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On the Evolutionary History of Philometridae (Nematoda: Dracunculoidea): Integrative Taxonomy Reveals Evidence of Character Diversification and Host–Parasite Cophylogenetic Patterns

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Abstract: (1) Background: Integrative taxonomy has been important in the comprehension of relationships among nematode parasites. Philometridae is a highly diverse family of these organisms, but poorly-known regarding genetic characterization and evolution. An integrative taxonomic analysis was performed to improve the knowledge of the evolutionary history of Philometridae. (2) Methods: Phylogenies were reconstructed based on genetic sequences alone and integrated with morphological/life history traits, which were phylogenetically mapped. The host–parasite cophylogeny was evaluated. (3) Results: Previously unpublished 28S rDNA sequences are given for some species. The phylogeny from this marker, although limited by data scarcity, showed similar patterns as that from 18S rDNA. Clades shared common features related to the structure of the esophagus and of the tail in males (especially the gubernaculum), site of infection, habitat, host taxa and geographic origin; most of these features were phylogenetically informative. The integrative phylogeny was better resolved. A cophylogenetic signal was present mainly in clades of freshwater species. (4) Conclusions: The speciation process in Philometridae is not unique or uniform; host capture, host–parasite co-evolution and allopatric (especially in freshwater) events may be occurring simultaneously in different lineages, places and times. Cases of plesiomorphy retention probably occur. Evolutionary convergence of poorly-informative characters is suggested, even though they are important for species diagnosis.

Keywords: fish parasite; morphology; genetic characterization; phylogeny; freshwater; marine; nematode

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1. Introduction

Philometridae Baylis and Daubney, 1926 (Nematoda: Dracunculoidea) is a family of parasitic nematodes exclusively from fishes, with a wide global distribution and importance as pathogenic organisms [1]. This highly diverse group of dracunculoids includes parasites infecting several marine, brackish and freshwater teleosts, with broad morphological variability and marked sexual dimorphism [1,2]. While males measure only a few millimeters and probably are short-lived, females are larger, measuring centimeters, long-lived and may occupy different sites of infection than that of males [1,2]. Consequently, most species are proposed based on the morphology of gravid females in addition to the host species and site of infection [1–4].

During the last decade, the morphological taxonomy of philometrids has improved considerably due to detailed analysis using scanning electron microscopy (SEM), judicious descriptions of new taxa and redescriptions of poorly-known species (see [5–11]). As a

result, the knowledge pertaining to these nematodes has been enriched. However, studies on the Philometridae are still scarce, making the systematics of the family one of the most unsatisfactory and difficult within the phylum Nematoda [1,4]. Moreover, studies are uneven across geographical regions, host taxa and habitat (marine vs. brackish vs. freshwater) [1,4].

Several genetic characterizations of philometrid nematodes have been published in the last two decades [4,12–17], which were important for the advancement of knowledge about their diversity [1,4]. However, despite this progress, most species are yet to be genetically characterized and need morphological revaluation [4]. Therefore, the evolutionary history and systematics of Philometridae remain blurry. For example, the largest genera within the family, namely, *Philometra* Costa, 1845 and *Philometroides* Yamaguti, 1935, have been polyphyletic in molecular phylogenies, suggesting their artificiality and the necessity of a revaluation of their generic diagnosis [4,14–17].

Recently, integrative taxonomic approaches have been very efficient for the resolution of systematic deadlocks related to nematode parasites of fishes (see [18–23]). These approaches allow the evaluation of morphological and life history traits in the evolutionary history of parasites and their importance during this process. Despite the importance of and the problems associated with Philometridae highlighted here, integrative taxonomy has never been used for studying this taxon. Therefore, in the present work we used an integrative taxonomic approach to evaluate the evolutionary history of Philometridae, as well as the importance of morphological and life history traits for this process. Moreover, host–parasite cophylogenetic patterns were also analyzed for the first time in Philometridae.

2. Materials and Methods

2.1. Genetic Data

The following tissue samples used for genetic sequencing were kindly provided by Dr. František Moravec: *Buckleyella ornata* Moravec, Diggles, Barnes and Macbeth, 2014, parasite in the mesentery of *Scomberoides commersonnianus* Lacepède, 1801 (Carangiformes: Carangidae); *Philometra australiensis* Moravec and Diggles, 2014, parasite in the swim bladder of *Polydactylus macrochir* (Günther, 1867) (Carangaria: Polynemidae); *Ph. johnii* Moravec and Ali, 2013, parasite in the ovary of *Johnius* sp. (Eupercaria: Sciaenidae), all from Australia; and *Ph. rubra* (Leidy, 1856), parasite in the mesentery of *Morone saxatilis* (Walbaum, 1792) (Eupercaria: Moronidae) from Canada. These samples were subjected to DNA isolation and PCR aiming to amplify the nuclear 28S rDNA, since 18S rDNA failed to amplify in all assays (see all related details in Supplement S1). Due to the low availability of sequences from the same genetic marker for philometrids in GenBank, an integrative analysis using 28S sequences would not make sense in the present context. Nonetheless, a phylogeny was reconstructed based only on 28S genetic data including the newly obtained sequences and those of *Ph. kotlani* (Molnár, 1969) (MH791050), *Ph. nattereri* Cárdenas, Moravec, Fernandes and Morais, 2012 (MH930985), *Ph. obturans* (Prennant, 1886) (MH791053), *Ph. rischta* Skrjabin, 1923 (MH791052), *Ps. acreanensis* Cavalcante, Moravec and Santos, 2018 (MH923192), *Ps. moraveci* Vismanis and Yunchis, 1994 (MH791048) and *Ps. tahieli* Montes, Plaul and Martorelli, 2016 (MN822003), using *Camallanus xenopodis* Jackson and Tinsley, 1995 (Camallanoidea: Camallanidae) (MG947389) as outgroup.

An extensive search of the GenBank database was conducted in order to evaluate the number, length and type of sequences from philometrid nematodes available. We found that the most complete dataset was that consisting of 18S sequences, because it included the highest number of representatives and greater genetic information. Therefore, these 18S sequences were used for the present integrative analysis and were selected based on the following criteria: originated from previously published taxonomic/phylogenetic papers in order to ensure the taxonomic identification (see [4]); with sequence length longer than 1600 bp to maximize genetic information. The sequence AB185161 (GenBank ID) of *Margolisianum bulbusum* Baylock and Overstreet, 1999 was not considered, because it is species inquirenda (see [23]); the sequence JX456388 of *Ph. tunisiensis* Moravec, Chaabane,

Neifar Gey et Justine, 2016 was not considered due to some taxonomic problems (see the discussion of this paper). The dracunculoid *Philonema onchorynchi* Kuitunen-Ekbaum, 1933 (Philonematidae) was used as outgroup, based on previous phylogenies of Philometridae/Dracunculoidea showing that this nematode, which is also parasitic in fish, represents a closely related basal lineage to Philometridae [4,12–15,17]. Information on these sequences associated with parasite and host taxa, habitat, geographic origin, site of infection of gravid females, GenBank ID and reference is given in Table 1.

Table 1. Information on 18S rDNA sequences from nematodes used in the present analysis.

GenBank ID	Parasite Taxa	Host Taxa (Order)	Habitat	Site of Infection [1]	Geographic Origin	Reference
JF803946	*Afrophilometra hydrocyoni* (Fahmy, Mandour and El-Nafar, 1976)	*Hydrocynus forskahlii* (Cuvier, 1819) (Characiformes)	Freshwater	Fins and nearby muscles	Kenya	[13]
DQ442672	*Alinema amazonicum* (Travassos, 1960)	*Callophysus macropterus* (Lichtenstein, 1819) (Siluriformes)	Freshwater	Abdominal cavity and mesentery	Peru	[12]
JF803939	*Caranginema americanum* Moravec, Montoya-Mendoza and Salgado-Maldonado, 2008	*Caranx hippos* (Linnaeus, 1766) (Carangiformes)	Marine	Subcutaneous tissues	USA	[13]
MZ274360	*Digitiphilometroides marinus* (Moravec et de Buron, 2009)	*Rachycentron canadum* (Linnaeus, 1766) (Carangiformes)	Marine	Body cavity	Australia	[4]
DQ442671	*Nilonema senticosum* (Baylis, 1927)	*Arapaima gigas* (Schinz, 1822) (Osteoglossiformes)	Freshwater	Abdominal cavity and swim bladder	Peru	[12]
MZ274352	*Philometra arafurensis* Moravec and Barton, 2018	*Lutjanus sebae* (Cuvier, 1816) (Eupercaria)	Marine	Ovary	Australia	[4]
JF803948	*Philometra bagri* (Khalil, 1965)	*Bagrus bajad* (Fabricius, 1775) (Siluriformes)	Freshwater	Subcutaneous tissues	Kenya	[13]
JF803943	*Philometra brevispicula* Moravec and Bakenhaster, 2010	*Lutjanus griseus* (Linnaeus, 1758) (Eupercaria)	Marine	Subcutaeous tissues	USA	[13]
DQ442675	*Philometra cyprinirutili* (Creplin, 1825)	*Abramis brama* (Linnaeus, 1758) (Cypriniformes)	Freshwater	Abdominal cavity	Czech Republic	[12]
JF803942	*Philometra diplectri* Moravec and Bakenhaster, 2010	*Diplectrum formosum* (Linnaeus, 1766) (Serranoidei)	Marine	Subcutaneous tissues	USA	[13]
JF803928	*Philometra floridensis* Moravec, Fajer-Ávila and Bakenhaster, 2010	*Sciaenops ocellatus* (Linnaeus, 1766) (Eupercaria)	Marine	Ovary	USA	[13]

