



Anisakis infections in lantern fish (Myctophidae) from the Arabian Sea: A dual role for lantern fish in the life cycle of *Anisakis brevispiculata*?

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ABSTRACT

Data on geographic distribution and life cycles of parasitic nematodes of the genus *Anisakis* in tropical oceanic regions are extremely scarce. Myctophids (lantern fishes) are a key component of mesopelagic trophic webs and could play a significant role in the transmission of *Anisakis* spp. in these regions. We examined *Anisakis* infections in a sample of 160 myctophids collected in three localities from the Indian Exclusive Economic Zone (Arabian Sea) in 2015–16. Due to the poor condition of fish, a combination of morphological data and molecular bar-coding was used to identify myctophids. Of the 104 specimens that could be identified at least to genus level, 75 (72.1%) were assigned to *Diaphus* spp. A total of 131 *Anisakis* larvae were collected from the fish sample (prevalence: 52.5%). One larva was identified as type I, 114 as type II, and 16 larvae could not be assigned to either type. The morphology of all type II larvae conformed to the recent diagnosis of *Anisakis brevispiculata*. DNA could be obtained only from four larvae, and phylogenetic analyses based on mtDNAcox2 confirmed the identification. *Anisakis brevispiculata* is specific to dwarf and pygmy sperm whales (Kogiidae), which feed on mesopelagic cephalopods and marginally on myctophids. Therefore, myctophids could play a dual role in the transmission of *A. brevispiculata*, as direct prey of kogiids and/or as prey of cephalopods that consume myctophids. The prevalence of *A. brevispiculata* varied significantly in myctophid samples that were just ~ 265 km apart, suggesting potential local effects on transmission rates.

1. Introduction

Parasitic nematodes of the genus *Anisakis* are ubiquitous in all oceans (Mattiucci and Nascetti, 2008; Klimpel et al., 2008; Klimpel and Palm, 2011). Their life cycle relies on trophic transmission and involves crustaceans as first intermediate hosts, fish and cephalopods as paratenic hosts, and marine mammals, particularly cetaceans, as definitive hosts (Klimpel et al., 2004; Mattiucci and Nascetti, 2008). To date, nine species, divided into two clades, have been identified based on a combination of morphological and molecular methods. The first clade includes six species typically infecting ziphiids (*A. ziphidarum*, *A. nascetti*), delphinids (*A. typica*), or a wide array of delphinoid odontocetes and mysticetes (species of the *A. simplex* complex, i.e. *A. simplex* s. s., *A. berlandi*, and *A. pegreffii*); the second clade (species of the *A. physeteris* complex) includes three species specific to the sperm whale, *Physeter macrocephalus* (i.e., *A. physeteris*) or dwarf and pigmy sperm whales (*A. brevispiculata* and *A. paggiae*) (Mattiucci and Nascetti, 2008; Klimpel

and Palm, 2011; Mattiucci et al., 2014a). Larvae of species from the first clade (type I larvae) can be distinguished from those of the second clade (type II larvae) based on morphological features, i.e., long vs. short ventriculus, and presence vs. absence of mucron at the tip of the tail (Berland, 1961; Mattiucci and Nascetti, 2008).

Previous investigation on the geographical distribution, life cycle, and ecology of *Anisakis* spp. has largely been driven by two factors. First, our knowledge on geographic distribution of *Anisakis* spp. is biased by the availability of research resources, with most records being concentrated in the North Atlantic and North Pacific oceans, and the Mediterranean Sea (see Kuhn et al., 2016). Conversely, there are just a handful of records of *Anisakis* spp. in the Indian Ocean, and all concern larvae infecting fishes (Mattiucci et al., 2002; Palm et al., 2017, and references therein). Second, there is a better understanding of the ecology and life cycles of *Anisakis* spp. infecting fish and cephalopod species of commercial value, which typically dwell in neritic areas (see Mattiucci and Nascetti, 2008; Klimpel et al., 2008; Klimpel and Palm,

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2011). In contrast, knowledge on *Anisakis* spp. inhabiting the deep-sea is far more limited (Klimpel et al., 2011) and, to our knowledge, only Klimpel et al. (2004) have reported on the complete life cycle of an species of *Anisakis* in the oceanic realm: in the Norwegian Deep, *A. simplex* (s.s.) uses a copepod (*Paraeuchaeta norvegica*) as first intermediate host, a mesopelagic fish (*Maurolicus muelleri*) as second intermediate (transport) host, and the harbor porpoise, *Phocoena phocoena*, as main definitive host.

Myctophids, or lanternfish, are mesopelagic fish that account for a high abundance and biomass in the continental slope and oceanic waters, with most species performing daily vertical migrations to feed on zooplankton (Catul et al., 2011; Olivar et al., 2012). Therefore, they play a critical role in the oceanic trophic webs by linking the zooplankton to top predators, such as large cephalopods, fish and cetaceans (Olivar et al., 2012). Accordingly, myctophids would be expected to play also an equally important role in the life cycle of oceanic species of *Anisakis* (Klimpel et al., 2010b). Unfortunately, evidence for this is very limited and concerns only three *Anisakis* species. Klimpel et al. (2010b) identified a possible key role of two myctophid species, namely, *Myctophum punctatum* and *Notoscopelus kroyeri*, in the transmission of *A. simplex* (s.s.) to piscivorous cetaceans in the Mid-Atlantic Ridge. Likewise, Mateu et al. (2015) identified a significant ecological function of *Notoscopelus elongatus* and *Ceratoscopelus maderensis* in the transmission of *A. pegreffii* and *A. physeteris* to piscivorous and teuthophagous cetaceans, respectively, in the western Mediterranean.

In this study we examined samples of myctophids collected from several localities from the Arabian Sea. Our goal was two-fold, i.e., to fill the enormous gap in the biogeography of *Anisakis* spp. in the Indian Ocean, and to shed light on the role of myctophids in the life cycle of *Anisakis* spp. in oceanic waters.

2. Materials and methods

2.1. Sample collection

Samples of myctophids were collected during 2015 and 2016 in three sampling stations of the Arabian Sea (Indian Ocean) by the Fishery Oceanographic Research Vessel *Sagar Sampada*, CMLRE, Ministry of Earth sciences, India, using a cosmos Krill trawl mid-water net (length: 49.5 m; codal end mesh size 10 mm) (Fig. 1, Table 1). The depth of collection varied between samples 1 vs. 2–3 likely because of vertical migration (epipelagic occurrence at night, mesopelagic at daytime). Sampling was opportunistic, i.e., it was not specifically devised to perform parasitological analysis, but to carry out censuses of mesopelagic resources. Therefore, fish samples were fixed and preserved depending on available resources in each trip. Fish from samples 1 and 2 were fixed and preserved on board in 70% ethanol, and those from sample 3 in 5% formalin. Once in the laboratory, fishes were measured (total length, TL) and individually preserved in 70% ethanol. Specimens were generally in poor state of preservation and, therefore, TL could only be obtained from 26 specimens of *Diaphus* spp. from sample 3 (see below).

