Annals of Parasitology 2021, 67(4), 723–731
doi: 10.17420/ap6704.389

Original paper

Molecular and morphological identification of *Adenocephalus pacificus* (Cestoda) isolated from South American sea lion *Otaria byronia* stranded on the northern Peruvian coasts

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ABSTRACT. The most frequent etiologic agent of diphyllobothriosis in South America and the only one confirmed by molecular data in human cases in Peru is *Adenocephalus pacificus* (syn. *Diphyllobothrium pacificum*). This cestode is transmitted by ingestion of the plerocercoids found in marine fish, causing a parasitic zoonosis. The objective of the present study was to identify two cestodes isolated from two specimens of the South American sea lion (*Otaria byronia*) stranded on the beaches of Huacho and Barranca cities, located on the northern Peruvian coasts, in the department of Lima. Tapeworms were confirmed by morphological characteristics due to the presence of transverse papilla-like tegumental protuberances in proglottids and small sized eggs, as well as by sequencing of the partial cytochrome *c* oxidase subunit 1 (mtDNA-COI) gene that are congruent with additional available *A. pacificus* sequences. Even though sea lions in Peru are distributed along the coast and in areas of difficult access, generally located in protected natural areas, the fortuitous finding represented an opportunity to confirm the presence of *A. pacificus* in South American sea lions. This report of tapeworm *A. pacificus* could allow future monitoring of the occurrence and geographical distribution of this causative agent in epidemiological studies, since it is one of the main species of zoonotic importance in Peru.

Keywords: diphyllobothriosis, tapeworm, sea lion, COI gene, South America

Introduction

The South American sea lion *Otaria byronia* (de Blainville, 1820) is a carnivorous mammal that is distributed along the coasts of South America (Argentina, Brazil, Chile, Uruguay and Peru) and solitary individuals have been reported in the Galapagos Islands [1]. According to Crespo et al. [2], this species has a northern distribution, reaching to Zorritos (4°00'S, 81°00'W) in Peru. Its diet is mainly made up of *Engraulis ringens* and *Trachurus picturatus* [3,4] but it includes many species of benthic and pelagic fish and invertebrates, several of them of commercial value. Miranda et al. [5] and Tantaleán [6] recorded *Diphyllobothrium pacificum* (Nybelin, 1931) Margolis, 1956 for the first time in *Otaria flavescens* (syn. *O. byronia*) on the Peruvian coasts of Trujillo (Isla Guañape) and Ica (San Juan de Marcona). In Punta San Juan, Ica, Peru, parasites of Peruvian fur seals (*Arctocephalus australis*) and *O. byronia* found dead were collected, where they reported the acanthocephalan *Corynosoma australe* isolated from the small intestine and the nematode *Contracaecum osculatum* isolated from the stomach, and no associated lesions were observed with these helminths [7]. Morphological identification studies of parasites in South American sea lion from Peru and Uruguay, reported different species of helminths such as nematodes, acanthocephalan, trematodes and cestodes of the genus *Diphyllobothrium* [8–10]. In Chile, studies have been carried out on parasitic fauna in South American sea lions analysing faecal samples, where they determined five zoonotic parasites (Diphyllobothriidae gen. sp., Anisakidae gen. sp., *Giardia*, *Cryptosporidium* and *Balantidium*) [11]. In Argentina, the intestinal content of 56 *O. byronia* was examined post-mortem, and several species of helminths were reported as part of their parasitic fauna, among them the *Diphyllobothrium* spp. [12]. A revision and redescription carried out based on an extensive material collected from the northern fur seal *Callorhinus ursinus* [13], from the island of St. Paul, Alaska, allowed the transfer of the cestode called *Diphyllobothrium pacificum* (Nybelin, 1931) Margolis, 1956 to its original genus *Adenocephalus* based on molecular and morphological evidence [14]. Kuchta and Scholz [15], reported 20 species of parasites exclusively of pinnipeds of the genus *Diphyllobothrium*, of which 8 species were molecularly characterized by Waeschenbach et al. [16]. In addition, "Reported incidences of parasitic infections in marine mammals from 1892 to 1978", contains detailed list information of parasites reported from marine mammals, including geographical locations of the host/parasite, covering the parasite groups Acanthocephala, Acarina, Anoplura, Cestoda, Nematoda, and Trematoda, and the host orders Pinnipedia (seals, sea lions, walruses), Cetacea (whales, dolphins), and Carnivora (sea otters) [17].