Table 1. *Cont.*

GenBank ID	Parasite Taxa	Host Taxa (Order)	Habitat	Site of Infection [1]	Geographic Origin	Reference
MZ274354	*Philometra globiceps* (Rudolphi, 1819)	*Uranoscopus scaber* Linnaeus, 1758 (Uranoscopoidei)	Marine	Ovary	Italy	[4]
MZ274362	*Philometra gracilis* Moravec and Barton, 2016	*Lutjanus johnii* (Bloch, 1792) (Eupercaria)	Marine	Tissue behind head	Australia	[4]
JF803916	*Philometra gymnosardae* Moravec, Lorber and Konečný, 2007	*Gymnosarda unicolor* (Rüppell, 1836) (Sombriformes)	Marine	Abdominal cavity	Maldives	[13]
MZ274349	*Philometra iraqiensis* Moravec, Ali, Abed and Shaker, 2016	*Planiliza klunzingeri* (Day, 1888) (=*Liza kluzingeri*) (Mugiliformes)	Marine	Ovary	Iraq	[4]
MH725819	*Philometra kotlani* (Molnár, 1969)	*Leuciscus aspius* (Linnaeus, 1758) (=*Aspius aspius*) (Cypriniformes)	Freshwater	Ovary	Russia	[15]
KP122959	*Philometra lagocephali* Moravec and Justine, 2008	*Lagocephalus lunaris* (Bloch and Schneider, 1801) (Tetraodontiformes)	Marine	Abdominal cavity	China	[24]
FJ161972	*Philometra lateolabracis* (Yamaguti, 1935)	*Lateolabrax japonicus* (Cuvier, 1828) (Acropomatiformes)	Marine	Ovary	Japan	[25]
JF803945	*Philometra lati* Moravec, Charo-Karisa and Jirků, 2009	*Lates niloticus* (Linnaeus, 1758) (Carangaria)	Freshwater	Abdominal cavity	Kenya	[13]
MZ274356	*Philometra longa* Moravec, Barton and Shamsi, 2021	*Hyporhamphus australis* (Steindachner, 1866) (Beloniformes)	Marine	Abdominal cavity	Australia	[4]
FJ161974	*Philometra madai* Quiazon, Yoshinaga and Ogawa, 2008	*Pagrus major* (Temminck and Schlegel, 1843) (Eupercaria)	Marine	Ovary	Japan	[26]
JF803933	*Philometra morii* Moravec, Bakenhaster and Fajer-Ávila, 2010	*Epinephelus morio* (Valenciennes, 1828) (Serranoidei)	Marine	Subcutaneous tissues	USA	[13]
MH930986	*Philometra nattereri* Cárdenas, Moravec, Fernandes and Morais, 2012	*Serrasalmus gibbus* Castelnau, 1855 (Characiformes)	Freshwater	Stomach wall	Brazil	[14]
FJ161975	*Philometra nemipteri* Luo, 2001	*Nemipterus virgatus* (Houttuyn, 1782) (Eupercaria)	Marine	Ovary	Japan	[25]
AY852267	*Philometra obturans* (Prennant, 1886	*Esox lucius* Linnaeus, 1758 (Esociformes)	Freshwater	Gill blood vessel	Czech Republic	[12]

Table 1. *Cont.*

GenBank ID	Parasite Taxa	Host Taxa (Order)	Habitat	Site of Infection [1]	Geographic Origin	Reference
JF803929	*Philometra ocularis* Moravec, Ogawa, Suzuki, Miyazaki and Donai, 2002	*Epinephelus areolatus* (Forsskål, 1775) (Serranoidei)	Marine	Tissue behind eye	New Caledonia	[13]
DQ442677	*Philometra ovata* (Zeder, 1803)	*Gobio gobio* (Linnaeus, 1758) (Cypriniformes)	Freshwater	Abdominal cavity	Czech Republic	[12]
LC536677	*Philometra pellucida* (Jägerskiöld, 1893)	*Arothron mappa* (Lesson, 1831) (Tetraodontiformes)	Marine	Abdominal cavity	Japan	[27]
MZ274353	*Philometra rara* Moravec, Chaabane, Neifar, Gey and Justine, 2017	*Hyporthodus haifensis* (Ben-Tuvia, 1953) (Serranoidei)	Marine	Ovary	Libya	[4]
MH725822	*Philometra rischta* Skrjabin, 1923	*Alburnus alburnus* (Linnaeus, 1758) (Cypriniformes)	Freshwater	Subcutaneous tissues	Russia	[15]
JF803920	*Philometra saltatrix* Ramachandran, 1973	*Pomatomus saltatrix* (Linnaeus, 1766) (Scombriformes)	Marine	Ovary	USA	[13]
FJ161971	*Philometra sciaenae* Yamaguti, 1941	*Nemipterus virgatus* (Houttuyn, 1782) (Eupercaria)	Marine	Ovary	Japan	[26]
JF803944	*Philometra spiriformis* Moravec, Charo-Karisa and Jirků, 2009	*Lates niloticus* (Linnaeus, 1758) (Carangaria)	Freshwater	Subcutaneous tissues	Kenya	[13]
JF803941	*Philometroides grandipapillatus* Moravec and Bakenhaster, 2010	*Caranx hippos* (Linnaeus, 1766) (Carangiformes)	Marine	Pectoral fin muscle	USA	[13]
MH714520	*Philometroides moraveci* Vismanis and Yunchis, 1994	*Perccottus glenii* Dybowski, 1877 (Gobiiformes)	Freshwater	Subcutaneous tissues	Russia	[15]
DQ442676	*Philometroides sanguineus* (Rudolphi, 1819)	*Carassius carassius* (Linnaeus, 1758) (Cypriniformes)	Freshwater	Fins and subcutaneous tissues	Czech Republic	[12]
FJ155811	*Philometroides seriolae* (Ishii, 1931)	*Seriola quinqueradiata* Temminck and Schlegel, 1845 (Carangiformes)	Marine	Muscles	Japan	[28]
MZ274350	*Philometroides stomachicus* Moravec and Barton, 2016	*Protonibea diacanthus* (Lacepède, 1802) (Eupercaria)	Marine	Stomach wall	Australia	[4]
JF803923	*Rumai rumai* Travassos, 1960	*Arapaima gigas* (Schinz, 1822) (Osteoglossiformes)	Freshwater	Abdominal cavity	Brazil	[13]
DQ442670 [2]	*Philonema oncorhynchi* Kuitunen-Ekbaum, 1933	*Oncorhynchus kisutch* (Walbaum, 1792) (Salmoniformes)	Freshwater	Abdominal cavity	Canada	[12]

[1] Site of infection of gravid females; [2] Outgroup.

2.2. Phylogenetic Reconstruction Using Genetic Data

Sequences were aligned using the multiple algorithm tool T-Coffee [29], then submitted to the transitive consistency score to verify the reliability of aligned positions and those scored as average to bad were automatically trimmed by the algorithm to optimize the phylogenetic topology [30].

Based on recent results indicating a good response of Bayesian inference in integrative taxonomic studies of nematode parasites of fishes with complicated taxonomy [20–23], the phylogenetic hypotheses in the present work were inferred using this approach in BEAST 2.5 [31]; the best-fit substitution model was chosen according to bModelTest [32]. The molecular clock model was relaxed (log exponential), defined using the nested sampling method [33] and the Yule tree prior, selected according to the posterior densities and the effective sample sizes (ESS), verified in Tracer [34]. This approach was chosen for its robustness, because it provides improved evolutionary pathways in phylogenetic reconstruction, without overestimating the nodal supports [31], making it more reliable to evaluate the evolutionary history of taxa. The posterior estimates of parameter densities and the ESS for each parameter of the model, as well as the posterior probability for nodal supports in the majority-rule consensus phylogenetic trees, were determined after running the Markov chain Monte Carlo (MCMC) analysis, always four chains in two runs, each run with 10×10^6 generations, saving the last 10,001 trees and 25% burn-in. The quality of the analysis (parameter densities, ESS and burn-in) and the chain convergence were examined in Tracer [34].

The definition of major clades in all phylogenies presented in this work follows the proposition of Barton et al. [4].

