Original Article

Morphological and molecular identification with frequency analysis of *Calicophoron microbothrioides* in central Chile

Identificação morfológica e molecular com análise de frequência de *Calicophoron microbothrioides* na região central do Chile

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Abstract

This research aimed to determine the presence of paramphistomids in cattle slaughtered in a slaughterhouse of the Ñuble Region of Chile, to identify flukes and to analyze the frequency of these parasites in the Maule, Ñuble, and Biobío administrative regions of Chile. Between October of 2016 and April of 2017, rumens of 494 cattle were examined for flukes in the forestomachs. Worms were identified morphologically and, in addition, molecular analysis of the internal transcriber spacer region 2 of the fluke's DNA was done and phylogenetic analyses were performed with Bayesian inference in 14 worms. The frequency was analyzed by locality (low- or highlands) and age. The overall frequency was 11.24%. The district with the highest frequency of presentation was Chillán Viejo (30.8%). Districts in the lowlands had similar frequencies to those in the mountain lands ($p=0.1$). The frequency of flukes was significantly higher in adult animals than in young ones ($p<0.01$). We obtained a 460 bp-length fragment of DNA that was identical to the sequences previously identified as *Paramphistomum cervi* and *Calicophoron microbothrioides*, and performed morphological analyses confirmed that our samples belonged to *C. microbothrioides*. This is the first published study of *C. microbothrioides* in Chile.

Keywords: Paramphistomidae, ruminal flukes, bovine, epidemiology, ITS-2, *Calicophoron microbothrioides*.

Resumo

Este trabalho teve como objetivo determinar a presença de paramphistomídeos em bovinos abatidos em um matadouro da Região do Ñuble do Chile, para identificar parasitas e analisar a frequência desses parasitos nas regiões administrativas de Maule, Ñuble e Biobío, no Chile. Entre outubro de 2016 e abril de 2017, rúmens de 494 bovinos foram examinados à procura de vermes no pré-estômago. Os vermes foram identificados morfologicamente e, além disso, a análise molecular da região interna do espaçador do transcritor 2 do DNA e análises filogenéticas foram realizadas com inferência bayesiana em 14 vermes. A frequência foi analisada pela altitude da localidade (baixa ou alta) e idade. A frequência geral foi de 11,24%. O distrito com as maiores frequências de parasitismo foi Chillán Viejo (30,8%). Os distritos das terras baixas tinham frequências semelhantes às encontradas nas terras das montanhas ($p=0,17$). A frequência foi significativamente maior em animais adultos do que em jovens ($p<0.01$). Obtivemos um fragmento de DNA de 460 pb que era idêntico às sequências anteriores identificadas como *Paramphistomum cervi* e *Calicophoron microbothrioides*, e realizamos análises morfológicas que permitiram confirmar que nossas amostras pertenciam a *C. microbothrioides*. Este é o primeiro estudo publicado sobre *C. microbothrioides* no Chile.

Palavras-chave: Paramphistomidae, vermes ruminais, bovino, epidemiologia, ITS-2, *Calicophoron microbothrioides*.

Introduction

Paramphistomids are ruminal trematodes with a worldwide distribution, they affect both domestic and wild ruminant (SANABRIA et al., 2011; HUSON et al., 2017; ALI et al., 2018; NARANJO-LUCENA et al., 2018; O'CONNOR et al., 2018).

The impact of this parasitosis is variable; some regions have reported it as a very pathogenic disease (DORNY et al., 2011), while others have considered it quite harmless (FOSTER et al., 2008; ZINTL et al., 2014). Highly intense infections can cause damage in the mucosa and submucosa of the forestomachs and duodenum (FUERTES et al., 2015). Also reported are age-associated variations in the prevalence of adult flukes, with higher values found in adult

animals than in young ones (GONZÁLEZ-WARLETA et al., 2013; SANCHÍS et al., 2013).

An increase of the prevalence of paramphistomids has been reported in several regions in Europe (Great Britain, Ireland, Belgium), and South America (Argentina) (MALRAIT et al., 2015; SANABRIA & ROMERO, 2008), as well as a high seroprevalence (29%) has been reported in Uruguay (SANCHÍS et al., 2013), which underline the importance of studying this group of parasite in new localities. Given that their life cycles are very similar to *Fasciola hepatica*, particularly in the free-living stages and snail-parasitizing stage, it is expected that both parasites have a similar distribution. However, despite the high frequency of *F. hepatica* in central Chile (MORALES et al., 2000), there are no published findings of paramphistomid trematodes in this country, except for an imported bull that was diagnosed shortly after its arrival (GONZÁLEZ & PLAZA, 1966).

