



Contents lists available at ScienceDirect

International Journal for Parasitology

journal homepage: www.elsevier.com/locate/ijpara

Independent host switching events by digenean parasites of cetaceans inferred from ribosomal DNA [☆]

Natalia Fraija-Fernández ^{a,*}, Peter D. Olson ^b, Enrique A. Crespo ^c, Juan A. Raga ^a, Francisco J. Aznar ^a, Mercedes Fernández ^a

^a Marine Zoology Unit, Cavanilles Institute of Biodiversity and Evolutionary Biology, Science Park, University of Valencia, PO Box 22085, 46071 Valencia, Spain

^b Department of Life Sciences, The Natural History Museum, Cromwell Road, London SW7 5BD, UK

^c Marine Mammal Laboratory (CENPAT/CONICET), Bvd. Brown 2915, 9120 Puerto Madryn, Argentina

ARTICLE INFO

Article history:

Received 14 August 2014

Received in revised form 26 September 2014

Accepted 13 October 2014

Available online xxx

Keywords:

Digenea

Cetacea

Molecular phylogeny

Host switching

ABSTRACT

Cetaceans harbour a unique fauna of digeneans whose origin and relationships have sparked considerable debate during recent decades. Disparity in the species reported indicates that they do not share close affinities, but their unusual morphology has made their taxonomic identities and phylogenetic positions uncertain. Here we use sequence data to investigate the phylogenetic relationships of the main species of flukes infecting cetaceans. We sequenced the 18S, 28S and internal transcribed spacer 2 rDNA of digenean species representing all known families reported from cetaceans: *Braunina cordiformis* (Brauninidae), *Ogmogaster antarcticus* (Notocotylidae), *Pholeter gastrophilus* (Heterophyidae), and *Campula oblonga*, *Nasitrema* sp. and *Oschmarinella rochebruni* (Brachycladiidae). The phylogenetic position of the taxa was estimated by Bayesian inference and maximum likelihood incorporating published sequences of 177 species of Digenea. Further Bayesian and maximum likelihood analyses were performed with sequences of 14 Heterophyidae and Opisthorchiidae taxa, incorporating new sequences of *P. gastrophilus*. Species nominally assigned to the Brachycladiidae formed a clade that was embedded among species of the Acanthocolpidae, thus making the latter family paraphyletic. *Braunina cordiformis* formed a sister lineage to the Strigeidae and Diplostomidae, whereas *O. antarcticus* was placed within the Notocotylidae, in agreement with the previous taxonomy of this genus. Similarly, *P. gastrophilus* was placed within the Heterophyidae as originally described. Our results suggest a paraphyletic relationship between the Heterophyidae and Opisthorchiidae, mirroring the uncertain taxonomic placement of *P. gastrophilus*, which has been assigned to both families in the past. The digenean families involved are parasites of fish-eating birds and mammals (i.e. Strigeidae, Diplostomidae and Heterophyidae), parasites of marine fish (i.e. Acanthocolpidae) and other herbivorous aquatic birds and mammals (i.e. Notocotylidae). The phylogenetic positions of these taxa indicate that the digenean fauna of cetaceans may have been acquired through independent host-capture events, with two clades showing subsequent diversification exclusively among marine mammals.

© 2014 Australian Society for Parasitology Inc. Published by Elsevier Ltd. All rights reserved.

1. Introduction

Parasitic flatworms of the subclass Digenea (Platyhelminthes: Neodermata) are obligate parasites that make up a significant component of ecosystems (Kuris et al., 2008). The complexity of their life cycles and their ubiquity among a diverse group of vertebrate

and invertebrate hosts give digeneans an ecologically important status (Cribb et al., 2001; Cribb et al., 2003). Cetaceans harbour a specific, relatively diverse fauna of digeneans that have been assigned to four families: Brachycladiidae, Brauninidae, Notocotylidae and Heterophyidae (Gibson, D.I., 2002, Trematodes in marine mammals: morphology, systematics and origins. In: Proceedings of the 10th International Congress of Parasitology: Symposia, Workshops and Contributed papers, August 4–9, Vancouver, Canada, pp. 59–63).

Species of the Brachycladiidae occur worldwide, parasitising the hepatic and pancreatic ducts, intestine, lungs and air sinuses of marine mammals (Gibson, 2005). After decades of taxonomic controversy, Gibson (2005) divided the family into two subfamilies

[☆] Nucleotide sequence data reported in this paper are available in GenBank under the accession numbers: KM258663, KM258664, KM258665, KM258666, KM258667, KM258668, KM258669, KM258670, KM258671, KM258672, KM258673, KM258674, KM258675.

* Corresponding author. Tel.: +34 963543685.

E-mail address: natalia.fraija@uv.es (N. Fraija-Fernández).

and 10 genera, seven of which are exclusive to cetaceans (*Brachycladium*, *Hunterotrema*, *Oschmarinella*, *Synthesium*, *Campula*, *Nasitrema* and *Cetitrema*), one exclusive to pinnipeds (*Zalophotrema*) and two that infect both cetaceans and pinnipeds (*Odhneriella* and *Orthosplanchnus*).

The Brauninidae is a monotypic family with *Braunina cordiformis* as its only species. This parasite exhibits a rather peculiar morphology (i.e. body cordiform with a caudal appendage and a holdfast organ which bears gonads, part of the uterus and caeca within it) (Niewiadomska, 2002a). It is found attached to the stomach wall of several odontocete species which occur mainly in the southern hemisphere (see Figueroa and Franjola, 1988; Berón-Vera et al., 2007; Romero et al., 2014).

The genus *Ogmogaster* (family Notocotylidae) is exclusive to marine mammals and currently contains six species (Barton and Blair, 2005). Members of this genus has a worldwide distribution and its species occur primarily in the intestine of baleen whales, mainly those of the Balaenopteridae and Eschrichtiidae (see Malatesta et al., 1998; Dailey et al., 2000; Leonardi et al., 2011). There are also reports, and one species described, i.e. *Ogmogaster heptalineatus*, in pinnipeds (see Beverley-Burton, 1972; Carvajal et al., 1983).

The genus *Pholeter* (family Heterophyidae) contains two species, one of which is exclusive to cetaceans (*Pholeter gastrophilus*) (Pearson, 2008). The other species, *Pholeter anterouterus*, is found encysted in the intestinal wall of fish-eating birds such as pelicans (Pearson and Courtney, 1977; Dronen et al., 2003). *Pholeter gastrophilus* lives in the stomach wall of odontocetes, surrounded by a fibrous nodule of host origin (Aznar et al., 2006). Sexually mature specimens have been reported in at least 17 cetacean species from coastal, oceanic and even freshwater habitats (Raga and Balbuena, 1993; Berón-Vera et al., 2001; Aznar et al., 2006; Quiñones et al., 2013).

The systematic affiliations of these taxa have been unstable or uncertain for decades (Fernández et al., 1998; Niewiadomska, 2002b; Gibson, 2005; Blair et al., 2008). For instance, the Brachycladiidae has been considered to be a close relative to both the Fasciolidae and Acanthocolpidae based on morphological traits (see Cable, 1974; Brooks et al., 1989). Fernández et al. (1998) found a closer relationship with Acanthocolpidae, which are parasites of fish, and this conclusion has been corroborated by more detailed studies on whole digenean phylogenies (e.g. Cribb et al., 2001; Olson et al., 2003; Bray et al., 2005). However, no attempt has been made to investigate the phylogenetic affinities of any other digenean taxa infecting cetaceans.