2.3. Morphological and Life History Traits

A matrix (referred as the morphological matrix from here) including 19 morphological and life history characters of philometrid nematodes, with 78 states in total, was constructed for the integrative phylogenetic analysis using the software Mesquite [35]. These characters were selected based on the morphological diagnoses of genera and species of Philometridae, as well as based on their life history importance [1,2,9,36–40]. Details of the matrix, characters and states are given in Supplement S2. The characters and their number of states are summarized as follows: (1) cuticle surface (two states); (2) cephalic outgrowth (two states); (3) number of outer-circle cephalic papillae (three states); (4) number of inner-circle cephalic papillae (three states); (5) peribuccal ring of teeth (two states); (6) esophageal teeth (two states); (7) esophagus structure (three states); (8) esophageal gland (three states); (9) ventriculus (two states); (10) vulva (two states); (11) protrusions on female tail (three states); (12) structure of gubernaculum distal end in males (seven states); (13) relative size of spicules in males (two states); (14) pair of far anterior precloacal papillae in males (two states); (15) structure of tail in males (four states); (16) site of infection of gravid females (nine states); (17) habitat (two states); (18) host order (seventeen states); (19) geographic origin (eight states). The systematic classification of hosts was checked in Froese and Pauly [41]. The geographic origin was defined according to the zoogeographic regions [42] for freshwater fish and according to the marine ecoregions [43,44] for marine fish. All original descriptions and redescriptions of species included in the analysis were consulted for data accuracy (details of these references are in Supplement S2).

2.4. Integrated Analysis of Genetic, Morphological and Life History Data, Character Evaluation and Mapping

The morphological matrix and the alignment of 18S sequences were imputed in BEAUti 2.5 (an implementation of BEAST 2.5) [31], partitioned according to the data type. The substitution model for 18S sequences partition was the same as previously described. The Markovian Mkv model of character change, which is adequate for categorical data (morphology and life history traits), was applied to the morphological matrix data [45] using the Morph-models package implemented in BEAST 2.5 [31]. The phylogeny of integrated

data was reconstructed as previously described, with the clock model, tree priors and MCMC parameters linked among the partitions and substitution models unlinked. To evaluate the degree of homoplasy of morphological and life history traits, their consistency indexes (CIs) were calculated based on the concatenation of the morphological matrix and genetic alignment imputed in Mesquite software, along with the majority-rule consensus phylogenetic tree [35]. The relation between the CI values ($0 < CI < 1$) and degree of homoplasy is indicated as follows: CI = 0 full homoplasy, CI = 1 lack of homoplasy, CI $\leq$ 0.6 presence of homoplasy (low phylogenetic information) [21]. The characters and their respective states were mapped on the phylogenetic tree using Mesquite software [35]. CIs were also calculated for the phylogenetic tree inferred from genetic data alone and for that inferred from the integrated dataset.

2.5. Host–Parasite Cophylogenetic Analysis

To evaluate any sign of phylogenetic congruence between philometrids and their hosts, a cophylogenetic analysis was performed using the package PACo, implemented in RStudio [46,47]. The packages ape, vegan and ggplot2 were used as auxiliary to the analysis [48–50]. For the phylogenetic reconstruction of hosts, we used partial sequences of *cox1* mtDNA (detailed information is given in Table S1), based on data availability and adequate phylogenetic information for the present purpose [51]. The host phylogeny was reconstructed as previously described for parasites. Then, a matrix associating hosts to their parasite species was constructed and imputed in RStudio along with the host and parasite phylogenies, which were transformed into genetic distance matrixes. PACo was run with 10,000 permutations and using both forced and unforced phylogenetic superimposition. To evaluate the contribution of host–parasite individual links to possible cophylogenetic signals, Procrustes jacknifed squared residuals were calculated for each link (with 95% confidence intervals), their median estimated and values below the median considered with possible cophylogenetic signal [46,52]. The Procrustes residuals of host–parasite links with supposed cophylogenetic signal were tested against those of host–parasite links without cophylogenetic signal using Welch's t-test in order to confirm the presence of the signal [46]. In this analysis, the host and parasite names were abbreviated as recommended by Hutchinson et al. [46] for better layout; the correspondence between names and abbreviations is in Table S2.

3. Results

3.1. New Genetic Sequences Obtained and Preliminary Phylogeny Using 28S rDNA Sequences

Partial sequences of 28S were obtained for *B. ornata* (887 bp; GenBank OQ858487), *Ph. australiensis* (818 bp; GenBank OQ858488), *Ph. johnii* (801 bp; GenBank OQ858489) and *Ph. rubra* (799 bp; GenBank OQ858490) for the first time (Figure 1). The genetic variability was higher in the alignment of 28S sequences when compared with that of 18S. The nucleotide substitution model used for 28S phylogenetic reconstruction was TN93 without equal base frequencies and other parameters similar to those used for 18S sequences (see materials and methods). In this phylogeny, *Ps. acreanensis* appeared as a basal lineage within Philometridae; *B. ornata* was sister group of a clade formed by the freshwater species *Ph. nattereri*, *Ph. obturans* and *Ph. Rubra*; a clade was formed by the two marine species from Australia, *Ph. australiensis* and *Ph. johnii*, and another formed by *Ph. kotlani* and *Ph. rischta*, both parasites of freshwater Cypriniformes from Russia; all these clades were fully supported (Figure 1). The species tended to group according to the clades defined by Barton et al. [4], similar to those observed in the 18S phylogenies (see the following text and Figures 1 and 2). *Philometra* and *Philometroides* were not monophyletic (Figure 1).

3.2. Phylogeny Based on 18S rDNA Genetic Data

The phylogeny of 18S sequences (alignment length 1700 bp; CI = 0.65) was inferred based on the model TN93 + I + G, with equal base frequencies, including 39 species of philometrids plus the outgroup (Figure 2). Philometridae formed a fully supported mono-

phyletic assemblage; within the family, there were four major, fully supported clades, named sensu Barton et al. [4]: Clade A, containing parasites of the body cavity and subcutaneous tissues of freshwater fishes from South America (Neotropical Region), occupying the most basal position; Clade B, containing parasites of freshwater Cypriniformes from Europe (Palearctic Region), mostly parasitizing the body cavity; Clade C, sister to Clade B, containing parasites of marine fish from different orders and ecoregions, mostly parasitizing the gonads (ovary), but also found in the body cavity; and Clade D, which was the most diverse regarding host order, habitat, site of infection of gravid females and geographic origin (Figure 2). Four groups within Clade D were identified as follows: group 1 (weakly supported), including parasites of subcutaneous tissues (including that of the head) of marine fish; group 2 (fully supported), including species with random characteristics (see the next section) composed of two *Philometroides* found in the head tissues of marine and freshwater fish and *Ph. diplectri* Moravec and Bakenhaster, 2010, all from different host orders and geographic origins; group 3 (fully supported), with random host orders, site of infection of gravid females and geographic origins, included *Philometra* spp. from freshwater and marine fish and the only representative of *Caranginema* Moravec, Montoya-Mendoza and Salgado-Maldonado, 2008, a parasite of marine fish, with the internal nodes weakly supported; group 4 (weakly supported), another clade with low resolution and random characteristics, but philometrid parasites of freshwater fish from the Afro-Tropical region tended to form an assemblage (although weakly supported) and parasites of marine Carangaria/Carangiformes formed a fully supported clade (Figure 2). *Afrophilometra hydrocyoni* (Fahmy, Mandour and El-Nafar, 1976), *Dentiphilometroides marinus* (Moravec and de Buron, 2009) and *Ph. lati* Moravec, Charo-Karisa and Jirků, 2009 were not assigned to any group within Clade D (Figure 2). However, *A. hydrocyoni* and *Ph. lati* were closer to representatives of group 4 (with low support) and *D. marinus* closer to representatives of group 2 (with moderate support) (Figure 2). *Philometra* and *Philometroides* were polyphyletic (Figure 2).

3.3. Phylogeny Based on 18S rDNA, Morphological and Life History Data

The general topology of the phylogeny using the integrated data was similar to that of the genetic tree; the major clades (A, B, C and D) were in the same position and fully to highly supported (Figures 2 and 3). However, the configuration of lineages within Clades C and D was different, and the phylogenetic resolution was better with higher nodal supports in the tree from the integrated data (see the following text) (Figures 2 and 3).

According to the CI values, the integrated data tree was phylogenetically informative and the most informative characters were the peribuccal ring of teeth, esophagus structure, esophageal gland, ventriculus, vulva, structure of gubernaculum distal end, pair of far anterior precloacal papillae, site of infection of gravid females and host order (Table 2). Even though the structure of tail in males, host habitat and geographic origin had relatively low CIs, character mapping indicated interesting relations between them and the phylogenetic patterns (Figure 3, Table 2).

Table 2. Consistency index (CI) values estimated for phylogenetic tree and morphological and life history traits of philometrid nematodes from the integrated dataset.

