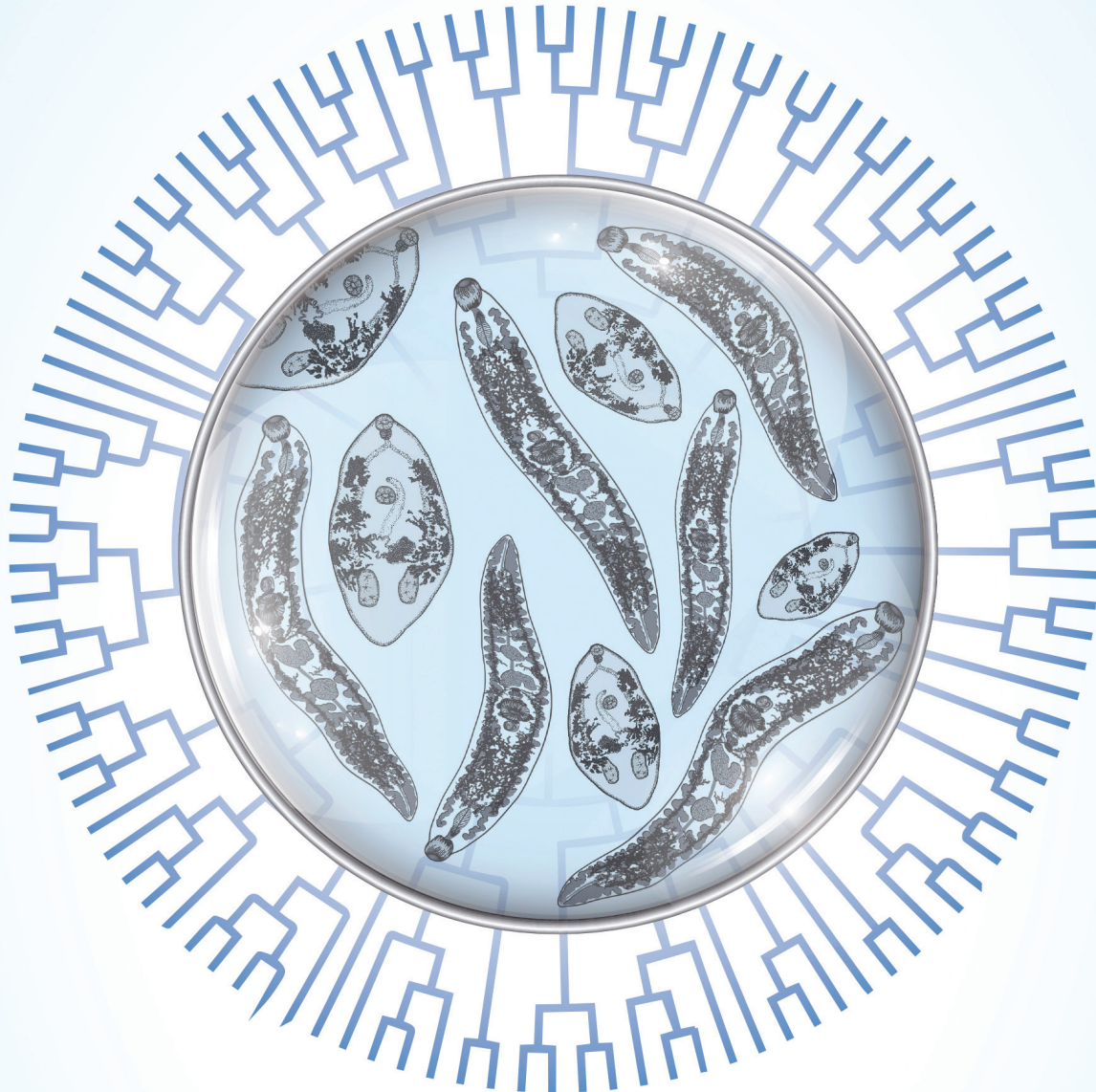


DIGENEANS OF CETACEANS: DIVERSITY, EVOLUTIONARY HISTORY AND HOST SPECIFICITY



Tesis Doctoral por: **Natalia Fraija Fernández**

Directores:

Dra. Mercedes Fernández Martínez

Dr. Francisco Javier Aznar Avendaño

Valencia, mayo 2016



VNIVERSITAT
DE VALÈNCIA



Facultat de
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PROGRAMA DE DOCTORADO
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D^a. María Mercedes Fernández Martínez, Profesora Titular del Departamento de Zoología de la Facultad de Ciencias Biológicas de la Universidad de Valencia y **D. Francisco Javier Aznar Avendaño**, Profesor Titular del Departamento de Zoología de la Facultad de Ciencias Biológicas de la Universidad de Valencia,

CERTIFICAN que **D^a. Natalia Fraija Fernández** ha realizado bajo nuestra dirección, y con el mayor aprovechamiento, el trabajo de investigación recogido en esta memoria, y que lleva por título “**DIGENEANS OF CETACEANS: DIVERSITY, EVOLUTIONARY HISTORY AND HOST SPECIFICITY**”, para optar al grado de Doctora en Ciencias Biológicas.

Y para que así conste, en cumplimiento de la legislación vigente, expedimos el presente certificado en Valencia, a 02 de mayo de 2016.

Firmado:

Firmado:

*A mi má,
a mis abues,
y a Juan*

Con amor y gratitud.

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SUMMARY



Parasitism is an extremely successful lifestyle among animals. In fact, every free-living organism is believed to harbour at least a parasite species, and cetaceans are not the exception. Host-parasite systems offer a suitable model for studying systematics, evolution, biogeography and ecology because the evolutionary fate of parasites is linked to that of their hosts. In particular, present-day associations between cetaceans and their parasites have been shaped by unique historical events. The parasites of the terrestrial ancestors of cetaceans likely faced an ecological barrier when their hosts colonized the marine environment; it is postulated that during the process terrestrial parasites went extinct, thus the current parasitic fauna of cetaceans was acquired through colonization events in the marine realm. Cetaceans harbour a trophically-transmitted helminth fauna that includes more than 100 species of acanthocephalans, cestodes, nematodes and digeneans. Records of helminths in cetaceans go back to the time when commercial whaling was allowed and material for scientific studies was available, and efforts for maintaining an updated list have been made. However, additional information from post-mortem examinations of stranded or by-caught animals, from rarely cetacean species, and from unexplored areas has become available over the last decades. In addition, new taxonomic rearrangements have been proposed and the use of molecular information has helped to unveil a significant portion of hidden biodiversity (i.e., cryptic species).

Digeneans represent a highly diverse and relatively specific group of helminths infecting cetaceans. Records in cetaceans include four families of highly specific taxa, i.e., the monotypic Brauninidae with *Braunina cordiformis* as its only species, species of *Ogmogaster* (Notocotylidae), *Pholeter gastrophilus* (Heterophyidae), and species of the family Brachycladiidae. The systematic affiliation of these digeneans has been controversial with limited phylogenetic information. Only for the Brachycladiidae, available data indicates that the origin of this group, and its association with marine mammals, likely resulted from a host-switching event. However, in order to understand biogeographical and historical scenarios in which host and parasites become associated specific cophylogenetic studies for each family are also needed.

The digeneans of cetaceans display sharp differences in host specificity, i.e., there are examples of highly specific species restricted to few host species (e.g., *Synthesium pontoporiae*, in the Franciscana dolphin, *Pontoporia blainvillei*), whereas other species are generalists and widely distributed among several host species (e.g., *Pholeter gastrophilus*, reported in at least 17 cetacean species). However, the number of hosts a parasite can exploit is not the only measure of host specificity, since hosts may differ in quality, and may not be equally compatible with each parasite species. There are examples of evident morphological and reproductive differences (i.e., dwarfism and impaired reproduction) when digeneans infect unsuitable cetacean species. Still, few studies have analysed how presumably equally suitable hosts and the environmental pressures where the parasite's life cycle develops would affect the host compatibility of a generalist digenean species.

The amount of gene flow between individuals likely contributes to the genetic cohesion of a species, and is highly determined by their capacity for dispersion, which in parasites depends on the

most mobile host. Cetaceans are long-ranging animals with some highly structured populations. Currently, few studies have analysed the genetic structure of helminths in cetaceans, including the generalist nematode *Anisakis typica*, and the specific digenean *Synthesium pontoporiae*. No attempt has been made to study the genetic structure and gene flow in any generalist digenean species.

This study addresses different aspects of the diversity, origin and diversification, and patterns of host specificity in digeneans of cetaceans, focusing on the following objectives:

- 1 – To update the systematic list of digeneans, and other helminths, infecting cetaceans.
- 2 – To describe a new digenean species found in cetaceans.
- 3 – To assess the taxonomic affiliation and to investigate the origin of the four families of digeneans found in cetaceans.
- 4 – To analyse the cophylogenetic relationship between the most diverse digenean family that parasitize cetaceans, i.e., the Brachycladiidae, and their marine mammal hosts.
- 5 – To investigate phylogeographical patterns of the most generalist species of digeneans infecting cetaceans, namely, *Pholeter gastrophilus*.
- 6 – To examine host specificity patterns in *Pholeter gastrophilus*, determining the extent to which the parasite's performance varies according to host species, and how host's habitat can influence the reproductive strategy of the parasite.

The following results and conclusions were obtained from this study:

Out of the 90 species currently recognised in the order Cetacea, 72 have been examined for helminths. The family Balaenopteridae among baleen whales and the family Delphinidae among toothed whales harbour the most diverse helminth fauna; this is partly accounted for by a higher sampling effort on these families. In contrast, the helminth fauna of the beaked whales (family Ziphiidae) is the least known since only 9 out of the 22 species in the family have been examined for parasites. Currently, there are 174 helminth species reported in cetaceans including nematodes (62 spp.), digeneans (54 spp.), cestodes (38 spp.) and acanthocephalans (20 spp.). However, 20 (11.5%) of these species represent accidental infections, which occur because cetaceans and other marine vertebrates (i.e., pinnipeds and marine birds) share a trophic guild. The families Anisakidae, Pseudaliidae and Tetrameridae (Nematoda), Brachycladiidae, Notocotylidae, Heterophyidae and Brauninidae (Digenea), Diphylobothriidae, Phyllobothriidae and Tetrabothriidae (Cestoda), and Polymorphidae (Acanthocephala) are the most diverse. The

update of the helminth diversity in cetaceans provides a baseline for future studies, stressing the biological importance of these organisms.

A new species of a brachycladiid is described from the bile ducts of a Gervais' beaked whale *Mesoplodon europaeus* (Ziphiidae) stranded on the North Atlantic coast of Florida. *Brachycladium parvulum* Fraija-Fernández, Aznar, Raga, Gibson et Fernández, 2014 differs from other species of the genus *Brachycladium* by the relative size of the oral and ventral suckers, the shape and size of the eggs and an extremely small body size. Four discriminating variables i.e., worm body area, oral sucker area, pharynx area and testes area were used in a canonical discriminant analysis between specimens of *B. parvulum* n. sp. (n = 17) and dwarf individuals of *B. atlanticum* (n = 16), which was considered the morphologically closest species. There were significant differences between the two samples, and a jack-knife classification showed that 96.2% of the specimens were correctly classified to their group.

Phylogenetic relationships of the main groups of digeneans infecting cetaceans were also investigated. We obtained sequences of the small subunit rDNA (SSU), the large subunit rDNA (LSU) and the internal transcribed spacer 2 (ITS2) rDNA of *Braunina cordiformis* (Brauninidae), *Ogmogaster antarcticus* (Notocotylidae), *Pholeter gastrophilus* (Heterophyidae), and *Campula oblonga*, *Nasitrema* sp. and *Oschmarinella rochebruni* (Brachycladiidae). The phylogenetic position of taxa was estimated by Bayesian inference and maximum likelihood (ML) incorporating published sequences of 177 species of Digenea. In the case of *P. gastrophilus*, further Bayesian and ML analyses were performed with additional sequences of 14 species of the Heterophyidae and Opisthorchiidae. Species nominally assigned to the Brachycladiidae formed a clade that was embed among species of the Acanthocolpidae, thus making the relationship between these families 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. However, our results suggest a paraphyletic relationship between the Heterophyidae and the Opisthorchiidae. The phylogenetic positions of these taxa indicate that the origin of digeneans in cetaceans occurred independently through host switching events from 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). Only two clades, i.e., the Brachycladiidae and species of *Ogmogaster* (Notocotylidae) subsequently diversified exclusively among marine mammals. Specific cophylogenetic studies for each family are needed to understand the complete biogeographic and historical scenarios that account for the association between host and parasites.

The association of the species from the largest family infecting cetaceans, the Brachycladiidae, and their hosts was studied under a cophylogenetic framework. Distance-based and event-based methods were used to explicitly test, for the first time, the coevolutionary events that have led to the current brachycladiid-marine mammal associations. The parasite phylogeny was constructed using sequences of the mtDNA NADH dehydrogenase, subunit 3 (ND3) of nine brachycladiid

species, and host phylogeny using cytochrome *b* sequences of 104 mammalian species. A total of 50 host-parasite links were identified. Distance-based methods supported the hypothesis of a global non-random association of host and parasite phylogenies. Significant individual links were identified in 24 out of 50 associations, which were related to *Campula oblonga*, *Nasitrema delphini*, *N. globicephalae* and *Brachycladium atlanticum* and their associated taxa from the Delphinoidea. Regarding event-based methods, 54 schemes were explored using different combinations of costs for each potential coevolutionary event, i.e. duplication, loss, failure-to-diverge and host-switching. Three equally likely coevolutionary scenarios were identified across all schemes, and in all cases the number of loss events (87 – 156) was the most numerous, followed by failure-to-diverge (40), duplication, (3 – 6), host-switching (0 – 3) and cospeciation (0 – 2) events. Failure-to-diverge and colonization with or without subsequent diversification were thus identified as decisive events for the establishment of the associations between brachycladiids and marine mammals.

Sequences from ribosomal (ITS2) and mitochondrial (COI and ND1) DNA were used to explore the phylogeography and historical demography of *Pholeter gastrophilus*. We used 68 specimens of *P. gastrophilus* collected from seven cetacean species in nine sampling localities from four regions, i.e., Mediterranean Sea, Central Eastern Atlantic, North Eastern Atlantic, and South Western Atlantic. Less than 1% of genetic variation in ribosomal and mitochondrial markers, and no reciprocal monophyly associated to geographic regions, suggest that *P. gastrophilus* is a single lineage in the Atlantic basin. The genetic cohesion of the species throughout the Atlantic Ocean and the Mediterranean Sea would be accounted for the ubiquity of *P. gastrophilus* in the food web, by infecting several species of invertebrates and vertebrates, which are used as intermediate hosts, and the extensive exploitation of long-ranging cetaceans as definitive hosts. Nevertheless, a significant pattern of isolation by distance associated to ecological barriers, and unique haplotypes for samples in the two most distant populations, i.e., South Atlantic Ocean and North Atlantic Ocean were found, indicating that the genetic structure of the population is accounted for samples from these areas. In fact, the results from AMOVA detected a significant population structure for *P. gastrophilus* ($\Phi_{ST} = 0.40, p < 0.001$), and a significant structure among regions ($\Phi_{CT} = 0.37, p = 0.005$). Most of the samples in the North Atlantic Ocean were collected from harbour porpoises, which is the only cetacean in this study restricted to coastal habitats. Thus, in this case the ecological isolation of hosts would be contributing to the genetic isolation of their parasites. Demographic analyses detected a recent population expansion for *P. gastrophilus*, which was estimated in 60,627 years ago. We raise the hypothesis that the population expansion in *P. gastrophilus* would be related to the origin of its association with cetaceans, which we postulate to have occurred via a host-switching event from fish-eating birds. Furthermore, the ubiquity of *P. gastrophilus* would be accounted for the extensive use of the food web through intermediate hosts and the exploitation of long-ranging cetaceans as definitive hosts.

Finally, we investigated how the combined effect of cryptic host specificity and local selective pressures could shape key life-history traits of *P. gastrophilus*. In the western Mediterranean, *P. gastrophilus* infects the oceanic striped dolphin, *Stenella coeruleoalba*, but also the coastal

bottlenose dolphin, *Tursiops truncatus*. The LSU rDNA, the ITS2 rDNA and the mtCOI indicated that worms collected from both dolphin species were conspecific. Also, no significant differences were observed in the infection parameters between both dolphin species. General Linear Mixed Models were used to analyse the influence of host species on four reproductive traits of *P. gastrophilus*: body size, maturity stage (non-gravid/gravid), egg size, and number of eggs *in utero*. AIC values were used to rank competing models, and *p* values to assess the effect of specific predictors. All worms collected were gravid and body size and egg size of individuals of *P. gastrophilus* were significantly larger in striped dolphins. The number of eggs *in utero* did not significantly differ between dolphin species but, for a given body size, worms in bottlenose dolphins harboured more eggs. A trade-off between egg size and egg number was found in worms from both dolphin species, with a higher slope in striped dolphins. Apparently, the striped dolphin is a more suitable host for *P. gastrophilus*, but reproductive investment seems to be adapted to the habitat where the life-cycle develops. Worms from striped dolphins likely face the problem of finding intermediate hosts in the oceanic realm and apparently investing into offspring size enhance the early survival of larvae and the potential to multiply asexually within the first intermediate host. The small-sized worms from bottlenose dolphins would be adapted to reproduce early because of higher adult mortality, generating smaller and numerous eggs in a coastal habitat where chances of transmission are presumably higher.

RESUMEN



DIVERSIDAD EN LOS PARÁSITOS

Entre los metazoos, el parasitismo puede considerarse como uno de los estilos de vida más exitosos, ya que probablemente cualquier organismo de vida libre alberga por lo menos una especie de parásito (Poulin y Morand, 2000). Los parásitos representan una porción significativa de la biodiversidad global. Sin embargo, suelen generar una percepción negativa por parte del público, lo que puede ocasionar un desconocimiento de su estilo de vida y una menor inversión de recursos para su investigación (Aznar et al., 2010; Gómez y Nichols, 2013). No obstante, los parásitos tienen un valor natural intrínseco sólo por el hecho de ser organismos, además de tener un valor instrumental, que se hace evidente en el uso que se les puede dar como marcadores naturales de las poblaciones de sus hospedadores (Mackenzie, 2002; Hudson et al., 2006). Por ejemplo, la capacidad de dispersión (Keeney et al., 2009), identificación de stocks (Balbuena et al., 1995), historias de vida (Kaliszewska et al., 2005) o condiciones de salud (Aznar et al., 2005) de algunas especies hospedadoras se han evaluado a partir de la información obtenida de sus parásitos. Este valor instrumental que tienen algunos parásitos refleja la íntima asociación con sus hospedadores, por lo que un sistema parásito-hospedador es un modelo ideal para estudiar aspectos de la sistemática, evolución y biogeografía de las especies (Brooks y Hoberg, 2000).

PATRONES Y PROCESOS EN LA DIVERSIDAD DE PARÁSITOS

Asociaciones parásito-hospedador

En todos los sistemas vivos el estudio de la interacción entre individuos o entre especies es fundamental para entender la dinámica ecológica de un ecosistema. Dichas interacciones pueden llegar a ejercer efectos evolutivos significativos entre las especies, por lo que no es posible concebir la historia evolutiva de cualquier taxón sin tener en cuenta la coevolución con otros (Charleston y Libeskind-Hadas, 2014). En un sistema parásito-hospedador, la asociación filogenética recíproca puede generar patrones de congruencia entre filogenias, denominados patrones de *coespeciación* (Brooks y McLennan, 1991; Johnson et al., 2003). No obstante, se han identificado cuatro procesos coevolutivos capaces de generar incongruencia entre las filogenias de parásitos y sus hospedadores: i) Cuando existe *duplicación*, los parásitos especian pero los hospedadores no; ii) la *incapacidad para especiar* implica que los parásitos no especian pero los hospedadores sí; iii) existen *pérdidas* cuando un parásito no aparece en la especie hospedadora; y iv) la *colonización* se da cuando un parásito se establece en una especie hospedadora diferente a la habitual (Paterson y Gray, 1997; Johnson et al., 2003; Banks y Paterson, 2005; Conow et al., 2010). Es de resaltar que el término *captura de hospedador* se ha usado para designar eventos de colonización exitosos, en el que los parásitos han especiado en la especie hospedadora colonizada (Ronquist, 1997). Por otra parte, el término *radiación* se refiere a la colonización sin especiación concomitante de un parásito en otros hospedadores diferentes al habitual, los cuales no necesariamente tienen que estar emparentados filogenéticamente con el hospedador “donante” (Charleston y Libeskind-Hadas, 2014) (Figura 1.1).

Existen dos objetivos principales en el estudio de los patrones cofilogenéticos. El primero es evaluar el nivel de congruencia entre las filogenias de los parásitos y de sus hospedadores, y el segundo es identificar los eventos coevolutivos que han dado origen a las actuales asociaciones entre parásitos y hospedadores. Para abordar el primer objetivo se usan los métodos basados en distancias, que usan información filogenética y distancias genéticas entre especies para establecer el nivel de congruencia entre las filogenias y la contribución de cada asociación parásito-hospedador a dicha congruencia (Legendre et al., 2002; Balbuena et al., 2013; De Vienne et al., 2013). Para abordar el segundo objetivo se utilizan los métodos basados en eventos, que asocian un determinado coste a cada evento evolutivo y los mapean sobre las filogenias teniendo en cuenta cada asociación entre los parásitos y los hospedadores. Dichos métodos permiten plantear posibles hipótesis sobre la historia de las asociaciones asumiendo el menor coste evolutivo (Desdevises, 2007; Conow et al., 2010; Charleston y Libeskind-Hadas, 2014).

La colonización ha sido considerada como uno de los principales eventos evolutivos que han dado origen a varios linajes de parásitos en el medio marino (Hoberg y Brooks, 2008). Para que se den colonizaciones exitosas se deben producir dos procesos de forma secuencial. Primero, los parásitos deben contactar con hospedadores potenciales, lo que depende de filtros temporales, espaciales o comportamentales. Segundo, tras el contacto con hospedadores potenciales, estos deben ser compatibles con los parásitos. En este caso, la compatibilidad podría verse afectada por condiciones morfológicas, inmunológicas o fisiológicas del hospedador (Kuris et al., 2007). Las posibilidades de éxito de los procesos de colonización pueden aumentar con la cercanía filogenética entre el hospedador “donante” y el “receptor”, y/o por una similitud ecológica entre los mismos, por ejemplo si los hospedadores tienen hábitos alimenticios similares (Figura 1.2). Un ejemplo de colonización exitosa es la de los cestodos tetrabotrídeos en cetáceos, pinnípedos y aves marinas. Los ancestros de los actuales tetrabotrídeos parasitaban arcosaurios marinos primitivos pero fueron capaces de colonizar aves marinas antes de la extinción de sus hospedadores originales (Hoberg y Brooks, 2008). Otro ejemplo son las especies de acantocéfalos de los géneros *Corynosoma* y *Bolbosoma* (familia Polymorphidae). Aparentemente, los ancestros de estas especies infectaban aves acuáticas y fueron capaces de colonizar y especiar en otros vertebrados, como cetáceos y pinnípedos, a través de hospedadores intermediarios comunes. En este caso, la probabilidad de colonización aumentó porque hospedadores “donantes” y “receptores” explotaban un mismo nivel trófico, y las larvas de los acantocéfalos pueden contactar otros hospedadores potenciales (García-Varela et al., 2013).

Flujo genético e identidad específica

La identidad de una especie está definida por la cantidad de flujo genético entre los individuos, y este depende de la capacidad de dispersión de estos (Morjan y Rieseberg, 2004). La estrategia vital de los parásitos restringe su capacidad de movimiento, en donde solo las fases larvarias de vida libre pueden tener cierta libertad de dispersión sin depender de sus hospedadores

(Blasco-Costa y Poulin, 2013). Estudios previos muestran cómo la estructura genética de los parásitos está directamente condicionada por la capacidad de dispersión de la especie hospedadora más móvil. Por ejemplo, se han observado diferencias en la estructura genética de dos especies de digeneos que usan peces y aves marinas como hospedadores definitivos en la costa nororiental del Océano Atlántico (Feis et al., 2015). Se detectó una estructura genética significativa en *Bucephalus minimus*, un parásito que infecta la lubina, *Dicentrarchus labrax* la cual tiene un rango de dispersión limitado; por el contrario, no se detectó ninguna estructura genética en *Gymnophallus choledochus*, que infecta varias especies de aves marinas con un mayor potencial de dispersión (Feis et al., 2015). En un caso aún más extremo, no se detectó estructura genética en el acantocéfalo *Profilicollis altmani* al comparar poblaciones de las costas Atlántica y Pacífica de Estados Unidos (Goulding y Cohen, 2014). La ausencia de estructura en esta especie podría estar relacionada con la capacidad del parásito de infectar varias especies de aves marinas, que usa como hospedadores definitivos. Algunas de estas especies harían movimientos transoceánicos y otras contribuirían con la dispersión de los huevos de *P. altmani* mediante migraciones temporales, que de forma escalada, irán conectando las líneas de costa distantes (Goulding y Cohen, 2014).

En los casos mencionados anteriormente el flujo genético entre poblaciones, permite la cohesión genética de la especie. No obstante, si se interrumpe el flujo genético pueden darse procesos de especiación. En este caso, las nuevas especies serían genéticamente diferentes, pero podrían ser morfológicamente indistinguibles, generando complejos de especies crípticas (Blasco-Costa et al., 2010; Pérez-Ponce de León y Nadler, 2010; Rosas-Valdez et al., 2011).

Especificidad en parásitos

La especificidad de un parásito está determinada por el número de especies hospedadoras en donde éste puede crecer y reproducirse, y puede variar entre especies especialistas y generalistas. Las especies especialistas infectan una o pocas especies hospedadoras; por el contrario, las generalistas infectan un mayor número de especies hospedadoras (Poulin et al., 2011). Una cuestión fundamental en el estudio de la especificidad es hasta qué punto las diferentes especies ofrecen un hábitat similar para el desarrollo de los parásitos, y si estos optimizan sus estrategias vitales de acuerdo a la especie que infectan (Lane et al., 2015). Existen ejemplos que muestran alteraciones en el crecimiento y la reproducción de algunos parásitos cuando estos infectan especies hospedadoras poco habituales (ver Perdiguero-Alonso et al., 2006; Aznar et al., 2012). Sin embargo, otros ejemplos señalan cómo algunas especies de parásitos pueden crecer y reproducirse, a pesar de que los hospedadores no sean los habituales. Este es el caso del acantocéfalo *Corynosoma australe*, que es relativamente específico de pinnípedos, de forma que los individuos encontrados en otras especies hospedadoras no acaban de desarrollarse y son generalmente inmaduros (Aznar et al., 2012). Sin embargo, recientemente, se ha visto que algunos individuos de *C. australe* maduran en el pingüino de Magallanes, *Spheniscus magellanicus*. Los

pingüinos comparten presas con los pinnípedos, a través de las que las larvas de acantocéfalos pueden migrar de un hospedador a otro, manteniendo la identidad de la especie a través del flujo genético entre individuos (Brandao, 2013).

DIVERSIDAD Y ORIGEN DE HELMINTOS EN CETÁCEOS

Diversidad de helmintos en cetáceos

La fauna helmíntica de cetáceos es de transmisión trófica y comprende 174 especies divididas en cuatro grandes grupos: nematodos (62 especies), digeneos (54 especies), cestodos (38 especies) y acantocéfalos (20 especies) (Figura 4.1). Las familias Pseudaliidae, Anisakidae y Tetrameridae tienen la mayor representación taxonómica entre los nematodos. En cuanto a los digeneos, esta se distribuye en las familias Brachycladiidae, Notocotylidae, Brauninidae y Heterophyidae. La fauna de cestodos está representada principalmente por tres familias: Tetrabothriidae, Diphylobothriidae y Phyllobothriidae. Finalmente, en la familia Polymorphidae se incluye la totalidad de las especies de acantocéfalos que infectan a cetáceos.

Diversidad de digeneos en cetáceos

Los digeneos son un grupo de parásitos que representan una porción significativa de la biosfera. Estos animales tienen ciclos de vida complejos en los que se combinan fases de reproducción sexual y asexual. El ciclo de vida general, incluye un primer hospedador intermediario, generalmente un molusco, otros invertebrados o vertebrados, que usa como segundos hospedadores antes de infectar su hospedador definitivo que casi invariablemente es un vertebrado (Cribb et al., 2001; 2003).

Existen, principalmente, cuatro taxones de digeneos en cetáceos:

1 – La familia monotípica Brauninidae, que incluye a *Braunina cordiformis* como su único representante. Esta especie tiene una morfología particular, con un apéndice caudal y un órgano de fijación (Niewiadomska, 2002a) (Figura 1.3 A). *Braunina cordiformis* se ha citado en varias especies de cetáceos odontocetos en el Atlántico Sur (Berón-Vera et al., 2007; Romero et al., 2014).

2 – Las especies del género *Ogmogaster* (familia Notocotylidae) se han descrito principalmente en el intestino de cetáceos misticetos, aunque existen algunos registros en pinnípedos (Beverley-Burton, 1972; Carvajal et al., 1983). Otras especies de la familia son parásitos de otros mamíferos y aves acuáticas (Barton y Blair, 2005) (Figura 1.3 B).

3 – El género *Pholeter* (familia Heterophyidae) contiene únicamente dos especies: *P. anterouterus*, que vive enquistada en la pared intestinal de aves piscívoras, como pelícanos

(Dronen et al., 2003), y *P. gastrophilus*, que vive igualmente enquistada en la pared estomacal de cetáceos odontocetos (Pearson, 2008) (Figura 1.3 F). Esta última especie puede considerarse como una de las más generalistas entre los digeneos de cetáceos, ya que se ha registrado, por lo menos, en 17 especies de odontocetos costeros, oceánicos e incluso de hábitats dulceacuícolas (Aznar et al., 2006).

4 – Las especies de la familia Brachycladiidae se ubican en los conductos hepatopancreáticos, senos aéreos, intestino y pulmones de mamíferos marinos. En la familia se incluyen actualmente 10 géneros, siete de los cuales tienen especies que son exclusivas de cetáceos (*Brachycladium*, *Hunterotrema*, *Oschmarinella*, *Synthesium*, *Campula*, *Nasitrema* y *Cetitrema*), uno exclusivo de pinnípedos (*Zalophotrema*), y dos con especies que infectan tanto cetáceos como pinnípedos (*Odhneriella* y *Orthosplanchnus*) (Gibson, 2005) (Figura 1.3 C-E). La afiliación taxonómica de la familia ha sido problemática, ya que hipótesis iniciales basadas en información morfológica planteaban que los braquicládidos podrían estar emparentados con digeneos terrestres de la familia Fasciolidae (Brooks et al., 1985), o bien con digeneos marinos de la familia Acanthocolpidae (Cable, 1974). Información molecular reciente apoya la última hipótesis (Fernández et al., 1998b; Bray et al., 2005) y sugiere que el ancestro de los braquicládidos, que posiblemente infectaba peces, colonizó y especió en los cetáceos a través de relaciones predador–presa (Fernández et al., 1998b).

No existe información sobre la afiliación taxonómica y origen de las demás familias de digeneos de cetáceos.

Origen y coevolución de las asociaciones entre helmintos y cetáceos

Hace unos 30–35 millones de años los ancestros de cetáceos experimentaron una radiación evolutiva asociada a un cambio de hábitat desde un ambiente terrestre a uno marino (Thewissen y Williams, 2002; Arnason et al., 2004) (Figura 1.4). Esta transición generó una barrera ecológica para sus parásitos, de modo que estos debían adaptarse al nuevo estilo de vida o extinguirse; al parecer, esta última opción fue lo más probable (Delyamure, 1955; Raga et al., 2009). De hecho, las actuales asociaciones entre acantocéfalos de la familia Polymorphidae (García-Varela et al., 2013), cestodos de la familia Diphylobothriidae (Hoberg y Adams, 2000), nematodos de la familia Anisakidae (Nadler y Hudspeth, 2000; Mattiucci y Nascetti, 2008) y digeneos de la familia Brachycladiidae (Fernández et al., 1998b; Bray et al., 2005) parece haberse establecido a través de procesos de colonización en el medio marino.

Aunque no son muy numerosos, se han llevado a cabo varios estudios sobre la historia evolutiva entre helmintos y mamíferos marinos (Mattiucci y Nascetti, 2006; Marigo et al., 2011). En un estudio sobre nematodos de la familia Anisakidae se descubrió que un evento coevolutivo ha dado origen a la asociación estricta entre el nematodo *Anisakis physeteris*, y el cachalote, *Physeter macrocephalus* (Mattiucci y Nascetti, 2006). En cuanto a los digeneos de la familia

Brachycladiidae, se ha sugerido que tras los eventos de captura de hospedador que dieron origen a la actual asociación con mamíferos marinos (Fernández et al., 1998b), se habrían dado capturas posteriores generando la expansión y diversificación de la familia entre misticetos y pinnípedos (Raga et al., 2009; Marigo et al., 2011). Sin embargo, ninguna de estas hipótesis se ha investigado utilizando una metodología específica en un contexto cofilogénico.

Flujo genético y dispersión de los helmintos de cetáceos

Dado que los helmintos tienen una capacidad de dispersión propia limitada a sus fases de vida libre, deben depender de la movilidad de sus hospedadores para promover su dispersión y flujo genético (Blasco-Costa y Poulin, 2013). Los cetáceos pueden tener una elevada capacidad de dispersión (Hoelzel, 2009) que se ve reflejada, en algunos casos, en la falta de estructura genética en algunas de sus poblaciones (ver Engelhaupt et al., 2009 para el cachalote, *Physeter macrocephalus*, o Moura et al., 2013 para el delfín común, *Delphinus delphis*). Sin embargo, algunas barreras geográficas pueden interrumpir el flujo genético entre individuos estructurando genéticamente sus poblaciones (ver Natoli et al., 2005 para el delfín mular, *Tursiops truncatus*, o Fontaine et al., 2007 para la marsopa común, *Phocoena phocoena*). Se han llevado a cabo algunos estudios sobre la estructura poblacional de helmintos en cetáceos (Mattiucci et al., 2002; Marigo et al., 2015). En el nematodo generalista *Anisakis typica* se ha detectado un elevado flujo genético y se ha postulado que la alta capacidad de dispersión de sus hospedadores definitivos (delfines) contribuirían a la dispersión y el flujo genético de estos individuos (Mattiucci et al., 2002). En algunas especies especialistas se ha observado este mismo patrón. Por ejemplo, en el digeneo *Synthesium pontoporiae* no se detectó ninguna estructura genética, a pesar de que su hospedador, la franciscana *Pontoporia blainvillei*, tiene stocks diferenciados genéticamente en su rango de distribución. La hipótesis que se plantea es que la mezcla genética de *S. pontoporiae* podría estar facilitada por la alta movilidad de sus hospedadores intermediarios (Marigo et al., 2015).

Hasta la fecha no se ha estudiado los patrones de dispersión y la estructura genética de ninguna especie de digeneo generalista en cetáceos.

Especificidad en los helmintos de cetáceos

Existen amplias diferencias en la especificidad de helmintos de cetáceos. Por ejemplo, hay especies muy específicas como el digeneo *Synthesium pontoporiae*, que se encuentra exclusivamente en la franciscana, *Pontoporia blainvillei*, en las zonas costeras de Argentina, Uruguay y Brasil en el océano Atlántico (Aznar et al., 1994). Otra especie de digeneo con una distribución geográfica limitada es *Braunina cordiformis*, que se distribuye en varias especies de odontocetos, casi todas en aguas costeras del océano Atlántico en el hemisferio sur (Berón-Vera et al., 2007; Romero et al., 2014). Por el contrario, existen especies muy generalistas, como

Synthesium tursionis o *Pholeter gastrophilus*, que se han detectado en al menos 15 especies de odontocetos con una amplia distribución geográfica en los océanos Atlántico y Pacífico, y los mares Mediterráneo, Báltico y Negro (Fernández, 1996; Aznar et al., 2006).

Sin embargo, el número de especies que un parásito infecta no es la única medida para determinar su grado de especificidad. La calidad y cantidad de recursos disponibles para que un parásito crezca y se reproduzca puede variar entre especies hospedadoras, afectando la compatibilidad entre parásito y hospedador (Kuris et al., 2007). De hecho, algunos estudios han detectado variaciones en la morfología y reproducción de algunos individuos en hospedadores poco habituales. Por ejemplo, el digeneo *Campula oblonga*, que infecta odontocetos de la familia Phocoenidae, se encontró en un tiburón zorro, *Alopias vulpinus*, siendo los ejemplares más pequeños de lo normal (Adams et al., 1998). En otro ejemplo, individuos de *Brachycladium atlanticum* encontrados en un delfín común, *Delphinus delphis*, resultaron ser más pequeños que los encontrados en el delfín listado *Stenella coeruleoalba*, lo que sugiere que esta última especie podría ser su hospedador habitual (Mateu et al., 2011).

Una cuestión que aún queda por resolver es hasta qué punto una especie de digeneo generalista, que explota de forma habitual varias especies hospedadoras puede desarrollar diferencias sutiles en sus estrategias vitales de acuerdo con las especies hospedadoras y el hábitat de las mismas.

JUSTIFICACIÓN Y OBJETIVOS

En esta tesis doctoral se exploran tres líneas de estudio en los digeneos en cetáceos: primero, se analizan los patrones de diversidad en los digeneos de cetáceos, incluyendo la descripción de una nueva especie; segundo, se investiga el origen y la historia evolutiva de las actuales asociaciones entre digeneos y cetáceos; finalmente, se examina la filogeografía y los patrones de especificidad de la especie de digeneo más generalista en cetáceos, *Pholeter gastrophilus*.

Los objetivos específicos que se plantean son los siguientes:

Objetivo 1

Actualizar el listado sistemático de especies de digeneos de cetáceos, incluyendo también otras especies de helmintos encontradas en estos hospedadores. Se incluirán únicamente nombres de especies aceptadas taxonómicamente y complejos de especies crípticas. Este objetivo se desarrolla en el Capítulo 4.

Objetivo 2

Describir taxonómicamente una nueva especie de digeneo de la familia Brachycladiidae encontrada en el zifio de Gervais, *Mesoplodon europaeus*. Este objetivo se desarrolla en el Capítulo 5.

Objetivo 3

Evaluar la afiliación taxonómica y el origen de las cuatro principales familias de digeneos en cetáceos: Brachycladiidae, Brauninidae, Notocotylidae y Heterophyidae, usando marcadores moleculares e información filogenética. Este objetivo se desarrolla en el Capítulo 6.

Objetivo 4

Investigar la relación cofilogenética entre especies de la familia más diversa de digeneos de cetáceos, Brachycladiidae, y sus hospedadores. Para ello se cuantifica el nivel de congruencia entre las filogenias de parásitos y hospedadores, y se identifica los eventos evolutivos que han dado origen a las actuales asociaciones entre braquicládidos y mamíferos marinos. Este objetivo se desarrolla en el Capítulo 7.

Objetivo 5

Investigar los patrones filogeográficos y la historia demográfica de la especie de digeneo más generalista en cetáceos, *Pholeter gastrophilus*. Este objetivo se desarrolla en el Capítulo 8.

Objetivo 6

Examinar los parámetros de reproducción de *Pholeter gastrophilus* en dos especies de delfines que viven en simpatria en el Mediterráneo occidental. Se evalúa el efecto de la especificidad y el ambiente en donde viven los hospedadores sobre las estrategias de vida del parásito. Este objetivo se desarrolla en el Capítulo 9.

MATERIALES Y MÉTODOS GENERALES

A continuación se detallan los materiales y métodos generales, así como los análisis filogenéticos que se usaron en este estudio. Los análisis estadísticos, cofilogenéticos y de diversidad genética se explicarán brevemente en el apartado correspondiente de cada capítulo en que fueron usados.

Muestreo y recolección de datos

Se usó un total de 358 especímenes, repartidos en 15 especies y cuatro familias de digeneos de cetáceos. En la Tabla 3.1 se presenta la información correspondiente a las especies de digeneos, sus hospedadores, localidades de muestreo y el nombre de la institución que proporcionó la muestra. Además, se colectaron digeneos de delfines varados en la costa de la Comunidad Valenciana (Mediterráneo occidental; 40°25'N, 0°26'E y 37°58'N, 0°41'O) de cuya gestión se encarga la “Red de varamientos de cetáceos y tortugas marinas de la Comunidad Valenciana”. Los delfines encontrados en un estado moderado de descomposición (menor a 3, según la escala propuesta por Geraci y Lounsbury, 2005) se transportaron a la Unidad de Zoología Marina del Instituto Cavanilles de Biodiversidad y Biología Evolutiva (ICBIBE) de la Universidad de Valencia para su necropsia (Figura 3.1). Posteriormente, se revisaron el estómago, hígado, páncreas e intestino de cada delfín. Los digeneos colectados se lavaron en solución salina al 0,9% y se fijaron en etanol al 70% para estudios morfológicos y al 96% para estudios moleculares.

Para la identificación de las especies se usaron claves taxonómicas y material bibliográfico específico para cada familia de digeneos. El material tipo descrito en esta tesis doctoral se depositó en la colección de parásitos del Museo de Historia Natural en Londres. El resto de material se conserva en la Unidad de Zoología Marina del ICBIBE.

Análisis morfológicos

Un total de 88 individuos se encontró en el hígado de un zifio de Gervais, *Mesoplodon europaeus*, que varó en la ensenada de Fort Pierce, Florida (costa occidental del Atlántico Norte) en 1976. Los especímenes fueron donados a la Unidad de Zoología Marina del ICBIBE y se usaron 20 individuos para la descripción de una especie nueva perteneciente al género *Brachycladium* (Capítulo 5). Por otra parte, se colectaron 140 individuos de *Pholeter gastrophilus* de cinco delfines listados, *Stenella coeruleoalba*, y 97 individuos de cinco delfines mulares, *Tursiops truncatus*, del Mediterráneo occidental. Estos parásitos se usaron para comparar las estrategias vitales de *P. gastrophilus* en las dos especies de delfines (Capítulo 9). En ambos casos, los parásitos se tiñeron con acetocarmín, se deshidrataron en un tren de alcoholes de pureza creciente y se aclararon con dimetil ftalato (DMP). Finalmente, se montaron en preparaciones permanentes

con bálsamo de Canadá. Se realizaron dibujos de cada individuo, que fueron procesados como imágenes para medir sus órganos con el programa Image Tool v.3.0.

Análisis moleculares: extracción, amplificación y secuenciación de ADN

Se siguieron tres procedimientos para la extracción de ADN genómico usando un solo espécimen en cada extracción: i) protocolo con tampón CTAB, ii) protocolo con fenol-cloroformo, y iii) kit de extracción QIAGEN DNeasy Blood and Tissue. En total, para esta tesis doctoral se obtuvieron 40 secuencias nuevas de digeneos de cetáceos (Tabla 3.3), que corresponden a tres regiones del ADN ribosómico (ADNr) (subunidades pequeña, 18S, y grande, 28S, y segundo espaciador interno transcrito, ITS2), y tres regiones del ADN mitocondrial (ADNmt) (subunidad 1 del citocromo oxidasa, COI, y las subunidades 1 y 3 del NADH deshidrogenasa, ND1 y ND3). Para ello, se usaron diferentes pares de cebadores (Tabla 3.2) y varios perfiles de termociclado (Figura 3.2). Los amplicones fueron purificados y secuenciados por el Servicio Central de Apoyo a la Investigación Experimental de la Universidad de Valencia, MacroGen Inc. Europa y Servicio de Secuenciación del Museo de Historia Natural de Londres. En todos los casos, la identidad de las secuencias se comprobó con la herramienta *online* BLAST y las nuevas secuencias obtenidas se depositaron en GenBank (ver la Tabla 3.3 para los números de acceso).

Análisis filogenéticos

En esta tesis doctoral se analizó un total de nueve conjuntos de datos nucleotídicos: i) para evaluar el origen de los digeneos en cetáceos se usaron secuencias concatenadas del ADNr 18S y 28S de 177 especies de digeneos (Capítulo 6); ii y iii) para explorar las relaciones cofilogenéticas entre braquicládidos y mamíferos marinos se usaron secuencias del ADNmt del citocromo *b* de 104 especies de mamíferos y secuencias de ADNmt ND3 de 11 especies de la familia Brachycladiidae, Acanthocolpidae y Paragonimidae (Capítulo 7). Los seis conjuntos de datos restantes hacen referencia a secuencias de *Pholeter gastrophilus*: iv) para evaluar las relaciones taxonómicas de esta especie y otras de la superfamilia Opisthorchioidea se usaron secuencias concatenadas del ADNr 18S e ITS2 de 14 especies de las familias Heterophyidae y Opisthorchiidae (Capítulo 6); v) y vi) para investigar la filogeografía y estructura genética de *P. gastrophilus* en un contexto geográfico global se usaron 68 secuencias del ADNr ITS2 y 68 secuencias concatenadas de ADNmt ND1 y ADNmt COI (Capítulo 8); y vii-ix) para evaluar la identidad genética de la especie se usaron ocho, cuatro y ocho secuencias del ADNr ITS2, 28S y ADNmt COI, respectivamente, (Capítulo 9). En todos los casos, las secuencias se alinearon manualmente o usando la versión *online* de Mafft.

Los modelos de evolución para cada alineamiento se seleccionaron usando el programa JModelTest (Darriba et al., 2012). Se utilizó máxima verosimilitud e inferencia Bayesiana para la reconstrucción filogenética.

RESUMEN DE RESULTADOS

LA DIVERSIDAD DE HELMINTOS EN CETÁCEOS

Capítulo 4: Fraija-Fernández N., Fernández M., Raga J.A., Aznar F.J. (en prensa) Advances in Marine Biology, Volume 1.

Se ha decidido empezar la sección de resultados de esta tesis con la diversidad de helmintos en cetáceos, ya que, para poder conocer cualquier proceso ecológico o evolutivo relacionado con la interacción entre parásitos y hospedadores, es necesario tener un conocimiento sobre su biodiversidad. El último catálogo publicado sobre la diversidad de helmintos en cetáceos lo realizó Raga (1994). Sin embargo, en la última década se han documentado más registros que merecen ser incluidos (p. ej., Lane et al., 2014; Tajima et al., 2015). Asimismo, se han propuesto cambios taxonómicos e identificado taxones como parte de complejos de especies crípticas (*Anisakis* spp.; Mattiucci et al., 2014).

En total, se han documentado 174 especies validas de helmintos en cetáceos, entre las que se encuentran 62 especies de nematodos, 54 especies de digeneos, 38 especies de cestodos y 20 especies de acantocéfalos (Tabla 4.1). Las familias con una mayor representación taxonómica son: Pseudaliidae (30 especies), Anisakidae (18 especies) y Tetrameridae (10 especies) (Nematoda); Brachycladiidae (43 especies), Notocotylidae (5 especies), Brauninidae (1 especie) y Heterophyidae (1 especie) (Digenea); Tetrabothriidae (20 especies), Diphyllobothriidae (13 especies) y Phyllobothriidae (4 especies) (Cestoda); y Polymorphidae (20 especies) (Acanthocephala). De las 174 especies de helmintos en cetáceos, 20 pueden considerarse infecciones accidentales. Este tipo de infecciones se dan porque los cetáceos y otros vertebrados como pinnípedos o aves marinas, comparten un mismo nivel trófico, y las larvas de los parásitos pueden intercambiarse entre hospedadores a través de presas comunes.

Actualmente, existe información de helmintos en 72 de las 90 especies validas de cetáceos (Committee on Taxonomy, 2014). La familia Balaenopteridae entre los misticetos, y la familia Delphinidae entre los odontocetos, albergan una mayor representación de especies de helmintos que el resto de las familias. Esto no es sorprendente, ya que en estas dos familias se concentra el mayor esfuerzo de muestreo hasta la fecha. Por el contrario, la familia Ziphiidae es de las que se tiene menos información parasitológica, ya que solo nueve de sus 22 especies se han estudiado a este nivel.

Los resultados de este capítulo se recogen en la Tabla 4.2, en donde la información de especies de helmintos citadas, ubicación geográfica y referencias para cada registro se organiza respecto a cada especie de cetáceo.

UNA NUEVA ESPECIE DE BRAQUICLÁDIDO (DIGENEA) EN EL ZIFIO DE GERVAIS *Mesoplodon europaeus* EN AGUAS DEL ATÁNTICO NOR-OCCIDENTAL

Capítulo 5: Fraija-Fernández N., Aznar F.J., Raga J.A., Gibson D. Fernández M. (2014) Acta Parasitologica 59, 510–517.

El zifio de Gervais, *Mesoplodon europaeus*, habita aguas profundas del océano Atlántico Norte donde se alimenta principalmente de cefalópodos y peces mesopelágicos. La mayoría de la información sobre la especie proviene de varamientos en las costas de Florida y Carolina del Norte, en la costa occidental del Atlántico Norte, aunque también existen registros puntuales en las costas del Atlántico oriental en Francia y España (ver Norman y Mead, 2001 y referencias asociadas). La información parasitológica sobre el zifio de Gervais es escasa y se limita a registros de nematodos del género *Anisakis*, el cestodo *Phyllobothrium delphini*, el acantocéfalo *Bolbosoma vasculosum* y el digeneo *Cetitrema meadi* (Martin et al., 1990; Raga, 1994; Dailey, 2007).

En 1976, un zifio de Gervais varó en la ensenada de Fort Pierce, en la costa de Florida. Tras su necropsia se recolectaron 88 digeneos que se encontraban en el hígado. Todos los parásitos eran adultos y se asignaron a la familia Brachycladiidae. Del número total de animales recolectados se usaron 20 (17 teñidos y montados en preparaciones permanentes y tres utilizados para obtener secciones transversales) para la descripción morfológica de la nueva especie. Además, se hicieron varios intentos para la extracción y amplificación del ADN de la especie; sin embargo, estos no fueron exitosos, probablemente porque los animales llevaban más de 20 años conservados en etanol al 70% y el ADN podría estar degradado.

Brachycladium parvulum n. sp. se diferencia de las otras especies de su género por el tamaño relativo de las ventosas oral y ventral, la forma y el tamaño de los huevos, así como por su tamaño corporal, siendo esta especie la de menor tamaño dentro del género (Tabla 5.1). Entre todas las especies del género *Brachycladium*, *B. atlanticum* fue la de mayor similitud morfológica respecto a *B. parvulum* n. sp. Dado que en *B. atlanticum* se han documentado patrones de variación morfológica, como enanismo (Mateu et al., 2011), y dado el pequeño tamaño de los ejemplares de *B. parvulum* n. sp., se realizó un análisis discriminante usando variables morfométricas entre los especímenes descritos como *B. parvulum* (n = 17), y los especímenes enanos de *B. atlanticum* (n = 16) colectados por Mateu et al. (2011) para determinar si se trataba o no de la misma especie. En este caso, se usaron las áreas corporales, de la ventosa oral, de la faringe y de los testículos como variables discriminantes. Se detectaron diferencias morfológicas significativas entre las dos especies (Lamda de Wilks 0,201; F = 35,349; 4 gl, $p < 0,005$) y en el 96,2% de los casos, los especímenes se asignaron de forma correcta a cada especie.

EVENTOS INDEPENDIENTES DE CAPTURA DE HOSPEDADOR EN DIGENEO DE CETÁCEOS, INFERIDOS A PARTIR DE ADN RIBOSÓMICO

Capítulo 6: Fraija-Fernández N., Olson P.D., Crespo E.A., Raga J.A., Aznar F.J. Fernández M. (2015) *International Journal for Parasitology* 45, 167–173.

Se considera que la captura de hospedador ha sido uno de los principales eventos que han dado origen a los helmintos en cetáceos (Hoberg y Adams, 2000; Mattiucci y Nascetti, 2008; García-Varela et al., 2013). Estudios previos han sugerido que los ancestros de digeneos de la familia Brachycladiidae inicialmente se encontraban en peces y expandieron su ciclo de vida colonizando cetáceos, en los que posteriormente especiaron (Fernández et al., 1998b). Sin embargo, no existe ningún otro estudio similar para las demás familias de digeneos de cetáceos.

En este estudio se obtuvieron nuevas secuencias del ADNr 18S y 28S de las siguientes especies de las cuatro familias de digeneos de cetáceos: *Braunina cordiformis* (Brauninidae), *Ogmogaster antarcticus* (Notocotylidae), *Pholeter gastrophilus* (Heterophyidae), y *Campula oblonga*, *Nasitrema* sp. y *Oschmarinella rochebruni* (Brachycladiidae). Estas secuencias se analizaron en un contexto filogenético previo (Olson et al., 2003) con otras 177 secuencias de digeneos que representaban la diversidad global del grupo. Asimismo, y dado que *P. gastrophilus* ha tenido una historia taxonómica incierta, ya que ha estado clasificado en las familias Troglotrematidae, Opisthorchiidae, Pholeteridae y Heterophyidae (Pearson y Courtney, 1977), se exploraron las relaciones filogenéticas de la especie con otras 14 de las familias Heterophyidae y Opisthorchiidae usando secuencias del ADNr 18S e ITS2.

Las relaciones filogenéticas de las cuatro familias de digeneos de cetáceos se resolvieron con valores altos de soporte en los nodos (Probabilidades posteriores = 100% y “bootstrap” de Máxima verosimilitud > 80%). *Braunina cordiformis* fue la única especie entre los digeneos de cetáceos ubicada en el orden Diplostomida, lo que se corresponde con su clasificación taxonómica en la superfamilia Diplostomoidea. Asimismo, el análisis filogenético resolvió la posición taxonómica de *B. cordiformis* con otras especies de las familias Strigeidae y Diplostomidae, que son parásitos de aves y mamíferos piscívoros (Niewiadomska, 2002b). Se detectó una relación parafilética entre las familias Brachycladiidae y Acanthocolpidae, que confirma estudios filogenéticos previos (Fernández et al., 1998b; Bray et al., 2005). *Ogmogaster antarcticus* se incluyó en un clado monofilético con otras especies de la familia Notocotylidae, las cuales se encuentran comúnmente en aves y pequeños mamíferos acuáticos (Barton y Blair, 2005). Por último, *P. gastrophilus* se ubicó con otras especies de la familia Heterophyidae, aunque esta y la familia Opisthorchiidae mostraron una relación parafilética, tal como se señaló en estudios filogenéticos previos (Thaenkhram et al., 2011; 2012). A nivel específico, *P. gastrophilus* estuvo estrechamente relacionado con parásitos de aves piscívoras como *Ascocotyle longa*, *Pygidiopsis genata* y *Centrocestus formosanus* (Sepúlveda et al., 1999; Scholz y Salgado-Maldonado, 2000; Dzikowski et al., 2004).

Las relaciones filogenéticas de los digeneos de cetáceos con parásitos de peces (Acanthocolpidae), de mamíferos y aves piscívoras (Strigeidae, Diplostomidae y Heterophyidae) y de mamíferos y aves acuáticas (Notocotyliidae) sugieren que el origen de digeneos en cetáceos ha sido el resultado de cuatro procesos evolutivos que sucedieron de forma independiente y estuvieron relacionados con capturas de hospedador. La probabilidad de que se dé este tipo de evento evolutivo aumenta por el uso de presas comunes entre hospedadores donantes y receptores (Hoberg y Klassen, 2002).

RELACIONES EVOLUTIVAS ENTRE DIGENEOS DE LA FAMILIA BRACHYCLADIIDAE ODHNER, 1905 Y SUS HOSPEDADORES MAMÍFEROS MARINOS: UN ESTUDIO COFILOGENÉTICO

Capítulo 7: Fraija-Fernández N., Aznar F.J., Fernández A., Raga J.A., Fernández M. (2016) Parasitology International 65, 209–217.

En un contexto cofilogenético, los estudios sobre las relaciones evolutivas entre parásitos y hospedadores deben determinar el nivel de congruencia entre sus filogenias. Un patrón de coespeciación estricta reflejaría una perfecta congruencia entre las filogenias de parásitos y hospedadores. Por el contrario, eventos como duplicación, incapacidad para especiar, pérdida, captura de hospedador y radiación generarán patrones de incongruencia entre las filogenias (Banks y Paterson, 2005). Las capturas de hospedador han sido consideradas como el principal evento que ha dado origen a asociaciones parásito hospedador en el ambiente marino, dado que varias especies pueden explotar recursos tróficos similares que actúan como potenciales hospedadores intermediarios para los parásitos (Hoberg y Brooks, 2008). En este trabajo se usa una metodología específica en un contexto cofilogenético para explorar los eventos coevolutivos que han dado origen a la actual asociación entre digeneos de la familia Brachycladiidae y sus hospedadores.

Para este estudio se obtuvieron nuevas secuencias de ADNmt NAD3 de *Brachycladium atlanticum*, *Campula oblonga*, *Nasitrema delphini*, *Oschmarinella rochebruni* y *Synthesium tursionis*. Adicionalmente se usaron secuencias de otras cuatro especies de braquicládidos obtenidas a partir de estudios previos (Fernández et al., 1998a; Marigo et al., 2011; Briscoe et al., 2016). Para elaborar la filogenia de los hospedadores se usaron 104 secuencias de especies de mamíferos de los ordenes Artiodactyla (1 especie), Cetacea (66 especies), Carnivora (35 especies) y Sirenia (2 especies), que fueron seleccionados a partir de estudios filogenéticos previos (May-Collado et al., 2006; Fulton and Strobeck, 2010; Meredith et al., 2011). Se identificaron 50 asociaciones entre braquicládidos y mamíferos marinos (ver Apéndice A3) y se usaron dos métodos específicos para estudios cofilogenéticos: métodos basados en distancias, a través de

los programas ParaFit (Legendre et al., 2002) y PACo (Balbuena et al., 2013), y métodos basados en eventos, a través del programa Jane 4.0 (Conow et al., 2010).

La topología del árbol filogenético de la familia Brachycladiidae fue congruente con estudios filogenéticos previos que usaron una menor cantidad de taxones (Fernández et al., 1998a; Marigo et al., 2011). Las especies *S. tursionis* y *S. pontoporiae* formaron un clado separado del resto de los braquicládidos, y se detectó una relación parafilética entre las especies *B. atlanticum* y *B. goliath*. En cuanto al análisis cofilogenético, se encontró una congruencia global entre las filogenias de los parásitos y la de los hospedadores (ParaFit Global = 33,5627, $p = 0,001$; valor global de $m^2 = 10,998$ $p = 0,011$). A esta congruencia contribuyeron de forma significativa las asociaciones entre *C. oblonga*, *N. delphini*, *N. globicephalae* y *B. atlanticum*, y sus respectivos hospedadores de las familias Delphinidae y Phocoenidae. Asimismo, se identificó una historia evolutiva compleja entre braquicládidos y mamíferos marinos que se caracterizó por tener entre 87 y 156 eventos de pérdida, 40 eventos de colonización sin especiación concomitante, entre 0 y 2 eventos de coespeciación, entre 3 y 6 duplicaciones y hasta 3 eventos de captura de hospedador.

Sugerimos que la colonización con especiación (captura de hospedador) o sin especiación (radiación) han sido procesos relevantes para el establecimiento de las actuales asociaciones entre las especies de la familia Brachycladiidae y los mamíferos marinos. A través de este estudio se propone que los ancestros de los braquicládidos colonizaron delfines y marsopas, y por lo menos, tres eventos de captura de hospedador posteriores permitieron la colonización de misticetos, pinnípedos y la franciscana, *Pontoporia blainvillei*. Los eventos de captura de hospedador pudieron producirse porque cetáceos y pinnípedos pueden explotar presas similares, incrementando así la probabilidad de traspaso de larvas entre posibles hospedadores.

VIAJEROS DE LARGA DISTANCIA: FILOGEOGRAFÍA DE UN DIGENEO GENERALISTA DE CETÁCEOS

*Capítulo 8: Fraija-Fernández N., Fernández M., Lehnert K., Raga J.A., Siebert U., Aznar F.J.
(En evaluación)*

En este estudio se evalúa por primera vez la filogeografía e historia demográfica de la especie más generalista entre los digeneos de cetáceos, *Pholeter gastrophilus*. Se colectaron 68 especímenes de siete especies de delfines del océano Atlántico y el mar Mediterráneo. Las muestras provenían de nueve regiones geográficas repartidas en cuatro regiones: i) Mediterráneo (Med), con muestras del golfo de Valencia y el Mar de Alborán; ii) Atlántico Sur (AtSu), con muestras de la costa de Argentina; iii) Atlántico Central (AtCe), con muestras de las islas Canarias, de la costa de Galicia y el golfo de Cádiz; y iv) Atlántico Norte (AtNo), con muestras del mar del Norte y

el mar Céltico. Se amplificó el ADNmt COI y ND1, y el ADNr ITS2 de cada ejemplar. La diversidad genética en *P. gastrophilus* se evaluó a partir de la diversidad y el número de haplotipos en cada región geográfica y la distribución de los mismos en una red de haplotipos. Asimismo, se calculó el número de sitios polimórficos y la diversidad nucleotídica de las secuencias estudiadas. Se exploró la posible existencia de un patrón de aislamiento por distancia a través de las correlaciones entre distancias genéticas y geográficas entre localidades, con una prueba de Mantel. De igual forma, se consideró el efecto de la estructuración geográfica de las localidades en el patrón de aislamiento por distancia, a través de una prueba de Mantel parcial. La estructura genética en *P. gastrophilus* se exploró a través de un Análisis de Varianza Molecular (AMOVA) (Excoffier et al., 1992). Finalmente, la historia demográfica de la especie se investigó mediante las pruebas de neutralidad de Tajima (Tajima, 1993), Fu (Fu, 1997) y R2 (Ramos-Onsins y Rozas, 2002), así como por un Análisis de Distribución en Desequilibrio (Rogers y Harpending, 1992).