Host switching via ecological mechanisms is thought to be the most common route by which helminth parasites have colonised marine mammals (see Hoberg, 1995, 1997 for cestodes; Mattiucci and Nascetti, 2008 for nematodes; and García-Varela et al., 2013 for acanthocephalans). For digeneans of cetaceans a similar situation has been previously observed only for the

Brachycladiidae. Based on the hypothesis of the close relationship between Acanthocolpidae and Brachycladiidae, Fernández et al. (1998) suggested that the ancestor of the brachycladiids expanded its host range to include cetaceans as new definitive hosts. Further information about the origin of the remaining digenean taxa in cetaceans is unavailable.

Here we assess the taxonomic affiliation of digeneans of cetaceans within the larger phylogeny of Digenea proposed by Olson et al. (2003) using new sequences of *Campula oblonga*, *Nasitrema* sp., *Oschmarinella rochebruni*, *B. cordiformis*, *Ogmogaster antarcticus* and *P. gastrophilus*. We show that at least four independent colonisation events occurred, two of which led to further diversification within marine mammals.

2. Materials and methods

2.1. Taxa and outgroups selected

Table 1 summarises the species, hosts, collectors' institutions and GenBank accession numbers for all new sequences. Specimens were preserved in absolute or 70% ethanol. Other GenBank accession numbers and species used in this study are detailed in Supplementary Table S1. Two datasets were used: the first contained 77 families and 177 taxa representing the broad diversity of the subclass Digenea (Trematoda) including taxa previously used by Olson et al. (2003) and Bray et al. (2005). Five species of Aspidogastrea (see Supplementary Table S1) were used to root the resultant tree (Olson et al., 2003). A second dataset was used based on available sequences of the Heterophyidae and Opisthorchiidae together with *P. gastrophilus*. This dataset included 14 taxa from the two families and *Echinostoma revolutum* (Echinostomatidae) as an outgroup, as previously used by Thaenkham et al. (2011, 2012). A lack of available sequences prevented the construction of family level alignments of other digenean families including parasites of cetaceans.

2.2. DNA extraction, amplification and sequencing

Genomic DNA from individual worms was extracted using a Qiagen DNeasy Blood and Tissue Kit (Qiagen, Germany), following the manufacturer's recommendations with two modifications: ethanol in the samples was replaced by TE buffer (0.001 M TrisHCl, pH 7.5, 0.001 M EDTA, pH 8) and the incubation period with proteinase K was extended overnight. Partial small subunit (SSU) rDNA was amplified with primers WormA + WormB (Littlewood and Olson, 2001) or 1600R (Lim et al., 1993), and in cases of poor amplification, semi-nested PCRs were made on primary amplicons using WormA + 1270R (Fernández et al., 1998) and 600F (Littlewood and Olson, 2001) + WormB/1600R primers. Partial large subunit (LSU) rDNA was amplified with primers LSU5 (Littlewood and Johnston, 1995) + 1200R (Lockyer et al., 2003) or 1500R (Olson et al., 2003)

Table 1
List of species, hosts, collectors' institutions and GenBank accession numbers of sequences generated as part of this study for the small subunit (SSU) rDNA, the large subunit (LSU) rDNA and the second internal transcribed spacer rDNA (ITS2).

Species name (Family)	Host (Common name)	Source (Institution)	GenBank accession number		
			SSU rDNA	LSU rDNA	ITS2
<i>Braunina cordiformis</i> (Brauninidae)	<i>Delphinus delphis</i> (Short beaked common dolphin)	CENPAT	KM258664	KM258670	–
<i>Ogmogaster antarcticus</i> (Notocotylidae)	<i>Balaenoptera borealis</i> (Sei whale)	CENPAT	KM258669	KM258675	–
<i>Pholeter gastrophilus</i> (Heterophyidae)	<i>Tursiops truncatus</i> (Bottlenose dolphin)	ICBIBE	KM258668	KM258674	KM258663
<i>Campula oblonga</i> (Brachycladiidae)	<i>Phocoena phocoena</i> (Harbour porpoise)	ZSL	KM258665	KM258671	–
<i>Nasitrema</i> sp. (Brachycladiidae)	<i>Feresa attenuata</i> (Pygmy killer whale)	ULPGC	KM258666	KM258672	–
<i>Oschmarinella rochebruni</i> (Brachycladiidae)	<i>Stenella coeruleoalba</i> (Striped dolphin)	ICBIBE	KM258667	KM258673	–

CENPAT, Marine Mammal Laboratory at the Centro Nacional Patagónico (CONICET, Argentina); ICBIBE, Cavanilles Institute of Biodiversity and Evolutionary Biology at the University of Valencia (Spain); ZSL, Zoological Society of London (United Kingdom); ULPGC, University of Las Palmas de Gran Canaria (Spain).

and, when necessary, semi-nested PCRs on primary amplicons were made using primers LSU5 + ECD2 and 300F (Littlewood et al., 2000) + 1200R/1500R. Additional internal primers used for sequencing were 930F, A27 (Littlewood and Olson, 2001) and 1420R (Caira et al., 2014) for SSU rDNA. The internal transcribed spacer 2 (ITS2) region was also amplified only for *P. gastrophilus* using primers 3S (Morgan and Blair, 1995) + ITS2.2 (Anderson and Baker, 1998). The thermocycling profile for SSU rDNA and LSU rDNA amplification was as follows: denaturation at 94 °C for 3 min, 40 cycles of 94 °C for 30 s, 56 °C for 30 s and 72 °C for 2 min, and a final extension at 72 °C for 7 min (Olson et al., 2003). The profile for ITS2 was denaturation at 95 °C for 3 min, 40 cycles at 94 °C for 50 s, 53.6 °C for 50 s and 72 °C for 1 min 20 s, and a final extension of 72 °C for 4 min (Blasco-Costa et al., 2010). Amplicons were purified with a GFX PCR DNA and Gel Band Purifying Kit (GE Healthcare Life Sciences, UK) and cycle sequenced on an Applied Biosystems 3730 DNA Analyser, using Big Dye version 1.1. Contigs were assembled using Sequencher 4.8 and BioEdit 7.0.5.3. Sequence identity was checked using the Basic Local Alignment Search Tool (BLAST).

2.3. Phylogenetic analyses

The first general dataset included concatenated SSU and LSU rDNA sequences from 177 digenean taxa including the newly generated sequences of digeneans of cetaceans. Alignments for each gene were made using the online version of Mafft (<http://mafft.cbrc.jp/alignment/server/>), using the E-INS-I algorithm recommended for sequences with multiple conserved domains and long gaps (Katoh et al., 2005). SSU and LSU rDNA concatenated sequences formed an alignment that consisted of 6,327 bp. ZORRO (Wu et al., 2012) was used to exclude sites with uncertain positional homology. Excluded sites represented 49% of the complete SSU and LSU rDNA alignment. The final working dataset was 3,252 bp long, of which 1,649 bp were parsimony informative. The second dataset included available sequences of the SSU and the ITS2 rDNA of 14 taxa from the Opisthorchioidea (only from the Opisthorchiidae and Heterophyidae; data from the Cryptogonimidae were not available), including new sequences from *P. gastrophilus*. This SSU + ITS2 alignment had 3,039 bp, of which 31% were excluded due to uncertain positional homology. The resulting alignment was 2,097 bp long in which 165 bp were parsimony informative. Nucleotide alignments are available in the TreeBASE repository (<http://www.treebase.org>), study ID 16416 for the SSU and LSU rDNA sequences, and study ID 16407 for the SSU and ITS2 rDNA sequences.