Identification of myctophids was attempted based on morphological traits, i.e. photophore position (Hulley, 1984; Nafpaktitis, 1984; Hulley and Paxton, 2016) and/or otolith morphology (Gago, 1993; Rivaton and Bourret, 1999; Schwarzhans, 2013). A sample of muscle was also collected and preserved in 100% ethanol for molecular identification. Then, the gills, gonads, stomach and intestine, liver, kidney, heart, brain, and epiaxial and hypoaxial muscles of each fish were placed in 9% saline, torn apart and examined under a stereomicroscope (20–100×). When larvae of *Anisakis* were found, a small piece of tissue was taken for each worm and preserved in 100% ethanol for molecular identification. Specimens were then submerged in glycerol for 15 min and morphologically identified as type I or type II larvae based on the length of ventriculus and the presence vs. absence of mucron at the tip of the tail (Berland, 1961; Mattiucci and Nascetti, 2008). Specimens

identified as type II larvae were further assigned to morphological subtypes based on the criteria given by Murata et al. (2011) (Table 2). These authors were apparently able to determine the specific identity of type II larvae using slight differences in the morphology of the caudal end (Table 2). To avoid potential confusion, subtypes of Type II larvae are coded with Arabic numerals.

2.2. DNA extraction, amplification and sequencing

Molecular analysis was carried out in order to ascertain/confirm the identity of myctophids and *Anisakis* larvae. Genomic DNA from myctophids and *Anisakis* larvae was extracted using the *DNeasy Blood and Tissue* (QIAGEN®) kit. The mitochondrial cytochrome oxidase subunit I (mtCOI) of myctophids was amplified using primers FishR2_T1 [5'-TGTAACGACGCGCCAGTCGACTAATCATAAAGATATCGGCAC-3'] and VF2_T1 [5'-TGTAACGACGCGCCAGTCAACCAACCACAAAGACAT TGGCAC-3'] (Ward et al., 2005). PCR was carried out by an initial denaturation at 94 °C for 7 min, followed by 35 cycles (denaturation at 94 °C for 45 s, chain alignment at 55 °C for 55 s and chain elongation at 72 °C for 1 min) and a final elongation process at 72 °C for 7 min (Pappalardo et al., 2015).

In the case of *Anisakis* larvae, we amplified the mitochondrial cytochrome oxidase subunit II (mtDNAcox2) and the small subunit rRNA (18 s rRNA). For mtDNAcox2, we used primers 210 R [5'-CACCAACTC TTAAATTATC-3'] and 211 F [5'-TTTCTAGTTATATAGATTGTTT-YAT-3'], and a thermocycling profile starting with a denaturation process at 94 °C during 3 min, followed by 34 cycles (94 °C denaturation for 30 s, 46 °C chain alignment for 1 min and elongation phase at 72 °C for 1 min 30 s), and a final process of elongation at 72 °C during 10 min (Valentini et al., 2006). Primers Philonema F [5'-GCCTATAATGGTGA AACCGCAAC-3'] and PhilPCRR [5'-CCGGTTCAAGCCACTGCGA TTA-3'] were used to amplify the 18 s rRNA, with the following profile: denaturation at 94 °C for 5 min, followed by 35 cycles at 94 °C for 40 s, 50 °C for 40 s and 72 °C for 2 min, finishing at 72 °C for 10 min (Černotíková et al., 2011).

Amplified products, for both myctophids and *Anisakis* larvae were purified using the NucleoSpin® and PCR Clean-up kit, and sequenced in both directions on an Applied Biosystems ABI 3730 XL automated sequencer at Macrogen Europe (The Netherlands). Sequences were manually edited and verified using BioEdit v. 7.0.5.3 (Hall, 1999) and Basic Local Alignment Search Tool (BLAST®) (Altschul et al., 1990).

2.3. Phylogenetic analyses

For the myctophid phylogenetic analysis we used all mtCOI sequences from *Diaphus* spp., *Symbolophorus* spp. and *Benthosema* spp. available from GenBank, and other 106 myctophid sequences previously used by Denton (2014) (Suppl. Table 1). Sequences from 4 species of the Neoscopelidae (*Neoscopelus microchir*, *N. porosus*, *Scopelengys tristis* and *Solivomer arenidens*) were used as outgroup according to Denton (2014). Alignment was made using the online version of MAFFT (Katoh et al., 2017), and intra and interspecific genetic distances based on the Maximum Composited Likelihood method (Tamura et al., 2004) were calculated using MEGA v. 7.0.26 (Kumar et al., 2015). Akaike's information criterion was used to select the most accurate evolutionary model for the aligned sequences using jModelTest v. 2.1.10 (Darriba et al., 2012). Phylogenetic relationships were inferred using Maximum Likelihood (ML) and Bayesian inference (BI) using the generalised time-reversible model (Tavaré, 1986) with gamma distribution rates with invariant sites (GTR+I+G). ML was carried on MEGA v. 7.0.26 and nodal support was assessed with 1000 bootstrap replicates. BI was conducted in MrBayes v. 3.2.6 (Ronquist and Huelsenbeck, 2003) using four Markov Chain Monte Carlo chains running for 10 million generations and sample frequency every 100 generations. The first 25% of each run was discarded as burn-in.