Los resultados de este estudio sugieren que *P. gastrophilus* comprende un solo linaje en el área estudiada. En primer lugar, no hubo variación genética en la región alineada del ADNr ITS2 de todas las secuencias analizadas. En segundo lugar, la máxima divergencia genética entre las secuencias del ADNmt ND1 y COI fue de 0,6%, que corresponde a la comparación entre muestras de las dos regiones geográficas más aisladas: AtSu y AtNo. Finalmente, no se detectó monofilia recíproca asociada a secuencias de una misma región geográfica. La cohesión genética detectada en *P. gastrophilus* sugiere que los parásitos tienen una alta capacidad de dispersión en el océano Atlántico y el mar Mediterráneo. La amplia explotación de la red trófica a través de varias especies de vertebrados e invertebrados que usan como hospedadores intermediarios, y el uso de cetáceos como hospedadores definitivos con una alta capacidad de movimiento (p. ej., el delfín común, *Delphinus delphis* [Moura et al., 2013], o el calderón tropical *Globicephala macrorhynchus* [Alves et al., 2013]), podrían contribuir a la alta dispersión de *P. gastrophilus*.

A pesar de haber encontrado un solo linaje para *P. gastrophilus*, parece existir una cierta estructuración genética asociada a la distribución geográfica de las muestras. Aunque se contó con pocas muestras en el AtSu, estas se agruparon en un solo clado y representaron un único haplotipo. Asimismo, se detectó una correlación significativa entre la variación genética y la distribución geográfica de las muestras mediante un patrón de aislamiento por distancia. Desafortunadamente, dadas las pocas secuencias en el AtSu y el hecho de que todas ellas provenían de una misma localidad, no fue posible elucidar si el patrón de aislamiento observado estaba causado por una barrera geográfica o por la distancia geográfica que hay entre esta localidad y el resto. *Pholeter gastrophilus* no se ha citado en el Caribe (Mignucci-Giannoni et al., 1998), y en el hemisferio sur su registro más septentrional es en la costa sur de Brasil (Valente y Ebert, 2014). Por lo tanto, cabría preguntarse si el Ecuador podría estar ejerciendo de barrera de aislamiento entre las poblaciones, de forma que los movimientos trans ecuatoriales de algunos cetáceos (ver Oremus et al., 2009) estarían contribuyendo a la cohesión genética de la especie en los dos hemisferios.

En el AtNo, las muestras de *P. gastrophilus* formaron un solo clado y se detectaron tres haplotipos únicos para esta zona. Además, se detectó una estructura genética significativa que en parte estaría asociada a la divergencia genética de estas muestras. Los resultados de la prueba de Mantel parcial sugiere que el aparente aislamiento de *P. gastrophilus* en el ATNo podría explicarse por una barrera ecológica. Por un lado, el golfo de Vizcaya se considera que es una barrera física y oceanográfica para varias especies de vertebrados e invertebrados (Spitz et al., 2006), incluyendo especies potenciales de hospedadores intermediarios para *P. gastrophilus*. Sin embargo, 21 de las 23 muestras de *P. gastrophilus* en el AtNo fueron recolectadas de la marsopa común, *Phocoena phocoena*, que es la única especie de cetáceo de nuestro estudio con un hábitat restringido a regiones costeras. Es posible que el aislamiento ecológico y genético de la marsopa común contribuye al aislamiento genético de sus parásitos.

Los resultados de la historia demográfica de *P. gastrophilus* sugieren que habría ocurrido una expansión poblacional hace 60.627 años. El patrón demográfico que se detectó es compatible con el origen de la asociación de *P. gastrophilus* y cetáceos, a partir de un proceso de captura de hospedador, y una posterior expansión a través de la ubiquidad en la red trófica. Esto, sin embargo, implicaría que el origen de *P. gastrophilus* fue en el océano Atlántico y que su expansión a otras regiones (p. ej. océano Pacífico) sucedió posteriormente. Esta hipótesis debería contrastarse incluyendo muestras de todo el rango de distribución de la especie.

COMPROMISOS VITALES EN UN DIGENEO GENERALISTA DE CETÁCEOS: EL PAPEL DE LA ESPECIFICIDAD Y FACTORES MEDIOAMBIENTALES

Capítulo 9: Fraija-Fernández N., Fernández M., Raga J.A., Aznar F.J. (2015) Parasites & Vectors 8, 659.

El mecanismo principal que conforma la especificidad de un parásito está definido por la probabilidad de encuentro y la compatibilidad entre este y sus hospedadores (Kuris et al., 2007). Una cuestión que llama la atención en las especies generalistas, dada su amplia explotación de especies hospedadoras, es hasta qué punto las diferentes especies ofrecen la misma calidad como hábitat para un parásito. Estudios previos han mostrado un efecto de la especie hospedadora sobre el crecimiento y la reproducción de un parásito generalista (George-Nascimento y Marin, 1992; Beck et al., 2015). Además, las presiones de selección ejercidas por el ambiente en donde se desarrolla el ciclo de vida pueden modificar la manera que los parásitos optimizan sus estrategias de vida (Poulin y Hamilton, 2000; Loot et al., 2008; Koehler et al., 2011).

En este estudio se exploró el efecto combinado de las presiones de selección ejercidas por las especies hospedadoras y por el ambiente en donde se desarrolla el ciclo de vida de un

digeneo generalista, *Pholeter gastrophilus*. Se usaron muestras de *P. gastrophilus* obtenidas de delfines listado, *Stenella coeruleoalba*, y delfines mular, *Tursiops truncatus*, que tienen hábitos predominantemente oceánicos y costeros, respectivamente, en el Mediterráneo occidental. Se comprobó que los parásitos colectados de las dos especies de delfines fueran la misma especie con ayuda de marcadores moleculares. Se compararon los parámetros de infección entre las dos especies de delfines estimando la prevalencia, intensidad y abundancia de *P. gastrophilus* (Rozsa et al., 2000; Neuhäuser and Poulin, 2004). Se exploró la compatibilidad entre parásitos y hospedadores mediante un análisis morfológico en el que se usó el tamaño corporal, el tamaño de los huevos y la cantidad de huevos en el útero de cada espécimen. A través de Modelos Mixtos Lineales Generales (GLMMs) se exploró el efecto de la especie hospedadora, el delfín individual y el tamaño corporal de *P. gastrophilus* sobre cada una de las variables estudiadas. Los mejores modelos fueron seleccionados por el menor valor de AIC (Criterio de Información de Akaike), y los demás modelos se organizaron en orden ascendente de acuerdo a las diferencias en sus valores de AIC. Los análisis estadísticos se llevaron a cabo en el programa SPSS para Macintosh, v. 19.0.

La divergencia genética en el ADNr y ADNmt de los individuos de *P. gastrophilus* en el delfín listado y en el delfín mular del Mediterráneo occidental fue mínima. Además, no hubo diferencias significativas en la prevalencia, intensidad y abundancia de *P. gastrophilus* en las dos especies de delfines. Sin embargo, los individuos de *P. gastrophilus* en el delfín listado eran más grandes y tenían huevos más grandes que los individuos en el delfín mular, lo que podría sugerir que el delfín listado representa un hábitat más apropiado para el desarrollo de los parásitos. La fecundidad de los parásitos no varió entre especies de delfines, aunque para un mismo tamaño corporal *P. gastrophilus* en el delfín mular producía relativamente más huevos que en el delfín listado. Tanto en el delfín listado como en el delfín mular se detectó un compromiso entre tamaño de huevo y fecundidad. De forma global, los resultados de este estudio sugieren que *P. gastrophilus* adopta diferentes estrategias de vida de acuerdo a la especie hospedadora que parasita.

A partir de los resultados de este estudio se propone que las diferencias en el hábitat de los delfines listados y mulares podrían ejercer presiones de selección en las estrategias vitales de *P. gastrophilus*. Esta hipótesis está asociada a la probabilidad de que el primer estadio infectivo del parásito encuentre el primer hospedador intermediario. Los ambientes oceánicos, en donde el delfín listado suele habitar, están caracterizados por ser menos productivos y tener menor densidad de organismos que los ambientes neríticos (Marcogliese, 1995; Moore, 2005). La hipótesis que planteamos es que en el delfín listado, *P. gastrophilus* estaría expuesto a un ambiente adverso para su transmisión, y producir huevos grandes es quizás una ventaja para la producción de miracidios más longevos (Morand, 1996; Trouvé et al., 1998), además de maximizar su multiplicación asexual en el primer hospedador intermediario (Poulin, 1997).

CONCLUSIONES

En esta tesis doctoral se estudiaron los patrones de diversidad, origen, diversificación y especificidad de digeneos en cetáceos, obteniéndose las siguientes conclusiones:

1 – Se han documentado 174 especies válidas de helmintos en cetáceos, de las cuales 54 son especies de digeneos. La familia Brachycladiidae es la más especiosa, con 43 especies; le sigue la familia Notocotylidae, con cinco especies del género *Ogmogaster*. Las familias Heterophyidae y Brauninidae incluyen una especie en cetáceos, *Pholeter gastrophilus* y *Braunina cordiformis*, respectivamente. Los demás helmintos que se han citado en cetáceos son nematodos (62 especies), cestodos (38 especies) y acantocéfalos (20 especies). En los nematodos, las familias con mayor representación taxonómica son Pseudaliidae (30 especies), Anisakidae (19 especies) y Tetrameridae (10 especies), en los cestodos, Tetrabothriidae (20 especies), Diphylobothriidae (13 especies) y Phyllobothriidae (4 especies), y en los acantocéfalos una única familia, Polymorphidae (20 especies).

2 – De las 54 especies de digeneos registradas en cetáceos, solo tres pueden considerarse infecciones accidentales, 14 son específicas de una especie de cetáceo y tres se han encontrado sólo una vez. Entre el resto de los helmintos en cetáceos, 17 especies representan infecciones accidentales, 24 son específicas de una especie de cetáceo y 14 se han encontrado solo una vez. Las infecciones accidentales en cetáceos se dan porque estos y otros vertebrados, como pinnípedos y aves marinas pueden compartir presas comunes, intercambiando larvas de helmintos.

3 – Se describió una nueva especie de digeneo de la familia Brachycladiidae, *Brachycladium parvulum* Fraija-Fernández, Aznar, Raga, Gibson *et* Fernández (2014), hallada en un zifio de Gervais, *Mesoplodon europaeus*. La nueva especie fue asignada al género *Brachycladium* por las siguientes características: extensión de las vitelógenas entre la ventosa ventral y la parte anterior del cuerpo, ciegos intestinales formando divertículos, cirro y metatremo sin espinas, ovario lobulado, y ventosa ventral ubicada en el segundo tercio del cuerpo. *Brachycladium parvulum* n. sp. se diferencia de otras especies del género por el tamaño relativo de las ventosas oral y ventral, la forma y el tamaño de los huevos y su pequeño tamaño corporal. Un análisis discriminante mostró diferencias significativas entre *B. parvulum* n. sp. e individuos enanos de *B. atlanticum*, siendo esta última la especie con mayor similitud morfológica.

4 – Se resolvió la afiliación taxonómica de las cuatro principales familias de digeneos de cetáceos: Brachycladiidae, Brauninidae, Notocotylidae y Heterophyidae, usando ADN ribosómico. *Braunina cordiformis* formó un clado con especies de las familias Strigeidae y Diplostomidae en la superfamilia Diplostomoidea. *Ogmogaster antarcticus* apareció relacionado con otras especies de la familia Notocotylidae. *Pholeter gastrophilus* se incluyó en un clado con otras especies de la familia Heterophyidae, principalmente *Ascocotyle longa* y *Pygidiopsis genata*. No obstante, se detectó una relación parafilética entre esta familia y la familia Opisthorchiidae. Finalmente, las

especies de Brachycladiidae se ubicaron entre especies de la familia Acanthocolpidae, formando una relación parafilética.

5 – Los digeneos de cetáceos se relacionaron filogenéticamente con otros parásitos típicos de aves y mamíferos piscívoros (Strigeidae, Diplostomidae y Heterophyidae), con parásitos de peces (Acanthocolpidae), y con parásitos de aves y mamíferos acuáticos y herbívoros (Notocotylidae). Por lo tanto, se propone que el origen de los digeneos en cetáceos tuvo lugar a partir de procesos de captura de hospedador de otros vertebrados con hábitos alimenticios similares. Las especies de la familia Brachycladiidae y del género *Ogmogaster* diversificaron posteriormente entre los cetáceos y otros mamíferos marinos.

6 – Se investigaron las relaciones filogenéticas de especies de la familia Brachycladiidae a partir de una filogenia parcial con las siguientes nueve especies: *Brachycladium atlanticum*, *B. goliath*, *Campula oblonga*, *Nasitrema delphini*, *N. globicephalae*, *Synthesium pontoporiae*, *S. tursionis*, *Orthosplanchnus fraterculus* y *Oschmarinella rochebruni*, las cuales representan seis de los 10 géneros de la familia. La topología del árbol filogenético fue similar a la de otros estudios filogenéticos previos que usaron una menor cantidad de especies. Se encontró una relación parafilética entre *B. atlanticum* y *B. goliath*, lo cual debería investigarse más a fondo.

7 – Un análisis cofilogenético usando métodos basados en distancias y 50 asociaciones entre especies de la familia Brachycladiidae y mamíferos marinos, detectó una congruencia global entre las filogenias de parásitos y hospedadores. Esta congruencia depende de 24 relaciones parásito-hospedador significativas entre las especies *Campula oblonga*, *Nasitrema delphini*, *N. globicephalae* y *Brachycladium atlanticum* y sus respectivas especies hospedadoras de las familias Delphinidae y Phocoenidae. También se detectó una porción de incongruencia significativa asociada a una historia evolutiva compleja.

8 – Los métodos cofilogenéticos basados en eventos mostraron tres escenarios para explicar la historia evolutiva entre especies de la familia Brachycladiidae y sus hospedadores. En ellos, los eventos de pérdidas fueron los más numerosos (entre 87 y 156), seguidos por eventos de colonización sin especiación concomitante (40), duplicaciones (entre 3 y 6), capturas de hospedador (hasta 3) y coespeciación (hasta 2). La colonización con especiación (captura de hospedador) y sin especiación fueron los eventos con mayor relevancia en la historia evolutiva de los braquicládidos y mamíferos marinos.

9 – La información genética y filogenética obtenida en esta tesis doctoral sugiere que la especie de digeneo más generalista entre los cetáceos, *Pholeter gastrophilus*, representa un solo linaje, al menos en la cuenca Atlántica. Se propone que el flujo genético es frecuente debido, por una parte, al uso de varias especies de cetáceos, que tienen una alta capacidad de dispersión, y por otra, a la amplia explotación de la red trófica a través de hospedadores intermediarios que, posiblemente, incluyen varias especies de invertebrados y vertebrados.

10 – El análisis de genética de poblaciones en *Pholeter gastrophilus* detectó 16 haplotipos entre individuos del océano Atlántico y el mar Mediterráneo. En el Atlántico Sur y en el Atlántico Norte se encontraron uno y tres haplotipos únicos, respectivamente; mientras que en el mar Mediterráneo y Atlántico Central se encontraron cuatro haplotipos comunes. Además, se detectó una estructura genética en la población de *P. gastrophilus* que puede atribuirse, probablemente, a la divergencia genética de las muestras del Atlántico Norte. Una barrera ecológica podría contribuir al aislamiento de *P. gastrophilus* en el Atlántico Norte. Es necesario un número mayor de muestras en el Atlántico Sur para tener un análisis más completo de la estructura genética de *P. gastrophilus* en esta área.

11 – Los análisis demográficos mostraron que la población de *Pholeter gastrophilus* se desvía de los supuestos de neutralidad, y que ha sufrido una reciente expansión hace, aproximadamente, 60.000 años. La expansión poblacional podría coincidir con el origen de la asociación de *P. gastrophilus* con los cetáceos.

12 – No se hallaron diferencias significativas en los niveles de infección ni en la fecundidad de *Pholeter gastrophilus* entre el delfín listado, *Stenella coeruleoalba*, y en el delfín mular, *Tursiops truncatus*, del Mediterráneo occidental. Sin embargo, los individuos en el delfín listado eran más grandes y capaces de producir huevos más grandes, lo que sugiere una mayor compatibilidad del parásito con esta especie de delfín. No obstante, los valores de fecundidad fueron similares en las dos especies de delfines, lo que hace suponer que *P. gastrophilus* optimiza de forma diferente su estrategia vital de acuerdo a la especie hospedadora.

13 – Planteamos que la inversión reproductiva de *Pholeter gastrophilus* podría variar de acuerdo al ambiente en donde se desarrolla su ciclo de vida. En el ambiente oceánico, donde habita el delfín listado, *P. gastrophilus* puede enfrentar la dificultad de encontrar hospedadores intermediarios, por lo que invertir en el tamaño de la descendencia puede ser una ventaja para la supervivencia de las larvas y la multiplicación asexual en el primer hospedador. Por el contrario, los parásitos en el delfín mular estarían adaptados a reproducirse antes, debido a una mayor tasa de mortalidad en los adultos, y a producir más huevos en un ambiente costero, donde las probabilidades de transmisión pueden ser más altas.

14 – Esta tesis contribuye al conocimiento molecular de los digeneos en cetáceos aportando nueva información genética de *Pholeter gastrophilus*, *Braunina cordiformis* y *Ogmogaster antarcticus*, y nuevas secuencias del ADNmt ND3 de *Brachycladium atlanticum* y *Nasitrema delphini*. De forma específica, se pone a disposición para futuros estudios nuevas secuencias del ADN ribosómico de *Braunina cordiformis* (Brauninidae), *Ogmogaster antarcticus* (Notocotylidae), *Pholeter gastrophilus* (Heterophyidae), y *Campula oblonga*, *Nasitrema* sp. y *Oschmarinella rochebruni* (Brachycladiidae), así como secuencias del ADNmt ND1 de *Pholeter gastrophilus* y el ADNmt ND3 de *Campula oblonga*, *Oschmarinella rochebruni* y *Synthesium tursionis* (Brachycladiidae).

GENERAL INTRODUCTION

01



1.1. PARASITE DIVERSITY

Parasitism is one of the most successful lifestyle on earth: almost one third to over half of the species on Earth are parasites, and most free-living metazoans have an associated parasitic organism (Poulin and Morand, 2000; Poulin, 2014). However, parasites have been somehow neglected as a research subject, having received fewer funds than other organisms, and no direct benefit from conservation policies (Aznar et al., 2010; Gómez and Nichols, 2013). This negative perception of parasites stems largely, among other reasons, from the pathogenic impact they exert on their hosts. Nonetheless, parasites have a positive instrumental value, i.e., they are capable of influencing the ecosystem functioning, and can provide information on biological features of their hosts (Mackenzie, 2002; Hudson et al., 2006). In fact, parasites have been used to unveil aspects of the dispersal ability (Keeney et al., 2009), stock identity (Balbuena et al., 1995), population histories (Kaliszewska et al., 2005), and health condition (Aznar et al., 2005) of host populations. Thus, a host-parasite model provides a suitable tool for studying the linkage of ecology, systematics, evolution and biogeography between organisms (Brooks and Hoberg, 2000).

1.2. PATTERNS AND PROCESSES IN PARASITE DIVERSITY

Host-parasite associations

In every living system, there are constant interactions between organisms and between species that may be ecological determinants in an evolutionary scale: this is the reason why the evolutionary history of species should be studied under a coevolutionary frame (Hoberg and Klassen, 2002; Charleston and Libeskind-Hadas, 2014). The evolutionary fate of parasites depends on that of their hosts, thus a host-parasite system offers a suitable model for studying coevolutionary relationships. Theoretically, *coespeciation* occurs when parasites are able to perfectly track the evolutionary history of its hosts, i.e., there are congruent portions of host and parasite phylogenies (Brooks and McLennan, 1991; Johnson et al., 2003). In contrast, incongruent host and parasite phylogenies are the result of the following evolutionary processes: parasite *duplication*, i.e., the parasite speciates in the absence of host speciation; *failure-to-diverge*, i.e., the parasite does not speciate in response to host speciation; *loss or lineage sorting*, i.e., the parasite species is absent in the host populations mainly because extinction, sampling error or “missing the boat”; and finally, *colonization* i.e., a parasite species invades a new host (Paterson and Gray, 1997; Johnson et al., 2003; Banks and Paterson, 2005; Conow et al., 2010). The term *host switching* has been used as a synonym for colonization: however, host switching occurs when the parasite is able to speciate in the acquired host (Ronquist, 1997). Recently, an evolutionary event has been designated as *spreading*, and refers to the process in which a parasite species colonizes but do not speciate in other hosts; this host might not be closely related to the current host (Charleston and Libeskind-Hadas, 2014) (Figure 1.1).

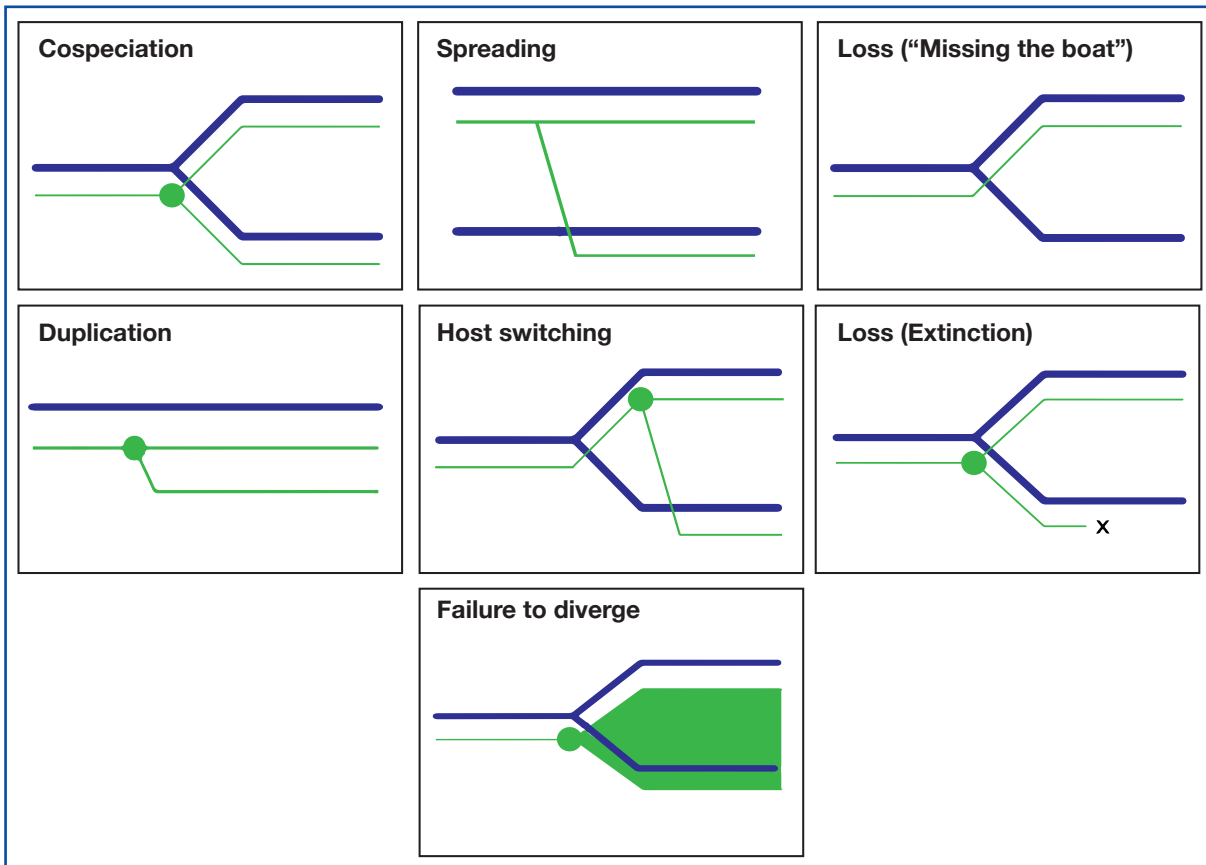


Figure 1.1. Coevolutionary processes in a host-parasite association. Blue lines represent host lineages, whereas green lines represent parasite lineages. Circles represent moments of diversification, and shaded areas represent gene flow between parasite populations. Modified from: <https://www.cs.hmc.edu/~hadas/jane/index.html>.

To test a cophylogenetic scenario, the degree of congruence between host and parasite phylogenies and the evolutionary events shaping the host-parasite association should be explored. There are available methods for studying host parasite associations in a cophylogenetic framework, which can be classified broadly into distance-based methods and event-based methods (for a review see Paterson and Banks, 2001; Stevens, 2004). Distance-based methods aim to quantify the degree of overall congruence by comparing genetic distances in the host and the parasite phylogenies, and identifies the relative contribution from each host-parasite link to the global congruence (Legendre et al., 2002; Balbuena et al., 2013; De Vienne et al., 2013). Event-based methods map the parasite phylogeny onto the host phylogeny, assigning a cost value to each evolutionary event, in order to reconstruct a putative historic scenario under a minimal cost (Desdevises, 2007; Conow et al., 2010; Charleston and Libeskind-Hadas, 2014).

In the marine realm, colonization has been considered a major process for the origin of host-parasite associations (Hoberg and Brooks, 2008). However, for a colonization to be successful, two sequential filters have to occur, i.e., first the parasite has to encounter the potential new host, and then they have to be compatible between each other. Opportunities for contact depend mostly on behavioural, ecological or geographical factors, whereas the compatibility between

host and parasite depends on morphological, immunological or physiological factors that allows or not the parasite to establish, mature and reproduce in the contacted host (Kuris et al., 2007). Parasite diversification via host switching increases with phylogenetic proximity between donor and target hosts, particularly if they share an ecological guild, e.g., trophic guild (Figure 1.2). For instance, species of tetrabothriid cestodes occur in seabirds, cetaceans and pinnipeds, and their evolutionary history is full of host switching events and opportunities for geographical expansion (Hoberg, 1989; Hoberg and Adams, 1992; 2000). Ancestors of the contemporary tetrabothriids were in the marine environment as parasites of early marine archosaurs. These parasites were able to switch to marine birds before the extinction of their ancestral hosts (other archosaurs) (Hoberg and Brooks, 2008). Similarly, acanthocephalans of the family Polymorphidae occur in fish-eating birds, waterfowl and marine mammals. The current phylogenetic hypothesis for the relationship among polymorphids suggests that aquatic birds are the ancestral definitive hosts, and the occurrence of polymorphids in marine mammals is the result of host-switching events (García-Varela et al., 2013). Host switching allows host expansion, so it is a fundamental mechanism for the study of processes generating host specificity (Hoberg and Brooks, 2008).

Gene flow and parasite dispersion

The amount of gene flow between individuals and their dispersal ability helps to define the identity of a species (Morjan and Rieseberg, 2004). Free-living stages of parasites have a limited dispersal capacity so they must depend on their hosts for dissemination (Blasco-Costa et al., 2013). Previous studies have suggested that the genetic structure of parasites is determined by the most vagile host. For instance, in the east coast of the North Atlantic Ocean, a strong population structure was detected in the digenean *Bucephalus minimus*, which infects the sea bass, *Dicentrarchus labrax*, a species with strong genetic structure due to homing behaviour (Feis et al., 2015). In contrast, no genetic structure was detected for the digenean *Gymnophallus choledochus*, which infects marine birds, which are highly vagile hosts (Feis et al., 2015). Other studies have explored the genetic structure of the acanthocephalan *Profillicollis altmani* in a large geographic range, i.e., between the Pacific and the Atlantic coast of North America. No genetic structure was detected in *P. altmani*, and it was suggested that the high mobility of its definitive hosts, i.e., marine birds, would contribute to the genetic homogeneity of the species (Goulding and Cohen, 2014). In this study, authors proposed that marine birds would contribute in a “stepping-stone” manner to the dispersal of *P. altmani* by seasonal migrations throughout the coastline (Goulding and Cohen, 2014 and references therein). In *P. altmani* a continuum of gene flow exists; however, if gene flow is interrupted, speciation may happen. Sometimes, the new species would be morphologically indistinguishable and reinterpreted as cryptic species (Poulin, 2011; Herrmann et al., 2014).



Figure 1.2. Marine birds and cetaceans usually share the same area, and often exploit the same trophic resources. Image used with previous authorization from author; credit of image: Cici Sayer from Offshore Blue Adventures (San Diego, CA, USA).

Host specificity

Host specificity is defined as the extent to which a parasite species is able to infect and reproduce in different host species. Host specificity is a matter of degree, because at one extreme, specialist parasites can exploit a single host species and, at the other extreme, generalist species can infect and reproduce in many host species (Poulin et al., 2011). Similarly as for colonization events, the encounter and compatibility paradigm modulates the extent of host specificity, i.e., parasites must reach potential hosts and escape from host's defenses in order to establish and reproduce in the contacted host (Combes, 2001; Kuris et al., 2007). A fundamental question in the study of host specificity is the extent to which different host species represent equal habitats for parasites, i.e., whether parasite's growth and/or reproduction varies according to the host species (Lane et al., 2015). Several studies have reported on a significant host species effect on fitness-related traits of parasites, such as dwarfism or impaired reproduction in presumably suboptimal hosts (e.g., Perdiguero-Alonso et al., 2006; Aznar et al., 2012). Other studies, however, have reported on the occurrence of mature parasites in unusual hosts. For instance, the acanthocephalan *Corynosoma australe* appears to be highly specific to pinnipeds, and records in non-pinniped hosts invariably include immature specimens only (see Aznar et al., 2012). However, a recent study has shown that individuals of *C. australe* are also able to reproduce in the Magellanic penguin, *Spheniscus magellanicus*. This penguin apparently shares a prey with pinnipeds and, therefore, larvae of *C. australe* are able to contact them, maintaining the identity of the species through gene flow between individuals (Brandao, 2013).

1.3. HELMINTH DIVERSITY IN CETACEANS

Cetaceans harbour a trophically-transmitted helminth fauna made up by 174 species, from which nematodes are the most speciose group (62 spp.), followed by digeneans (54 spp.), cestodes (38 spp.) and acanthocephalans (20 spp.) (Figure 4.1). Nematodes are represented by species of the families Pseudaliidae, Anisakidae and Tetrameridae. The bulk of digeneans infecting cetaceans include species of the Brauniniidae, Brachycladiidae, Notocotylidae and Heterophyidae. Three families of cestodes have species infecting cetaceans, namely, Tetrabothriidae, Diphylobothriidae, and Phyllobothriidae. Among the Acanthocephala, species of two genera of the family Polymorphidae namely, *Corynosoma* and *Bolbosoma* infect cetaceans. Records of helminths in cetaceans go back to the time when commercial whaling was allowed, and material for scientific purposes was available. More recently, helminths have become available through post-mortem examination of stranded or by-caught animals, and very recent studies have started to use faecal samples to identify helminths in wild free-ranging cetaceans (Kleinertz et al., 2014; Hermosilla et al., 2016).

The last host-parasite record list on the helminth diversity of cetaceans was elaborated by Raga (1994). However, an updated list is needed because additional information of helminths from rarely stranded cetaceans, or unexplored areas, has been collected over the last two decades (see Lane et al., 2014; Tajima et al., 2015). Similarly, some taxonomic rearrangements have been proposed, especially for digeneans (see Gibson, 2005), and the application of molecular techniques has shed light on the existence of previously unnamed species, many of which are part of cryptic species complexes (e.g., *Anisakis* spp.; see Mattiucci et al., 2005; 2014).

Digenean diversity in cetaceans

Digeneans are a highly diverse and successful group of parasitic flatworms (Cribb et al., 2001; 2003). Part of this success relies on their complex life cycle, which usually includes free-living and parasitic stages, and a combination of asexual and sexual reproduction (Cribb et al., 2003). Typically, adult worms release eggs into the external environment. Eggs hatch and a free-living miracidium emerges, which contact and penetrate a mollusc (rarely other invertebrates), the first intermediate host; alternatively, hatching occurs after the mollusc ingests the egg. Inside the mollusc the asexual phase develops, and miracidia reproduce asexually. These clones transform into tailed cercariae, which leave the mollusc and actively search for a second intermediate host, namely, an invertebrate or, more generally, a vertebrate. Within the second intermediate host, cercariae develops as a metacercarie and encyst until the second intermediate host is preyed by the definitive host, in which sexual maturation and reproduction occur (Cribb et al., 2003). Several authors have argued that the use of second intermediate hosts, coupled with the trophic transmission of larvae, are fundamental steps for the mixture of different clones, which benefits the expansion and evolution of digenean species (Rauch et al., 2005; Keeney et al., 2007; Leung et al., 2009).

Cetaceans harbour a unique digenean fauna made by four families:

1 – The monotypic Brauninidae with *Braunina cordiformis* as its only species. This parasite exhibits a singular morphology, i.e., a cordiform body with a caudal appendage and a holdfast organ (Niewiadomska, 2002a) (Figure 1.3 A). The systematic affiliation of *B. cordiformis* has been controversial, i.e., it was first assigned to the Cyathocotyliidae, which are parasites of reptiles, birds and mammals; however, a later taxonomic revision considered that the Brauninidae and the Cyathocotyliidae as independent families within the Diplostomoidea (Niewiadomska, 2002b).

2 – Species of *Ogmogaster* (Family Notocotyliidae) occur mainly in the intestine of baleen whales, although there are some reports in pinnipeds (e.g., Beverley-Burton, 1972; Carvajal et al., 1983). Other notocotyliids are commonly found in aquatic birds and mammals (Barton and Blair, 2005) (Figure 1.3 B).

3 – The genus *Pholeter* (Family Heterophyidae) contains two species. *Pholeter anterouterus* lives encysted in the intestinal wall of fish-eating birds like pelicans (Dronen et al., 2003), and *P. gastrophilus* lives encysted in the stomach wall of cetaceans, mainly odontocetes (Pearson, 2008) (Figure 1.3 F). The taxonomic affiliation of the species of this genus has been controversial; before their current assignation to the Heterophyidae they had been assigned to three families, i.e., the Troglotrematidae, the Pholeteridae and the Opisthorchidae, based on morphological traits (Blair et al., 2008).

4 – Species of the Brachycladiidae occur in the hepatopancreatic ducts, air sinuses, intestine and lungs of marine mammals (Figure 1.3 C-E). There are 10 genera currently recognised within the family, seven of which are exclusive to cetaceans (i.e., *Brachycladium*, *Hunterotrema*, *Oschmarinella*, *Synthesium*, *Campula*, *Nasitrema* and *Cetitrema*), one is exclusive to pinnipeds (i.e., *Zalophotrema*) and two infect both cetaceans and pinnipeds (i.e., *Odhneriella* and *Orthosplanchnus*). The taxonomic affiliation of this family has also been problematic. Previous phylogenetic analyses, based on morphology, suggested that brachycladiids were alternatively associated with terrestrial digeneans, i.e., the Fasciolidae (Brooks et al., 1985), and to marine digeneans infecting fish, i.e., the Acanthocolpidae (Cable, 1974). Recently, molecular phylogenetics have supported the latter hypothesis, suggesting that the origin of the Brachycladiidae likely resulted from a host-switching event from an ancestor occurring in fish that was able to switch to odontocetes through predator-prey relationships (Fernández et al., 1998b; Bray et al., 2005).

Information on the taxonomic affiliation and the origin of other families of digeneans in cetaceans is missing.

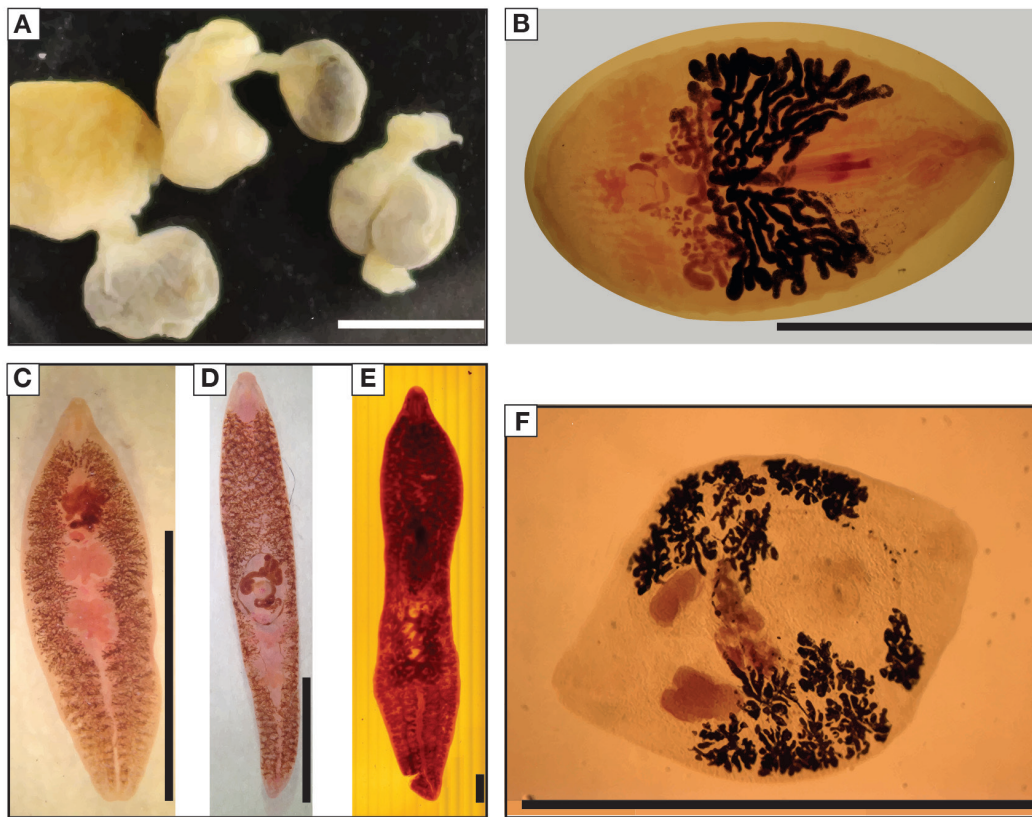


Figure 1.3. Representatives of digenean species in cetaceans. Specimens of **A)** *Brauinina cordiformis* (Brauninidae); **B)** *Ogmogaster antarcticus* (Notocotylidae); **C)** *Campula oblonga*, **D)** *Brachycladium atlanticum* and **E)** *Brachycladium goliath* (Brachycladiidae); and **F)** *Pholeter gastrophilus* (Heterophyidae). Scale bars: 5 mm.

Origin and coevolution of the association between helminths and cetaceans

Ancestors of cetaceans experienced an explosive evolutionary radiation from terrestrial to aquatic life 30 – 35 million years ago (MYA) (Thewissen and Williams, 2002; Arnason et al., 2004) (Figure 1.4). This transition from land to sea likely posed an environmental barrier to their parasites so that they had to adjust their life cycles or face extinction (Raga et al., 2009). In fact, it has been suggested that all helminths from cetaceans' ancestors went extinct, thus the current helminth fauna found in cetaceans was acquired from the aquatic environment through multiple colonization events (Delyamure, 1955; Raga et al., 2009). Opportunities for host switching arise when an ecological guild is shared (Figure 1.2). The origin of parasites of cetaceans via colonization has been substantiated for species of the Acanthocephala (see García-Varela et al., 2013), Nematoda (see Nadler and Hudspeth, 2000; Mattiucci and Nascetti, 2008), Cestoda (see Hoberg and Adams, 2000) and digeneans of the Brachycladiidae (see Fernández et al., 1998b; Bray et al., 2005). Available evidence indicates that teleost radiation (>100 MYA; Santini et al., 2009), seabird radiation (~65 MYA; Cooper and Penny, 1997), and pinniped radiation (~52 MYA; Pyenson et al., 2014) predates cetacean radiation (~35 MYA; Arnason et al., 2006) in the ocean,

so it is plausible to hypothesize that the ancestors of helminths of cetaceans were able to shift from former hosts, i.e., teleosts, seabirds and pinnipeds, to cetaceans (Aznar et al., 2001).

Studies under a cophylogenetic framework for each group of helminths and their cetacean hosts are needed to fully understand the biogeographical and historical scenarios in which host and parasites become associated. Previous studies have suggested that a coespeciation event occurred for the origin of the strict association between the nematode *Anisakis physeteris* and the

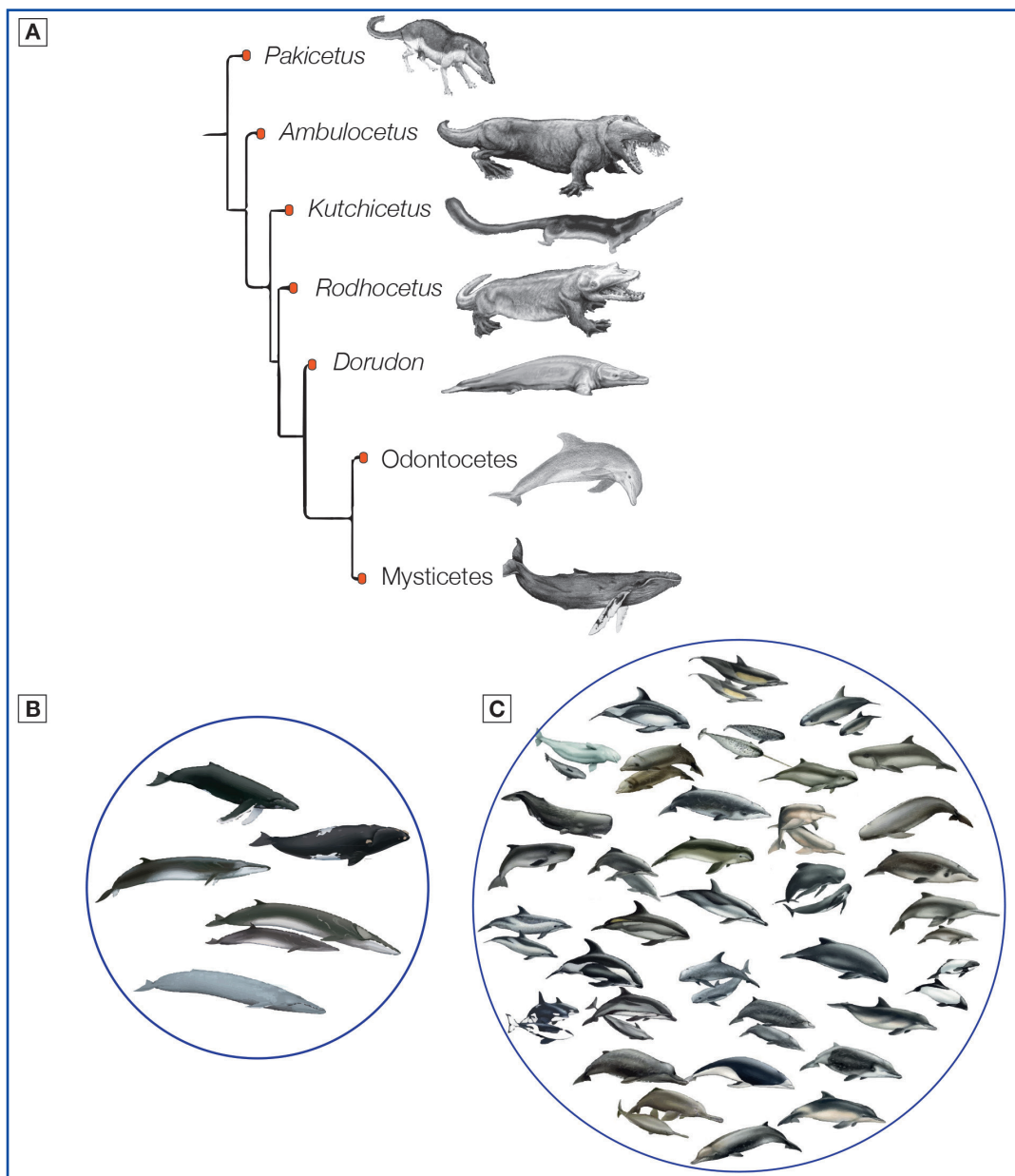


Figure 1.4. A) Cladogram showing the relationships between early whales (Archaeocetes), and the two extant groups (Odontocetes and Mysticetes). **B)** Images representing the diversity of whales; and **C)** the diversity of dolphins. Images modified from Thewissen and Williams (2002), and from the CMS (Convention on the Conservation of Migratory Species of Wild Animals) webpage.

sperm whale *Physeter macrocephalus* (Mattiucci and Nascetti, 2006). Other studies, however, have suggested that the colonization of brachycladiids occurred among toothed whales, and diversification among other marine mammals involved subsequent cospeciation and host switching events (Raga et al., 2009; Marigo et al., 2011). None of these hypotheses have been tested under a cophylogenetic framework.

Gene flow and dispersion in helminths of cetaceans

Helminths have a limited capacity for dispersion through the movement of their free-living stages. Therefore, they must depend on their hosts for their dispersion and gene flow between individuals (Blasco-Costa et al., 2013). Cetaceans are long-ranging animals that may undergo seasonal migrations between feeding and breeding grounds (Hoelzel, 2009). In some species, long-distance dispersal has resulted in little genetic structure of populations (e.g., Engelhaupt et al., 2009 for the sperm whale, *Physeter macrocephalus*; Moura et al., 2013 for the common dolphin, *Delphinus delphis*). Dispersion of other species, however, is influenced by changes in habitat and food availability, which would contribute to genetic structure within populations (e.g., Natoli et al., 2005 for the bottlenose dolphin, *Tursiops truncatus*; Fontaine et al., 2007 for the harbour porpoise, *Phocoena phocoena*). Few studies have explored the phylogeography and genetic structure of helminths in cetaceans. The nematode *Anisakis typica* infects several species of Delphinidae, Phocoenidae and Pontoporidae. A remarkable genetic homogeneity between larvae and adults of *A. typica* was detected by allozyme data despite their distant geographic origin (Mattiucci et al., 2002). Among digeneans, the only study describing the genetic structure of a species was that of *Synthesium pontoporiae* (Marigo et al., 2015). This digenean infects the Franciscana, *Pontoporia blainvillei*, which is an endemic species of the coasts of Southern Brazil, Uruguay and Argentina, and has a limited capacity for dispersal. In fact, various genetically-based stocks have been identified for the Franciscana in the distribution range of the species (Gariboldi et al., 2015). Interestingly, no genetic structure was found for *S. pontoporiae*, and it is suggested that the dispersal ability of intermediate hosts is what likely accounts for the genetic homogeneity of the species (Marigo et al., 2015).

Any attempt to explore the genetic structure of a generalist digenean in cetaceans over a wide geographical context has been made.

Host specificity in helminths of cetaceans

Helminths of cetaceans display large differences regarding host specificity. For instance, there are highly specific digeneans in cetaceans with a restricted habitat, e.g., *Synthesium pontoporiae* has been exclusively reported in the Franciscana, *Pontoporia blainvillei*, in the western South Atlantic (Aznar et al., 1994), or the digenean *Braunina cordiformis*, exhibits high specificity to odontocetes occurring mainly in the Southern Hemisphere (e.g., Berón-Vera et al.,

2007; Romero et al., 2014). In contrast, other species are generalists, being widely distributed among hosts; e.g., *Pholeter gastrophilus* or *Synthesium tursionis* have been reported in a large number of odontocete species (Fernández et al., 1998a; Aznar et al., 2006). There are records of these parasitic species in the Atlantic and Pacific Oceans, and the Mediterranean, Black and Baltic Seas (Fernández, 1996; Aznar et al., 2006).

However, the number of hosts a parasite can exploit is not the only measure of host specificity, because hosts may differ in their quality as habitat for the parasite, i.e., hosts are not equally compatible (Kuris et al., 2007). Some studies have reported impaired reproduction and dwarfism in individual parasites infecting unusual hosts. Among brachycladiid species, stunted individuals of *Campula oblonga*, which commonly occurs in phocoenids, have been found in the thresher shark, *Alopias vulpinus* (Adams et al., 1998). Also, individuals of *Brachycladium atlanticum* from a common dolphin, *Delphinus delphis*, were smaller and less fecund than those infecting striped dolphins, *Stenella coeruleoalba*. This suggests that the common dolphin is an suboptimal host for the species (Mateu et al., 2011).

A fundamental question is, therefore, to what extent different host species that are apparently equally exploited by a generalist digenean affect the development and life strategy of the parasite.

1.4. THIS STUDY

This thesis aims at analysing the diversity, origin and diversification, and patterns of host specificity in digeneans of cetaceans.

First, the systematic list of digeneans and other helminths of cetaceans is updated. In addition, a new digenean species of the family Brachycladiidae is described.

Second, to assess the taxonomic affiliation and origin of the four main families of digeneans in cetaceans, i.e., Brauninidae, Notocotylidae, Brachycladiidae and Heterophyidae, molecular data are used to place them within a phylogeny of the Digenea. Furthermore, to investigate diversification patterns, species of the most diverse family of digeneans of cetaceans, i.e., the Brachycladiidae, and their specific association with marine mammals are analysed under a cophylogenetic framework. Distance-based methods are applied to detect the phylogenetic congruence between the host and the parasite phylogenies, and event-based methods are used to identify the evolutionary events that have shaped the current host-parasite associations.

Finally, patterns of host specificity are examined for the most generalist digenean species among cetaceans, i.e., *Pholeter gastrophilus*. First, the phylogeography and demographic history of the species are explored through a population genetics approach; and second, reproductive parameters in this species are compared between two sympatric dolphin species to assess the role of host specificity in the reproductive strategy of *P. gastrophilus*.

This study has been supported by the Ministry of Economy and Competitiveness (Spain) through the projects “Origen y diversificación de los digeneos parásitos de cetáceos: aspectos coevolutivos y biogeográficos” (CGL2009-07465) and “Parásitos como indicadores de impacto ambiental a largo plazo en las poblaciones de cetáceos del Mediterráneo” (CGL2012-39545). Additional funding has been received from the local government of Valencia, Generalitat Valenciana (Spain), through the projects “Cambios a largo plazo en la abundancia poblacional, ecología trófica y fauna de metazoos simbioses, de delfines y tortugas marinas en aguas de la Comunidad Valenciana: conservación en un contexto de cambio global” (PROMETEO/2011/040) and “Biología y parasitología de vertebrados marinos del Mediterráneo occidental: aplicaciones en conservación y acuicultura” (PROMETEO II/2015/018).

AIM AND OBJECTIVES



AIM

The aim of this thesis is three-fold: first, to analyse diversity patterns of digeneans infecting cetaceans including description of new species; second, to investigate the origin and coevolutionary history of digeneans and their cetacean hosts; and third, to examine phylogeographic and host specificity patterns of the most generalist digenean specific to cetaceans.

SPECIFIC OBJECTIVES

The specific objectives of this study are the following:

Objective 1

To update the systematic list of digenean taxa, and other helminths of cetaceans, including taxonomically accepted names and cryptic species complexes. This objective is developed in Chapter 4.

Objective 2

To describe a new digenean species from the family Brachycladiidae found in a Gervais' beaked whale, *Mesoplodon europaeus*. This objective is developed in Chapter 5.

Objective 3

To assess the taxonomic affiliation and origin of the main families of digeneans found in cetaceans, i.e., the Brachycladiidae, Brauninidae, Notocotylidae and Heterophyidae, based on a phylogenetic analysis. This objective is developed in Chapter 6.

Objective 4

To investigate the cophylogenetic relationship between the most diverse digenean family that parasitize cetaceans, i.e., the Brachycladiidae, and their marine mammal hosts, by quantifying the degree of congruence between host and parasite phylogenies, and by identifying the coevolutionary events that have shaped the current host-parasite associations. This objective is developed in Chapter 7.

Objective 5

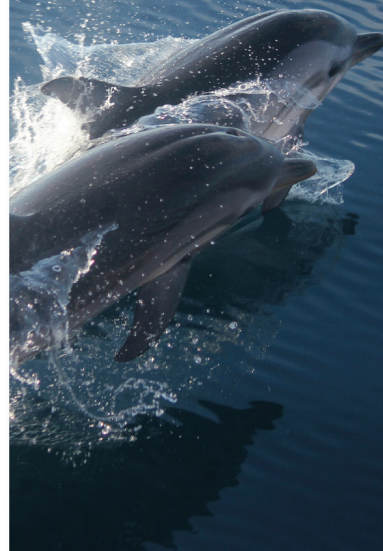
To investigate phylogeographical patterns and demographic history of the most generalist species of digeneans specific to cetaceans, namely, *Pholeter gastrophilus*. This objective is developed in Chapter 8.

Objective 6

To examine reproductive parameters of *Pholeter gastrophilus* in two sympatric cetacean species from the western Mediterranean, assessing the role of host specificity and host's habitat in shaping the life history strategy of this species. This objective is developed in Chapter 9.

GENERAL MATERIALS AND METHODS

03



In this section we provide a brief description of the different materials and methods used in this study. Detailed methodology will be explained in the corresponding chapters.

3.1. SAMPLES AND DATA COLLECTION

A total of 358 specimens from 15 species of digeneans from cetaceans were used in this study. Table 3.1 shows the list of the species of digeneans and their hosts, locality and institutions that kindly provided the specimens used. Digeneans were also collected from cetaceans that stranded along the Mediterranean coast of Spain (40°25'N, 0°26'E and 37°58'N, 0°41'W). Animals with a low to moderate level of decomposition, i.e., states 1 – 3 sensu Geraci and Lounsbury (2005) were transported to the Marine Zoology Unit of the Cavanilles Institute of Biodiversity and Evolutionary biology (ICBIBE) of the University of Valencia for necropsy (Figure 3.1). Full necropsies of cetacean carcasses were performed, and all organs were examined for parasites. Special attention was paid to organs where digeneans are commonly located, i.e., air sinuses, lungs, stomach, intestine, liver and pancreas. Air sinuses and lungs were observed in situ, whereas stomach, intestine, liver and pancreas were opened and cleaned over a 0.02 mm mesh. Parasites were isolated, cleaned in 0.9% saline solution and fixed in 70% ethanol for morphological analyses and 96% ethanol for molecular analyses.

Specific identification of parasites was carried out using available taxonomic keys for digeneans (Dailey, 1980; Niewiadomska, 2002b; Barton and Blair, 2005; Bray, 2008) and specific bibliography for the Brachycladiidae (Neiland et al., 1970; Yamaguti, 1971; Skrjabin, 1981; Abril et al., 1991; Gibson and Bray, 1997; Dailey, 2007), the Notocotyliidae (Rausch and Fay, 1966; Barton and Blair, 2005), the Heterophyidae (Pearson, 2008) and the Brauninidae (Niewiadomska, 2002a). Type-material of the species described in Chapter 5 is deposited in the Natural History Museum, London, UK. The rest of the material is stored in the Marine Zoology Unit at the ICBIBE (University of Valencia).

The updated list of helminths in cetaceans (Chapter 4) included bibliographic information collected from previous inventories, e.g., Baylis (1932), Price (1932), Delyamure (1955), Tomilin (1967), Yamaguti (1971), Dailey and Brownell (1972), Gibson and Harris (1979) and Raga (1994), and from studies published during the last decade. The taxonomic information of cetaceans follows the lists of species and subspecies of marine mammals elaborated by the Committee on Taxonomy (2014). Parasite species were verified with the WoRMS Editorial Board (2015) and the Global Biodiversity Information Facility database (www.gbif.org).

3.2. MORPHOLOGICAL ANALYSES

A total of 20 specimens of the genus *Brachycladium* that were collected from a Gervais' beaked whale, *Mesoplodon europaeus* were used to describe a new species (Chapter 5). A total of 140 specimens of *Pholeter gastrophilus* from striped dolphins, *Stenella coeruleoalba*,

Table 3.1. List of species of digenean and host species, collection place, source and number of parasite specimens (n) used in this study. The chapter in which each species is used is also included.

(Family) Parasite species	Host species (Common name)	Locality (Source)	n	Chapter
(Brachycladiidae)				
<i>Brachycladium atlanticum</i>	<i>Delphinus delphis</i> (Short beaked common dolphin)	Mediterranean Sea (Mateu et al., 2011)	16	5
	<i>Stenella coeruleoalba</i> (Striped dolphin)	Mediterranean Sea (CEGMA ² , Spain)	1	7
<i>Brachycladium goliath</i>	<i>Balaenoptera acutorostrata</i> (Minke whale)	North Atlantic Ocean (Briscoe et al., 2016*)	1	7
<i>Brachycladium parvulum</i>	<i>Mesoplodon europaeus</i> (Gervais' beaked whale)	North Atlantic Ocean (Dr. Daniel K. Odell*)	20	5
<i>Campula oblonga</i>	<i>Phocoena phocoena</i> (Harbour porpoise)	North Atlantic Ocean (ZSL ⁹ , UK)	2	6 and 7
<i>Nasitrema delphini</i>	<i>Delphinus delphis</i> (Short beaked common dolphin)	North Atlantic Ocean (ULPGC ⁸ , Spain)	1	7
<i>Nasitrema globicephalae</i>	<i>Globicephala melas</i> (Long-finned pilot whale)	South Pacific Ocean (Fernández et al., 1998a)	1	7
<i>Nasitrema</i> sp.	<i>Feresa attenuata</i> (Pygmy killer whale)	North Atlantic Ocean (ULPGC, Spain)	1	6
<i>Orthosplanchnus fraterculus</i>	<i>Enhydra lutris</i> (Sea otter)	North Pacific Ocean (Fernández et al., 1998a)	1	7
<i>Oschmarinella rochebruni</i>	<i>Stenella coeruleoalba</i> (Striped dolphin)	Mediterranean Sea (ICBIBE ⁵ , Spain)	2	6 and 7
<i>Synthesium pontoporiae</i>	<i>Pontoporia blainvillei</i> (Franciscana)	South Atlantic Ocean (Marigo et al., 2011)	1	7
<i>Synthesium tursionis</i>	<i>Tursiops truncatus</i> (Bottlenose dolphin)	Mediterranean Sea (ICBIBE, Spain)	1	7
<i>Zalophotrema hepaticum</i>	<i>Zalophus californianus</i> (California sea lion)	North Pacific Ocean (Fernández et al., 1998b)	1	6
(Brauninidae)				
<i>Braunina cordiformis</i>	<i>Delphinus delphis</i> (Short beaked common dolphin)	South Atlantic Ocean (CENPAT ⁴ , Argentina)	1	6
(Heterophyidae)				
<i>Pholeter gastrophilus</i>	<i>Delphinus delphis</i> (Short beaked common dolphin)	Mediterranean Sea (ICBIBE, Spain)	2	8
	<i>Globicephala melas</i> (Long-finned pilot whale)	Mediterranean Sea (CEGMA, Spain)	2	8
	<i>Globicephala macrorhynchus</i> (Short-finned pilot whale)	North Atlantic Ocean (CEMMA ³ , Spain)	5	8

Table 3.1. (Continued)

<i>P. gastrophilus</i> (continued)	<i>Lagenorhynchus acutus</i> (Atlantic white-sided dolphin)	North Atlantic Ocean (ITAWR ⁶ , Germany)	1	8
	<i>Phocoena phocoena</i> (Harbour porpoise)	North Atlantic Ocean (SBEES ⁷ , Ireland)	4	8
		North Atlantic Ocean (BMMB ¹ , Belgium)	6	8
		North Atlantic Ocean (ITAWR, Germany)	11	8
		<i>Stenella coeruleoalba</i> (Striped dolphin)	Mediterranean Sea (ICBIBE, Spain)	150
	<i>Tursiops truncatus</i> (Bottlenose dolphin)	North Atlantic Ocean (ULPGC, Spain)	1	8
		North Atlantic Ocean (SBEES, Ireland)	1	8
		Strait of Gibraltar (CEGMA, Spain)	11	8
		Mediterranean Sea (ICBIBE, Spain)	110	6, 8 and 9
	(Notocotyliidae)	<i>Ogmogaster antarcticus</i> <i>Balaenoptera borealis</i> (Sei whale)	South Atlantic Ocean (CENPAT, Argentina)	3
South Atlantic Ocean (CENPAT, Argentina)			1	6

* Dr. Andrew Briscoe, from the Natural History Museum (London, UK), made the unpublished sequence of *Brachycladium goliath* from GenBank available for this study; it was subsequently published at the time of writing this thesis.

* Dr. Daniel K. Odell, from the Hubbs-Sea World Research Institute, collected the material from which the description of *Brachycladium parvulum* was based, and made it available for this study.

¹ **BMMB**, Belgian Marine Mammal Biobank (Belgium).

² **CEGMA**, Centro de Gestión del Medio Marino Andaluz del Estrecho (Spain).

³ **CEMMA**, Coordinadora para o Estudio dos Mamíferos Mariños (Spain).

⁴ **CENPAT**, Laboratorio de Mamíferos Marinos del Centro Nacional Patagónico (CONICET, Argentina).

⁵ **ICBIBE**, Institut Cavanilles de Biodiversitat i Biologia Evolutiva, University of Valencia (Spain).

⁶ **ITAWR**, Institute for Terrestrial and Aquatic Wildlife Research, University of Veterinary Medicine (Germany).

⁷ **SBEES**, School of Biological, Earth and Environmental Sciences, University College Cork (Ireland).

⁸ **ULPGC**, University of Las Palmas de Gran Canaria (Spain).

⁹ **ZSL**, Zoological Society of London (United Kingdom).

and 97 from bottlenose dolphins, *Tursiops truncatus*, were used for a comparison of life-history traits (Chapter 9). All specimens were collected from their hosts, cleaned and stained with iron acetocarmine (Georgiev et al., 1986). Excess of carmine was removed with HCl in 70% ethanol. Specimens were then dehydrated through a graded ethanol series, cleared with dimethyl phthalate

and mounted as permanent preparations in Canada balsam. Drawings of individual worms were made with the aid of a drawing tube connected to a stereomicroscope. Images were processed with the program Image Tool v.3.0 (University of Texas Health Science Centre at San Antonio; downloaded from <http://compdent.uthscsa.edu/dig/download.html>).

