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NOTE

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# Larvae of *Clistobothrium grimaldii* (Cestoda: Phyllobothriidea) from a Cape fur seal (*Arctocephalus pusillus pusillus*) kept in a zoo in Japan

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**ABSTRACT.** The larval form of the Phyllobothriidea cestode was found in the blubber of a Cape fur seal (*Arctocephalus pusillus pusillus*) from a zoo in Japan. Bladder-bearing larval cestodes with a scolex have been occasionally reported from blubbers of pinnipeds and morphologically identified as *Clistobothrium delphini* (formerly known as *Phyllobothrium delphini*) or rarely *Clistobothrium grimaldii* (*Monorygma grimaldii*). Although the larvae here morphologically resembled *C. delphini*, the 28S rDNA sequence was 100% (1,430/1,430 bp) homologous to the registered sequence of *C. grimaldii* (GenBank Accession No. KU724058). This discrepancy between morphological and molecular analyses confirms the difficulty of identifying *C. delphini* and *C. grimaldii* larvae based solely on morphology, and the need for molecular data to elucidate the morphological variations in *Clistobothrium* parasites.

**KEYWORDS:** Cape fur seal, *Clistobothrium*, Phyllobothriidea

The order Phyllobothriidea currently includes 21 genera [5, 16], and is known to parasitize elasmobranch and holocephalan species as definitive hosts. The estimated diversity in this order is 669 species, but only approximately 20% of these have been discovered thus far [16]. Larval forms of Phyllobothriidea cestodes with a scolex and a bladder, generally referred to as merocercoids (terminology as in [7]), have been occasionally reported in the blubber of pinniped species and identified as *Phyllobothrium delphini* Bosc, 1802 or *Monorygma grimaldii* Moniez, 1889 [3, 9, 11, 13, 15, 17–19]. Recent phylogenetic analyses using 28S ribosomal RNA gene (rDNA) have invalidated the genus combination for both *P. delphini* and *M. grimaldii*, and both species have now been transferred to the genus *Clistobothrium* [1, 6, 14]. Although the great white shark (*Carcharodon carcharias*) is the most plausible definitive host for *Clistobothrium delphini* and *C. grimaldii* due to its regular consumption of large marine mammals, adult forms have not yet been found and its complete life cycle remains to be elucidated [6]. Here, we identified larval cestodes with an everted scolex and bladder from the subcutaneous adipose tissues of a >20-year-old captive Cape fur seal from a zoo in Japan. Morphological and molecular examinations of the larvae were conducted to provide additional information on the poorly understood *Clistobothrium* species.

A female Cape fur seal (*Arctocephalus pusillus pusillus*) kept at Tobu Zoo in Saitama, Japan was necropsied in January 2022. The seal was originally caught on the South African coast and brought to the Zoo in May 2000. Necropsy revealed that the cause of death was a cardiovascular complication possibly related to filarial infection. In addition to pathological findings directly related to death, large cavities containing larval cestodes within the subcutaneous adipose tissue were found and collected during necropsy. The collected larvae were washed with saline and preserved in 70% ethanol for morphological and molecular analyses. Morphological measurements were recorded for two larvae under a stereomicroscope using cellSens® Standard software (Olympus, Tokyo, Japan), according to the metrics defined by Agustí *et al*. [1].

DNA was extracted from one of the collected larva using a DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The D1-D3 region of the 28S rDNA gene was amplified using primer pairs LSU-5 (5′-TAGGTCGACCCGCTGAAYTTA-3′) [12] and LSU-1500R (5′-GCTATCCTGGAGGGAAACTTCG-3′) [20]. PCR was performed in a 50-µL reaction volume, including 1 µL of DNA template, 1.25 U of Takara Ex Taq®, 1× Ex Taq® Buffer, 0.2 mM dNTP mixture (Takara Bio Inc., Kusatsu, Japan), and 0.2 µM primers (FASMAC Co., Ltd., Atsugi, Japan). The reaction conditions included initial denaturation at 94°C for 3 min, followed by 40 cycles of denaturation at 94°C for 30 sec, annealing at 52°C for 30 sec, and extension at 72°C for 2 min, with a final extension at 72°C for 7 min [20]. The amplified products were visualized on

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1.5% agarose gel. Positive PCR products were purified using the NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's protocol and directly sequenced at a sequencing facility (FASMAC Co., Ltd.) using the same primers used for the PCR and additional primers LSU-55F (5′-AACCAGGATTCCCCTAGTAACGGC-3′) [4] and LSU-1200R (5′-GCATAGTTCACCATCTTTCGG-3′) [12]. The obtained sequences were compared with those available in GenBank (National Center for Biotechnology Information) using the nucleotide BLAST program (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The sequence obtained in this study was deposited in the DNA Data Bank of Japan (DDBJ) under accession number LC718556.