For the phylogenetic analysis of *Anisakis* species, the sequences

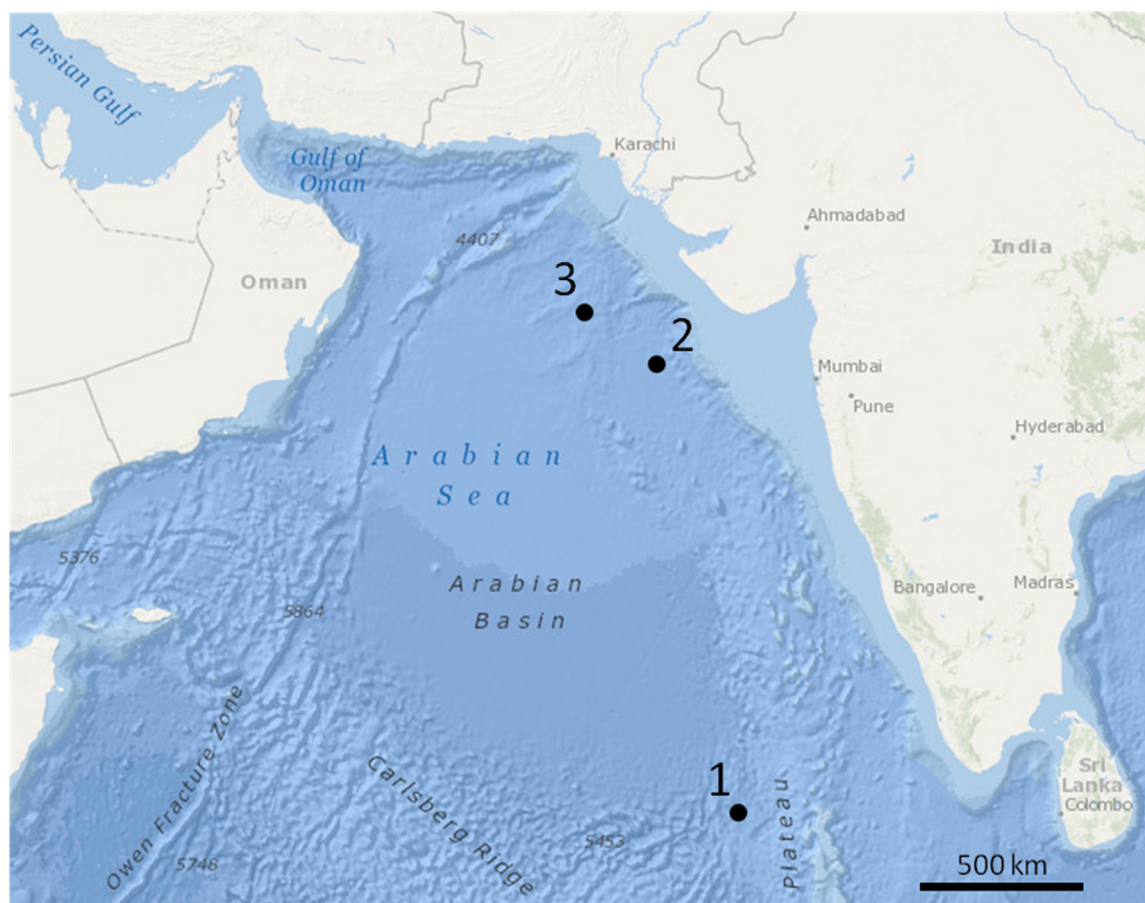


Fig. 1. Sampling localities in the Arabian Sea where three samples of myctophids were collected.

obtained in the present study were aligned with other sequences of *Anisakis* spp. available from GenBank, following previous analyses made by Di Azevedo et al. (2014, 2015), Mattiucci et al. (2014a) and Chen and Shih (2015). *Hysterothylacium aduncum* was used as outgroup according to Di Azevedo et al. (2015). ML and Bayesian phylogenetic analyses were performed using the generalised time-reversible model with gamma-distributed rates (GTR + G) according to JModelTest. ML bootstrap values were calculated after 1000 generations, and BI posterior probabilities (PP) were calculated after 1 million generations and a burn-in of 25%.

2.4. Statistical analysis

Infection parameters, including prevalence, mean abundance and mean intensity, were calculated using the online software Quantitative Parasitology (QP3.0) (<http://www.zoologia.hu/qp/qp.html>) (Reiczigel and Rózsa, 2005). The 95% confidence interval for prevalence was calculated based on Sterne's exact method, and that for mean abundance and mean intensity using bootstrap with 20,000 resamples. Fisher's exact tests were used to compare prevalence of *Anisakis* larvae

between fish samples, whereas bootstrap *t*-test and Kruskal-Wallis test were used for two-sample and multiple-sample comparisons of intensity, respectively. Spearman correlation coefficient was employed to investigate the association between fish body size and the number of *Anisakis* larvae.

Statistical significance was set at $p < 0.05$. Analyses were carried out by SPSS v.22 (SPSS Inc., Chicago, Illinois, USA).

3. Results

3.1. Host identification

Based on external appearance all specimens could be assigned to the family Myctophidae. Most specimens had lost the skin and some photophores, and their otoliths were extremely friable and could seldom be recovered intact. However, several taxa could be identified at least at genus level (Table 3): in sample 1, 33 of 44 specimens (75.0%) were identified, including *Diaphus* spp. ($n = 26$), *Benthosema* sp. (3), *Diogenichthys* spp. (2), *Myctophum obtusirostre* (1) and *Symbolophorus evermanni* (1); in sample 2, 2 of 38 specimens (5.3%), including *Diaphus*

Table 1

Date, time, no. of fish collected, depth and linear distance covered by the linear trawling transect, from initial to final coordinate positions, for the three samples myctophid fishes collected in the Arabian Sea, Indian Ocean.

Sample	Date	Time	N	Coordinates		Distance (m)	Depth range (m)
				initial	final		
1	09.09.15	9:45	43	07°45'26" N 70°50'41" E	08°05'13" N 71°52'39" E	119,592	304–600
2	23.01.16	19:11	38	20°08'40" N 68°24'24" E	20°07'12" N 68°23'21" E	3275	60–100
3	25.01.16	19:15	79	21°45'28" N 66°29'52" E	21°45'23" N 66°32'19" E	4225	65–90

Table 2

Diagnostic features to identify L3 larvae of *Anisakis* spp. Subdivision into larval types follows Berland (1969); subdivision of larva type II into subtypes follows Murata et al. (2011). Relative length refers to the ratio tail length/body length.^a

Larval type	Larval subtype	Species	Boring tooth	Ventriculus		Caudal end		
				Length	Junction with intestine	Mucron	Relative length (%)	Shape
Type I	–	<i>A. simplex</i> s.s., <i>A. simplex</i> C, <i>A. pegreffii</i> , <i>A. typica</i> , <i>A. nascetii</i> , <i>A. ziphidarum</i>	Clearly tilted	Long	Tilted	Yes	–	Rounded
Type II	2	<i>A. physeteris</i>	Parallel or slightly tilted	Short	Horizontal	No	Long (0.87)	Conical and tapering
	3	<i>A. brevispiculata</i>	Slightly tilted	Short	Horizontal	No ^b	Short (0.38)	Rounded
	4	<i>A. paggiae</i>	Slightly tilted	Short	Horizontal	No	Medium (0.59)	Conical and pointed

^a Murata et al. (2011) give the ratio body/tail length.

^b The tail of some individuals bear a tiny spine-like mucron.

Table 3

Taxa identified at least at generic level, based on morphological traits (photophores, otoliths) and/or mtCOI in the three samples of myctophids from the Arabian Sea. Note that, in sample 1, the specimens of *Diaphus* spp. identified by molecular markers were a subset of those identified by morphology and, in sample 2, *Diaphus* sp. and *Symbolophorus evermanni* were identified by morphology and then re-identified with molecular markers. No sample of DNA could be obtained from sample 3.

Sample	n	Morphology	mtCOI	No. unidentified specimens
1	43	<i>Benthosema</i> spp. (3) <i>Diaphus</i> spp. (27) <i>Myctophum obtusirostre</i> (1) <i>Symbolophorus evermanni</i> (1) <i>Diogenichthys</i> spp. (2)	<i>Diaphus</i> sp. (13)	9
2	38	<i>Diaphus</i> spp. (1) <i>Symbolophorus evermanni</i> (1)	<i>Diaphus</i> sp. (35) <i>Benthosema</i> sp. (1) <i>S. evermanni</i> (1)	1
3	79	<i>Diaphus</i> spp. (27) <i>Myctophum</i> spp. (6)	–	46

spp. (1) and *S. evermanni* (1); in sample 3, 33 of 79 (41.8%), including *Diaphus* spp. (27) and *Myctophum* spp. (6).

We attempted DNA extraction of all fish specimens, but could obtain mtCOI sequences (range: 505–687 bp-long) for 13 and 33 specimens of *Diaphus* spp. from samples 1 and 2, respectively, and *Benthosema* sp. and *S. evermanni* from sample 2 (Table 3). Sequence alignment with other myctophid species was 324 bp-long. In the case of *Diaphus* spp. average genetic divergence ($\pm$ SD) between individuals was 1.3%, ranging from 0% to 8.6% (Suppl. Table 1). Three fishes exhibited comparatively higher divergence than the remaining and, when they were excluded from calculations, average divergence ($\pm$ S.D.) dropped to 0.9 (0.5)%, with a range from 0% to 2.3% (Suppl. Table 2). Average divergence of our specimens from those available from 22 *Diaphus* spp. ranged from 8.9% to 14.1% (Suppl. Table 2).