Tree/Characters	CI
Phylogenetic tree	0.70
Cuticle surface	0.19
Cephalic outgrowth	1.0
Number of outer-circle cephalic papillae	0.43
Number of inner-circle cephalic papillae	0.38
Peribuccal ring of teeth	1.0
Esophageal teeth	0.33
Esophagus structure	1.0

Table 2. *Cont.*

Tree/Characters	CI
Esophageal gland	1.0
Ventriculus	1.0
Functional vulva	1.0
Protrusions on female tail	0.27
Structure of gubernaculum distal end	0.85
Relative size of spicules	0.25
Pair of far anterior precloacal papillae	1.0
Structure of male tail	0.44
Site of infection of gravid females	0.68
Habitat	0.48
Host order	0.68
Geographic origin	0.47

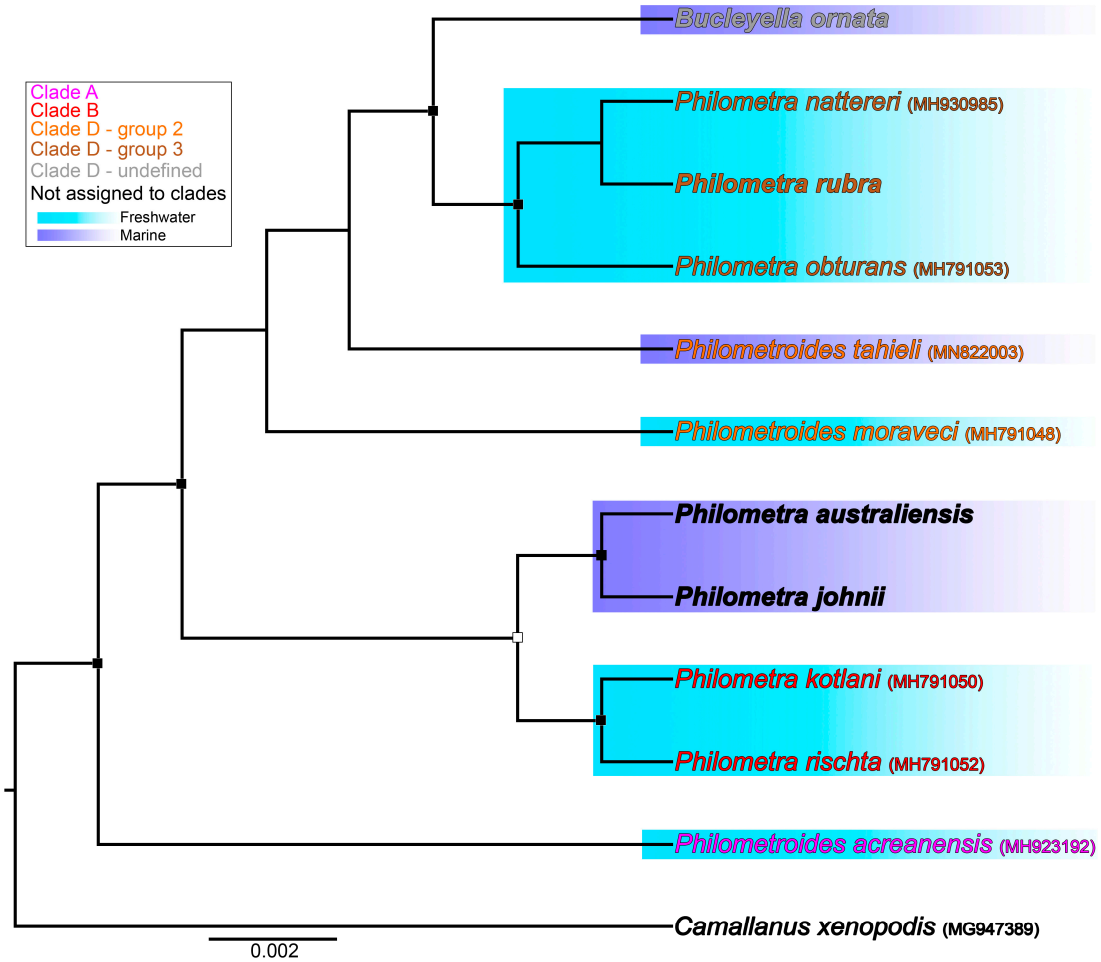

Figure 1. Phylogeny reconstructed using Bayesian inference based on 28S rDNA sequences from philometrid nematodes, with GenBank accession numbers in parentheses. Font colors indicate clades and groups sensu Barton et al. [4]. Colors shading the clades indicate aquatic habitat. Sequences generated in the present study are in bold. Nodal supports were estimated as Bayesian posterior probability (BPP) indicated as follows: black squares, BPP = 1 (full support); white squares, $0.96 \leq$ BPP ≤ 0.99 (high support); white circles, $0.90 \leq$ BPP < 0.96 (moderate support).

Alinema amazonicum (Travassos, 1960) was the only taxon with a peribuccal ring of teeth and a functional vulva present (Figure 3). An esophagus without an anterior expansion, formed by anterior smaller muscular and posterior larger muscular–glandular portions, appeared as a basal state in the outgroup, as well as in the basal philometrid

lineages *Nilonema senticosum* (Baylis, 1927) and *Rumai rumai* Travassos, 1960 (Figure 3). A muscular esophagus with an anterior expansion, without a glandular portion, but with an esophageal gland was the most common state within Philometridae, in which *A. amazonicum* was the only taxon with a multinucleated esophageal gland and the inner lineages *C. americanum* Moravec, Montoya-Mendoza and Salgado-Maldonado, 2008 and *Ph. nattereri* showed an anterior expansion of the esophagus markedly separated from the rest of the organ (Figure 3). The absence of a well-developed ventriculus in the esophagus–intestinal junction also appeared as a basal state present in the outgroup, in *N. senticosum* and in *R. rumais*, whereas a well-developed ventriculus was common among the other philometrids (Figure 3).

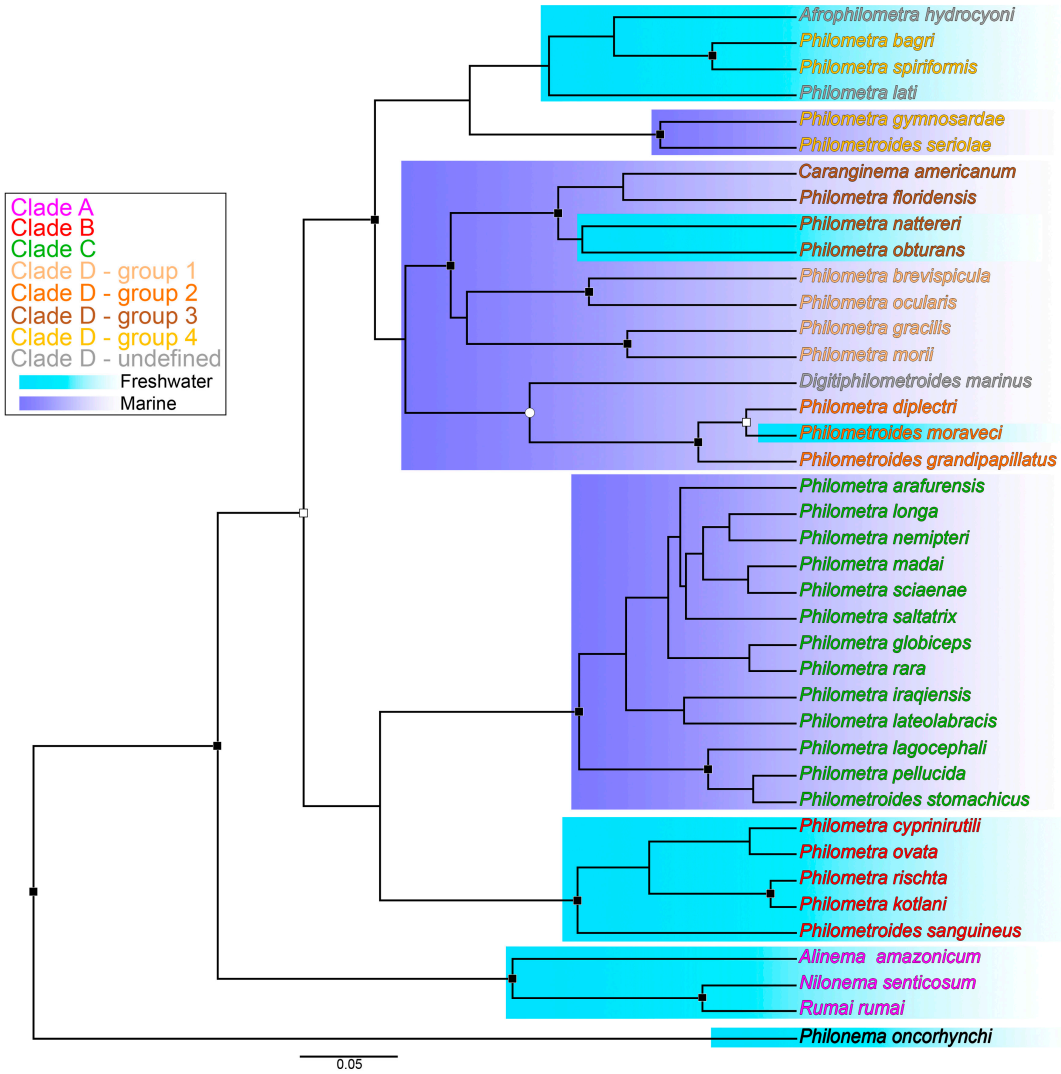

Figure 2. Phylogeny reconstructed using Bayesian inference based on 18S rDNA sequences from philometrid nematodes. Font colors indicate clades and groups sensu Barton et al. [4]. Colors shading the clades indicate aquatic habitat. Nodal supports were estimated as Bayesian posterior probability (BPP) indicated as follows: black squares, BPP = 1 (full support); white squares, $0.96 \leq \text{BPP} \leq 0.99$ (high support); white circles, $0.90 \leq \text{BPP} < 0.96$ (moderate support).

