sp. A few sequences for other diclidophorids and the mazocraeid *Kuhnia scombri* (Kuhn, 1829) available in the GenBank database were also included in the analysis.

MATERIALS AND METHODS

In 2006, 50 specimens of the Pacific sandperch, *Prolatilus jugularis* (Perciformes: Pinguipedidae), were obtained from local fishermen near Coquimbo, northern Chile (30°56'S; 71°20'W); of those 38 were infected by specimens of a hitherto unknown diclidophorid. Worms were removed from the gills, fixed in AFA (ethanol: formalin: acetic acid), stained with hematoxyline-eosin or Gomori's trichrome, dehydrated in alcohol series from 70% to 100%, cleared in acid alcohol and mounted

Table 1. List of sequences analysed.

Parasite species	Family and subfamily*	Host species	Origin	GenBank accession	Gene	Source
<i>Parapedocotyle prolatili</i> sp. n.	DICL, PARA	<i>Prolatilus jugularis</i> (Valenciennes)	Chile	KJ397731	28S	Present study
<i>Parapedocotyle prolatili</i> sp. n.	DICL, PARA	<i>Prolatilus jugularis</i>	Chile	KJ397725	18S	Present study
<i>Parapedocotyle prolatili</i> sp. n.	DICL, PARA	<i>Prolatilus jugularis</i>	Chile	KJ794212, KJ794214, KJ794216, KJ794218, KJ794219	cox1	Present study
<i>Pedocotyle annakohni</i> Luque-Alejos et Iannacone-Oliver, 1989	DICL, PEDO	<i>Stellifer minor</i> (Tschudi)	Peru	KJ397728	28S	Present study
<i>Pedocotyle annakohni</i>	DICL, PEDO	<i>Stellifer minor</i>	Peru	KJ397722	18S	Present study
<i>Pedocotyle bravoii</i> Luque-Alejos et Iannacone-Oliver, 1989	DICL, PEDO	<i>Stellifer minor</i>	Peru	KJ397729	28S	Present study
<i>Pedocotyle bravoii</i>	DICL, PEDO	<i>Stellifer minor</i>	Peru	KJ397723	18S	Present study
<i>Pedocotyle bravoii</i>	DICL, PEDO	<i>Stellifer minor</i>	Peru	KJ794208–KJ794211	cox1	Present study
<i>Paraeuryorchis sarmientoi</i> (Tantalean, 1974)	DICL, EURY	<i>Seriolella violacea</i> Guichenot	Chile	KJ397730	28S	Present study
<i>Paraeuryorchis sarmientoi</i>	DICL, EURY	<i>Seriolella violacea</i>	Chile	KJ397724	18S	Present study
<i>Paraeuryorchis sarmientoi</i>	DICL, EURY	<i>Seriolella violacea</i>	Chile	KJ794213–KJ794217	cox1	Present study
<i>Choricotyle anisotremi</i> Oliva, 1987	DICL, CHOR	<i>Anisotremus scapularis</i> (Tschudi)	Chile	KJ397727	28S	Present study
<i>Choricotyle anisotremi</i>	DICL, CHOR	<i>Anisotremus scapularis</i>	Chile	KJ397721	18S	Present study
<i>Choricotyle anisotremi</i>	DICL, CHOR	<i>Anisotremus scapularis</i>	Chile	KJ794205–KJ794207	cox1	Present study
<i>Choricotyle</i> cf. <i>chrysophryi</i> (Beneden et Hesse, 1863)	DICL, CHOR	<i>Pagellus acarne</i> (Risso)	France	AF311705	28S	Jovelín and Justine (2001)
<i>Diclidophora minor</i> (Olsson, 1876)	DICL, DNAE	<i>Micromesistius poutassou</i> (Risso)	UK	AF382048	28S	Olson et al. (2002)
<i>Diclidophora denticulata</i> (Olsson, 1876)	DICL, DNAE	<i>Pollachius virens</i> (Linnaeus)	UK	AF382047	28S	Olson et al. (2002)
<i>Diclidophora luscae capelanii</i> (van Beneden et Hesse, 1863)	DICL, DNAE	<i>Trisopterus luscus capelanus</i> (Lacepede)	France	AF311704	28S	Jovelín and Justine (2001)
<i>Paramicrocotyle</i> sp.	MICR, MNAE	<i>Pinguipes chilensis</i> Valenciennes	Chile	KJ794215	cox1	Present study
<i>Kuhnia scombri</i> (Kuhn, 1829)	MAZO, MNAE	<i>Scomber scombrus</i> Linnaeus	UK	AF382044	28S	Olson et al. (2002)
<i>Kuhnia scombri</i>	MAZO, MNAE	<i>Scomber scombrus</i>		AJ228783	18S	Littlewood et al. (1998)