In both the ML and Bayesian phylogenetic trees, all individuals of *Diaphus* spp. from our sample grouped together in a high-supported clade (ML bootstrap = 100% and PP = 1.0). The ML analysis could not resolve the relationships of *Diaphus* spp. with other myctophids (Suppl. Fig. 1), but the Bayesian analysis recovered a well-supported relationship with *D. schmidtii* and *D. garmani* (Suppl. Fig. 2).

The mtCOI sequence of two specimens from sample 2 were most closely related to species from the genera *Benthosema* and *Symbolophorus*; in particular, *B. fibulatum* (7.0% of average divergence) and *S. evermanni* (12.5%), respectively. The topology of the BI tree confirmed that one of our specimens clustered with sequences of *B. fibulatum*, and the other clustered with sequences of *S. evermanni* + *S.*

rufinus, forming a polytomy in both cases (Suppl. Fig. 2).

3.2. Parasitological analysis

A total of 131 *Anisakis* larvae were collected from liver and body cavity of myctophid specimens. Sixteen larvae could not be further morphologically identified. One larva was morphologically identified as type I larva and was collected from an unidentified myctophid from sample 3. The remaining larvae were identified as type II (subtype 3) larvae *sensu* Murata et al. (2011), i.e., *Anisakis brevispiculata* (Table 2). DNA extraction was attempted for all worms, but it could be obtained only for the 4 type II (subtype 3) larvae from sample 2 (GenBank accession numbers: MH669506–MH669509). The 18S rRNA sequence, identical for all specimens, was not informative. Average divergence ($\pm$ SD) in the mtDNAcox2 among specimens was 1.85% (0.73), and ranged between 0.5 and 2.7. The closest *Anisakis* species was *A. brevispiculata* (average divergence (SD): 2.21% (0.83), range: 1.4–3.2%). In both ML and BI, sequences of our specimens clustered together with sequences of *A. brevispiculata* in a high-supported monophyletic clade (ML bootstrap = 94%; PP = 1.0) (Fig. 2).

Infection parameters for type II (subtype 3) larvae per host and sample are shown in Table 4. In sample 1, there were not significant differences of prevalence (Fisher test, $p = 0.537$) and mean intensity (bootstrap t -test, $t = 0.213$, $p = 0.8328$) between *Diaphus* spp. and unidentified myctophids. Likewise, prevalence (Fisher test, $p = 0.202$) did not differ between *Diaphus* spp. and unidentified myctophids in sample 2, and all infected fish harbored just 1 worm. However, we found differences of prevalence between *Diaphus* spp., *Myctophum* spp. and unidentified myctophids in sample 3 (Fisher test, $p = 0.006$). A pairwise comparison revealed that only *Myctophum* spp. differed from unidentified myctophids (sequential Bonferroni-corrected $p = 0.01$) and the mean intensity was similar between *Diaphus* spp. and unidentified myctophids ($t = 0.483$, $p = 0.642$). The correlation between fish body length and total number of type II (subtype 3) larvae in sample 3 was not significant (Spearman correlation, $r_s = -0.228$, $n = 26$, $p = 0.263$).

The geographical comparison indicated highly significant differences of prevalence of type II (subtype 3) larvae in *Diaphus* spp. among samples (Fisher test, $p < 0.001$). The *post hoc* comparison revealed that sample 2 differed from the others (Fisher test, $p < 0.001$), which did not differ one another ($p = 1$). The comparison of prevalence between samples considering as a whole myctophid taxa other than *Diaphus* spp. indicated differences close to significance (Fisher test, $p = 0.069$), with the value for sample 2 being lower than that from the other samples (Table 4). The intensity of type II (subtype 3) larvae did not differ between samples, considering separately *Diaphus* spp. and other myctophids as a group (Kruskal-Wallis tests, $p > 0.05$).