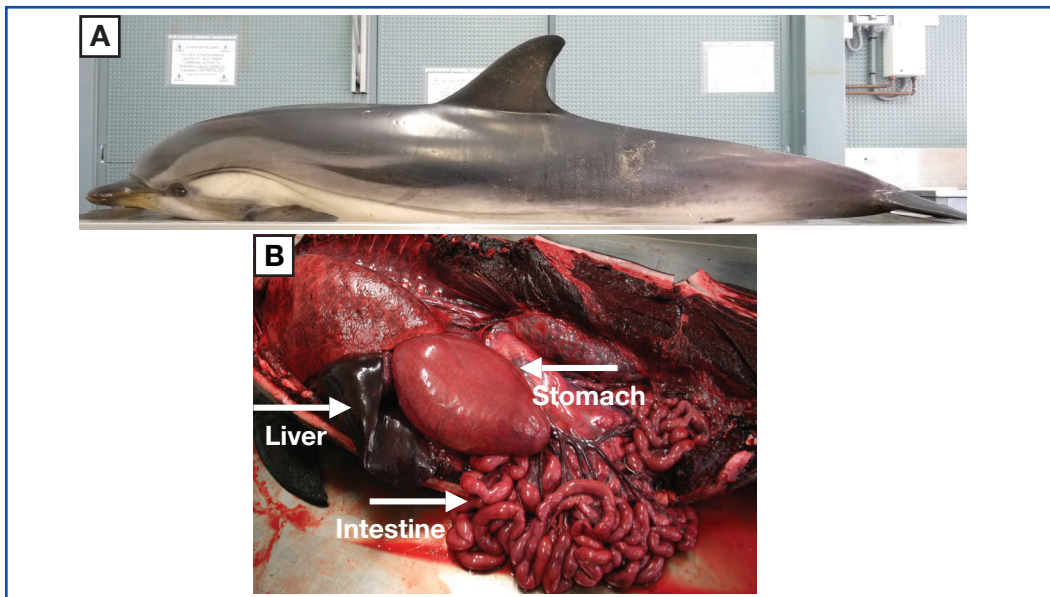


Figure 3.1. A) Carcass of a stranded striped dolphin, *Stenella coeruleoalba*, previous to necropsy at the Marine Zoology Unit (ICBIBE, University of Valencia). **B)** Dolphin's body cavity exposed during necropsy. In an anterior-posterior direction: left lung, liver, stomach, kidney and intestine.

3.3. STATISTICAL ANALYSES

Statistical analyses were used particularly in Chapters 5 and 9. First, a canonical discriminant analysis was performed to investigate morphometric differences between “dwarf” individuals of *Brachycladium atlanticum* from a common dolphin reported in Mateu et al. (2011), and the specimens here described as *B. parvulum* from a Gervais' beaked whale. Worms collected by Mateu et al. (2011) were measured following the same methodology used for *B. parvulum*. Body area, oral sucker area, pharynx area and testes area were selected as the discriminating variables. Jack-knife cross-validation was used to investigate the stability of discrimination.

Life-history traits of *Pholeter gastrophilus* were compared between specimens collected from striped and bottlenose dolphins from the Western Mediterranean (Chapter 9). Log-transformed variables for three reproductive traits, namely, body size, egg size, and number of eggs *in utero* were analysed through General Linear Mixed Models (GLMMs), with type III sum of squares, to analyse the influence of different predictors on the selected variables (Singer, 1998). We used Akaike Information Criteria (AIC) values to rank competing models with different numbers of fixed and random parameters. The model with minimum AIC was considered the “best model”, and the rest of the models were ranked according to differences in their AIC values. GLMMs were implemented with SPSS for Macintosh, v. 19.0.

3.4. MOLECULAR ANALYSES: DNA EXTRACTION, AMPLIFICATION AND SEQUENCING

Genomic DNA was extracted from single digenean individuals using three different extraction methods: i) a modified protocol using CTAB extraction buffer (Chapter 5), ii) a standard phenol-chloroform protocol (Chapters 5 and 9), and iii) the QIAGEN DNeasy Blood and Tissue Kit (GE Healthcare Life Sciences) (Chapters 5, 6, 7, 8). Partial sequences were obtained for the small subunit (SSU) rDNA, the large subunit (LSU) rDNA, the internal transcribed spacer 2 (ITS2) region and three mitochondrial (mt) regions, namely, the cytochrome *c* oxidase subunit 1 (COI), the DNA NADH dehydrogenase, subunit 1 (ND1) and the DNA NADH dehydrogenase subunit 3 (ND3), using different pair of primers (Table 3.2). Thermocycling profiles for each gene amplification are shown in Figure 3.2. Amplicons were purified and cycle sequenced by the Central Service for Experimental Research (SCSIE) of the University of Valencia, Macrogen Inc. Europe, Netherlands and the Sequencing Unit of the Natural History Museum (London). Sequence identity was checked using the Basic Local Alignment Search Tool (BLAST). All new sequences were deposited in GenBank (see Table 3.3 for accession numbers).

3.5. PHYLOGENETIC ANALYSES

A total of nine nucleotidic datasets were used for this study: i) concatenated SSU and LSU rDNA sequences from 177 digenean taxa, to infer the phylogenetic relationships between the bulk of digeneans of cetaceans and the rest of the Digenea (Chapter 6); ii and iii) mtCytochrome *b* sequences from 104 species of mammals, and mtND3 sequences from 11 species of the Brachycladiidae, the Acanthocolpidae and the Paragonimidae, to explore the cophylogenetic relationship between the Brachycladiidae and their marine mammal hosts (Chapter 7). The remaining six datasets correspond to sequences exclusive to *P. gastrophilus*, i.e., iv) concatenated SSU rDNA and ITS2 rDNA sequences from 14 species of the Heterophyidae and the Opisthorchiidae, to assess the taxonomic relationship of *P. gastrophilus* with other taxa of the Opisthorchioidea (Chapter 6); v and vi) 68 sequences of the ITS2 region, and 68 sequences of the concatenated mtND1 and mtCOI genes to explore the phylogeography of *P. gastrophilus* in a global geographical context (Chapter 8); and vii-ix) eight sequences of the ITS2 rDNA, four sequences of the LSU rDNA, and eight sequences of the mtCOI gene of *P. gastrophilus* to ascertain the genetic identity of the species (Chapter 9). In all cases sequences were manually aligned or aligned using the online version of Mafft (<http://mafft.cbrc.jp/alignment/server/>). Nucleotide alignments from Chapters 6 and 7 were deposited in the TreeBASE repository (<http://www.treebase.org>). Study IDs for each sequence alignment are detailed in their corresponding chapter.

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, 2002) and

MEGA 6 (Tamura et al., 2013). Specific parameters applied for each phylogenetic inference will be indicated in each chapter.

Table 3.2. List of primers used in this study, its target digenean gene, definition, direction and number of chapter in which they were used.

Gene fragment	Primer name	Primer definition 5' – 3' (Forward or Reverse) (Source)	Chapter
SSU rDNA	Worm A	GCG AAT GGC TCA TTA AAT CAG (F) (Littlewood and Olson, 2001)	6
	Worm B	CTT GTT ACG ACT TTT ACT TCC (R) (Littlewood and Olson, 2001)	6
	1600R	GGG CAT CAC AGA CCT G (R) (Lim et al., 1993)	6
	1270R	ACT TAA AGG AAT AGA CGG (R) (Fernández et al., 1998b)	6
	600F	GGT GCC AGC MCG GGC G (F) (Littlewood and Olson, 2001)	6
	930F	GCA TGG AAT AAT GGA ATA GG (F) (Littlewood and Olson, 2001)	6
	A27	CCA TAC AAA TGC CCC CGT CTG (R) (Littlewood and Olson, 2001)	6
	1420R	TAA CCA GAC AAA TCG CTC C (R) (Caira et al., 2014)	6
	LSU rDNA	LSU5	TAG GTC GAC CCG CTG AAY TTA AGC A (F) (Littlewood and Johnston, 1995)
1200R		GCA TAG TTC ACC ATC TTT CGG (R) (Lockyer et al., 2003)	6
1500R		GCT ATC CTG AGG GAA ACT TCG (R) (Olson et al., 2003)	6 and 9
ECD2		CTT GGT CCG TGT TTC AAG ACG GG (R) (Littlewood et al., 2000)	6
300F		CAA GTA CCG TGA GGG AAA GTT G (F) (Littlewood et al., 2000)	6 and 9
ITS2 rDNA	3S	GGT ACC GGT GGA TCA CGT GGC TAG TG (F) (Morgan and Blair, 1995)	6, 8 and 9
	ITS2.2	CCT GGT TAG TTT CTT TTC CTC CGC (R) (Anderson and Barker, 1998)	6, 8 and 9
mtDNA COI	JB3	TTT TTT GGG CAT CCT GAG GTT TAT (F) (Morgan and Blair, 1998)	8 and 9
	JB4.5	TAA AGA AAG AAC ATA ATG AAA ATG (R) (Razo-Mendivil et al., 2008)	8 and 9
mtDNA ND3	ND3F	GCT TAA TTK KTA AAG CYT TGR ATT CTT ACT (F) (Fernández et al., 2000)	7
	ND3R Primer 4	CTA CTA GTC CCA CTC AAC (G/A)TA ACC (T/C)T (R) (Fernández et al., 1998a)	7
mtDNA ND1	JB11	AGA TTC GTA AGG GGC CTA ATA (F) (Morgan and Blair, 1998)	8
	NDJ12a	CTT CAG CCT CAG CAT AAT (R) (Kostadinova et al., 2003)	8

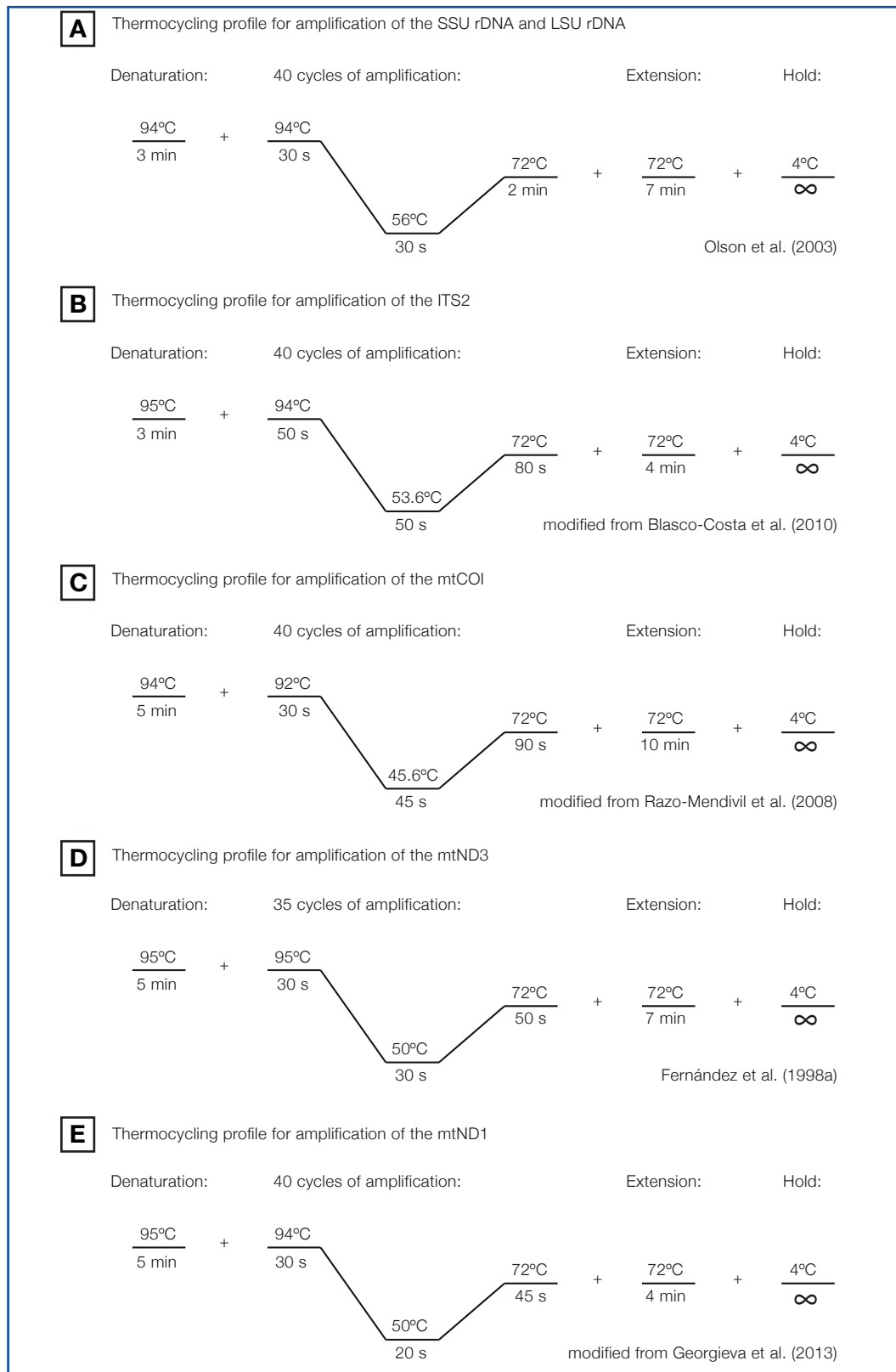


Figure 3.2. Thermocycling profiles for the amplification of the **A**) small subunit rDNA (SSU rDNA) and the large subunit rDNA (LSU rDNA); **B**) the internal transcribed spacer 2 (ITS); **C**) the mitochondrial cytochrome *c* oxidase subunit 1 (mtCOI); **D**) the mitochondrial DNA NADH dehydrogenase subunit 3 (mtND3); and **E**) the mitochondrial DNA NADH dehydrogenase subunit 1 (mtND1).

Table 3.3. List of the species of digeneans sequenced in this study, their hosts and GenBank accession numbers for the small subunit (SSU) rDNA, the large subunit (LSU) rDNA, the internal transcribed spacer 2 (ITS2) region, and the mitochondrial regions of the cytochrome c oxidase subunit 1 (mtCOI) and the mitochondrial DNA NADH dehydrogenase, subunits 3 (mtND3) and 1 (mtND1).

Targeted gene	GenBank accession number	Parasite species	Host species
SSU rDNA	KM258664	<i>Braunina cordiformis</i>	<i>Delphinus delphis</i>
	KM258665	<i>Campula oblonga</i>	<i>Phocoena phocoena</i>
	KM258666	<i>Nasitrema</i> sp.	<i>Feresa attenuata</i>
	KM258667	<i>Oschmarinella rochebruni</i>	<i>Stenella coeruleoalba</i>
	KM258668	<i>Pholeter gastrophilus</i>	<i>Tursiops truncatus</i>
	KM258669	<i>Ogmogaster antarcticus</i>	<i>Balaenoptera borealis</i>
LSU rDNA	KT883852	<i>Pholeter gastrophilus</i>	<i>Stenella coeruleoalba</i>
	KT883853	<i>Pholeter gastrophilus</i>	<i>Tursiops truncatus</i>
	KM258670	<i>Braunina cordiformis</i>	<i>Delphinus delphis</i>
	KM258671	<i>Campula oblonga</i>	<i>Phocoena phocoena</i>
	KM258672	<i>Nasitrema</i> sp.	<i>Feresa attenuata</i>
	KM258673	<i>Oschmarinella rochebruni</i>	<i>Stenella coeruleoalba</i>
	KM258674	<i>Pholeter gastrophilus</i>	<i>Tursiops truncatus</i>
ITS2 rDNA	KM258675	<i>Ogmogaster antarcticus</i>	<i>Balaenoptera borealis</i>
	KT883854	<i>Pholeter gastrophilus</i>	<i>Stenella coeruleoalba</i>
	KT883855	<i>Pholeter gastrophilus</i>	<i>Tursiops truncatus</i>
mtCOI	KM258663	<i>Pholeter gastrophilus</i>	<i>Tursiops truncatus</i>
	KT883856	<i>Pholeter gastrophilus</i>	<i>Stenella coeruleoalba</i>
mtND3	KT883857	<i>Pholeter gastrophilus</i>	<i>Tursiops truncatus</i>
	KT180214	<i>Campula oblonga</i>	<i>Phocoena phocoena</i>
mtND1	KT180215	<i>Oschmarinella rochebruni</i>	<i>Stenella coeruleoalba</i>
	KT180216	<i>Nasitrema delphini</i>	<i>Delphinus delphis</i>
	KT180217	<i>Brachycladium atlanticum</i>	<i>Stenella coeruleoalba</i>
	KT180218	<i>Synthesium tursionis</i>	<i>Tursiops truncatus</i>
	KX059397	<i>Pholeter gastrophilus</i> (Haplotype 1)	<i>Stenella coeruleoalba</i>
	KX059398	<i>Pholeter gastrophilus</i> (Haplotype 2)	<i>Stenella coeruleoalba</i>
	KX059399	<i>Pholeter gastrophilus</i> (Haplotype 3)	<i>Tursiops truncatus</i>
	KX059400	<i>Pholeter gastrophilus</i> (Haplotype 4)	<i>Tursiops truncatus</i>
	KX059401	<i>Pholeter gastrophilus</i> (Haplotype 5)	<i>Delphinus delphis</i>
	KX059402	<i>Pholeter gastrophilus</i> (Haplotype 6)	<i>Delphinus delphis</i>
	KX059403	<i>Pholeter gastrophilus</i> (Haplotype 7)	<i>Stenella coeruleoalba</i>
	KX059404	<i>Pholeter gastrophilus</i> (Haplotype 8)	<i>Stenella coeruleoalba</i>
	KX059405	<i>Pholeter gastrophilus</i> (Haplotype 9)	<i>Tursiops truncatus</i>
	KX059406	<i>Pholeter gastrophilus</i> (Haplotype 10)	<i>Stenella coeruleoalba</i>
	KX059407	<i>Pholeter gastrophilus</i> (Haplotype 11)	<i>Phocoena phocoena</i>
	KX059408	<i>Pholeter gastrophilus</i> (Haplotype 12)	<i>Phocoena phocoena</i>
KX059409	<i>Pholeter gastrophilus</i> (Haplotype 13)	<i>Stenella coeruleoalba</i>	
KX059410	<i>Pholeter gastrophilus</i> (Haplotype 14)	<i>Globicephala macrorhynchus</i>	
KX059411	<i>Pholeter gastrophilus</i> (Haplotype 15)	<i>Globicephala macrorhynchus</i>	
KX059412	<i>Pholeter gastrophilus</i> (Haplotype 16)	<i>Phocoena phocoena</i>	

3.6. COPHYLOGENETIC ANALYSES

In Chapter 7, host and parasite relationships were analysed for digeneans of the Brachycladiidae and their marine mammal hosts under a cophylogenetic framework. Each brachycladiid-marine mammal association was defined by records of brachycladiid species reported in each marine mammal species (see Appendix A3). On the one hand, the distance-based methods ParaFit (Legendre et al., 2002) and PACo (Balbuena et al., 2013) were used to test the null hypothesis of a random association between host and parasite phylogenies (Legendre et al., 2002; Balbuena et al., 2013). Both ParaFit and PACo were implemented under the public domain statistical software R (R Core Team, 2015). On the other hand, a coevolutionary hypothesis between marine mammals and brachycladiids was investigated with the event-based software Jane 4.0 (Conow et al., 2010).

3.7. GENETIC DIVERSITY ANALYSES

In Chapter 8, the phylogeography of *P. gastrophilus* was investigated using 68 sequences of the ITS2 rDNA and 68 sequences from the mtND1 and mtCOI genes. Samples were obtained from nine sampling localities, which were hierarchically organised in four regions, i.e., i) Mediterranean Sea (Med), with samples from the Alboran Sea and the Western Mediterranean, ii) South Western Atlantic (SWA), with samples from the coast of Argentina, iii) Central Eastern Atlantic (CEA), with samples from the coast of Galicia (North-West Spain), the Strait of Gibraltar and the Canary Islands, and iv) North Eastern Atlantic (NEA), with samples from the Celtic and the North Sea (see Figure 8.1). Genetic diversity for the complete dataset and for each population was estimated as the number of segregating or polymorphic sites, the number of unique haplotypes, haplotype diversity, and nucleotide diversity as implemented in DNAsp (Librado and Rozas, 2009). A haplotype network using the Minimum Spanning Tree was constructed to illustrate the connections between haplotypes using Population Analysis with Reticulate Trees (PopART), available at <http://popart.otago.ac.nz> (Leigh and Bryant, 2015). Pairwise genetic distances were calculated using MEGA 6. A pattern of isolation by distance was explored using a Mantel test and a partial Mantel test that examined the correlation between genetic distances of mitochondrial sequences and geographical distances along each sampling point, taking into account the geographic structure of samples, as implemented in Vegan under the software R (Oksanen et al., 2009; R Core Team, 2015).

The partition of genetic variation within sequences was investigated with an Analysis of Molecular Variance (AMOVA) (Excoffier et al., 1992). The genetic structure in *P. gastrophilus* was partitioned into variation from all samples considering the nine localities (“within the population” Φ_{ST} estimate), the variation among four regions, i.e., SWA, NEA, Med and CEA (“among regions” Φ_{CT} estimate) and the variation among localities within regions (“among localities, within regions” Φ_{SC} estimate). Significance associated with the fixation indices was evaluated through 2,520 and 1,260 random permutations for a four-grouped and a three-grouped structure, respectively, as implemented in Arlequin v. 3.5. (Excoffier and Lischer, 2010). Demographic events in *P.*

gastrophilus were explored by testing the null hypothesis of neutrality within the population. Accordingly, the statistical tests of Tajima's D and Fu's and their significance were estimated with Arlequin v. 3.5, based on 1,000 simulated re-sampling replicates (Excoffier and Lischer, 2010). In addition, the neutrality test R2 (Ramos-Onsins and Rozas, 2002) was calculated for 10,000 coalescent simulations using Population and Evolutionary Genetics Analysis System (pegas) (Paradis, 2010), implemented in the software R (R Core Team, 2015). A mismatch distribution was used to explore the distribution of pairwise nucleotide differences between pairs of individuals (Rogers and Harpending, 1992) as implemented in Arlequin. The smoothness of the observed distribution was quantified with the Raggedness Index with 10,000 bootstrap replicates. The approximate time (t) since population expansion was estimated as $t = \tau/2u$, where τ is the time of expansion in units of mutational time, given as an output of the mismatch distribution, and u is the mutation rate of the DNA region calculated as $m_r\mu$, where m_r is the length of the sequences under studied, and μ the mutation rate per nucleotide (Rogers and Harpending 1992). The rate was considered 2% per million years for the mtDNA, according to Galtier et al. (2009).

HELMINTH DIVERSITY OF CETACEANS: AN UPDATE

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ABSTRACT

Parasitism is an extraordinary life-strategy that largely influences that of most free-living organisms, including cetaceans. Parasites of cetaceans have a positive intrinsic and instrumental value, but also a potential pathogenic impact on host populations and, therefore, they should be considered in any biodiversity or conservation program. Yet, a starting point for understanding the relationships between cetaceans and their parasites is to have a detailed account on parasite diversity. The knowledge of the parasite fauna of cetaceans goes back to the time when commercial whaling was allowed and material for scientific purposes was available. Over the last decades, however, parasites have been obtained mainly from stranded or by-caught animals, and regular updates of host-parasite lists have been published until the 1990's. In this chapter we provide an updated review of the helminth fauna of cetaceans. The data here presented constitutes a baseline for future diversity surveys and a way to encourage researchers towards a greater awareness about the biological importance of the helminth fauna of cetaceans. Out of the 90 species currently recognized in the order Cetacea, 72 have hitherto been examined for helminths. The family Balaenopteridae among baleen whales and the family Delphinidae among toothed whales harbour the most diverse helminth fauna, which is partly accounted for by a higher sampling effort. In contrast, the helminth fauna of the beaked whales (family Ziphiidae) is the least known since only 9 out of the 22 species in the family have been examined for parasites. Currently, there are 174 helminth species reported in cetaceans, from which nematodes is the most speciose group (62 spp.), followed by digeneans (54 spp.), cestodes (38 spp.) and acanthocephalans (20 spp.). However, 20 (11.5%) of these species represent accidental infections that occur because cetaceans and other marine vertebrates (i.e., pinnipeds and marine birds) share common prey. The families Anisakidae, Pseudaliidae and Tetrameridae (Nematoda), Brachycladiidae, Notocotylidae, Heterophyidae and Brauninidae (Digenea), Diphylobothriidae, Phyllobothriidae and Tetrabothriidae (Cestoda), and Polymorphidae (Acanthocephala) have a major species-representation in the helminth fauna of cetaceans.

PARASITISM IN CETACEANS

Parasitism is an extraordinary and widespread life-strategy because parasites are able to infect almost every living organism, including cetaceans. Previous studies have highlighted the importance of including parasites in biodiversity and conservation programs for their cetacean hosts (Aznar et al., 2010). Like any other organism, parasites have an intrinsic value and should be considered as an integral part of the Biosphere. Parasites also have an instrumental value, and have commonly been used as natural tags for their host populations. Studies concerning cetacean social behaviour (Balbuena and Raga, 1994), diet (Blazekovic et al., 2015), distribution (Aznar et al., 1995), stock identity (Marigo et al., 2015) and health status (Aznar et al., 2005) have benefited from the use of parasites as biomarkers. For instance, Blazekovic et al. (2015) found a relationship between the prevalence of three nematode species, i.e., *Anisakis simplex* sensu stricto, *A. physeteris* and *A. pegreffii* and the population structure of four toothed whales in the eastern Adriatic. On the other hand, heavy parasite loads are often detected in apparently healthy cetaceans (Rogan et al., 1997), but some species may have a clear pathogenic impact on cetacean populations. For instance, digeneans of the genus *Nasitrema*, which usually occur in the pterygoid sinuses of some dolphin species, may cause brain injuries (Arbelo et al., 2013). Details about the negative impact of parasites on cetacean populations have been explored in previous studies (see e.g., Aznar et al., 2001a; Raga et al., 2009).

In any event, our understanding of the historical and present-day interactions between cetaceans and their parasites require a detailed and updated account of its biodiversity. Helminths, i.e., acanthocephalans, cestodes, nematodes and digeneans, constitute the most significant portion of the metazoan parasitic fauna of cetaceans (Figure 4.1). Records of helminths in cetaceans go back to the early 1900's, and efforts for updating data have regularly been made until the 1990's (e.g., Baylis, 1932; Price, 1932; Delyamure, 1955; Tomlin, 1967; Yamaguti, 1971; Dailey and Brownell, 1972; Gibson and Harris, 1979; Raga, 1994). In this chapter we provide the reader with an updated review of the helminth fauna occurring in cetaceans. This goal is justified for several reasons, including, i) an increase, over the last two decades, of the sampling effort on poorly known cetaceans and unexplored areas; ii) significant taxonomic changes and rearrangements of helminth taxa; and iii) an increased use of molecular markers, which have helped to unveil a significant portion of hidden biodiversity (i.e., cryptic species). In the next section, we outline major diversity patterns of the helminth fauna of cetaceans. A host-parasite list is then presented in Tables 4.1 and 4.2.

HELMINTHS OF CETACEANS

Currently, there are 174 helminth species reported from cetaceans, which can be grouped into 4 major taxa as follows: Acanthocephala (20 spp.), Cestoda (38 spp.), Nematoda (62 spp.) and Digenea (54 spp.) (Table 4.1). However, a total of 20 species from these taxa represent accidental infections, which occur because cetaceans and other marine vertebrates (e.g., marine birds or pinnipeds) share prey and parasite larvae are exchanged among hosts through the trophic webs.

Accidental infections include 7 species of acanthocephalans, 4 of cestodes, 6 of nematodes and 3 of digeneans (Table 4.1). Most of these taxa are typical from pinnipeds and marine birds. Interestingly, several helminth species that reproduce in cetaceans also have congeneric taxa infecting pinnipeds and/or marine birds, i.e., *Corynosoma* spp. (Acanthocephala), *Tetrabothrius* spp., *Diplogonoporus* spp. and *Diphyllobothrium* spp. (Cestoda), and *Orthosplanchnus* spp. and *Ogmogaster* spp. (Digenea). Also, *Anisakis simplex* sensu lato, which is typical from cetaceans, has also been reported as adult in some pinnipeds (Bratley and Stenson, 1993). These observations illustrate the high potential for host-switching events between marine mammals and birds (see Fraija-Fernández et al., 2015 and references therein).

A total of 72 out of 90 species currently recognized within the order Cetacea have ever been examined for helminths. The family Balaenopteridae among baleen whales, and the family Delphinidae among toothed whales, have the most diverse helminth fauna, which is not particularly surprising because they concentrate the highest sampling effort (Table 4.2). The helminth fauna of beaked whales (family Ziphiidae), on the other hand, is very poorly known, and not a single parasitological datum exists for 9 out of the 22 species included in this family.

As noted above, the helminth fauna of cetaceans is, as a whole, highly specific. At a lower taxonomic scale, highly specific taxa have a major representation among digeneans (14 spp. reported in single cetacean species), followed by nematodes (13 spp.), cestodes (8 spp.) and acanthocephalans (3 spp.) (Table 4.1). Only 19 species (10.9% of the total) has been reported just once. Among the species of Acanthocephala, only species from two genera of the family Polymorphidae, namely *Bolbosoma* and *Corynosoma*, occur in the intestine, and occasionally in the stomach, of cetaceans (Table 4.2). Three families of cestodes have species infecting cetaceans, namely Tetrabothriidae, Diphyllobothriidae, and Phyllobothriidae, whereas nematodes are represented by taxa of three families, i.e., Pseudaliidae, Anisakidae and Tetrameridae. Finally, the bulk of digeneans reported in cetaceans belong to four families: Brachycladiidae, Notocotyliidae, Heterophyidae and Brauninidae (Table 4.2).

In the Tables 4.1 and 4.2 below, information for helminth species, locality and references for each record is organized according to each cetacean species. Cetacean taxonomy follows the lists of species and subspecies of marine mammals made by the Committee on Taxonomy (2014). Only taxonomically accepted parasite name species are included as they were verified with the WoRMS Editorial Board (2015) and the Global Biodiversity Information Facility database (www.gbif.org). Parasite taxa not identified to the species level are not included, except in the cases in which the genus of the named species has not been previously recorded in the cetacean species. References are organized in chronologically order and are identified by numbers in the reference list. The information here presented may constitute a baseline for diversity studies. We encourage researchers to carry out parasite surveys of poorly studied cetacean species and areas, paying special attention to cryptic diversity.

Table 4.1. List of the helminth species and their families found in cetaceans. Abbreviations: 1) Accidental infections: not adult specimens reported in cetaceans; the species typically infect other vertebrates (i.e., fish, birds, pinnipeds); 2) Specific to a single cetacean species; reported more than once; 3) single records

Acanthocephala		Cestoda			
Species	Family	Species	Family	Species	Family
<i>Bolbosoma balaenae</i>	Polymorphidae	<i>Diphyllobothrium fuhrmanni</i>	Diphyllobothriidae	<i>Strobilocephalus triangularis</i>	Tetrabothriidae
<i>Bolbosoma brevicolle</i>	Polymorphidae	<i>Diphyllobothrium lanceolatum</i>	Diphyllobothriidae	<i>Tentacularia</i> sp. ¹	Tentacularidae
<i>Bolbosoma capitatum</i>	Polymorphidae	<i>Diphyllobothrium latum</i> ^b	Diphyllobothriidae	<i>Tetrabothrius affinis</i>	Tetrabothriidae
<i>Bolbosoma hamiltoni</i>	Polymorphidae	<i>Diphyllobothrium orcini</i> ³	Diphyllobothriidae	<i>Tetrabothrius arsenyevi</i> ²	Tetrabothriidae
<i>Bolbosoma nipponicum</i>	Polymorphidae	<i>Diphyllobothrium phocarum</i> ^{1c}	Diphyllobothriidae	<i>Tetrabothrius curilensis</i> ²	Tetrabothriidae
<i>Bolbosoma tuberculata</i>	Polymorphidae	<i>Diphyllobothrium polyrugosum</i>	Diphyllobothriidae	<i>Tetrabothrius diplosoma</i> ³	Tetrabothriidae
<i>Bolbosoma turbinella</i>	Polymorphidae	<i>Diphyllobothrium stemmacephalum</i>	Diphyllobothriidae	<i>Tetrabothrius egregius</i> ²	Tetrabothriidae
<i>Bolbosoma vasculosum</i> ^a	Polymorphidae	<i>Diplogonoporus balaenopterae</i>	Diphyllobothriidae	<i>Tetrabothrius forsteri</i>	Tetrabothriidae
<i>Corynosoma alaskensis</i> ²	Polymorphidae	<i>Hexagonoporus calyptocephalus</i> ²	Diphyllobothriidae	<i>Tetrabothrius hobergi</i> ³	Tetrabothriidae
<i>Corynosoma australe</i> ¹	Polymorphidae	<i>Hexagonoporus giganticus</i> ²	Diphyllobothriidae	<i>Tetrabothrius ruudi</i>	Tetrabothriidae
<i>Corynosoma bullosum</i> ¹	Polymorphidae	<i>Hexagonoporus physeteris</i> ³	Diphyllobothriidae	<i>Tetrabothrius schaeferi</i> ²	Tetrabothriidae
<i>Corynosoma cameroni</i> ^a	Polymorphidae	<i>Multiductus physeteris</i> ³	Diphyllobothriidae	<i>Tetrabothrius wilsoni</i>	Tetrabothriidae
<i>Corynosoma cetaceum</i>	Polymorphidae	<i>Plicobothrium globicephalae</i>	Diphyllobothriidae	<i>Trigonocotyle globicephalae</i>	Tetrabothriidae
<i>Corynosoma curilense</i> ²	Polymorphidae	<i>Monorygma grimaldii</i> ^d	Phyllobothriidae	<i>Trigonocotyle lintoni</i>	Tetrabothriidae
<i>Corynosoma wegneri</i> ¹	Polymorphidae	<i>Phyllobothrium delphini</i> ^d	Phyllobothriidae	<i>Trigonocotyle prudhoei</i> ^e	Tetrabothriidae
<i>Corynosoma reductum</i> ¹	Polymorphidae	<i>Phyllobothrium physeteris</i> ^{3d}	Phyllobothriidae	<i>Trigonocotyle sextesticulae</i> ³	Tetrabothriidae
<i>Corynosoma semerme</i> ¹	Polymorphidae	<i>Scolex pleuronectis</i> ^d	Phyllobothriidae	<i>Trigonocotyle spasskyi</i> ²	Tetrabothriidae
<i>Corynosoma septentrionalis</i> ²	Polymorphidae	<i>Anophryocephalus</i> sp. ¹	Tetrabothriidae		
<i>Corynosoma strumosum</i> ¹	Polymorphidae	<i>Priapocephalus eschrichtii</i> ²	Tetrabothriidae		
<i>Corynosoma validum</i> ¹	Polymorphidae	<i>Priapocephalus grandis</i>	Tetrabothriidae		
		<i>Priapocephalus minor</i>	Tetrabothriidae		

Table 4.1 (Continued)

Nematoda					
Species	Family	Species	Family	Species	Family
<i>Anisakis berlandi</i>	Anisakidae	<i>Procamallanus</i> sp. ¹	Camallanidae	<i>Stenurus auditivus</i>	Pseudaliidae
<i>Anisakis brevispiculata</i>	Anisakidae	<i>Halocercus brasiliensis</i>	Pseudaliidae	<i>Stenurus australis</i> ²	Pseudaliidae
<i>Anisakis insignis</i> ²	Anisakidae	<i>Halocercus dalli</i> ²	Pseudaliidae	<i>Stenurus globicephalae</i>	Pseudaliidae
<i>Anisakis nascettii</i>	Anisakidae	<i>Halocercus delphini</i>	Pseudaliidae	<i>Stenurus minor</i>	Pseudaliidae
<i>Anisakis paggiae</i>	Anisakidae	<i>Halocercus hyperoodoni</i> ³	Pseudaliidae	<i>Stenurus nanjingensis</i> ³	Pseudaliidae
<i>Anisakis pegreffii</i>	Anisakidae	<i>Halocercus invaginatus</i>	Pseudaliidae	<i>Stenurus ovatus</i>	Pseudaliidae
<i>Anisakis physeteris</i>	Anisakidae	<i>Halocercus kirbyi</i>	Pseudaliidae	<i>Stenurus truei</i> ³	Pseudaliidae
<i>Anisakis simplex sensu lato</i> ^f	Anisakidae	<i>Halocercus kleinenbergi</i>	Pseudaliidae	<i>Stenurus yamagutii</i> ³	Pseudaliidae
<i>Anisakis simplex sensu stricto</i>	Anisakidae	<i>Halocercus lagenorhynchi</i>	Pseudaliidae	<i>Torynurus convolutus</i>	Pseudaliidae
<i>Anisakis typica</i>	Anisakidae	<i>Halocercus monoceris</i>	Pseudaliidae	<i>Torynurus dalli</i> ²	Pseudaliidae
<i>Anisakis ziphidarum</i>	Anisakidae	<i>Halocercus pingi</i>	Pseudaliidae	<i>Hysterothylacium aduncum</i> ¹	Rhaphidascarididae
<i>Contraecaecum lobulatum</i> ¹	Anisakidae	<i>Halocercus sunameri</i> ²	Pseudaliidae	<i>Crassicauda anthonyi</i>	Tetrameridae
<i>Contraecaecum multipapillatum</i> ¹	Anisakidae	<i>Halocercus taurica</i>	Pseudaliidae	<i>Crassicauda boopis</i>	Tetrameridae
<i>Goezia</i> sp. ¹	Anisakidae	<i>Pharurus alatus</i>	Pseudaliidae	<i>Crassicauda carbonelli</i> ²	Tetrameridae
<i>Phocascaris</i> sp. ¹	Anisakidae	<i>Pharurus asiaorientali</i> ²	Pseudaliidae	<i>Crassicauda costata</i> ³	Tetrameridae
<i>Pseudoterranova ceticola</i>	Anisakidae	<i>Pharurus pallasii</i> ²	Pseudaliidae	<i>Crassicauda crassicauda</i>	Tetrameridae
<i>Pseudoterranova decipiens</i> ¹	Anisakidae	<i>Pharurus sunameri</i> ²	Pseudaliidae	<i>Crassicauda delamureana</i> ³	Tetrameridae
<i>Pseudoterranova kogiae</i> ²	Anisakidae	<i>Pseudalius inflexus</i>	Pseudaliidae	<i>Crassicauda gillakiana</i>	Tetrameridae
<i>Terranova</i> sp. ^{3g}	Anisakidae	<i>Skrjabinalius cryptocephalus</i>	Pseudaliidae	<i>Crassicauda grampicola</i>	Tetrameridae
<i>Porrocaecum</i> sp. ⁹	Ascarididae	<i>Skrjabinalius guevarai</i>	Pseudaliidae	<i>Crassicauda magna</i> ²	Tetrameridae
<i>Odontobius ceti</i>	Monhysteridae	<i>Stenurus arctomarinus</i> ²	Pseudaliidae	<i>Placentonema gigantissimum</i> ²	Tetrameridae
Trematoda					
Species	Family	Species	Family	Species	Family
<i>Brachycladium atlanticum</i>	Brachycladiidae	<i>Nasitrema gondo</i>	Brachycladiidae	<i>Synthesium seymouri</i> ²	Brachycladiidae
<i>Brachycladium curilense</i>	Brachycladiidae	<i>Nasitrema lanceolatum</i>	Brachycladiidae	<i>Synthesium subtile</i>	Brachycladiidae
<i>Brachycladium delphini</i>	Brachycladiidae	<i>Nasitrema spathulatum</i> ²	Brachycladiidae	<i>Synthesium tursionis</i>	Brachycladiidae
<i>Brachycladium goliath</i>	Brachycladiidae	<i>Nasitrema stenosoma</i>	Brachycladiidae	<i>Braunina cordiformis</i>	Brauninidae
<i>Brachycladium nipponicum</i>	Brachycladiidae	<i>Nasitrema sunameri</i> ²	Brachycladiidae	<i>Galactosomum erinaceum</i> ^h	Heterophyidae
<i>Brachycladium pacificum</i>	Brachycladiidae	<i>Odhneriella arctica</i> ²	Brachycladiidae	<i>Pholeter gastrophilus</i>	Heterophyidae
<i>Brachycladium palliatum</i>	Brachycladiidae	<i>Odhneriella elongata</i>	Brachycladiidae	<i>Ogmogaster antarctica</i>	Notocotylidae

Table 4.1 (Continued)

Trematoda					
Species	Family	Species	Family	Species	Family
<i>Brachycladium parvulum</i> ³	Brachycladiidae	<i>Orthosplanchnus arcticus</i> ²	Brachycladiidae	<i>Ogmogaster grandis</i>	Notocotylidae
<i>Brachycladium petrowi</i>	Brachycladiidae	<i>Orthosplanchnus pygmaeus</i> ²	Brachycladiidae	<i>Ogmogaster pentalineata</i> ²	Notocotylidae
<i>Campula folium</i> ²	Brachycladiidae	<i>Orthosplanchnus sudarikovi</i> ²	Brachycladiidae	<i>Ogmogaster plicata</i>	Notocotylidae
<i>Campula gondo</i>	Brachycladiidae	<i>Oschmarinella albamarina</i>	Brachycladiidae	<i>Ogmogaster trilineata</i> ²	Notocotylidae
<i>Campula oblonga</i>	Brachycladiidae	<i>Oschmarinella laevicaecum</i>	Brachycladiidae	<i>Amphimerus lancea</i> ^b	Opisthorchiidae
<i>Cetitrema foliforme</i> ²	Brachycladiidae	<i>Oschmarinella macrorchis</i> ²	Brachycladiidae	<i>Delphinicola tenuis</i>	Opisthorchiidae
<i>Cetitrema meadi</i> ³	Brachycladiidae	<i>Oschmarinella rochebruni</i>	Brachycladiidae	<i>Opisthorchis tenuicollis</i> ^b	Opisthorchiidae
<i>Hunterotrema caballeroi</i> ²	Brachycladiidae	<i>Oschmarinella sobolevi</i>	Brachycladiidae		
<i>Hunterotrema macrosoma</i> ³	Brachycladiidae	<i>Synthesium delamurei</i>	Brachycladiidae		
<i>Nasitrema attenuatum</i>	Brachycladiidae	<i>Synthesium elongatum</i>	Brachycladiidae		
<i>Nasitrema dalli</i>	Brachycladiidae	<i>Synthesium mironovi</i>	Brachycladiidae		
<i>Nasitrema delphini</i>	Brachycladiidae	<i>Synthesium nipponicum</i>	Brachycladiidae		
<i>Nasitrema globicephalae</i>	Brachycladiidae	<i>Synthesium pontoporiae</i> ²	Brachycladiidae		

^a. Described only in cetaceans as immature individuals; adults unknown.

^b. Probably accidental.

^c. Synonym: *Pyramicocephalus phocarum*.

^d. Collective name for larval forms of species of tetraphyllideans whose adult stage infects elasmobranchs; cetaceans act as intermediate hosts for some species.

^e. Unclear taxonomical status (see Hoberg, 1990).

^f. Name used for a species complex.

^g. Synonym of *Pseudoterranova* (?).

^h. Listed as "indetermined species" by Dailey et al. (2002). Likely accidental.

ⁱ. Species name not included in the taxonomic database searched.

Table 4.2. List of helminth taxa reported in cetaceans. Abbreviations: Ant, Antarctic; AO, Atlantic Ocean; AR, Amazon River; Arc, Arctic; BaS, Baltic Sea; BIS, Black Sea; GR, Ganges River; IO, Indian Ocean; MS, Mediterranean Sea; PO, Pacific Ocean; RS, Red Sea; SA, Region of South Africa; YR, Yangtze River; (\$) Geographic location not specified by the authors; (*) See Table 4.1 for specific considerations

Acanthocephala	Locality	Ref.	Cestoda	Locality	Ref.	Nematoda	Locality	Ref.	Trematoda	Locality	Ref.
Mysticeti: Family Balaenidae											
<i>Balaena mysticetus</i> Linnaeus, 1758. Bowhead whale											
<i>Bolbosoma balaenae</i>	AO	[31, 76, 306]	<i>Mononygma grimaldii</i> *	\$	[17]	<i>Anisakis simplex</i> s.l.*	\$	[137]	<i>Brachycladium goliath</i>	AO, PO	[31, 76, 314, 306, 272, 229]
			<i>Phyllobothrium delphini</i> *	AO	[31, 76, 263, 84, 229]	<i>Odontobius ceti</i>	\$	[274]	<i>Ogmogaster antarctica</i>	Arc	[258]
			<i>Phyllobothrium physeteris</i> *	\$	[31]	<i>Crassicauda crassicauda</i>	AO	[76, 306, 270]	<i>Ogmogaster plicata</i>	AO, PO	[285, 306, 124, 229]
			<i>Scolex pleuronectis</i> *	AO	[306]						
<i>Eubalaena australis</i> (Desmoulins, 1822). Southern right whale											
-	-	-	<i>Priapocephalus grandis</i>	Ant, SA	[31]	<i>Crassicauda costata</i> *	SA	[131]	-	-	-
			<i>Mononygma grimaldii</i> *	\$	[17]	<i>Odontobius ceti</i>	\$	[31]			
			<i>Phyllobothrium delphini</i> *	\$	[17]						
<i>Eubalaena glacialis</i> (Müller, 1776). North Atlantic right whale											
<i>Bolbosoma balaenae</i>	\$	[306]	<i>Diplogonoporus balaenopterae</i>	\$	[306]	<i>Odontobius ceti</i>	PO	[262, 274]	<i>Ogmogaster antarctica</i>	\$	[306]
<i>Bolbosoma brevicolle</i>	AO	[76]	<i>Mononygma grimaldii</i> *	\$	[17]						
<i>Bolbosoma nipponicum</i>	PO	[262, 306]	<i>Scolex pleuronectis</i> *	\$	[306]						
<i>Bolbosoma turbinella</i>	AO, PO	[84, 270, 306]	<i>Priapocephalus grandis</i>	\$	[31, 76, 84, 306]						
			<i>Tetrabothis affinis</i>	\$	[306]						
			<i>Tetrabothis ruudi</i>	PO	[78, 306]						

Table 4.2 (Continued)

Acanthocephala	Locality	Ref.	Cestoda	Locality	Ref.	Nematoda	Locality	Ref.	Trematoda	Locality	Ref.
Family Balaenopteridae											
<i>Balaenoptera acutorostrata</i> Lacépède, 1804. Common minke whale											
<i>Bolbosoma balaenae</i>	AO	[31, 76, 306, 115]	<i>Diplogonoporus balaenopterae</i>	PO	[140, 115]	<i>Anisakis physeteris</i>	PO	[137, 209]	<i>Brachycladium goliath</i>	AO, PO, Ant	[42, 206, 31, 219, 76, 314, 306, 272, 229, 44]
<i>Bolbosoma brevicolle</i>	AO	[31, 76, 306, 84]	<i>Monorygma grimaldii</i> *	§	[17]	<i>Anisakis simplex</i> s.l.*	AO	[31, 76, 73, 306, 170, 116]	<i>Ogmogaster antarctica</i>	Arc	[239]
<i>Bolbosoma nipponicum</i>	PO	[307, 76, 262, 313, 306]	<i>Phyllobothrium delphini</i> *	§	[17]	<i>Anisakis simplex</i> s.s.	AO	[181, 180]	<i>Ogmogaster plicata</i>	AO, PO	[31, 219, 74, 76, 84, 306]
			<i>Tetrabothrius</i> sp.	Ant	[70]	<i>Pseudoterranova decipiens</i> *	§	[76, 306]			
						<i>Odontobius ceti</i>	§	[274]			
						<i>Hysterothylacium aduncum</i> *	Arc	[211]			
						<i>Crassicauda crassicauda</i>	AO	[76, 306, 271]			
<i>Balaenoptera borealis</i> Lesson, 1828. Sei whale											
<i>Bolbosoma balaenae</i>	AO	[31, 76, 306]	<i>Diphyllobothrium</i> sp.	PO	[171]	<i>Anisakis simplex</i> s.l.*	AO, PO, Ant	[31, 172, 73, 171, 306, 115, 70, 201, 157]	<i>Brachycladium goliath</i>	AO, PO, Ant	[42, 206, 31, 76, 172, 289, 314, 171, 306, 272, 70, 200]
<i>Bolbosoma brevicolle</i>	AO	[31, 76, 306, 84]	<i>Diplogonoporus balaenopterae</i>	AO, Arc, Ant	[31, 76, 177, 312, 238, 306, 85, 229, 70]	<i>Crassicauda boopis</i>	AO, Ant	[14]	<i>Ogmogaster antarctica</i>	AO, PO, Arc	[239, 306, 200, 157, 109]
<i>Bolbosoma nipponicum</i>	PO	[76, 313, 306]	<i>Monorygma grimaldii</i> *	§	[17]	<i>Crassicauda crassicauda</i>	AO, Ant	[31, 76, 162, 306, 271, 70, 200]	<i>Ogmogaster grandis</i>	Ant	[265, 306]

Table 4.2 (Continued)

Acanthocephala	Locality	Ref.	Cestoda	Locality	Ref.	Nematoda	Locality	Ref.	Trematoda	Locality	Ref.
<i>Balaenoptera borealis</i> (Continued)											
<i>Bolbosoma tuberculata</i>	Ant	[266, 306]	<i>Scolex pleuronectis</i> *	Ant	[306]	<i>Crassicauda delamureana</i> *	Ant	[131]	<i>Ogmogaster plicata</i>	AO, PO, Arc	[31, 219, 74, 76, 172, 239, 314, 171, 306, 115]
<i>Bolbosoma turbinella</i>	AO, PO, Ant, SA	[30, 122, 31, 110, 76, 172, 171, 306, 270, 115, 70, 200, 201]	<i>Priapocephalus grandis</i>	AO, Ant	[31, 76, 177, 84, 306, 115, 229, 70]	<i>Odontobius ceti</i>	PO	[245, 274]			
			<i>Priapocephalus minor</i>	AO	[23, 31, 76, 306, 229]						
			<i>Tetrabothrius affinis</i>	AO, Ant, SA	[31, 76, 306, 115, 229, 70, 157]						
			<i>Tetrabothrius arsenyevi</i> *	Ant	[76, 312, 306]						
			<i>Tetrabothrius wilsoni</i>	AO, PO, Ant	[156, 31, 177, 115, 63, 229, 70]						
<i>Balaenoptera edeni</i> Anderson, 1879. Bryde's whale											
<i>Bolbosoma capitatum</i>	AO	[166]	-	-	-	<i>Anisakis simplex</i> s.l.*	PO	[306, 115, 201]	<i>Brachycladium petrowi</i>	PO	[268, 306]
<i>Bolbosoma tuberculata</i>	IO	[266]				<i>Crassicauda crassicauda</i>	AO	[306]	<i>Cetitrema foliforme</i> *	IO	[267, 314, 306]
<i>Bolbosoma turbinella</i>	PO	[201]							<i>Ogmogaster grandis</i>	IO	[306]
<i>Balaenoptera musculus</i> (Linnaeus, 1758). Blue whale											
<i>Bolbosoma balaenae</i>	AO, PO	[31, 76, 306]	<i>Diplogonoporus balaenopterae</i>	AO	[177, 238, 306, 115, 189]	<i>Anisakis simplex</i> s.l.*	AO, PO, IO	[31, 189, 76, 73, 306, 115]	<i>Brachycladium goliath</i>	AO	[306]
<i>Bolbosoma brevicolle</i>	AO, SA	[31, 76, 96, 306, 115]	<i>Monorygma grimaldii</i> *	§	[17]	<i>Pseudoterranova decipiens</i> *	§	[76]	<i>Ogmogaster antarctica</i>	Arc	[76, 239, 314, 306]

Table 4.2 (Continued)

Acanthocephala	Locality	Ref.	Cestoda	Locality	Ref.	Nematoda	Locality	Ref.	Trematoda	Locality	Ref.
<i>Balaenoptera musculus</i> (Continued)											
<i>Bolbosoma hamiltoni</i>	AO	[30, 31, 76, 313, 306, 115]	<i>Phyllobothrium delphini</i> *	§	[17]	<i>Crassicauda boopis</i>	AO	[113, 115, 189]	<i>Ogmogaster grandis</i>	AO	[265, 306]
<i>Bolbosoma nipponicum</i>	PO	[78, 306]	<i>Priapocephalus grandis</i>	AO, PO, SA	[31, 76, 177, 220, 306, 115, 229]	<i>Crassicauda crassicauda</i>	AO, PO	[31, 76, 162, 306, 271, 113, 115]	<i>Ogmogaster plicata</i>	AO	[31, 219, 76]
<i>Bolbosoma turbinella</i>	AO, PO	[31, 76, 270, 306]	<i>Tetrabothrius affinis</i>	AO, PO	[31, 76, 220, 177, 229, 306, 115]	<i>Odontobius ceti</i>	PO	[31, 262]			
			<i>Tetrabothrius schaeferi</i> *	AO	[189, 177, 306, 115]						
			<i>Tetrabothrius wilsoni</i>	AO	[177, 115]						
<i>Balaenoptera physalus</i> (Linnaeus, 1758). Fin whale											
<i>Bolbosoma balaenae</i>	AO	[76, 306, 115]	<i>Diplogonoporus balaenopterae</i>	Arc	[76, 177, 238, 306, 115]	<i>Anisakis simplex</i> s.l.*	AO, PO, Ant	[305, 285, 73, 115, 170, 201]	<i>Brachycladium goliath</i>	AO, PO, Ant	[76, 172, 314, 171, 306, 272]
<i>Bolbosoma brevicolle</i>	Ant, SA	[31, 76, 84, 306, 115]	<i>Mononygma grimaldii</i> *	Ant	[269, 306]	<i>Odontobius ceti</i>	§	[31, 274]	<i>Brachycladium petrowi</i>	AO	[306]
<i>Bolbosoma hamiltoni</i>	Ant	[31, 76, 54, 313, 306, 115]	<i>Phyllobothrium delphini</i> *	AO, PO	[172, 285, 171, 306, 170]	<i>Crassicauda boopis</i>	AO	[76, 113, 306, 115, 149, 116]	<i>Ogmogaster antarctica</i>	AO, PO, Arc, MS, SA	[76, 143, 239, 314, 171, 306, 115, 229, 167, 200, 201]
<i>Bolbosoma nipponicum</i>	PO	[76, 313, 306]	<i>Priapocephalus grandis</i>	AO, Ant, SA	[76, 177, 306, 115, 229]	<i>Crassicauda crassicauda</i>	AO, Ant, SA	[31, 76, 162, 306, 113, 271, 115, 200]	<i>Ogmogaster grandis</i>	Ant	[265, 306]
<i>Bolbosoma turbinella</i>	AO, PO	[76, 270, 306]	<i>Priapocephalus minor</i>	AO	[76, 306, 229]				<i>Ogmogaster plicata</i>	AO, PO, Arc	[31, 74, 76, 172, 262, 239, 314, 171, 306, 229]

Table 4.2 (Continued)

Acanthocephala	Locality Ref.	Cestoda	Locality Ref.	Nematoda	Locality Ref.	Trematoda	Locality	Ref.
<i>Balaenoptera physalus</i> (Continued)								
		<i>Tetرابothrius affinis</i>	Ant	[161, 76, 306, 115]		<i>Ogmogaster trilineata</i> *	PO	[242, 314, 171]
		<i>Tetرابothrius egregius</i>	Ant	[276, 306]				
		<i>Tetرابothrius ruudi</i>	Ant	[31, 76, 177, 306, 115]				
Megaptera novaeangliae (Borowski, 1781). Humpback whale								
<i>Bolbosoma balaenae</i>	Ant	<i>Diplogonoporus balaenopterae</i>	PO, Arc	[245, 238, 171, 306]	\$	<i>Anisakis simplex</i> s.l.*	AO	[73]
<i>Bolbosoma nipponicum</i>	PO	<i>Monorygma grimaldii</i>	\$	[17]	\$	<i>Odontobius ceti</i>	\$	[31, 274]
<i>Bolbosoma turbinella</i>	AO, PO	<i>Phyllobothrium delphini</i>	\$	[306]	AO	<i>Pharurus alatus</i>	AO	[229]
					PO	<i>Crassicauda boopsis</i>	PO	[31, 76, 162, 270, 306, 115]
					AO	<i>Crassicauda crassicauda</i>	AO	[76, 285, 306, 271]
Family Eschrichtiidae								
<i>Eschrichtius robustus</i> (Lilljeborg, 1861). Gray whale								
<i>Bolbosoma balaenae</i>	PO	<i>Monorygma grimaldii</i>	\$	[17]	PO	<i>Anisakis simplex</i> s.l.*	PO	[246, 66]
<i>Corynosoma sermerae</i>	Arc	<i>Phyllobothrium delphini</i>	\$	[17]			PO	[246, 65, 171, 66]
<i>Corynosoma septentrionalis</i> *	Arc	<i>Priapocephalus eschrichtii</i>	Arc	[202, 246, 306]		<i>Ogmogaster pentalineata</i> *	PO, Arc	[239, 242, 314, 246, 306, 171, 115, 66]
<i>Corynosoma strumosum</i> *	Arc					<i>Ogmogaster plicata</i>	Arc	[246, 306]
<i>Corynosoma validum</i> *	Arc					<i>Orthosplanchnus pygmaeus</i> *	PO	[316, 246, 306]
Family Neobalaenidae								
<i>Caperea marginata</i> (Gray, 1846). Pygmy right whale								
					SA	<i>Anisakis pegreffii</i>	SA	[181]

Table 4.2 (Continued)

Acanthocephala	Locality	Ref.	Cestoda	Locality	Ref.	Nematoda	Locality	Ref.	Trematoda	Locality	Ref.
Odontoceti: Family Delphinidae											
<i>Cephalorhynchus commersonii</i> (Lacépède, 1804). Commerson's dolphin											
-	-	-	<i>Monorygma grimaldii</i>	§	[17]	<i>Anisakis simplex</i> s.l.*	AO	[115, 36]	<i>Braunina cordiformis</i>	AO	[115, 36]
			<i>Phyllobothrium delphini</i> *	§	[17]	<i>Halocercus brasiliensis</i>	AO	[115, 190]	<i>Pholeter gastrophilus</i>	AO	[36]
			<i>Strobilocephalus triangularis</i>	AO	[36]				<i>Synthesium delamurei</i>	AO	[35]
<i>Cephalorhynchus eutrophia</i> (Gray, 1846). Chilean dolphin											
<i>Corynosoma cetaceum</i>	PO	[287, 20]	-	-	-	<i>Anisakis</i> sp.	PO	[287]	<i>Braunina cordiformis</i>	PO	[105, 287]
						<i>Pseudoterranova</i> sp.	PO	[287]	<i>Nasitrema globicephalae</i>	PO	[43]
									<i>Synthesium tursionis</i>	PO	[287]
<i>Cephalorhynchus heavisidii</i> (Gray, 1828). Haviside's dolphin											
-	-	-	<i>Monorygma grimaldii</i> *	AO	[17]	<i>Anisakis pegreffii</i>	SA	[181]	-	-	-
			<i>Phyllobothrium delphini</i> *	AO	[17]						
<i>Cephalorhynchus hectori</i> (Van Beneden, 1881). Hector's dolphin											
<i>Corynosoma</i> sp.	PO	[188]	<i>Monorygma grimaldii</i>	PO	[17]	<i>Contracecum</i> sp.	PO	[188]	<i>Campula</i> sp.	§	[128]
			<i>Phyllobothrium delphini</i> *	PO	[188, 17]	<i>Phocascaris</i> sp.*	PO	[188]	<i>Braunina cordiformis</i>	PO	[188]
<i>Delphinus capensis</i> Gray, 1828. Long-beaked common dolphin											
-	-	-	<i>Monorygma grimaldii</i>	§	[17]	<i>Anisakis simplex</i> s.l.*	AO, PO	[194, 147]	-	-	-
			<i>Phyllobothrium delphini</i> *	§	[17]						

Table 4.2 (Continued)

Acanthocephala	Locality	Ref.	Cestoda	Locality	Ref.	Nematoda	Locality	Ref.	Trematoda	Locality	Ref.
<i>Bolbosoma vasculosum</i> *	MS, BIS	[295, 76, 77, 306]	<i>Diphyllobothrium lanceolatum</i>	§	[218]	<i>Anisakis pegreffii</i>	AO, MS	[181]	<i>Amphimerus lancea</i> *	AO	[166]
<i>Corynosoma cetaceum</i>	AO, IO, BIS	[134, 76, 77, 313, 254, 306, 34]	<i>Diphyllobothrium sternmacephalum</i>	BIS	[31, 77, 38]	<i>Anisakis simplex</i> s.l.*	AO, PO, BIS, SA	[76, 77, 73, 306, 71, 115, 1, 116, 38, 21, 34]	<i>Brachycladium atlanticum</i>	MS	[178]
<i>Corynosoma strumosum</i> *	§	[218]	<i>Monorygma grimaldii</i>	AO, PO, BIS	[23, 76, 77, 306, 281, 62, 71, 115, 67, 229, 1, 116]	<i>Anisakis simplex</i> s.s.	AO	[181]	<i>Brachycladium delphini</i>	AO, MS, BIS	[31, 219, 76, 77, 127, 314, 306, 229]
			<i>Phyllobothrium delphini</i> *	AO, PO, BIS	[31, 119, 76, 77, 263, 303, 171, 306, 281, 62, 71, 115, 67, 229, 1, 116]	<i>Anisakis typica</i>	AO, PO, BIS	[31, 76, 77, 73, 306, 71]	<i>Brachycladium palliatum</i>	AO, BIS	[31, 219, 76, 77, 314, 306, 229, 116, 38]
			<i>Scolex pleuronectis</i> *	PO, MS	[222, 71]	<i>Crassicauda crassicauda</i>	AO	[306]	<i>Braunina cordiformis</i>	AO, BIS	[76, 77, 275, 289, 314, 306, 229, 34, 109]
			<i>Strobilocephalus triangularis</i>	PO	[67]	<i>Goezia</i> sp.*	§	[237]	<i>Campula oblonga</i>	AO	[5, 116]
			<i>Tetrabothrius diplosoma</i> *	AO	[306]	<i>Halocercus delphini</i>	AO, PO, MS, BIS	[31, 94, 310, 76, 77, 283, 306, 115, 1, 116, 278]	<i>Galactosomum erinaceum</i> *	AO, BIS	[31, 76, 77, 306, 229]
			<i>Tetrabothrius forsteri</i>	PO, MS, BIS	[31, 119, 76, 77, 306, 67, 222]	<i>Halocercus invaginatus</i>	§	[218, 13]	<i>Nasitrema delphini</i>	PO	[204, 314, 171, 12, 71, 62, 278]

Table 4.2 (Continued)