JModelTest 2.1.4 (Darriba et al., 2012) was used to choose the model of evolution independently for each gene in each dataset under the Akaike Information Criterion (AIC). Phylogenetic trees were constructed by Bayesian inference (BI) using MrBayes 3.1 (Ronquist and Huelsenbeck, 2003) and maximum likelihood (ML) using PAUP* 4.0b10 (Swofford, D.L., 2002, PAUP*. Phylogenetic Analysis Using Parsimony (* and other methods). Version 4. Sinauer Associates, Sunderland, Massachusetts, USA). For BI, likelihood parameters for the SSU + LSU rDNA dataset were set to the GTR + I + G. For the SSU + ITS2, parameters were set separately for each gene; HKY + I + G showed the best fit for the SSU and GTR + G for ITS2. Posterior probabilities (PP) for each dataset were obtained after four Markov Chain Monte Carlo (MCMC) chains ran for 1,000,000 generations with one topology saved for every 100 generations. Consensus trees were constructed using a burn-in of 990 for estimating sumt and sump after the average S.D. of split frequencies was <0.01.

ML analyses were performed based on a successive approximation approach following Waeschenbach et al. (2007), starting on a tree estimated by Neighbour-Joining. A heuristic search was

performed using model parameters estimated from the previous analysis based on nearest-neighbour-interchange (NNI) first, subtree-pruning-regrafting (SPR) second, and tree-bisection-reconnection (TBR) last, until the topology remained stable. ML bootstrap values for 100 replicates were estimated using Genetic Algorithm for Rapid Likelihood Inference (GARLI 0.942) (Zwickl, D.J., 2006, Genetic algorithm approaches for the phylogenetic analysis of large biological sequence datasets under the maximum likelihood criterion. Ph.D. dissertation, The University of Texas at Austin, USA) using default settings except for the number of generations which was 10,000. Clades were considered to have high nodal support when PP were >90% and ML bootstrap values were >80%.

3. Results

For the Digenea dataset, all major clades were found to be congruent with the results previously published by Olson et al. (2003). The two main clades of the Digenea, Diplostomida and Plagiorchiida were recovered with high bootstrap and PP values (100% in both cases) (Fig. 1). Thus all taxonomic entities as defined in Olson et al. (2003) were supported.

The phylogenetic positions of all species from cetaceans were resolved with a high degree of support (PP = 100% and ML bootstrap > 80%) (Fig. 1). Within the Diplostomida, *B. cordiformis* formed a sister clade to the Strigeidae + Diplostomidae clade (Fig. 1). All other taxa, i.e. species of Brachycladiidae, *O. antarcticus* and *P. gastrophilus* were consistently placed in the Plagiorchiida (Fig. 1). Species of the Brachycladiidae were grouped in a monophyletic clade with high nodal support (PP and ML bootstrap = 100%) and it was strongly nested within the Acanthocolpiidae (Fig. 1) which resulted in a paraphyletic group.

Ogmogaster antarcticus was placed in a monophyletic and well-supported clade (PP and ML bootstrap = 100%) with species of the Notocotylidae, *Notocotylus* sp., being placed as its sister taxon with strong nodal support (Fig. 1). The Notocotylidae was placed in a clade together with the Labicolidae, in the superfamily Pronocephaloidea, with members of the Rhabdiopoeidae, the Opisthotrematidae and the Pronocephalidae (Fig. 1).

Using the SSU and LSU rDNA sequences, *P. gastrophilus* was placed among the Heterophyidae in a clade together with *Centrocestus formosanus* (Fig. 1). The clade *P. gastrophilus* + *C. formosanus* was supported by a high PP (100%), indicating that this clade occurred in all the topologies sampled in the Bayesian MCMC. However, the ML bootstrap value was only 59%, indicating that there is not enough character support for this clade (see García-Sandoval, 2014). In the SSU and ITS2 rDNA analysis, *P. gastrophilus* was placed together with two species of the Heterophyidae, i.e. *Ascocotyle longa* and *Pygidiopsis genata*, in a better supported clade (PP = 100% and ML bootstrap = 99%) (Fig. 2). Unfortunately, there were no LSU rDNA sequences for *A. longa* and *P. genata* available in GenBank to be included in the first analysis; neither was the ITS2 sequence for *C. formosanus* to be included in the second analysis. The paraphyletic condition of the Heterophyidae was evident in both analyses (Figs. 1 and 2). This family, together with members of the Opisthorchiidae and Cryptogonimidae, were grouped in the superfamily Opisthorchioidea (Fig. 1).

4. Discussion

Braunina cordiformis is the only digenean of cetaceans placed in the order Diplostomida. When described, the genus *Braunina* was not assigned to a supra-specific taxonomic rank (Niewiadomska, 2002a), reflecting its unusual morphology. Later, Yamaguti (1971) suggested that the Brauniniidae was related to the