Considering the above, in this study, we searched for the presence of this parasite in the Chilean administrative regions of Maule, Ñuble and Biobío, and in the southern Maule region, located between 35° 25' 6.95" S and 38° 29' 38.14" S. This is a zone of central Chile where *F. hepatica* is frequently found. These regions have a transitional climate that falls somewhere between the classifications of warm Mediterranean and wet temperate. This territory includes two mountain ranges that run north–south: Los Andes (which separates the region from Argentina) and La Costa (which is west of Los Andes), and two flat lowlands: the Central Valley (between the mountain ranges) and the coastal plain (a narrow strip of land between the La Costa range and the Pacific Ocean). Given that cattle (*Bos taurus taurus*) constitute a species that can present higher prevalence rates of paramphistomids than other domestic ruminants (e.g. JONES et al., 2017; PLOEGER et al., 2017), and with good traceability in Chile, we chose this species

to identify the origin of inspected animals in this study. The area studied harbors around 12% of the total cattle population in Chile, comprising about 460,000 animals (ODEPA, 2016). There is a greater likelihood of the presence of stagnant water, which favors the abundance of the intermediate host (snails) in the Central Valley and coastal plain when compared with the mountain ranges, therefore, we expected a higher prevalence of paramphistomids in districts located in the Central Valley and coastal plain rather than in districts with more mountainous terrain. The identifications of paramphistomids can be based on morphological attributes, but misidentifications have been reported (SANABRIA et al., 2009). On the other hand, the internal transcriber spacer 2 (ITS-2) fragment has been used for the identification of the paramphistomid species previously, showing that is a good marker for this purpose (SANABRIA et al., 2011).

Thus, this research aimed to determine the presence of paramphistomids in beef cattle slaughtered in a slaughterhouse of the Ñuble Region of Chile, to identify the flukes and to analyze the frequency of these parasites in the Maule, Ñuble, and Biobío regions.

Materials and Methods

Sampling

Between October of 2016 and April of 2017, 494 *B. taurus taurus* (sample size calculated following Vallejo et al. (2013), with an expected proportion of 50%, an accepted error <5%, and a confidence level of 95%) slaughtered in the Ñuble Region were examined for flukes in the forestomachs. The details related to the origins of the animals are documented in Table 1. The animals included in the study correspond to all animals slaughtered in

Table 1. Frequency of presentation of *Calicophoron microbothrioides* in forestomachs of cattle by province and district in the Maule, Ñuble and Biobío regions of Chile. October of 2016 - April of 2017. The codes added to the district name are that used in Figure 1.

Province	District/Code	Examined Animals	Frequency of presentation [%] (95%, confidence interval)	Mountain or flat lowlands district
Cauquenes	Cauquenes/1	1	100 (5 – 100)	Mountain
Linares	Linares/2	3	66.67 (13.32 – 100)	Mountain
Punilla	Ñiquén/3	21	28.57 (9.25 – 47.89)	Lowland
	San Carlos/4	178	11.80 (7.06 – 19.54)	Lowland
	San Nicolás/5	26	3.85 (0.0 - 11.24)	Lowland
	Coihueco/6	24	25 (7.68 - 42.32)	Mountain
Diguillín	Chillan/7	7	0 (0 - 34.8)	Lowland
	Chillan Viejo/8	13	30.77 (5.68 - 55.86)	Lowland
	Pinto/9	7	0 (0 - 34.8)	Mountain
	Bulnes/10	73	4.11 (0.0 - 8.66)	Lowland
	San Ignacio/11-El Carmen/12	10	0 (0 - 25.9)	Mountain
Itata	Quirihue/13	12	16.67 (9.25 - 47.89)	Mountain
	Ninhue/14-Coelemu/15	7	14.29 (0 - 40.21)	Mountain
	Portezuelo/16-Quillón/17	17	11.76 (0 - 27.08)	Lowland
Concepción	San Pedro de la Paz/18	2	0 (0 - 77.64)	Lowland
Arauco	Arauco/19	3	0 (0 - 63.16)	Lowland
Biobío	Cabrero/20	4	0 (0 - 52.71)	Lowland
	Los Ángeles/21	59	6.78 (0.36 - 13.19)	Lowland
	Mulchén/22-Santa Bárbara/23-Tucapel/24	27	11.11 (0 - 22.97)	Mountain
	Total	**494**	**11.34 (8.54 – 14.13)**	

each day of sampling, regardless of their origin, in such a way that the number of animals from each district was not previously determined.