Acanthocephala	Locality	Cestoda	Locality	Ref.	Nematoda	Locality	Ref.	Trematoda	Locality	Ref.
<i>Delphinus delphis</i> (Continued)										
					<i>Halocercus kleinenbergi</i>	BIS, SA	[76, 77, 306, 38]	<i>Nasitrema globicephalae</i>	PO	[62, 71, 67]
					<i>Halocercus lagenorhynchi</i>	AO, IO	[190, 286]	<i>Nasitrema lanceolatum</i>	PO	[62]
					<i>Pseudoterranova decipiens</i> *	§	[218]	<i>Nasitrema stenosoma</i>	PO	[71]
					<i>Skrjabinaius cryptocephalus</i>	PO, BIS	[76, 77, 278, 38]	<i>Oschmarinella rochebruni</i>	AO, PO, BIS	[31, 219, 76, 77, 314, 247, 306, 62, 71, 67, 229, 116, 34]
					<i>Skrjabinaius guevarai</i>	AO, MS	[227, 190]	<i>Pholeter gastrophilus</i>	AO, BIS	[306, 92, 213, 229, 116, 38, 34]
					<i>Stenurus minor</i>	§	[218]	<i>Synthesium delamurei</i>	MS	[222]
								<i>Synthesium tursionis</i>	PO	[280]
Feresa attenuata Gray, 1874. Pygmy killer whale										
<i>Bolbosoma vasculosum</i> *	PO	[194]	<i>Monorygma grimaldii</i> *	PO	[194]	<i>Anisakis simplex</i>	PO	[319]	<i>Nasitrema lanceolatum</i>	PO
			<i>Phyllobothrium delphinii</i> *	§	[17]	s.l.				
			<i>Scolex pleuronectis</i> *	PO	[106]	<i>Anisakis typica</i>	PO	[319, 184]		
			<i>Tetrabothrius forsteri</i>	PO	[106]	<i>Terranova sp.</i> *	PO	[194]		
			<i>Trigonocotyle sexfisticulæ</i> *	PO	[126, 194]	<i>Halocercus brasiliensis</i>	PO	[120]		
						<i>Stenurus globicephalæ</i>	PO	[106, 194]		

Table 4.2 (Continued)

Acanthocephala	Locality	Ref.	Cestoda	Locality	Ref.	Nematoda	Locality	Ref.	Trematoda	Locality	Ref.
<i>Bolbosoma capitatum</i>	AO	[194, 55]	<i>Globicephala macrocephalus</i> sp.	§	[226]	<i>Anisakis simplex</i> s.l.*	AO, SA	[138, 137, 115, 55]	<i>Brachycladium nipponicum</i>	PO	[308, 314, 306]
			<i>Diphyllobothrium</i> sp.	AO	[47]	<i>Anisakis simplex</i> s.s.	AO	[48]	<i>Campula gondo</i>	PO	[308, 314, 306]
			<i>Monorygma grimaldii</i>	AO	[194, 55]	<i>Anisakis typica</i>	AO	[194, 181, 47, 48]	<i>Nasitrema attenuatum</i>	PO	[204]
			<i>Phyllobothrium delphini</i>	AO	[17, 47]	<i>Crassicauda</i> sp.	AO	[194]	<i>Nasitrema globicephalae</i>	AO, PO	[204, 314, 171, 12, 196]
			<i>Plicobothrium globicephalae</i>	§	[315]	<i>Halocercus kleinenbergi</i>	AO	[47]	<i>Nasitrema gondo</i>	PO	[309, 314, 306]
			<i>Trigonocotyle globicephalae</i>	§	[312]	<i>Stenurus globicephalae</i>	AO	[94, 306, 196, 194, 55, 47]	<i>Nasitrema lanceolatum</i>	PO	[204, 314, 171]
			<i>Trigonocotyle lintoni</i>	PO	[306]	<i>Stenurus minor</i> <i>Tonymurus convolutus</i>	AO, PO	[196] [94]			
Globicephala melas (Traill, 1809). Long-finned pilot whale											
<i>Bolbosoma capitatum</i>	AO, MS	[31, 76, 313, 59, 306, 115, 97, 27, 232, 28, 25]	<i>Diphyllobothrium polyrugosum</i>	AO	[27]	<i>Anisakis berlandi</i>	SA	[181, 180]	<i>Campula gondo</i>	PO	[76]
			<i>Diphyllobothrium stemmacephalum</i>	AO	[26]	<i>Anisakis pegreffii</i>	PO	[180]	<i>Nasitrema globicephalae</i>	PO	[63, 99]
			<i>Monorygma grimaldii</i> *	AO, MS	[76, 306, 229, 232]	<i>Anisakis simplex</i> s.l.*	AO	[88, 232, 182, 1, 116]	<i>Orthosplanchnus arcticus</i> *	AO	[59, 229, 232]
			<i>Phyllobothrium delphini</i> *	AO, MS	[119, 76, 263, 303, 306, 288, 115, 229, 232, 1]	<i>Anisakis simplex</i> s.s.	AO, SA	[115, 182, 181, 180]	<i>Pholeter gastrophilus</i>	AO, MS	[237, 229, 232]

Table 4.2 (Continued)

Acanthocephala	Locality	Ref.	Cestoda	Locality	Ref.	Nematoda	Locality	Ref.	Trematoda	Locality	Ref.
<i>Globicephala melas</i> (Continued)											
			<i>Plicobothrium globicephalae</i>	AO	[241, 229, 232]	<i>Anisakis typica</i>	PO	[31, 76, 73, 306, 184]	<i>Synthesium delamurei</i>	AO, MS	[230, 229, 232, 28]
			<i>Scolex pleuronectis</i> *	AO	[59]	<i>Crassicauda carbonelli</i> *	MS	[231, 232]	<i>Synthesium subtile</i>	AO	[29, 232, 28, 35]
			<i>Trigonocotyle globicephalae</i>	AO, MS, SA	[23, 306, 115, 27, 28]	<i>Stenurus globicephalae</i>	AO, MS	[31, 76, 59, 306, 13, 115, 237, 232, 1, 116]			
			<i>Trigonocotyle lintoni</i>	AO, MS	[76, 59, 306, 229]	<i>Torynurus convolutus</i>	AO, PO	[31, 76, 306, 13]			
Grampus griseus (G. Cuvier, 1812). Risso's dolphin											
			<i>Monorygma grimaldii</i> *	PO	[69, 237, 229, 58]	<i>Anisakis pegreffii</i>	MS	[39]	<i>Brachycladium palliatum</i>	AO	[318, 229]
			<i>Phyllobothrium delphini</i> *	AO	[31, 76, 263, 303, 318, 306, 229, 1, 194, 58]	<i>Anisakis physeteris</i>	PO	[147]	<i>Nasitrema gondo</i>	PO	[197]
			<i>Scolex pleuronectis</i> *	MS	[98, 100, 9, 17]	<i>Anisakis simplex s.l.</i> *	PO, SA	[115, 147]	<i>Synthesium delamurei</i>	MS	[98]
			<i>Tetrabothrius forsteri</i>	MS	[98]	<i>Crassicauda grampicola</i>	AO, PO, MS	[135, 76, 162, 306, 235, 225, 197, 279]	<i>Synthesium tursionis</i>	AO	[318]
			<i>Trigonocotyle globicephalae</i>	MS	[98]	<i>Pseudoterranova ceticola</i>	PO	[147]	<i>Pholeter gastrophilus</i>	AO, MS	[318, 229, 58, 98]
						<i>Stenurus globicephalae</i>	AO, MS	[13, 237, 98]			
						<i>Stenurus minor</i>	AO	[285, 306, 190]			

Table 4.2 (Continued)

Acanthocephala	Locality	Ref.	Cestoda	Locality	Ref.	Nematoda	Locality	Ref.	Trematoda	Locality	Ref.
<i>Bolbosoma</i> sp.	PO	[284]	<i>Monorygma grimaldii</i>	AO, IO	[186, 294, 194, 55, 47]	<i>Anisakis pegreffii</i>	AO	[48]	<i>Campula</i> sp.	IO	[186]
			<i>Phyllobothrium delphini</i> *	AO	[281, 294, 229, 194, 55, 47]	<i>Anisakis simplex</i> s.l.*	AO, IO	[186, 294]	<i>Nasitrema</i> sp.	PO	[278]
			<i>Strobilocephalus triangularis</i>	AO	[223, 294, 229]	<i>Anisakis typica</i>	AO	[48]			
			<i>Tetрабоthrius forsteri</i>	AO	[194]	<i>Stenurus ovatus</i>	PO	[186, 278]			
Lagenorhynchus acutus (Gray, 1828). Atlantic white-sided dolphin											
<i>Bolbosoma</i> sp.	AO	[37]	<i>Diphylobothrium stemmacephalum</i>	AO	[208]	<i>Anisakis simplex</i> s.l.*	AO	[137, 115, 237, 116]	<i>Oschmarinella laevicaecum</i>	AO	[37]
			<i>Monorygma grimaldii</i>	AO	[23, 31, 76, 115, 229, 248, 116]	<i>Anisakis typica</i>	AO	[137]	<i>Pholeter gastrophilus</i>	AO	[37, 229, 248]
			<i>Phyllobothrium delphini</i> *	AO	[23, 306, 248, 116]	<i>Pseudalius inflexus</i>	AO	[115, 190]			
			<i>Strobilocephalus triangularis</i>	AO	[23, 31, 76, 306, 229]	<i>Stenurus globicephalae</i>	AO	[306, 13, 37, 248]			
			<i>Tetрабоthrius forsteri</i>	AO	[37, 229]	<i>Torynurus convolutus</i>	AO	[115]			
						<i>Crassicauda grampicola</i>	AO	[112]			
Lagenorhynchus albirostris (Gray, 1846). White-beaked dolphin											
-	-	-	<i>Monorygma grimaldii</i>	§	[17]	<i>Anisakis simplex</i> s.l.*	AO	[31, 76, 73, 306, 116]	<i>Pholeter gastrophilus</i>	AO	[116]
			<i>Phyllobothrium delphini</i> *	§	[17]	<i>Anisakis simplex</i> s.s.	AO	[182]			
			<i>Plicobothrium globicephalae</i>	AO	[170]	<i>Halocercus lagenorhynchi</i>	AO	[31, 94, 310, 76, 306]			
						<i>Pseudoterranova</i> sp.	§	[15]			
Lagenorhynchus australis (Peale, 1848). Peale's dolphin											
-	-	-	<i>Trigonocotyle pruchoei</i> *	AO	[177, 115]	-	-	-	-	-	-

Table 4.2 (Continued)

Acanthocephala	Locality	Ref.	Cestoda	Locality	Ref.	Nematoda	Locality	Ref.	Trematoda	Locality	Ref.
Lagenorhynchus cruciger (Quoy and Gaimard, 1824). Hourglass dolphin											
-	-	-	<i>Phyllobothrium delphini</i> *	AO	[103]	<i>Anisakis simplex</i> s.l.*	AO	[76, 306, 103]	<i>Oschmarinella</i> sp.	AO	[103]
-	-	-	<i>Trigonocotyle prudhoei</i> *	Ant	[306]	<i>Anisakis typica</i>	AO	[76, 306]	<i>Synthesium subtile</i>	AO	[35]
Lagenorhynchus obliquidens Gill, 1865. Pacific white-sided dolphin											
-	-	-	<i>Diphylobothrium fuhrmanni</i>	PO	[26]	<i>Anisakis simplex</i> s.l.*	PO	[62, 71]	<i>Brachycladium</i> sp.	PO	[247]
-	-	-	<i>Monorygma grimaldii</i> *	PO	[67, 71]	<i>Crassicauda</i> sp.	PO	[67]	<i>Nasitrema globicephalae</i>	PO	[204, 171, 12, 62, 71, 67]
-	-	-	<i>Phyllobothrium delphini</i> *	PO	[136, 171, 71, 67]						
-	-	-	<i>Scolex pleuronectis</i> *	PO	[71]						
-	-	-	<i>Strobilocephalus triangularis</i>	PO	[71]						
-	-	-	<i>Tetrabothis sp.</i>	PO	[71]						
Lagenorhynchus obscurus (Gray, 1828). Dusky dolphin											
<i>Corynosoma australe</i> *	AO	[72]	<i>Monorygma grimaldii</i> *	§	[17]	<i>Anisakis simplex</i> s.l.*	AO	[134, 72, 21]	<i>Braunina cordiformis</i>	AO, PO	[297, 72]
-	-	-	<i>Phyllobothrium delphini</i> *	AO, PO, SA	[115, 297, 201, 160]	<i>Anisakis typica</i>	SA	[31, 73, 115]	<i>Nasitrema</i> sp.	PO	[297]
-	-	-	<i>Scolex pleuronectis</i> *	PO	[177, 297]				<i>Pholeter gastrophilus</i>	AO, PO	[297, 72]
-	-	-	<i>Trigonocotyle prudhoei</i> *	AO	[177, 115]				<i>Synthesium subtile</i>	AO	[35]
Lissodelphis borealis (Peale, 1848). Northern right-whale dolphin											
-	-	-	<i>Monorygma grimaldii</i> *	PO	[71]	<i>Anisakis berlandi</i>	PO	[181]	<i>Nasitrema globicephalae</i>	PO	[204, 314, 171, 12, 62, 71]
-	-	-	<i>Phyllobothrium delphini</i> *	§	[17]	<i>Anisakis simplex</i> s.l.*	PO	[62, 71]			
-	-	-	<i>Scolex pleuronectis</i> *	PO	[199]						

Table 4.2 (Continued)

Acanthocephala	Locality	Ref.	Cestoda	Locality	Ref.	Nematoda	Locality	Ref.	Trematoda	Locality	Ref.
-	-	-	<i>Lissodelphis peronii</i> (Lacépède, 1804). Southern right-whale dolphin	-	-	-	-	-	<i>Delphinicola tenuis</i>	PO	[201]
-	-	-	<i>Strobilocephalus triangularis</i>	PO	[201]	-	-	-	-	-	-
-	-	-	<i>Tetrabothrius forsteri</i>	PO	[201]	-	-	-	-	-	-
-	-	-	<i>Orcaella brevirostris</i> (Owen in Gray, 1866). Irrawaddy dolphin	-	-	-	-	-	<i>Amphimerus lancea</i>	PO, IO	[31, 219, 76, 306]
<i>Orcinus orca</i> (Linnaeus, 1758). Killer whale											
<i>Bolbosoma nipponicum</i>	PO	[262, 306]	<i>Diphyllobothrium fuhmanni</i>	PO	[257]	<i>Anisakis simplex</i> s.s.	PO	[181]	<i>Oschmarinella albamarina</i>	AO	[114, 116]
<i>Bolbosoma capitatum</i>	AO, PO	[262, 306, 114, 116]	<i>Diphyllobothrium orcini</i>	PO	[123]	<i>Anisakis simplex</i> s.i.*	AO, PO	[76, 73, 171, 306, 114, 116]	<i>Synthesium subtile</i>	AO, PO, Arc	[261, 314, 306, 292, 35]
-	-	-	<i>Diphyllobothrium polyrugosum</i>	AO	[83, 306, 115, 229, 114, 116]	-	-	-	-	-	-
-	-	-	<i>Monorygma grimaldii</i>	AO	[89, 229]	-	-	-	-	-	-
-	-	-	<i>Phyllobothrium delphini</i>	AO	[115, 229]	-	-	-	-	-	-
-	-	-	<i>Trigonocotyle spasskyi</i>	PO	[76, 306]	-	-	-	-	-	-
<i>Peponocephala electra</i> (Gray, 1846). Melon-headed whale											
<i>Bolbosoma capitatum</i>	AO	[55]	<i>Diphyllobothrium</i> sp.	AO	[194]	<i>Anisakis physeteris</i>	PO	[147]	<i>Nasitrema gondo</i>	PO	[195]
-	-	-	<i>Monorygma grimaldii</i>	AO	[55, 47]	<i>Anisakis simplex</i> s.i.*	PO	[147]	-	-	-
-	-	-	<i>Phyllobothrium delphini</i>	AO	[47]	<i>Anisakis typica</i>	AO	[129, 184]	-	-	-
-	-	-	<i>Strobilocephalus triangularis</i>	AO	[47]	<i>Halocercus</i> sp.	PO	[278]	-	-	-
-	-	-	-	-	-	<i>Stenurus globicephalae</i>	AO, PO	[194, 190, 278, 47]	-	-	-

Table 4.2 (Continued)

Acanthocephala	Locality Ref.	Cestoda	Locality Ref.	Nematoda	Locality Ref.	Trematoda	Locality Ref.
<i>Bolbosoma capitatum</i>	AO, IO [31, 76, 313, 306, 115, 97, 11]	-	-	<i>Pseudorca crassidens owenii</i> , 1846	PO [182]	<i>Nasitrema attenuatum</i>	PO [204, 314, 171]
				<i>Anisakis berlandi</i>			
				<i>Anisakis simplex</i> s.l.*	AO [76, 73, 318, 306, 115, 182, 11]	<i>Nasitrema globicephalae</i>	PO [204, 314, 171, 12]
				<i>Anisakis simplex</i> s.s.	PO [182]	<i>Nasitrema gondo</i>	PO [198]
				<i>Anisakis typica</i>	AO, PO [318, 209]	<i>Odhneriella elongata</i>	AO, PO [229]
				<i>Sternurus auditivus</i>	AO [318]	<i>Synthesium elongatum</i>	AO [318]
				<i>Sternurus globicephalae</i>	AO [190, 320]		
Sotalia fluviatilis (Gervais and Deville in Gervais, 1853). Tucuxi							
-	-	<i>Mononygma grimaldii</i>	§ [17]	<i>Anisakis simplex</i> s.l.*	AO [21]	<i>Amphimerus lancea</i> *	AO, AR [31, 219, 76, 289, 115]
		<i>Phyllobothrium delphini</i> *	§ [17]	<i>Anisakis typica</i>	AO [253, 184, 192, 317]	<i>Braunina cordiformis</i>	AO [253, 192]
				<i>Halocercus brasiliensis</i>	AO [115, 253, 190, 192]	<i>Nasitrema attenuatum</i>	AO [115]
Sotalia guianensis (Van Bénédén, 1864). Guiana dolphin							
-	-	-	-	<i>Anisakis typica</i>	AO [130, 163]	<i>Braunina cordiformis</i>	AO [163, 176, 93]
				<i>Halocercus brasiliensis</i>	AO, AO [94, 310, 76, 306, 192, 176, 47, 163, 120, 93]	<i>Nasitrema attenuatum</i>	AO [95]
						<i>Synthesium tursionis</i>	AO [176]

Table 4.2 (Continued)

Acanthocephala	Locality	Ref.	Cestoda	Locality	Ref.	Nematoda	Locality	Ref.	Trematoda	Locality	Ref.
-	-	-	<i>Sousa chinensis</i> (Osbeck, 1765). Indo-Pacific humpback dolphin	PO	[17]	<i>Halocercus pingi</i>	PO	[212]	-	-	-
-	-	-	<i>Monorygma grimaldii</i>	PO	[17]	-	-	-	-	-	-
-	-	-	<i>Phyllobothrium delphini</i> *	PO	[17]	-	-	-	-	-	-
<i>Stenella attenuata</i> (Gray, 1846). Pantropical spotted dolphin											
<i>Bolbosoma balaenae</i>	PO	[68]	<i>Monorygma grimaldii</i> *	PO	[68, 67, 207]	<i>Anisakis simplex</i> s.l.*	PO	[65, 68]	<i>Brachycladium pacificum</i>	PO, SA	[68, 115, 67]
<i>Bolbosoma vasculosum</i> *	PO	[68]	<i>Phyllobothrium delphini</i> *	PO	[68, 67]	<i>Anisakis typica</i>	AO	[181, 48]	<i>Braunina cordiformis</i>	PO	[76, 314, 306]
-	-	-	<i>Scolex pleuronectis</i> *	PO	[207]	<i>Halocercus delphini</i>	PO	[68]	<i>Nasitrema globicephalae</i>	PO	[67, 207]
-	-	-	<i>Strobilocephalus triangularis</i>	PO	[68, 67]	-	-	-	<i>Nasitrema stenosoma</i>	PO	[204]
-	-	-	<i>Tetrabothis forsteri</i>	PO	[68, 67, 207]	-	-	-	<i>Oschmarinella rochebruni</i>	PO, SA	[68, 115, 67]
-	-	-	-	-	-	-	-	-	<i>Synthesium elongatum</i>	SA	[115]
<i>Stenella clymene</i> (Gray, 1850). Clymene dolphin											
<i>Bolbosoma</i> sp.	AO	[47]	<i>Monorygma grimaldii</i> *	AO	[17, 47]	<i>Anisakis</i> sp.	AO	[47]	-	-	-
-	-	-	<i>Phyllobothrium delphini</i> *	AO	[17, 47]	<i>Anisakis typica</i>	AO	[129]	-	-	-
-	-	-	<i>Scolex pleuronectis</i> *	AO	[47]	<i>Halocercus brasiliensis</i>	AO	[47, 120]	-	-	-
-	-	-	<i>Strobilocephalus triangularis</i>	AO	[47]	<i>Skjrabinalius guevarai</i>	AO	[6]	-	-	-
-	-	-	<i>Tetrabothis forsteri</i>	AO	[47]	-	-	-	-	-	-
-	-	-	<i>Trigonocotyle</i> sp.	AO	[47]	-	-	-	-	-	-

Table 4.2 (Continued)

Acanthocephala	Locality	Ref.	Cestoda	Locality	Ref.	Nematoda	Locality	Ref.	Trematoda	Locality	Ref.
<i>Stenella coeruleoalba</i> (Meyen, 1833). Striped dolphin											
<i>Bolbosoma capitatum</i>	AO	[251]	<i>Monorygma grimaldii</i> *	AO, PO, MS	[115, 67, 233, 229, 169, 1, 116, 58, 17, 47, 207]	<i>Anisakis pegreffii</i>	MS	[181, 180, 39]	<i>Brachycladium atlanticum</i>	AO, MS	[4, 99, 178]
<i>Bolbosoma turbinella</i>	AO	[251]	<i>Phyllobothrium delphini</i> *	AO, PO, MS, SA	[92, 318, 115, 229, 169, 1, 116, 58, 17, 47, 207]	<i>Anisakis simplex</i> s.l.*	AO, PO, MS	[138, 73, 318, 169, 1, 116]	<i>Brachycladium delphini</i>	AO, MS	[127, 92, 229]
<i>Bolbosoma vasculosum</i> *	MS	[233, 169, 8, 179]	<i>Scolex pleuronectis</i> *	AO, PO, MS	[8, 251, 9, 17, 207, 159]	<i>Anisakis simplex</i> s.s.	AO	[181, 39]	<i>Brachycladium pacificum</i>	PO	[67]
			<i>Strobilocephalus triangularis</i>	AO, PO, MS	[68, 69, 233, 169, 229, 8, 207, 179]	<i>Anisakis typica</i>	AO, MS, SA	[73, 115, 3, 184, 48]	<i>Brachycladium palliatum</i>	AO, PO, MS	[233, 158, 229, 169, 207]
			<i>Tetрабоthrius forsteri</i>	AO, PO, MS, SA	[115, 233, 229, 169, 8, 207, 179]	<i>Halocercus brasiliensis</i>	AO	[251]	<i>Nasitrema globicephalae</i>	PO	[67, 207]
			<i>Trigonocotyle globicephalae</i>	MS	[179]	<i>Halocercus delphini</i>	AO, MS	[299, 237]	<i>Oschmarinella rochebruni</i>	AO, MS	[224, 229, 169, 99, 116, 178, 109]
						<i>Halocercus legenorhynchi</i>	AO, PO, SA	[252, 115, 116, 207]	<i>Pholeter gastrophilus</i>	AO, MS	[92, 233, 234, 229, 169, 168, 58, 159]
						<i>Skriabinailius guevarai</i>	AO, MS	[233, 227, 169]			
						<i>Stenurus minor</i>	MS	[16]			
						<i>Stenurus ovatus</i>	MS	[169]			

Table 4.2 (Continued)

Acanthocephala	Locality	Ref.	Cestoda	Locality	Ref.	Nematoda	Locality	Ref.	Trematoda	Locality	Ref.
<i>Stenella frontalis</i> (G. Cuvier, 1829). Atlantic spotted dolphin											
<i>Bolbosoma vasculosum</i> *	AO	194	<i>Phyllobothrium delphini</i> *	AO	[194, 47]	<i>Anisakis typica</i>	AO	[194]	<i>Brachycladium palliatum</i>	AO	[318]
			<i>Strobilocephalus triangularis</i>	AO	[47]	<i>Halocercus delphini</i>	AO	[318]	<i>Braunina cordiformis</i>	AO	[318]
									<i>Pholeter gastrophilus</i>	AO	[318]
<i>Stenella longirostris</i> (Gray, 1828). Spinner dolphin											
<i>Bolbosoma balaenae</i>	PO	[68]	<i>Diphyllobothrium fuhrmanni</i>	PO	[76, 306]	<i>Anisakis simplex</i> s.l.*	PO	[68]	<i>Brachycladium nipponicum</i>	PO	[308, 76, 306]
<i>Bolbosoma hamiltoni</i>	PO	[7]	<i>Diphyllobothrium stemmacephalum</i>	PO	[26]	<i>Anisakis typica</i>	AO, PO	[7, 48, 129]	<i>Brachycladium pacificum</i>	PO	[76, 68, 67, 7, 207]
<i>Bolbosoma vasculosum</i> *	PO	[68]	<i>Monorygma grimaldii</i>	AO, PO	[68, 67, 55, 47]	<i>Halocercus brasiliensis</i>	AO	[47]	<i>Oschmarinella laevicaecum</i>	PO	[308, 76, 314, 306]
			<i>Phyllobothrium delphini</i> *	AO, PO	[68, 67, 47]	<i>Halocercus delphini</i>	PO	[68]	<i>Oschmarinella rochebruni</i>	PO	[68, 67, 7]
			<i>Scolex pleuronectis</i> *	AO, PO	[7, 47]				<i>Synthesium tursionis</i>	PO	[7]
			<i>Strobilocephalus triangularis</i>	AO, PO	[68, 67, 7, 47]						
			<i>Tetrabothrius forsteri</i>	AO, PO	[68, 67, 47]						
			<i>Trigonocotyle</i> sp.	AO	[47]						
<i>Steno bredanensis</i> (G. Cuvier in Lesson, 1828). Rough-toothed dolphin											
<i>Bolbosoma capitatum</i>	AO, MS	[31, 76, 313, 306, 115]	<i>Phyllobothrium delphini</i> *	AO	[55]	<i>Anisakis simplex</i> s.l.*	PO	[138, 73, 209, 147]	<i>Brachycladium palliatum</i>	AO	[107]
			<i>Strobilocephalus triangularis</i>	AO	[76, 306, 229]	<i>Anisakis simplex</i> s.s.	AO	[48]	<i>Nasitrema attenuatum</i>	AO	[95]
			<i>Tetrabothrius forsteri</i>	AO	[23, 31, 76, 306, 107]	<i>Anisakis typica</i>	AO	[107, 55, 48, 129]	<i>Nasitrema globicephalae</i>	AO	[95]
			<i>Trigonocotyle prudhoei</i> *	AO	[177, 306, 115]				<i>Synthesium tursionis</i>	AO	[107]
									<i>Braunina cordiformis</i>	AO	[253]
									<i>Pholeter gastrophilus</i>	AO	[107]

Table 4.2 (Continued)

Acanthocephala	Locality	Ref.	Cestoda	Locality	Ref.	Nematoda	Locality	Ref.	Trematoda	Locality	Ref.
<i>Tursiops aduncus</i> (Ehrenberg, 1833). Indo-Pacific bottlenose dolphin											
-	-	-	<i>Diphyllobothrium fuhrmanni</i>	RS	[142]	<i>Anisakis typica</i>	RS	[142]	<i>Brachycladium curilense</i>	RS	[142]
						<i>Crassicauda</i> sp.	IO	[151]	<i>Nasitrema attenuatum</i>	RS	[142]
						<i>Halocercus lagenorhynchi</i>	IO	[286]	<i>Pholeter gastrophilus</i>	RS	[142]
						<i>Stenurus ovatus</i>	IO	[286]			
<i>Tursiops truncatus</i> (Montagu, 1821). Common bottlenose dolphin											
<i>Bolbosoma</i> sp.	§	[40]	<i>Diphyllobothrium fuhrmanni</i>	PO	[257]	<i>Anisakis pegreffii</i>	MS, SA	[182, 39]	<i>Brachycladium atlanticum</i>	AO	[222]
<i>Corynosoma australe</i> *	AO	[250]	<i>Diphyllobothrium stemmacephalum</i>	BIS	[81, 86, 38]	<i>Anisakis simplex</i> s.l.*	AO, PO, BIS	[76, 115, 40, 1, 116, 38, 201, 250]	<i>Brachycladium delphini</i>	AO	[318]
<i>Corynosoma cetaceum</i>	AO, IO, BIS	[134, 76, 77, 313, 254, 306, 250]	<i>Monorygma grimaldii</i> *	AO, PO, MS, BIS	[31, 76, 77, 89, 306, 229, 298, 1, 116, 58, 207]	<i>Anisakis simplex</i> s.s.	AO	[48, 39]	<i>Brachycladium palliatum</i>	AO	[304, 318, 229]
			<i>Phyllobothrium delphini</i> *	AO, PO, MS, BIS	[31, 76, 77, 89, 263, 303, 306, 229, 298, 1, 116, 58, 55]	<i>Anisakis typica</i>	AO, PS, MS	[318, 73, 298, 184, 55, 48]	<i>Braunina cordiformis</i>	AO, PO, BIS	[76, 275, 255, 136, 314, 318, 171, 306, 118, 298, 253, 38, 250]
			<i>Scolex pleuronectis</i> *	AO	[17, 222]	<i>Contracaecum multipapillatum</i> *	AO	[48]	<i>Campula oblonga</i>	AO	[318]
			<i>Strobilocephalus triangularis</i>	MS	[222]	<i>Crassicauda crassicauda</i>	MS, BIS	[31, 76, 77, 162, 306]	<i>Nasitrema attenuatum</i>	AO	[145, 115]

Table 4.2 (Continued)

Acanthocephala	Locality	Ref.	Cestoda	Locality	Ref.	Nematoda	Locality	Ref.	Trematoda	Locality	Ref.
<i>Tursiops truncatus</i> (Continued)											
			<i>Tetrabothrius forsteri</i>	PO	[298, 222]	<i>Crassicauda grampicola</i>	BIS	[38]	<i>Nasitrema dalli</i>	SA	[115]
						<i>Halocercus delphini</i>	AO	[1, 190]	<i>Nasitrema delphini</i>	AO	[318]
						<i>Halocercus lagenorhynchi</i>	AO, PO, BIS	[135, 76, 77, 282, 304, 193, 318, 306, 115, 278]	<i>Nasitrema globicephalae</i>	PO	[298]
						<i>Pharurus alatus</i>	IO	[286]			
						<i>Pseudoterranova decipiens</i> *	AO	[250]	<i>Pholeter gastrophilus</i>	AO, PO, MS, BIS	[304, 193, 92, 115, 234, 229, 298, 38, 222, 250, 109]
						<i>Skrjabinalius cryptocephalus</i>	PO, BIS	[40, 190, 278]	<i>Synthesium tursionis</i>	AO, PO, MS, BIS	[31, 219, 76, 77, 306, 229, 298, 102, 101, 99, 194, 280, 38, 174, 222, 250]
						<i>Skrjabinalius guevarai</i>	MS	[111, 237, 190]			
						<i>Stenurus minor</i>	AO, PO, BIS	[190]			
						<i>Stenurus ovatus</i>	AO, PO, MS, BIS, IO	[31, 94, 75, 76, 77, 306, 115, 298, 278, 38, 286]			

Table 4.2 (Continued)

Acanthocephala	Locality	Ref.	Cestoda	Locality	Ref.	Nematoda	Locality	Ref.	Trematoda	Locality	Ref.
Family Iniidae											
<i>Inia geoffrensis</i> (Blainville, 1817). Amazon river dolphin											
-	-	-	-	-	-	<i>Anisakis insignis</i> *	AR	[31, 76, 152, 115, 163, 317]	<i>Hunterotrema caballeri</i> *	AR	[187, 152, 304, 317]
-	-	-	-	-	-	<i>Hunterotrema macrosoma</i> *	AR	[60]			
-	-	-	-	-	-	<i>Pholeter gastrophilus</i>	AR	[317]			
Family Kogliidae											
<i>Kogia breviceps</i> (Blainville, 1838). Pygmy sperm whale											
<i>Bolbosoma vasculosum</i> *	§	[215, 15]	<i>Monorygma grimaldii</i> *	AO, IO	[23, 31, 115, 229, 1, 47]	<i>Anisakis brevispiculata</i>	AO, SA	[2, 181, 48]	-	-	-
			<i>Phyllobothrium delphini</i> *	AO, MS [∞] , SA	[76, 263, 91, 303, 318, 306, 281, 115, 229, 1, 194, 55, 47]	<i>Anisakis paggiae</i>	AO, SA	[183, 48]			
			<i>Scolex pleuronectis</i> *	AO	[9]	<i>Anisakis physeteris</i>	AO, PO, SA	[115, 194, 147, 48]			
						<i>Anisakis simplex s.l.</i> *	AO, PO, SA	[296, 73, 115, 1, 147, 48]			
						<i>Anisakis simplex s.s.</i>	AO	[48]			
						<i>Anisakis typica</i>	AO, SA	[318, 115, 129]			
						<i>Pseudoterranova ceticola</i>	AO, PO	[194, 147, 2, 48]			
						<i>Pseudoterranova kogiae</i> *	SA	[76, 91, 306, 115]			
						<i>Crassicauda magna</i> *	AO, PO	[76, 162, 91, 306, 1]			

[∞]Single record of the host species in the Mediterranean Sea

Table 4.2 (Continued)

Acanthocephala	Locality Ref.	Cestoda	Locality Ref.	Nematoda	Locality Ref.	Trematoda	Locality Ref.
-	-	<i>Monorygma grimaldii</i> [*]	§ [17]	<i>Anisakis brevispiculata</i>	AO [55, 48, 221]	-	-
-	-	<i>Phyllobothrium delphini</i> [*]	AO, SA [318, 115, 55, 47]	<i>Anisakis paggiae</i>	AO [181, 55, 48, 221]	-	-
-	-	-	-	<i>Anisakis physeteris</i>	PO, SA [115, 147]	-	-
-	-	-	-	<i>Anisakis simplex</i> s.l. [*]	AO, PO, SA [115, 147, 48]	-	-
-	-	-	-	<i>Anisakis typica</i>	AO [318, 48, 221]	-	-
-	-	-	-	<i>Anisakis ziphidarum</i>	AO [221]	-	-
-	-	-	-	<i>Pseudoterranova ceticola</i>	AO, PO [147, 55, 48]	-	-
Family Monodontidae							
<i>Delphinapterus leucas</i> (Pallas, 1776). Beluga							
<i>Bolbosoma</i> sp.	AO [191]	<i>Diphylobothrium lanceolatum</i>	Arc [82, 80, 306]	<i>Anisakis simplex</i> s.l. [*]	AO, Arc [31, 164, 76, 73, 306, 115, 301, 191]	<i>Ochmeriella arctica</i> [*]	Arc [82, 141, 80, 314, 306, 292, 301]
<i>Corynosoma cameroni</i> [*]	AO, Arc [295, 203, 306, 293]	<i>Diphylobothrium phocarum</i> [*]	Arc [82, 306]	<i>Anisakis simplex</i> s.s.	AO [182]	<i>Orthosplanchnus sudarikovi</i> [*]	Arc [290, 306, 292, 293]
<i>Corynosoma wegeneri</i> [*]	Arc [203, 84]	<i>Monorygma grimaldii</i> [*]	§ [17]	<i>Crassicauda gilakiana</i>	PO [76, 162, 80, 306]	<i>Oschmarinella albamarina</i>	Arc [291, 314, 306, 292, 293]
<i>Corynosoma reductum</i> [*]	AO [295]	<i>Phyllobothrium delphini</i> [*]	§ [17]	<i>Halocercus monoceris</i>	AO, Arc [191, 148]	<i>Synthesium mironovi</i>	AO, PO [219, 144, 76, 141, 285, 80, 314, 306]
<i>Corynosoma semerme</i> [*]	PO, Arc [203, 171, 293, 301]	-	-	<i>Halocercus taurica</i>	AO [191, 148]	<i>Synthesium seymouri</i> [*]	AO, PO, Arc [31, 309, 76, 141, 80, 171, 306, 292, 293, 301, 191, 174]

Table 4.2 (Continued)

Acanthocephala	Locality Ref.	Cestoda	Locality Ref.	Nematoda	Locality Ref.	Trematoda	Locality Ref.
<i>Delphinapterus leucas</i> (Continued)							
<i>Corynosoma strumosum</i> *	AO, PO, Arc [164, 76, 203, 171, 306, 293, 301]		AO, PO, Arc [76, 306, 13, 293, 301, 191]	<i>Pharurus pallasi</i> *	AO, PO, Arc [76, 306, 13, 293, 301, 191]	<i>Synthesium subtile</i>	PO, Arc [261, 306, 292]
				<i>Porrocaecum</i> sp.*	AO [82, 256]		
				<i>Pseudoterranova decipiens</i> *	Arc [32, 80, 306]		
				<i>Stenurus arctomarinus</i> *	AO, Arc [306, 13, 293, 301, 191, 148]		
				<i>Stenurus minor</i>	AO [80, 306]		
Monodon monoceros Linnaeus, 1758. Narwhal							
				<i>Anisakis simplex</i> s.l.*	AO [31, 76, 73, 306]		
				<i>Pseudoterranova decipiens</i> *	AO [76, 306]		
				<i>Halocercus monoceris</i>	Arc [302, 165]		
				<i>Pharurus alatus</i>	Arc [31, 94, 76, 306, 13]		
Family Phocoenidae							
Neophocaena phocaenoides (G. Cuvier, 1829). Indo-Pacific finless porpoise							
<i>Corynosoma</i> sp.	PO [146]	<i>Diphylobothrium fuhrmanni</i>	PO, IO [306, 76, 133]	<i>Anisakis simplex</i> s.l.*	PO [147]	<i>Campula follum</i> *	PO [210, 76, 314, 306, 133]
		<i>Monorygma grimaldii</i> *	\$ [17]	<i>Anisakis typica</i>	\$ [133]	<i>Campula oblonga</i>	PO [146, 133]
		<i>Phyllobothrium delphini</i> *	\$ [17]	<i>Halocercus pingi</i>	PO [31, 94, 310, 76, 306, 146, 212, 133]	<i>Delphinicola tenuis</i>	PO [146, 133]

Table 4.2 (Continued)

Acanthocephala	Locality	Ref.	Cestoda	Locality	Ref.	Nematoda	Locality	Ref.	Trematoda	Locality	Ref.
<i>Neophocaena phocaenoides</i> (Continued)											
						<i>Halocercus sunameri</i> *	PO	[310, 306, 146, 133]	<i>Nasitrema spathulatum</i> *	PO	[210, 309, 76, 314, 306, 146, 133]
						<i>Halocercus taurica</i>	PO	[146, 133]	<i>Nasitrema sunameri</i> *	PO	[309, 314, 306, 146, 133]
						<i>Pharurus asiaeorientalis</i> *	PO, YR	[146, 133]	<i>Odhneriella elongata</i>	PO	[210, 314]
						<i>Pharurus sunameri</i> *	PO	[310, 306, 146, 133]	<i>Synthesium elongatum</i>	PO	[210, 76, 306, 133]
						<i>Stenurus auditivus</i>	PO	[94, 76, 306]	<i>Synthesium nipponicum</i>	PO	[146, 133]
						<i>Stenurus nanjingensis</i> *	PO	[133]	<i>Synthesium tursionis</i>	IO	[121, 133]
<i>Phocoena dioptrica</i> Lahille, 1912. Spectacled porpoise											
<i>Corynosoma australe</i> *	AO	[33]	<i>Scolex pleuronectis</i> *	AO	[33, 205]	<i>Anisakis simplex</i>	AO	[217, 33]	-	-	-
			<i>Tetrabothrius</i> sp.	AO	[205]	s.l.					
<i>Phocoena phocoena</i> (Linnaeus, 1758). Harbor porpoise											
<i>Bolbosoma capitatum</i>	AO	[116]	<i>Diphyllobothrium lanceolatum</i>	AO, BIS	[76, 77, 306, 229]	<i>Anisakis simplex</i> s.l.*	AO, PO, BIS, BaS	[31, 76, 77, 73, 306, 115, 53, 236, 170, 24, 41, 45, 125, 116, 1, 155, 153]	<i>Brachycladium nipponicum</i>	PO	[306]
<i>Corynosoma alaskensis</i> *	PO	[117, 171, 306]	<i>Diphyllobothrium latum</i> *	BIS	[76, 77, 306, 38]	<i>Anisakis simplex</i> s.s.	AO, PO	[182]	<i>Campula oblonga</i>	AO, PO, Arc, MS, BIS, BaS	[31, 219, 76, 77, 51, 314, 171, 306, 115, 229, 237, 53, 236, 170, 24, 41, 45, 249, 104, 116, 155, 109, 153]

Table 4.2 (Continued)

Acanthocephala	Locality Ref.	Cestoda	Locality Ref.	Nematoda	Locality Ref.	Trematoda	Locality Ref.
<i>Phocoena phocoena</i> (Continued)							
<i>Corynosoma semerme</i> *	[76, 77, 306]	<i>Diphyllobothrium phocarum</i> *	PO [240, 171]	<i>Anisakis typica</i>	AO, BIS [31, 76, 77, 73, 306, 184]	<i>Opisthorchis tenuicollis</i> *	AO, Arc [76]
<i>Corynosoma strumosum</i> *	[76, 77, 306]	<i>Diphyllobothrium stemmacephalum</i>	AO, BIS [31, 76, 77, 312, 79, 306, 53, 229, 41, 45, 125, 116, 38]	<i>Phocascaris</i> sp.*	AO [41]	<i>Pholeter gastrophilus</i>	AO, BIS, BaS [31, 76, 77, 306, 92, 115, 229, 24, 125, 116, 38, 153]
		<i>Monorygma grimaldii</i> *	AO [17, 155]	<i>Pseudoterranova decipiens</i> *	AO [76, 32, 306]	<i>Synthesium mironovi</i>	PO [69]
		<i>Phyllobothrium delphini</i> *	AO [17, 155]	<i>Porrocaecum</i> sp.*	AO [256]	<i>Synthesium nipponicum</i>	PO [51, 171, 69, 170]
		<i>Tetrabothrius</i> sp.	AO [41]	<i>Halocercus invaginatus</i>	AO, PO, Arc, BIS, BaS [94, 76, 77, 171, 306, 13, 115, 24, 195, 249, 1, 116, 38, 154, 155, 153, 214]		
				<i>Halocercus kirbyi</i>	PO [190]		
				<i>Halocercus taurica</i>	AO, PO, BIS [76, 77, 306, 13, 116, 38, 214]		
				<i>Pseudalium inflexus</i>	AO, PO, BIS, BaS [31, 94, 76, 77, 304, 306, 13, 115, 53, 236, 24, 45, 249, 116, 154, 153]		
				<i>Stenurus minor</i>	AO, PO, BIS, BaS [31, 76, 77, 171, 306, 13, 115, 237, 53, 236, 24, 45, 249, 116, 38, 154, 155, 153, 214]		
				<i>Torynurus convolutus</i>	AO, PO, BIS, BaS [31, 76, 77, 171, 306, 13, 115, 236, 24, 45, 249, 116, 154, 153]		
				<i>Hysterothylacium aduncum</i>	AO [125]		
				<i>Crassicauda</i> sp.	AO, PO [69, 170, 116]		

Table 4.2 (Continued)

Acanthocephala	Locality	Ref.	Cestoda	Locality	Ref.	Nematoda	Locality	Ref.	Trematoda	Locality	Ref.
-	-	-	-	-	-	-	-	-	<i>Synthesium tursionis</i>	PO	[150]
-	-	-	-	-	-	-	-	-	-	-	-
<i>Phocoena spinipinnis</i> Burmeister, 1865. <i>Burmeister's porpoise</i>											
<i>Corynosoma australe</i> *	AO, PO	[287, 33]	<i>Monorygma grimaldii</i> *	§	[17]	<i>Anisakis simplex</i> s.l.*	AO, PO	[287, 21, 33]	<i>Braunina cordiformis</i>	PO	[244]
<i>Corynosoma cetaceum</i>	AO, PO	[287, 57, 33]	<i>Phyllobothrium delphini</i> *	§	[17]	<i>Anisakis typica</i>	PO	[244]	<i>Nasitrema globicephalae</i>	PO	[244]
-	-	-	-	-	-	<i>Pseudalius inflexus</i>	AO, PO	[57, 244, 280, 50]	<i>Pholeter gastrophilus</i>	AO, PO	[57, 244, 33]
-	-	-	-	-	-	<i>Pseudoterranova</i> sp.	PO	[287]	<i>Synthesium tursionis</i>	PO	[287, 244, 280]
-	-	-	-	-	-	<i>Stenurus australis</i> *	AO, PO	[280, 190, 320, 201]	-	-	-
-	-	-	-	-	-	<i>Stenurus minor</i>	AO, PO	[57, 280]	-	-	-
<i>Phocoenoides dalli</i> (True, 1885). <i>Dall's porpoise</i>											
-	-	-	<i>Monorygma grimaldii</i> *	§	[17]	<i>Anisakis simplex</i> s.l.*	PO	[138, 61, 171, 115, 132, 170]	<i>Campula oblonga</i>	PO	[309, 51, 314, 61, 193, 306, 71, 56, 132, 170]
-	-	-	<i>Phyllobothrium delphini</i> *	PO	[17, 300]	<i>Crassicauda boopis</i>	PO	[279]	<i>Nasitrema dalli</i>	PO	[309, 314, 61, 306, 71, 132]
-	-	-	<i>Tetrabothis forsteri</i>	PO	[311, 306]	<i>Halocercus dalli</i> *	PO	[310, 306, 56, 132]	<i>Nasitrema delphini</i>	PO	[62]
-	-	-	-	-	-	<i>Halocercus kirbyi</i>	PO	[76, 171, 306, 115]	<i>Nasitrema globicephalae</i>	PO	[62]
-	-	-	-	-	-	<i>Halocercus pingi</i>	PO	[190]	<i>Synthesium nipponicum</i>	PO	[309, 314, 306]
-	-	-	<i>Placentonema</i> sp.	§	[132]	-	-	[132]	-	-	-
-	-	-	<i>Stenurus minor</i>	PO	[136, 193, 171, 62, 71, 132]	-	-	[136, 193, 171, 62, 71, 132]	-	-	-
-	-	-	<i>Stenurus truei</i> *	PO	[190]	-	-	[190]	-	-	-
-	-	-	<i>Stenurus yamagutii</i> *	PO	[190]	-	-	[190]	-	-	-
-	-	-	<i>Torynurus dalli</i> *	PO	[306, 13, 71, 132, 170]	-	-	[306, 13, 71, 132, 170]	-	-	-

Table 4.2 (Continued)

Acanthocephala	Locality	Ref.	Cestoda	Locality	Ref.	Nematoda	Locality	Ref.	Trematoda	Locality	Ref.
Family Physeteridae											
<i>Physeter macrocephalus</i> Linnaeus, 1758. Sperm whale											
<i>Bolbosoma brevicolle</i>	AO	[31, 76, 306, 84]	<i>Diphyllobothrium</i> sp.	Ant	[70]	<i>Anisakis pegreffii</i>	§	[182]	<i>Brachycladium curilense</i>	PO	[76, 314, 306, 84]
<i>Bolbosoma capitatum</i>	AO, MS, Ant	[31, 70, 76, 306, 84]	<i>Diplogonoporus</i> sp.	§	[84]	<i>Anisakis physeteris</i>	AO, PO, Ant, MS, SA	[31, 76, 172, 138, 73, 171, 306, 115, 170, 70, 200, 147, 181, 201, 33, 48]			
<i>Bolbosoma tuberculata</i>	AO, IO	[266, 273]	<i>Hexagonoporus calyptocephalus</i> *	PO	[306, 86]	<i>Anisakis simplex</i> s.l.*	AO, PO, SA	[76, 172, 138, 73, 171, 306, 115, 170]			
<i>Bolbosoma turbinella</i>	PO	[84, 306]	<i>Hexagonoporus giganteus</i> *	Ant	[264, 306, 273]	<i>Crassicauda anthonyi</i>	AO	[229]			
<i>Corynosoma bullosum</i> *	AO, Ant	[70, 33]	<i>Hexagonoporus physeteris</i> *	PO	[76, 306, 84]	<i>Placentonema gigantissimum</i> *	AO, PO	[76, 245, 263, 84, 171, 306, 115, 70, 170]			
<i>Corynosoma curilense</i> *	PO	[76, 306, 84]	<i>Monorygma grimaldii</i> *	PO, Ant	[269, 306, 115, 70]	<i>Stenurus ovatus</i>	AO	[229]			
<i>Corynosoma strumosum</i> *	PO	[84, 305]	<i>Multiductus physeteris</i> *	PO	[115]						
			<i>Phyllobothrium delphini</i>	AO, PO, Ant, MS, SA	[31, 76, 171, 243, 261, 302, 84, 268, 305, 170, 115, 227, 70, 116, 190, 47]						
			<i>Priapocephalus grandis</i>	AO, Ant	[243, 177, 52, 84, 306, 115, 229, 70]						
			<i>Scolex pleuronectis</i> *	PO	[177, 306, 9]						
			<i>Tentacularia</i> sp.*	§	[273]						
			<i>Tetrabothrius affinis</i>	AO, PO, Ant	[177, 84, 306, 115]						
			<i>Tetrabothrius curilensis</i> *	Ant	[76, 306, 277, 70]						
			<i>Tetrabothrius wilsoni</i>	Ant	[243, 54, 70]						
			<i>Trigonocotyle</i> sp.	PO	[76]						

Table 4.2 (Continued)

Acanthocephala	Locality	Ref.	Cestoda	Locality	Ref.	Nematoda	Locality	Ref.	Trematoda	Locality	Ref.
Family Platanistidae											
<i>Platanista gangetica</i> (Lebeck, 1801). South Asian river dolphin											
-	-	-	-	GR	[31, 76, 216, 65]	<i>Contracaecum lobulatum</i> *	GR	[31, 76, 216, 65]	<i>Campula oblonga</i>	GR	[31, 76, 65]
Family Pontoporiidae											
<i>Pontoporia blainvilliei</i> (Gervais and d'Orbigny, 1844). Franciscana											
<i>Bolbosoma turbinella</i>	AO	[10]	<i>Monorygma grimaldii</i>	AO	[17]	<i>Anisakis simplex</i> s.l.*	AO	[18, 21]	<i>Pholeter gastrophillus</i>	AO	[18]
<i>Corynosoma australe</i> *	AO	[10, 20]	<i>Phyllobothrium delphini</i> *	AO	[17]	<i>Anisakis typica</i>	AO	[139, 22, 10, 192]	<i>Synthesium pontoporiae</i> *	AO	[18, 228, 10, 175, 35, 174, 173]
<i>Corynosoma cetaceum</i>	AO	[254, 10, 19, 20]				<i>Procammallanus</i> sp.*	AO	[22]			
Family Ziphiidae											
<i>Berardius bairdii</i> Stejneger, 1883. Baird's beaked whale											
-	-	-	<i>Phyllobothrium delphini</i> *	PO	[245, 171]	<i>Anisakis simplex</i> s.l.*	PO	[172, 171, 306]	<i>Oschmarinella sobolevi</i>	PO	[306]
						<i>Crassicauda gilliakiana</i>	PO	[306, 279]			
						<i>Halocercus hyperooodni</i> *	PO	[190]			
Hyperooodon ampullatus (Forster, 1770). Northern bottlenose whale											
<i>Bolbosoma balaenae</i>	AO	[76, 306]	<i>Strobilocephalus triangularis</i>	AO	[23, 31, 76, 306, 229]	<i>Anisakis physeteris</i>	§	[73, 84]	<i>Oschmarinella sobolevi</i>	AO	[260, 76, 314]
<i>Bolbosoma turbinella</i>	AO	[31, 76, 270, 306]				<i>Anisakis simplex</i> s.l.*	AO	[31, 76, 73]			
						<i>Anisakis typica</i>	§	[137]			
						<i>Crassicauda gilliakiana</i>	§	[76]			
Hyperooodon planifrons Flower, 1882. Southern bottlenose whale											
-	-	-	-	-	-	<i>Crassicauda gilliakiana</i>	Ant	[306]	-	-	-

Table 4.2 (Continued)

Acanthocephala	Locality Ref.	Cestoda	Locality Ref.	Nematoda	Locality Ref.	Trematoda	Locality Ref.
<i>Bolbosoma vasculosum</i> *	AO, MS [295, 76, 306]	<i>Phyllobothrium delphini</i> <i>Strobilocephalus triangularis</i> <i>Tetرابothrius forsteri</i>	AO, MS [76, 263, 303, 306, 229] AO [76, 229] MS [∞] [23, 31, 76, 306, 229]	<i>Anisakis simplex</i> s.l.*	AO [31, 76, 73, 306, 115]	-	-
∞Single record of the host species in the Mediterranean Sea							
Mesoplodon bowdoini Andrews, 1908. Andrews' beaked whale							
-	-	-	-	<i>Anisakis nascettii</i> <i>Anisakis ziphidarum</i>	PO [185] PO [185]	-	-
Mesoplodon densirostris (Blainville, 1817). Blainville's beaked whale							
<i>Bolbosoma vasculosum</i> *	AO [318, 55]	<i>Phyllobothrium delphini</i> <i>Tetرابothrius</i> sp.	AO [318] AO [116]	<i>Anisakis simplex</i> s.l.* <i>Anisakis ziphidarum</i> <i>Crassicauda</i> sp.	AO [318, 116] SA [181] PO [279]	-	-
Mesoplodon europaeus (Gervais, 1855). Gervais' beaked whale							
-	-	<i>Monorygma grimaldii</i> <i>Phyllobothrium delphini</i> *	§ [17] § [17]	<i>Anisakis simplex</i> s.s. <i>Anisakis ziphidarum</i>	AO [48] AO [181, 48]	<i>Brachycladium parvulum</i> <i>Cetitrema meadi</i> *	AO [108] AO [64]
Mesoplodon grayi von Haast, 876. Gray's beaked whale							
-	-	<i>Phyllobothrium delphini</i> <i>Scolex pleuronectis</i> *	AO [33] AO [33]	<i>Anisakis nascettii</i> <i>Anisakis ziphidarum</i>	PO [185] § [181]	-	-
Mesoplodon hectori (Gray, 1871). Hector's beaked whale							
<i>Bolbosoma</i> sp.	AO [46]	<i>Tetرابothrius hobergi</i> *	AO [205]	<i>Anisakis</i> sp.	AO [46]	<i>Braunina cordiformis</i>	AO [46]
Mesoplodon layardii (Gray, 1865). Layard's beaked whale							
-	-	-	-	<i>Anisakis nascettii</i> <i>Anisakis berlandi</i> <i>Anisakis ziphidarum</i>	PO [185] SA [182] SA [185]	-	-

Table 4.2 (Continued)

Acanthocephala	Locality	Ref.	Cestoda	Locality	Ref.	Nematoda	Locality	Ref.	Trematoda	Locality	Ref.
			Mesoplodon mirus True, 1913. True's beaked whale								
-	-	-	<i>Monorygma grimaldii</i>	§	[17]	<i>Anisakis nascettii</i>	SA	[185]	-	-	-
			<i>Phyllobothrium delphini</i> *	§	[17]	<i>Anisakis simplex</i> s.l.*	SA	[115]			
						<i>Crassicauda anthonyi</i>	AO	[49, 306]			
			Mesoplodon stejnegeri True, 1885. Stejneger's beaked whale								
-	-	-	<i>Tetraphothrius forsteri</i>	Arc	[259]	<i>Crassicauda</i> sp.	PO	[279]	<i>Oschmarinella macrorchis</i>	PO	[87]
			Ziphius cavirostris G. Cuvier, 1823. Cuvier's beaked whale								
<i>Bolbosoma vasculosum</i> *	MS	[100]	<i>Monorygma grimaldii</i>	AO	[55]	<i>Anisakis pegreffii</i>	MS	[182, 39]	<i>Oschmarinella albamarina</i>	PO	[207]
			<i>Phyllobothrium delphini</i> *	AO	[194, 55]	<i>Anisakis physeteris</i>	MS	[162, 39]			
			<i>Scolex pleuronectis</i> *	MS	[9]	<i>Anisakis simplex</i> s.l.*	PO	[147]			
			<i>Tetraphothrius</i> sp.	MS	[100]	<i>Anisakis typica</i>	MS	[229]			
						<i>Anisakis ziphidarum</i>	AO, MS, SA	[181, 55]			
						<i>Crassicauda anthonyi</i>	AO, PO, MS	[49, 90, 306, 194, 55, 207]			
						<i>Crassicauda boopis</i>	MS	[31, 162]			
						<i>Crassicauda crassicauda</i>	AO, MS	[76, 162, 115]			

No helminths reported from the following cetacean species:

Family Balaenidae, North Pacific right whale, *Eubalaena japonica* (Lacépède, 1818).

Family Balaenopteridae, Antarctic minke whale, *Balaenoptera bonaerensis* Burmeister, 1867.

Family Delphinidae, Australian snubfin dolphin, *Orcaella heinsohni* Beasley, Robertson and Arnold, 2005.

Indian Ocean humpback dolphin, *Sousa plumbea* (G. Cuvier, 1829).

Family Lipotidae, Baiji, *Lipotes vexillifer* (Miller, 1918).

Family Phocoenidae, Narrow-rigged finless porpoise, *Neophocaena asiakorintalis* (Pilleri and Gühr, 1972).

Family Ziphiidae, Arnoux' beaked whale, *Berardius arnuxi* Duvernoy, 1851.

Ginkgo-toothed beaked whale, *Mesoplodon ginkgodens* Nishiwaki and Kamiya, 1958.

Perrin's beaked whale, *Mesoplodon perrini* Dalebout, Mead, Baker, Baker and van Heiden, 2002.

Pygmy beaked whale, *Mesoplodon peruvianus* Reyes, Mead and Van Waerebeek, 1991.

Shepherd's beaked whale, *Tasmacetus shepherdi* Oliver, 1937.

Omura's whale, *Balaenoptera omurai* Wada, Oishi and Yamada, 2003.

Atlantic humpback dolphin, *Sousa teuszii* (Kükenthal, 1892).

Australian humpback dolphin, *Sousa sahulensis*.

Longman's beaked whale, *Indopacetus pacificus* (Longman, 1926).

Hubbs' beaked whale, *Mesoplodon carlhubbsi* Moore, 1963.

Deraniyagala's beaked whale, *Mesoplodon hotaula* Deraniyagala, 1963.

Spade-toothed whale, *Mesoplodon traversii* (Gray, 1874).

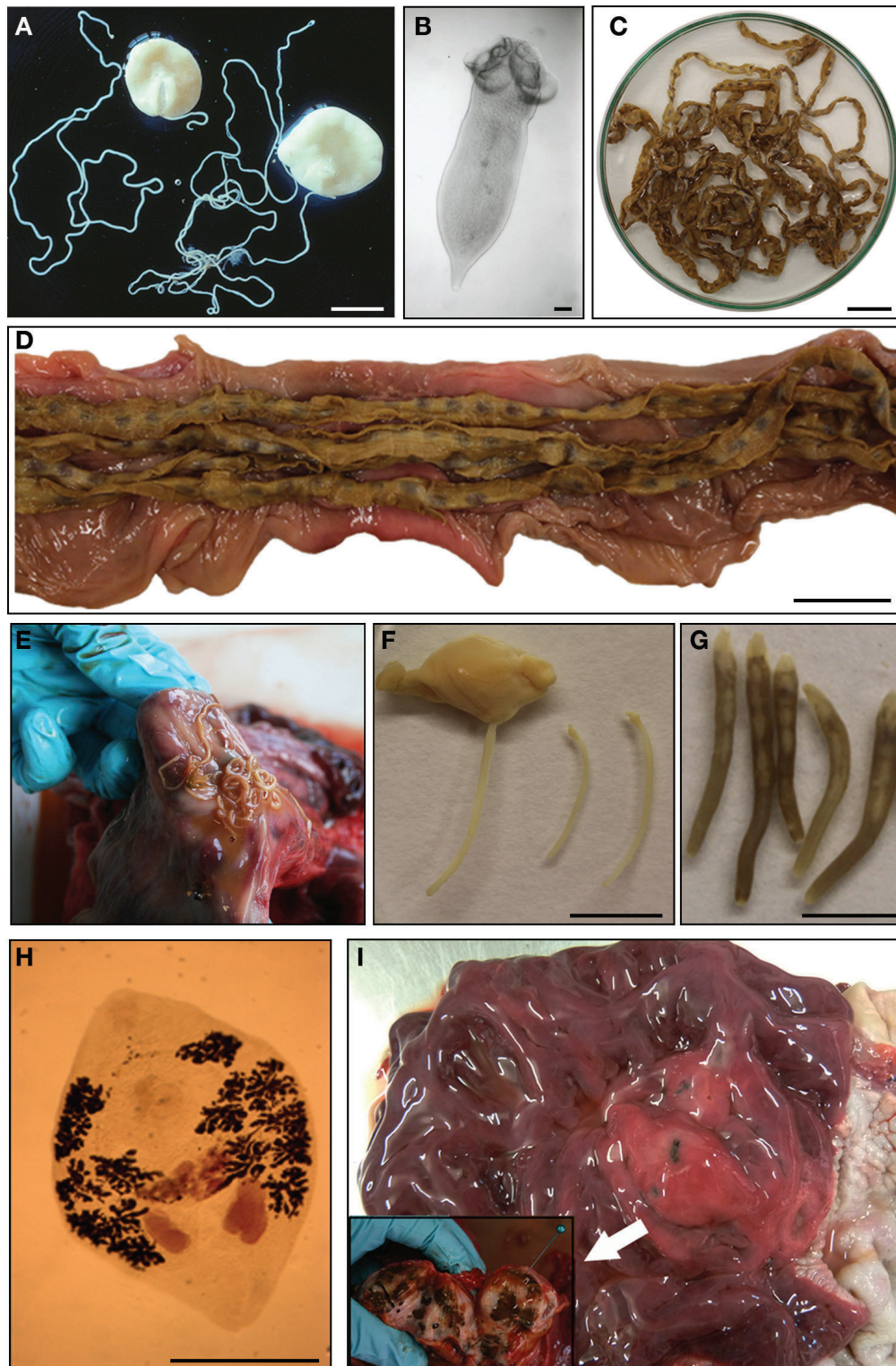


Figure 4.1. Representatives of helminth species in cetaceans. **A)** *Monorygma grimaldii* (Cestoda) and its capsule. Scale bar: 1 cm. **B)** *Scolex pleuronectis* (Cestoda). Scale bar: 0.1 mm. **C)** and **D)** *Diphyllobothrium* sp. (Cestoda) in the intestine of a bottlenose dolphin, *Tursiops truncatus*. Scale bars: 2 cm. **E)** *Anisakis simplex* sensu lato (Nematoda) in the stomach of a striped dolphin, *Stenella coeruleoalba*. **F)** Two specimens of *Bolbosoma capitatum* (Acanthocephala) and one embedded in host tissue. Scale bar: 1 cm. **G)** *Oschmarinella rochebruni* (Digenea). Scale bar: 1 cm. **H)** *Pholeter gastrophilus* (Digenea). Scale bar: 1 cm. **I)** Cyst of *P. gastrophilus* in the stomach of a striped dolphin.