* Abbreviations: DICL – Diclidophoridae, PARA – Parapedocotylinae, PEDO – Pedocotylinae, EURY – Euryorchini, CHOR – Choricotylinae, DNAE – Diclidophorinae, MICR – Microcotylidae, MCAE – Microcotylinae, MAZO – Mazocraeidae, MNAE – Mazocraeinae.

in Entellan. Two specimens were serially sectioned (6 µm in thickness) and stained with hematoxyline-eosin.

Drawings were made with the aid of a drawing tube. Measurements are in micrometres (µm) unless otherwise indicated and are given as range with standard deviation in parentheses. For scanning electron microscopy (SEM), two live specimens were fixed in alcohol (80%), dehydrated through a graded ethanol series (80%, 90%, absolute alcohol), critically point dried (liquid CO₂ as intermediate liquid) in a Samdri-780 A Tousimis and sputter-coated with gold. Samples were examined using a JEOL-JSM-T300 at an accelerating voltage of 15 kV. Terminology of clamp sclerites was used as defined for other diclidophorids (Euzet and Suriano 1975).

Newly collected specimens for molecular study were fixed with 99% ethanol. The 28S rRNA gene, the V4 region of the 18S rDNA gene as well as the mitochondrial *cox1* gene were sequenced from five specimens of *Parapedocotyle prolatili*, four of *Pedocotyle bravoii* and four of *P. annakohni* from the type host the minor stardrum *Stellifer minor* (Sciaenidae). Available sequences from *Choricotyle chrysophryi* (van Beneden et Hesse, 1863), *Diclidophora minor* (Olsson, 1876), *Diclidophora denticulata* (Olsson, 1876), *Diclidophora luscae capelanii* (van Beneden et

Hesse, 1863) as well *Kuhnia scombri* were also included in the analysis (see Table 1 for GenBank accession numbers).

DNA was extracted from newly collected specimens using the QIAampTissue Kit (Qiagen, Hilden, Germany) following manufacturer's instructions. The DNA was then eluted into 50 µl of nuclease-free water.

The 28S rDNA gene was amplified by polymerase chain reaction (PCR) using the forward primer C1 (5'-ACCCGCTGAATT-TAAGCAT-3') and the reverse primer D2 (5'-TGGTCCGTGTT-TCAAGAC-3') (Chisholm et al. 2001). The 18S rRNA gene was amplified with forward primer Sb3a (5'-GGAGGGCAAGTCT-GGTGC-3') and the reverse primer A27a (5'-CCATACAAAT-GCCCCCGTCTG-3') following the protocol described by Hall et al. (1999). The *cox1* gene was amplified with forward L-CO1 (5'-TTTTTGGGCATCCTGAGGTTTAT-3') and the reverse primer H-CO1 (5'-TAAAGAAAGAACATAATGAAATG-3') (Verneau et al. 2009).

Each PCR reaction had a final volume of 35 µl including: 5 standard units of GoTaq DNA polymerase (Promega, Madison, USA), 7 µl 5× PCR buffer, 5.6 µl MgCl₂ (25 mM), 2.1 µl BSA (10 mg/ml), 0.7 µl of deoxynucleotide triphosphate (dNTP) (10 mM), 10 pM of each primer and 7 µl template DNA. Amplification for each molecular marker was done according to the

protocols described by Chisholm et al. (2001), Hall et al. (1999) and Verneau et al. (2009) for the 28S rDNA, 18S rDNA gene and *cox1* gene, respectively. Double-stranded PCR products were cleaned using an E.Z.N.A.[®] Cycle-Pure Kit (Omega Bio-Tek, Inc., Atlanta, USA), and both DNA strands were directly sequenced (Macrogen, Seoul, Korea; <http://www.macrogen.com>).