Currently, a new species of the genus *Diphyllobothrium* has been described which parasitizes the intestine of California sea lions *Zalophus californianus* from the Pacific coast of the USA and South American sea lions *Otaria flasvencens* (syn. *O. byronia*) from Peru and Argentina [18].

In the present study, we carry out a brief morphological and morphometric characterization, as well as a molecular analysis identifying *A. pacificus* parasitizing the intestine of the South American sea lion *O. byronia*. Supporting the information registered on parasitism in this otariids of the Peruvian coasts, this study is an important contribution for the southern hemisphere.

Materials and Methods

Collection of specimens

The *Adenocephalus pacificus* tapeworms were collected between September and October 2015, from two stranded male specimens of *O. byronia* that were found on the beaches of Huacho (11°06'S and 77°36'W) and Barranca (10°45'S and 77°46'W), department of Lima, and measured 1.25 m and 1.40 m length, respectively. Head and body injuries were observed, probably due to anthropogenic activity. Necropsies of the South American sea lions were performed *in situ* and one specimen was collected from gastrointestinal tract of each animal, which were washed separately with 0.75% NaCl, fixed in hot water and stored in 70% ethanol, kept refrigerated at 4°C for subsequent morphological and molecular analyses.

Morphological analyses

Proglottids were stained with Semichon's acetic carmine and mounted in Canada balsam according to Eiras et al. [19]. The two scolices unstained were taken with a Leica EZ 4HD stereomicroscope with an incorporate camera in a phase contrast. The eggs were examined with a Leica DM750 compound microscope and were photomicrographed. The isolated tapeworms were deposited and encoded in the Collection of Zoonotic Parasites of the Laboratory of Parasitology of Wildlife and Zoonoses of the Faculty of Biological Sciences of the Universidad Nacional Mayor de San Marcos, with the codes LPFSZ1 and LPFSZ2.

Molecular data

Genomic DNA of the hologenophore was isolated according to Pleijel et al. [20] using the Qiagen DNeasy Blood and Tissue kit as per the manufacturer's protocol. Purity and concentration of the DNA was checked by Nanodrop 2000C spectrophotometer (Thermo Scientific). The mtDNA-COI fragments were amplified with primers DipPaCO1r (reverse, 5′-ATGATAAGGGA YAGGRGCYCA-3′) common for all *Diphyllobothrium* and DipPaCO1f (forward, 5′-ACATGTG TGTAGTAACC TTGGC-3′) specific to *A. pacificus*, both designed by Wicht et al. [21]. PCR reactions were performed in 50 µl mixtures containing 500 ng genomic DNA, 0.5 µM each of