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A NEW BRACHYCLADIID SPECIES (DIGENEA) FROM GERVAIS' BEAKED WHALE *Mesoplodon europaeus* IN NORTH-WESTERN ATLANTIC WATERS

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ABSTRACT

A new species of the digenean family Brachycladiidae Odhner, 1905 is described from the bile ducts of a Gervais' beaked whale *Mesoplodon europaeus* Gervais (Ziphiidae) stranded on the North Atlantic coast of Florida. These parasites were assigned to *Brachycladium* Looss, 1899 and differed from other species of the genus in the relative size of the oral and ventral suckers, the form and size of the eggs and their extremely small body size. A canonical discriminant analysis was used to examine differences between these specimens and the smallest available individuals of *B. atlanticum* (Abril, Balbuena *et* Raga, 1991) Gibson, 2005, considered the morphologically closest species. The overall results indicated significant differences between the two samples and a jack-knife classification showed that 96.2% of the specimens were correctly classified to their group. In view of evidence from morphological data, the specimens from *M. europaeus* are considered as new to science and are designated as *Brachycladium parvulum* n. sp.

INTRODUCTION

Beaked whales (family Ziphiidae Gray) of the genus *Mesoplodon* Gervais are among the least known groups of cetaceans, and most of the data on their biology have been obtained from stranded animals (Pitman, 2009). Gervais' beaked whale, *Mesoplodon europaeus* Gervais, inhabits deep waters of the western North Atlantic, where it feeds mainly on mesopelagic cephalopods and fish. Most reports of this species come from stranding events on the Atlantic coast of Florida and North Carolina, where this species occurs regularly. However, there are also occasional records in the Eastern Atlantic on the coasts of France, Spain (including the Canary Islands) and West Africa (Norman and Mead, 2001, and references therein). Parasitological data from *M. europaeus* are scarce, being limited to reports of the larval cestode *Phyllobothrium delphini* Bosc, 1802, nematode species of the genus *Anisakis* Dujardin, 1845, the acanthocephalan *Bolbosoma vasculosum* (Rudolphi, 1819) (Martin et al., 1990; Raga, 1994) and the brachycladiid digenean *Cetitrema meadi* Dailey, 2007. The latter was reported from *M. europaeus* off the Atlantic coast of North Carolina (Dailey, 2007).

During early 1976 an adult male Gervais' beaked whale was found stranded near Fort Pierce on the North Atlantic coast of Florida. Its organs were processed and numerous mature specimens of a digenean trematode were collected from the liver. These flukes were assigned to the family Brachycladiidae Odhner, 1905 (=Campulidae Odhner, 1926) and keyed down to *Brachycladium* Looss, 1899, a genus with no previous records from any ziphiid species. The present study describes these specimens as belonging to a new species of this genus based on morphological analyses.

We also tried to perform molecular analyses, but DNA could not be extracted or amplified likely because worms had been preserved for more than twenty years in 70% ethanol. We attempted three different protocols to extract genomic DNA and amplify the second internal transcribed spacer (ITS2), i.e., i) extraction of gDNA with a modified protocol using CTAB extraction buffer, ii) the QIAGEN DNeasy Blood and Tissue Kit, and iii) a standard phenol-chloroform protocol. Amplification of DNA was attempted with primers used before for digeneans (Miller and Cribb, 2007; Blasco-Costa et al., 2010). All attempts were unsuccessful.

MATERIALS AND METHODS

Sample collection

On the 7th of January, 1976 an adult male Gervais' beaked whale (4.13 m long) was stranded near Fort Pierce Inlet, Saint Lucie County, Florida, USA. We have no information on its condition. The necropsy was performed by Dr Daniel K. Odell, who inspected the liver for parasites. Eighty-eight digeneans were collected and fixed in 70% ethanol. This material was kindly passed to the Marine Zoology Unit, Cavanilles Institute of Biodiversity and Evolutionary Biology, Science Park, University of Valencia, for study.

Morphological analyses

Seventeen specimens were stained with iron acetocarmine and differentiated using HCl in 70% ethanol. Specimens were dehydrated through a graded ethanol series, cleared with dimethyl phthalate and mounted as permanent preparations in Canada balsam. Serial sections (10 µm) were cut from three specimens and stained in Mayer's haematoxylin and eosin (the stain was not taken up well, making the sections difficult, but not impossible, to interpret). A drawing of each worm was made with the aid of a microscope drawing tube, and diagnostic morphometric measurements were obtained using the program Image Tool v.3.0 (University of Texas Health Science Centre at San Antonio; downloaded from <http://compdent.uthscsa.edu/dig/download.html>). This program uses a spatial calibration based on a known measurement and calculates the number of pixels contained in a polygon defined by the shape of the structure to be measured.

The specimens from Gervais' beaked whale were morphologically similar to some of the specimens of *Brachycladium atlanticum* (Abril, Balbuena *et* Raga, 1991) Gibson, 2005 reported by Mateu *et al.* (2011) in the western Mediterranean. These authors described two forms of *B. atlanticum*: "normal-size" individuals from striped dolphins *Stenella coeruleoalba* Meyen and "dwarf" individuals from common dolphins *Delphinus delphis*. We had access to these samples and the worms were processed and measured in the same way as our specimens. A canonical discriminant analysis (DA) was used to investigate morphometric differences between "dwarf" specimens of *B. atlanticum* from common dolphins and specimens from Gervais' beaked whale. Given that the overall sample was small ($n = 26$), only four discriminating variables were selected to avoid the risk of over-fitting, i.e. body area, oral sucker area, pharynx area and testes area, which could be measured in all specimens. Jack-knife cross-validation was used to ensure the stability of discrimination. The DA was performed with SPSS v. 19.

***Brachycladium parvulum* n. sp.** (Figures 5.1 – 5.4)

Type-host: Gervais' beaked whale, *Mesoplodon europaeus* Gervais (Ziphiidae).

Site: Bile ducts.

Locality: Fort Pierce Inlet, North Atlantic Ocean, off St. Lucie County, Florida, USA.

Type-material: Holotype and paratypes deposited in the Natural History Museum, London, UK (BMNH 2013.7.19.1 holotype, 2013.7.19. 2–14 paratypes); vouchers at the Marine Zoology Unit at the Cavanilles Institute of Biodiversity and Evolutionary Biology, University of Valencia (11676, UV/Zoomar/EF2_11597–11599).

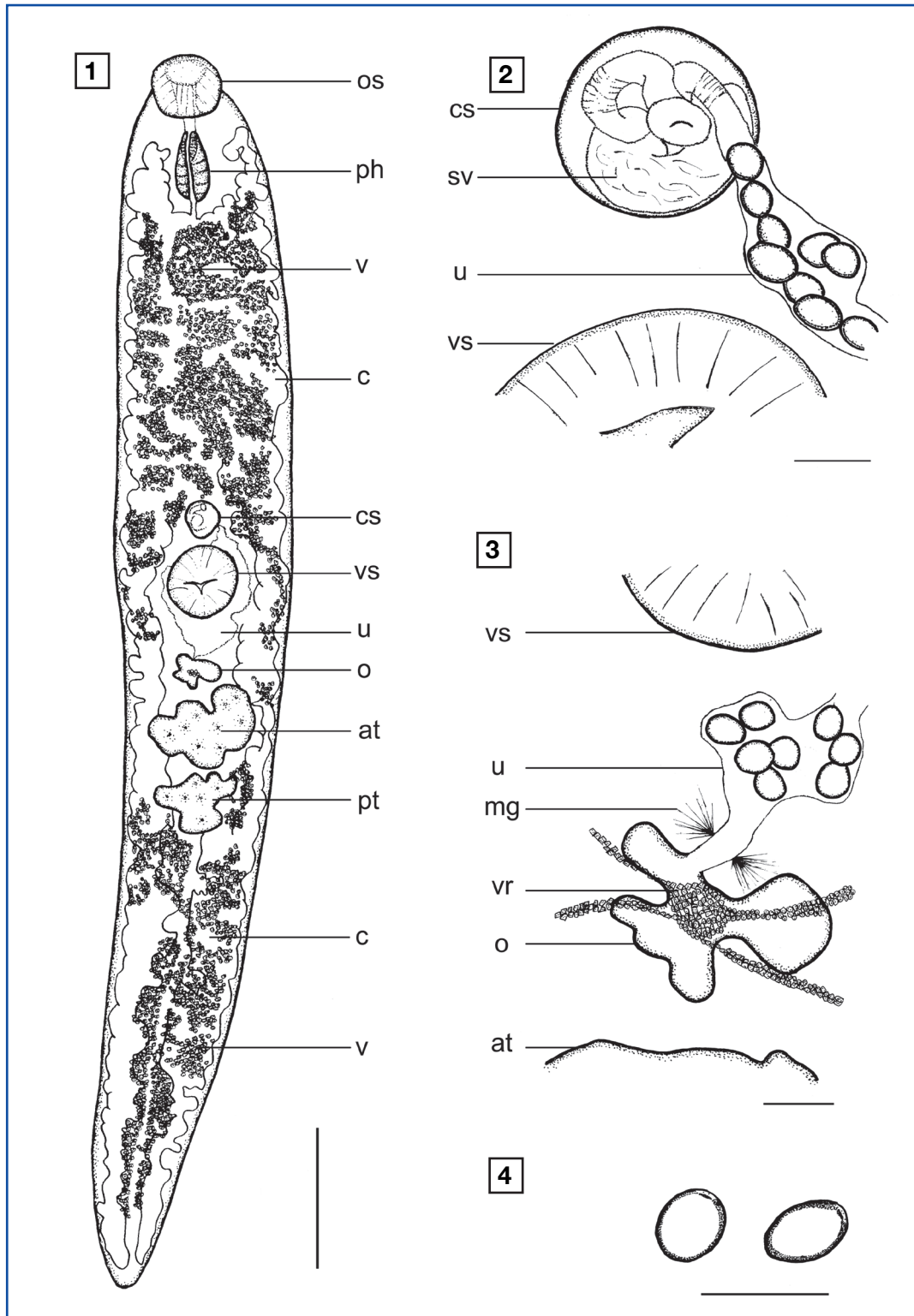
Etymology: The appellation "parvulum", a Latin adjective meaning very small, is appended to the new taxon, reflecting the fact that it is the smallest species of the genus.

DESCRIPTION

[Observations and measurements based on 17 whole-mounted and 3 serially sectioned specimens. Measurements (length × width) are shown as the range, with the mean in parentheses followed by the standard deviation, and are expressed in micrometres, unless otherwise stated; see Table 5.1]. Body small, elongate, fusiform, relatively stout, dorso-ventrally flattened, 4.9–8.4 (6.4 ± 0.9) × 0.9–1.2 (1.1 ± 0.1) mm. Anterior extremity more rounded than posterior. Body spines apparently lost. Forebody long, but shorter than hindbody. Oral sucker ventro-subterminal, 360–530 (427 ± 53) × 330–520 (405 ± 53). Ventral sucker located in anterior region of middle third of body, similar in size to oral sucker, 390–550 (449 ± 63) × 360–530 (425 ± 59). Oral sucker/ventral sucker length ratio 1:1.05. No distinct prepharyngeal pouch, but pharynx often protruding forwards into lumen of distinct prepharynx. Pharynx pyriform, 350–520 (437 ± 45) × 170–260 (214 ± 28). Oesophagus short. Intestine branches, giving rise to anterior and posterior caeca on each side of body; anterior caeca reach level of anterior margin of pharynx; posterior caeca reach close to posterior extremity of body; all caeca exhibit deep, globular diverticula on median and lateral sides. Uroproct absent.

Testes large, deeply lobed to elliptical, with or without notches, tandem, located in posterior region of middle third of body, irregular in shape; maximum dimensions: anterior testis 360–840 (623 ± 133) × 220–630 (379 ± 106); posterior testis 280–780 (570 ± 126) × 210–700 (482 ± 130). Cirrus-sac short, usually antero-dorsal to ventral sucker, contains seminal vesicle, pars prostatica and ejaculatory duct. Seminal vesicle long, broad, saccular, unipartite, forms single large loop occupying most of cirrus-sac. Wide pars prostatica and ejaculatory duct present. Cirrus small, papilla-like, apparently unarmed. Genital atrium small. Genital pore ventro-medial in first part of second third of body, just anterior to ventral sucker.

Ovary lobed, irregular in form, median, located between ventral sucker and anterior testis, 190–400 (269 ± 72) × 110–240 (170 ± 44). Uterine seminal receptacle and Laurer's canal present. Mehlis' gland, with widely dispersed gland cells, surrounds oviduct. Uterus coiled, intercaecal, dorsal to ventral sucker, extends between anterior region of vitelline reservoir and genital pore. Metraterm present, apparently unarmed, opens into genital atrium. Eggs oval, 49–61 (53 ± 4) × 35–49 (41 ± 3), round in cross-section. Vitellarium follicular; follicles arranged in dendritic, moniliform system extending through-out most of region between levels of posterior margin of pharynx and c.75% of post-testicular field; lateral fields confluent in forebody and in post-testicular region. Lateral vitelline collecting ducts extend throughout length of vitellarium, uniting to form small vitelline reservoir just posterior to ovary. Excretory pore terminal; excretory vesicle l-shaped, reaches anterior testis, where it gives rise to main lateral collecting ducts which pass ventrally to caeca into forebody.



Figures 5.1–5.4. *Brachycladium parvulum* n. sp. from *Mesoplodon europaeus*. **5.1.** Paratype, ventral view. **5.2.** Detail of male reproductive system. **5.3.** Detail of female reproductive system. **5.4.** Detail of eggs. Scale-bars: 1 mm (5.1), 0.1 mm (5.2, 5.3 and 5.4). Abbreviations: at anterior testis, c caeca, cs cirrus sac, mg Mehlis' gland, o ovary, os oral sucker, ph pharynx, pt posterior testis, sv seminal vesicle, u uterus, v vitelline follicles, vr vitelline reservoir, vs ventral sucker.

Remarks

The specimens here described can be assigned to the genus *Brachycladium* based on the following characters (Gibson, 2005): vitellarium reaching at least as far as the ventral sucker and extensive in the forebody; prepharyngeal pouch and uroproct absent; intestinal caeca diverticulate; cirrus and metraterm unarmed; ovary lobed; and forebody long, with ventral sucker in middle third of body.

Eight species are currently included in *Brachycladium*, i.e. *B. delphini* (Poirier, 1886) Looss, 1899, *B. nipponicum* (Yamaguti, 1942) n. comb.¹, *B. atlanticum*, *B. pacificum* (Dailey and Perrin, 1973) Gibson, 2005, *B. curilense* (Gubanov in Delyamure, 1955) Dailey, 2007, *B. goliath* (van Beneden, 1858) n. comb. (syn. *B. spinosum* [Margolis and Pike, 1955] Dailey, 2007), *B. petrowi* (Skrjabin, 1970) n. comb. and *B. palliatum* (Looss, 1885) Looss, 1899. Specimens from *B. parvulum* n. sp. can be distinguished morphologically from other species of the genus by a combination of the relative size of the oral and ventral suckers, the size and shape of the eggs and body size (Table 5.1). It differs from all species, except *B. delphini*, *B. nipponicum*, *B. atlanticum*, *B. pacificum* and *B. curilense*, in that the oral and ventral suckers are similar in size, and from all species, except for *B. curilense*, *B. pacificum* and *B. atlanticum*, in that the eggs are round in cross-section rather than triangular. In terms of general morphology, size and distribution, *B. atlanticum* appears to be the species most similar to *B. parvulum*.

Specimens of *B. parvulum* n. sp. are the smallest of all *Brachycladium* spp. (Table 5.1). However, dwarfism has been recorded in brachycladiids, including *B. atlanticum*, occurring in unusual host species (Adams et al., 1998; Mateu et al., 2011). Body areas, obtained from the available material of Mateu et al. (2011), were $26.42 \pm 8.73 \text{ mm}^2$ for specimens, which these authors described as “normal” *B. atlanticum* (ex *Stenella coeruleoalba*) and $15.49 \pm 6.82 \text{ mm}^2$ for specimens, which they described as “dwarf” *B. atlanticum* (ex *Delphinus delphis*). The discriminant analysis between “dwarf” specimens of *B. atlanticum* and specimens of *B. parvulum* indicated that there are significant morphometrical differences between these two species (Wilk’s Lambda = 0.201; F = 35.349, 4 df, $p < 0.005$) (Table 5.2). The jack-knife classification showed that 96.2% of the specimens were correctly classified to their group (Figure 5.5).

¹- Note that some of the combinations have been implied but have not previously been made in print.

Table 5.1. Mean (range) of morphological measurements of *Brachycladium parvulum* n. sp. compared to other members of the genus. Measurements are given as length × breadth in micrometres unless otherwise stated.

Species	<i>B. parvulum</i> n. sp.	<i>B. palliatum</i>	<i>B. delphini</i>	<i>B. goliath</i>	<i>B. nipponicum</i>	<i>B. petrowi</i>	<i>B. curilense</i>	<i>B. atlanticum</i>	<i>B. pacificum</i>
Source		(after Delyamure, 1955)	(after Skrijabin, 1948)	(after Skrijabin, K.L., 1948 and Skrijabin, A.S., 1970)	(after Yamaguti, 1942)	(after Skrijabin, 1970)	(after Delyamure, 1955)	(after Abril et al., 1991)	(after Dailey and Perrin, 1973)
No. of specimens	(n= 17)	(n= 72)	(n=2 ⁺⁺)	(n= 2)	(n= 2)	(n= 17)	(n=2 ⁺⁺⁺)	(n= 4)	(n= 34)
Body length (mm)	6.4 (4.9–8.4)	(10.8–18.7)	14	(60–90)	(9–27)	(25–40)	(18–22.5)	20.2 (18.8–24.1)	10 (8.3–12.6)
Body width (mm)	1.1 (0.9–1.2)	(2.5–3.1)	2	(8–9)	(1.4–3.3)	(5.7–7.2)	(5–7)	2.6 (2.4–3.1)	1.7 (1.4–2.0)
Oral sucker	427 (360–530) × 405 (330–520)	509–622 × 810–940	700 ⁺	2,300 ⁺	600–1,250 × 800–1,600	1,200–1,320 ⁺	200 × 600–640	1,096 (951–1,208) × 1,111 (1,002–1,237)	535 (398–592) × 575 (439–673)
Ventral sucker	449 (390–550) × 425 (360–530)	420–890 × 520–950	700 ⁺	1,300–1,800 ⁺	600–1,450 ⁺	1,110–1,220 ⁺	800–900 ⁺	1,137 (925–1,413) × 1,041 (882–1,285)	600 ⁺ (520–710)
Position of ventral sucker	Anterior of 2/3 of body	Anterior of 2/3 of body	Middle of 2/3 of body	Anterior of 2/3 of body	Anterior of 2/3 of body	Anterior of 2/3 of body	Middle of 1/3 of body	Anterior of 2/3 of body	Anterior of 2/3 of body
Sucker length ratio ⁺⁺⁺	1: 1.05	1: 0.82	1: 0.56	1: 1	1: 1	1: 0.92	1: 1.33	1: 1.04	1: 1.12
Pharynx	437 (350–520) × 214 (170–260)	460–610 × 420–830	700 × 400	1,500 × 700–950	410–1,000 × 330–750	1,020–1,070 × 680– 760	540–560 × 400–440	639 (518–659) × 423 (391–464)	494 (398–541) × 314 (255–367)
Ovary	269 (190–400) × 170 (110–240)	339–377 × 829–999	1,050–1,400 × 1,800–2,100	270–820 × 500– 1250	930–1360 × 1340– 1700	500–1,200 × 1,500–1,900	694 (591 874) × 990 (889–1,156)	333 (235–418) × 477 (310–765)	
Anterior testis	623 (360–840) × 379 (220–630)	720–830 × 1,030–1,830	6,300–7,900 × 6,300–6,800	500–2,600 × 650– 2,500	2,700–4,700 × 3,300–4,800	2,000–5,000 × 2,500–4,100	1,658 (1,440–979) × 1,619 (1,516–1,850)		
Posterior testis	570 (280–780) × 482 (210–700)	810–1160 × 1500–1630	60 × 45	60–72 × 45–54	2,400–6,000 × 2,600–3,600	2,549 (2,008–3,224) × 1,552 (1,184–1,669)			
Eggs	53 (49–61) × 41 (35–49)	63–66 × 36–47	104–120 × 62–75	60–72 × 45–54	80–90 × 50–60	68–78 × 45–53	58 (54–61) × 43 (40–47)		61 × 42
Shape of eggs in cross-section	Circular	Triangular	Triangular	Triangular	Triangular	Triangular	Circular	Circular	Circular

⁺ Diameter of sucker. ⁺⁺ No information available. ⁺⁺⁺ Ratio obtained using the length of oral and ventral suckers, except for *B. curilense*, where the width of the suckers was used instead, since, in the original description, the length of the oral sucker does not resemble that in the illustration of the specimen (see Delyamure, 1955).

DISCUSSION

The Brachycladiidae is a relatively small family of digeneans, the members of which occur worldwide, mainly in the hepatic ducts, intestine, air sinuses and lungs of marine mammals (Delyamure, 1955; Gibson, 2005). Its taxonomy has been controversial, in part because specimens, often in poor condition, are collected from long-dead, stranded or frozen hosts (Gibson, 2002; 2005). The group was first considered as a sub-family, the Brachycladiinae Odhner, 1905, by Odhner (1905) based on *Brachycladium palliatum*; later Faust (1929) raised the Brachycladiinae to full family status. In between, Odhner (1926) used the name Campulidae, considering that *Brachycladium* was a synonym of *Campula* Cobbold, 1858. Recently, Gibson (2005) recognized *Brachycladium* as a valid genus distinct from *Campula* and accepted Brachycladiidae as the family name on the basis of priority. In his revision of the family, Gibson (2005) recognized two subfamilies and 10 genera: the Brachycladiinae, including *Brachycladium*, *Campula*, *Hunterotrema* McIntosh, 1960, *Odhneriella* Skrjabin, 1915, *Oschmarinella* Skrjabin, 1947, *Orthosplanchnus* Odhner, 1905, *Synthesium* Stunkard and Alvey, 1930 and *Zalophotrema* Stunkard and Alvey, 1929; and the Nasitrematinae Ozaki, 1935, including *Nasitrema* Ozaki, 1935 and *Cetitrema* Skrjabin, 1970.

The relative size of the oral and ventral suckers has been considered a diagnostic trait for differentiating brachycladiid species (Delyamure, 1955; Gibson, 2005) and indeed for digeneans in general. This criterion is supported by studies on the static allometry of brachycladiids, suggesting that the relationship between the dimensions of the suckers is proportional (Fernández et al., 1995). Neiland et al. (1970) used body size as a criterion for differentiating species of *Nasitrema*. These authors indicated that this feature could vary among the various species within 50% of the mean. However, body size has not always proved to be a reliable diagnostic character in brachycladiids, because dwarfism has been reported when a species infects an unusual host (Adams et al., 1998; Mateu et al., 2011). Although we consider that body size must be used with caution as a diagnostic character in isolation for differentiating *Brachycladium* spp., the huge range of body (and organ) sizes within the genus suggests that there must be limits to host-induced differences. Note that *B. goliath*, which is 60–90 mm in length (Skrjabin, 1948), is 7–8 times larger than *B. pacificum*, which measures 8.3–12.6 mm (Delyamure, 1955; Dailey and Perrin, 1973) (Table 5.1). We consider that the extremely small body size of *B. parvulum* n. sp. is probably diagnostic, because it is consistent with other morphological differences used for differentiating most species of *Brachycladium*.

The eight previously known species of *Brachycladium* infect a wide array of cetaceans: two are apparently specific to baleen whales, i.e. *B. goliath* and *B. petrowi*, and six have been reported only from toothed whales, i.e. *B. delphini*, *B. palliatum*, *B. nipponicum*, *B. atlanticum* and *B. pacificum* from the Delphinoidea, and *B. curilense* from the Physeteroidea. The new species, *B. parvulum*, is the first member of the genus to be reported from the Ziphioidea.

Species of *Brachycladium* have a wide geographical range, occurring extensively in the Pacific and Atlantic Oceans. In the Pacific Ocean, five species are known, i.e. *B. nipponicum*, *B.*

curilense, *B. petrowi*, *B. pacificum* and *B. goliath*, some of them, i.e. *B. curilense* and *B. petrowi*, from single records (Yamaguti, 1942; Delyamure, 1955; Skrjabin, 1970; Dailey and Perrin, 1973; Gibson and Harris, 1979). In the Atlantic Ocean, four species have been reported, i.e. *B. goliath*, *B. delphini*, *B. palliatum* and *B. atlanticum* (Margolis and Pike, 1955; Zam, 1971; Raga and

Table 5.2. Sizes of various areas (in mm² unless otherwise stated) of “dwarf specimens” of *Brachycladium atlanticum* and specimens of *B. parvulum* n. sp.

	<i>Brachycladium atlanticum</i> ex <i>Delphinus delphis</i> n = 16		<i>Brachycladium parvulum</i> n. sp. ex <i>Mesoplodon europaeus</i> n = 17	
	Mean (S.D.)	Range	Mean (S.D.)	Range
Body length (mm)	11.35 (2.23)	8.21–15.11	6.36 (0.93)	4.88–8.42
Body width (mm)	1.7 (0.43)	1.14–2.71	1.09 (0.09)	0.88–1.22
Body area ⁺	15.49 (6.82)	8.25–30.35	5.45 (1.35)	3.18–8.39
Oral sucker area ⁺	0.19 (0.03)	0.14–0.25	0.13 (0.03)	0.09–0.20
Ventral sucker area	0.25 (0.05)	0.19–0.35	0.15 (0.04)	0.11–0.22
Ovary area	0.09 (0.03)	0.05–0.13	0.03 (0.02)	0.01–0.05
Pharynx area ⁺	0.12 (0.02)	0.09–0.17	0.07 (0.02)	0.04–0.1
Testes area ⁺	0.37 (0.1)	0.19–0.55	0.16 (0.06)	0.05–0.27
Egg area (µm ²)	1599 (154)	1320–1948	1682 (208)	1472–2268

⁺ Organs used for the comparison between the species in the discriminant analysis.

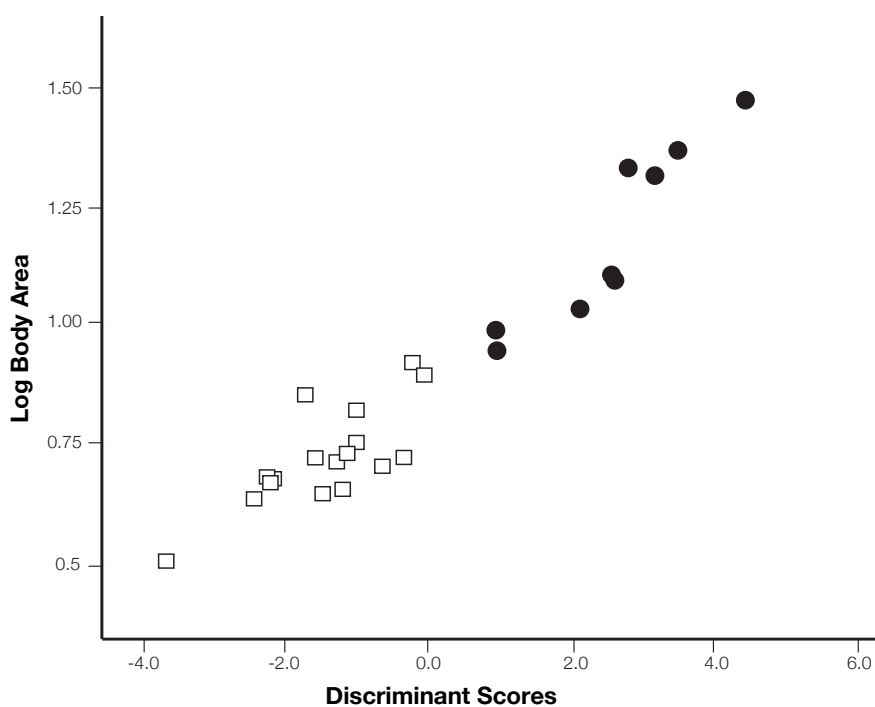


Figure 5.5. Scatterplot showing discriminant scores from the function (x-axis) plotted against the log₁₀-transformed body area (y-axis) from “dwarf” specimens of *Brachycladium atlanticum* from *Delphinus delphis* (circles) and specimens of *B. parvulum* n. sp. from *Mesoplodon europaeus* (squares). Standardized canonical discriminant function coefficients for each variable are as follows: body area, 0.783; oral sucker area, –0.339; pharynx area, 0.472 and testes area, 0.390.

Balbuena, 1987; Gibson et al., 1998), and now *B. parvulum*. *Brachycladium atlanticum* has also been reported in the Mediterranean Sea (Mateu et al., 2011; Quiñones et al., 2013). In the waters off the coast of Florida, only *B. delphini*, from *Tursiops truncatus* Montagu, and *B. palliatum*, from *Grampus griseus* Gray, *Stenella frontalis* Cuvier, *Steno bredanensis* Lesson and *T. truncatus*, have previously been reported (Woodard et al., 1969; Zam, 1971; Forrester and Robertson, 1975).

In view of the evidence from morphological data and, in addition, the geographical and host-related information outlined above, we have no hesitation in considering the specimens recovered from *M. europaeus* as new to science, and for which we assign the name *Brachycladium parvulum* n. sp.

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INDEPENDENT HOST SWITCHING EVENTS BY DIGENEAN PARASITES OF CETACEANS INFERRED FROM RIBOSOMAL DNA

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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 (ITS2) rDNA of digenean species representing all known families reported from cetaceans: *Braunina cordiformis* (Brauniniidae), *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 (ML) incorporating published sequences of 177 species of Digenea. Further Bayesian and ML 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.

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, 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, 2002).

Species of the Brachycladiidae occur worldwide, parasitizing 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 and 10 genera, seven of which are exclusive to cetaceans (*Brachycladium*, *Hunterotrema*, *Oschmarinella*, *Synthesium*, *Campula*, *Nasitrema* and *Cetitrema*), one exclusive to pinnipeds (*Zalophotrema*) and three 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). 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 Beverly-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.

MATERIALS AND METHODS

Taxa and outgroups selected

Table 6.1 summarizes 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 Appendix A1. 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 Appendix A1) 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.

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:

Table 6.1. 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 ¹	KM258664	KM258670	---
<i>Ogmogaster antarcticus</i> (Notocotyliidae)	<i>Balaenoptera borealis</i> (Sei whale)	CENPAT	KM258669	KM258675	---
<i>Pholeter gastrophilus</i> (Heterophyidae)	<i>Tursiops truncatus</i> (Bottlenose dolphin)	ICBIBE ²	KM258668	KM258674	KM258663
<i>Campula oblonga</i> (Brachycladiidae)	<i>Phocoena phocoena</i> (Harbour porpoise)	ZSL ⁴	KM258665	KM258671	---
<i>Nasitrema</i> sp. (Brachycladiidae)	<i>Feresa attenuata</i> (Pygmy killer whale)	ULPGC ³	KM258666	KM258672	---
<i>Oschmarinella rochebruni</i> (Brachycladiidae)	<i>Stenella coeruleoalba</i> (Striped dolphin)	ICBIBE	KM258667	KM258673	---

¹ **CENPAT**, Laboratorio de Mamífero Marinos, Centro Nacional Patagónico (CONICET, Argentina).

² **ICBIBE**, Institut Cavanilles de Biodiversitat i Biologia Evolutiva, University of Valencia (Spain).

³ **ULPGC**, University of Las Palmas de Gran Canaria (Spain).

⁴ **ZSL**, Zoological Society of London (United Kingdom).


ethanol in the samples was replaced by TE buffer (0.001M TrisHCl, pH 7.5, 0.001M 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) 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 Barker, 1998) (see definition of primers in Table 3.2). 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).

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 (Kato 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, 2002). 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, 2006) 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%.

Figure 6.1. (See next page) Bayesian inference (BI) of the small subunit (SSU) and large subunit (LSU) 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 BI (posterior probabilities) $\geq 90\%$ and Maximum Likelihood (ML) (bootstrap, $n = 100$) $\geq 80\%$. For the detailed trees, BI (posterior probabilities) and ML bootstrap for each node are shown as the first and second numbers, respectively. 

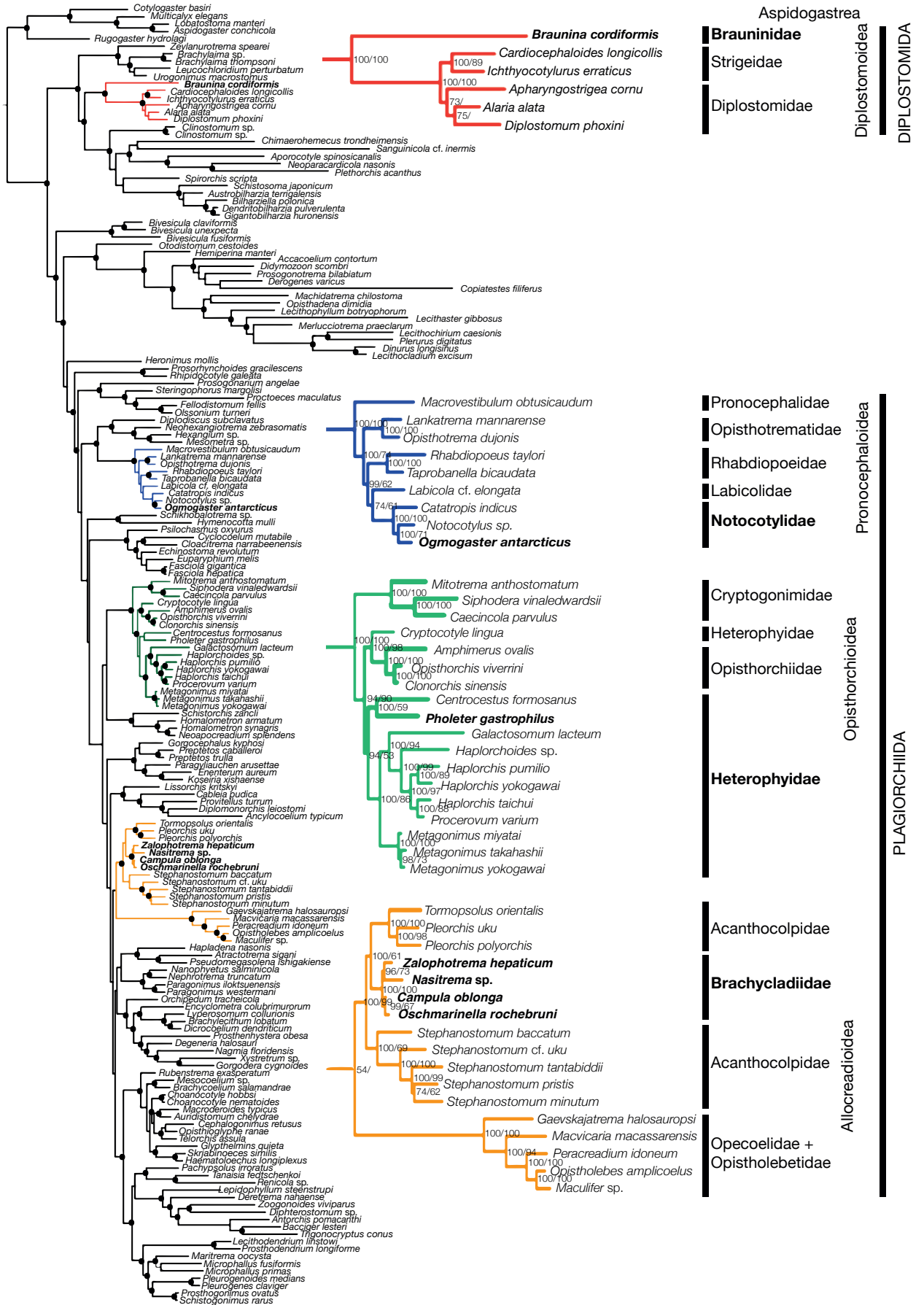
RESULTS

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) (Figure 6.1). Thus all taxonomic entities as defined in Olson et al. (2003) were supported.

The phylogenetic positions of all species of digeneans from cetaceans were resolved with a high degree of support (PP = 100% and ML bootstrap $> 80\%$) (Figure 6.1). Within the Diplostomida, *B. cordiformis* formed a sister clade to the Strigeidae + Diplostomidae clade (Figure 6.1). All other taxa, i.e. species of Brachycladiidae, *O. antarcticus* and *P. gastrophilus* were consistently placed in the Plagiorchiida (Figure 6.1). 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 Acanthocolpidae (Figure 6.1) 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 (Figure 6.1). 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 (Figure 6.1).

Using the SSU and LSU rDNA sequences, *P. gastrophilus* was placed among the Heterophyidae in a clade together with *Centrocestus formosanus* (Figure 6.1). 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%) (Figure 6.2). 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 (Figures 6.1 and 6.2). This family, together with members of the Opisthorchiidae and Cryptogonimidae, were grouped in the superfamily Opisthorchioidea (Figure 6.1).



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 Brauninidae was related to the 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 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 Dronen et al., 1999; Sepúlveda et al., 1999; Niewiadomska, 2002b; Sanmartín et al., 2005; Diaz et al., 2010). 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. 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*) (Figure 6.1). 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* (Figure 6.1). Species of the genus *Notocotylus* are defined as a cosmopolitan group parasitizing 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 *Ascocotyle longa* and *Pygidiopsis 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 Troglotrematidae, 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 colonization 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

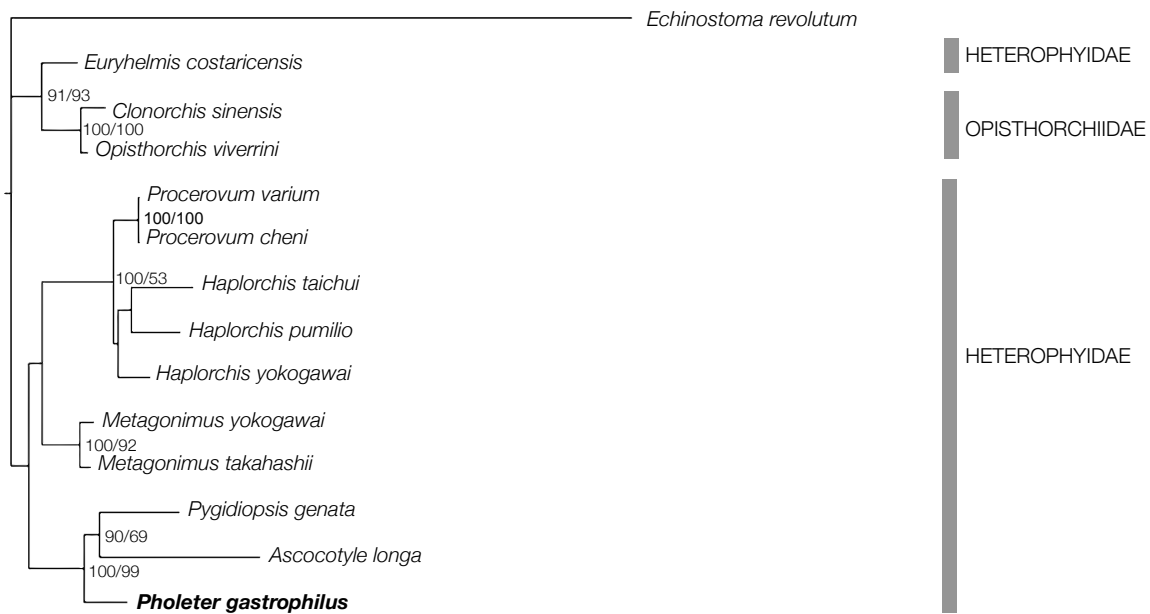


Figure 6.2. Bayesian analysis of the small subunit and internal transcribed spacer 2 (ITS2) rDNA sequences from 14 species of the families Heterophyidae and Opisthorchiidae. Nodal support values for each node are indicated as posterior probabilities (Bayesian inference) and Maximum Likelihood (bootstrap, n = 100), respectively. Bold letters indicate parasitic species in cetaceans.

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 colonization.

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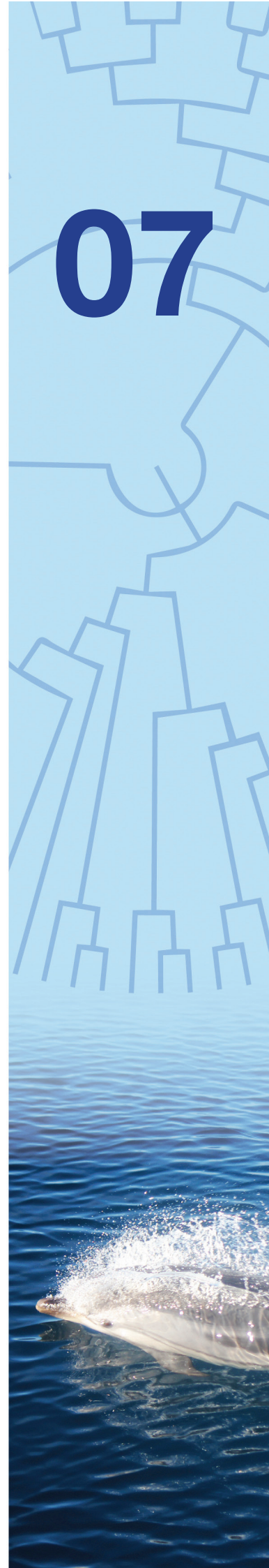
EVOLUTIONARY RELATIONSHIPS BETWEEN DIGENEANS OF THE FAMILY BRACHYCLADIIDAE ODHNER, 1905 AND THEIR MARINE MAMMAL HOSTS: A COPHYLOGENETIC STUDY

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ABSTRACT

Cophylogenetic studies examine the congruence between host and parasite phylogenies. There are few studies that quantify the relative contribution of coevolutionary events, i.e. duplication, loss, failure-to-diverge, host switching and spreading in trophically-transmitted parasites at the marine realm. We addressed this issue in the Brachycladiidae, a cosmopolitan digenean family specific to marine mammals. We used, for the first time, distance-based and event-based methods to explicitly test the coevolutionary events that have shaped the current brachycladiid-marine mammal associations. Parasite phylogeny was constructed using mtDNA ND3 sequences of nine brachycladiid species, and host phylogeny using cytochrome *b* sequences of 104 mammalian species. A total of 50 host-parasite links were identified. Distance-based methods supported the hypothesis of a global non-random association of host and parasite phylogenies. Significant individual links (i.e., 24 out of 50) were those related to *Campula oblonga*, *Nasitrema delphini*, *N. globicephalae* and *Brachycladium atlanticum* and their associated taxa from the Delphinoidea. Regarding event-based methods, we explored 54 schemes using different combinations of costs for each potential coevolutionary event. Three coevolutionary scenarios were identified across all schemes and in all cases the number of loss events (87–156) was the most numerous, followed by failure-to-diverge (40) duplication, (3–6), host-switching (0–3) and cospeciation (0–2). We developed a framework to interpret the evolution of this host-parasite system and confirmed that failure-to-diverge and colonization with or without subsequent diversification could have been decisive in the establishment of the associations between brachycladiids and marine mammals.

INTRODUCTION

Host-parasite systems are complex associations that can be studied under an ecological, historical and biogeographical context in order to reveal the processes that have shaped the current faunal structure (Hoberg and Klassen, 2002). Congruence between host and parasite phylogenies indicates the degree to which parasites and their hosts occupy corresponding positions in their phylogenetic trees (Brooks, 1979), and perfect congruence is a good indicator of host and parasite cospeciation (Legendre et al., 2002). Congruence is usually a sort of “null hypothesis” in many coevolutionary studies (Paterson and Banks, 2001). However, several processes can lead to incongruent phylogenies (Johnson et al., 2003; Banks and Peterson, 2005; Charleston and Libeskind-Hadas, 2014): i) duplication, when parasites independently speciate from their hosts; ii) loss or lineage sorting, when the parasite is absent in a host lineage due to processes such as extinction or missing the boat; iii) failure-to-diverge, when a host speciates but the parasite does not, remaining in both new host species and iv) colonization, when a parasite successfully invades a new host lineage. Colonization has sometimes been synonymized to host-switching (e.g. Brooks and McLennan, 1991; Hoberg and Klassen, 2002), although some authors reserve the term host-switching for the event by which parasites, after colonizing a newly acquired host, are able to speciate in it (e.g., Brooks, 1979; Ronquist, 1997; Conow et al., 2010). In order to be consistent with the terminology used by the event-based tools employed in this study, we will consider “colonization” as an event in which a parasite successfully invades a new host lineage; “host-switching” as a colonization event in which the parasite speciates in the new host; “failure-to-diverge” as an event in which a parasite fails to speciate in closely related host species, and “spreading” as a colonization event in which a parasite fails to speciate in distantly related species (Brooks and McLennan, 1991; Hoberg and Klassen, 2002; Charleston and Libeskind-Hadas, 2014).

In the marine realm, colonization has been suggested to be a major process for the origin of host-parasite associations, particularly when hosts share a common trophic guild (Hoberg and Klassen, 2002; Hoberg and Brooks, 2008). Indeed, the origin of the associations between helminths and cetaceans seems to be the result of several colonization events with subsequent specific diversification (see Hoberg and Adams, 1992 for Cestoda; Hoberg and Adams, 2000 and Fraija-Fernández et al., 2015 for Digenea; Mattiucci and Nascetti, 2008 and Nadler et al., 2013 for Nematoda; and García-Varela et al., 2013 for Acanthocephala). One example is that of the Brachycladiidae Odhner, 1905. This family of digeneans occurs in the hepatopancreatic ducts, air sinuses and intestine of cetaceans, pinnipeds and the sea otter, *Enhydra lutris*. It currently includes ten genera and more than 50 species, i.e., *Brachycladium* (9 species), *Campula* (3 species), *Cetitrema* (2 species), *Hunterotrema* (1 species), *Nasitrema* (10 species), *Odhneriella* (4 species), *Orthosplanchnus* (7 species), *Oschmarinella* (5 species), *Synthesium* (8 species) and *Zalophotrema* (2 species) (Cribb and Gibson, 2015). Brachycladiids represent a suitable model for evaluating associations with their hosts, as they are a widespread family, with some species occurring among many sympatric marine mammals (e.g., Adams and Rausch, 1989; Oliveira et al., 2011), whereas other species are restricted to single host species (e.g., Dailey, 1971; Yablokov

et al., 1972; Dailey, 2007). No intermediate hosts have been identified to date in any brachycladiid species. However, based on the broad ecological distribution of the definitive hosts of members of this family, it may be postulated that molluscs could act as first intermediate hosts and a wide array of invertebrates and fish as second intermediate hosts. The fact that brachycladiids are trophically-transmitted parasites that presumably exploit food webs extensively may have provided opportunities for colonization of new, unrelated hosts, during their evolution.

Previous studies on the Brachycladiidae have analysed partial phylogenetic relationships within the family, suggesting hypotheses about their origin and diversification (Fernández et al., 1998a; 1998b; 2000; Marigo et al., 2011). Brachycladiids likely originated through a host-switching event, when their ancestor, having marine fish as former definitive hosts, was able to shift to odontocetes through predator-prey relationships (Fernández et al., 1998a; Bray et al., 2005). After the initial colonization and diversification in toothed whales, brachycladiids were able to switch also to baleen whales and pinnipeds (Fernández et al., 2000; Aznar et al., 2001; Raga et al., 2009). However, no cophylogenetic study has explicitly addressed these hypotheses.

The aim of this study is to extensively investigate the cophylogenetic relationships between species of the Brachycladiidae and their marine mammal hosts. Distance-based methods were used to quantify the degree of overall congruence between host and parasite phylogenies, identifying also the relative contribution from each host-parasite link to the general congruence (Legendre et al., 2002; Balbuena et al., 2013). Event-based methods, in which the combination of evolutionary events is assumed to occur under a minimal cost, were used to reconstruct a putative historic scenario for the brachycladiid-marine mammal system (Conow et al., 2010). The combination of both types of methods allowed us to identify potential coevolutionary events that shaped the current associations between brachycladiids and marine mammals, also assessing the relative importance of colonization events in the coevolutionary history of these digeneans.

MATERIALS AND METHODS

Parasite collection and taxa selection

The parasite dataset used for this study was composed of nine species of the Brachycladiidae (Table 7.1), five of which, i.e., *Brachycladium atlanticum*, *Oschmarinella rochebruni*, *Nasitrema delphini*, *Synthesium tursionis* and *Campula oblonga* were collected from marine mammals that became stranded along the coasts of Spain and United Kingdom during 2011, 2013 and 2014. Data from *N. globicephalae* and *Orthosplanchnus fraterculus*, and from *S. pontoporiae* were obtained from available studies i.e., Fernández et al. (1998a), and Marigo et al. (2011), respectively. Data from *B. goliath* were made available by Briscoe et al. (2016) (Table 7.1).

Table 7.1. List of species, hosts, places of collection, collector's institution or bibliographic source and GenBank accession numbers of the mitochondrial ND3 sequences of the parasite species used in this study. New sequences generated in this study are indicated with an asterisk (*).

	Host species (Common name)	Locality (Source)	GenBank accession no.
Acanthocolpidae			
<i>Tormopsolus orientalis</i>	<i>Seriola dumerili</i> (Greater amberjack)	Mediterranean Sea (Bartoli et al., 2004)	KT180219*
Paragonimidae			
<i>Paragonimus westermani</i>	Data not available	Data not available	NC_002354
Brachycladiidae			
<i>Brachycladium atlanticum</i>	<i>Stenella coeruleoalba</i> (Striped dolphin)	Mediterranean Sea (CEGMA ¹)	KT180217*
<i>Brachycladium goliath</i>	<i>Balaenoptera acutorostrata</i> (Minke whale)	North Sea (Briscoe et al., unpub. data)	KR703278
<i>Campula oblonga</i>	<i>Phocoena phocoena</i> (Harbour porpoise)	North Sea (ZSL ⁴)	KT180214*
<i>Nasitrema delphini</i>	<i>Delphinus delphis</i> (Common dolphin)	Off Canary Islands (ULPGC ³)	KT180216*
<i>Nasitrema globicephalae</i>	<i>Globicephala melas</i> Long-finned pilot whale	Southern Pacific Ocean (Fernández et al., 1998a)	AF034557
<i>Orthosplanchnus fraterculus</i>	<i>Enhydra lutris</i> (Sea otter)	North Pacific Ocean (Fernández et al., 1998a)	AF034555
<i>Oschmarinella rochebruni</i>	<i>Stenella coeruleoalba</i> (Striped dolphin)	Mediterranean Sea (ICBIBE ²)	KT180215*
<i>Synthesium tursionis</i>	<i>Tursiops truncatus</i> (Bottlenose dolphin)	Mediterranean Sea (ICBIBE)	KT180218*
<i>Synthesium pontoporiae</i>	<i>Pontoporia blainvillei</i> (Franciscana)	Off Brazilian coast (Marigo et al., 2011)	FJ829472

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Molecular analyses: DNA extraction, amplification and sequencing

Newly generated sequences of the mitochondrial DNA ND3 (NADH dehydrogenase, subunit 3) were obtained for *B. atlanticum*, *C. oblonga*, *N. delphini*, *O. rochebruni* and *S. tursionis*; sequences of the four remaining brachycladiid species used in this study were available from GenBank (Table 7.1). Genomic DNA was extracted using Qiagen DNeasy Blood and Tissue Kit (Qiagen, Germany) following manufacturer recommendations with two modifications: ethanol in the samples was replaced by TE buffer (0.001M TrisHCl pH 7.5, 0.001M EDTA pH 8) and the incubation period with proteinase K was extended overnight. Mitochondrial DNA ND3 sequences were amplified using the following pair of primers specifically designed for brachycladiids: the ND3F (5' GCT TAA TTK KTA AAG CYT TGR ATT CTT ACT 3') (Fernández et al., 2000) and the ND3 Primer 4 (5' CTA CTA GTC CCA CTC AAC (G/A)TA ACC (T/C)T 3') (Fernández et al., 1998a). The thermocycling profile was as follows: initial denaturation at 95° C for 5 min, 35 cycles of 95° C for 30 s, 50° C for 30 s and 72° C for 50 s, and a final extension at 72° C for 7 min (Fernández et al., 1998a). Amplicons were purified with 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. (Macrogen Europe, Netherlands). Contigs were assembled using BioEdit v.7.0.5.3 and the sequence quality was verified using the Basic Local Alignment Search Tool (BLAST).

Parasite phylogenetic analyses

Mitochondrial DNA ND3 sequences of nine species of the Brachycladiidae, one species of the family Paragonimidae, *Paragonimus westermani* and one from the family Acanthocolpidae, *Tormopsolus orientalis*, were aligned using the online version of Mafft (<http://mafft.cbrc.jp/alignment/server/>). The species of the Paragonimidae and the Acanthocolpidae were selected as outgroups based on the phylogenetic relationships previously described for the family Brachycladiidae (see Fernández et al., 1998b; Bray et al., 2005; Fraija-Fernández et al., 2015). The aligned dataset of 375 base pairs (bp) had 125 parsimony-informative characters and it was analysed under the Akaike Information Criterion to select the appropriate model of evolution implemented in JModelTest 2.1.4 (Darriba et al., 2012). Nucleotide alignment is available online from the TreeBASE repository (<http://www.treebase.org>) study ID 17815. The phylogenetic tree was constructed using MrBayes 3.2.5 (Ronquist and Huelsenbeck, 2003) under the HKY+I+G model. Posterior probabilities (PP) were obtained after four Markov Chain Monte Carlo (MCMC) chains ran for 500,000 generations, with one topology saved for every 100 generations. Consensus trees were constructed using a “burn in” of 1,900 for estimating “sumt” and “sump” after the average standard deviation of split frequencies was < 0.01. A Maximum Likelihood (ML) analysis was conducted using MEGA 6 (Tamura et al., 2013) under the HKY+I+G model and bootstrap values were estimated for 100 replicates. The ML heuristic tree searching strategy was Subtree-Pruning-Regrafting (SPR). Clades were considered highly supported when PP were > 90% and ML bootstrap values were > 80%.

Host phylogenetic analyses

A total of 102 species of mammals represented by the orders Artiodactyla (one species), Cetacea (66 species) and Carnivora (35 species) were used for reconstructing the host phylogeny. Taxa were selected according to resolved phylogenies for mammals (Meredith et al., 2011), and specifically for cetaceans (May-Collado and Agnarsson, 2006) and pinnipeds (Fulton and Strobeck, 2010). Two species of the order Sirenia, namely *Trichechus manatus* and *Dugong dugon*, were used as outgroups (Meredith et al., 2011). Sequences of the cytochrome *b* gene from all mammal species were downloaded from GenBank (see Appendix A2) and they were aligned using the online version of Mafft. From the 1140 bp-aligned sites, 584 were parsimony-informative characters. Total alignment was analysed through Bayesian inference under the GTR+I+G model of evolution implemented in MrBayes (Ronquist and Huelsenbeck, 2003). Each codon position of the cytochrome *b* was treated separately with a substitution rate partitioning. After 2,000,000 generations, ran in four MCMC, values for PP were obtained with one topology saved for every 100 generations. The consensus tree was constructed using a “burn in” of 10,800 after the average standard deviation of split frequencies was < 0.01. The complete nucleotide alignment is available online from the TreeBASE repository (<http://www.treebase.org>) study ID 18098. A ML analysis was conducted in MEGA 6 (Tamura et al., 2013) under the GTR+I+G model and bootstrap values were obtained for 100 replicates. The ML heuristic tree searching strategy was Subtree-Pruning-Regrafting (SPR).

Cophylogenetic analyses

Based on the host and the parasite phylogenies, a tanglegram was constructed using the available information obtained from bibliographic sources for each host-parasite association. Each association was defined by records of each brachycladiid species reported in each marine mammal species (see Appendix A3). The distance-based methods ParaFit (Legendre et al., 2002) and PACo (Balbuena et al., 2013) were used to test the null hypothesis of a random association between host and parasite phylogenies (Legendre et al., 2002; Balbuena et al., 2013; De Vienne et al., 2013). Both methods compare genetic distances and randomize host-parasite links to statistically quantify the degree of global congruence between phylogenies, and they support uneven number of host and parasite species, multi-host parasitism and not completely resolved phylogenies. The individual contribution of each host-parasite link to the global congruence of the cophylogenetic structure was assessed. Both ParaFit and PACo were implemented under the public domain statistical software R (R Core Team, 2015).

A coevolutionary hypothesis between marine mammals and brachycladiids was investigated with the event-based software Jane 4.0 (Conow et al., 2010). This program maps the parasite phylogeny onto the host phylogeny considering the association between host and parasites under a cost regime assigned to each potential coevolutionary event. Jane 4.0 deals with cospeciation, duplication, duplication and host switch, loss, and failure-to-diverge (Conow et al., 2010), but neither Jane 4.0 nor any other available event-based mapping system includes spreading events

(Charleston and Libeskind-Hadas, 2014). Ancillary information is required to distinguish between failure-to-diverge and spreading events (see Banks and Paterson, 2005). Jane 4.0 generates a high number of possible scenarios aiming at reducing total cost of events. Since the assignment of accurate costs for each evolutionary event is difficult, we selected three cost values (0, 1 and 2) for each event except cospeciation (whose cost was always 0), and explored all possible combinations of costs (for a similar procedure, see Ronquist, 1997; Desdés et al., 2002; Millanes et al., 2014; Santiago-Alarcon et al., 2014). The number of different solutions at each iteration, or population size, was set to 200 and the number of iterations made by the algorithm, or generations, was set to 100 (see Conow et al., 2010 for details). The statistical significance of the total costs for each cost scheme was tested using a null distribution of cost values based on 500 randomly generated trees. If the total costs were significantly lower ($p < 0.05$) than that assumed under the null distribution, then a global cospeciation signal would be detected under the specified cost regime (Conow et al., 2010).

RESULTS

Phylogenetic analyses

Within the family Brachycladiidae two clusters were recovered (PP = 95%, ML bootstrap = 86%) grouping the two species of *Synthesium* in a separate clade from all other brachycladiids (Figure 7.1). A paraphyletic clade was obtained for the two *Brachycladium* species. A singular clade grouped *Orthosplanchnus fraterculus* and *Oschmarinella rochebruni* as sister species, which together with *C. oblonga*, formed a clade closely related to species of *Nasitrema* (Figure 7.1).

Host phylogeny was congruent with previous studies made for carnivores (see Arnason et al., 2006; Fulton and Strobeck, 2010) and for cetaceans (see May-Collado and Agnarsson, 2006), with some exceptions. There was a polytomy within the Carnivora involving members of the Mustelidae, in which their phylogenetic relationships were not fully resolved (see Appendix A4). In the order Cetacea, the clade formed by Physteridae and Kogiidae was not consistently placed together with other toothed whales (suborder Odontoceti) and, within toothed whales, the clade formed by Platanistidae was not fully resolved. Consistently with previous studies, species of the suborder Pinnipedia were recovered in a highly supported monophyletic clade (PP = 100%, ML bootstrap = 100%). The Odobenidae, a monotypic family, was recovered as sister taxa of the Otariidae (PP = 100%, ML bootstrap = 100%). Members of the Phocidae were also recovered in a highly supported monophyletic clade (PP = 100%, ML bootstrap = 99%). In the case of cetaceans, baleen whales (Suborder Mysticeti) formed a monophyletic clade (PP = 100%, ML bootstrap = 90%), and five main lineages of toothed whales were recovered, i.e., i) the sperm whale, *Physeter macrocephalus* (Physteridae) and the dwarf sperm whales, *Kogia breviceps* and *Kogia sima* (Kogiidae), ii) beaked whales (Ziphiidae), iii) riverine dolphins of the genus *Platanista* (Platanistidae), iv) other riverine and coastal dolphins (Lipotidae, Iniidae and Pontoporidae) and iv) members of the superfamily Delphinoidea (Monodontidae, Phocoenidae and Delphinidae) (see Appendix A4).