A 820 bp fragment of the 28S rDNA, 450 bp fragment of the 18S rDNA and 440 bp fragment for *cox1* were edited using ProSeq v 2.9 beta (Filatov 2002) and aligned with Clustal X (Larkin et al. 2007) using the default parameters. The distance analysis (p-distance and number of differences) was estimated using Mega v.5.0 (Tamura et al. 2011).

For the phylogenetic analyses, sequences of some diclidophorids available in the GenBank database, as well our own sequences from some diclidophorids from Chile, were included in the analysis (Table 1). Sequences of the mazocraeid monogenean *Kuhnia scombri* (28S and 18S rDNA) and *Paramicrocotyle* sp. (*cox1*) were used as outgroups. Since sequences from the GenBank database were always shorter than the sequences obtained in the present study, only 521 bp fragment for the 28S rDNA, 441 bp for the 18S rDNA and 415 bp for *cox1* genes, respectively, were used.

The phylogenetic trees were constructed using the maximum likelihood (ML) method in Mega v.5.0 (Tamura et al. 2011) and by Bayesian inference (BI) using MrBayes v3.1.2 (Huelsenbeck and Ronquist 2005). To determine the model that yielded the best fit to our dataset, Mega v.5.0 (Tamura et al. 2011) was employed.

The results from the model test indicated that the HKY + G model was the most appropriate evolution model for this dataset. Consequently, we incorporated this model of nucleotide evolution in ML and BI analyses. For the ML tree, a heuristic search using nearest neighbour interchange and bootstrap re-sampling (Felsenstein 1985) was applied to assess the support for individual nodes using 1 000 bootstrap replicates. Bayesian phylogenetic analyses were conducted using four simultaneous Markov chains running 5 000 000 generations sampling each 1 000 generations. The first 1 250 generations (burn-in) were discarded as suggested by Felsenstein (1985) and the 1 250 random generations also were discarded. The remaining 2 500 trees sampled were used to construct a 50% majority-rule consensus tree. Nodal support values (posterior probabilities) were calculated as the percentage of the 2 500 sampled trees containing the node.

RESULTS

Family Diclidophoridae Cerfontaine, 1895

Parapedocotylineae new subfamily

Diagnosis. Body cylindrical. Haptor with four pairs of clamp of diclidophorid type. First pair of clamps in posterior extension of haptor, non-pedunculate. Three pairs of pedunculate clamps (pairs 2–4) different in sclerite armature from pair 1. Genital corona present. Oesophagus short, intestinal crura extending into haptor or not. Testes in two rows in median field posterior to ovary. Genital atrium armed. Ovary tubular, curved from right to left (inverted U-shaped). Seminal receptacle preovarian. Vagina absent. Genitointestinal canal present. Vitellarium not extending to haptor. Parasite of gills of marine fish (Pinguipedidae).

Remarks. Among the Diclidophoridae, the Pedocotylineae is unique by having a haptoral projection bearing the first pair of clamps. All nominal species of *Pedocotyle* are parasites of marine sciaenid fishes and are characterised by a non-functional first pair of clamps with pairs 2–4 functional; sclerite structure for the first pair of clamp cannot be resolved (Sproston 1946, Luque-Alejos and Iannacone-Oliver 1989). In contrast, the first pair is well developed and functional in the Parapedocotylineae, whereas pairs 2–4 are apparently non-functional. The seminal receptacle is preovarian in the new subfamily rather than postovarian in the Pedocotylineae. The morphological characteristics of our specimens as well as the results of molecular analyses (see below) justify the erection of this new subfamily.

Parapedocotyle gen. n.

Diagnosis. With characteristics of Parapedocotylineae. Body long, fusiform, bearing extended haptor. Four pairs of clamps of diclidophorid type. First pair of clamp non-pedunculate at end of haptoral appendix. Clamp pairs 2 to 4 near proximal end of haptor, pedunculate, strongly modified. Sclerites As (axial sclerite of anterior jaw) and Al (lateral sclerite of anterior jaw) bearing lamellar projections covering almost all posterior quadrant. Intestinal caeca and vitellarium not extending to haptor. Testes arranged in two rows. Copulatory organ anterior to intestinal bifurcation, armed with curved hooks with bifid base. Seminal receptacle preovarian.