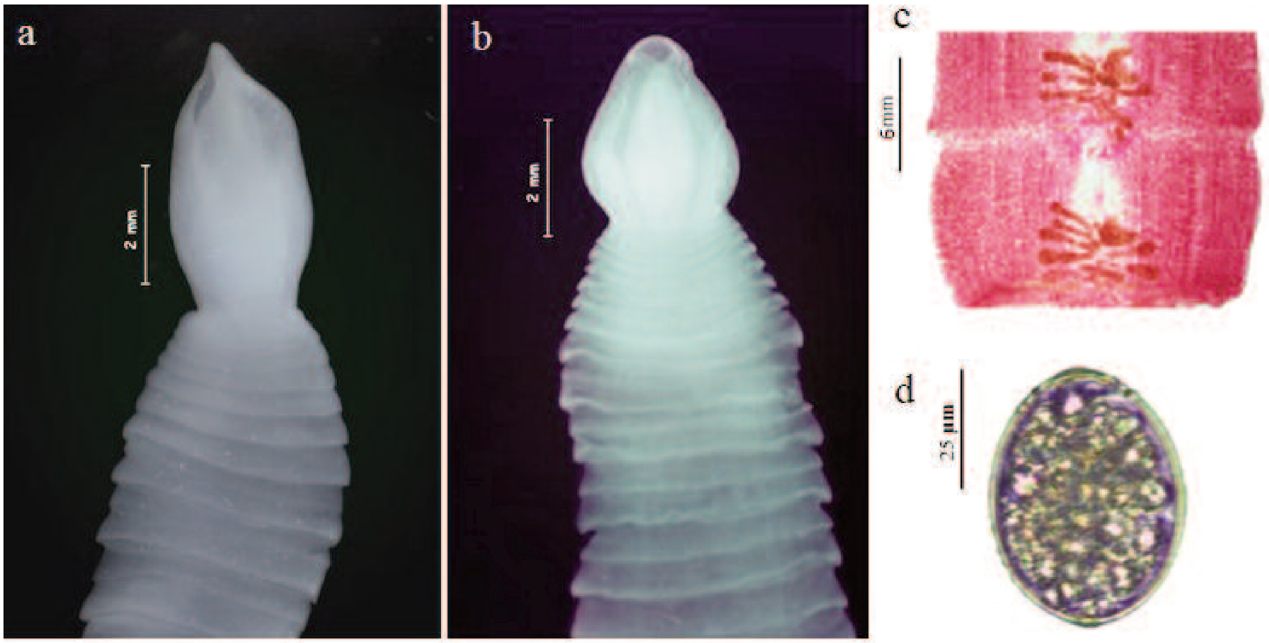

Figure 1. Morphological characteristics of *Adenocephalus pacificus*. (a,b) Scolices, lateral view, photographed unstained; (c) Mature proglottid, ventral view, stained with Semichon's acetic carmine; (d) Non-embryonated egg isolated from gravid proglottids

the two primers, 200 µM of each of the dNTPs, 1×PCR buffer (with 1.5 mM MgCl2), 2.5 U of Hot Start Taq DNA polymerase (Qiagen, Germany) in Veriti™96-well Thermal Cycler (Applied Biosystems, USA) under the following conditions: 95°C for 5 min, followed by 40 cycles of 94°C for 30 s, 50°C for 90 s and 72°C for 2 min 30 s with a final extension at 72°C for 10 min. The amplified fragments were visualised on 1% agarose gel.

Specific amplified products were eluted from the gel, using the QIAquick Gel Extraction Kit and QIAquick PCR Purification Kit. Purified products were sent to Macrogen (South Korea) for automated sequencing. The sequence was manually checked and edited for accuracy using Bioedit Software [22]. Contigs were assembled using the CAP3 interface in the bioedit software. Sequence data from samples LPFSZ1 and LPFSZ2 were submitted to GenBank, with accession numbers MK500873 and MK500874, respectively.

Phylogenetic analysis

Phylogenetic relationships of *A. pacificus* was assessed on the basis of genetic marker COI, sequences were aligned with homologous sequences using Clustal W2 alignment software [23]. The phylogenetic relationships were evaluated with maximum likelihood (ML) in the MEGA version X program [24], using the Kimura 2-parameter model and the nodal support values were calculated by running 1000 bootstrap replicates [25]. Bayesian inference criteria (BIC) were analysed in the Bayesian Evolutionary Analysis program by Sampling Trees (BEAST) version 1.7 [26]. The BIC model selected was HKY + G + I running a chain of 20 million generations and sampling tree topologies every 1000 generations. The burning fraction was set at 10%.