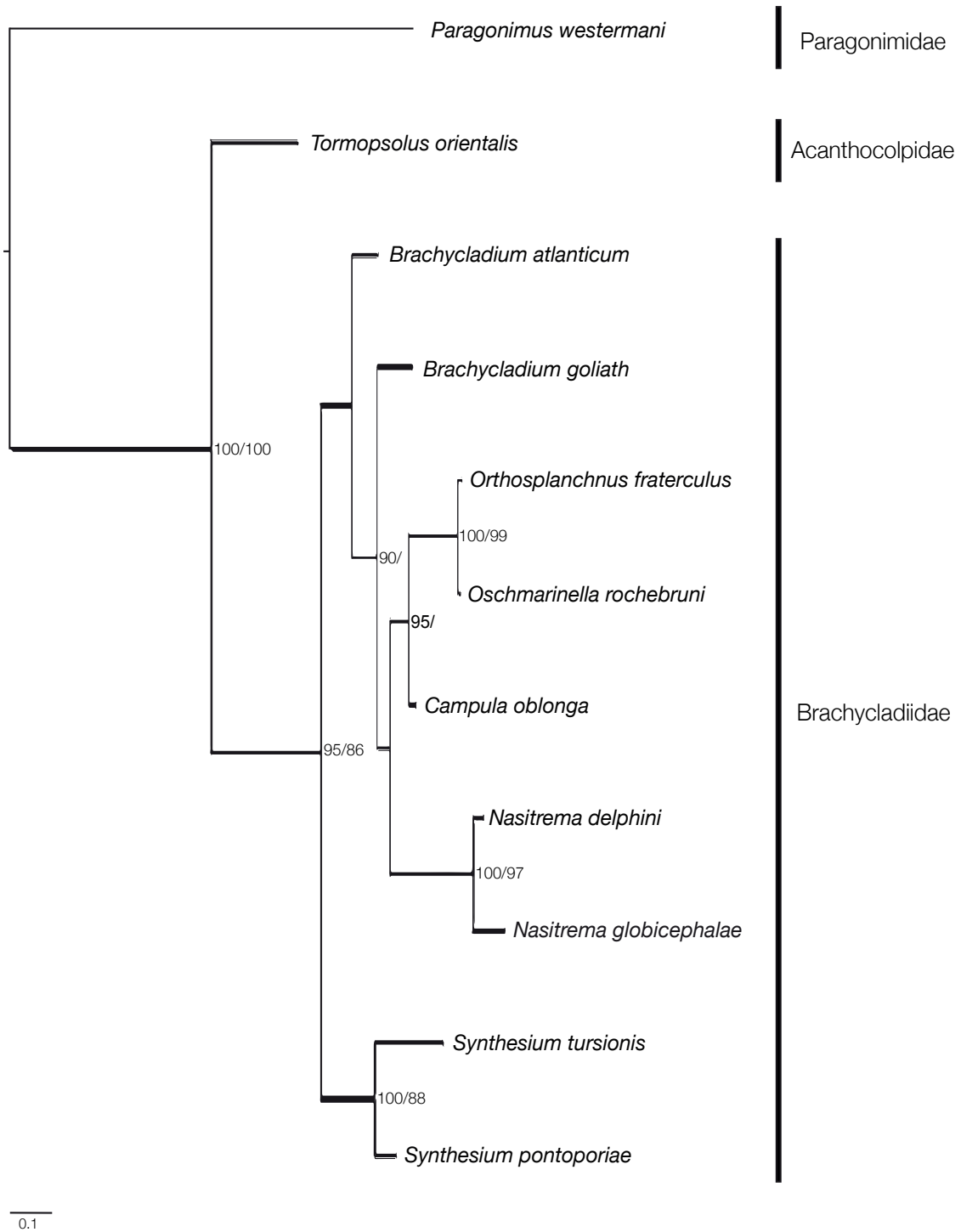


Figure 7.1. Bayesian analysis for the phylogenetic relationships inferred from the mitochondrial ND3 sequences of 9 species of the Brachycladiidae. Support values for each node are expressed as posterior probabilities and Maximum Likelihood (bootstrap n = 100); scores below 80% are not shown. Branch length scale bars indicate number of substitutions per site.

Cophylogenetic analyses


Both ParaFit and PACo rejected the null hypothesis of random association between host and parasite phylogenies (ParaFitGlobal = 32.6527, $p = 0.001$; m^2 global value = 10.998, $p = 0.011$). According to ParaFit, 24 out of the 50 individual host-parasite links showed a significant contribution to the global congruence between host and parasite associations ($p < 0.04$ in all cases). Significant individual links were all those related to *C. oblonga*, *N. delphini*, *N. globicephalae* and *B. atlanticum* and their associated taxa from the Delphinoidea clade. The species *C. oblonga*, *N. delphini* and *N. globicephalae* were each associated to different species from both the Delphinidae and the Phocoenidae, whereas *B. atlanticum* was only associated with species from the Delphinidae. These multi-hosts associations, according to ParaFit and PACo, were the ones that most contributed to the global congruence detected in the cophylogenetic analysis and are observed more frequently than expected by chance (Figure 7.2 and Appendix A5).

A total of 54 cost schemes were explored using all possible combinations of costs between 0 and 2 for each evolutionary event considered in Jane 4.0 (Appendix A6). A complex coevolutionary scenario between brachycladiids and marine mammals was found and three different scenarios were identified across the different cost schemes, which were repeated throughout the analysis (Figure 7.3). Every cost scenario was statistically analysed and 45 out of the 54 scenarios found had significantly lower costs than expected by chance ($p < 0.001$), whereas the other nine were not ($p = 1.000$) (Figure 7.3 and Appendix A6). The high number of possible coevolutionary scenarios suggests that the outcome of the analysis may vary accordingly to the cost assignment, as previously suggested by Du Toit et al. (2013) and Millanes et al. (2014). In all cases the number of loss (87–156) and failure-to-diverge (40) events were the most numerous across all cost schemes. The number of cospeciation events varied between 0 and 2; between 3 and 6 duplication events occurred in the speciation of the Brachycladiidae among the superfamily Delphinoidea, and up to 3 duplications and host-switching events occurred across all the cost schemes examined (Figure 7.3 and Appendix A6).

DISCUSSION

Our results show that the phylogenetic relationship of the family Brachycladiidae inferred by Bayesian analysis was congruent with previous studies of the family that used other methods for phylogenetic reconstruction i.e., neighbour joining, maximum parsimony and maximum likelihood (Fernández et al., 1998a; 2000; Marigo et al., 2011). In the last taxonomic revision of the family Brachycladiidae, Gibson (2005) proposed to resurrect the genus *Brachycladium* and to consider the genus *Lecithodesmus* its synonym. However, the paraphyletic relationship between *B. goliath* (= *Lecithodesmus goliath*) and *B. atlanticum* found in this study demands further morphological and molecular studies in order to clarify their taxonomic relationship.

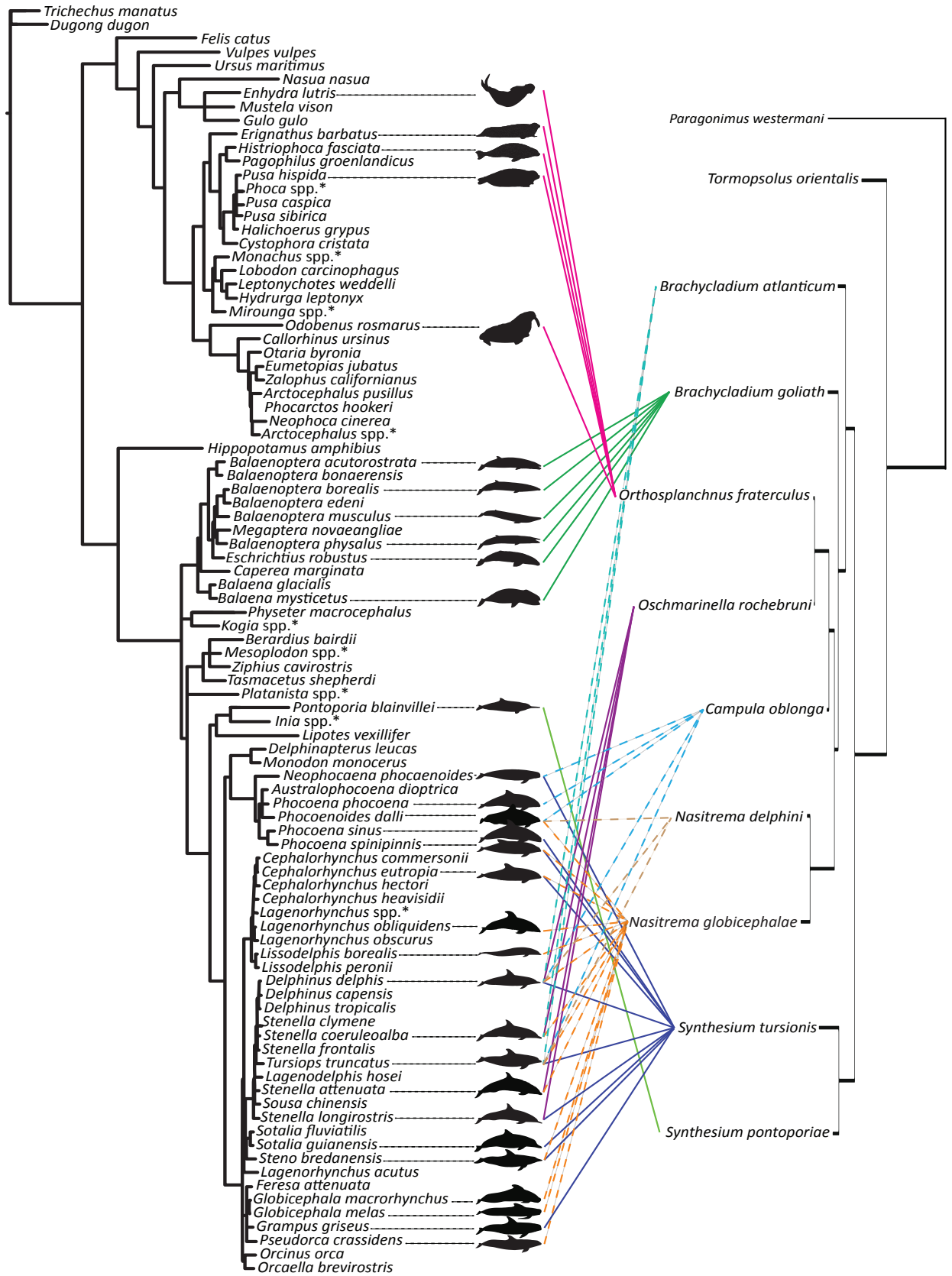
Distance-based cophylogenetic methods do not support the hypothesis that the association between brachycladiids and marine mammals was random, and rather suggest a global

Figure 7.2. (See next page) Tanglegram for the association between digeneans of the family Brachycladiidae (tree on the right) and their marine mammal hosts (tree on the left). Lines in the host tree link the specific association between hosts and parasites. Dashed lines represent the significant individual links ($p < 0.05$) for the result from ParaFit individual host-parasite analysis. Trees were constructed through Bayesian inference using sequences of the cytochrome *b* gene from 104 mammalian species for the host tree, and mitochondrial ND3 sequences from 11 species for the parasite tree. Asterisks (*) represent collapsed branches for the host tree. Branch length scale bars indicate the number of substitutions per site. Marine mammal silhouettes were modified from Jefferson et al. (1993). 

congruence between phylogenies. Such congruence is supported by the significant individual host-parasite links occurring among delphiniids and phocoeniids, suggesting that the ancestor of brachycladiids was able to colonize and diversify among the Delphinoidea. Traditionally, congruent phylogenies have been accounted for by cospeciation events (Clayton et al., 2004), however “pseudo-cospeciation” could have also had a role in generating congruence. Pseudo-cospeciation occurs when parasites adaptively radiate on a range host species by multiple host-switches followed by speciation events (De Vienne et al., 2007). This process could have occurred among closely related members of the Delphinoidea because they are a highly diverse host clade of species that belong to a common trophic guild (i.e., they are mainly piscivorous and/or teuthophagous) and may share the same habitat, two conditions that facilitate contacts between hosts and parasites (see Charleston and Robertson, 2002; Banks and Paterson, 2005).

Our event-based analysis suggests a complex evolutionary history between brachycladiids and their marine mammal hosts, where losses and failure-to-diverge events were the two most frequent evolutionary events, followed by host-switching, duplication and cospeciation. More than 80 loss events were detected in the cophylogenetic analysis (Appendix A6). There are several factors that may account for these events. First, some of the detected losses could have just resulted from sampling biases. Parasites are collected from stranded marine mammals that are sometimes dead for a long time before beaching, which contribute to parasite decomposition and undetection. In addition, parasite data are likely to be underrepresented from marine mammal species that rarely become stranded, e.g., beaked whales (Delyamure, 1955; Gibson, 2005; Chapter 4 of this thesis). Second, evolutionary causes for loss events include extinction and “missing the boat” (Page and Charleston, 1998; Paterson et al., 1999). Given the protracted history of association between brachycladiids and marine mammals, the occurrence of loss events would not be particularly surprising. The establishment and evolution of helminth communities in marine mammals have suffered important ecological filters due to their hosts’ evolution and the different aquatic environments they may have occupied (Raga and Balbuena, 1993; Aznar et al., 1994). For instance, the low diversity of the brachycladiid fauna found in the Franciscana dolphin, *Pontoporia blainvillei*, could be related to the repeated ecological shifts that their ancestors suffered between freshwater and marine habitats (see Aznar et al., 1994).

Our results show that failure-to-diverge has been a common event in the coevolutionary history of brachycladiids and marine mammals. This is obviously related to the fact that the same brachycladiid species are found in several host species (Figure 7.2), which might suggest,



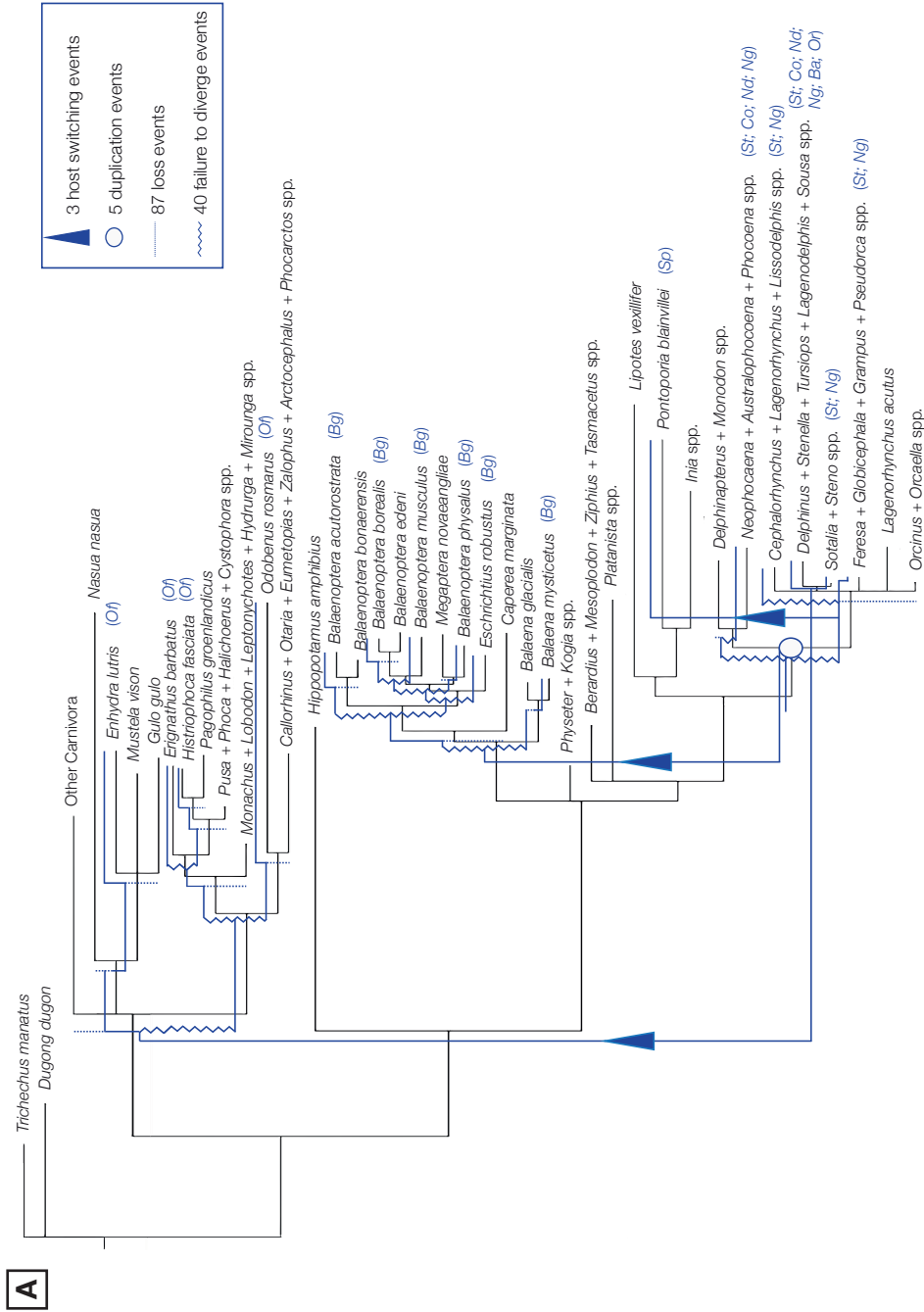


Figure 7.3. Modified output of Jane 4.0 representing the three topologies **A, B, C**, obtained from the cophylogenetic analysis on the Brachycladiidae and marine mammal host-parasite system. The black tree represents the host phylogeny, whereas the blue tree represents the parasite phylogeny. Filled circles at the nodes indicate cospeciation events, empty circles indicate parasite duplication events, arrows indicate host-switching events, dotted lines indicate loss events, and zigzag lines indicate failure-to-diverge events. Letters in parentheses indicate parasite species of each corresponding host species. Parasite abbreviations: **Of**, *Orthosplanchnus fraterculus*; **Bg**, *Brachycladium goliath*; **Sp**, *Synthesium pontoporiae*; **St**, *Synthesium tursionis*; **Co**, *Campula oblonga*; **Nd**, *Nasitrema delphini*; **Ng**, *Nasitrema globicephalae*; **Ba**, *Brachycladium atlanticum*; **Or**, *Oschmarinella rochebruni*.

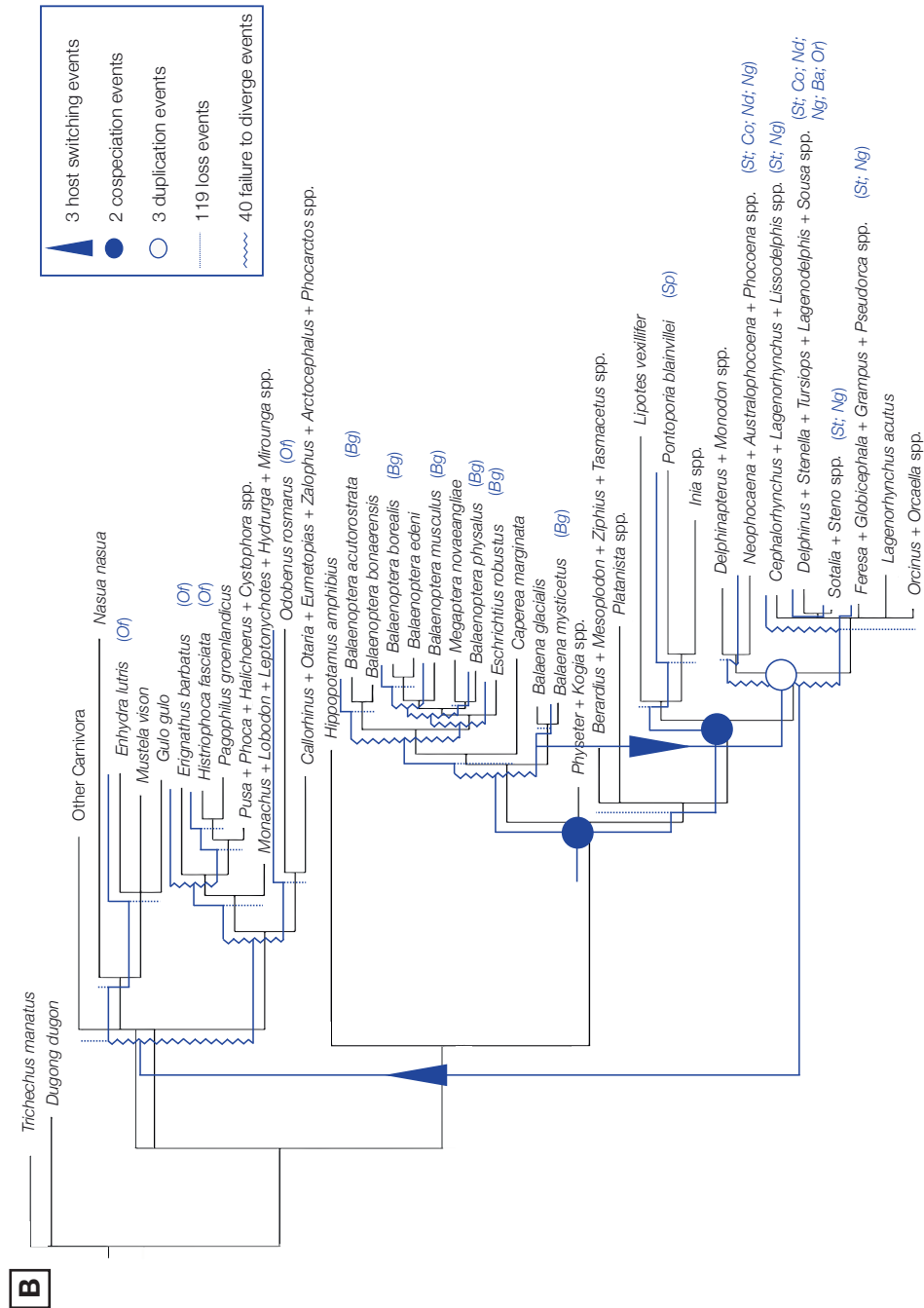


Figure 7.3. (Continued) Modified output of Jane 4.0 representing the three topologies **A, B, C**, obtained from the cophylogenetic analysis on the Brachycladiidae and marine mammal host-parasite system. The blue tree represents the host phylogeny, whereas the black tree represents the parasite phylogeny. Filled circles at the nodes indicate cospeciation events, empty circles indicate duplication events, arrows indicate host-switching events, dotted lines indicate loss events, and zigzag lines indicate failure-to-diverge events. Letters in parentheses indicate parasite species of each corresponding host species. Parasite abbreviations: **Of**, *Orthosplanchnus fraterculus*; **Bg**, *Brachycladium goliath*; **Sp**, *Synthesium pontoporiae*; **St**, *Synthesium tursionis*; **Co**, *Campula oblonga*; **Nd**, *Nasitrema delphini*; **Ng**, *Nasitrema globicephalae*; **Ba**, *Brachycladium atlanticum*; **Or**, *Oschmarinella rochebruni*.

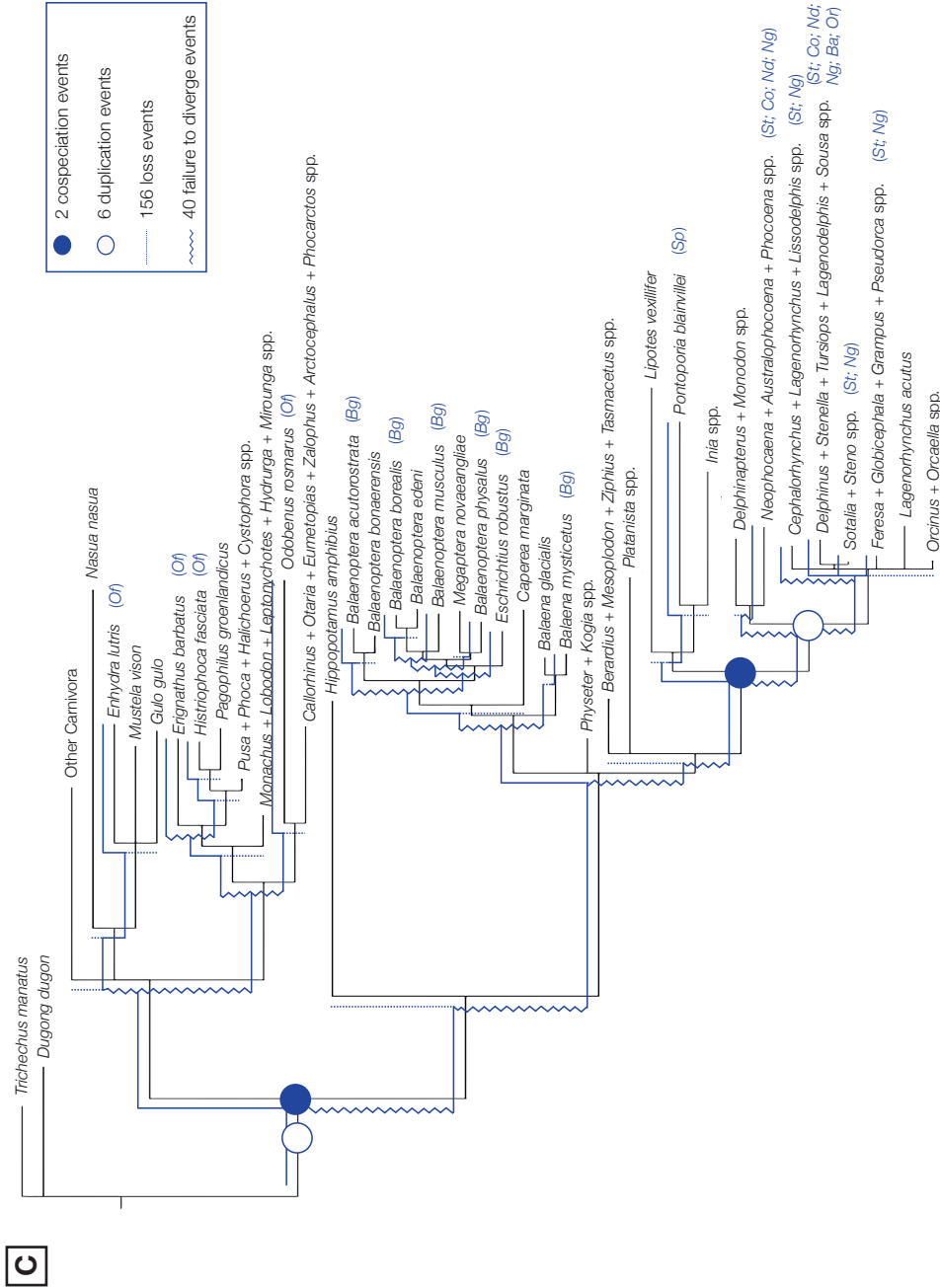


Figure 7.3. (Continued) Modified output of Jane 4.0 representing the three topologies **A, B, C**, obtained from the cophylogenetic analysis on the Brachycladiidae and marine mammal host-parasite system. The black tree represents the host phylogeny, whereas the blue tree represents the parasite phylogeny. Filled circles at the nodes indicate cospeciation events, empty circles indicate duplication events, arrows indicate host-switching events, dotted lines indicate loss events, and zigzag lines indicate failure-to-diverge events. Letters in parentheses indicate parasite species of each corresponding host species. Parasite abbreviations: **Of**, *Orthoplanchinus fraterculus*; **Bg**, *Brachycladium goliath*; **Sp**, *Synthesium pontoporiae*; **St**, *Synthesium tursionis*; **Co**, *Campula oblonga*; **Nd**, *Nasitrema delphini*; **Ng**, *Nasitrema globicephalae*; **Ba**, *Brachycladium atlanticum*; **Or**, *Oschmarinella rochebruni*.

prima facie, that parasites failed to speciate in closely related species. However, as noted in the introduction, spreading events can also cause multi-host parasitism, and neither Jane 4.0, nor any other event-based method includes such type of events (Charleston and Libeskind-Hadas, 2014). Therefore, the question remains as to how many of the so considered as failure-to-diverge events by Jane 4.0 actually are spreading events. This is difficult to ascertain because for both events, failure-to-diverge and spreading, a minimum genetic flow may exist to maintain the integrity of the parasite population (Johnson et al., 2003; Banks and Paterson, 2005). Since brachycladiids are food-transmitted parasites, any common trophic guild shared by sympatric marine mammals would promote the gene flow needed that allows failure-to-diverge and spreading events.

In some cases, however, ancillary information may help to elucidate the most plausible scenario. For instance, Jane 4.0 considered that the association between *O. fraterculus* and, both pinnipeds and the sea otter resulted from a failure-to-diverge event (Figure 7.3). However, sea otters are distantly related to pinnipeds (see Appendix A4), and the restricted geographic distribution of *O. fraterculus* to North Pacific pinnipeds and sea otters strongly suggest that this parasite has been transferred from pinnipeds to sea otter through shared feeding habitats without concomitant speciation (Margolis et al., 1997). This process is actually very common in helminths of marine mammals. For instance, some acanthocephalan species of the genus *Corynosoma* are able to reproduce among not closely related Antarctic pinnipeds that shared infected prey (Zdzitowiecki, 1986). Similarly, the nematode *Anisakis simplex* sensu stricto infects at least nine cetacean species of the Northern Hemisphere, and uses different fish and squid species as intermediate hosts in order to get to their definitive hosts (Mattiucci and Nascetti, 2008). In any event, the extensive host and/or geographical occurrence of some of these “generalist” helminth species, including brachycladiids (e.g. *S. tursionis*, *C. oblonga* or *Nasitrema* spp. in dolphins and porpoises) also raises the possibility that they actually represent examples of cryptic species, which will require further analyses.

Colonization has been recognised as a major evolutionary force for the origin of helminths in cetaceans. In fact, previous hypotheses suggested that the diversification of the Brachycladiidae in pinnipeds and baleen whales has been the result of host-switching events (Fernández et al., 2000; Aznar et al., 2001). Our study confirms this hypothesis: brachycladiids have diversified in whales and pinnipeds from odontocetes through host-switching; an additional host-switching event is proposed for the origin of *S. pontoporiae* in the Franciscana dolphin. Successful host-switching events cannot be predicted but require long-lasting ecological associations that promote not only colonization, but also the subsequent diversification within the new host lineage (Hoberg and Brooks, 2008). It is plausible to assume that the common trophic guild that involves whales, dolphins and pinnipeds provided the opportunities for host-switching and spreading in brachycladiids.

Our study is based on a partial phylogeny for the Brachycladiidae, therefore taxa from four genera are missing: species of *Hunterotrema*, lung parasites of riverine dolphins, species of *Zalophotrema*, hepatopancreatic parasites specific to pinnipeds, species of *Odhneriella*, mainly

occurring in pinnipeds and species of *Cetitrema*, cetacean parasites of the hepatopancreatic ducts. Nevertheless, our results show a complex coevolutionary history between brachycladiid parasites and their marine mammal hosts. We developed a framework to interpret the evolution of this host-parasite system and the relative contribution of coevolutionary events, such as colonization with speciation (i.e., host-switching), colonization without speciation (i.e., spreading) and failure-to-diverge. In this context, it is essential to provide further molecular sampling to elucidate the phylogenetic relationships of those missing taxa from our Brachycladiidae phylogeny.

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* The sequence was published at the time of writing this thesis.

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LONG-DISTANCE TRAVELLERS: PHYLOGEOGRAPHY OF A GENERALIST DIGENEAN (*Pholeter gastrophilus*) FROM CETACEANS

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ABSTRACT

We studied the phylogeography and historical demography of the most generalist digenean from cetaceans, *Pholeter gastrophilus*, exploring how the effect of hosts' dispersal ability and specificity could shape population structure of this parasite. The ITS2 rDNA, and the mitochondrial COI and ND1 from 68 individuals was analysed. Worms were collected from seven oceanic and coastal cetacean species from the South Western Atlantic (SWA), Central Eastern Atlantic, North Eastern Atlantic (NEA), and Mediterranean Sea. *Pholeter gastrophilus* was considered a single lineage because no genetic variation was observed in the ITS2 and less than 1% in the mtDNA, and no reciprocal monophyly was detected in the ML cladogram of all individuals. The genetic cohesion of *P. gastrophilus* could result from extensive ecological and geographic dispersal of infective stages through the food web and the high vagility of definitive hosts. However, unique haplotypes were detected in SWA and NEA, and an AMOVA revealed significant population structure associated to the genetic variation in these regions. Although a general pattern of isolation by distance was detected, partial Mantel tests revealed that at least the significant isolation of NEA populations actually resulted from geographic clustering. Apparently, oceanographic conditions limit host exchange with southern populations. Also, the coastal habits of harbour porpoises, *Phocoena phocoena* (the host from which most NEA samples were collected) could contribute to significant ecological isolation of *P. gastrophilus*. Historical demographic analyses suggested a recent population expansion for *P. gastrophilus* in the Atlantic Ocean, perhaps linked to initial association and subsequent spreading in cetaceans.

INTRODUCTION

Digeneans are parasitic organisms that have complex life cycles including free living and parasitic stages, and alternation of asexual and sexual reproduction. Typically, there is a free-living stage, which has a limited capacity for dispersal, whereas all other stages use invertebrates (usually molluscs) as first intermediate hosts, other invertebrates or vertebrates as second intermediate hosts, and vertebrates as definitive hosts (Cribb et al., 2003). Consequently, gene flow and dispersal in digeneans are largely determined by the dispersal ability of the most mobile host, which usually is the definitive host (Blasco-Costa and Poulin, 2013). This is particularly true in the marine environment where few barriers for gene flow presumably exist (Palumbi, 1994) and, therefore, the dispersal role of hosts is particularly important. Very few studies have investigated phylogeographic patterns of marine digeneans (Blasco-Costa and Poulin, 2013), but some of them have demonstrated fundamental genetic structure differences according to the degree of vagility of host species. For instance, genetic differentiation was observed in *Bucephalus minimus* in the North Atlantic Ocean, thus suggesting that all of its hosts should also be isolated (Feis et al., 2015). In fact, the most vagile host in the life cycle of this digenean, namely, the sea bass (*Dicentrarchus labrax*) has a significant population structure in the northeast Atlantic associated to homing behaviour (Feis et al., 2015 and references therein). In contrast, little genetic structure was detected in the case of *Gymnophallus choledochus*, a parasite of marine birds. Marine birds generally behave as highly vagile definitive hosts, which offer digeneans ample opportunities for dispersal (Feis et al., 2015).

Cetaceans are long-ranging animals that often undergo seasonal migrations between feeding and breeding grounds (Hoelzel, 2009). Their high dispersal capacity is mirrored by little genetic differentiation of many oceanic species, e.g., sperm whale, *Physeter macrocephalus* (Engelhaupt et al., 2009); common dolphin, *Delphinus delphis* (Moura et al., 2013) or Gray's beaked whale, *Mesoplodon grayi* (Thompson et al., 2016), although geographical barriers may contribute to population differentiation in some cases, e.g., the Mediterranean vs. Atlantic populations of fin whale, *Balaenoptera physalus*, and striped dolphin, *Stenella coeruleoalba* (Bérubé et al., 1998; García-Martínez et al., 1999). Cetacean species that are more restricted to neritic waters are expected to experience also ecological barriers that tend to promote more isolation and local adaptation of populations; this is the case, e.g., of the harbour porpoise, *Phocoena phocoena* (Fontaine et al., 2007), the bottlenose dolphin, *Tursiops truncatus* (Natoli et al., 2005), or the Franciscana dolphin, *Pontoporia blainvillei* (Aznar et al., 1995; Gariboldi et al., 2015).

As far as we are aware, there is a single study that has investigated the phylogeography and genetic structure of a digenean from cetaceans. Marigo et al. (2015) described the genetic structure of *Synthesium pontoporiae*, which is specific to the Franciscana dolphin. This largely sedentary cetacean has a relatively restricted geographic range occurring along the temperate coasts of Southern Brazil, Uruguay and Argentina. Previous studies have shown a significant genetic subdivision of Franciscanas at a regional scale (Gariboldi et al., 2015). Unexpectedly, no genetic differentiation was found for *S. pontoporiae*, which suggests that the dispersal capacity

of the (unknown) intermediate hosts would most likely have overcome the low vagility of definitive hosts and contributed to the genetic mixing of *S. pontoporiae* populations (Marigo et al., 2015).

Pholeter gastrophilus is the most generalist and geographically widespread digenean exclusive to cetaceans; it has been reported in at least 17 species of dolphins and porpoises in the Atlantic and Pacific Oceans in both Hemispheres, as well as in the Mediterranean, Black and Baltic Seas (Table 8.1). Given the potential high vagility of definitive hosts and the putative use of many host species through the food web, one could expect little population structure of this species. However, *P. gastrophilus* infects both oceanic and neritic cetaceans, and some species with a highly migratory behaviour but others are largely sedentary. Thus, an open question is the extent to which isolation by distance, and/or the operation of ecological or geographic barriers may limit gene flow and help to generate population structure in this digenean species. In this study we specifically explore the genetic structure of *P. gastrophilus* infecting seven oceanic and coastal cetacean species across nine sampling localities from the South Atlantic Ocean to the North Sea, ca. 8000 nautical miles apart. We used phylogenetic and population genetic approaches to ascertain, firstly, whether *P. gastrophilus* represents a single species. Then, we elucidated patterns of genetic structure and demographic history of the species. The later goal could also shed light on the origin of the association of *P. gastrophilus* with cetaceans.

MATERIALS AND METHODS

Parasite collection and study design

A total of 68 specimens of *P. gastrophilus* was collected from the stomach of 55 stranded individuals of seven cetacean species, i.e., striped dolphin (*Stenella coeruleoalba*); bottlenose dolphin (*Tursiops truncatus*); short-beaked common dolphin (*Delphinus delphis*); short-finned pilot whale (*Globicephala macrorhynchus*); long-finned pilot whale, (*G. melas*); Atlantic white-sided dolphin (*Lagenorhynchus acutus*), and harbour porpoise (*Phocoena phocoena*), in nine localities (Table 8.2). Permission to collect dead stranded dolphins was given by the Wildlife Service of regional governments in Argentina, Belgium, Germany, Ireland and Spain. Worms were collected from the stomach of cetaceans and stored in either 70% or 96% ethanol for further molecular analyses. The nine sampling localities are hierarchically organised into four regions according to their proximity, i.e., i) Mediterranean Sea (Med), which included samples from the Alboran Sea and the western Mediterranean (Spain); ii) South Western Atlantic (SWA), with samples from the coast of Argentina; iii) North Eastern Atlantic Ocean (NEA), with samples from the Celtic Sea and the North Sea (Ireland, Belgium and Germany); and iv) Central Eastern Atlantic (CEA), with samples from the coast of Galicia (North-West Spain), the Strait of Gibraltar, and the Canary Islands (Spain) (Figure 8.1). The coast of Galicia was included in the CEA because all worms were collected from short-finned pilot whales, after an exceptional mass stranding event. The distribution of this cetacean species is largely restricted to warm temperate and tropical waters (Olson, 2009).

Table 8.1. Reports of cetacean species infected by the digenean *Pholeter gastrophilus*. Abbreviations for habitat: **A**, Amazon basin; **C**, Costal; **O**, Oceanic. Abbreviations for locality: **A**, Amazon basin; **AO**, Atlantic Ocean; **BaS**, Baltic Sea; **BS**, Black Sea; **MS**, Mediterranean Sea; **PO**, Pacific Ocean; **RS**, Red Sea; **SA**, South Australia.

Host species	Habitat	Locality	References
Delphinidae			
<i>Cephalorhynchus commersonii</i> (Commerson's dolphin)	C	AO	[5]
<i>Delphinus delphis</i> (Short-beaked common dolphin)	O	AO; BS; SA	[6]; [9]; [15]; [20]; [26]; [27]; [29]; [41]
<i>Globicephala macrorhynchus</i> (Short-finned pilot whale)	O	AO	[36]
<i>Globicephala melas</i> (Long-finned pilot whale)	C, O	AO; MS	[29]; [30]; [32]
<i>Grampus griseus</i> (Risso's dolphin)	O	AO; MS	[11]; [16]; [29]; [43]
<i>Lagenorhynchus acutus</i> (Atlantic white-sided dolphin)	C, O	AO	[8]; [29]; [34]
<i>Lagenorhynchus albirostris</i> (White-beaked dolphin)	C	AO	[20]
<i>Lagenorhynchus obscurus</i> (Dusky dolphin)	C	AO; PO	[12]; [39]
<i>Stenella frontalis</i> (Atlantic spotted dolphin)	O	AO	[43]
<i>Stenella coeruleoalba</i> (Striped dolphin)	C, O	AO; MS	[2]; [15]; [18]; [24]
<i>Steno bredanensis</i> (Rough-toothed dolphin)	O	AO	[17]; [37]
<i>Tursiops aduncus</i> (Indo-Pacific bottlenose dolphin)	C	RS	[22]
<i>Tursiops truncatus</i> (Common bottlenose dolphin)	C, O	AO; PO; MS; BS; SA;	[2]; [9]; [15]; [18]; [19]; [25]; [27]; [28]; [29]; [31]; [35]; [38]; [40];
Iniidae			
<i>Inia geoffrensis</i> (Amazon river dolphin)	A	A	[42]
Phocoenidae			
<i>Phocoena phocoena</i> (Harbour porpoise)	C	AO; BS; BaS	[3]; [4]; [9]; [13]; [14]; [15]; [19]; [20]; [21]; [23]; [29]; [41]
<i>Phocoena spinipinnis</i> (Burmeister's porpoise)	C	AO; PO	[7]; [10]; [33]
Pontoporiidae			
<i>Pontoporia blainvillei</i> (Franciscana)	C	AO	[1]

References: [1] Aznar et al. (1994); [2] Aznar et al. (2006); [3] Baker and Martin (1992); [4] Baylis (1932); [5] Berón-Vera et al. (2001); [6] Berón-Vera et al. (2007); [7] Berón-Vera et al. (2008); [8] Beverly and Burton (1978); [9] Birkun (2002); [10] Corcuera et al. (1995); [11] Cornaglia et al. (2000); [12] Dans et al. (1999); [13] Delyamure (1955); [14] Delyamure (1957); [15] Dollfus (1973/74); [16] Fernández et al. (2003); [17] Forrester and Robertson (1975); [18] Fraija-Fernández et al. (2015a); [19] Gibson and Harris (1979); [20] Gibson et al. (1998); [21] Herreras et al. (1997); [22] Kleinertz et al. (2014); [23] Lehnert et al. (2005); [24] Loizaga de Castro et al. (2011); [25] Migaki et al. (1971); [26] Pearson and Courtney (1977); [27] Pers. Comm. from Ms. Jo Wood (South Australian Museum); [28] Quiñones et al. (2013); [29] Raga and Balbuena (1987); [30] Raga and Balbuena (1993); [31] Raga et al. (1985); [32] Raga et al. (1987); [33] Reyes and Van Waerebeek (1993); [34] Rogan et al. (1997); [35] Romero et al. (2014); [36] This study; [37] Valente and Ebert (2014); [38] Van Waerebeek et al. (1990); [39] Van Waerebeek et al. (1993); [40] Woodard et al. (1969); [41] Yablokov et al. (1972); [42] Zam et al. (1970); [43] Zam et al. (1971).

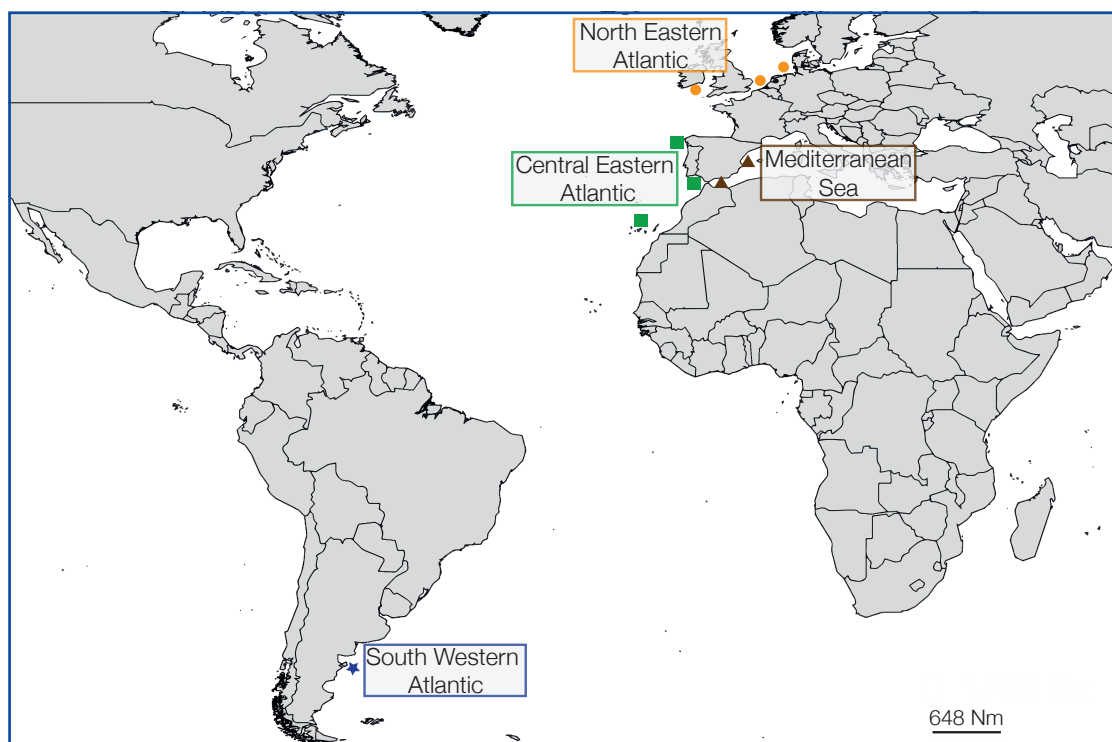


Figure 8.1. Sampling sites of the digenean *Pholeter gastrophilus*, identified by regions. Argentina: **South Western Atlantic** (stars); Galicia, Strait of Gibraltar and the Canary Islands: **Central Eastern Atlantic** (squares); Alboran Sea and western Mediterranean: **Mediterranean Sea** (triangles); and North Sea and Celtic Sea: **North Eastern Atlantic** (circles). Nm = Nautical miles.

Molecular analyses: DNA extraction, amplification and sequencing

All collected worms were used for molecular analysis. Previous to DNA extraction, ethanol in the samples was replaced by 500µl of TE buffer (0.001M TrisHCl, pH 7.5, 0.001M EDTA, pH 8). Genomic DNA was extracted from individual worms using the QIAGEN DNeasy Blood and Tissue Kit (QIAGEN, Germany), following the manufacturer's recommendations, except for the incubation period, which was extended overnight. We amplified the internal transcribed spacer 2 (ITS2 rDNA) using primers 3S (Morgan and Blair, 1995) and ITS2.2 (Anderson and Barker, 1998). In addition, the mitochondrial DNA cytochrome c oxidase subunit 1 (mtCOI) was amplified using primers JB3 (Morgan and Blair, 1998) and JB4.5 (Razo-Mendivil et al., 2008) and the mitochondrial DNA NADH dehydrogenase subunit 1 (mtND1) using primers JB11 (Morgan and Blair, 1998) and NDJ2a (Kostadinova et al., 2003) (see definition of primers in Table 3.2). The thermocycling profile for the ITS2 rDNA amplification was as follows: denaturation at 95° C for 3 min, 40 cycles at 94° C for 50 s, 56° C for 50 s and 72° C for 1 min 20 s, and a final extension at 72° C for 4 min. The profile for the mtCOI amplification was denaturation at 94° C for 5 min, 40 cycles at 92° C for 30 s, 45.6° C for 45 s and 72° C for 90 s, and a final extension at 72° C for 10 min. The profile for mtND1 amplification was denaturation at 95° C for 5 min, 40 cycles at 94° C for 30 s, 50° C for 20 s and 72° C for 45 s, and a final extension at 72° C for 4 min. Amplicons were purified with GFX PCR DNA and Gel Band Purifying Kit (GE Healthcare Life Sciences, UK)

and sequenced on an Applied Biosystems ABI 3730 XL automated sequencer by Macrogen Inc. Europe (The Netherlands). Contigs were assembled using BioEdit 7.0.5.3 and sequence identity was checked using the Basic Local Alignment Search Tool (BLAST).

Table 8.2. Localities, host species, number of dolphins sampled (N), number of worms collected (n) and institutions responsible for the collection of specimens of the digenean *Pholeter gastrophilus* used for this study.

Locality (Country or region)	Host species (Common name)	N (n)	Collection institution
Mediterranean Sea (Alboran Sea and Western Mediterranean)	<i>Stenella coeruleoalba</i> (Striped dolphin)	15 (14)	ICBIBE ⁵
	<i>Tursiops truncatus</i> (Bottlenose dolphin)	5 (7)	ICBIBE
	<i>Delphinus delphis</i> (Common dolphin)	1 (2)	ICBIBE
	<i>Globicephala melas</i> (Long-finned pilot whale)	1 (2)	CEGMA ²
South Western Atlantic (Argentina)	<i>Tursiops truncatus</i> (Bottlenose dolphin)	1 (3)	CENPAT ⁴
Central Eastern Atlantic (Canary Islands, Cádiz and Galicia)	<i>Stenella coeruleoalba</i> (Striped dolphin)	1 (1)	ULPGC ⁸
	<i>Stenella coeruleoalba</i> (Striped dolphin)	9 (11)	CEGMA
	<i>Globicephala macrorhynchus</i> (Short-finned pilot whale)	3 (5)	CEMMA ³
North Eastern Atlantic (Germany, Belgium and Ireland)	<i>Phocoena phocoena</i> (Harbour porpoise)	11 (11)	ITAWR ⁶
	<i>Lagenorhynchus acutus</i> (Atlantic white-sided dolphin)	1 (1)	ITAWR
	<i>Phocoena phocoena</i> (Harbour porpoise)	4 (6)	BMMB ¹
	<i>Phocoena phocoena</i> (Harbour porpoise)	2 (4)	SBEES ⁷
	<i>Stenella coeruleoalba</i> (Striped dolphin)	1 (1)	SBEES

¹ **BMMB**, Belgian Marine Mammal Biobank (Belgium).

² **CEGMA**, Centro de Gestión del Medio Marino Andaluz del Estrecho (Spain).

³ **CEMMA**, Coordinadora para o Estudo dos Mamíferos Mariños (Spain).

⁴ **CENPAT**, Laboratorio de Mamíferos Marinos, Centro Nacional Patagónico (Argentina).

⁵ **ICBIBE**, Institut Cavanilles de Biodiversitat i Biologia Evolutiva, University of Valencia (Spain).

⁶ **ITAWR**, Institute for Terrestrial and Aquatic Wildlife Research, University of Veterinary Medicine (Germany).

⁷ **SBEES**, School of Biological, Earth and Environmental Sciences, University College Cork (Ireland).

⁸ **ULPGC**, University of Las Palmas de Gran Canaria (Spain).

Phylogenetic analyses

The first dataset used in this study included 68 sequences of the ITS2 rDNA of *P. gastrophilus*, and sequences from *Pygidiopsis genata* and *Ascocotyle longa* (Heterophyidae), which were

downloaded from GenBank and used as outgroups (GenBank accession numbers AY245710 and AY245703, respectively). Outgroups were selected according to previous phylogenetic studies for the family Heterophyidae (Fraija-Fernández et al., 2015b). Sequences were aligned using the online version of MAFFT, available at <http://mafft.cbrc.jp/alignment/server/>. Complete alignment was analysed and uncorrected pairwise p distance matrices were obtained using MEGA 6 (Tamura et al., 2013).

An additional dataset included 68 concatenated sequences of each corresponding mtND1 and mtCOI sequence from each worm, and sequences of *Metagonimus yokogawai* (Heterophyidae) and *Opisthorchis felineus* (Opisthorchiidae), which were downloaded from GenBank and used as outgroups (GenBank accession numbers NC011127 and NC023249, respectively). Previous to concatenation, mitochondrial sequences were aligned independently for each gene using the online version of MAFFT, and were analysed using JModelTest 4.1.2 (Darriba et al., 2012). The complete dataset was run on MEGA 6 (Tamura et al., 2013) for a ML analysis under the Hasegawa, Kishino and Yano model with gamma distribution (HKY+G), which was selected as the best model that fit both, mtCOI and mtND1 alignments. Bootstrap values were obtained after 1000 replicates and the heuristic tree searching strategy was Subtree-Pruning-Regrafting (SPR).

Genetic diversity analyses

For population analyses, the 68 sequences of the concatenated regions of the mtND1 and mtCOI of *P. gastrophilus* were arranged according to the four geographic regions defined previously, i.e., Med, SWA, CEA and NEA (Figure 8.1). Genetic diversity for the complete dataset ($n = 68$) and for each population were estimated as the number of unique haplotypes, the number of segregating or polymorphic sites (S), haplotype diversity (H_d) (i.e., the probability that two randomly sampled haplotypes are different), and nucleotide diversity (π) (i.e., the average number of nucleotide differences per site), without considering missing data, as implemented in DNAsp (Librado and Rozas, 2009). A haplotype network using Minimum Spanning Tree was constructed to illustrate the connections between haplotypes using Population Analysis with Reticulate Trees (PopART) available at <http://popart.otago.ac.nz> (Leigh and Bryant, 2015).

Uncorrected pairwise p distance matrices for the aligned mitochondrial dataset were calculated with MEGA 6. To examine whether genetic differentiation in samples from *P. gastrophilus* might be influenced by a pattern of isolation by distance, Mantel tests were explored as implemented in the library VEGAN from the public domain statistical software R (Oksanen et al., 2015; R Core Team, 2015). Genetic distances were calculated as Φ_{ST} between pairs of localities in the Isolation by Distance Web Service, available at <http://ibdws.sdsu.edu/~ibdws> (Jensen et al., 2005). Geographic distances, expressed as nautical miles (nm), were estimated to the nearest port using the online platform <http://ports.com>. An effect of isolation by distance would be indicated, prima facie, by a positive and significant correlation between geographic and genetic distances. However, a Mantel test fails to differentiate between patterns resulting from geographical clustering, and those from isolation by distance where there is equal migration

among populations (Meirmans, 2012; Diniz-Filho et al., 2013). Therefore, a partial Mantel test was used to explore whether the correlation between genetic and geographic distances of samples was affected by a spatially structured effect. For the partial Mantel tests a third matrix was included in the correlation with the information on the geographical structuring of localities, i.e., whether pairs of localities were from the same region or not. We are aware of recent debate on the probability of type I error in partial Mantel tests (see Guillot and Rousset, 2013), thus results were interpreted with caution, and the null hypothesis of isolation by distance was rejected only if p was < 0.001 (Oden and Sokal, 1992; Diniz-Filho et al., 2013). In the partial Mantel test we permuted localities within regions (Meirmans, 2012), thus samples from SWA were excluded from the analysis because all samples were collected from a single locality. A graphical representation of the pattern of isolation by distance was made using the Isolation by Distance Web Service (Jensen et al., 2005).

Population structure analyses

The partitioning of genetic variation within sequences of *P. gastrophilus* was investigated with an Analysis of Molecular Variance (AMOVA) (Excoffier et al., 1992). AMOVA estimates the genetic variance at different levels of a hierarchical division of the population and test the null hypothesis of no genetic structure between regions using a permutational approach (Excoffier et al., 1992). The power for detection of genetic structure between regions decreases with few samples per locality in the population (Fitzpatrick, 2009). Following Fitzpatrick's (2009) approach, we estimated the number of possible permutations and the minimum p value according to the region structure designed for *P. gastrophilus*, i.e., one locality for SWA, two localities for Med, three localities for CEA, and three localities for NEA (see Figure 8.1). The genetic structure in *P. gastrophilus* was partitioned into variation from all samples considering the nine localities ("within the population" Φ_{ST} estimate), the variation among four regions, i.e., SWA, NEA, Med and CEA ("among regions" Φ_{CT} estimate), and the variation among localities within regions ("among localities, within regions" Φ_{SC} estimate). To assess "among regions" variation, three alternative scenarios were considered according to geographic proximity. First, samples from the Celtic Sea were grouped with CEA (i.e., the AMOVA included one locality for SWA, two localities for Med, four localities for CEA and two localities for NEA). Second, samples from SWA were grouped with CEA (i.e., four localities for SWA+CEA, two localities for Med and three localities for NEA). Third, samples from the Strait of Gibraltar were grouped with Med (i.e., one locality for SWA, three localities for Med, two localities for CEA and three localities for NEA). P values associated with the fixation indices were evaluated through 2520 and 1260 random permutations for a four-grouped and a three-grouped structure, respectively, as implemented in Arlequin v. 3.5. (Excoffier and Lischer, 2010).

Demographic history analyses

Tajima's D and Fu's tests were used to assess whether sequences of *P. gastrophilus* departed from the assumption of neutrality (Tajima, 1993; Fu, 1997). Positive or negative values of Tajima's D and Fu's would imply the operation of non-neutral processes (e.g., natural selection or population expansion/contraction), whereas significant values would be consistent with population expansion (Tajima, 1993). Tajima's D and Fu's tests and their significance values were calculated with Arlequin v. 3.5, based on 1000 simulated re-sampling replicates (Excoffier and Lischer, 2010). The significant threshold for Tajima's D was set at 0.05, and for Fu's at 0.02. The R2 neutrality test was also used because it seems to be more powerful for detecting population growth with small sample sizes (Ramos-Onsins and Rozas, 2002; Ramírez-Soriano et al., 2008). R2 values and its significance, under 10,000 coalescent simulations, were calculated with the Population and Evolutionary Genetics Analysis System (PEGAS), implemented in the software R (Paradis, 2010; R Core Team, 2015).


Growth or decline episodes in a population leave signals in the distribution of pairwise nucleotide differences between pairs of individuals, which can be analysed through a mismatch distribution (Rogers and Harpending, 1992). Unimodal and smooth wave-like distributions suggest recent population expansion, whereas irregular multimodal distributions would occur in stationary or shrinking populations (Harpending, 1994). Mismatch distribution was computed in Arlequin v.5.3, and the smoothness of the observed distribution was quantified with the Harpending's Raggedness Index under 10,000 bootstrap replicates. The approximate time (t) since population expansion was estimated as $t = \tau/2u$, where τ is the time of expansion in units of mutational time, given as an output of the mismatch distribution, and u is the mutation rate of the DNA region calculated as $m_{\tau}\mu$, where m_{τ} is the length of the sequences under study and μ the mutation rate per nucleotide (Rogers and Harpending, 1992). The mutation rate for the mtDNA was considered 2% per million years according to Galtier et al. (2009).

RESULTS

Phylogenetic analyses

We obtained 68 sequences from the ITS2 rDNA from specimens of *P. gastrophilus*, which were between 407 base pairs (bp) and 501 bp long. Pairwise distance comparison for the aligned portion of the ITS2 rDNA indicated no genetic variation among specimens of *P. gastrophilus* across the sampled regions.

The 68 mtND1 sequences of *P. gastrophilus* were between 407 bp and 504 bp long, whereas the 68 mtCOI sequences were between 341 bp and 441 bp long. The alignment of the concatenated sequences of the mtND1 and the mtCOI was 701 bp long excluding sites with missing data. The ML hypothesis suggests that all worms collected from the four regions corresponded to a single and well-supported lineage (ML bootstrap = 100%; Figure 8.2). The

Figure 8.2. (See next page) Maximum Likelihood analysis for the phylogenetic relationships inferred from the mtND1 and mtCOI sequences of 68 individuals of the digenean *Pholeter gastrophilus*, collected from seven host species and four geographical regions. Support values for each node were calculated as ML bootstrap after 1000 replicates. Sequences are labelled according to their sampling localities as follows: Central Eastern Atlantic, **CEA** (Canary Islands, **CI**; Galicia North-West Spain, **Gal**; Strait of Gibraltar, **SoG**); Mediterranean Sea, **Med** (Alboran Sea, **AS**; western Mediterranean, **WM**); North Eastern Atlantic, **NEA** (Celtic Sea, **CS**; North Sea, **NS**); South Western Atlantic, **SWA**. 

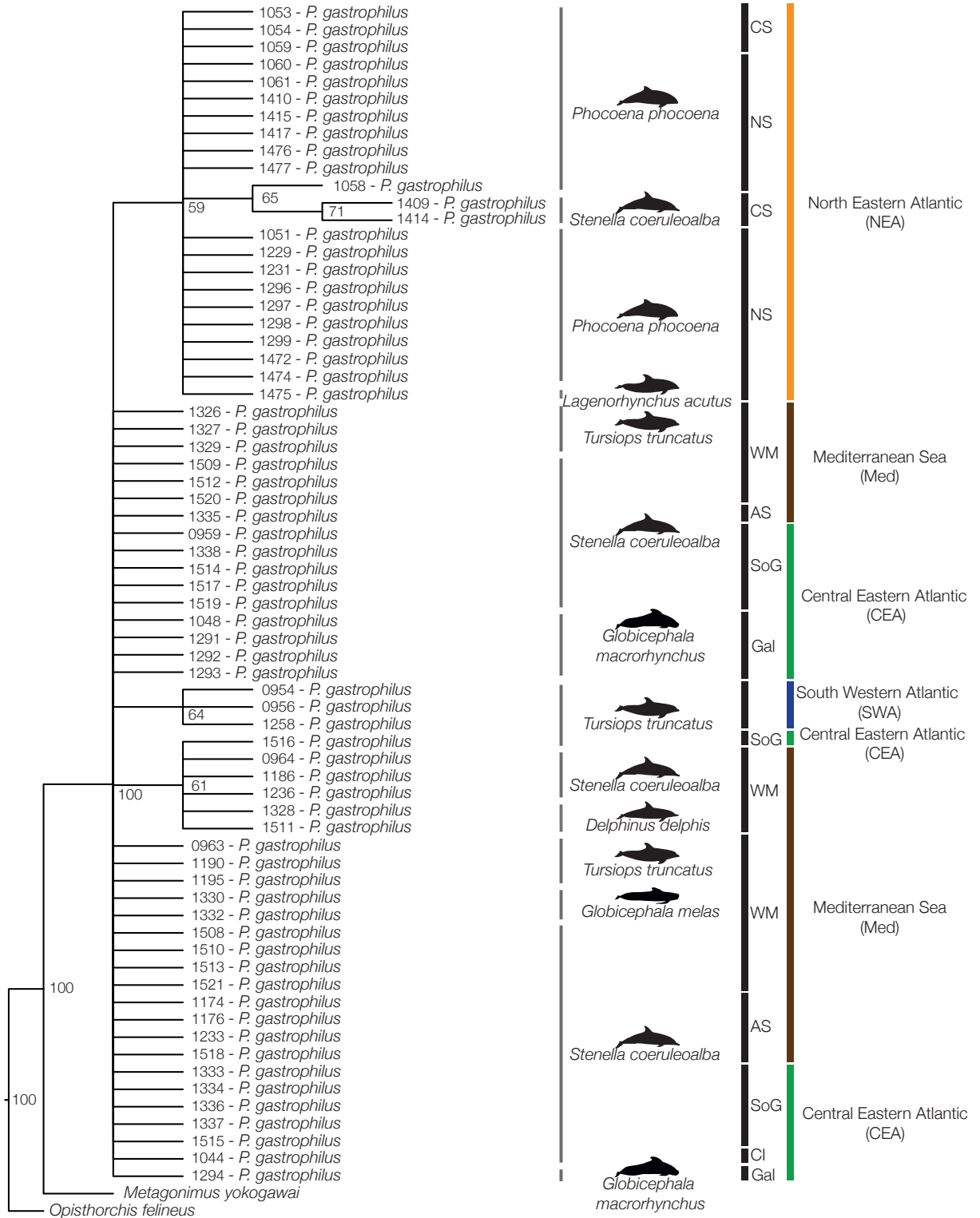
intraspecific topology of the ML hypothesis did not support reciprocally monophyletic groups of samples. No internal node received bootstrap support $\geq 71\%$, although some clades received support $\geq 59\%$, including samples for NEA (59%) and SWA (64%). An additional clade was also found, grouping five and one sequences from Med and CEA, respectively (ML bootstrap = 61%) (Figure 8.2).