Even though the males of some species are still unknown, some characters of these parasites could be evaluated, showing interesting patterns. The outgroup had a completely different structure in the posterior end of males, showing a conical shape and numerous pre- and postcloacal papillae (Figure 3). In philometrids, the posterior end of males was rounded, with a reduced number of caudal papillae, tending to be grouped (Figure 3). A pair of far

anterior conspicuous papillae was present only in *A. amazonicum* (Figure 3). The outgroup lacked a gubernaculum, whereas this structure was present in all known philometrid males; the morphology of this organ showed interesting variation in the phylogeny, in which a harpoon shape with a distal barb was present in the basal *A. amazonicum* and kept in Clade B (Figure 3). A gubernaculum lacking a distal barb but with lamellae-like structures was exclusive of males in Clade C (Figure 3). In the terminal lineages of Clade D, *C. americanum*, *Ph. brevispicula* Moravec and Bakenhaster, 2010 and *Ph. diplectri*, the structure of gubernaculum was similar to that of males in Clade C, except for the lamellae-like structures, which are absent in species of Clade D (Figure 3).

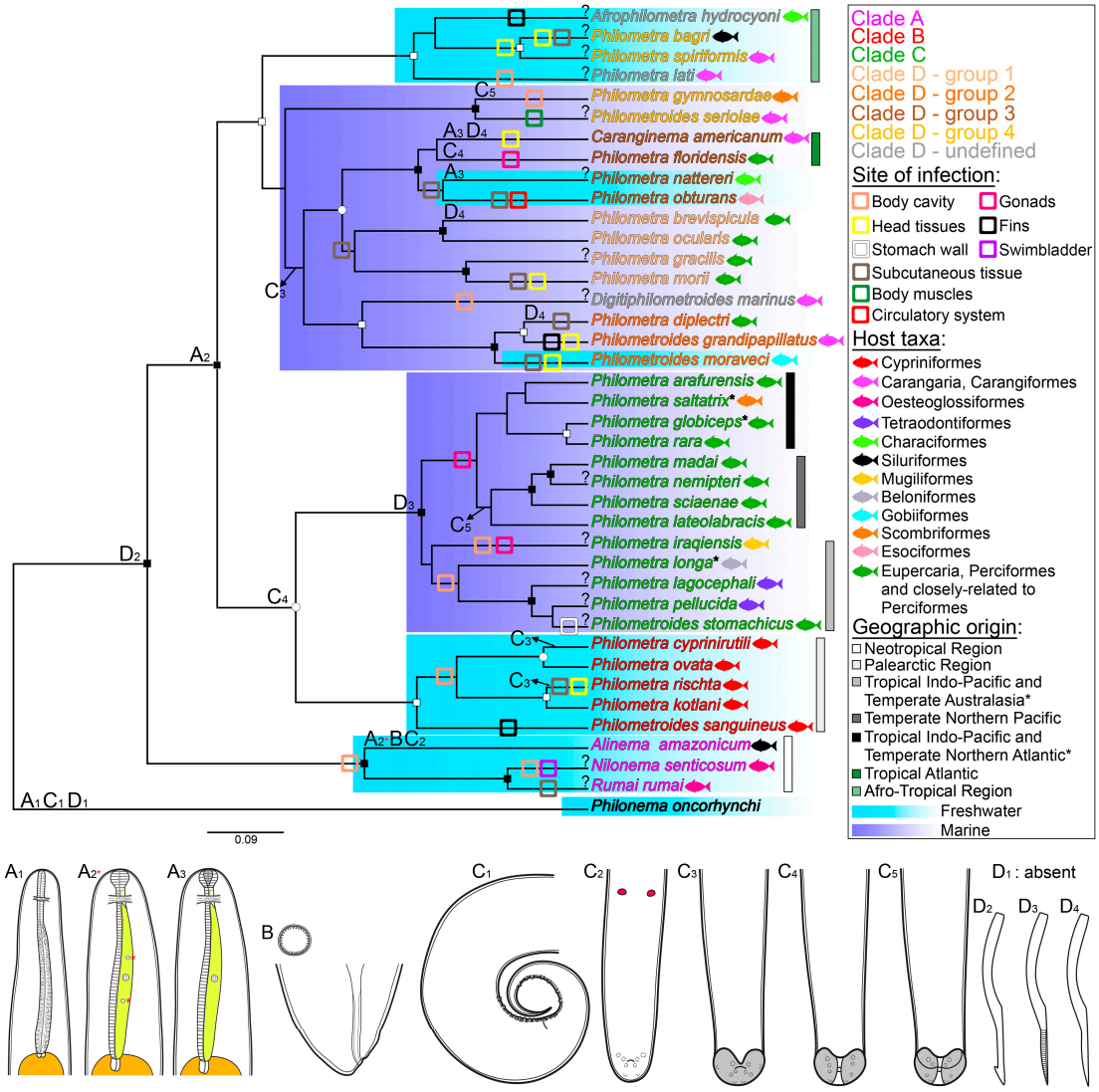

Figure 3. Phylogeny reconstructed using Bayesian inference based on 18S rDNA sequences and morphological and life history traits from philometrid nematodes. Font colors indicate clades and groups sensu Barton et al. [4]. Most relevant characters are mapped. Morphological schemes: (**A**): Esophagus structures; esophageal gland (yellow) may surpass nerve ring (red asterisks indicate multinucleated gland present in *A. amazonicum*). (**B**): Peribuccal ring of teeth and functional vulva present in *A. amazonicum*. (**C**): Posterior end of males with caudal mound in gray and far anterior precloacal papillae in red (simple representation of caudal papillae as dotted line). (**D**): Gubernaculum of males. Question marks (?) indicate unknown males. Asterisks (*) indicate correspondence between species and geographic origin. Nodal supports were estimated as Bayesian posterior probability (BPP) indicated as follows: black squares, BPP = 1 (full support); white squares, $0.96 \leq$ BPP ≤ 0.99 (high support); white circles, $0.90 \leq$ BPP < 0.96 (moderate support).

Alinema amazonicum was the only species with males lacking a caudal mound; this structure was non-lobulated in most males of Clade D and also present in some terminal lineages of Clade B (Figure 3). A caudal mound separated into two lobes was common among individuals of Clade C and present in two lineages of Clade B and in *Ph. floridensis* Moravec, Fajer-Avila and Bakenhaster, 2009 of Clade D (Figure 3). A caudal mound separated into four lobes was shared by males of the species forming a weakly supported assemblage within Clade C and also present in *Ph. gymnosardae* Moravec, Lorber and Konecný, 2007 of Clade D (Figure 3).

The site of infection of gravid philometrid females was similar in several assemblages, although the nodal supports were low (Figure 3). The body cavity, subcutaneous tissues and gonads (ovary) were common among closely related lineages, for example, within Clade A, Clade B and especially Clade C (Figure 3). The patterns relating these sites of infection and species grouping in Clade D were not clear (Figure 3).

There was a strong relationship between the host order (Cypriniformes) and the assembling of Clade B (Figure 3). In Clade A, a fully supported group was formed by parasites of Osteoglossiformes (i.e., *N. senticosum* and *R. rumai*) (Figure 3). In Clade C, one of the two major outer clades was formed by eight species, of which seven are parasites of Eupercaria, Perciformes or closely related taxa to Perciformes [53]; however, the nodal support of this clade was weak (Figure 3). A similar situation was observed in group 1 of Clade D (Figure 3).

Philometrid species also tended to group according to habitat and geographic origin. Clades A and B were formed by parasites of freshwater fish from the Neotropical and Palearctic regions, respectively (Figure 3). Clade C was formed by parasites of marine fish, mostly from the tropical Indo-Pacific; a weakly supported group was formed by species from the temperate Northern Pacific; and two species from the temperate Northern Atlantic and one from temperate Australasia were also present in this clade (Figure 3). In Clade D, philometrids of freshwater fishes from the Afro-Tropical region formed a highly supported group; the remaining species were mostly parasites of marine fishes from different geographic origins, except for *Ph. nattereri*, *Ph. obturans* and *Ps. moraveci*, which occur in freshwater (Figure 3).