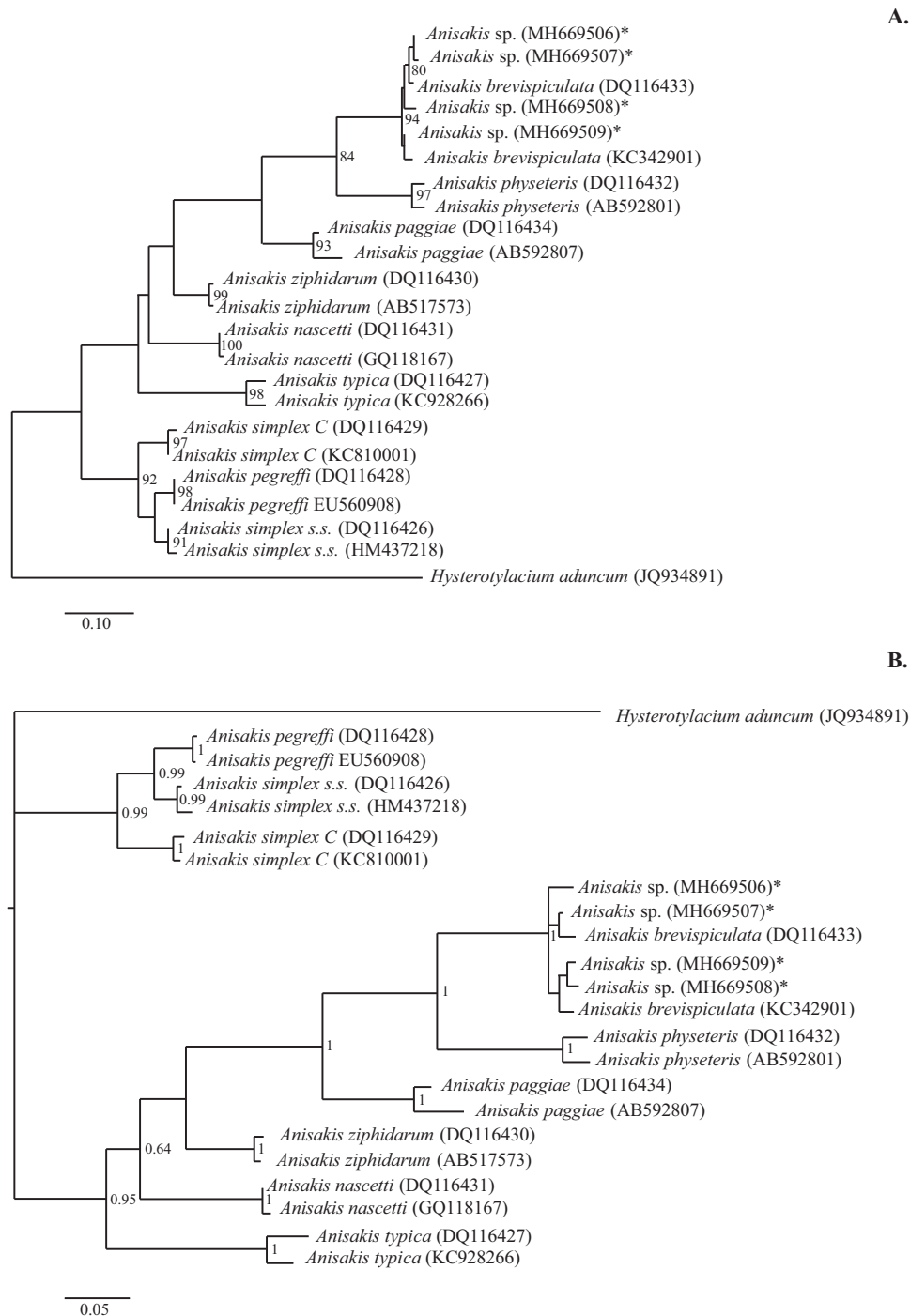


Fig. 2. Phylogenetic relationships inferred from the mtDNAcox2 sequences of species of the genus *Anisakis*, including four specimens collected in myctophids from the Arabian Sea (labelled as *Anisakis* sp. and marked with an asterisk). A. Tree inferred by Maximum Likelihood analysis. B. Tree inferred by Bayesian inference. Support values for each node are expressed as Maximum Likelihood (bootstrap $n = 1000$) and posterior probabilities; bootstrap values < 80 and posterior probabilities < 0.9 are not shown. Branch length scale bars indicate number of substitutions per site.

4. Discussion

Mesopelagic fish, including myctophids, have weak bodies that are frequently damaged during capture, often resulting in a loose of diagnostic structures, e.g., photophores (Kenchington et al., 2017). Although we managed to identify a sizeable portion (ca. 64%) of our myctophid sample to at least generic level based on morphology, only some individuals ($< 2\%$) could be identified at species level. Molecular methods (i.e. barcoding) could alleviate this problem if voucher sequences are available for comparison. Fishes from sample 3 had been

preserved in formalin and could not be analyzed by molecular methods, but we could use barcoding to shed light on the identity of myctophids from samples 1 and 2. We were particularly interested in *Diaphus* spp. because this taxon made up over 50% of our myctophid sample by number. A molecular analysis using mtCOI sequences suggests that all specimens analyzed in our sample belong to a single species. The average divergence among these specimens was similar to that from 23 identified species of *Diaphus*, and far lower than that observed between species. In addition, our specimens clustered together in a well-supported clade of the phylogenetic tree of the Myctophidae.

Table 4Infection parameters (with 95% C.I. in parentheses) of type II (subtype 3) larvae of *Anisakis* spp. in the three samples of myctophids collected from the Arabian Sea.

Host	n	n infected	Prevalence (%)	Mean intensity	Mean abundance
Sample 1					
<i>Benthosema</i> spp.	3	1	33.3	1	0.3
<i>Diaphus</i> spp.	27	15	55.6 (36.9–73.1)	1.67 (1.17–2.20)	0.93 (0.56–1.37)
<i>Myctophum obtusirostre</i>	1	0	0.0	–	–
<i>Symbolophorus evermanni</i>	1	0	0.0	–	–
<i>Diogenichthys</i> spp.	2	1	50.0	3	1.5
Myctophidae spp.	9	5	55.6 (25.1–83.1)	1.40 (1.00–1.80)	0.78 (0.22–1.44)
Sample 2					
<i>Diaphus</i> spp.	36	3	8.3 (2.3–21.9)	1	0.08 (0.00–0.17)
<i>Symbolophorus evermanni</i>	1	1	100	1	1
<i>Benthosema</i> sp.	1	0	0.0	–	–
Myctophidae spp.	1	0	0.0	–	–
Sample 3					
<i>Diaphus</i> spp.	27	16	59.3 (40.2–76.3)	1.56 (1.19–2.25)	0.93 (0.56–1.44)
<i>Myctophum</i> spp.	6	1	16.7 (0.8–58.8)	1	0.17 (0.00–0.33)
Myctophidae spp.	46	36	78.3 (64.2–88.4)	1.42 (1.19–1.69)	1.1 (0.87–1.37)

Unfortunately, the specific identity of these specimens remains undetermined. Three of the 5 *Diaphus* species that are common in the sampling area of this study, i.e., *D. fragilis*, *D. aliciae* and *D. signatus* (Karupphasamy et al., 2006, 2010) were included in the phylogenetic analysis, but our specimens did not cluster with any of them (Suppl. Table 1, Suppl. Figs. 1, 2). These drawbacks obviously impede a proper quantification of specific host-parasite associations, but we think they have little impact on the inferences about geographical distribution and life cycle that we will discuss below.

All except one larva of *Anisakis* from our sample belonged to species from the *Anisakis physeteris* species complex. Murata et al. (2011) recently established morphological criteria for discrimination between the three species of this complex (Table 2). In particular, larvae having a relatively short, rounded tail, sometimes with a tiny spine-like mucron, had Internal Transcribed Spacer (ITS) sequences nearly identical to those of *A. brevispiculata* from pygmy sperm whale, *Kogia breviceps* (only 3 base substitutions) and, in a phylogeny based on the mtDNA *cox2* gene, they clustered with *A. brevispiculata* with a 99% ML support (Murata et al., 2011). In the present study, all but one larva were found to be morphologically identical to type II (subtype 3), with 11.0% of them having a spine-like mucron. In addition, the 4 larvae for which mtCOX2 sequences could be obtained were identified as *A. brevispiculata*, thus lending support to Murata et al.'s (2011) diagnostic criteria. Accordingly, there is the possibility that all the larvae of our sample from the *Anisakis physeteris* species complex are *A. brevispiculata*. The

present study reports this species for the first time in the Indian Ocean (Table 5).

The definitive hosts for *A. brevispiculata* are kogiid cetaceans, namely, the dwarf sperm whale, *Kogia sima*, and the pigmy sperm whale *Kogia breviceps* (Table 5). Both species occur in tropical and temperate waters from the Atlantic, Pacific and Indian Oceans (Mcalpine, 2009), favoring waters along the continental shelf break and over the continental slope (Bloodworth and Odell, 2008), with *K. breviceps* apparently spreading also out into oceanic waters (Wang et al., 2002). Although data on dwarf and pigmy sperm whales in the Indian Ocean are scarce, the two species have been reported in the Arabian Sea: *Kogia sima* seems to be abundant near the Maldives (Ballance et al., 1998; Anderson, 2005) and Sri Lanka (Ballance and Pitman, 1998), *K. breviceps* in waters of Gujarat (Singh, 2003), and both species in the Indian Exclusive Economic Zone (Afsal et al., 2008). Moreover, predictive maps of occurrence derived from the online tool AquaMaps (Kaschner et al., 2016) indicate that the study area is a 'hotspot' for *Kogia* spp. Remarkably, the geographic distribution of definitive hosts was recently found to be a key factor in predictive models for the distribution of *Anisakis* spp. at a global scale (Kuhn et al., 2016). These models provide reasonably good predictions for the probability of occurrence of *A. brevispiculata* in the study area, such as we have now confirmed.

Reports of larvae of *A. brevispiculata* grossly reflect the geographic and ecological distribution of its definitive hosts (see Kuhn et al., 2011, 2016), with records in at least 12 fish species from the mesopelagic

Table 5Definitive and intermediate hosts of *A. brevispiculata* reported in the literature. Data on habitat and bathymetric distribution (in parentheses) of hosts are also shown.