Table 8.3. Frequency and GenBank accession numbers of the 16 haplotypes identified for the mtND1 and mtCOI sequences of the digenean *Pholeter gastrophilus* in different geographic regions.

Region	Haplotype no.	Frequency	GenBank accession no.
Mediterranean Sea (Med)	2	1	KX059398
	5	1	KX059401
	6	1	KX059402
	8	1	KX059404
	10	1	KX059406
Central Eastern Atlantic (CEA)	13	1	KX059409
	14	1	KX059410
	15	1	KX059411
South Western Atlantic (SWA)	9	3	KX059405
North Eastern Atlantic (NEA)	11	20	KX059407
	12	1	KX059408
	16	2	KX059412
Med + CEA	1	10 (Med: 8, CEA: 2)	KX059397
	3	4 (Med: 3, CEA: 1)	KX059399
	4	15 (Med: 7, CEA: 8)	KX059400
	7	5 (Med: 2, CEA: 3)	KX059403

Genetic diversity analyses

A total of 15 polymorphic sites and 16 different haplotypes were found in the 701 bp-alignment of the concatenated mtND1 and mtCOI sequences (Table 8.3). The haplotype network showed a relatively simple, star-like topology (Figure 8.3). Haplotype 4, occurring in Med and CEA, was located centrally. Three haplotypes, namely 1, 3 and 7, stemmed from haplotype 4 and were also shared between Med and CEA. Five haplotypes occurred exclusively in the Med population (haplotypes 2, 5, 6, 8 and 10, see Figure 8.3), where the highest haplotype and nucleotide diversity was observed ($H_d = 0.82$; $\pi = 0.0021$, Table 8.4), followed by the CEA, with three exclusive haplotypes, i.e., 13, 14 and 15 (Figure 8.3), and $H_d = 0.76$; $\pi = 0.0016$. Three



0.6

unique haplotypes stemming from the central haplotype were found among individuals of NEA (haplotypes 11, 12 and 16, Figure 8.3; $Hd = 0.24$; $\pi = 0.0006$), whereas a single haplotype was found in individuals from SWA (haplotype 9, Figure 8.3; $Hd = 0$; $\pi = 0$). Single mutational steps established the connection between all haplotypes. The mtND1 showed the highest number of polymorphic sites representing, 14 out of the 16 defined haplotypes, whereas only two haplotypes were found in the mtCOI (data not shown).

Genetic pairwise distances for the mtND1 were 0.3% (CEA vs. Med), 0.4% (SWA vs. Med, SWA vs. CEA, and CEA vs. NEA), 0.5% (NEA vs. Med) and 0.6% (NEA vs. SWA), whereas for the mtCOI, a maximum of 0.1% genetic distance was found among sequences from all geographic regions. The plot for the correlation between genetic and geographic distances is shown in Figure 8.4. There was a significant pattern of isolation by distance, suggesting an increase of genetic distances together with geographic distances (Mantel test, $r = 0.5952$; $p = 0.001$). However, when the spatial structure of localities was considered a non-significant partial Mantel test was obtained (Partial Mantel test, $r = 0.5836$; $p = 0.007$), suggesting that the geographical clustering of localities, and not the isolation by distance, would account for the pattern observed in samples from the CEA, Med and NEA.

Table 8.4. Number of polymorphic sites (S), number of haplotypes (H), haplotype diversity (Hd), and nucleotide diversity (π) for the digenean *Pholeter gastrophilus* in different geographical regions. “N” is the sample size and (*) represents the number of shared haplotypes.

Region	N	S	H	Hd	π
Mediterranean Sea (Med)	25	8	5+4*	0.82	0.0021
Central Eastern Atlantic (CEA)	17	6	3+4*	0.76	0.0016
North Eastern Atlantic (NEA)	23	2	3	0.24	0.0006
South Western Atlantic (SWA)	3	0	1	0.00	0.0000
TOTAL	68	15	16	0.84	0.0023

Population structure analyses

The AMOVA showed a significant genetic differentiation in *P. gastrophilus* at different scales. The highest amount of variation was found within the population, which explained 59.71% of the overall variation ($\Phi_{ST} = 0.40$, $p < 0.001$, Table 8.5). A significant “among regions” variation ($\Phi_{CT} = 0.37$, $p = 0.005$) was also found, which accounted for 37.1% of the overall variation. However, only 3.2% of the overall variation was explained by “among localities, within regions” variation ($\Phi_{SC} = 0.05$, $p = 0.129$) (Table 8.5). In an alternative arrangement, we grouped samples of the Celtic Sea with those from CEA, and “among localities, within regions” variation increased to 10.3%, and the “among regions” variation decreased to 29.1%. A similar pattern was observed when sequences of SWA were grouped with CEA. In this case, the “among localities, within

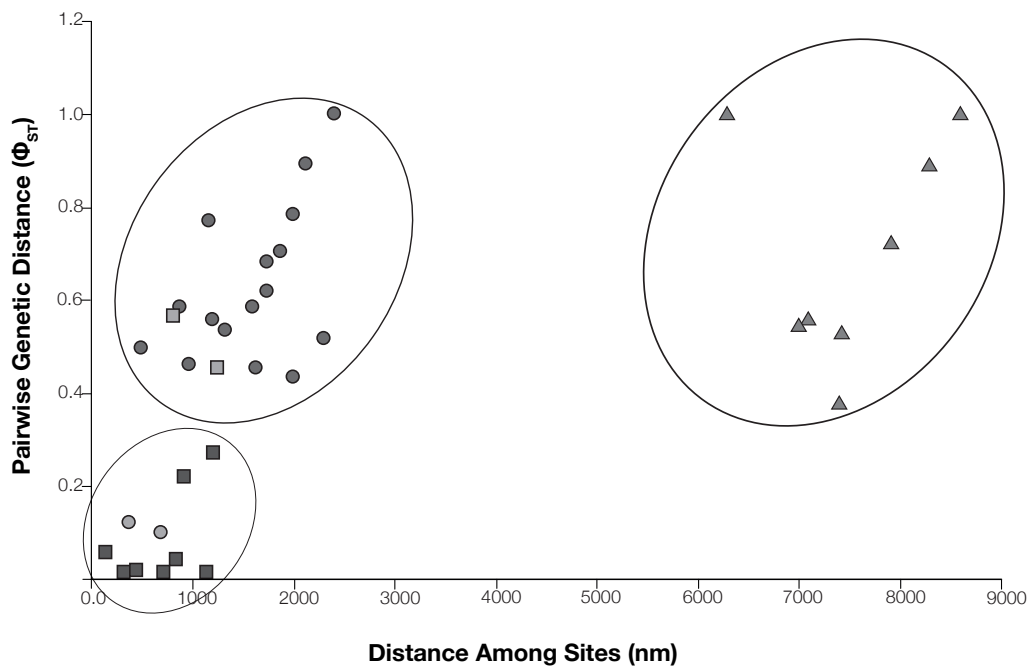


Figure 8.4. Graphical representation of the isolation by distance analysis among populations of the digenean *Pholeter gastrophilus*. The relationship between pairwise genetic distance (Φ_{ST}) and geographical distance (nm) between sampling sites are shown. Triangles represent comparisons of samples from the South Western Atlantic (SWA); squares represent comparison of samples with the Mediterranean Sea (Med) and the Central Eastern Atlantic (CEA); and circles represent comparison of samples with the North Eastern Atlantic (NEA).

Demographic history analyses

Values from both Tajima's D (-1.45, $p = 0.042$) and Fu's (-9.22, $p < 0.001$) tests were negative and statistically significant. The R^2 was low and significant ($R^2 = 0.049$, $p = 0.031$). Overall, these results suggest that sequences from *P. gastrophilus* have a bias towards few segregating sites and few haplotypes compared to what would be expected from the neutral theory, supporting a population expansion of *P. gastrophilus*.

The mismatch distribution analysis showed a unimodal distribution (Figure 8.5), with a low and significant Harpending's Raggedness Index ($r = 0.08$, $p < 0.001$), which suggests a good fit between the distribution of the observed and the expected values. The pattern of distribution and the small value of τ ($\tau = 1.7$) indicate that the population expansion was recent and started from a small population ($\theta = 0$). Based on the value of τ obtained in the mismatch distribution, and assuming that the mutation rate for the mtDNA is 2% per million years (Galtier et al., 2009), we infer that *P. gastrophilus* would have gone through an event that reduced sequence variation, followed by a recent population expansion, which was estimated to start 60,627 years ago.

Table 8.5. Analysis of Molecular Variance (AMOVA) of sequences of mtND1 and mtCOI from 68 individuals of the digenean *Pholeter gastrophilus*. Three scenarios are shown according to different arrangements of geographical regions. Abbreviations: **Arg**: Argentina coast; **AS**: Alboran Sea; **CEA**: Central Eastern Atlantic; **CI**: Canary Islands; **CS**: Celtic Sea; **Gal**: Galicia; **Med**: Mediterranean Sea; **NEA**: North Eastern Atlantic; **NS**: North Sea; **SoG**: Strait of Gibraltar; **SWA**: South Western Atlantic; **WM**: Western Mediterranean.

1) Geographical regions				
Med (AS + WM); SWA (Arg); CEA (Gal + SoG + CI); NEA (CS + NS)				
Source of variation	d.f.	Percentage of variation (%)	Fixation indices	<i>p</i> value
Among regions	3	37.14	Φ_{CT} 0.37	0.005
Within regions	5	3.15	Φ_{SC} 0.05	0.129
Within population	59	59.71	Φ_{ST} 0.40	<0.001
2) Geographical regions				
Med (AS + WM); SWA (Arg); CEA (Gal + CI + SoG + CS); NEA (NS)				
Source of variation	d.f.	Percentage of variation (%)	Fixation indices	<i>p</i> value
Among regions	3	29.11	Φ_{CT} 0.29	0.023
Within regions	5	10.25	Φ_{SC} 0.14	0.011
Within population	59	60.65	Φ_{ST} 0.39	<0.001
3) Geographical regions				
Med (AS + WM); SWA + CEA (Arg + Gal + CI + SoG + CS); NEA (CS + NS)				
Source of variation	d.f.	Percentage of variation (%)	Fixation indices	<i>p</i> value
Among regions	2	28.73	Φ_{CT} 0.28	0.025
Within regions	6	11.12	Φ_{SC} 0.15	0.001
Within population	9	60.15	Φ_{ST} 0.39	<0.001
4) Geographical regions				
Med (AS + WM + SoG); SWA (Arg); CEA (Gal + CI); NEA (CS + NS)				
Source of variation	d.f.	Percentage of variation (%)	Fixation indices	<i>p</i> value
Among regions	3	41.28	Φ_{CT} 0.41	<0.001
Within regions	5	2.34	Φ_{SC} 0.04	0.161
Within population	59	56.38	Φ_{ST} 0.44	<0.001

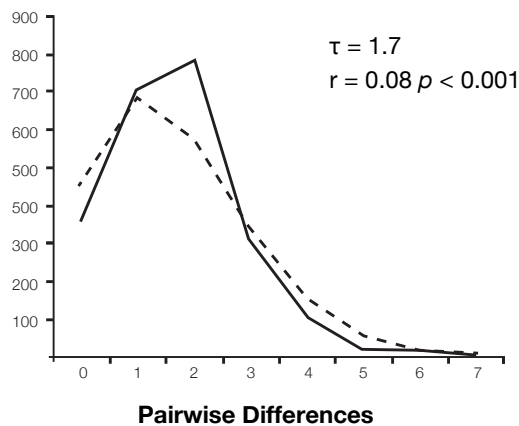


Figure 8.5. Mismatch distribution curve for the digenean *Pholeter gastrophilus* showing the expected (dashed line) and the observed values (continuous line) under the expanding population model. The time of expansion in units of mutational time (τ) and the Harpending's Raggedness Index (r) are shown.

DISCUSSION

Results from this study support the hypothesis that *P. gastrophilus* represents a single species. No variation was found in the ITS2 rDNA sequences from all samples, and a maximum genetic difference of just 0.6% was observed in the mtND1 and mtCOI between worms from the two most distant regions (SWA vs. NEA). Previous studies have suggested that the maximum intraspecific variation in digeneans to recognise separate species would be ~1% for ITS2 rDNA (Nolan and Cribb, 2005) and ~5% for mtDNA (Vilas et al., 2005), well above the observed values for *P. gastrophilus*. Although the use of this “genetic yardstick” has been criticised (see, e.g., Nadler, 2002), the absence of reciprocal monophyly also confirms that *P. gastrophilus* is a single lineage, with no deep genetic differentiation associated to a geographical or an ecological context. Reciprocal monophyly has been considered as a suitable alternative method for species delimitation (Nadler, 2002; Kizirian and Donnelly, 2004; De Queiroz, 2007).

The genetic cohesion of *P. gastrophilus* should be determined by the movement of individuals allowing gene flow across the broad distribution range of this species. However, the free-living stage of digeneans has small dispersal ability and, therefore, genetic exchange between digenean populations must rely on the vagility of their hosts (Blasco-Costa and Poulin, 2013; Feis et al., 2015). In the case of *P. gastrophilus*, both intermediate and definitive hosts might be contributing to large-scale dispersal. The life cycle of *P. gastrophilus* is expected to include an invertebrate host (likely a mollusc) as first intermediate host, and fish and/or cephalopods as second intermediate hosts (see Cribb et al., 2003). Unfortunately, the identity of the intermediate hosts of *P. gastrophilus* is unknown, and attempts to find infective stages in the main preys of pelagic cetaceans have been unsuccessful (Mateu et al., 2015). However, given the extensive occurrence of this parasite in coastal and oceanic habitats, and in both piscivorous and teuthophagous cetaceans (see Pauly et al., 1998 and Table 8.1), it is likely that *P. gastrophilus* may use a wide array of coastal and mesopelagic invertebrates and vertebrates as intermediate hosts (Fraija-Fernández et al., 2015a), thus promoting transmission and genetic mixture of the infective stages (see Rauch et al., 2005).

In this context, the dispersal role of definitive hosts should not be underestimated. In the Atlantic Ocean, *P. gastrophilus* has been reported in at least six species of pelagic cetaceans (Table 8.1). Most of these species exhibit high dispersal abilities between breeding and feeding grounds, and vast geographic ranges (Gowans et al., 2007; Hoelzel, 2009; Steiner et al., 2012). The ability of cetaceans to move over long distances has been documented for several species including the short-beaked common dolphin (Mirimin et al., 2009; Moura et al., 2013), the oceanic populations of the bottlenose dolphin (Stevick et al., 2002; Torres et al., 2003), the dusky dolphin (*Lagenorhynchus obscurus*) (Cassens et al., 2005), and the short-finned pilot whale (González et al., 2000; Alves et al., 2013). Moreover, data of tracked individuals have shown long-distance migrations of Risso’s dolphin (Wells et al., 2009), bottlenose dolphin (Wells et al., 1999), and striped dolphin (Loizaga de Castro et al., 2011). These data support the hypothesis that the genetic cohesion among populations of *P. gastrophilus* from the Atlantic Ocean would be maintained, at least in part, by the extensive exploitation of wide-ranging and highly mobile definitive hosts,

and a wide array of intermediate hosts that would allow the completion of the parasite's life cycle in many localities. A similar lack of structure has been observed for the nematode species of *Anisakis* spp., which extensively occur as larvae in different species of fish, and as adults in several cetacean species. The genetic homogeneity across distant geographic areas has also been attributed to the generalist condition of these nematodes (Mattiucci et al., 2002). Little genetic structure seems also to be common to parasites of other marine pelagic vertebrates with long-ranging movements (e.g., Goulding and Cohen, 2014; Feis et al., 2015). For instance, no genetic structure was detected for populations of the acanthocephalan *Profilicois altmani* occurring in the Pacific coast of South America and the Atlantic coast of North America. In this case, marine birds act as definitive hosts and help to disperse parasite's eggs through seasonal migrations, in a stepping-stone manner (Goulding and Cohen, 2014).

However, we detected significant population structure in the Atlantic population of *P. gastrophilus*, which was mainly accounted for the samples from the two most distant regions, i.e., SWA and NEA. Sequences of worms from SWA shared a unique haplotype and formed a single clade in the ML cladogram. However, the small sample size precluded the possibility of exploring whether this difference was linked to the existence of geographical or ecological barriers or simply resulted from isolation by distance through a stepping-stone process (Meirmans, 2012). In the Southern Hemisphere, the northernmost record of *P. gastrophilus* occurs off the southern coast of Brazil (Valente and Ebert, 2014), and the species has not been recorded in the Caribbean (Mignucci-Giannoni et al., 1998). This opens the question of whether the Equator could be a significant geographical barrier for the dispersal of *P. gastrophilus*, such as it has been observed in other invertebrates and vertebrates (see Kaliszewska et al., 2005; Plouviez et al., 2010). If so, trans-equatorial movements of large vertebrates (see Castro et al., 2007; Gonzalez et al., 2008) such as cetaceans (Oremus et al., 2009) could contribute to genetic cohesion between northern and southern populations of *P. gastrophilus*.

A single clade and three unique haplotypes were detected for specimens from NEA. Moreover, the AMOVA indicated that the genetic structure in *P. gastrophilus* might partly be attributed to differences between the NEA and all other regions. In addition, results from the partial Mantel test indicated that these differences could primarily result from the existence of a geographical barrier isolating NEA samples. In fact, strong oceanographic barriers have been identified for the transition zone between the boreal and the subtropical regions in the Bay of Biscay (Pingree and LeCann, 1990; Southward et al., 1995). Changes in the climatological conditions contribute to the formation of barriers that influences prey availability for cetaceans (Casey and Pereiro, 1995; Spitz et al., 2006; Fontaine et al., 2014), which would be potential intermediate hosts for *P. gastrophilus*. However, 21 out of the 23 samples from the NEA were collected from harbour porpoises, which is the only cetacean in our study that is restricted to coastal habitats. The relative sedentary behaviour of harbour porpoises, and their dependency of coastal prey for feeding, appear to generate a strong population structure in Northern European waters when compared to southern regions (Fontaine et al., 2007; Alfonsi et al., 2012; Fontaine et al., 2014). Thus, one can wonder if coastal isolation of the harbour porpoise might also contribute to the

population differentiation of *P. gastrophilus* in NEA. Interestingly, Fraija-Fernández et al. (2015a) recently found different life strategies of *P. gastrophilus* between coastal vs. oceanic cetaceans, suggesting a potential ecological isolation of hosts.

The neutrality tests rendered negative and significant values, suggesting that the population of *P. gastrophilus* has suffered either an expansion or an evolutionary event that removed variation in the population (e.g., selective sweeps or bottleneck events) (Tajima, 1993). Note that Fu's and R2 tests are more powerful than Tajima's (Ramírez-Soriano et al., 2008), particularly when sample size is small (Ramos-Onsins and Rozas, 2002). Moreover, results from the mismatch distribution suggested that this population expansion began ca. 60,000 years ago. This pattern is compatible with the hypothesis that *P. gastrophilus* suffered a population bottleneck in the Atlantic Ocean. Alternatively, the observed demographic structure could reflect the origin and subsequent spreading of *P. gastrophilus* in cetaceans. It has been hypothesized that the ancestor of *P. gastrophilus* colonised cetaceans from marine birds via trophic guilds (Fraija-Fernández et al., 2015b). Since colonization via host switching should start from a small population, the population of *P. gastrophilus* would have had undergone a population expansion by exploiting different cetacean species. This hypothesis, however, would imply that the origin of the association between *P. gastrophilus* and cetaceans occurred in the Atlantic, its occurrence in the Pacific basin being more recent (see Table 8.1). This hypothesis should be tested in the future by including samples from the entire geographic range of *P. gastrophilus*.

In summary, results from this study indicate that in the Atlantic Ocean and Mediterranean Sea *P. gastrophilus* appears to represent a single species. Patterns for gene flow are likely linked to its recent demographic history, and are related to the extensive exploitation of vertebrates and invertebrates as intermediate hosts, which allows a dissemination of infective stages through the food web, and the high mobility of (at least) its definitive hosts. However, geographic and/or ecological barriers would have promoted significant population structure in the Atlantic.

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LIFE-HISTORY TRADE-OFFS IN A GENERALIST DIGENEAN FROM CETACEANS: THE ROLE OF HOST SPECIFICITY AND ENVIRONMENTAL FACTORS

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ABSTRACT

Adults and larvae of generalist parasites are exposed to diverse hosts and local environmental conditions throughout their life cycles, thus local adaptation is expected to occur through phenotypic plasticity and/or natural selection. We investigated how the combined effect of cryptic host specificity and local selective pressures could shape reproductive traits of a putative generalist parasite in the oceanic realm. The LSU rDNA, ITS2 and the mtCOI of individuals of the digenean *Pholeter gastrophilus* (Kossack, 1910) Odhner, 1914 (Heterophyidae Leiper, 1909) from oceanic striped dolphins, *Stenella coeruleoalba* Meyen, and coastal bottlenose dolphins, *Tursiops truncatus* Montagu, in the western Mediterranean were used to elucidate whether worms were conspecific. Infection parameters were compared between both dolphin species. General Linear Mixed Models were used to analyse the influence of host species on four reproductive traits of *P. gastrophilus*: body size, maturity stage (non-gravid/gravid), egg size, and number of eggs *in utero*. AIC values were used to rank competing models, and *p* values to assess the effect of specific predictors. Evidence indicated that worms collected from both dolphin species were conspecific. All worms collected were gravid and infection parameters did not differ between dolphin species. However, body size and egg size of individuals of *P. gastrophilus* were significantly larger in striped dolphins. The number of eggs *in utero* did not significantly differ between dolphin species but, for a given body size, worms in bottlenose dolphins harboured more eggs. A trade-off between egg size and egg number was found in worms from both dolphin species, with a higher slope in striped dolphins. Apparently, striped dolphin is a more suitable host for *P. gastrophilus*, but reproductive investment seems to be adapted to the habitat where the life-cycle develops. Worms from striped dolphins likely face the problem of finding intermediate hosts in the oceanic realm and apparently invest into offspring size to enhance the early survival of larvae and the potential to multiply asexually within the first intermediate host. The small-sized worms from bottlenose dolphins would be adapted to reproduce early because of higher adult mortality, generating smaller and numerous eggs in a coastal habitat where chances of transmission are presumably higher.

INTRODUCTION

Host specificity is a measure of the degree to which parasites species can exploit different host species. Differences of specificity are a matter of degree rather than kind: at one extreme, specialist parasites can exploit only one or very few host species; at the other extreme, generalist parasites can infect and reproduce in a number of host species (Poulin et al., 2011). According to the encounter/compatibility paradigm, the degree of specificity of any parasite is determined by the action of two sequential filters. The ‘encounter’ filter prevents infections of potential hosts that cannot contact the parasite, whereas the ‘compatibility’ filter excludes contacted hosts in which the parasite cannot find the appropriate resources and/or escape or deter the host’s defenses (Combes, 2001; Kuris et al., 2007). If the contacted hosts are suitable but suboptimal, the compatibility filter can still negatively affect fitness components of the parasite.

A fundamental question regarding host specificity is the extent to which a putative generalist parasite performs equally well in all exploited host species, namely, whether these hosts are all equally compatible for the parasite (Lane et al., 2015). This question has important implications, not only for understanding the evolution of specificity, but also for population dynamics and epidemiology of generalist parasites (Beck et al., 2015). In some cases, there is little evidence that sympatric hosts impact differently the performance of a putative generalist parasite (e.g. Beck et al., 2015). Other studies, however, have reported on significant host species effects on fitness-related traits of the parasite such as dwarfism and impaired reproduction in presumably suboptimal hosts (e.g. Adams et al., 1998; Perdiguero-Alonso et al., 2006; Mateu et al., 2011). Other studies have discovered more subtle effects on parasite’s life history traits (e.g. fecundity) of host species that, at first glance, seemed to be equally suitable for the parasite (e.g., George-Nascimento and Marin, 1992; Riquelme et al., 2006; Lootvoet et al., 2013). It should be stressed, however, that some putative generalist parasites have later been re-interpreted, based on molecular evidence, as cryptic species complexes, each species being adapted to a different host (Jousson et al., 2000; Blasco-Costa et al., 2010).

Differences of performance of a generalist parasite among host species usually result from the diverse conditions each host species provides (Krasnov et al., 2003; Beck et al., 2014). For instance, a parasite can be adapted to some host species, but is also able to reproduce, with reduced success, in related hosts because, in the latter, the parasite suffers a shortage of trophic resources or harsh physiological conditions, or incur higher costs in the face of host immune responses (Sorci et al., 2003; Krasnov et al., 2004). However, individual life history traits are constrained, not only by phylogenetic, physical or developmental factors, but also by trade-offs with other traits (Stearns, 1992). Selection pressures operate on the whole life-cycle and, therefore, trade-offs are optimised for the specific environment where the life-cycle develops (Poulin and Hamilton, 2000; Loot et al., 2008; Koehler et al., 2011). Therefore, it is important to consider not only the microhabitat conditions each host species provides, but also where each host lives. To our knowledge, few studies have analysed how the combined effect of host species

constraints, and local selective pressures on the life-cycle, could shape life history traits of a generalist parasite (e.g. Beck et al., 2015).

In this paper, we investigate this issue in a digenean infecting two cetacean species that occur in different habitats, i.e. coastal and oceanic. *Pholeter gastrophilus* (Kossack, 1910) Odhner, 1914 is a member of the family Heterophyidae Leiper, 1909 that has been reported as adult in at least 17 odontocete species inhabiting coastal, oceanic and even freshwater habitats worldwide (Frajia-Fernández et al., 2015). Worms live encysted inside fibrotic nodules in the wall of glandular chambers of the stomach (Aznar et al., 2006). Eggs are released to the stomach lumen through a narrow duct that stems from the cyst (Migaki et al., 1971; Gibson et al., 1998). The life-cycle of *P. gastrophilus* is not known but, based on the broad ecological distribution of its definitive hosts and evidence on the life-cycles of other heterophyids, it can be postulated that molluscs act as first intermediate hosts, and a wide array of invertebrates and/or fish are second intermediate hosts (Gibson et al., 1998; Aznar et al., 2006).

In the western Mediterranean, *P. gastrophilus* has been reported in four sympatric cetacean species, i.e. the Risso's dolphin, *Grampus griseus* G. Cuvier, the long-finned pilot whale, *Globicephala melas* Traill and, especially, the bottlenose dolphin, *Tursiops truncatus* Montagu, and the striped dolphin, *Stenella coeruleoalba* Meyen (Raga et al., 1985; Raga and Balbuena, 1987; 1993; Fernández et al., 2003; Quiñones et al., 2013). Gravid specimens of *P. gastrophilus* have been found in all these host species, but a more rigorous analysis, of the extent to which host species may affect growth and reproduction of the parasite, has never been carried out. Interestingly, the Mediterranean hosts of *P. gastrophilus* live in ecologically distinct habitats. In particular, striped dolphins, Risso's dolphin and long-finned pilot whales are primarily oceanic species, whereas bottlenose dolphins favour more coastal waters (Cañadas and Sagarminaga, 2000; Gómez de Segura et al., 2008). The extent to which environmental factors associated to oceanic vs. coastal realm also influence the reproductive strategies of *P. gastrophilus* is an open, interesting question.

In this study, we examined patterns of host specificity of *P. gastrophilus* collected from bottlenose and striped dolphins in the western Mediterranean with four specific aims. First, we ascertained if worms collected from both host species belong to a single generalist taxon or represent a species complex. Second, we compared infection parameters of *P. gastrophilus* between both dolphin species. Third, we analysed host-parasite compatibility based on a comparison of four reproduction-related traits, i.e. body size, presence/absence of eggs, number of eggs *in utero*, and egg size. Finally, we explored whether a phenotypic trade-off between egg number and egg size occurred (Poulin, 1995a; Morand and Sorci, 1998; Trouvé et al., 1998), and whether it was optimised differently for each host species. We acknowledge from the outset that the amount of data that can be gathered from this system is limited. However, the results obtained strongly suggest how host suitability and environmental conditions can modulate some key features of the life history strategy of an oceanic parasite.

Ethical Approval

Permission and funding to collect stranded dolphins was given by the Wildlife Service of the Valencian Regional Government, Spain, which is the official institution in charge of managing and protecting wildlife in the region. No ethics board was involved because animals were collected after their natural death.

MATERIALS AND METHODS

Sample collection

A total of 39 striped dolphins and 21 bottlenose dolphins stranded on the Mediterranean coast of Spain (40°25'N, 0°26'E and 37°58'N, 0°41'W) were collected during 1990–2005. Only well-preserved carcasses (state 1–3 sensu Geraci and Lounsbury, 2005) were selected for analysis. Animals were brought to the laboratory and immediately necropsied; the stomach was removed and frozen at 20°C for later examination. After thawing, each stomach chamber was examined separately for the presence of *P. gastrophilus*. Nodules were detected through visual and tactile screening and incisions were made on each nodule to confirm the presence of *P. gastrophilus*. When positive, the nodule was removed and carefully cut into slices to collect and count all worms. Finally, the stomach content was filtered over a sieve with 0.02 mm mesh spacing to collect worms that were free in the lumen. Specimens of *P. gastrophilus* were washed in 0.9% saline and fixed in absolute ethanol for molecular analysis or 70% ethanol for morphometric analysis.

Molecular analyses

Single individuals of *P. gastrophilus* collected from five bottlenose dolphins, and six worms collected from five striped dolphins were used for molecular analysis to elucidate whether all worms were conspecific. Genomic DNA was extracted from individual worms using a standard phenol-chloroform protocol. Partial large subunit (LSU) rDNA was amplified in two specimens of *P. gastrophilus* from each dolphin species using primers LSU5 (Littlewood and Johnston, 1995) and LSU1500R (Olson et al., 2003). The ITS2 rDNA was amplified in four worms from each dolphin species using primers 3S (Morgan and Blair, 1995) and ITS2.2 (Anderson and Barker, 1998). The mitochondrial COI was amplified from five and three worms from striped and bottlenose dolphins, respectively, using primers JB3 (Morgan and Balir, 1998) and JB4.5 (Razo-Mendivil et al., 2008). An additional primer, 300F (Littlewood et al., 2000) was used as an internal primer for sequencing the LSU rDNA (see definition of primers in Table 3.2). Thermocycling profiles for gene amplification were as follows: for the LSU rDNA, initial denaturation at 94°C for 3 min, 40 cycles of 94°C for 30 s, 56°C for 30 s, 72°C for 2 min, and a final extension at 72°C for 7 min (Olson et al., 2003); for the ITS2 rDNA, initial denaturation at 95°C for 3 min, 40 cycles of 94°C for 50 s, 56°C for 50 s, 72°C for 80 s, and a final extension at 72°C for 4 min (Blasco-Costa et al., 2010); and for the mitochondrial COI gene, initial denaturation at 94°C for 5 min, 40 cycles of 92°C for

30 s, 45.6°C for 45 s, 72° for 90 s, and a final extension at 72°C for 10 min (Razo-Mendivil et al., 2008). Amplicons were purified with a GFX PCR DNA and Gel Band Purifying Kit (GE Healthcare Life Sciences, Buckinghamshire, UK) and cycle sequenced on an Applied Biosystems 3730 DNA Analyser, using Big Dye version 1.1. Contiguous sequences were assembled and analysed using BioEdit v.7.0.5.3 (Hall, 1999). Sequences are available online in the GenBank with accession numbers as follows: sequences for *P. gastrophilus* from striped dolphins KT883852 (LSU rDNA); KT883854 (ITS2 rDNA); KT883856 (mt COI) and sequences of *P. gastrophilus* from bottlenose dolphins KT883853 (LSU rDNA); KT883855 (ITS2 rDNA); KT883857 (mt COI).

Comparison of infection parameters

The 95% confidence intervals (CI) for prevalence of *P. gastrophilus* in striped and bottlenose dolphins was calculated with Sterne's exact method (Reiczigel, 2003), and for mean values of intensity, with the bias-corrected and accelerated bootstrap method using 20,000 replications (Rózsa and Reiczigel, 2000). Prevalence was compared between both dolphin species with Fisher's exact test and mean intensity with bootstrap t-tests (Rózsa and Reiczigel, 2000). Also, a Brunner-Munzel test was used to compare the probability that the intensity of *P. gastrophilus* in individual hosts from one dolphin species was higher than that in individual hosts from the other dolphin species (Neuhäuser and Poulin, 2004). These analyses were carried out with the free software Quantitative Parasitology v. 3 (Rózsa and Reiczigel, 2000).

Comparison of life-history traits

Individuals of *P. gastrophilus* collected from five freshly dead striped dolphins (n = 140 worms) and five bottlenose dolphins (n = 97) were used for comparison of four reproductive traits: body size, maturity stage (gravid/non-gravid), egg size, and number of eggs *in utero*. Parasites were stained with iron acetocarmine; excess of carmine was removed with HCl in 70% ethanol. Specimens were dehydrated through a graded ethanol series, cleared with dimethyl phthalate and mounted as permanent preparations in Canada balsam. Body area and uterine area filled with eggs were drawn for each individual using a stereomicroscope (×40) connected to a drawing tube. In platyhelminths, areas provide a good proxy of body size and the size of irregular elements (Mateu et al., 2011; Lefebvre and Poulin, 2005; Valero et al., 2006). Empty portions of the uterus could not be observed because the uterine wall is very faint and frequently obscured by vitelline follicles. The area of 10 randomly selected eggs was also drawn using a light microscope (×200) connected to a drawing tube (Figure 9.1). A single value of egg area per worm was obtained by averaging measurements from 10 eggs. Egg areas were preferred over egg volumes (calculated assuming regular shapes) for consistency with the other measurements. All areas were calculated with the program Image Tool v.3.0 (Willcox et al., 2015). The number of eggs *in utero* was calculated by dividing the uterine area filled with eggs by the average egg area for each parasite. We confirmed that this method was a good proxy for the number of eggs *in utero* as follows. After calculating the number of eggs *in utero* as described above, we de-mounted 10 randomly chosen worms from striped dolphins (2 per individual host) and 10 from bottlenose dolphins (2

per individual host). Each worm was torn apart in 2000 μl of saline to release all eggs. The solution was homogenised with a magnetic stirrer and eggs from 3 samples of 20 μl were counted with the aid of a Bürker chamber following the manufacturer's recommendations (OptikLabor, Lancing, UK). The average of the three counts was used as a measure of the number of eggs per μl . The relationship between the number of eggs *in utero* calculated with the two methods was linear, fairly strong and highly significant (Pearson's correlation, $r = 0.67$, $p = 0.001$).

General Linear Mixed Models (GLMMs) with type III sum of squares were used to analyse the influence of different predictors on body area, number of eggs *in utero* and egg area of *P. gastrophilus* (Singer, 1998). Morphometric variables were log₁₀-transformed prior to analysis. We used the values of Akaike Information Criterion (AIC) to rank competing models with different numbers of fixed and random parameters. The model with minimum AIC was considered the "best model", and the rest of the models were ranked according to differences in their AIC values (Johnson and Omland, 2004). Models with values of $\Delta\text{AIC} \leq 2$ were considered to have substantial empirical support, whereas those having $\Delta\text{AIC} > 4$ were assumed to have much less support (Burnham and Anderson, 2002). It was also assumed that models with Akaike weights (w_i) ≤ 0.01 were unlikely to be the "true" model (Burnham and Anderson, 2002) and, therefore, they are not shown in the tables. Fixed parameters in all candidate models, excluding the intercept, were also tested for statistical significance using F- or t-tests.

In models accounting for variability in worm body area, "host species" (factor), "intensity" (covariate), and "host species*intensity" were considered as potential predictors, i.e. fixed factors. "Intensity" was included to investigate possible crowding effects on body size (Valero et al., 2006). "Individual host" was considered as a potential random parameter, i.e. a random intercepts model. In models accounting for both variability in number of eggs *in utero* and egg area, "host species", "worm body area" (covariate), and "host species*worm body area" were considered as potential predictors. "Individual host" and "worm body area" were considered as potential random parameters. In this case, the random part of the models were allowed to include "individual host" (i.e. a random intercepts model), "individual host" + "worm body area" (i.e. a random intercepts and random slopes model), and "individual host" + "worm body area" + covariance between intercepts and slopes (i.e. an unstructured model; see Singer, 1998).

The trade-off between egg size and egg number was also assessed through GLMMs. In this case we used Type I sum of squares since we wanted to control for the effect of parasite body area on either number of eggs *in utero* and egg area (Hector et al., 2010). "Worm body area" (covariate), "egg area" (covariate), "host species", "host species*worm body area" and "host species*egg area" were entered sequentially in the models as potential predictors. "Individual host", "worm body area" and "egg area" were considered as potential random parameters. In this case, the random part of the models were allowed to include i) "individual host"; ii) "individual host" + "worm body area"; iii) "individual host" + "egg area"; iv) "individual host" + "worm body area" + "egg area"; and v) "individual host" + "worm body area" + "egg area" + covariance between intercepts and slopes. GLMMs were implemented with SPSS for Macintosh, v. 19.0.

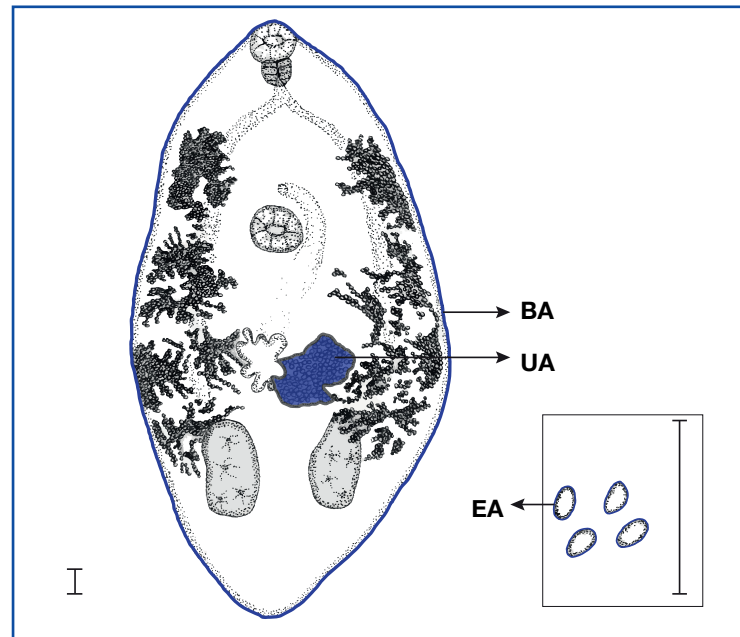


Figure 9.1. Schematic drawing of a specimen of *Pholeter gastrophilus* and its eggs (inset). Colour lines represent measurements taken for the study. Abbreviations: BA, body area; EA, egg area; UA, uterine area covered by eggs. Scale-bars: 0.1 mm.

RESULTS

Molecular analyses

We obtained four partial LSU rDNA sequences for *P. gastrophilus* (816 - 1,285 bp long). The eight sequences from each, the ribosomal ITS2 spacer and the mitochondrial COI gene varied between 466 and 515 bp, and between 396 and 446 bp, respectively. Comparison of pairwise divergence for each gene showed that the aligned portions of the sequences of all specimens of *P. gastrophilus* were identical.

Infection parameters

The prevalences (95% CI) of *P. gastrophilus* in striped and bottlenose dolphins were 56.4% (40.5 – 71.3) and 57.1% (35.4 – 76.7), respectively; the difference was not significant (Fisher's test, $p = 1$). Mean intensity of *P. gastrophilus* also did not differ between both host species: 77.7 (50.7 – 134.6) worms per infected host in striped dolphins vs. 249.2 (82.9 – 644.4) worms per infected host in bottlenose dolphins ($t = 1.321$, $p = 0.277$). In addition, the Brunner-Munzel test was not significant ($p = 0.847$).

Comparison of life-history traits

All individuals of *P. gastrophilus* collected from both, striped dolphins and bottlenose dolphins, were gravid. Mean values of worm body area, number of eggs *in utero* and egg area

are shown in Table 9.1. The best GLMM for worm body area included only “host species” and “host individual” effects; any other model received substantially less empirical support (Table 9.2). In the subset of models with $w_i > 0.01$, “host species” but not “intensity”, was found to be a highly significant predictor of worm body area (Table 9.2). The average body area of *P. gastrophilus* in striped dolphins was nearly twice that found in bottlenose dolphins, and the difference was consistent regardless of host individual (Table 9.1).

Table 9.1. Mean values (\pm standard deviation, SD) and coefficient of variation (CV in %) of body area, egg area and number of eggs *in utero* of individuals of *Pholeter gastrophilus* collected from five striped dolphins, *Stenella coeruleoalba*, and five bottlenose dolphins, *Tursiops truncatus*, stranded along the Mediterranean coast of Spain.

Host	Intensity	Body area (mm ²)	CV	Egg area (μm ²)	CV	Egg number	CV	Uterus area (mm ²)	CV
<i>Stenella coeruleoalba</i>									
Host 1	34	3.01 ± 0.77	25.6	227 ± 46	20.3	4,170 ± 2,391	57.3	0.94 ± 0.53	56.8
Host 2	27	2.51 ± 0.42	15.5	224 ± 44	19.5	2,148 ± 2,183	101.6	0.43 ± 0.40	92.8
Host 3	35	3.63 ± 0.86	23.6	220 ± 39	17.8	5,076 ± 3,414	67.3	1.07 ± 0.68	63.5
Host 4	24	4.09 ± 0.77	18.8	204 ± 30	14.6	6,814 ± 2,945	43.2	1.36 ± 0.57	42.2
Host 5	20	4.31 ± 0.86	20.0	237 ± 36	15.0	5,984 ± 1,556	26.0	1.43 ± 0.44	31.0
Total	140	3.44 ± 0.98	28.4	222 ± 41	18.3	4,719 ± 3,040	64.4	1.02 ± 0.64	62.6
Average per host	28	3.5 ± 0.75	21.43	222 ± 12	5.4	4,838 ± 1,799	37.2	1.05 ± 0.40	38.1
<i>Tursiops truncatus</i>									
Host 1	33	2.22 ± 0.96	43.2	233 ± 39	16.9	3,914 ± 3,068	78.4	0.88 ± 0.68	77.4
Host 2	5	0.92 ± 0.33	36.1	185 ± 20	10.6	2,125 ± 879	41.4	0.40 ± 0.18	44.7
Host 3	18	1.47 ± 0.23	15.6	149 ± 15	10.4	3,209 ± 1,741	54.2	0.48 ± 0.26	53.0
Host 4	19	1.27 ± 0.28	22.0	154 ± 17	11.2	1,480 ± 1,370	92.2	0.23 ± 0.23	96.5
Host 5	22	2.51 ± 0.53	21.3	169 ± 26	15.3	8,100 ± 2,168	26.8	1.37 ± 0.40	29.2
Total	97	1.89 ± 0.82	43.5	185 ± 45	24.4	4,164 ± 3,236	77.7	0.77 ± 0.61	80.2
Average per host	19.4	1.68 ± 0.66	39.3	178 ± 34	19.1	3,765 ± 2,599	69.0	0.67 ± 0.46	68.7

Table 9.2. General Linear Mixed Models with type III sum of squares accounting for the effect of host species (factor) and trematode intensity (covariate) on body area of individuals of *Pholeter gastrophilus* collected from striped and bottlenose dolphins. Models are arranged by increase of Akaike information criterion (AIC) and decrease of Akaike weight (w_i). Models with $w_i < 0.01$ are not shown. The probability associated to each fixed effect is also given; significant values are in bold. Abbreviations: HS, host species; HI, host individual; RI, random intercept; I, intensity.

Model	ΔAIC	w_i	Predictor	t	df	p		
Fixed effects	Random effects	Covariance structure						
Intercept + HS	HI	RI	0.00	0.922	HS	3.85	7.61	0.005
Intercept	HI	RI	6.39	0.038	-	-	-	-
Intercept + HS + I	HI	RI	6.63	0.034	HS	2.84	6.80	0.026
					I	1.38	7.31	0.209

The best GLMM for the number of eggs *in utero* included “host species”, “worm body area” and the interaction between both variables as fixed predictors, and “host individual” as a random factor (Table 9.3). However, three competing models received also substantial support ($\Delta\text{AIC} < 4$; $w_i > 0.10$), and all included both “host species” and “worm body area” as fixed factors (Table 9.3). These two factors also were significant predictors in all models ($p \leq 0.015$), but their interaction was not (Table 9.3). These results indicate that larger worms harboured more eggs regardless of host species and, for a given body size, the number of eggs *in utero* was significantly higher in worms from bottlenose dolphins (Figure 9.2 A). Note, however, that in all the models excluding “worm body area” (all with $\Delta\text{AIC} > 4$), the effect of “host species” was not significant ($p > 0.05$). In other words, absolute fecundity was similar between worms from both host species (Figure 9.2 A).

Table 9.3. General Linear Mixed Models with type III sum of squares accounting for the effect of host species (factor) and worm body area (covariate) on the number of eggs *in utero* of *Pholeter gastrophilus* collected from striped and bottlenose dolphins. Models are arranged by increase of Akaike information criterion (AIC) and decrease of Akaike weight (w_i). Models with $w_i < 0.01$ are not shown. The probability associated to each fixed effect is also given; significant values are in bold. Abbreviations: HS, host species; BA, worm body area; HI, host individual; RI, random intercept; RS, random slope.

Model			ΔAIC	w_i	Predictor	t	df	p
Fixed effects	Random effects	Covariance structure						
Intercept + HS + BA + HS*BA	HI	RI	0.00	0.355	HS	-2.58	32.68	0.015
					BA	6.24	190.29	<0.005
					HS*BA	0.64	190.29	0.526
Intercept + HS + BA	HI	RI	0.07	0.342	HS	-2.97	9.82	0.014
					BA	9.32	189.55	<0.005
Intercept + HS + BA + HS*BA	HI + BA	RI + RS	2.00	0.130	HS	-2.58	32.68	0.015
					BA	6.24	190.29	<0.005
					HS*BA	0.64	190.29	0.526
Intercept + HS + BA	HI + BA	RI + RS	2.07	0.126	HS	-2.97	9.82	0.014
					BA	9.32	189.55	<0.005
Intercept + BA	HI	RI	4.67	0.034	BA	8.70	149.45	<0.005

The best GLMM for egg size included only “body area” as a fixed, highly significant predictor, and “host individual” as a random factor (Table 9.4). Two additional models also received substantial empirical support ($\Delta\text{AIC} < 4$; $w_i > 0.10$), and one of them also included “host species” as an additional predictor (Table 9.4). The effect of “body area”, but not “host species”, was statistically significant (Table 9.4). When “body area” was removed from models, empirical support decreased substantially ($\Delta\text{AIC} \geq 6$) and the effect of “host species” was statistically significant ($t = 2.74$, $df = 8.28$, $p = 0.025$). Accordingly, the size of the eggs in *P. gastrophilus* increased with worm body size, and the difference of egg size between host species resulted from corresponding differences in worm body size, being larger in striped dolphins (Figure 9.2 B). This pattern was consistent across host individuals, except in the case of one individual bottlenose dolphin that harboured worms with similar values as those observed in striped dolphins (Table 9.1).

Table 9.4. General Linear Mixed Models with type III sum of squares accounting for the effect of host species (factor) and worm body area (covariate) on egg area of individuals of the digenean *Pholeter gastrophilus* collected from striped and bottlenose dolphins. Models are arranged by increase of Akaike information criterion (AIC) and decrease of Akaike weight (w_i). Models with $w_i < 0.01$ are not shown. The probability associated to each fixed effect is also given; significant values are in bold. Abbreviations: BA, worm body area; HI, host individual; RI, random intercept; RS, random slope; HS, host species.

Model			Δ AIC	w_i	Predictor	t	df	p
Fixed effects	Random effects	Covariance structure						
Intercept + BA	HI	RI	0	0.506	BA	3.66	165.54	<0.005
Intercept + BA	HI + BA	RI + RS	2.00	0.186	BA	3.66	165.53	<0.005
Intercept + HS + BA	HI	RI	2.33	0.158	HS	1.58	10.05	0.145
					BA	2.98	223.38	<0.005
Intercept + HS + BA	HI + BA	RI + RS	4.33	0.058	HS	1.58	10.04	0.145
					BA	2.98	223.35	<0.005
Intercept + HS + BA + HS*BA	HI	RI	5.51	0.032	HS	1.40	23.44	0.175
					BA	2.27	222.45	0.024
					HS*BA	-0.23	222.45	0.820
Intercept + HS	HI	RI	6.45	0.020	HS	2.74	8.28	0.025

When evaluating a trade-off between number of eggs *in utero* and egg area, five models received substantial empirical support (Δ AIC $\leq 2w_i > 0.085$) (Table 9.5). “Worm body area” and “egg area” were included in all models and also received strong statistically significant support ($p \leq 0.005$). After controlling for body area, a negative relationship was found between the egg number and egg area in all five models (Figure 9.3). Parameter for “egg area” in the best model (SE) was -0.88 (0.47); in the other four models values ranged from -0.95 (0.46) to -0.82 (0.48). “Host species” and “host species*egg area” were also selected in all five best supported models; apparently, the negative relationship between “number of eggs” and “egg area” was steeper in worms from striped dolphins (Figure 9.3). However, “host species”, but not “host species*egg area”, was statistically significant (Table 9.5).

DISCUSSION

Pholeter gastrophilus is considered a generalist species with a large range of hosts and a worldwide distribution. This raises the question as to whether this parasite might actually represent a complex of cryptic species (Aznar et al., 2006), a phenomenon that has been observed in other digeneans (e.g., Georgieva et al., 2014). The lack of variation in the sequences of the LSU and ITS2 rDNA and mtCOI, respectively, suggests that individuals of *P. gastrophilus* collected from the striped and bottlenose dolphins are conspecific. Ribosomal genes exhibit some variable sites with phylogenetic information for congeneric taxa (Blasco-Costa et al., 2010) whereas mitochondrial genes usually accumulate much higher nucleotide substitutions than rDNA, thus being especially useful for discriminating closely related species (Vilas et al., 2005; Perkins et al., 2011). The absence of genetic differences in *P. gastrophilus* associated with host species could

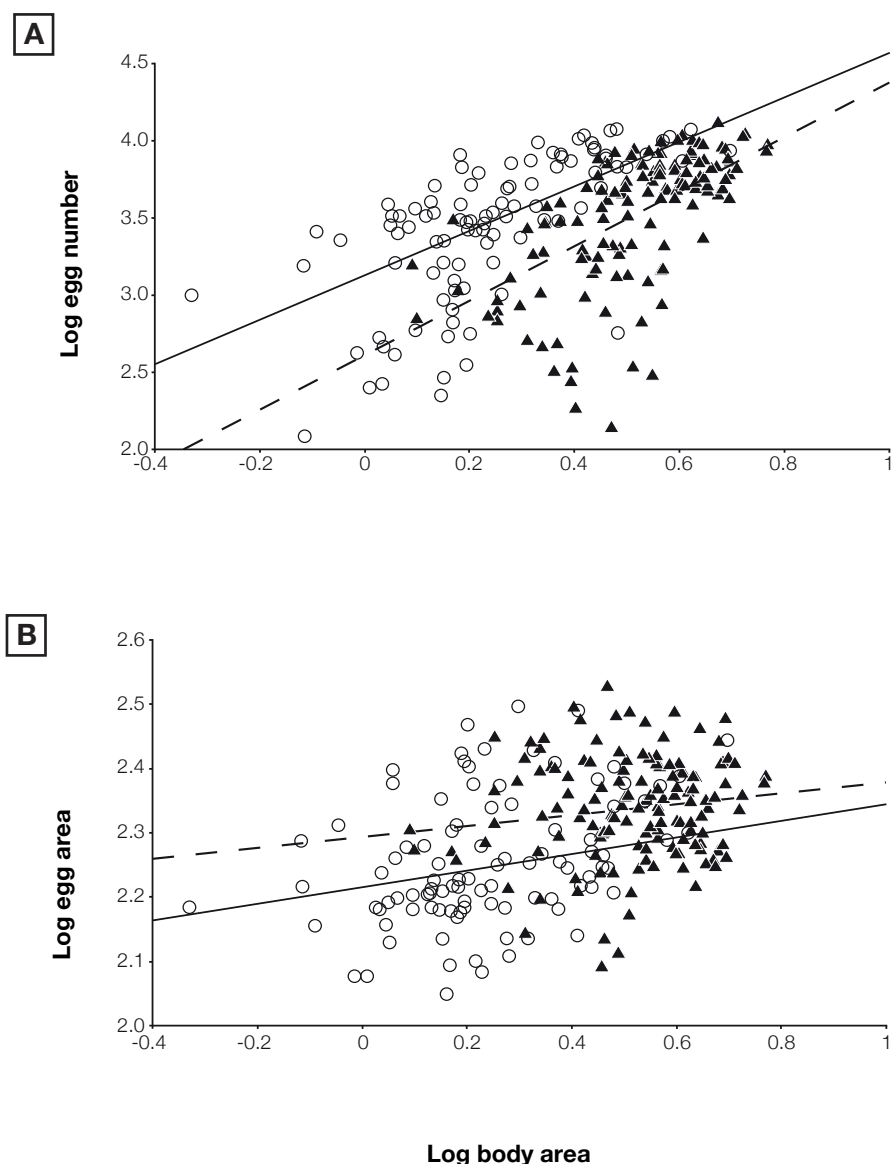


Figure 9.2. Scatterplots showing the relationship of life-history traits in *Pholeter gastrophilus*. Scatterplots showing the relationship between log₁₀-transformed values of **(A)** body area and number of eggs *in utero* and **(B)** body area and egg area of 237 individuals of the digenean *Pholeter gastrophilus* in five striped dolphins, *Stenella coeruleoalba* (triangles; n = 140) and five bottlenose dolphins, *Tursiops truncatus* (circles; n = 97) from the western Mediterranean. Regression lines were obtained from the best model that fit the data for striped dolphins (dashed line) and bottlenose dolphins (solid line).

be related to the fact that striped and bottlenose dolphins utilise partially overlapping habitats (Gómez de Segura et al., 2008) and share some preys in the study area (Blanco et al., 1995; 2001), thus enabling gene flow in the parasite population. Nevertheless, significant population structure might still occur in the neritic vs. oceanic populations of *P. gastrophilus* (see below). To detect intraspecific structure at this spatial scale, sequences of a much larger number of worms, and use of additional genetic markers are required (see, e.g. Blasco-Costa et al., 2012).

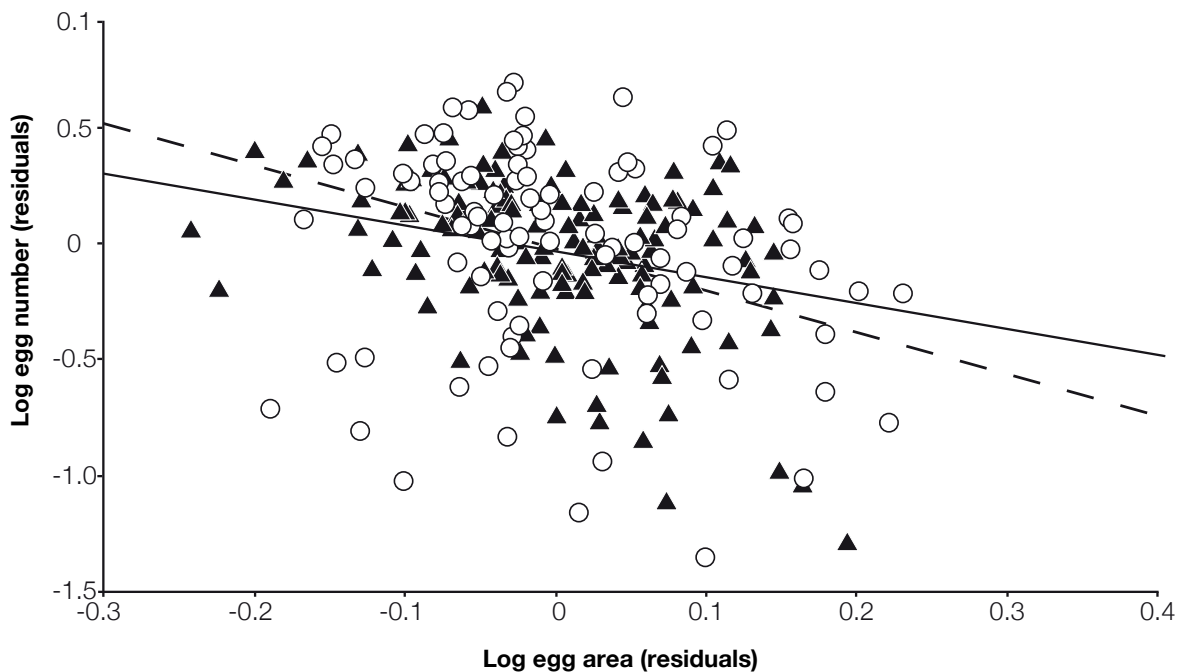


Figure 9.3. Scatterplot showing the relationship between body-size corrected residuals of egg area and number of eggs *in utero* of the digenean *Pholeter gastrophilus* in striped dolphins, *Stenella coeruleoalba* (triangles) and bottlenose dolphins, *Tursiops truncatus* (circles) from the western Mediterranean. Regression lines were obtained from the best model that fit the data for striped dolphins (dashed line) and bottlenose dolphins (solid line).

Infection levels of *P. gastrophilus* were similar in both striped and bottlenose dolphins; prevalence was virtually identical, and differences in worm intensity were not significant. To understand how the contact and/or compatibility filters generate this similarity, we would need information about i) the diet of each dolphin species; ii) the life cycle of *P. gastrophilus*; and iii) the potential effect of hosts' physiological or immunological factors on mortality rates of parasites. The latter factor cannot be ascertained unless experimental infections are carried out. Concerning host-parasite contact, in the study area, striped dolphins feed primarily on oceanic mesopelagic fish and cephalopods (Blanco et al., 1995), although they may consume some neritic fish, e.g. juvenile hake, *Merluccius merluccius*, or cephalopods, e.g. *Illex coindetii* (Aznar et al., 2015). In contrast, bottlenose dolphins feed largely on demersal neritic fish (especially hake) and, to a lesser extent, on benthic cephalopods (Blanco et al., 2001). Thus, it is puzzling how *P. gastrophilus* is able to infect a sizeable proportion of two dolphin species with such a small overlap in habitat and diet. The problem is compounded because, in the study area, this parasite also infects other oceanic cetaceans that feed almost exclusively on mesopelagic cephalopods (Raga and Balbuena, 1993; Fernández et al., 2003). Unfortunately, parasitological surveys have failed to find infective stages of *P. gastrophilus* in the main prey of striped and bottlenose dolphins (see Ferrer-Maza et al., 2014; Mateu et al., 2015). Based on data from other digeneans infecting pelagic vertebrates (Marcogliese, 1995; Daponte et al., 2006; Diaz Briz et al., 2012) we can postulate that *P. gastrophilus* extensively exploits the food web to reach its definitive hosts, but it is unclear to what extent the transmission routes are different depending on each cetacean species.

Table 9.5. General Linear Mixed Models with type I sum of squares accounting for the trade-off between egg number and egg area of individuals of *Pholeter gastrophilus* collected from striped and bottlenose dolphins, controlling for worm body area (covariate). Models are arranged by increase of Akaike information criterion (AIC) and decrease of Akaike weight (w_i). Models with $w_i < 0.01$ are not shown. The probability associated to each fixed effect is also given; significant values are in bold. Abbreviations: BA, worm body area; EA, egg area; HS, host species; HI, host individual; RI, random intercept; RS, random slope.