Ethics approval

The study was approved by the Research Ethical Committee of Universidad Ricardo Palma, Faculty of Biological Sciences (Certificate 005-2015), in compliance with institutional, national, or international guidelines that were followed for using animals in the study.

Results

The gravid cestode collected from the intestine of *O. byronia* from Barranca beach (LPFSZ1) has a lanceolate scolex, 4.46 mm long by 2.46 mm wide. The strobila is 135 cm long and 0.4 cm wide. The specimen from the Huacho area (LPFSZ2) has an oval-shaped scolex 3.1 mm long by 2.7 mm width. The strobila is 67 cm long by 0.3 cm wide. Both cestodes have a short neck (Fig. 1). On the ventral surface of the stained gravid proglottids, 5 and 7 transverse papilla-like tegumental protuberances decreasing in size anteriorly, were observed. The

Table 1. List of species, hosts species, localities, stage, molecular IDs and GenBank accession numbers for sequences mitochondrial cytochrome c oxidase subunit 1 (COI) gene. Newly generated sequences are presented

Species	Hosts species	Locality	Molecular ID	Stage	Access. No.
OUTGROUP					
Spirometra erinaceieuropaei	*Canis lupus familiaris*	Latvia	W12	adult	MT951153
Spirometra mansoni	*Ptyas korros*	Laos	Laos -Khammouan-Se3	plerocercoid	KM099124
INGROUP					
Diphyllobothriidae gen. n. sp. n.	*Trematomus bernacchii*	Antarctica	PBI-980	plerocercoid	KY552888
Diplogonoporus balaenopterae	*Homo sapiens*	Japan	–	adult	AB822370
Diplogonoporus balaenopterae	*Homo sapiens*	Japan	PBI-590	adult	KY552884
Dibothriocephalus latus	*Homo sapiens*	Chile: Santiago	–	adult	AB511963
Dibothriocephalus latus	*Homo sapiens*	Switzerland: Geneva	–	adult	AM712906
Dibothriocephalus latus	*Homo sapiens*	Chile: Santiago	–	adult	AB504899
Dibothriocephalus latus	*Homo sapiens*	Canada: Manitoba	PBI-975	adult	KY552871
Dibothriocephalus nihonkaiensis	*Oncorhynchus gorbuscha*	USA	US361b	plerocercoid	KY000483
Dibothriocephalus nihonkaiensis	*Homo sapiens*	Switzerland: Geneva	PBI-594	adult	AM412559
Dibothriocephalus ursi	*Ursus arctos middendorffi*	USA	No. 49355	adult	AB605763
Dibothriocephalus ditremus	*Hypomesus pretiosus japonicus*	Japan: Hokkaido	5dcox1	plerocercoid	AB979518
Dibothriocephalus ditremus	*Oncorhynchus tshawytscha*	USA	PBI-974	plerocercoid	KY552872
Dibothriocephalus dendriticus	*Coregonus lavaretus*	Scotland: Loch Lomond	PBI-596	plerocercoid	KY552870
Dibothriocephalus dendriticus	*Homo sapiens*	Czech Republic	CZ49	adult	KC812047
Diphyllobothrium stemmacephalum	*Homo sapiens*	Japan: Kochi	–	adult	LC042231
Diphyllobothrium stemmacephalum	*Tursiops trunchus*	USA: Minnesota	PBI-978	adult	KY552885
Diphyllobothrium sprakeri	*Otaria byronia*	Argentina: Chubut	ARG27	adult	MW596661
Diphyllobothrium sprakeri	*Zalophus californianus*	USA: central California	ZC1	adult	MW596662
Diphyllobothrium sprakeri	*Zalophus californianus*	USA: central California	ZC4	adult	MW596665
Diphyllobothrium sprakeri	*Otaria byronia*	Peru: Callao	PERU63	adult	MW596677
Diphyllobothrium sprakeri	*Otaria byronia*	Peru: Callao	PERU64	adult	MW596678
Diphyllobothrium sprakeri	*Otaria byronia*	Peru: Callao	PERU66	adult	MW596680
Adenocephalus pacificus	*Homo sapiens*	Peru	Dp-Hs4	adult	AB548654
Adenocephalus pacificus	*Arctocephalus pusillus*	Australia	AU11	adult	KR269745