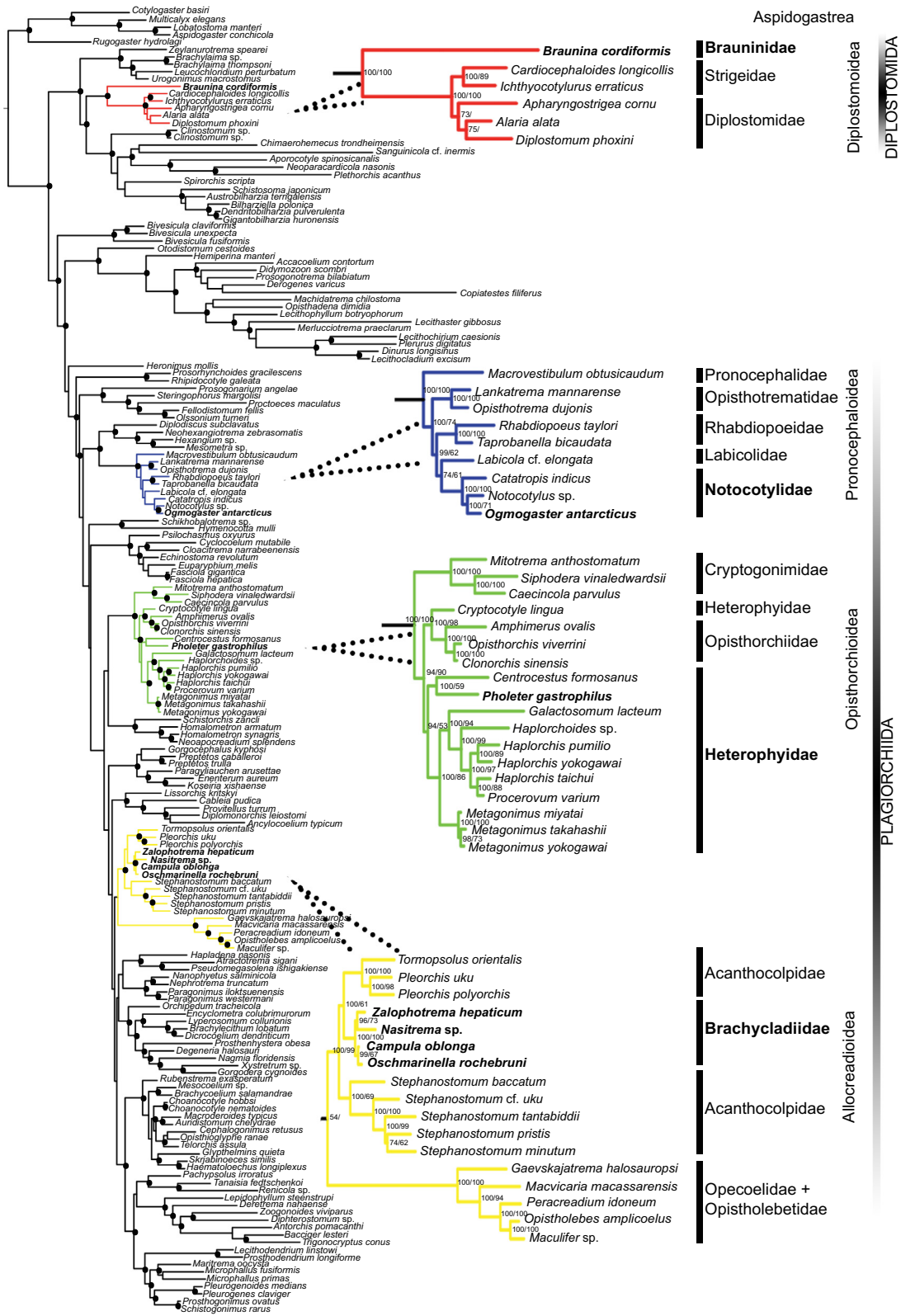


Fig. 1. Bayesian inference of the small subunit and large subunit rDNA sequences from 177 species of the subclass Digenea. Bold letters indicate species, or group of species, parasitic in cetaceans. A closer view of each segment of the tree with digeneans of cetaceans is also shown. Classification of superfamilies and suborders follows Olson et al. (2003). Black circles in the general tree indicate nodal supports of Bayesian inference (posterior probabilities) $\geq 90\%$ and maximum likelihood (bootstrap, $n = 100$) $\geq 80\%$. For the detailed trees, Bayesian inference (posterior probabilities) and maximum likelihood bootstrap for each node are shown as the first and second numbers, respectively. No bootstrap value means that the node was not resolved by maximum likelihood.

Cyathocotylidae, which are parasites of reptiles, birds and mammals. Unfortunately, available SSU and LSU rDNA sequences of the Cyathocotylidae were too short to be included in our analyses

and evaluation of the mentioned relationship between the two families was not possible. In the latter taxonomic revision based on morphology, both the Brauninidae and the Cyathocotylidae

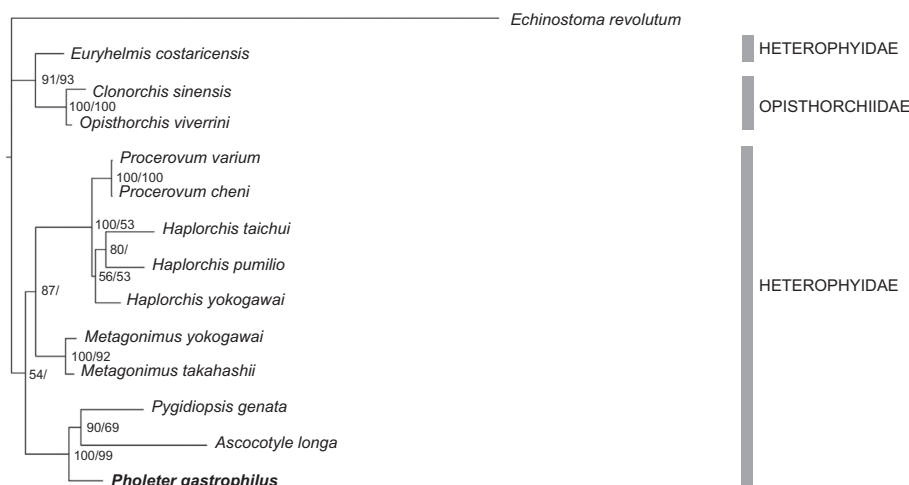


Fig. 2. Bayesian analysis of the small subunit and internal transcribed spacer 2 rDNA sequences from 14 species of the families Heterophyidae and Opisthorchiidae. Nodal support values for each node are indicated as posterior probabilities (Bayesian inference) and maximum likelihood (bootstrap, $n = 100$), respectively. Bold letters indicate parasitic species in cetaceans. No bootstrap value means that the node was not resolved by maximum likelihood.

were assigned to the superfamily Diplostomoidea (Niewiadomska, 2002b). Our results support the clade formed by the Diplostomoidea, placing *B. cordiformis* in a well supported clade together with members of the Strigeidae and the Diplostomidae, parasites of fish-eating birds and mammals (see Sepulveda et al., 1999; Niewiadomska, 2002b; Sanmartín et al., 2005; Diaz et al., 2010; Dronen et al., 1999). The taxonomic affiliation of *B. cordiformis* is consistent with the fact that its delphinoid hosts are mainly fish-eating mammals (see Berón-Vera et al., 2007; Romero et al., 2014).

The taxonomy of the Brachycladiidae has been controversial, in part because specimens are often collected in poor condition from long dead, stranded or frozen hosts, making it difficult to carry out reliable taxonomic studies based on morphological traits (Gibson, D.I., 2002, Trematodes in marine mammals: morphology, systematics and origins. In: Proceedings of the 10th International Congress of Parasitology: Symposia, Workshops and Contributed papers, August 4–9, Vancouver, Canada, pp. 59–63). Phylogenetic studies using only a few sequences, i.e. three taxa in Fernández et al. (1998) and only one in Bray et al. (2005), concluded that the Brachycladiidae forms a sister clade to the Acanthocolpidae, parasites of fish. In particular, Bray et al. (2005), using only sequences from *Zalophotrema hepaticum*, found a paraphyletic relationship and suggested that these two families should be considered synonyms. Our study includes three taxa from Brachycladiidae in a larger dataset, and provides evidence of the mentioned paraphyletic relationship between Brachycladiidae and Acanthocolpidae given the following clade: (((*Pleorchis*, *Tormopsolus*) Brachycladiidae) *Stephanostomum*) (Fig. 1). Further studies including more taxa from both families will help to circumscribe more natural groupings of these taxa.