Model			Δ AIC	w_i	Predictor	F	df	p
Fixed effects	Random effects	Covariance structure						
Intercept + BA + EA + HS + HS*BA + HS*EA	HI	RI	0	0.231	BA	24.93	1, 9.6	<0.005
					EA	31.18	1, 120.4	<0.005
					HS	5.66	1, 9.7	0.040
					HS*BA	0.27	1, 195.8	0.601
					HS*EA	2.45	1, 198.2	0.119
Intercept + BA + EA + HS + HS*EA	HI	RI	0.36	0.193	BA	24.49	1, 9.3	<0.005
					EA	31.03	1, 119.2	<0.005
					HS	5.85	1, 9.5	0.037
					HS*EA	1.99	1, 199.0	0.160
Intercept + BA + EA + HS + HS*BA + HS*EA	HI + EA	RI + RS	1.66	0.101	BA	24.42	1, 9.4	<0.005
					EA	29.53	1, 114.1	<0.005
					HS	5.51	1, 9.5	0.042
					HS*BA	0.19	1, 196.0	0.661
					HS*EA	2.67	1, 192.1	0.104
Intercept + BA + EA + HS + HS*EA	HI + EA	RI + RS	1.92	0.089	BA	24.09	1, 9.2	<0.005
					EA	29.35	1, 113.0	<0.005
					HS	5.70	1, 9.4	0.040
					HS*EA	2.24	1, 191.4	0.136
Intercept + BA + EA + HS + HS*BA + HS*EA	HI + BA	RI + RS	2.00	0.085	BA	24.93	1, 9.6	<0.005
					EA	31.18	1, 120.4	<0.005
					HS	5.66	1, 9.7	0.040
					HS*BA	0.27	1, 195.8	0.601
					HS*EA	2.45	1, 198.2	0.119
Intercept + BA + EA + HS + HS*EA	HI + BA	RI + RS	2.36	0.071	BA	24.49	1, 9.3	<0.005
					EA	31.03	1, 119.2	<0.005
					HS	5.85	1, 9.5	0.037
					HS*EA	1.99	1, 199.0	0.160
Intercept + BA + EA + HS	HI	RI	3.00	0.052	BA	22.76	1, 9.1	<0.005
					EA	39.23	1, 137.1	<0.005
					HS	4.89	1, 9.2	0.054
Intercept + BA + EA + HS + HS*BA	HI	RI	3.14	0.048	BA	22.74	1, 9.2	<0.005
					EA	39.41	1, 137.9	<0.005
					HS	4.65	1, 9.3	0.058
					HS*BA	0.30	1, 201.1	0.583
Intercept + BA + EA + HS + HS*BA + HS*EA	HI + BA + EA	RI + RS	4.65	0.023	BA	27.79	1, 3133.1	<0.005
					EA	33.21	1, 352.7	<0.005
					HS	6.29	1, 3119.3	0.012
					HS*BA	0.27	1, 323.9	0.605
					HS*EA	2.66	1, 298.4	0.104
Intercept + BA + EA + HS	HI + EA	RI + RS	4.84	0.021	BA	23.29	1, 9.0	<0.005
					EA	36.91	1, 91.0	<0.005
					HS	4.36	1, 9.2	0.066

In parasitic platyhelminths, a large body size has been linked to a suite of life history traits including low mortality, long maturation time, slow growth rate and high reproductive output (Trouvé et al., 1998). In *P. gastrophilus*, a larger body size was correlated with both a higher number of eggs *in utero* and larger eggs, similarly as in other digeneans (e.g. Oliva and Alvarez, 2011; González et al., 2013). Also, individuals of *P. gastrophilus* were able to mature and reproduce in both dolphin hosts, but worms were significantly larger in striped dolphins and also harboured larger eggs. These differences were consistent regardless of substantial variability associated to host individual, and did not appear to be confounded by differential density-dependence (Beck et al., 2015). In fact, bottlenose dolphins provided a larger microhabitat (stomach area: 520 cm² vs. 297 cm² in striped dolphins, F.J. Aznar, unpub. obs.) but harboured smaller parasites that produced smaller eggs.

The larger investment in body size and egg size of *P. gastrophilus* in striped dolphins tentatively suggests that they are more suitable hosts than bottlenose dolphins. The absolute number of eggs *in utero* did not differ between hosts, but this measure of fecundity is just a “snapshot” of the total egg output that a digenean can produce throughout its lifetime (Whittington, 1997). In digeneans, the adult size has a significant positive effect on the total reproductive output (Trouvé et al., 1998), thus we cannot rule out that worms from striped dolphins also have a higher overall fecundity. On the other hand, if bottlenose dolphins impose higher adult mortality rates of *P. gastrophilus*, shorter parasite maturation times and smaller body sizes should be favoured by natural selection (see Morand and Sorci, 1998). This would explain why, for a given body size, worms from bottlenose dolphins had a relatively higher number of eggs *in utero*. Although the reasons for such contrasting host suitability are unknown, the possibility that differences in host’s immune response play a role should not be underestimated, since infection with *P. gastrophilus* elicit a significant immune reaction associated to the formation of fibrotic nodules (Howard et al., 1983).

According to the above discussion, individuals of *P. gastrophilus* seem to adopt different reproductive strategies depending on the host species, i.e. larger worms in the seemingly suitable hosts (striped dolphins) allocate resources for both somatic growth and production of large eggs, whereas smaller worms in the presumably suboptimal hosts (bottlenose dolphins) primarily allocate resources for egg production. The reasons why the absolute number of eggs *in utero* was not significantly higher in worms from striped dolphins deserve further attention. Perhaps there are spatial constraints on the amount of eggs that the uterus can harbour (Herrerias et al., 2007), so the larger body size (= uterus) in worms from striped dolphins would be inconsequential for harbouring more eggs if eggs are also larger. A second, non-exclusive hypothesis is that a trade-off could exist between egg size and egg number so that worms in striped dolphins produces larger eggs at the expense of lowering fecundity (see Poulin, 1996; 1997). In fact, we obtained evidence of such trade-off, not only in striped dolphins, but also in bottlenose dolphins. This would empirically confirm that parasites could not equally invest into both quantity and quality of offspring (Jennings and Calow, 1975; Poulin, 1995b). Apparently, individuals of *P. gastrophilus* are at their maximum reproductive capacity (see Cavaleiro and Santos, 2014), thus the higher allocation in egg size observed in worms from striped dolphins should involve higher costs for

egg production. In support of this, the slope of the egg size-egg number regression, corrected for body size, seemed to be steeper in worms from striped dolphins, and indeed the best models examining the trade-off included the interaction between host species and egg area. However, this interaction was not statistically significant, which suggests that a great deal of unexplained variability also exist among individual hosts.

The previous discussion raises the question as to why adults of *P. gastrophilus* in the most suitable host opt to produce larger eggs rather than more numerous but smaller eggs. A possible explanation is that in an aquatic environment, the offspring can potentially be exposed to elevated levels of mortality (see, e.g., Poulin and Hamilton, 2000; Loot et al., 2008; Loot et al., 2011). In particular, the first infective stage (which, depending on the putative life-cycle of *P. gastrophilus*, could be the egg, or the free-living miracidium that emerges from the egg) must face the challenge to contact the first intermediate host, and subsequent infective stages must make their way through the trophic web to infect a top predator. As noted above, Mediterranean striped and bottlenose dolphins favour different habitats, namely, oceanic vs. neritic (Forcada et al., 2004; Gómez de Segura et al., 2008), which could pose different selective pressures. Oceanic ecosystems are widely recognised as having significantly lower levels of productivity than neritic ones (Moore, 2005), which also results in much lower density of organisms (Marcogliese, 1995; Huston, 1998). From the perspective of *P. gastrophilus*, the oceanic habitat would therefore be much more adverse for transmission. The production of large eggs would therefore be an adaptive response because large eggs generate large, long-lived miracidia (Morand, 1996; Trouvé et al., 1998), thus enhancing the likelihood of finding the first intermediate host (see Koehler et al., 2012). In addition, the potential to multiply asexually within the first intermediate host could also be proportional to larval size (Poulin, 1997), which would increase further transmission opportunities in the most adverse environment. Interestingly, a similar phenomenon has been reported in the case of the digenean *Proctoeces lintoni* Siddiqui and Cable, 1960: compared with pristine areas, parasite individuals from human-disturbed areas exhibited larger eggs because harvesting by humans had reduced the density of intermediate and definitive hosts (Loot et al., 2008). Assuming that differences in the reproductive traits of *P. gastrophilus* in each dolphin species are adaptive, they could result from two potential processes, i.e. i) phenotypic plasticity, so that the reproductive strategy of *P. gastrophilus* could vary according to the cues emanated from the definitive host (see Loot et al., 2008), or ii) local adaptation by natural selection, so that differences in reproductive allocation between coastal vs. oceanic populations of *P. gastrophilus* could have at least a partial genetic component. The operation of these two processes should be explored in future studies.

In summary, the results from the present study suggest that the reproductive strategy of *P. gastrophilus* could be differently optimised depending on the suitability of the host species and the local habitat where the life-cycle develops. As far as we are aware, this is the first study to document subtle differences in the reproductive strategy of a generalist helminth from marine mammals, illustrating how constraints and natural selection shape life history traits. Future research should explore whether differences between neritic and oceanic habitats have similar impact on the life-cycles of other trophically-transmitted helminths.

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CONCLUSIONS



This thesis aims at the study of diversity, origin and evolutionary history, and host specificity patterns of digeneans from cetaceans. As a result, the following conclusions have been reached:

1 – There are 174 valid species of helminths reported up to date in cetaceans, from which there are 54 digenean species mainly distributed in four families. The family Brachycladiidae is the most speciose (43 spp.), followed by the Notocotylidae (5 spp.), from which only species of *Ogmogaster* have records in cetaceans; the Heterophyidae, from which only *Pholeter gastrophilus* has been reported in cetaceans; and the Brauninidae, a monotypic family with *Braunina cordiformis* as its only species. The remaining species of helminths reported in cetaceans are distributed in nematodes (62 spp.), cestodes (38 spp.) and acanthocephalans (20 spp.). The families Anisakidae (19 spp.), Pseudaliidae (30 spp.) and Tetrameridae (10 spp.) (Nematoda); Diphylobothriidae (13 spp.), Phyllobothriidae (4 spp.) and Tetrabothriidae (20 spp.) (Cestoda); and Polymorphidae (20 spp.) (Acanthocephala) have a major species-representation in the helminth fauna of cetaceans.

2 – Out of the 54 digenean species reported in cetaceans, 14 are considered highly specific, i.e., they have been reported in single cetacean species, and three species are considered single records, i.e., they have been reported just once. Other 24 species of helminth reported in cetaceans are highly specific, and 14 species are single records. A total of 20 species of helminths in cetaceans (three of which are digeneans) are parasites of other vertebrates. Records of these species in cetaceans are considered accidental infections. Cetaceans and other marine vertebrates (i.e., pinnipeds or marine birds) share common prey, and helminth larvae might be exchanged between hosts through trophic guilds.

3 – A new digenean species is described from a Gervais' beaked whale, *Mesoplodon europaeus*. Specimens were assigned to the genus *Brachycladium* based on the following diagnostic traits: vitellarium reaching at least as far as ventral sucker and extending in the forebody, prepharyngeal pouch and uroproct absent, intestinal caeca diverticulate, cirrus and metatrem unarmed, ovary lobed, and ventral sucker in middle third of the body. The new species, *Brachycladium parvulum* Fraija-Fernández, Aznar, Raga, Gibson *et* Fernández, 2014 differs from other species of the genus by the relative size of oral and ventral suckers, the size and shape of the eggs and its extremely small body size. A discriminant analysis also showed significant morphometric differences between *Brachycladium parvulum* n. sp. and dwarf specimens of its most similar species, *Brachycladium atlanticum*.

4 – The taxonomic affiliation of the main four families of digeneans infecting cetaceans, namely, Brachycladiidae, Brauninidae, Notocotylidae and Heterophyidae, was resolved based on ribosomal DNA. *Braunina cordiformis* formed a sister clade with other species of the Strigeidae and Diplostomidae. *Ogmogaster antarcticus* was placed within the Notocotylidae, in agreement

with the current taxonomic arrangement of the genus. *Pholeter gastrophilus* was placed within the Heterophyidae, close to *Ascocotyle longa* and *Pygidiopsis genata*, but this family was found to form a paraphyletic clade with the Opisthorchiidae. Species of the Brachycladiidae were placed among species of the Acanthocolpidae, making the relationship between these families paraphyletic.

5 – Digeneans of cetaceans were related to families typical from fish-eating birds and mammals (Strigeidae, Diplostomidae and Heterophyidae), fish (Acanthocolpidae) and herbivorous aquatic birds and mammals (Notocotyliidae). Accordingly, digeneans in cetaceans would have been acquired from other vertebrates through host switching events. Two clades, i.e., the Brachycladiidae, and species of *Ogmogaster*, have undergone subsequent diversification among marine mammals.

6 – A partial phylogenetic analysis of the most diverse family of digeneans from cetaceans, the Brachycladiidae, was made using nine species, namely, *Brachycladium atlanticum*, *B. goliath*, *Campula oblonga*, *Nasitrema delphini*, *N. globicephalae*, *Synthesium pontoporiae*, *S. tursionis*, *Orthosplanchnus fraterculus*, and *Oschmarinella rochebruni*, which represent six out of 10 genera of the family. The cladogram conformed that obtained in previous studies, dealing with a smaller number of species. A paraphyletic relationship was found between *Brachycladium atlanticum* and *Brachycladium goliath*, which deserves further attention when new molecular and morphological data become available.

7 – A cophylogenetic analysis based on distance-based methods, and using 50 host-parasite associations between species of the Brachycladiidae and their marine mammal hosts, revealed a global congruence between both phylogenies. This was supported by the 24 significant host-parasite associations between *Campula oblonga*, *Nasitrema delphini*, *N. globicephalae* and *Brachycladium atlanticum*, and their associated taxa from the Delphinoidea. However, incongruence between host and parasite phylogenies was also detected, suggesting a complex coevolutionary history identified by event-based methods.

8 – Event-based methods revealed three potential scenarios for the coevolution between species of the Brachycladiidae and their marine mammal hosts, in which the number of loss events (87 – 156) was the most numerous, followed by failure to diverge (40), duplication (3 – 6), host switching (0 – 3), and cospeciation (0 – 2). Colonization with speciation, i.e., host switching, and colonization without speciation, i.e., failure to diverge and spreading, were recognised as the main coevolutionary events for the establishment of the associations between brachycladiids and marine mammals.

9 – Genetic information and phylogenetic inference supports the hypothesis that the most generalist digenean species exclusive to cetaceans, *Pholeter gastrophilus*, represents a single lineage. It is postulated that the occurrence of *P. gastrophilus* in long-ranging cetaceans species, and the putative use of many vertebrates and invertebrates as intermediate hosts, would account for the ubiquity of this species in the trophic web, providing opportunities for cohesive gene flow.

10 – Population genetic analyses revealed a total of 16 haplotypes for *Pholeter gastrophilus* across four populations, i.e., North Eastern Atlantic, South Western Atlantic, Mediterranean Sea and Central Eastern Atlantic. One unique haplotype exclusively occurred in samples from the South Western Atlantic, and three unique haplotypes in samples from the North Eastern Atlantic. A significant population structure was detected, which might be attributed to variation between samples from the North Eastern Atlantic and all other regions. Most of the worms from the North Eastern Atlantic were collected from the harbour porpoise, *Phocoena phocoena*, so habitat division and coastal isolation of hosts would contribute to the observed population structure. The small sample size in the South Western Atlantic precluded an interpretation of population structure in this area.

11 – Demographic analyses showed that the genetic variation in the population of *Pholeter gastrophilus* studied was deviated from neutrality, suggesting that a recent population expansion took place, apparently from the Mediterranean Sea and the Central Eastern Atlantic. According to the results from the mismatch distribution, the time since the population expansion was estimated to be, approximately, 60,000 years ago, which likely coincide with the origin of the association between *P. gastrophilus* and cetaceans.

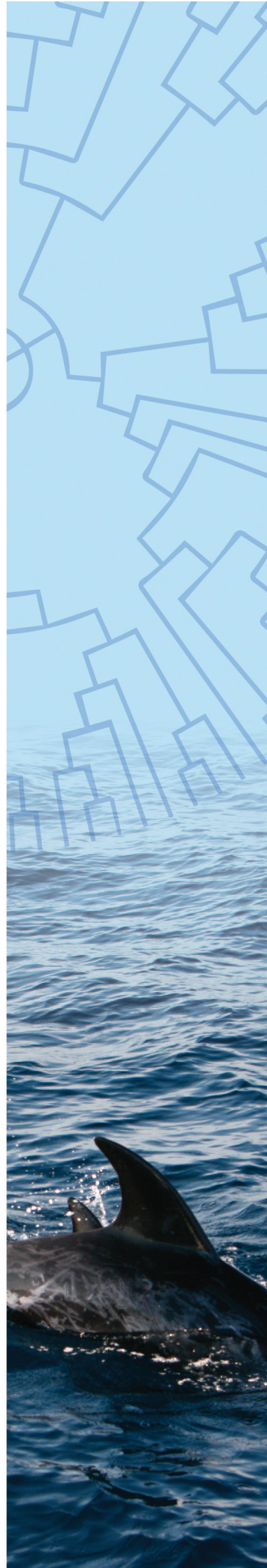
12 – In the Western Mediterranean, *Pholeter gastrophilus* occurred in the oceanic striped dolphin, *Stenella coeruleoalba*, and in the more costal bottlenose dolphin, *Tursiops truncatus*, with similar levels of infection, and no significant differences in the worm's absolute fecundity were observed. However, worms from striped dolphin were larger and produced larger eggs than in the bottlenose dolphin, which suggests that the striped dolphin is, apparently, a more suitable host for *P. gastrophilus*. Still, the similar level of fecundity in worms from both host species suggests that *P. gastrophilus* develops different reproductive strategies depending on the suitability of the host species and the local habitat where the life cycle occurs.

13 – The reproductive investment of *P. gastrophilus* is associated to the environment where the life cycle develops. Worms in striped dolphins likely face the problem of finding intermediate hosts in the oceanic realm, so apparently to invest into offspring size attempt to enhance early survival of larvae, and potential for asexual multiplication in the first intermediate host. In contrast, small-bodied worms in bottlenose dolphins would be adapted to reproduce early because higher

adult mortality, which would generate smaller and numerous eggs in a coastal habitat were chances for transmission are presumably higher.

14 – This thesis provides new molecular information for *Braunina cordiformis*, *Ogmogaster antarcticus* and *Pholeter gastrophilus* and new sequences of the mtDNA ND3 gene for *Brachycladium atlanticum* and *Nasitrema delphini*. Specifically, new sequences of the ribosomal DNA were obtained from species of the four families of digeneans in cetaceans, namely, *Braunina cordiformis* (Brauninidae), *Ogmogaster antarcticus* (Notocotyliidae), *Pholeter gastrophilus* (Heterophyidae), and *Campula oblonga*, *Nasitrema* sp. and *Oschmarinella rochebruni* (Brachycladiidae). Additional sequences of the mtDNA ND3 gene for *Campula oblonga*, *Oschmarinella rochebruni* and *Synthesium tursionis* (Brachycladiidae) and new sequences of the mtDNA ND1 gene for *Pholeter gastrophilus* were provided.

APPENDICES



APPENDIX A1

List of species and GenBank accession numbers of small subunit (SSU) 18S, large subunit (LSU) 28S and internal transcribed spacer 2 (ITS2) rDNA sequences used for the phylogenetic inference of digeneans (Chapter 6).

		GenBank accession number		
		SSU 18S	LSU 28S	ITS2
Class Trematoda				
Subclass Aspidogastrea				
Order Aspidogastrida				
Aspidogastridae	<i>Aspidogaster conchicola</i>	AJ287478	AY222162	---
	<i>Cotylogaster basiri</i>	AY222082	AY222164	---
	<i>Lobatostoma manteri</i>	L16911	AY157177	---
Multicalycidae	<i>Multicalyx elegans</i>	AJ287532	AY222163	---
Rugogastridae	<i>Rugogaster hydrolagi</i>	AJ287573	AY157176	---
Subclass Digenea				
Order Diplostomida				
Aporocotylidae	<i>Aporocotyle spinosicanalis</i>	AJ287477	AY222177	---
	<i>Chimaerohemecus trondheimensis</i>	AY157213	AY157239	---
	<i>Neoparacardicola nasonis</i>	AY222097	AY222179	---
	<i>Plethorchis acanthus</i>	AY222096	AY222178	---
	<i>Sanguinicola cf. inermis</i>	AY222098	AY222180	---
Brachylaimidae	<i>Zeylanurotrema spearei</i>	AY222088	AY222170	---
	<i>Brachylaima sp.</i>	AY222084	AY222167	---
	<i>Brachylaima thompsoni</i>	AY222085	AF184262	---
Clinostomidae	<i>Clinostomum sp.</i>	AY222094	AY222175	---
	<i>Clinostomum sp.</i>	AY222095	AY222176	---
Diplostomidae	<i>Alaria alata</i>	AY222091	AF184263	---
	<i>Diplostomum phoxini</i>	AY222090	AY222173	---
Leucochloridiidae	<i>Leucochloridium perturbatum</i>	AY222087	AY222169	---
	<i>Urogonimus macrostomus</i>	AY222086	AY222168	---
Schistosomatidae	<i>Austrobilharzia terrigalensis</i>	AY157223	AY157249	---
	<i>Bilharziella polonica</i>	AY157214	AY157240	---
	<i>Dendritobilharzia pulverulenta</i>	AY157215	AY157241	---
	<i>Gigantobilharzia huronensis</i>	AY157216	AY157242	---
	<i>Schistosoma japonicum</i>	AY157226	AY157607	---
Spirorchidae	<i>Spirorchis scripta</i>	AY222093	AY222174	---
Strigeidae	<i>Apharyngostrigea cornu</i>	AY222092	AF184264	---
	<i>Cardiocephaloides longicollis</i>	AY222089	AY222171	---
	<i>Ichthyocotylurus erraticus</i>	AJ287526	AY222172	---
Order Plagiorchiida				
Acanthocolpidae	<i>Cableia pudica</i>	AJ287486	AY222251	---
	<i>Stephanostomum baccatum</i>	AJ287577	AY222256	---
	<i>Stephanostomum cf. uku</i>	DQ248206	DQ248219	---
	<i>Stephanostomum tantabiddii</i>	DQ248207	DQ248220	---
	<i>Stephanostomum pristis</i>	DQ248209	DQ248222	---
	<i>Stephanostomum minutum</i>	DQ248211	DQ248224	---
	<i>Tormopsolus orientalis</i>	DQ248204	DQ248217	---
	<i>Pleorchis uku</i>	DQ248203	DQ248216	---
	<i>Pleorchis polyorchis</i>	DQ248202	DQ248215	---

Appendix A1. (Continued)

Accacoelidae	<i>Accacoelium contortum</i>	AJ287472	AY222190	---
Apocreadiidae	<i>Homalometron armatum</i>	AY222130	AY222241	---
	<i>Homalometron synagris</i>	AJ287523	AY222243	---
	<i>Neoapocreadium splendens</i>	AJ287543	AY222242	---
	<i>Schistorchis zanclic</i>	AY222129	AY222240	---
Atractotrematidae	<i>Atractotrema sigani</i>	AJ287479	AY222267	---
Auridistomidae	<i>Auridistomum chelydrae</i>	AY222159	AY116872	---
Azygiidae	<i>Otodistomum cestoides</i>	AJ287553	AY222187	---
Bivesiculidae	<i>Bivesicula claviformis</i>	AJ287485	AY222182	---
	<i>Bivesicula unexpecta</i>	AY222099	AY222181	---
	<i>Bivesicula fusiformis</i>	AY222100	AY222183	---
Brachycladiidae	<i>Zalophotrema hepaticum</i>	AJ224884	AY222255	---
Brachycoeliidae	<i>Brachycoelium salamandrae</i>	AY222160	AF151935	---
	<i>Mesocoelium</i> sp.	AJ287536	AY222277	---
Bucephalidae	<i>Prosorhynchoides gracilescens</i>	AJ228789	AY222224	---
	<i>Rhipidocotyle galeata</i>	AY222119	AY222225	---
Callodistomidae	<i>Prosthenhystera obesa</i>	AY222108	AY222206	---
Cephalogonimidae	<i>Cephalogonimus retusus</i>	AJ287489	AY222276	---
Choanocotylidae	<i>Choanocotyle hobbsi</i>	AY116868	AY116865	---
	<i>Choanocotyle nematoides</i>	AY116867	AY116862	---
Cryptogonimidae	<i>Caecicola parvulus</i>	AY222123	AY222231	---
	<i>Siphodera vinalwardsii</i>	AY222122	AY222230	---
	<i>Mitotrema anthostomatum</i>	AJ287542	AY222229	---
Cyclocoelidae	<i>Cyclocoelum mutabile</i>	AJ287494	AY222249	---
Derogenidae	<i>Derogenes varicus</i>	AJ287511	AY222189	---
	<i>Hemiperina manteri</i>	AY222105	AY222196	---
Dicrocoeliidae	<i>Brachylecithum lobatum</i>	AY222144	AY222260	---
	<i>Dicrocoelium dendriticum</i>	Y11236	AY222261	---
	<i>Lyperosomum collurionis</i>	AY222143	AY222259	---
Didymozoidae	<i>Didymozoon scomбри</i>	AJ287500	AY222195	---
Diplodiscidae	<i>Diplodiscus subclavatus</i>	AJ287502	AY222212	---
Echinostomatidae	<i>Echinostoma revolutum</i>	AY222132	AY222246	AF067850
	<i>Euparyphium melis</i>	AY222131	AF151941	---
Encyclometridae	<i>Encyclometra colubrimurorum</i>	AY222142	AF184254	---
Enenteridae	<i>Enenterum aureum</i>	AY222124	AY222232	---
	<i>Koseiria xishaense</i>	AY222125	AY222233	---
Eucotylidae	<i>Tanaisia fedtschenkoi</i>	AY222154	AY116870	---
Fasciolidae	<i>Fasciola gigantica</i>	AJ011942	AY222245	---
	<i>Fasciola hepatica</i>	AJ004969	AY222244	---
Faustulidae	<i>Antorchis pomacanthi</i>	AJ287476	AY222268	---
	<i>Bacciger lesteri</i>	AJ287482	AY222269	---
	<i>Trigonocryptus conus</i>	AJ287584	AY222270	---
Fellodistomidae	<i>Fellodistomum fellis</i>	Z12601	AY222282	---
	<i>Olssonium turneri</i>	AJ287548	AY222283	---
	<i>Proctoeces maculatus</i>	AY222161	AY222284	---
	<i>Steringophorus margolisi</i>	AJ287578	AY222281	---
Gorgocephalidae	<i>Gorgocephalus kyphosi</i>	AY222126	AY222234	---
Gorgoderidae	<i>Degeneria halosauri</i>	AJ287497	AY222257	---
	<i>Gorgodera cygnoides</i>	AJ287518	AY222264	---
	<i>Nagmia floridensis</i>	AY222145	AY222262	---
	<i>Xystretrum</i> sp.	AJ287588	AY222263	---

Appendix A1. (Continued)

Gyliauchenidae	<i>Paragyliauchen arusettae</i>	AY222127	AY222235	---
Haploporidae	<i>Hapladena nasonis</i>	AY222146	AY222265	---
	<i>Pseudomegasolena ishigakiense</i>	AJ287569	AY222266	---
Haplospalchnidae	<i>Hymenocotta mulli</i>	AJ287524	AY222239	---
	<i>Schikhobalotrema</i> sp.	AJ287574	AY222238	---
Hemiuridae	<i>Dinurus longisinus</i>	AJ287501	AY222202	---
	<i>Lecithochirium caesionis</i>	AJ287528	AY222200	---
	<i>Lecithocladium excisum</i>	AJ287529	AY222203	---
	<i>Machidatrema chilostoma</i>	AY222106	AY222197	---
	<i>Merluciotrema praeclarum</i>	AJ287535	AY222204	---
	<i>Opisthadena dimidia</i>	AJ287549	AY222198	---
	<i>Plerurus digitatus</i>	AJ287562	AY222201	---
Heronimidae	<i>Heronimus mollis</i>	AY222118	AY116878	---
Heterophyidae	<i>Ascocotyle longa</i>	AY245703	---	AY245703
	<i>Centrocestus formosanus</i>	HQ874608	HQ874609	---
	<i>Cryptocotyle lingua</i>	AJ287492	AY222228	---
	<i>Euryhalmis costaricensis</i>	AB521800	---	AB521800
	<i>Galactosomum lacteum</i>	AY222120	AY222227	---
	<i>Haplorchoides</i> sp.	AJ287521	AY222226	---
	<i>Haplorchis taichui</i>	HM004197	HM004181	HM004155
	<i>Haplorchis pumilio</i>	HM004194	HM004173	HM004161
	<i>Haplorchis yokogawai</i>	HM004207	HM004177	HM004158
	<i>Metagonimus miyatai</i>	HQ832624	HQ832633	---
	<i>Metagonimus takahashii</i>	HQ832627	HQ832636	HQ832618
	<i>Metagonimus yokogawai</i>	HQ832630	HQ832639	HQ832621
	<i>Procerovum cheni</i>	HM004212	---	HM004164
	<i>Procerovum varium</i>	HM004199	HM004182	HM004167
	<i>Pygidiopsis genata</i>	AY245710	---	AY245710
	Labicolidae	<i>Labicola</i> cf. <i>elongata</i>	AY222115	AY222221
Lecithasteriidae	<i>Lecithaster gibbosus</i>	AJ287527	AY222199	---
	<i>Lecithophyllum botryophorum</i>	AY222107	AY222205	---
Lecithodendriidae	<i>Lecithodendrium linstowi</i>	AY222147	AF151919	---
	<i>Prosthodendrium longiforme</i>	AY222148	AF151921	---
Lepocreadiidae	<i>Preptetos caballeri</i>	AJ287563	AY222236	---
	<i>Preptetos trulla</i>	AY222128	AY222237	---
Lissorchiidae	<i>Lissorchis kritskyi</i>	AY222136	AY222250	---
Macroderoididae	<i>Macroderoides typicus</i>	AY222158	AF433673	---
Mesometridae	<i>Mesometra</i> sp.	AJ287537	AY222216	---
Microphallidae	<i>Maritrema oocysta</i>	AJ287534	AY220630	---
	<i>Microphallus fusiformis</i>	AJ287531	AY220633	---
	<i>Microphallus primas</i>	AJ287541	AY220627	---
Microscaphidiidae	<i>Hexangium</i> sp.	AJ287522	AY222215	---
	<i>Neohexangiotrema zebrasomatis</i>	AJ287544	AY222214	---
Monorchiidae	<i>Ancylocoelium typicum</i>	AJ287474	AY222254	---
	<i>Diplomonorchis leiostomi</i>	AY222137	AY222252	---
	<i>Provitellus turrum</i>	AJ287566	AY222253	---
Notocotylidae	<i>Catatropis indicus</i>	AY222114	AY222220	---
	<i>Notocotylus</i> sp.	AJ287547	AY222219	---
Omphalometridae	<i>Rubenstrema exasperatum</i>	AJ287572	AY222275	---
Opecoelidae	<i>Gaevskajatrema halosauropsi</i>	AJ287514	AY222207	---
	<i>Macvicaria macassarensis</i>	AJ287533	AY222208	---
	<i>Peracreadium idoneum</i>	AJ287558	AY222209	---

Appendix A1. (Continued)

Opistholebetidae	<i>Maculifer</i> sp.	AY222109	AY222211	---
	<i>Opistholebes amplicoeelus</i>	AJ287550	AY222210	---
Opisthorchiidae	<i>Amphimerus ovalis</i>	AY222121	AY116876	---
	<i>Clonorchis sinensis</i>	AF408144	JF823989	EF688144
	<i>Opisthorchis viverrini</i>	AF408145	JF823990	AY584735
Opisthotrematidae	<i>Lankatrema mannarense</i>	AY222116	AY222222	---
	<i>Opisthotrema dujonis</i>	AY222117	AY222223	---
Orchipedidae	<i>Orchipedium tracheicola</i>	AJ287551	AY222258	---
Pachypsolidae	<i>Pachypsolus irroratus</i>	AJ287554	AY222274	---
Paragonimidae	<i>Paragonimus iloktsuenensis</i>	AY222141	AY116875	---
	<i>Paragonimus westermani</i>	AY222140	AY116874	---
Philophthalmidae	<i>Cloacitrema narrabeenensis</i>	AY222134	AY222248	---
Plagiorchiidae	<i>Haematoloechus longiplexus</i>	AJ287520	AY222280	---
	<i>Glypthelmins quieta</i>	AJ287517	AY222278	---
	<i>Skrjabinoeces similis</i>	AJ287575	AY222279	---
Pleurogenidae	<i>Pleurogenes claviger</i>	AY222152	AF151925	---
	<i>Pleurogenoides medians</i>	AY222151	AF433670	---
Pronocephalidae	<i>Macrovestibulum obtusicaudum</i>	AY222111	AY116877	---
Prosthogonimidae	<i>Prosthogonimus ovatus</i>	AY222149	AF151928	---
	<i>Schistogonimus rarus</i>	AY222150	AY116869	---
Psilostomidae	<i>Psilochasmus oxyurus</i>	AY222135	AF151940	---
Renicolidae	<i>Renicola</i> sp.	AY222155	AY116871	---
Rhabdiopoeidae	<i>Rhabdiopoeus taylori</i>	AY222113	AY222218	---
	<i>Taprobanella bicaudata</i>	AY222112	AY222217	---
Sclerodistomidae	<i>Prosogonotrema bilabiatum</i>	AJ287565	AY222191	---
Syncoelidae	<i>Copiatestes filiferus</i>	AJ287490	AY222188	---
Tandanicolidae	<i>Prosogonarium angelae</i>	AJ287564	AY222285	---
Telorchidae	<i>Opisthioglyphe ranae</i>	AY222157	AF151929	---
	<i>Telorchis assula</i>	AY222156	AF151915	---
Troglotrematidae	<i>Nanophyetus salminicola</i>	AY222138	AY116873	---
	<i>Nephrotrema truncatum</i>	AY222139	AF151936	---
Zoogonidae	<i>Deretrema nahaense</i>	AJ287498	AY222273	---
	<i>Diphtherostomum</i> sp.	AY222153	AY222272	---
	<i>Lepidophyllum steenstrupi</i>	AJ287530	AY157175	---
	<i>Zoogonoides viviparus</i>	AJ287590	AY222271	---

APPENDIX A2

List of the mammal species, common names and GenBank accession numbers for the sequences of the mitochondrial cytochrome *b* gene used in Chapter 7.

Order Family	Species (Common name)	GenBank Accession nro.	
Carnivora			
Canidae	<i>Vulpes vulpes</i> (Red fox)	JX013648	
Felidae	<i>Felis catus</i> (Domestic cat)	U20753	
Mustelidae	<i>Enhydra lutris</i> (Sea otter)	AB051244	
	<i>Gulo gulo</i> (Wolveine)	DQ206375	
	<i>Mustela vison</i> (American mink)	AF057129	
Odobenidae	<i>Odobenus rosmarus</i> (Walrus)	GU174611	
Otariidae	<i>Arctocephalus australis</i> (South American fur seal)	AY377329	
	<i>Arctocephalus forsteri</i> (New Zealand fur seal)	X82293	
	<i>Arctocephalus pusillus</i> (Cape fur seal)	AM181018	
	<i>Arctocephalus townsendi</i> (Guadalupe fur seal)	AM181021	
	<i>Callorhinus ursinus</i> (Northern fur seal)	AM181016	
	<i>Eumetopias jubatus</i> (Steller sea lion)	FJ948490	
	<i>Neophoca cinerea</i> (Australian sea lion)	AM181020	
	<i>Otaria byronia</i> (South American sea lion)	AY377328	
	<i>Phocarcos hookeri</i> (New Zealand sea lion)	AM181019	
	<i>Zalophus californianus</i> (California sea lion)	AM181017	
	Phocidae	<i>Cystophora cristata</i> (Hooded seal)	AY140981
		<i>Erignathus barbatus</i> (Bearded seal)	AY140982
		<i>Halichoerus grypus</i> (Gray seal)	GU167293
<i>Histiophoca fasciata</i> (Ribbon seal)		AY140980	
<i>Hydrurga leptonyx</i> (Leopard seal)		AY377323	
<i>Leptonychotes weddelli</i> (Weddell seal)		AY377324	
<i>Lobodon carcinophagus</i> (Crabeater seal)		AY377321	
<i>Mirounga angustirostris</i> (Northern elephant seal)		AY377325	
<i>Mirounga leonine</i> (Southern elephant seal)		AY377326	
<i>Monachus schauinslandi</i> (Hawaiian monk seal)		GU174610	
<i>Monachus monachus</i> (Mediterranean monk seal)		AY377327	
<i>Pagophilus groenlandicus</i> (Harp seal)		GU174609	
<i>Phoca largha</i> (Spotted seal)		AB510445	
<i>Phoca vitulina</i> (Harbor seal)		AB510422	
<i>Pusa caspica</i> (Caspian seal)		GU167295	
<i>Pusa hispida</i> (Ringed seal)	AM181036		
<i>Pusa sibirica</i> (Baikal seal)	AY140977		
Procyonidae	<i>Nasua nasua</i> (South American coati)	GQ214530	
Ursidae	<i>Ursus maritimus</i> (Polar bear)	U18898	
Cetariodactyla			
Hippopotamidae	<i>Hippopotamus amphibious</i> (Common hippopotamus)	Y08813	

Appendix A2. (Continued)

Balaenidae	<i>Balaena glacialis</i> (North Atlantic right whale)	X75587
	<i>Balaena mysticetus</i> (Bowhead whale)	KF056914
Balaenopteridae	<i>Balaenoptera acutorostrata</i> (Common minke whale)	X75753
	<i>Balaenoptera bonaerensis</i> (Antarctic minke whale)	X75581
	<i>Balaenoptera borealis</i> (Sei whale)	X75582
	<i>Balaenoptera edeni</i> (Bryde's whale)	X75583
	<i>Megaptera novaeangliae</i> (Humpback whale)	GQ353256
	<i>Balaenoptera musculus</i> (Blue whale)	X72204
	<i>Balaenoptera physalus</i> (Fin whale)	NC001321
Neobalaenidae	<i>Caperea marginata</i> (Pygmy right whale)	X75586
	<i>Eschrichtius robustus</i> (Gray whale)	X75585
Delphinidae	<i>Cephalorhynchus commersonii</i> (Commerson's dolphin)	AF084073
	<i>Cephalorhynchus eutropia</i> (Chilean dolphin)	AF084072
	<i>Cephalorhynchus heavisidii</i> (Heaviside's dolphin)	AF084070
	<i>Cephalorhynchus hectori</i> (Hector's dolphin)	AF084071
	<i>Delphinus capensis</i> (Long-beaked common dolphin)	AF084087
	<i>Delphinus delphis</i> (Short-beaked common dolphin)	AF084085
	<i>Delphinus tropicalis</i> (Indo-Pacific common dolphin)	AF084088
	<i>Feresa attenuata</i> (Pygmy killer whale)	AF084052
	<i>Globicephala macrorhynchus</i> (Short-finned pilot whale)	AF084055
	<i>Globicephala melas</i> (Long-finned pilot whale)	AF084056
	<i>Grampus griseus</i> (Risso's dolphin)	AF084059
	<i>Lagenodelphis hosei</i> (Fraser's dolphin)	AF084099
	<i>Lagenorhynchus australis</i> (Peale's dolphin)	AF084069
	<i>Lagenorhynchus acutus</i> (Atlantic white-sided dolphin)	AF084075
	<i>Lagenorhynchus cruciger</i> (Hourglass dolphin)	AF084068
	<i>Lagenorhynchus obliquidens</i> (Pacific white-sided dolphin)	AF084067
	<i>Lagenorhynchus obscurus</i> (Dusky dolphin)	AY257161
	<i>Lissodelphis borealis</i> (Northern right-whale dolphin)	AF084064
	<i>Lissodelphis peronei</i> (Southern right-whale dolphin)	AF084065
	<i>Orcaella brevirostris</i> (Irrawaddy dolphin)	AF084063
	<i>Orcinus orca</i> (Killer whale)	AF084061
	<i>Pseudorca crassidens</i> (False killer whale)	AF084057
	<i>Sotalia fluviatilis</i> (Tucuxi)	AF304067
	<i>Sotalia guianensis</i> (Guiana dolphin)	DQ086827
	<i>Sousa chinensis</i> (Indo-Pacific humpback dolphin)	AF084080
<i>Stenella attenuata</i> (Pantropical spotted dolphin)	AF084096	
<i>Stenella clymene</i> (Clymene dolphin)	AF084083	
<i>Stenella coeruleoalba</i> (Striped dolphin)	AF084082	
<i>Stenella frontalis</i> (Atlantic spotted dolphin)	AF084090	
<i>Stenella longirostris</i> (Spinner dolphin)	AF084103	
<i>Steno bredanensis</i> (Rough-toothed dolphin)	AF084077	
<i>Tursiops truncatus</i> (Common bottlenose dolphin)	AF084095	

Appendix A2. (Continued)

Iniidae	<i>Inia boliviensis</i> (Bolivian river dolphin)	AF334487
	<i>Inia geoffrensis</i> (Boto)	AF334485
	<i>Inia g. humboldtiana</i> (Orinoco bufeo)	AF521110
Kogiidae	<i>Kogia breviceps</i> (Pygmy sperm whale)	U72040
	<i>Kogia simus</i> (Dwarf sperm whale)	AF304072
Lipotidae	<i>Lipotes vexillifer</i> (Yangtze river dolphin)	AF304071
Monodontidae	<i>Delphinapterus leucas</i> (Beluga)	U72037
	<i>Monodon monocerus</i> (Narwhal)	U72038
Phocoenidae	<i>Australophocoena dioptica</i> (Spectacled porpoise)	U09681
	<i>Neophocaena phocaenoides</i> (Indo-Pacific finless porpoise)	AF334489
	<i>Phocoena phocoena</i> (Harbour porpoise)	U72039
	<i>Phocoena sinus</i> (Vaquita)	AF084051
	<i>Phocoena spinipinnis</i> (Burmeister's porpoise)	U09676
	<i>Phocoenoides dalli</i> (Dall's porpoise)	U09679
Physeteridae	<i>Physeter macrocephalus</i> (Sperm whale)	X75589
Platanistidae	<i>Platanista gangetica</i> (Ganges river dolphin)	AF304070
	<i>Platanista minor</i> (Indus river dolphin)	X92543
Pontoporiidae	<i>Pontoporia blainvillei</i> (Franciscana)	NC005277.1
Ziphiidae	<i>Berardius bairdii</i> (Baird's beaked whale)	X92541
	<i>Mesoplodon bidens</i> (Sowerby's beaked whale)	X92538
	<i>Mesoplodon densirostris</i> (Blainville's beaked whale)	X92536
	<i>Tasmacetus shepherdi</i> (Shepherd's beaked whale)	AF334484
	<i>Ziphius cavirostris</i> (Cuvier's beaked whale)	AF304075
Sirenia		
Dugongidae	<i>Dugong dugon</i> (Dugong)	U07564
Trichechidae	<i>Trichechus manatus</i> (West Indian manatee)	JF489120

APPENDIX A3


Individual host parasite links between digeneans of the family Brachycladiidae and their marine mammal hosts used in Chapter 7, with at least one reference supporting such association. The complete references are at the General References section.

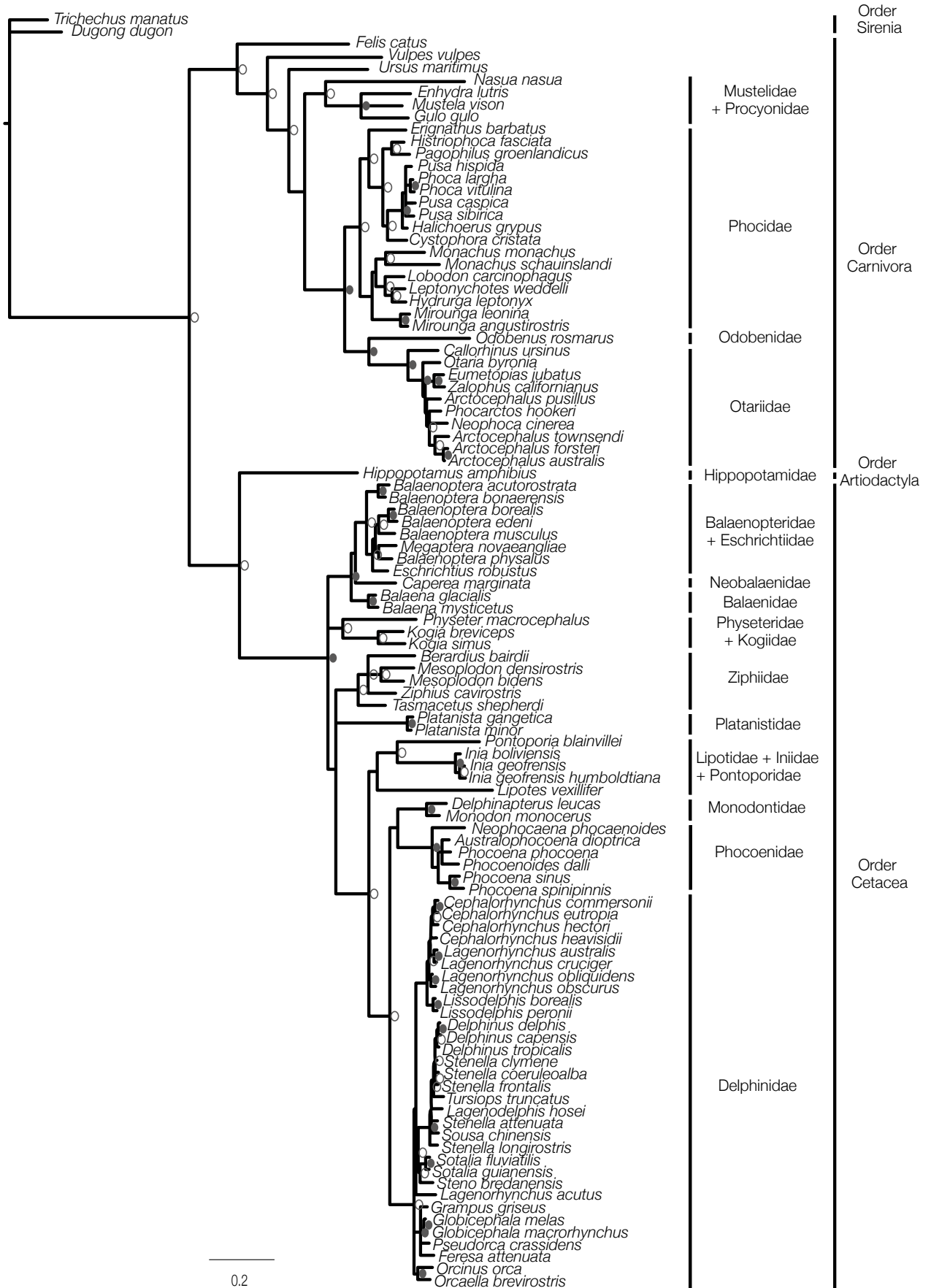
Parasite species	Host species (Common name)	Reference
<i>Brachycladium atlanticum</i>	<i>Delphinus delphis</i> (Short-beaked common dolphin)	Mateu et al. (2011)
	<i>Stenella coeruleoalba</i> (Striped dolphin)	Mateu et al. (2011)
	<i>Tursiops truncatus</i> (Common bottlenose dolphin)	Quiñones et al. (2013)
<i>Brachycladium goliath</i>	<i>Balaena mysticetus</i> (Bowhead whale)	Delyamure (1955)
	<i>Balaenoptera acutorostrata</i> (Common minke whale)	Raga and Balbuena (1987)
	<i>Balaenoptera borealis</i> (Sei whale)	Muniz-Pereira et al. (1999)
	<i>Balaenoptera musculus</i> (Blue whale)	Yablokov et al. (1972)
	<i>Balaenoptera physalus</i> (Fin whale)	Skrjabin (1974)
	<i>Eschrichtius robustus</i> (Gray whale)	Rice and Wolman (1971)
<i>Campula oblonga</i>	<i>Delphinus delphis</i> (Short-beaked common dolphin)	Gibson et al. (1998)
	<i>Neophocaena phocaenoides</i> (Indo-Pacific finless porpoise)	Jefferson and Hung (2004)
	<i>Phocoena phocoena</i> (Harbour porpoise)	Gibson et al. (1998)
	<i>Phocoenoides dalli</i> (Dall's porpoise)	Margolis and Arai (1989)
	<i>Tursiops truncatus</i> (Common bottlenose dolphin)	Zam et al. (1971)
<i>Nasitrema delphini</i>	<i>Delphinus delphis</i> (Short-beaked common dolphin)	Spratt (2002)
	<i>Phocoenoides dalli</i> (Dall's porpoise)	Dailey (1978)
	<i>Tursiops truncatus</i> (Common bottlenose dolphin)	Zam et al. (1971)
<i>Nasitrema globicephalae</i>	<i>Cephalorhynchus eutropia</i> (Chilean dolphin)	Brieva and Oporto (1991)
	<i>Delphinus delphis</i> (Short-beaked common dolphin)	Dailey and Otto (1982)
	<i>Globicephala macrorhynchus</i> (Short-finned pilot whale)	Morales-Vela and Olivera-Gómez (1993)
	<i>Globicephala melas</i> (Long-finned pilot whale)	Fernández et al. (1998)
	<i>Lagenorhynchus obliquidens</i> (Pacific white-sided dolphin)	Dailey and Otto (1982)
	<i>Lissodelphis borealis</i> (Northern right-whale dolphin)	Dailey and Walker (1978)
	<i>Phocoena spinipinnis</i> (Burmeister's porpoise)	Reyes and Van Waerebeek (1995)

Appendix A3. (Continued)

<i>Nasitrema globicephalae</i> (Continued)	<i>Phocoenoides dalli</i> (Dall's porpoise)	Dailey (1978)
	<i>Pseudorca crassidens</i> (False killer whale)	Margolis and Dailey (1972)
	<i>Stenella attenuata</i> (Pantropical spotted dolphin)	Oliveira et al. (2011)
	<i>Stenella coeruleoalba</i> (Striped dolphin)	Oliveira et al. (2011)
	<i>Steno bredanensis</i> (Rough-toothed dolphin)	Ebert and Schfino (2013)
	<i>Tursiops truncatus</i> (Common bottlenose dolphin)	Van Waerebeek et al. (1990)
	<i>Orthosplanchnus fraterculus</i>	<i>Enhydra lutris</i> (Sea otter)
<i>Erignathus barbatus</i> (Bearded seal)		Margolis and Dailey (1972)
<i>Histiophoca fasciata</i> (Ribbon seal)		Shults and Frost (1988)
<i>Odobenus rosmarus</i> (Walrus)		Adams and Rausch (1989)
<i>Pusa hispida</i> (Ringed seal)		Raga (1992)
<i>Oschmarinella rochebruni</i>	<i>Delphinus delphis</i> (Short-beaked common dolphin)	Berón-Vera et al. (2007)
	<i>Stenella attenuata</i> (Pantropical spotted dolphin)	Dailey and Otto (1982)
	<i>Stenella coeruleoalba</i> (Striped dolphin)	Mateu et al. (2011)
	<i>Stenella longirostris</i> (Spinner dolphin)	Aguilar-Aguilar et al. (2001)
<i>Synthesium pontoporiae</i>	<i>Pontoporia blainvillei</i> (Franciscana)	Marigo et al. (2008)
<i>Synthesium tursionis</i>	<i>Cephalorhynchus eutropia</i> (Chilean dolphin)	Torres et al. (1992)
	<i>Delphinus delphis</i> (Short-beaked common dolphin)	Tantalean and Cabrera (1999)
	<i>Grampus griseus</i> (Risso's dolphin)	Zam et al. (1971)
	<i>Neophocaena phocaenoides</i> (Indo-Pacific finless porpoise)	Jefferson and Hung (2004)
	<i>Phocoena sinus</i> (Vaquita)	Lamothe-Argumedo (1987)
	<i>Phocoena spinipinnis</i> (Burmeister's porpoise)	Tantalean and Cabrera (1999)
	<i>Sotalia guianensis</i> (Guiana dolphin)	Marigo et al. (2010)
	<i>Stenella longirostris</i> (Spinner dolphin)	Aguilar-Aguilar et al. (2001)
	<i>Steno bredanensis</i> (Rough-toothed dolphin)	Forrester and Robertson (1975)
<i>Tursiops truncatus</i> (Common bottlenose dolphin)	Romero et al. (2014)	

APPENDIX A4

(See next page) Bayesian hypothesis inferred from the mitochondrial cytochrome *b* gene sequences from 104 species of mammals used in Chapter 7. Classification of families, superfamily and orders follows May-Collado and Agnarsson (2006). Nodal support values are expressed like grey circles when Bayesian inference (posterior probabilities) > 80% and Maximum Likelihood (bootstrap, $n = 100$) > 70%, whereas white circles indicate only nodal support of Bayesian inference (posterior probabilities) > 80%; scores below those values are not indicated. Branch length scale bars indicate the number of substitutions per site. 



APPENDIX A5

Results of the individual host-parasite analysis obtained from ParaFit in Chapter 7. Significant p values (< 0.05) are indicated with an asterisk (*).

Parasite species	Host species	ParaFitLink1	p value
<i>Brachycladium atlanticum</i>	<i>Delphinus delphis</i>	0.40	0.002*
	<i>Stenella coeruleoalba</i>	0.40	0.002*
	<i>Tursiops truncatus</i>	0.41	0.001*
<i>Campula oblonga</i>	<i>Delphinus delphis</i>	0.67	0.036*
	<i>Neophocaena phocaenoides</i>	0.67	0.028*
	<i>Phocoenoides dalli</i>	0.69	0.037*
	<i>Phocoena phocoena</i>	0.67	0.043*
	<i>Tursiops truncatus</i>	0.68	0.033*
<i>Nasitrema delphini</i>	<i>Delphinus delphis</i>	0.68	0.033*
	<i>Phocoenoides dalli</i>	2.27	0.013*
	<i>Tursiops truncatus</i>	2.35	0.015*
<i>Nasitrema globicephalae</i>	<i>Cephalorhynchus eutropia</i>	2.61	0.015*
	<i>Delphinus delphis</i>	2.72	0.010*
	<i>Globicephala macrorhynchus</i>	2.48	0.022*
	<i>Globicephala melas</i>	2.47	0.019*
	<i>Lagenorhynchus obscurus</i>	2.57	0.012*
	<i>Lissodelphis borealis</i>	2.60	0.013*
	<i>Phocoena spinipinnis</i>	2.72	0.012*
	<i>Phocoenoides dalli</i>	2.81	0.010*
	<i>Pseudorca crassidens</i>	2.58	0.019*
	<i>Stenella attenuata</i>	2.68	0.014*
	<i>Stenella coeruleoalba</i>	2.70	0.011*
	<i>Steno bredanensis</i>	2.58	0.014*
<i>Tursiops truncatus</i>	2.77	0.016*	
<i>Brachycladium goliath</i>	<i>Balaenoptera acutorostrata</i>	0.40	0.087
	<i>Balaenoptera borealis</i>	0.42	0.065
	<i>Balaenoptera musculus</i>	0.42	0.074
	<i>Balaena mysticetus</i>	0.36	0.106
	<i>Balaenoptera physalus</i>	0.40	0.086
	<i>Eschrichtius robustus</i>	0.40	0.087
<i>Orthosplanchnus fraterculus</i>	<i>Enhydra lutris</i>	-0.60	0.921
	<i>Erignathus barbatus</i>	-0.64	0.954
	<i>Histiophoca fasciata</i>	-0.64	0.942
	<i>Odobenus rosmarus</i>	-0.75	0.965

Appendix A5. (Continued)

<i>Orthosplanchnus fraterculus</i> (Continued)	<i>Pusa hispida</i>	-0.66	0.944
<i>Oschmarinella rochebruni</i>	<i>Delphinus delphis</i>	0.45	0.194
	<i>Stenella attenuata</i>	0.44	0.198
	<i>Stenella coeruleoalba</i>	0.44	0.211
<i>Oschmarinella rochebruni</i> (continues)	<i>Stenella longirostris</i>	0.43	0.208
<i>Synthesium pontoporiae</i>	<i>Pontoporia blainvillei</i>	0.69	0.117
<i>Synthesium tursionis</i>	<i>Cephalorhynchus eutropia</i>	1.29	0.084
	<i>Delphinus delphis</i>	1.32	0.076
	<i>Grampus griseus</i>	1.26	0.082
	<i>Neophocaena phocaenoides</i>	1.48	0.067
	<i>Phocoena sinus</i>	1.40	0.074
	<i>Phocoena spinipinnis</i>	1.44	0.058
	<i>Sotalia guianensis</i>	1.27	0.088
	<i>Stenella longirostris</i>	1.31	0.075
	<i>Steno bredanensis</i>	1.29	0.079
	<i>Tursiops truncatus</i>	1.34	0.067

APPENDIX A6

Costs, number of events (in parenthesis) and p values for each cost combination used in the cophylogenetic analysis in Chapter 7. Results are organised by the three tree topologies that were constantly found throughout the analysis. Cost schemes in which the cost of duplication was greater than the cost of duplication and host-switch were not considered.

Cospeciation	Duplication	Duplication and host switch	Loss	Failure to diverge	Global cost	p value
0 (0)	0 (5)	0 (3)	0 (112)	0 (40)	0	1.000
0 (0)	0 (5)	0 (3)	0 (112)	1 (40)	40	1.000
0 (0)	0 (5)	0 (3)	0 (112)	2 (40)	80	1.000
0 (0)	0 (5)	0 (3)	1 (87)	0 (40)	87	<0.001
0 (0)	0 (5)	1 (3)	1 (87)	0 (40)	90	<0.001
0 (0)	0 (5)	2 (3)	1 (87)	0 (40)	93	<0.001
0 (0)	1 (5)	1 (3)	1 (87)	0 (40)	95	<0.001
0 (0)	1 (5)	2 (3)	1 (87)	0 (40)	98	<0.001
0 (0)	0 (5)	0 (3)	1 (87)	1 (40)	127	<0.001
0 (0)	0 (5)	1 (3)	1 (87)	1 (40)	130	<0.001
0 (0)	0 (5)	2 (3)	1 (87)	1 (40)	133	<0.001
0 (0)	1 (5)	1 (3)	1 (87)	1 (40)	135	<0.001
0 (0)	1 (5)	2 (3)	1 (87)	1 (40)	138	<0.001
0 (0)	0 (5)	0 (3)	1 (87)	2 (40)	167	<0.001
0 (0)	0 (5)	1 (3)	1 (87)	2 (40)	170	<0.001
0 (0)	0 (5)	2 (3)	1 (87)	2 (40)	173	<0.001
0 (0)	0 (5)	0 (3)	2 (87)	0 (40)	174	<0.001
0 (0)	1 (5)	1 (3)	1 (87)	2 (40)	175	<0.001
0 (0)	0 (5)	1 (3)	2 (87)	0 (40)	177	<0.001
0 (0)	1 (5)	2 (3)	1 (87)	2 (40)	178	<0.001
0 (0)	0 (5)	2 (3)	2 (87)	0 (40)	180	<0.001
0 (0)	1 (5)	1 (3)	2 (87)	0 (40)	182	<0.001
0 (0)	1 (5)	2 (3)	2 (87)	0 (40)	185	<0.001
0 (0)	2 (5)	2 (3)	2 (87)	0 (40)	190	<0.001
0 (0)	0 (5)	0 (3)	2 (87)	1 (40)	214	<0.001
0 (0)	0 (5)	1 (3)	2 (87)	1 (40)	217	<0.001
0 (0)	0 (5)	2 (3)	2 (87)	1 (40)	220	<0.001
0 (0)	1 (5)	1 (3)	2 (87)	1 (40)	222	<0.001
0 (0)	1 (5)	2 (3)	2 (87)	1 (40)	225	<0.001
0 (0)	0 (5)	0 (3)	2 (87)	2 (40)	254	<0.001
0 (0)	0 (5)	1 (3)	2 (87)	2 (40)	257	<0.001
0 (0)	0 (5)	2 (3)	2 (87)	2 (40)	260	<0.001
0 (0)	1 (5)	1 (3)	2 (87)	2 (40)	262	<0.001
0 (0)	1 (5)	2 (3)	2 (87)	2 (40)	265	<0.001
0 (0)	2 (5)	2 (3)	2 (87)	2 (40)	270	<0.001
0 (0)	2 (5)	2 (3)	2 (87)	1 (40)	230	<0.001

Appendix A6. (Continued)

Cospeciation	Duplication	Duplication and host switch	Loss	Failure to diverge	Global cost	<i>p</i> value
0 (2)	1 (3)	1 (3)	0 (119)	0 (40)	6	<0.001
0 (2)	2 (3)	2 (3)	0 (119)	0 (40)	12	0.020
0 (2)	1 (3)	1 (3)	0 (119)	1 (40)	46	<0.001
0 (2)	2 (3)	2 (3)	0 (119)	1 (40)	52	<0.001
0 (2)	1 (3)	1 (3)	0 (119)	2 (40)	86	<0.001
0 (2)	2 (3)	2 (3)	0 (119)	2 (40)	92	<0.001
0 (2)	2 (3)	2 (3)	1 (90)	0 (40)	102	<0.001
0 (2)	2 (3)	2 (3)	1 (90)	1 (40)	142	<0.001
0 (2)	2 (3)	2 (3)	1 (90)	2 (40)	182	<0.001
0 (2)	1 (3)	1 (3)	0 (119)	0 (40)	6	<0.001
0 (2)	2 (3)	2 (3)	0 (119)	0 (40)	12	0.020
0 (2)	1 (3)	1 (3)	0 (119)	1 (40)	46	<0.001
0 (2)	2 (3)	2 (3)	0 (119)	1 (40)	52	<0.001
0 (2)	1 (3)	1 (3)	0 (119)	2 (40)	86	<0.001
0 (2)	2 (3)	2 (3)	0 (119)	2 (40)	92	<0.001
0 (2)	2 (3)	2 (3)	1 (90)	0 (40)	102	<0.001
0 (2)	2 (3)	2 (3)	1 (90)	1 (40)	142	<0.001
0 (2)	2 (3)	2 (3)	1 (90)	2 (40)	182	<0.001
0 (2)	1 (3)	1 (3)	0 (119)	0 (40)	6	<0.001

Cospeciation	Duplication	Duplication and host switch	Loss	Failure to diverge	Global cost	<i>p</i> value
0 (2)	0 (6)	1 (0)	0 (156)	0 (40)	0	1.000
0 (2)	0 (6)	2 (0)	0 (156)	0 (40)	0	1.000
0 (2)	1 (6)	2 (0)	0 (156)	0 (40)	6	<0.001
0 (2)	0 (6)	1 (0)	0 (156)	1 (40)	40	1.000
0 (2)	0 (6)	2 (0)	0 (156)	1 (40)	40	1.000
0 (2)	1 (6)	2 (0)	0 (156)	1 (40)	46	<0.001
0 (2)	0 (6)	1 (0)	0 (156)	2 (40)	80	1.000
0 (2)	0 (6)	2 (0)	0 (156)	2 (40)	80	1.000

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This thesis addresses different aspects on the origin, diversification and host specificity of digeneans of cetaceans. It provides an updated list on the systematic information of digeneans and other helminths infecting cetaceans, hypotheses for the origin and coevolutionary history of digeneans and marine mammals, and the phylogeography and patterns of host specificity of the most generalist digenean specific to cetaceans, namely, *Pholeter gastrophilus*.