Type and only species: *Parapedocotyle prolatili* sp. n.

Etymology: *Parapedocotyle* refers to morphological similarity with *Pedocotyle*.

Parapedocotyle prolatili sp. n.

Figs. 1–7

Diagnosis (based on 16 stained and mounted specimens and 2 serially sectioned specimens): Body fusiform, 4.9–12.9 mm (7.8 ± 2.1 mm) in total length, 0.52–1.65 mm (0.92 ± 0.33 mm) in maximum width at level of ovary (Fig. 1). Haptor 2.0–5.4 mm (3.4 ± 0.9 mm) long and 110–330 (210 ± 60) wide, bearing four pairs of clamps (Fig. 2). Clamp pair 1 non-pedunculate at terminal end of haptor, 115–207 (155 ± 26) long and 130–212 (170 ± 30) wide. Structure of first pair of clamps typical of diclidophorids (Fig. 3). Clamps of pairs 2 to 4 at end of short peduncle, 118–215 (150 ± 18) long and 130–230 (160 ± 34) wide and modified, sclerites O (median sclerite of posterior jaw), Ps2 (sclerite bordering axial portion of the posterior jaw) and Pl2 (sclerite bordering lateral portion of posterior jaw) typical for diclidophorids, but sclerites As (axial sclerite of anterior jaw) and Al (lateral sclerite of anterior jaw) strongly modified, bearing well developed lamellar projection covering almost all posterior quadrants (Fig. 4). Two pairs of larval hooks of different sizes at end of haptor, between non-pedunculate clamps (Fig. 5). First pair of clamp serving a main attachment organ, pair 2 to 4 almost non-functional (*in situ* observation of live specimens).

Fig. 1. *Parapedocotyle prolatili* gen. n. et sp. n. from *Prolatilus jugularis*, holotype (USNPC 101156), ventral view. **Fig. 2.** Scanning electron micrographs of the haptor and clamp pair 1 (inset). **Fig. 3.** Armature of clamp pair 1. **Fig. 4.** Armature of clamp pairs 2–4. **Fig. 5.** Larval hooks. *Abbreviations:* Al – lateral sclerite of anterior jaw; ALLp – lamellar projection of sclerite Al; As – axial sclerite of anterior jaw; AsLp – lamellar projection of sclerite As; Mp – posterior section of median sclerite; O – median sclerite of posterior jaw; PI1 – sclerite bordering lateral portion of posterior jaw (dorsal); PI2 – sclerite bordering lateral portion of posterior jaw (ventral); Ps1 – sclerite bordering the axial portion of posterior jaw (dorsal); Ps2 – sclerite bordering axial portion of posterior jaw (ventral).

Oral suckers oval, 45–93 (71 ± 15) long and 34–67 (56 ± 9) wide. Mouth terminal; pharynx ovoid, 54–102 (77 ± 4) long, 45–83 (65 ± 11) wide. Intestinal bifurcation posterior to male copulatory organ. Testes 21–27, in 2 rows, 88–120 (105 ± 11) in diameter on average, spherical, postovarian, intercecal. Male copulatory organ 15–52 (31 ± 10) in diameter, armed with 6 to 9 (mode = 7) curved hooks, each having bifid base (Fig. 6). Ovary tubular, curved from right to left. Ootype postovarian (Fig. 7). Oviduct originating from left side of ovary, with short ascending section, descending near midline of body proper, receiving main vitelline and seminal ducts.

Seminal receptacle ovate, preovarian. Genitointestinal canal originating anterior to junction of oviduct and vitelline duct. Vitelline follicles coextensive with intestinal caeca. Eggs not observed.

Type host: Pacific sandperch *Prolatilus jugularis* (Valenciennes) (Perciformes: Pinguipedidae).

Site of infection: Gill filaments.

Type locality: Coquimbo ($30^{\circ}56'S$; $71^{\circ}20'W$), Province of Coquimbo, northern Chile.