3.4. Host–Parasite Cophylogenetic Analysis

Significant cophylogenetic signal was observed between fishes and their philometrid parasites, as indicated by both superimposed (m^2_{XY} = 55.17, $p < 0.001$, n = 10,000) and non-superimposed (m^2_{XY} = 0.45, $p < 0.001$, n = 10,000) models. Based on the global sum of squared residuals (m^2_{XY}), the non-superimposed model had the highest cophylogenetic signal (see [46]) (Figure 4). There were 43 host–parasite links, in which 20 indicated possible cophylogenetic signals (Figure 4A,B). The links that contributed the most to the cophylogenetic signal were between the philometrids of Clade B and their freshwater Cyprinoformes hosts; between *N. senticosum* and *R. rumai* of Clade A and their freshwater Osteoglossiformes host; between *Ph. iraqiensis* Moravec, Ali, Abed and Shaker, 2016 of Clade C and the marine fish *Planiliza klunzigeri* (Day, 1888); and between *D. marinus* of Clade D and the marine fish *Rachycentron canadum* (Linaeus, 1766) (Carangiformes) (Figure 4A). The links between *Ph. lati* and *Lates niloticus* (Linnaeus, 1758) (freshwater) and *Ps. seriolae* (Ishii, 1931) and *Seriola quinqueradiata* Temminck and Schlegel, 1845 (marine) had cophylogenetic signals weaker than those previously mentioned (Figure 4A). The links between the parasites *Ph. brevispicula* and *Ph. gracilis* Moravec and Barton, 2016 and their marine lutjanid (Eupercaria: Lutjanidae) hosts also showed cophylogenetic signal, but very weak (Figure 4A). The jacknifed Procrustes squared residuals of host–parasite interactions with cophylogenetic signal were significantly lower than those with non-cophylogenetic signal ($p < 0.001$) (Figure 4C).

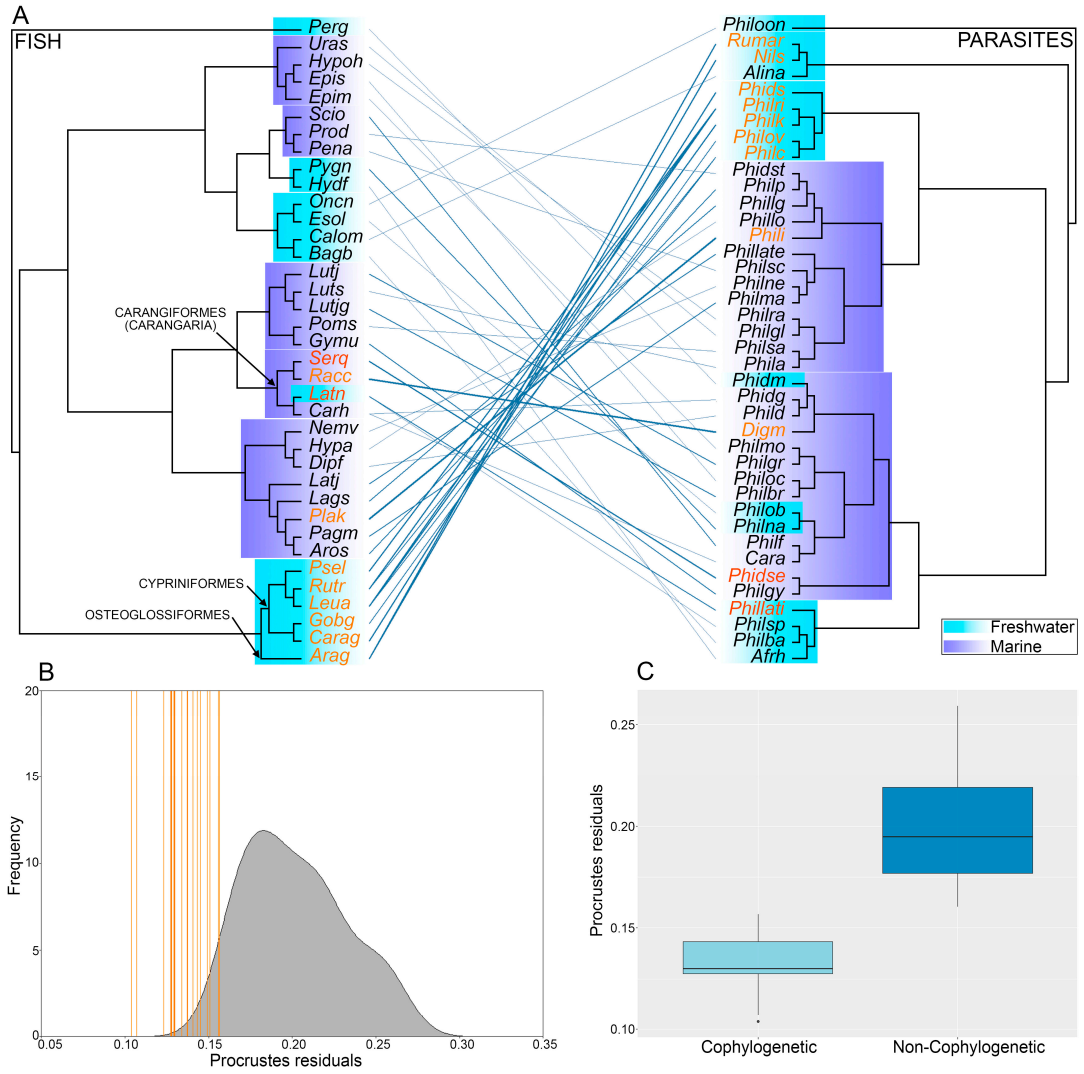

Figure 4. Results of cophylogenetic analysis between philometrid nematodes and their fish hosts. (**A**): Interaction network between parasite and fish host phylogenies; the wider the line the greater the cophylogenetic signal; taxon names in orange represent links that contributed most to cophylogenetic signal; taxon names in red represent taxa that were also important for cophylogenetic signal; colors shading the clades indicate aquatic habitat. (**B**): Curve plot showing the distribution of jacknifed Procrustes residuals (gray curve); orange lines represent interactions with cophylogenetic signal, in which those to the left of 0.15 on the X axis have the strongest signal. (**C**): Boxplot showing jacknifed Procrustes residuals of cophylogenetic and non-cophylogenetic interactions, which were statistically different ($p < 0.001$).

4. Discussion

Working on the evolutionary history of Philometridae is not an easy task. Only a few sequences from few genetic markers of these nematodes are available in the databases, and possible misidentifications of sequenced specimens further complicate the situation (see [4]). For solid phylogenetic results it is desirable to include as many species as possible in the analysis, but sequences with incipient genetic information (too short) and unconfirmed taxonomic accuracy (normally not originating from scientific publications) decreased the number of representatives in the present dataset. Nevertheless, the integrative approach included 39 philometrids, of which 28 were assigned to *Philometra*, five to *Philometroides* and one each to *Afrophilometra*, *Alinema*, *Caranginema*, *Digitiphilometroides* and *Nilonema*. It is also important to highlight that 28S sequences of *B. ornata*, *Ph. australiensis*, *Ph. johnii* and *Ph. rubra* are given for the first time and will be important in further work. Moreover,

Ph. Johnii, which has been found in Iraq [5], is reported in Australian waters for the first time, expanding its geographic occurrence.

Even though the current database has few 28S sequences of philometrids and the phylogenetic reconstruction generated from these sequences was limited to 11 species belonging to three genera (*Philometra*, *Philometroides* and *Buckleyella*), some important results could be observed. Similar to the phylogenies of 18S [4,14,15,17], that of 28S showed no monophyly of *Philometra* and *Philometroides*, the basal position of *Ps. acreanensis,* the monophyly of components of Clade B (*Ph. kotlani* and *Ph. rischta*) and the close relatedness of *B. ornata* and *Ph. rubra* with components of Clade D (*Ph. nattereri* and *Ph. obturans*) (see [4]). These observations confirm the congruence between 18S and 28S genetic markers, reinforce some phylogenetic patterns within Philometridae and highlight the need to complement the genetic database of these parasites. Moreover, the present 28S phylogeny showed better resolution than that observed in a previous study [17] because it included longer sequences (>799 bp) and, consequently, more genetic information. The present results also confirmed the higher nucleotide substitution rate of 28S sequences when compared with those of 18S, as has been observed for nematode parasites of fish [14,18–22,54]. It may be plausible that the 28S nuclear sequences of philometrid nematodes should help further studies on species delimitation approaches (see [18] as an example), until a greater diversity of sequences (including mitochondrial genes) is available. However, it is still early to discuss the suitability of genetic markers for the identification of species of philometrids due to the current fragmented database.