Species of *Ogmogaster* were assigned to the family Notocotylidae, being the only genus of the family infecting marine mammals (Rausch and Fay, 1966; Barton and Blair, 2005). The present study provides molecular evidence of the taxonomic position of *O. antarcticus* within the family Notocotylidae in the superfamily Pronocephaloidea. Pronocephaloids are otherwise found in marine and freshwater reptiles, birds, mammals and marine mammals of the order Sirenia (Barton and Blair, 2005). The phylogenetic position of *O. antarcticus* is close to *Notocotylus* sp. and *Catatropis indicus* (Fig. 1). Species of the genus *Notocotylus* are defined as a cosmopolitan group parasitising waterfowl and small aquatic mammals (Boyce et al., 2012), whereas species of *Catatropis* are all parasites of birds and *C. indicus* specifically of ducks (Koch, 2002).

The SSU + ITS2 dataset placed *P. gastrophilus* in the family Heterophyidae with strong support, together with *A. longa* and *P. genata*, whereas SSU + LSU placed it in a poorly supported clade united with *Centrocestus formosanus*. Unfortunately, no sequence of *P. anterouterus*, the only other member of the genus *Pholeter*, was available to be included in the analysis. All four of these species, including *P. anterouterus*, are parasites from fish-eating birds (Pearson and Courtney, 1977; Sepulveda et al., 1999; Scholz and Salgado-Maldonado, 2000; Dzikowski et al., 2004; Simoes et al., 2010). Our results support the paraphyletic relationship between the Heterophyidae and the Opisthorchiidae, which had previously been suggested (Thaenkham et al., 2011, 2012). These taxa are similar in their morphology and life cycle (Thaenkham et al., 2011). Heterophyids and opisthorchiids share the same second intermediate (fish) and definitive host (fish-eating birds and mammals) (Bray, 2008). The phylogenetic uncertainties that define these two families mirror the unclear taxonomic history of *P. gastrophilus*.

This species was first described as belonging to the Troglotreumatidae, as it lives encysted in host tissue. Later it was suggested that, given the artificial condition of troglotrematids as a taxon, the genus *Pholeter* should be included in a specific family, i.e. Pholeteridae. However, the family was subsequently reduced to a subfamily status and assigned to the Opisthorchiidae. Finally, and based on morphological affinities with other heterophyids, the genus *Pholeter* was assigned to the Heterophyidae (Yamaguti, 1958; Pearson and Courtney, 1977; Blair et al., 2008).

Host colonisation in the marine realm is driven by stochastic mechanisms and it has been proposed as a major process that increases parasitic biodiversity when food resources overlap between actual and potential new hosts in evolutionary time (Gibson and Bray, 1994; Hoberg and Klassen, 2002; Palm and Klimpel, 2006; Raga et al., 2009). In fact, studies of several helminth taxa, including cestodes, nematodes and acanthocephalans, strongly suggest that the helminth fauna of cetaceans has resulted from host switching events (Hoberg, 1995, 1997; Mattiucci and Nascetti, 2008; García-Varela et al., 2013). For instance, aquatic birds were the ancestral definitive host for acanthocephalans of the Polymorphidae, and the genera *Bolbosoma* and *Corynosoma* secondarily colonised marine mammals, which evolved into a strict association (García-Varela et al., 2013). Among marine mammals, co-evolutionary studies of nematodes of the genera *Anisakis* in cetaceans and *Contracaecum* in pinnipeds have shown that co-speciation and host-switching events have shaped the

evolutionary history of these parasites among their hosts (Mattiucci and Nascetti, 2008).

Specific co-phylogenetic studies for each digenean family of cetaceans are needed to understand the biogeographic and historical scenarios in which host and parasites became associated. However, given the evolutionarily recent radiation of cetaceans and because seabird and teleost fish radiation predate the presence of marine mammals in the ocean (McGowen et al., 2014; Pyenson et al., 2014), it is plausible that host-switching events occurred between parasites of marine mammals and other marine hosts. Results from the present study indicate that the digenean fauna of cetaceans was formed from at least four separate host-switching events, with only the Brachycladiidae and the genus *Ogmogaster* showing further radiation among cetacean hosts. Brachycladiids are related to fish parasites, and all other digeneans of cetaceans belong to families in which aquatic birds, in the case of *Ogmogaster* spp., and other fish-eating birds are definitive hosts. For the family Brachycladiidae, evidence suggests that other host-switching events may have occurred once that initial association with cetaceans was established. Some studies have suggested that baleen whales and pinnipeds acquired brachycladiids from toothed whales, and sea otters from pinnipeds (Fernández et al., 2000; Hoberg and Adams, 2000; Aznar et al., 2001).

The phylogenetic hypothesis proposed in this study is the first, to our knowledge, using combined information from the SSU, LSU and ITS2 rDNA from digeneans of cetaceans and the first providing phylogenetic evidence about taxonomic affiliation for *B. cordiformis*, *O. antarcticus* and *P. gastrophilus*. Overall, our findings suggest that the digenean fauna in cetaceans originated by colonisation.

Acknowledgments

The authors kindly thank the following persons who either helped to obtain or provided samples used during the study: Nestor A. García from the Marine Mammal Laboratory at Centro Nacional Patagónico (Puerto Madryn, Argentina), Rob Deaville from the UK Cetacean Strandings Investigation Programme, Zoological Society of London, Antonio Fernández from University of Las Palmas de Gran Canaria (Spain) and personnel from the Marine Zoology Unit of the Cavanilles Institute of Biodiversity and Evolutionary Biology (University of Valencia, Spain). We thank the staff at the Natural History Museum (London, UK) Sequencing Unit for running the automated sequencer. Significant comments on phylogenetic analysis from Andrea Waeschenbach were appreciated. Authors thank two anonymous reviewers for their suggestions that substantially improved a previous version of this article. This study is supported by grants CGL/2009/07465 and CGL/2012/39545 (Science and Innovation Ministry of Spain-MICINN) and PROMETEO/2011/040 (Generalitat Valenciana, Spain). Marine mammals along the Argentine coast were collected with support from the Agencia Nacional de Promoción Científica y Tecnológica (PICT 2110/2008). N. Fraija-Fernández held a visiting studentship to the NHM (London, UK) BEFPI/2013/073 (Generalitat Valenciana).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.ijpara.2014.10.004>.