Species	Hosts species	Locality	Molecular ID	Stage	Access. No.
Adenocephalus pacificus	*Homo sapiens*	Peru	TS06/30	adult	KR269743
Adenocephalus pacificus	*Homo sapiens*	Peru	TS05/16	adult	KR269742
Adenocephalus pacificus	*Sarda chiliensis*	Peru	PERU8	plerocercoid	KR269747
Adenocephalus pacificus	*Callorhynus ursius*	USA	SAM3/6a	adult	KR269748
Adenocephalus pacificus	*Homo sapiens*	Australia	DP	adult	KU519704
Adenocephalus pacificus	*Arctocephalus pusillus*	Australia: Victoria	PBI-606	adult	KY552867
Adenocephalus pacificus	*Otaria byronia*	Chile	C-01-LM	adult	MN967011
Adenocephalus pacificus	*Homo sapiens*	Peru: Lima	LPFSZ3	adult	MN127948
Adenocephalus pacificus	*Homo sapiens*	Peru: Lima	LPFSZ4	adult	MN127949
Adenocephalus pacificus	*Homo sapiens*	Peru: Lima	LPFSZ5	adult	MN127950
Adenocephalus pacificus	*Otaria byronia*	Peru, Callao	PERU65AP	adult	MW596679
Adenocephalus pacificus	*Otaria byronia*	Peru, Callao	PERU67AP	adult	MW596681
Adenocephalus pacificus	*Otaria byronia*	Peru, Callao	PERU56AP	adult	MW596674
Adenocephalus pacificus	*Delphinus delphis*	Argentina	E157	plerocercoid	MW546058
Adenocephalus pacificus	*Otaria byronia*	Peru, Barranca	LPFSZ1	adult	MK500873*
Adenocephalus pacificus	*Otaria byronia*	Peru, Huacho	LPFSZ2	adult	MK500874*

* present study

male gonopore is in a pre-equatorial position. The uterus has several bilateral branches (6–8), filled with small capped eggs located posterior to the female gonopore (Fig. 1), twenty-five eggs from each sample were measured with a range of 52–55 by 38–40 µm (LPFSZ1) and 51–55 by 38–41 µm (LPFSZ2) (Fig. 1). The two newly-generated mtDNA-COI sequences were compared with sequences of *A. pacificus* reported from otariids, human cases and plerocercoids obtained of the GenBank (Tab. 1). In addition, the two cox1 sequences were generated and aligned with published sequences from other molecularly characterized *Diphyllobothrium* species (Tab. 1). According to the *cox*1 sequences, we found that the two tapeworms isolated from *O. byronia* from the northern coasts of Peru formed a well-supported clade with *A. pacificus* sequences. Partial COI gene sequences (accession number MK500873 and MK500874) showed 99% similarity with *Adenocephalus pacificus* Nybelin, 1931 reference sequences (Fig. 2).

Discussion

In the southern hemisphere, *A. pacificus* has been reported in the Southern Ocean of Antarctic, temperate waters of the Australia, southern Africa and South America (Argentina, Australia, Chile, Ecuador, Namibia, New Zealand, Peru, South Africa, and Uruguay) [14]. The Pacific tapeworm *A. pacificus* has coexisted with humans since the early Neolithic period, as evidenced by the findings of coprolite eggs at the Los Gavilanes coastal site in Peru, dating from 2850 to 2700 B.C. [27], besides, there are several records from the pre-Inca times (Chiribaya culture, 800–1400 B.C.) and Inca (1476–1534 B.C.) in Peru and northern Chile [28]. The first human case caused by *A. pacificus* in Peru was reported by Baer et al. [29], and since that time around 1000 cases of human with these tapeworms have been reported between Peru, Chile and Ecuador as well as imported cases to Europe through trade in fresh or frozen products from