The larval cestodes collected in this study were composed of a bladder connected to a filament ending with an everted scolex (Fig. 1). An anterior apical organ surrounded by four bothridia was observed on the scolex of one larva (Fig. 2A). The apical organ on the scolex of another larva was not visible. Each bothridium consisted of a foliate margin and an accessory sucker in the middle (Fig. 2B). Morphological measurements of the larvae are presented in Table 1 along with data from previous studies [1, 9, 13, 17]. According to a key to marine cestode larval types by Jensen and Bullard [8], the present larvae were identified as Type XV, which includes species historically referred to as *Phyllobothrium delphini* Bosc, 1802, and *Monorygma grimaldii* Moniez, 1889. These two species have been reported in cetaceans and pinnipeds and are distinguished by the morphology of the scolex and the length of the filament that connects the scolex and the bladder [1]. However, as described earlier, both *P. delphini* and *M. grimaldii* have recently been transferred to the genus *Clistobothrium* [1, 6, 14]. Morphological characteristics of the larval cestodes collected in this study were similar to those of *C. delphini* with shorter filaments and foliate bothrium (Figs. 1 and 2A, Table 1), although measurements of the bothridial accessory sucker length matched that of *C. grimaldii* (Table 1).

Larval cestodes found in the blubbers of pinniped species were identified solely based on morphology until the first molecular data were reported by Klotz *et al*. [9]. Although the morphology of the larvae in their study resembled that of *C. delphini*, DNA sequences (GenBank Accession No. KU724058) identified the larvae as *C. grimaldii* [6], displaying a lack of congruence between the results of molecular and morphological analyses. Such discrepancies between molecular and morphological analyses of larvae observed in pinnipeds have not been reported in cetacean host species [1]. Therefore, the larvae of *Clistobothrium* species have been hypothesized to develop different morphologies in different intermediate hosts [6, 9, 19], suggesting that the identification of *C. delphini* and *C. grimaldii* larvae solely based on morphology is unreliable. Larval cestodes collected in this study also resembled those of *C. delphini*, but the 28S rDNA sequence was 100% (1,430/1,430 bp) identical to that of *C. grimaldii* from Klotz *et al*. [9]. Considering the possibility that *Clistobothrium* larvae obtained from different host species exhibit morphological variation, molecular data were used to identify the larvae found in this study as *C. grimaldii*.

Several morphological characteristics of the larvae in this study, including filament length and bothridial accessory sucker length, did not match completely with those of the larvae described by Klotz *et al*. [9] (Table 1). Notably, the larvae in the present study had an everted scolex and filament, whereas the majority of the larval cestodes reported from pinnipeds, including those by Klotz *et al*. [9], had an invaginated scolex and a filament, generally referred to as a merocercoid [7, 9, 11, 19]. Mendonca [13] reported both everted and invaginated larval types from the same Cape fur seal and attributed these variations to different developmental stages. Such morphological variations, whether caused by different host species or developmental stages, re-affirm the difficulty of species identification based solely on morphology, highlighting the need for additional molecular information.

The life cycle of *C. grimaldii* is still unknown, although it is speculated to cycle between crustacean species as the first intermediate hosts, pinnipeds as the second intermediate hosts, and elasmobranch species as the definitive hosts [6, 9]. The coast of South Africa is a habitat of diverse elasmobranch species and a hot spot for the great white shark population, a possible candidate for the definitive host, as it regularly consumes large marine mammals [6, 10]. Indeed, 24.5% (13/53) of Cape fur seals on the eastern Cape coast of South Africa harbored *Clistobothrium* merocercoids [18], indicating that *Clistobothrium* parasites had been prevalent in the area where the seal was caught. The larvae collected in the present study seemed to be intact, considering the years of infection, although their infectivity to the final host is unknown. As suggested by Aznar *et al*. [2], it is reasonable that *Clistobothrium* parasites have adapted to

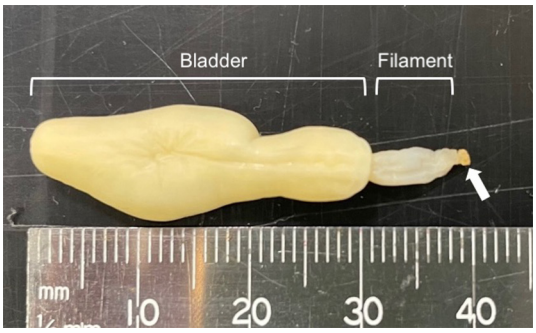


**Fig. 1.** Larval cestodes collected in this study composed of a bladder connected to a filament ending with an everted scolex (arrow).