According to Moravec et al. [50], the 18S sequence JX456388, identified as *Ph. lateolabracis* (Yamaguti, 1935), most likely belongs to *Ph. tunisiensis*. The arguments by these authors for such a conclusion are strong and based on morphology and host specificity [55]. However, since this sequence could not be linked to a precise taxonomic identification, which gives a chance for misdiagnosis, we refrained from including the sequence JX456388 in the present analysis. This exclusion was based on the fact that wrong entries in the morphological/life history matrix may generate inaccurate results. It is worth mentioning the high genetic similarity (99.88%; only two polymorphisms) between the sequence JX456388 and that identified as *Ph. lateolabracis* (FJ161972), which has accurate taxonomic identification [25]. Moreover, *Ph. tunisiensis* shares several features with species (including *Ph. lateolabracis*) in Clade C, e.g., host order (Perciformes), habitat (marine), morphology of male tail (caudal mound divided into two lobes) and gubernaculum (with distal lamellae) [50]. The close relatedness between the sequences attributed to *Ph. lateolabracis* and *Ph. tunisiensis* has also been observed in previous studies [4,14,15,17].

Philometroides seriolae, the type species of the genus, is a sister lineage of *Ph. gymnosardae* forming a strongly supported relationship, documented in several phylogenetic reconstructions from 18S sequences [4,14,15,17]. Some authors suggest that the isolation source of the 18S sequence identified as *Ps. seriolae* may represent a different species [4], putting the artificiality of *Philometra* and *Philometroides* in check. Although *Ps. seriolae* sequence misidentification could be possible, there is strong evidence in favor of the polyphyly of *Philometra* and *Philometroides* [4,14,15,17]. In this sense, one of the main features used for differentiating these genera (presence/absence of cuticular bosses) showed a high degree of homoplasy (low phylogenetic information) in the present results (see further discussion). Recently, Choe and Eom [56] provided additional genetic characterization for *Ps. seriolae*, but associated with poorly detailed morphological identification. A manner to improve the accuracy of taxonomic identification of genetic sequences is the deposition of hologenophores in referenced biological collections. Unfortunately, this practice is very rare for nematode parasites.

The general topology of the phylogenies inferred from the 18S sequences and from the integrated data was very similar, but the internal nodes were better-supported in the integrated data tree. These results are supported by the CI value, which was slightly higher for the phylogeny from the integrated dataset (0.70 vs. 0.65). This is interesting and indicates that the inclusion of morphological and life history traits improved the

phylogenetic resolution, highlighting the importance of integrative taxonomic approaches. In this sense, the clade of parasites from freshwater fish of the Afro-Tropical region showed better support in the integrative data tree, mostly due to the same characteristics in habitat and geographic origin. A similar situation was observed in the clade of parasites from marine Eupercaria (except *Ph. lateolabracis*) of the temperate Northern Pacific (in Clade C), which was fully supported in the integrated data tree, influenced by the same characteristics of habitat, host taxa and geographic origin.

Even though habitat and geographic origin were associated with low CI values, it was possible to observe well-supported assemblages of freshwater parasites from the same geographic origin, i.e., from the Neotropics (Clade A), from the Palearctic (Clade B) and from the Afro-Tropical region (part of Clade D). The probable reduction in CI values for habitat and geographic origin was caused by the assemblage in Clade D, formed by species from marine and freshwater habitats with a high diversity of geographic origins. Although CI is a good measure of the phylogenetic information of a character, it must be interpreted carefully, since the results can be skewed by the nature of the dataset, as has been observed in integrative taxonomic approaches on nematode parasites [21,54]. Therefore, habitat and geographic origin are indeed important for most, but not all, assemblages of philometrids. The close relatedness among freshwater philometrids from the same geographic origin may be a consequence of geographic isolation, suggesting that the radiation of each of these clades occurred after geological compartmentalization of the areas that are currently represented by the Neotropical, Afro-Tropical and Palearctic regions.

Philonema represents a basal group to Philometridae, and Clade A is the most basal in the family [13–15]. Interestingly, the esophagus lacking a gland and ventriculus, divided into anterior muscular and posterior glandular–muscular portions, was present in the outgroup and in the species of Clade A, *N. senticosum* and *R. rumai*. Most likely, this esophageal morphology represents an ancestral state among philometrids, kept in basal lineages and differentiating into a muscular esophagus with anterior bulbous expansion, well-developed gland and ventriculus, which is very common in the Philometridae. Some terminal lineages have developed specializations in the esophageal bulb as, for example, in *C. americanum* and *Ph. nattereri*; however, it is not clear at the moment whether or not these specializations evolved independently. It should be mentioned that a multinucleate esophageal gland is not common among philometrids and was present only in the basal species *A. amazonicum* (component of Clade A). However, it is not possible to determine if this state is basal or not, because it has been described in other philometrids (see [57]) that have no genetic characterization. The esophageal morphology of nematode parasites has been important for their systematics [58] and is also crucial to their establishment, since efficient nutrition guarantees an adequate energetic supply [59]. Therefore, the importance of the phylogenetic information associated with the esophagus structure in the Philometridae was an expected result.

A sclerotized peribuccal ring of teeth and functional vulva were exclusive states of *A. amazonicum*, consequently showing high phylogenetic information. However, similar peribuccal structures are observed in *Dentiphilometra* Moravec and Wang, 2002 [60], a genus apparently not monophyletic [4,14,17]. Therefore, we can consider such results biased by the database composition. On the other hand, the presence of a functional vulva is exclusive to the monotypic genus *Alinema* Rasheed, 1963 and may represent an ancestral state that was retained or an evolutionary reversion, since it is present in Skrjabillanidae Shigin and Shigina, 1958 and Daniconematidae Moravec and Køie, 1987 (both Dracunculoidea) [2,58], both basal families in relation to Philometridae [13–15].

The difficulty of finding philometrid males represents a great taxonomic challenge, since these specimens have important diagnostic features [1,4]. The present results corroborate this assertion, in which characteristics present in the posterior end of males showed relevant phylogenetic information. Unfortunately, the males of 15 parasite species out of the 39 included in this work are still unknown. However, since the algorithm used for phylogenetic reconstruction does not attribute weight to unknown data, interesting

patterns could be observed associated with masculine morphological characteristics. All philometrid males had a rounded posterior end and gubernaculum present, indicating the constancy of these features in the Philometridae. A pair of caudal papillae located far anterior to the cloacal opening and the absence of a caudal mound, were present only in *A. amazonicum*, representing basal states. Males of the species *N. senticosum* and *R. rumai*, which form a sister clade to *A. amazonicum*, are unknown. However, it is worth noting that males of *Ps. acreanensis* show similar characters as those mentioned for *A. amazonicum* [14], and these species have formed strongly supported assemblages in previous phylogenetic reconstructions [4,14,17].

The mapping of states related to the male caudal mound showed interesting patterns. The present results indicated that an undivided caudal mound could be an ancestral state, retained in some lineages (e.g., in group 1 of Clade D) or representing an evolutionary reversion, as in *Ph. cyprinirutili* (Creplin, 1825) and *Ph. rischta*. From an undivided state, the caudal mound may have separated into two lobes, as observed in several species of Clade C and in *Ph. floridensis* of Clade D. Following a bilobed state, each lobe may have been subdivided (forming four lobes), as in some terminal lineages of Clade C (e.g., *Ph. lateolabracis*, *Ph. sciaenae* Yamaguti, 1941, *Ph. nemipteri* Luo, 2001 and *Ph. madai* Quiazon, Yoshinaga and Ogawa, 2008). These results suggest the possible independent evolution of the caudal mound structure during different points and times of the evolutionary history of Philometridae, possibly resulting in evolutionary convergence, for example, regarding some components of Clade C and *Ph. gymnosardae* of Clade D (all with four lobes in the caudal mound). Such evolutionary convergence may generate low CI values, as observed for structures of the tail in males.

The gubernaculum morphology had high phylogenetic information, in which the states of this character were similar among males from the same major clade. A harpoon-shaped gubernaculum (with a distal barb) seems to be ancestral in Philometridae, since it appears in *A. amazonicum* (also in *Ph. acreanensis*; see [14]) of Clade A, being retained in Clade B. The gubernaculum may have lost dorsal barbs and developed lamellae-like structures at the distal end, as in species of Clade C, or simply have lost the dorsal barbs without developing ornamentations, as in the terminal lineages of Clade D, namely, *C. americanum*, *Ph. brevispicula* and *Ph. diplectri*. The masculine reproductive characteristics have also been crucial for the systematics of nematode parasites and are functionally important for efficient breeding [58,59]. Therefore, similar to the observations for esophageal morphology, good phylogenetic information is expected from masculine reproductive characters, as shown by the present results.