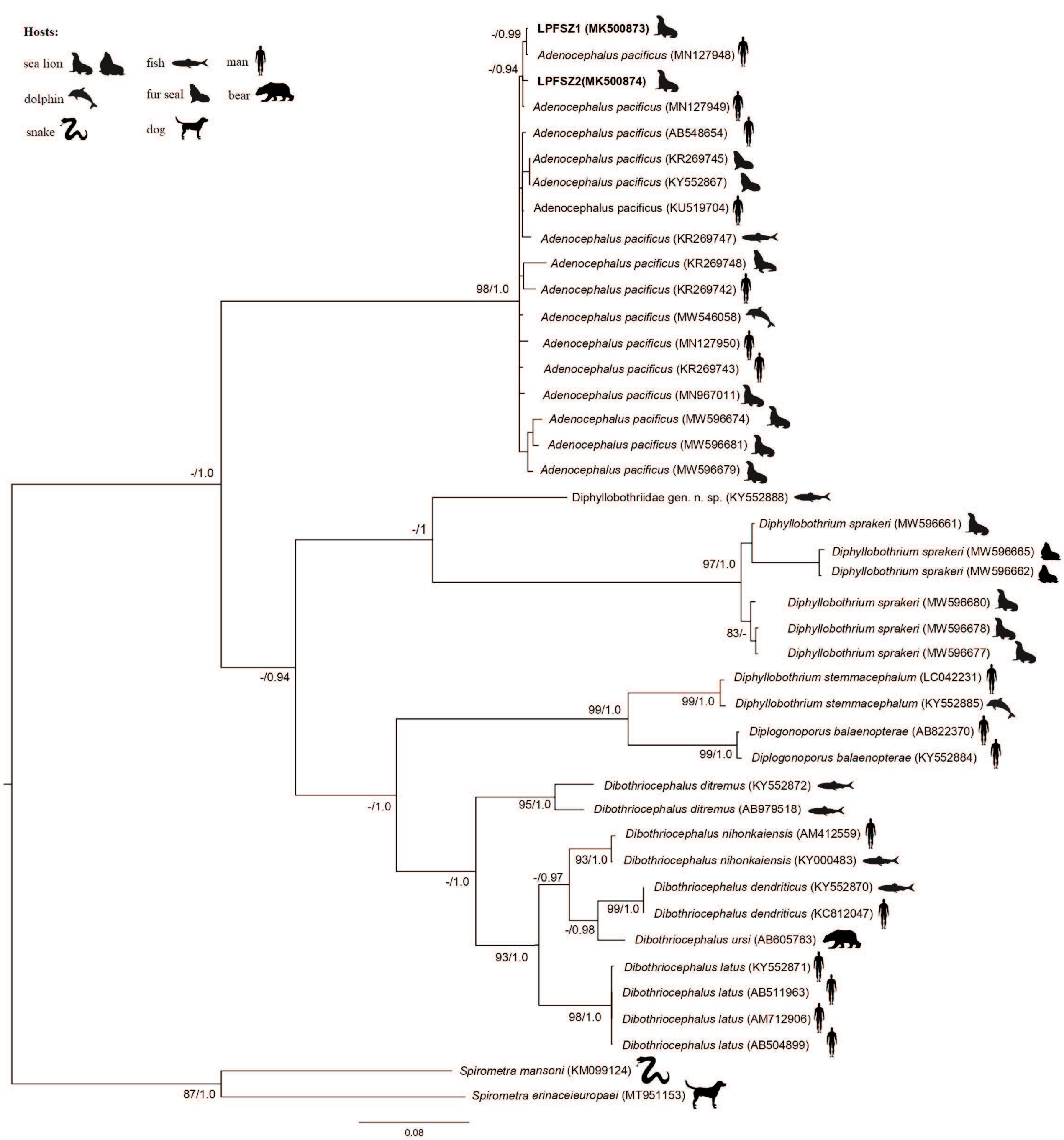

Figure 2. Inferred phylogenetic relationships of the partial sequences of the mtDNA-COI gene of the samples of *Adenocephalus pacificus* isolated from *Otaria byronia*. The numbers on the branches represent the starting value of maximum likelihood (ML) and Bayesian inference (BI), respectively. Branch length scale bars indicate number of substitutions per site. Newly obtained sequences are shown in bold type. *Spirometra erinaceieuropaei* and *Spirometra mansoni* is used as an outgroup

marine fish, travel or migration of humans [30]. Cabrera et al. [31], recorded the first finding of diphyllobothriosis of an adult specimen of *A. pacificus* in *Canis lupus familiaris* (considered an accidental host) on the southern coast of Peru. This species has been already recorded from *O. byronia* in the Gulf of Arauco and in the city Valdivia, Chile [11,32], as well as in *Arctocephalus australis* in Brazil [33]. Furthermore, specimens of *Diphyllobothrium* spp. were also reported in *O. byronia* and *A. australis* in northern Patagonia, Argentina [12], in California sea lion (*Zalophus* *californianus*), Steller sea lion (*Eumetopias jubatus*) in California, USA [34–36] and *C. ursinus* from St. Paul Island, Alaska, USA [13,37]. A first report in Chile of the gastrointestinal endoparasitic fauna by Hermosilla et al. [10] on an „urban" colony of 40 individuals of *O. byronia*, mention a species of the Diphyllobothriidae family with a parasitic prevalence of 13%. All these authors identified the adult cestode through a morphological study of the scolex, proglottids and, in other cases, through the eggs obtained from faecal samples. Currently, based on *cox*1 sequences, four diphyllobothriid tapeworms from *O. flavescens* in Peru were found to be conspecific with *Adenocephalus pacificus* Nybelin, 1931 [18]. Likewise, the ML and BI