Flukes identification

Flukes were fixed and conserved in 70% ethanol in 2 mL vials immediately after collecting in the slaughterhouse, and they were then identified morphologically and molecularly. For the morphological identification, three worms from San Carlos and one from each district with infected animals, total 15 worms, were stained with hydrochloric carmine and summited to increasing concentrations of ethanol until 100%, then they were cleared in methyl salicylate, immersed in xylol and finally mounted in Permount ®. The identification was performed following Eduardo (1982; 1983) and Jones (2005). For the histological study, worms were fixed in 10% neutral formalin for 48 h at room temperature, processed routinely, and embedded in paraffin; 3-μm serial sections were stained with hematoxylin and eosin. The ranges (mean ± standard deviation) of measures are given in results. For the molecular identification, a fragment of the internal transcriber spacer – 2 (ITS-2) region of the DNA was amplified and a phylogenetic analysis was performed in two worms from San Carlos and one from each district with infected animals, total 14 worms. These 14 Chilean DNA samples were genetically identical among them (null variability) and agreed with the morphological identification (see results), hence we did not include more samples for the analysis. DNA was extracted using the DNA Blood and Tissue Extraction Kit (Qiagen®, Hilden, Germany) following the manufacturer' instructions. Polymerase chain reaction (PCR) was performed following the method of Sanabria et al. (2011). PCR products were summited to electrophoresis in 1.2% agarose gel, DNA was extracted from the gel with E.Z.N.A® Gel Extraction Kit, sequenced at Austral-Omics (ABI 3500 Genetic Analyzer, Applied Biosystems, capillary analyzer system, Universidad Austral de Chile) and the sequences were included in GenBank with accession numbers MK683484–MK683497. For a first molecular identification, the sequences were analyzed by means of the Basic Local Alignment Search Tool (BLAST) in GenBank.

Phylogenetic and statistical analysis

For phylogenetic analyses only two of the 14 Chilean isolates were used given the 100% identity among them. The same criterion was used for including in the analysis other sequences with 100% identity when they come from the same study. Sequences were aligned with ClustalW method and a pairwise distance matrix was constructed with Mega 7 Software. Phylogenetic analysis was performed by Bayesian inference. For the latter, the best model and the parameters for tree construction were obtained by means of j Model Test 2.1.10 software, where AIC criteria were selected, and the phylogenetic tree was constructed with Mr. Bayes 3.2.6. This analysis was subjected to 2,000,000 bootstraps to test the

reliability of individual branches within the generated tree. Sequences of most Paramphistomidae species were included in the analysis. These were obtained from GenBank, with preference for those that had a publication with a PubMed Identifier (PMID) code included in the GenBank sequence record. GenBank codes of the included sequences are shown in the identification of the sequences in the phylogenetic tree in results. Some sequences included in the analysis did not have a linked publication, and they were identified with a hyphen between the GenBank code and the species name in the tree. *F. hepatica* and *F. gigantica* (different family) were used as the external group for this analysis.

Fisher's exact test (significance level = 0.05) was used to compare the frequencies of parasite presentation between districts belonging to the Central Valley and coastal plain and those that extend into the Los Andes and La Costa mountain ranges (see Table 1 and Figure 1 for the details of districts belonging to each category), as well as between adult animals (2 or more years old) and young animals (less than 2 years old). 95% confidence intervals (CI) of the frequencies of presentation were estimated with the online Working in Epidemiology platform (http://www.winepi.net/sp/index.htm), and they are reported in Table 1.