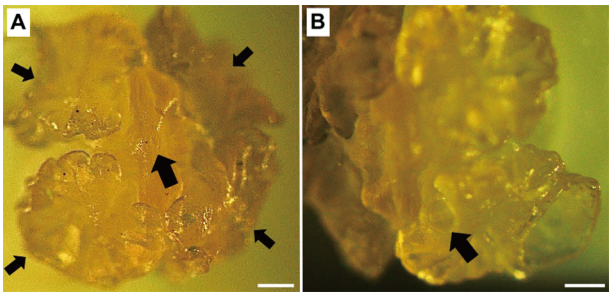


**Fig. 2.** (**A**) Reduced anterior glandular apical sucker (large arrow) surrounded by four bothridia (small arrow) is observed on the everted scolex. (**B**) Each bothridium consists of a foliate margin and an accessory sucker in the middle (arrow). Scale bars=200 µm.

**Table 1.** Comparison of morphological measurements (range for more than two samples) of the *Clistobothrium* larvae from the present and previous studies

| Host | *Arctocephalus pusillus pusillus* | *Arctocephalus pusillus pusillus* | *Arctocephalus pusillus pusillus* | *Arctocephalus australis* | *Stenella coeruleoalba*[d] | *Stenella coeruleoalba*[d] |
|---|---|---|---|---|---|---|
| Sample number | n=2 | n=2 | not reported | n=5 | n=20 | n=20 |
| Morphological identification | *delphini* | *delphini* | *delphini* | *delphini* | *delphini* | *grimaldii* |
| Molecular identification | *grimaldii* | *grimaldii* | ND | ND | *delphini* | *grimaldii* |
| Bladder length (mm) | 24.5, 31.0 | 35.0, 37.5 | 14.0–22.3 | 12.0–18.0 | 5.0–15.1 (10.3 ± 2.5) | 5.7–27.3 (13.7 ± 5.4) |
| Bladder width (mm) | 6.1, 8.9 | 7.0, 7.5 | 5.0–9.0 | 6.0–10.0 | 2.3–9.3 (5.9 ± 1.9) | 3.9–11.5 (7.7 ± 2.3) |
| Filament length (mm) | 6.9, 8.6 | 13.6, 18.2 | 5.0–12.0 | 12.0–14.0 | 1.5–12.9 (7.4 ± 2.7) | 3.0–415.7 (151.8 ± 122.9) |
| Filament width (mm) | 1.7, 3.3 | 1.5, 2.5 | 1.2–2.8 | 2.0–4.0 | 0.8–2.6 (1.6 ± 0.5) | 1.8–3.9 (2.7 ± 0.6) |
| Bothridium length (mm) | 0.6–0.7 (0.7), 0.8–1.2 (1.0)[a] | 1.5, ND | 0.9* | 1.6* | 1.2–2.1 (1.5 ± 0.2) | 3.8–6.0 (4.7 ± 0.6) |
| Bothridium width (mm) | 0.4–0.7 (0.6), 0.8–1.1 (0.9) [a] | ND | 1.0* | 1.6* | 1.0–1.9 (1.3 ± 0.2) | 2.2–4.8 (3.1 ± 0.6) |
| Accessory sucker length (μm) | 79.1–84.2 (81.7), 122.5–137.2 (129.0) [a] | 400, 350 | 277* | 133–200* | 220–325 (274 ± 29) | 107–196 (148 ± 22) |
| Accessory sucker width (μm) | 85.2–95.7 (89.9), 134.4–148.7 (142.3) [a] | 500, 400 | 321* | 242–250* | 230–360 (288 ± 33) | 133–248 (172 ± 25) |
| Diameter of the apical organ (μm) | ND[b], 87 | ND[c] | 240–400 | 120–150 | 50–170 (91 ± 30) | 53–131 (84 ± 20) |
| Reference | This study | [9] | [13] | [17] | [1] | |

[a] Range for four bothridia per scolex. Mean value in the parenthesis. [b] Apical organ not visible. [c] Although the study mentions the presence of an apical organ, description of the measurements were unavailable. [d] Mean ± Standard deviation described in the parenthesis. * Values measured by Agustí *et al*. [1] from published drawing by [13] and [17].

and taken advantage of the longevity and body size of large marine mammals as intermediate hosts, instead of fish and cephalopods, to increase the chance of development and survival.

Here, we provide both morphological and molecular data for *C. grimaldii* from a Cape fur seal caught on the coast of South Africa. Molecular data for *Clistobothrium* species from pinnipeds are especially valuable, as the only DNA sequence data available to date are those by Klotz *et al*. [9]. Furthermore, the morphological features did not completely agree with those of *C. grimaldii* reported by Klotz *et al.* [9], although the 28S rDNA sequence was 100% identical. These results suggest that morphological variations exist, even among *C. grimaldii* larvae collected from the same host species, highlighting the importance of molecular data. Thus, to gain a better understanding of the development of *Clistobothrium* parasites, further molecular analyses in relation to morphological variations need to be conducted in future studies.

CONFLICT OF INTEREST. The authors declare that there are no known conflicts of interest associated with this publication.

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