analyzes of the *cox*1 data set resulted in four newly generated sequences from *Zalophus californianus* isolates and 14 new sequences from *O. byronia* (13 from Peru and 1 from Argentina) in a well-supported clade confirmed a new species denominate *Diphyllobothrium sprakeri* n.sp., additionally it is the first coinfection of two species of diphyllobotriids in sea lions from the southern hemisphere [18]. We evaluated the morphology witnessing 5 and 7 transverse papilla-like tegumental protuberances anterior to the male gonopore on the ventral surface of the proglottids, a characteristic that allows *A. pacificus* to be distinguished from *Diphyllobothrium* species, as also pointed out by Hernández-Orts et al. [14,18]. We reported the presence of *A. pacificus* in *O. byronia* in 2 locations on the northern Peruvian coast, based on both ML and BI analyses characterized by the mtDNA-COI gene. Although in this study only two specimens of tapeworms were analysed, it was possible to make the appropriate morphological descriptions thanks to the good quality of the samples and to the subsequent DNA sequencing. Recently, three human cases caused by *A. pacificus* were reported in Peru [37]. The Pacific broad tapeworm *A. pacificus* is considered the most important causative agent of diphyllobothriosis among humans in South America, predominantly in Peru, where human infections are associated with the habits of consuming raw or undercooked marine fishes [38]. The present study molecularly confirmed the presence of *Adenocephalus pacíficus* in sea lions stranded on the northern coast of the Peruvian Sea.

Acknowledgements

This work was supported by the "Vicerrectorado de Investigación of the Universidad Nacional Mayor de San Marcos" (VRI-UNMSM) under grant number B17100721.

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Received 28 June 2021
Accepted 02 August 2021