The Ethics Committee of the Veterinary Sciences Faculty at the Universidad de Concepción approved and certified this study.
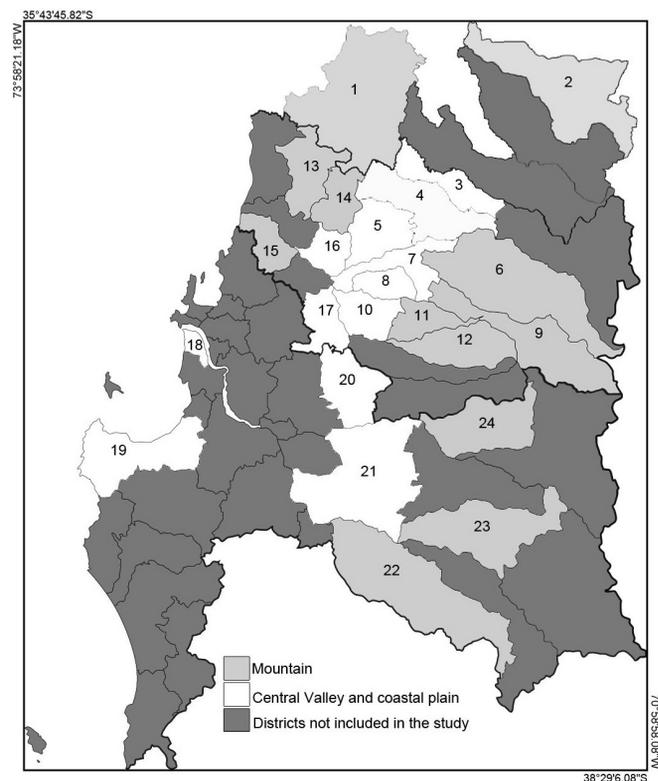

Figure 1. Map of the southern part of the Maule Region, Ñuble Region and Biobío Region. The names of the districts included in this study are detailed in the Table 1 and correspond to each number (code) included in this map.

Results

Statistical reports

A total of 56 of 494 forestomachs (11.3%) were found to be colonized with flukes. When considering districts with more than 10 examined animals, those with the highest frequencies of fluke presentation were Chillán Viejo (30.8%, n=13), Ñiquén (28.6%, n=21), and Coihueco (25%, n=24; Table 1).

Only 384 animals were recorded as adult or young animal. The frequency of infection was significantly higher in adult cattle than in young cattle (35.8%, n=53 and 5.4%, n=331, respectively; $p<0.01$). The frequencies with which the animals were infected in the Central Valley and coastal plain districts

(10.2%, n=403 animals) were not significantly different from those districts that extended into to the Los Andes and La Costa mountain ranges (16.5%, n=91; $p=0.1$).