Host	Habitat	Locality	Ref.
Definitive			
<i>K. breviceps</i>	Shelf break and slope, oceanic (epi- and mesopelagic)	Florida, South Africa, NE Atlantic, Philippines, Brazil	1, 6, 12, 13
<i>K. sima</i>	Shelf and slope (epi- and mesopelagic)	Philippines, Brazil, Australia	7, 13, 14
Intermediate			
<i>Pinguipes brasilianus</i>	Coastal (demersal)	Brazil	2
<i>Scomber australasicus</i>	Coastal, oceanic (epipelagic)	Taiwan	2,8
<i>Eptatretus burgeri</i>	Shelf (benthic)	Taiwan	11
<i>Gadus chalcogrammus</i>	Shelf and slope (benthic-pelagic)	Japan	4
<i>Beryx splendens</i>	Shelf and slope (benthic-pelagic)	Japan	6
<i>Merluccius merluccius</i>	Shelf and slope (demersal)	East Atlantic, Mediterranean	1
<i>Hoplostethus cadonati</i>	Slope (benthic-pelagic)	Mauritius	2
<i>Xiphias gladius</i>	Oceanic, coastal (epipelagic)	NW, E and Central Atlantic	1,9
<i>Aphanopus carbo</i>	Oceanic (Bathypelagic [adult]; mesopelagic [juvenile])	E Atlantic	1
<i>Polyipnus clarus</i>	Oceanic (mesopelagic)	Gulf of Mexico	3,10
<i>Pollichthys mauli</i>	Oceanic (mesopelagic)	Gulf of Mexico	10
<i>Argyropelecus aculeatus</i>	Oceanic (mesopelagic)	Gulf of Mexico	10

1. Mattiucci et al. (2001); 2. Kuhn et al. (2013); 3. Sardella (2016); 4. Quiazon et al. (2009); 5. Chen and Shih (2015); 6. Murata et al. (2011); 7. Quiazon et al. (2013); 8. Chen et al. (2014); 9. Mattiucci et al. (2014b); 10. Andres et al. (2016); 11. Luo et al. (2016); 12. Quiazon (2016); 13. Di Azevedo et al. (2017); 14. Shamsi et al. (2012).

realm (Table 5). The present study reports infections in myctophids for the first time. The role of these fish in the life cycle of *A. brevispiculata* is interesting because pygmy and dwarf sperm whales are mostly teuthophagous. In all geographical regions surveyed to date, the diet of both species is made up mostly by mesopelagic cephalopods (~90% by number) (e.g., Plön, 2004; Beatson, 2007; West et al., 2009). However, a modest amount of fish (especially myctophids and other mesopelagic fish), have also been found in the stomach contents. Thus, there is the possibility that myctophids can directly infect dwarf and pigmy sperm whales, and/or help bridging the trophic gap in the life-cycle of *A. brevispiculata* by being prey of cephalopods that are consumed by these cetaceans. Interestingly, a similar dual role was proposed for myctophids in the case of *A. physeteris*, which infects the sperm whale, *Physeter macrocephalus* (Mateu et al., 2015). The indirect role of myctophids in the life cycle of *A. brevispiculata* is supported by two observations. First, cephalopods act as intermediate hosts for many *Anisakis* spp., including species from the Clade II (Pardo-Gandarillas et al., 2009; Culurgioni et al., 2010; Angelucci et al., 2011; Ibáñez et al., 2015). Second, some of the cephalopod species that are frequently found in the diet of both *K. breviceps* and *K. sima*, i.e. *Histioteuthis* spp., *Taonius* spp. (Plön, 2004; Santos et al., 2006; Beatson, 2007; Elwen et al., 2013; Staudinger et al., 2014 and references therein), are known to feed mostly on myctophids (Quetglas et al., 2010).

At a small geographic scale, we found significant heterogeneity of prevalence of *A. brevispiculata* between myctophid samples; interestingly, the highest difference was found between the closest localities (samples 2 and 3, ~ 265 km apart), whereas infection levels were similar between samples that were far apart (1 and 3, ~ 1500 km) (Fig. 1). Two non-exclusive factors could account for this pattern. First, species composition may differ among myctophid samples; just a few specimens could be identified at species level, and there were significant differences of prevalence between myctophid groups in one of the samples. Thus, we cannot rule out that infection levels of *A. brevispiculata* may differ between myctophid species, such as it has been observed for other *Anisakis* spp. in Mediterranean communities of myctophids (Mateu et al., 2015). However, at least part of the variability in prevalence could be spatial in nature. Samples 1 and 2 were collected just a few months apart and had a roughly similar composition of myctophid taxa (Table 2), yet the prevalence of *A. brevispiculata* was significantly different. These putative geographic differences in infection levels in localities are intriguing due to the lack of obvious physical barriers in mesopelagic communities (see Mateu et al., 2015).

In two of the three samples collected, the prevalence of *A. brevispiculata* was moderately high (> 50%) compared with those from *Anisakis* spp. hitherto reported in myctophids, which ranged from 1.4% to 22.7% (Rohde, 2008; Rokicki et al., 2009; Klimpel et al., 2010a; Mateu et al., 2015). There is an instructive exception, however: in the Mid-Atlantic Ridge (MAR), Klimpel et al. (2008) found 100% prevalence for *Anisakis simplex* (s.s) in *Myctophum punctatum* and *Notoscopelus kroyeri* (see also Klimpel et al., 2010b). These authors noted that the MAR is highly productive due to upwelling phenomena, sustaining dense populations of mesopelagic fishes and cetaceans. This scenario should increase the local population size of hosts, thus the likelihood of transmission, of *Anisakis* spp. It would thus be interesting to explore, in our study area, the hypothesis that local phenomena may affect the distribution of *Kogia* spp., or impact key abiotic factors (e.g., temperature, depth, salinity, or primary production), which that are known to influence the occurrence of *Anisakis* spp. (Kuhn et al., 2016).

5. Conclusions

In conclusion, this study reports for the first time *A. brevispiculata* in myctophids, and in the Indian Ocean. The finding of *A. brevispiculata* conforms to predictions from global models for this species. In addition, the prevalence of this species was comparatively high (> 50%) in some myctophid samples, suggesting that local phenomena, perhaps the local

density of definitive hosts, may boost the transmission of the parasite. Finally, our data suggest that myctophids could play a dual role as intermediate hosts in the life cycle of *A. brevispiculata*, bridging, directly or indirectly, the trophic gap between first intermediate hosts and dwarf and pygmy sperm whales, such as it has been suggested for the allied species *A. physeteris*.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.dsr.2018.08.004.

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Supplemental Table 1. GenBank accession numbers for the mtCOI sequences used for the phylogenetic analysis of the family Myctophidae in the present study. Four species of Neoscopelidae were used as outgroup.