Specimens deposited: Holotype (1 stained, whole-mounted specimen) in the United States National Parasite Collection, Beltsville, USA (USNPC 101156); paratypes

Fig. 6, 7. *Parapedocotyle prolatili* gen. n. et sp. n. from *Prolatulus jugularis* **Fig. 6.** Male copulatory organ. **Fig. 7.** Composition of female genitalia (ventral view) from serial sections. *Abbreviations:* Ut – uterus; SR – seminal receptacle; VD – vitelline ducts; Ov – ovary; Od – oviduct; GI – genito-intestinal canal; MG – Mehlis' gland.

(2 stained, whole-mounted specimens) in USNPC (101157) and in the Helminthological Collection, Institute of Parasitology, Biology Centre of the Czech Academy of Science, České Budějovice, Czech Republic (IPCAS M-549).

Etymology: Specific name refers to host genus.

Molecular analysis

No mutations were detected among the five sequences of *Parapedocotyle prolatili*, four sequences of *Pedocotyle bravoii* and *Pedocotyle annakohni* of both, the 18S and 28S rRNA genes. Intraspecific variability in *cox1* sequences of *P. prolatili* and *P. bravoii* was low: 0.4 and 0.6%, respectively (amplification of the *cox1* gene for *P. annakohni* was unsuccessful).

For 18S rDNA the lower divergence was observed between *P. prolatili* and *P. sarmientoi* (4.7%, i.e. 20 bp) compared to *P. prolatili* and *K. scombri* (17.8%, i.e. 76 bp). Divergence between the new species and *P. bravoii* and *P. annakohni* was 12.0% (51 bp) and 12.2% (52 bp), respectively. Lower divergence for 28S rDNA was found

Fig. 8. Phylogenetic tree based on 28S rDNA (A), 18S rDNA (B) and *cox1* (C) gene. All trees were inferred by Mr Bayes (left value in nodal support) and maximum likelihood with HKG + G model (right value in nodal support).

between *P. prolatili* and *Choricotyle* cf. *chrysophryii* (7.4%, i.e. 35 bp) and the higher between *P. prolatili* and *Kuhnia scombri* (29%, i.e. 137 bp). Divergence between the new species and *P. bravoii* and *P. annakohni* was 16.3% (77 bp) and 15.7% (74 bp), respectively. The lower divergence for the *cox1* gene was between *P. prolatili* and *Choricotyle anisotremi* (20.6%, 84–88 bp) compared to *P. prolatili* and *Paramicrocotyle* sp. (25.3%, 104–106 bp). Divergence between the new species and *P. bravoii* reached 23.1% (94–98 bp) (Fig. 8).

DISCUSSION

Among the Diclidophoridae, the Pedocotylineae is unique by the peculiar structure of the haptor, bearing the first pair of clamps non-pedunculate, situated on the posterior extension of the haptor. In the only known genus of this subfamily, *Pedocotyle*, the first pair is not functional and defined as in an immature and unemerged condition (see Sproston 1946), whereas pairs 2–4 are well developed and functional, but an accessory sucker is absent

and vitelline follicles extend to the haptor (Sproston 1946, Luque-Alejos and Iannacone-Oliver 1989).

In contrast, the specimens described herein have a well developed first pair of clamps, whereas pairs 2–4 are apparently not functional, as indicated by the small size of these structures and also confirmed by observation of live specimens *in situ*. In addition, vitelline follicles do not extend to the haptor. The armature of clamps pair 2–4 is unique among the diclidophorids; specifically the lamellar projection of sclerites As and Al covering almost entirely the posterior quadrant has not been described (see Mamaev 1976).

The reported for any other diclidophorid species of the Pedocotylineae are gill parasites of sciaenid fishes (Spro-

ston 1946, Hargis 1955, Luque-Alejos and Iannacone-Oliver 1989), whereas the new species is a parasite of a pin-guipedid. Its characteristics, in particular the structure of the haptor, which are unique among the Diclidophoridae, justify the erection of the new subfamily. Molecular data inferred from partial sequences of three genes also demonstrate that species of the Pedocotylineae and Parapedocotylineae are not closely related.

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