References

- Anderson, G.R., Baker, S.C., 1998. Inference of phylogeny and taxonomy within the Didymozoidae (Digenea) from the second internal transcribed spacer (ITS2) of ribosomal DNA. *Syst. Parasitol.* 41, 87–94. <http://dx.doi.org/10.1023/A:1006024128098>.
- Aznar, F.J., Balbuena, J.A., Fernández, M., Raga, J.A., 2001. Living together: the parasites of marine mammals. In: Evans, P.G.H., Raga, J.A. (Eds.), *Marine Mammals: Biology and Conservation*. Kluwer Academic/Plenum Publishers, New York, pp. 385–421.
- Aznar, F.J., Fognani, P., Balbuena, J.A., Pietrobelli, M., Raga, J.A., 2006. Distribution of *Pholeter gastrophilus* (Digenea) within the stomach of four odontocete species: the role of the diet and digestive physiology of hosts. *Parasitology* 133, 369–380. <http://dx.doi.org/10.1017/S0031182006000321>.
- Barton, D.P., Blair, D., 2005. Superfamily Pronocephaloidea Looss, 1899. In: Jones, A., Bray, R.A., Gibson, D.I. (Eds.), *Keys to the Trematoda*, vol. 2. CABI Publishing and The Natural History Museum, Wallingford, UK, pp. 357–411.
- Berón-Vera, B., Pedraza, S.N., Raga, J.A., Gil de Pertierra, A., Crespo, E.A., Alonso, M.K., Goodall, R.N.P., 2001. Gastrointestinal helminths of Commerson's dolphins *Cephalorhynchus commersonii* from central Patagonia and Tierra del Fuego. *Dis. Aquat. Organ.* 47, 201–208. <http://dx.doi.org/10.3354/dao047201>.
- Berón-Vera, B., Crespo, E.A., Raga, J.A., Fernández, M., 2007. Parasite communities of common dolphins (*Delphinus delphis*) from Patagonia: the relation with host distribution and diet and comparison with sympatric hosts. *J. Parasitol.* 93, 1056–1060. <http://dx.doi.org/10.1645/GE-1070.R.1>.
- Beverley-Burton, M., 1972. Trematodes from the Weddell Seal, *Leptonychotes weddelli* (Lesson, 1826) with a description of *Orthosplanchnus weddelli* n. sp. (Trematoda: Campulidae). *Zool. Anz.* 189, 49–60.
- Blair, D., Tkach, V.V., Barton, D.P., 2008. Family Troglotrematidae Odhner, 1914. In: Bray, R.A., Gibson, D.I., Jones, A. (Eds.), *Keys to the Trematoda*, vol. 3. CABI Publishing and The Natural History Museum, Wallingford, UK, pp. 277–289.
- Blasco-Costa, I., Balbuena, J.A., Raga, J.A., Kostadinova, A., Olson, P.D., 2010. Molecules and morphology reveal cryptic variation among digeneans infecting sympatric mullets in the Mediterranean. *Parasitology* 137, 287–302. <http://dx.doi.org/10.1017/S0031182009991375>.
- Boyce, K., Hide, G., Craig, P.S., Harris, P.D., Reynolds, C., Pickles, A., Rogan, M.T., 2012. Identification of a new species of digenean *Notocotylus malhamensis* n. sp. (Digenea: Notocotylidae) from the bank vole (*Myodes glareolus*) and the field vole (*Microtus agrestis*). *Parasitology* 139, 1630–1639. <http://dx.doi.org/10.1017/S0031182012000911>.
- Bray, R.A., 2008. Superfamily Opisthorchioidea Looss, 1899. In: Bray, R.A., Gibson, D.I., Jones, A. (Eds.), *Keys to the Trematoda*, vol. 3. CABI Publishing and The Natural History Museum, Wallingford, UK, pp. 7–142.
- Bray, R.A., Webster, B.L., Bartolli, P., Littlewood, D.T.J., 2005. Relationships within the Acanthocolpidae Lühe, 1906 and their place among the Digenea. *Acta Parasitol.* 50, 281–291.
- Brooks, D.R., Bandoni, S.M., Macdonald, C.A., O'Grady, R.T., 1989. Aspects of the phylogeny of the Trematoda Rudolphi, 1808 (Platyhelminthes: Cercaria). *Can. J. Zool.* 67, 2609–2624. <http://dx.doi.org/10.1139/z89-370>.
- Caira, J.N., Jensen, K., Waeschenbach, A., Olson, P.D., Littlewood, D.T.J., 2014. Orders out of chaos – molecular phylogenetics reveals the complexity of shark and stingray tapeworm relationships. *Int. J. Parasitol.* 44, 55–73. <http://dx.doi.org/10.1016/j.ijpara.2013.10.004>.
- Cable, R.M., 1974. Phylogeny and taxonomy of the trematodes with references to marine species. In: Vernberg, W.B. (Ed.), *Symbiosis in the Sea*. University of South California Press, South Carolina, USA, pp. 173–193.
- Carvajal, J., Durán, L.E., George-Nascimento, M., 1983. *Ogmogaster heptalineatus* n. sp. (Trematoda: Notocotylidae) from the Chilean sea lion *Otaria flavescens*. *Syst. Parasitol.* 5, 169–173. <http://dx.doi.org/10.1007/BF00009349>.
- Cribb, T.H., Bray, R.A., Littlewood, D.T.J., Pichelin, S.P., Herniou, E.A., 2001. The Digenea. In: Littlewood, D.T.J., Bray, R.A. (Eds.), *Interrelationships of the Platyhelminthes*. Taylor and Francis, London, UK, pp. 168–185.
- Cribb, T.H., Bray, R.A., Olson, P.D., Littlewood, D.T.J., 2003. Life cycle evolution in the Digenea: a new perspective from phylogeny. *Adv. Parasitol.* 54, 197–254.
- Dailey, M.D., Gulland, F.M., Lowenstine, L.J., Silvagni, P., Howard, D., 2000. Prey, parasites and pathology associated with the mortality of a juvenile gray whale (*Eschrichtius robustus*) stranded along the northern California coast. *Dis. Aquat. Organ.* 42, 111–117. <http://dx.doi.org/10.3354/dao042111>.
- Darriba, D., Taboada, G.L., Doallo, R., Posada, D., 2012. JModelTest 2: more models, new heuristics and parallel computing. *Nat. Methods* 9, 772. <http://dx.doi.org/10.1038/nmeth.2109>.
- Diaz, J.I., Cremonese, F., Navone, G.T., 2010. Helminths of the Magellanic penguin, *Spheniscus magellanicus* (Sphenisciformes), during the breeding season in Patagonian coast, Chubut, Argentina. *Comp. Parasitol.* 77, 172–177. <http://dx.doi.org/10.1654/4441.1>.
- Dronen, N.O., Tehrany, M.R., Wardle, W.J., 1999. Diplostomes from the Brown Pelican, *Pelecanus occidentalis* (Pelecanidae), from the Galveston, Texas area, including two new species of *Bursacetabulus* gen. n. *J. Helminthol. Soc. Wash.* 66, 21–24.
- Dronen, N.O., Blend, C.K., Anderson, C.K., 2003. Endohelminths from the Brown Pelican, *Pelecanus occidentalis*, and the American White Pelican, *Pelecanus erythrorhynchus*, from Galveston Bay, Texas, U.S.A., and Checklist of Pelican Parasites. *Comp. Parasitol.* 70, 140–154. [http://dx.doi.org/10.1654/1525-2647\(2003\)070\[0140:EFTBPP\]2.0.CO;2](http://dx.doi.org/10.1654/1525-2647(2003)070[0140:EFTBPP]2.0.CO;2).
- Dzikowski, R., Levy, M.G., Poore, M.F., Flowers, J.R., Paperna, I., 2004. Use of rDNA polymorphism for identification of Heterophyidae infecting freshwater fishes. *Dis. Aquat. Organ.* 59, 35–41. <http://dx.doi.org/10.3354/dao059035>.
- Fernández, M., Littlewood, D.T.J., Latorre, A., Raga, J.A., Rollinson, D., 1998. Phylogenetic relationships of the family Campulidae (Trematoda) based on 18S rRNA sequences. *Parasitology* 117, 383–391. <http://dx.doi.org/10.1017/S0031182098003126>.