The site of infection, here defined according to the location of gravid females in the fish host, because the males of several species are unknown, is assumed as an important driver of the speciation process in Philometridae [1,4]. In fact, phylogenetic reconstructions (including the present) have shown species grouping according to the site of infection, for example, in Clade C and in group 1 of Clade D [4,14,15,17]. With an integrative taxonomic perspective, it was confirmed and close relatedness was observed among parasite species infecting the body cavity, subcutaneous tissues, gonads and head tissues of fishes. Based on the present results, it is possible to assume that parasitism in Philometridae may have started in the host body cavity and moved to the subcutaneous tissues, once these states appear in the most basal group (Clade A) and in Clade B. These sites of infection are indeed very common for gravid philometrid females. From the body cavity of fishes, parasites may have infected the gonads (mainly ovary) or the external tissues of the digestive system, as observed in Clade C, or moved through the subcutaneous tissues of the body to the head tissues, as in assemblages of Clade D (groups 1 and 2). The circulatory system, body muscles and fins seem to be more derived states of the site of infection of gravid females, since they only appear in terminal lineages. *Nilonema senticosum* was the only parasite of the swim bladder in the present analysis; in addition, the species also infects the body cavity, which reinforces the previous argument that these parasites have moved from the body cavity to adjacent tissues. It is important to note that such theories are preliminary,

yet the site of infection of gravid females is a strong driver of the evolutionary process in Philometridae. Moreover, there was phylogenetic logic in the states of this character starting in the body cavity, passing through the subcutaneous tissues and gonads, and ending in the head tissues of the fish.

Equal CI values were observed for the site of infection of gravid females and host taxa. These taxa were defined at the order level, since families are very diverse and would overshadow the phylogenetic information. One of the most complicated factors in analyzing the host taxa here is the ever-changing classification of bony fishes, especially that of Perciformes (see [41,53,61]). Based on this difficulty, the terms "Eupercaria" and "closely related to Perciformes" were adopted following the major phylogenetic reconstructions of these vertebrates [53,61] in order to evidence possible phylogenetic information. Clade B was the only major clade in which all species parasitize the same host taxon (Cypriniformes). Clade C was mostly formed by parasites of Eupercaria (sensu [53]), with a tendency for several sister lineages to parasitize hosts of the same taxon, although the inner nodes generally had weak support. The only assemblage in Clade D formed by parasites of fish from the same taxon was group 1, but also with low support. These results do not allow further conclusions than that the host taxon is important in the speciation process of philometrids, but the patterns are not fully clear [4,14,15,17]. In order to move further and following the recommendation by Barton et al. [4], a host–parasite cophylogenetic analysis was performed and is discussed in the following text.

In this discussion, we choose to emphasize the morphological and life history traits with major importance in the phylogeny of Philometridae. It does not mean that the other features are not useful for the taxonomy of the group. The characters discussed as follows seem to be more relevant for species diagnosis; however, here they will be approached from the perspective of phylogenetic information for Philometridae.

The presence of cephalic outgrowths was considered here as in the diagnosis of the genus *Rumai* [40]. This feature also occurs in the genus *Dentirumai* Quiazon and Moravec, 2013 [1], which has no genetic characterization. Conspicuous cephalic projections, but with somewhat different morphology, are also present in *D. marinus* (Moravec and de Buron, 2009) and *Ph. rischta* [1,9,62]. A similar situation is related to the ornamentations on the cuticular surface, which are important for the diagnosis of *Philometroides*, but are also present in *Nilonema* and *Alinema*, exhibiting varied forms [1,37]. These characters, along with esophageal teeth, protrusions on the female tail and the relative size of spicules were highly homoplastic, appearing in a random pattern among the terminal lineages of Philometridae. These results may indicate the independent evolution of these characters, occurring in different times of the evolutionary history. It should be mentioned that during the course of the present work, the number and arrangement of caudal papillae in males were added as characters to the dataset, but were deleted based on the almost complete absence of phylogenetic information. Finally, the characters that included cephalic papillae, also homoplastic and randomly exhibited by terminal lineages, were hard to define with accuracy, since these structures are complex and very small, requiring detailed observations using SEM [1]. Therefore, a discussion regarding such characters would be premature and speculative.

The host–parasite cophylogenetic signal was better when a non-superimposed model was used [46]. This means that parasite evolution is not strictly shaped by their hosts, but some lineages of hosts and their parasites may be evolving together [46]. Another explanation is that the present host–parasite interaction net is a reflection of stochastic host shifting due to environmental stress that occurred in the past evolutionary time [63,64], in some lineages of the Philometridae. Regardless, the existence of cophylogenetic signal between philometrids and their fish hosts was proved with statistical tests. The host–parasite links that most contributed to the overall cophylogenetic signal were present in Clade B and in *N. senticosum* and *R. rumai* of Clade A (all freshwater species). In addition, there was significant cophylogenetic signal between one species from the Afro-Tropical region (Clade D) and its freshwater carangarian host. Geographic isolation is a key factor for al-

lopatric speciation [63–67], especially in freshwater environments where spatial restriction is higher than in oceans. Therefore, the probability of host–parasite co-evolution is higher in the groups formed by freshwater species, such as Clade B and Neotropical Clade A. Cophylogentic signals were also observed between marine Carangiformes/Carangaria fish and other philometrids of Clade D, as well as between one parasite of Clade C and its marine mugiliform host. These seemingly random patterns of cophylogenetic signal observed in marine species could be related to the greater vagility and sympatry of marine fish in comparison with those from freshwater. For example, in Clade C, between *Pomatomus saltatrix* (Linnaeus, 1766) (Scombriformes: Pomatomidae), which has a circumglobal distribution, and its parasite *Ph. saltatrix* Ramachandran, 1973, the cophylogenetic signal was very weak [41]. It should be mentioned that the links between freshwater catfishes from the Neotropical and Afro-Tropical regions and their philometrid parasites (Clade A and D) there was no cophylogenetic signal. This finding may be indicative of more recent host-capture events, which may have occurred after the continental drift. Another possible indication of a host-capture event is the link between the most basal lineage in the fish cladogram, *Perccottus glenii* Dybowski, 1877, and the philometrid *Ps. moraveci*, which is a derived lineage in the parasite cladogram. It must be considered that the current lack of genetic data on philometrid nematodes is hiding other host–parasite cophylogenetic signals. However, it is possible to assume that in the Philometridae, the speciation process is not singular or linear, but may be occurring simultaneously in different lineages, places and times, as a consequence of host capture, co-evolution and allopatric events.

5. Conclusions

New sequences of 28S were provided for the first time for *B. ornata*, *Ph. australiensis*, *Ph. johnii* and *Ph. rubra*, and the finding that phylogenetic patterns using this genetic marker are similar to those observed in 18S phylogenies, represents an important advance for further genetic studies on Philometridae. Here, the major clades in the family were similar to those defined by Barton et al. [4], in which Clade A seems to be the most basal. The integrative analysis improved the phylogenetic resolution in comparison with the one generated from genetic data, which reinforces the importance of integrative approaches. In this sense, similar states of different characters were clearly shared by lineages within the major clades, especially in Clades A, B and C. Clade D showed the greatest randomness of characters and states among its assemblages, possibly lowering the CI values of some characters and reducing their phylogenetic information. Therefore, the interpretation of CI must be cautious. The phylogenetic resolution in Clade D is largely unresolved, although the freshwater species from the Afro-Tropical region seem to form a consistent group. In fact, most parasite species from closely related freshwater hosts formed well-supported assemblages, which was confirmed with the cophylogenetic analysis. The host–parasite links in Clades A and B that are exclusive of freshwater showed the strongest cophylogenetic signals. Most assemblages (well-supported or not) shared similar geographic origins, especially those from freshwater, where geographic isolation is more restrictive. As expected, the site of infection of gravid females showed good phylogenetic information in the Philometridae; these parasites may have moved from the body cavity to other tissues. The structure of the esophagus, the tail of males and the gubernaculum were also congruent with several phylogenetic assemblages. However, the evolution of male caudal morphology could not be elucidated yet due to the scarcity of both morphological and genetic data. The importance of male morphology is then highlighted in the systematics of Philometridae. The speciation processes in the Philometridae are probably diverse, in which host capture, host–parasite co-evolution and allopatric (especially in freshwater) events most likely are occurring simultaneously in different lineages, places and times. Cases of plesiomorphy retention probably occur in Philometridae. Similarly, the evolutionary convergence of poorly-informative characters was suggestive; these convergent characters are important for specific diagnosis. Finally, authors need to be very careful when dealing with the GenBank database, since there are several taxonomic misidentifications of sequences. The present results represent

an important advance towards a better understanding of the highly diverse and relevant family Philometridae, serving as a starting point for discussions regarding the evolution, morphology and life history of these parasites in an integrative perspective.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/d15060763/s1, Supplement S1: Polymerase chain reaction (PCR) conditions and primers used to amplify the domains D2 and D3 of the nuclear 28S rRNA gene in the present study; Supplement S2: Details of the definition of morphological/life history characters and states used in the present study; Table S1: Species of fish hosts used in the cophylogenetic analysis associated with their order (or misc), family and GenBank accession numbers; Table S2: Correspondence between abbreviations and species names from fish hosts and philometrid parasites used in the cophylogenetic analysis (see Figure 4A of the manuscript) [68–77].

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