Family	Species	Accession number
Neoscopelidae	<i>Neoscopelus microchir</i>	KJ555446, KJ555447
	<i>Neoscopelus porosus</i>	KJ555448
	<i>Scopelogadus tristicus</i>	KJ555461, KJ555462
	<i>Solivomer arenidens</i>	KJ555464
Myctophidae	<i>Diaphus</i> spp. (this study)	XXXXXXXXXXXXXXXXXX
	<i>Benthosema</i> sp. (this study)	XXXXXXXXXXXXXXXXXX
	<i>Symbolophorus evermanni</i> (this study)	XXXXXXXXXXXXXXXXXX
	<i>Benthosema fibulatum</i>	KR231835, KR231834, KR231833, KR231831, KR231830, KR231829, KR231828, KR231832, KJ555324, KJ555325
	<i>Benthosema glaciale</i>	KY033572, KY033571, X008540, EU148098, EU148097, GU071748, HQ167646, KF768167, KC616369, KC616368, KC616367, KC616366, KC616365
	<i>Benthosema panamense</i>	MF956525, MF956524, MF956523, MF956522, MF956521, MF956518, MF956517, MF956520, MF956519, KJ555326
	<i>Benthosema pterotum</i>	KC442071, KY676153, KY676031, KY675856, KY675504, KU943105, KU943104, KU943102, KU943101, KJ555327
	<i>Benthosema suborbitale</i>	MF041720, MF041719, MF041682, MF041629, MF041533, MF041407, MF041393, MF041319, MF041229, MF041010, KU902902
	<i>Bolinichthys longipes</i>	KJ555328, KJ555329
	<i>Bolinichthys supralateralis</i>	KJ555330
	<i>Centrobranchus andreae</i>	KJ555331, KJ555332, KJ555333
	<i>Centrobranchus choerocephalus</i>	KJ555335
	<i>Centrobranchus nigroocellatus</i>	KJ555336
	<i>Ceratoscopelus maderensis</i>	KJ555337
	<i>Ceratoscopelus townsendi</i>	KJ555338
	<i>Ceratoscopelus warmingi</i>	KJ555339, KJ555340, KJ555341,

	KJ555342
<i>Diaphus adenomus</i>	KJ555343
<i>Diaphus aliciae</i>	KJ555344
<i>Diaphus anderseni</i>	KJ555345, KJ555346
<i>Diaphus antonbruuni</i>	KJ555347
<i>Diaphus bertelseni</i>	MF041374, EU148145,
<i>Diaphus brachycephalus</i>	MF041612, MF041398, MF041390, MF041286, MF041161, KU902931, GU071733, KJ555348
<i>Diaphus coeruleus</i>	MF123857
<i>Diaphus effulgens</i>	KF929816, EU148148, EU148147, EU148146, KJ190058, KJ555350
<i>Diaphus danae</i>	KC136590, KC136589, KC136588, KC136587, KC136586, KC136585,
<i>Diaphus dumerilii</i>	KY033610, MF041514, MF041495, MF041347, MF041263, MF041210, MF041178, MF041175, GU224895, GU224894, GU224892, KF929815, KF768169, MF041152, FJ918944,
<i>Diaphus fragilis</i>	MF041507, KU943111, MF041585, KJ555351, KJ555352
<i>Diaphus fulgens</i>	KJ555353, KP266803
<i>Diaphus garmani</i>	KU943109, KU885655, KR231846, KR231845, KR231844, KR231843, KR231842, KR231841, KJ555354
<i>Diaphus holti</i>	KJ709514, KC616390, KU902907, KC616391, KC616389, KC616388
<i>Diaphus lucidus</i>	MF041707, MF041667, MF041429, MF041238, MF041213, MF041150, MF041436, MF041345
<i>Diaphus luetkeni</i>	KU885654
<i>Diaphus lobatus</i>	KJ555355
<i>Diaphus malayanus</i>	KJ555356
<i>Diaphus meadi</i>	KJ555357
<i>Diaphus metopoclampus</i>	KJ709515, KJ709750, KJ709749, EU148151, EU148150, EU148149, HQ167648, KJ555358
<i>Diaphus mollis</i>	KY033611, MF041522, MF041481, MF041417, MF041416, MF041353, MF041276, MF041205, MF041094, MF041076, MF041028, MF040972, KJ555359, KJ555360
<i>Diaphus parri</i>	KJ555361, KJ555362, KJ555363
<i>Diaphus perspicillatus</i>	KJ555364, KJ555365
<i>Diaphus rafinesquii</i>	MF041607, KU902908, EU148154,

	EU148153, EU148152
<i>Diaphus richardsoni</i>	KC136593, KC136592, KC136591, KJ555366
<i>Diaphus schmidtii</i>	KJ555367
<i>Diaphus signatus</i>	KJ555368
<i>Diaphus splendidus</i>	KJ968048, KC136596, KC136595, KC136594, KU943106, KJ555372, KJ555373
<i>Diaphus suborbitalis</i>	KU943110
<i>Diaphus subtilis</i>	MF041662, GU071745, MF041073
<i>Diaphus theta</i>	JQ354073, FJ164561, FJ164560, FJ164559, FJ164558, FJ164557, FJ164556, KJ190057, HQ010052, KJ555374
<i>Diaphus thiollierei</i>	KR231840, KR231839, KR231838, KR231837, KR231836, KJ555375, KJ555376
<i>Diaphus watasei</i>	KC442072, KP267585, KU943107, KU885653, KR231855, KR231854, KR231853, KR231852, KR231851, KR231850, KR231849, KR231848, KR231847, KJ555377, KJ555378
<i>Diaphus whitleyi</i>	KJ555379
<i>Diogenichthys atlanticus</i>	KJ555380, KJ555381
<i>Diogenichthys laternatus</i>	KJ555383
<i>Electrona carlsbergi</i>	KJ555384
<i>Electrona paucirastra</i>	KJ555385, KJ555386
<i>Electrona risso</i>	KJ555387, KJ555388, KJ555389
<i>Gonichthys cocco</i>	KJ555390
<i>Gonichthys tenuiculus</i>	KJ555391
<i>Gymnoscopelus bolini</i>	KJ555392
<i>Gymnoscopelus braueri</i>	KJ555393
<i>Gymnoscopelus hintonoides</i>	KJ555394
<i>Gymnoscopelus nicholsi</i>	KJ555395
<i>Gymnoscopelus piabilis</i>	KJ555396
<i>Hygophum atratum</i>	KJ555397
<i>Hygophum hanseni</i>	KJ555398
<i>Hygophum hygomii</i>	KJ555399
<i>Hygophum proximum</i>	KJ555400, KJ555401
<i>Hygophum reinhardtii</i>	KJ555402
<i>Kefftichthys anderssoni</i>	KJ555403, KJ555404
<i>Lampadena atlantica</i>	KJ555407
<i>Lampadena luminosa</i>	KJ555405, KJ555406