- Fernández, M., Aznar, F.J., Raga, J.A., Latorre, A., 2000. The origin of *Lecithodesmus* (Digenea: Campulidae) based on ND3 gene comparison. *J. Parasitol.* 86, 850–852. [http://dx.doi.org/10.1645/0022-3395\(2000\)086\[0850:TOOLDLC\]2.0.CO;2](http://dx.doi.org/10.1645/0022-3395(2000)086[0850:TOOLDLC]2.0.CO;2).
- Figuerola, L., Franjola, R., 1988. *Braunina cordiformis* Wolf, 1903 (Digenea: Brauninidae) en delfin chileno *Cephalorhynchus eutropia* Gray, 1846 (Cetacea: Odontoceti). *Bol. Chil. Parasitol.* 43, 71–72.
- García-Sandoval, R., 2014. Why some clades have low bootstrap frequencies and high Bayesian posterior probabilities. *Isr. J. Ecol. Evol.* 60, 41–44. <http://dx.doi.org/10.1080/15659801.2014.937900>.
- García-Varela, M., Pérez-Ponce de León, G., Aznar, F.J., Nadler, S.A., 2013. Phylogenetic relationships among genera of Polymorphidae (Acanthocephala), inferred from nuclear and mitochondrial gene sequences. *Mol. Phylogenet. Evol.* 68, 176–184. <http://dx.doi.org/10.1016/j.ympev.2013.03.029>.
- Gibson, D.I., 2005. Family Brachycladiidae Odhner, 1905. In: Jones, A., Bray, R.A., Gibson, D.I. (Eds.), *Keys to the Trematoda*, vol. 2. CABI Publishing and The Natural History Museum, Wallingford, UK, pp. 641–652.
- Gibson, D.I., Bray, R.A., 1994. The evolutionary expansion and host–parasite relationships of the Digenea. *Int. J. Parasitol.* 24, 1213–1226. [http://dx.doi.org/10.1016/0020-7519\(94\)90192-9](http://dx.doi.org/10.1016/0020-7519(94)90192-9).
- Hoberg, E.P., 1995. Historical biogeography and modes of speciation across high-latitude seas of the Holarctic: concepts for host–parasite coevolution among the Phocini (Phocidae) and Tetrabotheiidae (Eucestoda). *Can. J. Zool.* 73, 45–57. <http://dx.doi.org/10.1139/z95-006>.
- Hoberg, E.P., 1997. Phylogeny and historical reconstruction: host–parasite systems as keystones in biogeography and ecology. In: Reaka-Kudla, M., Wilson, D.E., Wilson, E.O. (Eds.), *Biodiversity II: Understanding and Protecting Our Resources*. Joseph Henry Press, National Academy of Sciences, Washington, DC, USA, pp. 243–261.
- Hoberg, E.P., Adams, A., 2000. Phylogeny, history and biodiversity: understanding faunal structure and biogeography in the marine realm. *Bull. Scand. Soc. Parasitol.* 10, 19–37.
- Hoberg, E.P., Klassen, G.J., 2002. Revealing the faunal tapestry: co-evolution and historical biogeography of hosts and parasites in marine systems. *Parasitology* 124, S3–S22. <http://dx.doi.org/10.1017/S0031182002001841>.
- Katoh, K., Kuma, K., Toh, H., Miyata, T., 2005. MAFFT version 5: improvement in accuracy of multiple sequence alignment. *Nucleic Acids Res.* 33, 511–518. <http://dx.doi.org/10.1093/nar/gki198>.
- Koch, M., 2002. First record and description of *Catantropis indicus* Srivastava 1935 (Digenea: Notocotylidae), in Australia. *Mem. Queensl. Mus.* 48, 147–153.
- Kuris, A.M., Hechinger, R.F., Shaw, J.C., Whitney, K.L., Aguirre-Macedo, L., Boch, C.A., Dobson, A.P., Dunham, E.J., Fredensborg, B.L., Huspeni, T.C., Lorda, J., Mababa, L., Mancini, F.T., Mora, A.B., Pickering, M., Talhouk, N.L., Torchin, M.E., Lafferty, K.D., 2008. Ecosystem energetic implications of parasite and free-living biomass in three estuaries. *Nature* 454, 515–518. <http://dx.doi.org/10.1038/nature06970>.
- Leonardi, M.S., Grandi, M.F., García, N.A., Svendsen, G., Romero, M.A., González, R., Crespo, E.A., 2011. A stranding of *Balaenoptera borealis* (Lesson 1828) from Patagonia, Argentina, with notes on parasite infestation and diet. *Afr. J. Mar. Sci.* 33, 177–179. <http://dx.doi.org/10.2989/1814232X.2011.572384>.
- Lim, E.L., Amaral, L.A., Caron, D.A., DeLong, E.F., 1993. Application of rRNA-based probes for observing marine nanoplanktonic protists. *Appl. Environ. Microb.* 59, 1647–1655.
- Littlewood, D.T.J., Johnston, D.A., 1995. Molecular phylogenetics of the four *Schistosoma* species groups determined with partial 28S ribosomal RNA gene sequences. *Parasitology* 111, 167–175. <http://dx.doi.org/10.1017/S00311820006491X>.
- Littlewood, D.T.J., Olson, P.D., 2001. SSU rDNA and the Platyhelminthes: signal, noise, conflict and compromise. In: Littlewood, D.T.J., Bray, R.A. (Eds.), *Interrelationships of the Platyhelminthes*. Taylor and Francis, London, UK, pp. 262–278.
- Littlewood, D.T.J., Curini-Galletti, M., Herniou, E.A., 2000. The interrelationships of Proseriata (Platyhelminthes: Seriata) tested with molecules and morphology. *Mol. Phylogenet. Evol.* 16, 449–466. <http://dx.doi.org/10.1006/mpev.2000.0802>.
- Lockyer, A.E., Olson, P.D., Littlewood, D.T.J., 2003. Utility of complete large and small subunit rRNA genes in resolving the phylogeny of the Neodermata (Platyhelminthes): implications and a review of the cercomer theory. *Biol. J. Linn. Soc.* 78, 155–171. <http://dx.doi.org/10.1046/j.1095-8312.2003.00141.x>.
- Malatesta, T., Frati, R., Cerioni, S., Agrimi, U., Di Guardo, G., 1998. *Ogmogaster antarcticus* Johnston, 1931 (Digenea: Notocotylidae) in *Balaenoptera physalus* (L.): first record in the Mediterranean Sea. *Syst. Parasitol.* 40, 63–66. <http://dx.doi.org/10.1023/A:1005955200911>.
- Mattiucci, S., Nascetti, G., 2008. Advances and trends in the molecular systematics of anisakid nematodes, with implications for their evolutionary ecology and host–parasite co-evolutionary processes. *Adv. Parasitol.* 66, 47–148. [http://dx.doi.org/10.1016/S0065-308X\(08\)00202-9](http://dx.doi.org/10.1016/S0065-308X(08)00202-9).
- McGowen, M.R., Gatesy, J., Wildman, D.E., 2014. Molecular evolution tracks macroevolutionary transitions in Cetacea. *Trends Ecol. Evol.* 29, 336–346. <http://dx.doi.org/10.1016/j.tree.2014.04.001>.
- Morgan, J.A., Blair, D., 1995. Nuclear rDNA ITS sequence variation in the trematode genus *Echinostoma*: an aid to establishing relationships within the 37-collar spine group. *Parasitology* 111, 609–615. <http://dx.doi.org/10.1017/S003118200007709X>.
- Niewiadomska, K., 2002a. Family Brauninidae Wolf, 1903. In: Gibson, D.I., Jones, A., Bray, R.A. (Eds.), *Keys to the Trematoda*, vol. 1. CABI Publishing and The Natural History Museum, Wallingford, UK, pp. 199–200.
- Niewiadomska, K., 2002b. Superfamily Diplostomoidea Poirier, 1886. In: Gibson, D.I., Jones, A., Bray, R.A. (Eds.), *Keys to the Trematoda*, vol. 1. CABI Publishing and The Natural History Museum, Wallingford, UK, pp. 159–241.
- Olson, P.D., Cribb, T.H., Tkach, V.V., Bray, R.A., Littlewood, D.T.J., 2003. Phylogeny and classification of the Digenea (Platyhelminthes: Trematoda). *Int. J. Parasitol.* 33, 733–755. [http://dx.doi.org/10.1016/S0020-7519\(03\)00049-3](http://dx.doi.org/10.1016/S0020-7519(03)00049-3).
- Palm, H.W., Klimpel, S., 2006. Evolution of parasitic life in the ocean. *Trends Parasitol.* 23, 10–12. <http://dx.doi.org/10.1016/j.pt.2006.11.001>.
- Pearson, J., 2008. Family Heterophyidae Leiper, 1909. In: Bray, R.A., Gibson, D.I., Jones, A. (Eds.), *Keys to the Trematoda*, vol. 3. CABI Publishing and The Natural History Museum, Wallingford, UK, pp. 113–141.
- Pearson, J.C., Courtney, C.H., 1977. *Pholeter anterouterus* Fischthal and Nasir, 1974 (Digenea: Opisthorchiidae) redescribed, together with remarks on the genera *Pholeter* Odhner, 1914 and *Phocitrema* Goto and Ozaki, 1930 and their relationship to the centrocestine heterophyids. *Parasitology* 74, 255–271. <http://dx.doi.org/10.1017/S0031182000047880>.
- Pyenson, N.D., Kelley, N.P., Parham, J.F., 2014. Marine tetrapod macroevolution: physical and biological drivers on 250 Ma of invasions and evolution in ocean ecosystems. *Palaeogeogr. Palaeoclimatol. Palaeoecol.* 400, 1–8. <http://dx.doi.org/10.1016/j.palaeo.2014.02.018>.
- Quiñones, R., Giovannini, A., Raga, J.A., Fernández, M., 2013. Intestinal helminth fauna of bottlenose dolphin *Tursiops truncatus* and common dolphin *Delphinus delphis* from the western Mediterranean. *J. Parasitol.* 99, 576–579. <http://dx.doi.org/10.1645/GE-3165.1>.
- Raga, J.A., Balbuena, J.A., 1993. Parasites of the long-finned pilot whale *Globicephala melas* (Trail, 1809) in European waters. Reports of the International Whaling Commission Special Issue 14, 391–406.
- Raga, J.A., Fernández, M., Balbuena, J.A., Aznar, F.J., 2009. Parasites. In: Perrin, W.F., Theewissen, H.G.M., Würsig, B. (Eds.), *Encyclopedia of Marine Mammals*, second ed. Academic Press/Elsevier Inc., San Diego, USA, pp. 821–830.
- Rausch, R.L., Fay, F.H., 1966. Studies on the helminth fauna of Alaska. XLIV. Revision of *Ogmogaster* Jägerskiöld, 1891, with a description of *O. pentalinatus* sp. n. (Trematoda: Notocotylidae). *J. Parasitol.* 52, 26–38.
- Romero, M.A., Fernández, M., Dans, S.L., García, N.A., González, R., Crespo, E.A., 2014. Gastrointestinal parasites of bottlenose dolphins *Tursiops truncatus* from the extreme southwestern Atlantic, with notes on diet composition. *Dis. Aquat. Organ.* 108, 61–70. <http://dx.doi.org/10.3354/dao02700>.
- Ronquist, F., Huelsenbeck, J.P., 2003. MRBAYES 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* 19, 1572–1574. <http://dx.doi.org/10.1093/bioinformatics/btg180>.
- Sanmartín, M.L., Cordeiro, J.A., Alvarez, M.F., Leiro, J., 2005. Helminth fauna of the yellow-legged gull *Larus cachinnans* in Galicia, North-West Spain. *J. Helminthol.* 79, 361–371. <http://dx.doi.org/10.1079/JOH2005309>.
- Scholz, T., Salgado-Maldonado, G., 2000. The introduction and dispersal of *Centrocestus formosanus* (Nishigori, 1924) (Digenea: Heterophyidae) in Mexico: a review. *Am. Midl. Nat.* 143, 185–200. [http://dx.doi.org/10.1674/0003-0031\(2000\)143\[0185:TIADOC\]2.0.CO;2](http://dx.doi.org/10.1674/0003-0031(2000)143[0185:TIADOC]2.0.CO;2).
- Sepulveda, M.S., Spalding, M.G., Kinsella, J.M., Forrester, D.J., 1999. Parasites of the great Egret (*Ardea albus*) in Florida and a review of the helminths reported for the species. *J. Helminthol. Soc. Wash.* 66, 7–13.
- Simoes, S.B., Barbosa, H.S., Santos, C.P., 2010. The life cycle of *Ascoctyle (Phagicola) longa* (Digenea: Heterophyidae), a causative agent of fish-borne trematodosis. *Acta Trop.* 113, 226–233. <http://dx.doi.org/10.1016/j.actatropica.2009.10.020>.
- Thaenkham, U., Nawa, Y., Blair, D., Pakdee, W., 2011. Confirmation of the paraphyletic relationship between families Opisthorchiidae and Heterophyidae using small and large subunit ribosomal DNA sequences. *Parasitol. Int.* 60, 521–523. <http://dx.doi.org/10.1016/j.parint.2011.07.015>.
- Thaenkham, U., Blair, D., Nawa, Y., Waikagul, J., 2012. Families Opisthorchiidae and Heterophyidae: are they distinct? *Parasitol. Int.* 61, 90–93. <http://dx.doi.org/10.1016/j.parint.2011.06.004>.
- Waeschenbach, A., Webster, B.L., Bray, R.A., Littlewood, D.T.J., 2007. Added resolution among ordinal level relationships of tapeworms (Platyhelminthes: Cestoda) with complete small and large subunit nuclear ribosomal RNA genes. *Mol. Phylogenet. Evol.* 45, 311–325. <http://dx.doi.org/10.1016/j.ympev.2007.03.019>.
- Wu, M., Chatterji, S., Eisen, J.A., 2012. Accounting for alignment uncertainty in phylogenomics. *PLoS ONE* 7, e30288. <http://dx.doi.org/10.1371/journal.pone.0030288>.
- Yamaguti, S., 1958. *Systema Helminthum. The Digenetic Trematodes of Vertebrates*, vol. 1. Interscience Publishers, New York, USA.
- Yamaguti, S., 1971. *Synopsis of the Digenetic Trematodes of Vertebrates*, vol. 1. Keigaku Publishing Co., Tokyo, Japan, 687–824.