<i>Lampadena urophaos urophaos</i>	KJ555408, KJ555409, KJ555410
<i>Lampanyctus hubbsi</i>	KJ555411
<i>Lampanyctus intricarius</i>	KJ555412
<i>Lampanyctus jordani</i>	KJ555413
<i>Lampanyctus macdonaldi</i>	KJ555414
<i>Lampanyctus pusillus</i>	KJ555415
<i>Lampanyctus turneri</i>	KJ555416
<i>Lepidophanes guentheri</i>	KJ555417, KJ555418
<i>Lobianchia dofleini</i>	KJ555419
<i>Lobianchia gemellarii</i>	KJ555420
<i>Loweina rara</i>	KJ555421
<i>Metelectrona ventralis</i>	KJ555422, KJ555423, KJ555424
<i>Myctophum affine</i>	KJ555425
<i>Myctophum asperum</i>	KJ555426
<i>Myctophum aurolaternatum</i>	KJ555427
<i>Myctophum brachygnathum</i>	KJ555428
<i>Myctophum lychnobium</i>	KJ555429
<i>Myctophum nitidulum</i>	KJ555430
<i>Myctophum obtusirostre</i>	KJ555431
<i>Myctophum orientale</i>	KJ555432
<i>Myctophum phengodes</i>	KJ555433
<i>Myctophum punctatum</i>	KJ555434
<i>Myctophum selenops</i>	KJ555435, KJ555436, KJ555437
<i>Myctophum spinosum</i>	KJ555438, KJ555439
<i>Nannobrachium achirus</i>	KJ555440
<i>Nannobrachium atrum</i>	KJ555441
<i>Nannobrachium bristori</i>	KJ555442
<i>Nannobrachium fernae</i>	KJ555443
<i>Nannobrachium lineatum</i>	KJ555444
<i>Nannobrachium regale</i>	KJ555445
<i>Notolychnus valdiviae</i>	KJ555449
<i>Notoscopelus caudispinosus</i>	KJ555450
<i>Notoscopelus resplendens</i>	KJ555451
<i>Parvilux ingens</i>	KJ555452
<i>Protomyctophum andreashevi</i>	KJ555453, KJ555454
<i>Protomyctophum bolini</i>	KJ555455, KJ555456
<i>Protomyctophum crockeri</i>	KJ555457
<i>Protomyctophum gemmatum</i>	KJ555458, KJ555459
<i>Protomyctophum thompsoni</i>	KJ555460
<i>Scopelopsis multipunctatus</i>	KJ555463
<i>Stenobrachius leucopsarus</i>	KJ555465

<i>Symbolophorus barnardi</i>	JF494617, JF494616, JF494615
<i>Symbolophorus boops</i>	JF494622, JF494621, JF494620, JF494619, JF494618
<i>Symbolophorus californiensis</i>	GU440539, KJ190072, KJ555466, KJ555467
<i>Symbolophorus evermanni</i>	EU489719, GU440540, KJ555468
<i>Symbolophorus reversus</i>	KJ555469
<i>Symbolophorus rufinus</i>	KJ555470
<i>Symbolophorus veranyi</i>	KY033768, KJ709643, KU902925, EU148340, HQ167654, KF143897, KY033769, KY033767, KY033766, KY033765, EU148339, EU148338
<i>Taaningichthys bathyphilus</i>	KJ555471
<i>Taaningichthys minimus</i>	KJ555472
<i>Tarletonbeania crenularis</i>	KJ555473, KJ555474
<i>Triphoturus mexicanus</i>	KJ555475
<i>Triphoturus nigrescens</i>	KJ555476
<i>Triphoturus oculeum</i>	KJ555477

	D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	D11	D12	D13	D14	D15	D16	D17	D18	D19	D20	D21	D22	D23
1. <i>Diaphus</i> sp.	0.013																						
2. <i>D. garmani</i>	0.101	0.043																					
3. <i>D. richardsoni</i>	0.125	0.110	0.003																				
4. <i>D. brachycephalus</i>	0.141	0.121	0.039	0.004																			
5. <i>D. mollis</i>	0.118	0.114	0.079	0.083	0.000																		
6. <i>D. theta</i>	0.120	0.128	0.114	0.127	0.094	0.050																	
7. <i>D. subtilis</i>	0.124	0.114	0.078	0.104	0.076	0.074	0.002																
8. <i>D. holti</i>	0.118	0.110	0.071	0.085	0.076	0.090	0.054	0.032															
9. <i>D. rafinesquii</i>	0.118	0.100	0.083	0.089	0.068	0.089	0.063	0.069	0.006														
10. <i>D. thiollierei</i>	0.102	0.088	0.096	0.114	0.086	0.108	0.088	0.094	0.088	0.001													
11. <i>D. fragilis</i>	0.099	0.100	0.101	0.117	0.085	0.111	0.094	0.089	0.103	0.055	0.054												
12. <i>D. lucidus</i>	0.089	0.118	0.124	0.142	0.106	0.097	0.097	0.107	0.100	0.076	0.083	0.006											
13. <i>D. danae</i>	0.104	0.102	0.108	0.108	0.093	0.110	0.098	0.103	0.097	0.079	0.081	0.091	0.005										
14. <i>D. effulgens</i>	0.106	0.106	0.128	0.140	0.111	0.103	0.116	0.112	0.102	0.077	0.083	0.083	0.080	0.024									
15. <i>D. watasei</i>	0.100	0.105	0.113	0.125	0.103	0.119	0.116	0.107	0.099	0.071	0.087	0.090	0.103	0.086	0.043								
16. <i>D. luetkeni</i>	0.117	0.123	0.116	0.139	0.109	0.116	0.106	0.099	0.107	0.092	0.083	0.085	0.111	0.097	0.093	(-)							
17. <i>D. suborbitalis</i>	0.103	0.105	0.111	0.125	0.101	0.107	0.100	0.094	0.106	0.089	0.081	0.091	0.093	0.086	0.090	0.076	(-)						
18. <i>D. splendidus</i>	0.106	0.117	0.139	0.135	0.111	0.132	0.132	0.121	0.113	0.087	0.085	0.120	0.077	0.101	0.108	0.097	0.099	0.012					
19. <i>D. bertelseni</i>	0.129	0.152	0.137	0.128	0.108	0.122	0.123	0.125	0.128	0.107	0.102	0.102	0.100	0.108	0.112	0.101	0.114	0.102	0.007				
20. <i>D. metopoclampus</i>	0.126	0.133	0.115	0.119	0.111	0.121	0.119	0.105	0.114	0.097	0.095	0.100	0.106	0.096	0.109	0.107	0.096	0.117	0.118	0.001			
21. <i>D. dumerilii</i>	0.122	0.123	0.106	0.104	0.122	0.122	0.113	0.114	0.103	0.119	0.121	0.118	0.112	0.129	0.113	0.127	0.126	0.125	0.131	0.140	0.002		
22. <i>D. coeruleus</i>	0.089	0.100	0.108	0.121	0.101	0.114	0.117	0.104	0.094	0.071	0.087	0.085	0.102	0.084	0.026	0.091	0.082	0.111	0.117	0.110	0.106	(-)	
23. <i>D. fulgens</i>	0.107	0.106	0.106	0.119	0.108	0.115	0.105	0.095	0.101	0.077	0.083	0.090	0.091	0.072	0.056	0.095	0.087	0.118	0.105	0.088	0.119	0.048	(-)

Supplemental Table 2. Estimates of Evolutionary Divergence of mtCOI over Sequence Pairs between species of *Diaphus*. The values of intraspecific divergence are in bold.

The number of base substitutions per site from averaging over all sequence pairs between groups are shown. Analyses were conducted using the Maximum Composite Likelihood model [1]. The analysis involved 170 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 344 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 [2].

1. Tamura K., Nei M., and Kumar S. (2004). Prospects for inferring very large phylogenies by using the neighbor-joining method. *Proceedings of the National Academy of Sciences (USA)* 101:11030-11035.
2. Kumar S., Stecher G., and Tamura K. (2016). MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. *Molecular Biology and Evolution* 33:1870-1874.

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