Morphological identification

The specimens were characterized by a conical and ventrally curved body that was 5.88–6.68 mm (6.28 ± 0.57 mm) in length, with a subterminal oral aperture (Figure 2). The pharynx, measuring 687.8–748.5 µm (718.15 ± 42.92 µm) by 597.8–686.8 µm (642.3 ± 62.93 µm) was a calicophoron-type with small papillae on its surface at the sagittal section. The esophagus was characterized by the presence of an esophageal bulb, 735.4 µm length. The caeca were coiled, reaching the acetabulum. The genital pore opening
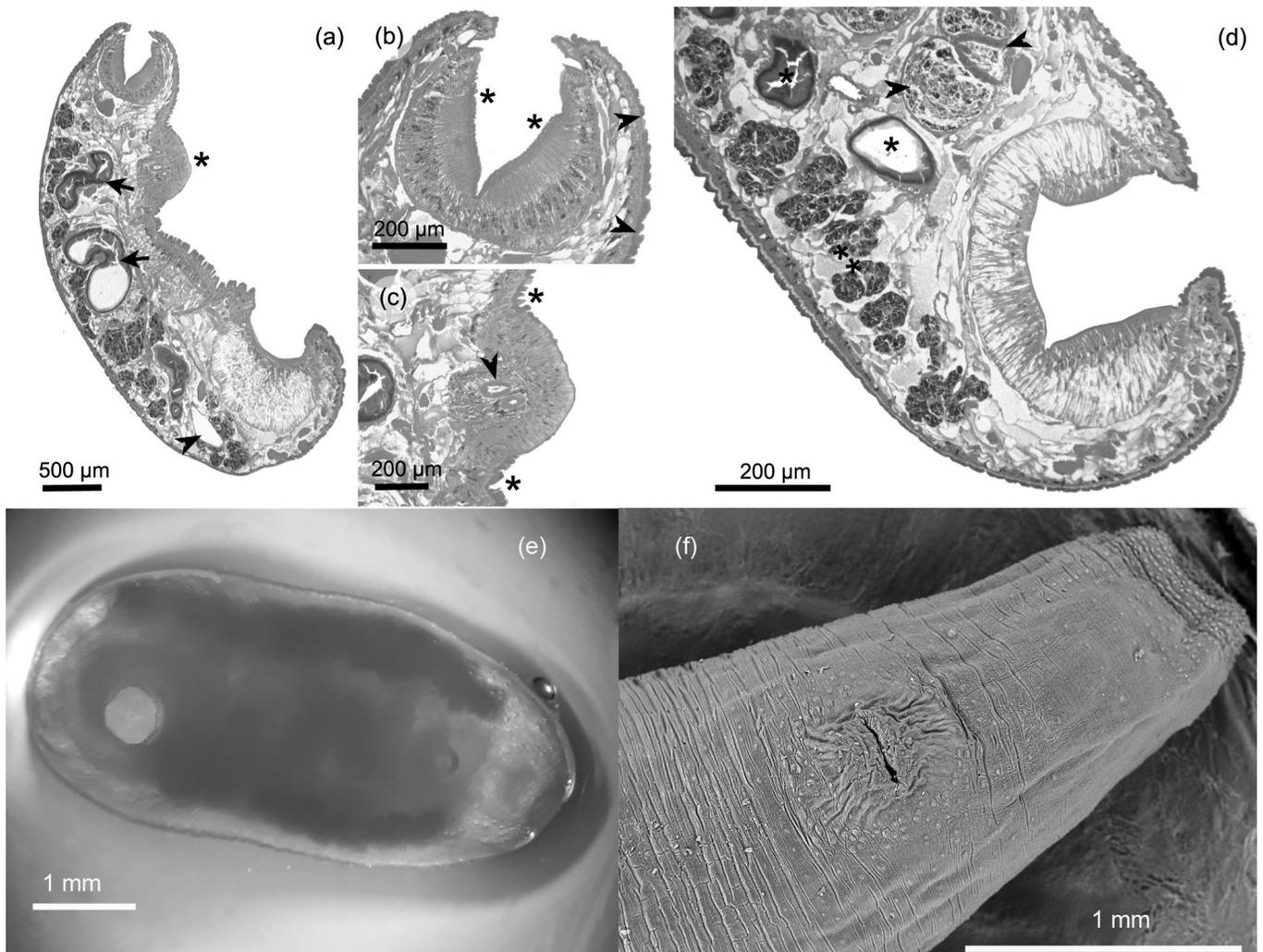
Figure 2. (a-d) *In toto* sagittal section of fluke *Calicophoron microbothrioides*. (a) Note pharynx located in first third of body and medium-size acetabulum in caudal third of body. There is also a prominent swelling over ventral surface around genital pore area, typical for this species (*), sections of uterine coils (arrows) and excretory vesicle in dorsal part of body, with the latter located over acetabulum (arrow head). (b) *Calicophoron*-type pharynx covered with small papillae over its surface (*) and papillae increasing in size from cranial to caudal direction over tegument (arrow heads). (c) Swelling in ventral surface of body; note terminal genitalium which will open to the genital pore (arrow head), there are notorious papillae over tegument (*). (d) Sub-median section of caudal third of body; note pisum-type acetabulum being surrounded by lobulated testis (arrow heads), coils of uterus in the dorsal part of body (asterisks) and vitellaria (**). (e) External view of *C. microbothrioides* in a stereomicroscope with oblique light. (f) Scanning electron microscope view of the anterior end of a *C. microbothrioides*, with a zoom on the genital pore and surrounding papillae.

was at the ventral surface, located caudal to the caecal bifurcation and surrounded by a prominent semicircular swelling at the sagittal section. Vitellaria were organized in two lateral fields, beginning caudal to the oral sucker and reaching the anterior border of the acetabulum, not converging at either the anterior or posterior end. The testes were located at the midline of the body and were tandem and deeply lobulated; further, the anterior testis was 0.73–1.15 mm (0.94 ± 0.30 mm) in length and the posterior testis was 0.77–1.17 mm (0.97 ± 0.28 mm) in length. The ovary was subspherical and measured 330.4–376.1 µm (353.25 ± 32.31 µm) by 312.4–327.4 µm (319.9±10.61 µm), and Mehlis' gland was close to it. Both were submedian and post-testicular. The excretory vesicle was dorsal to the acetabulum, ovary, and Mehlis' gland at the sagittal section. The uterus was filled with translucent oval eggs measuring 115.80–131.20 µm (122.79 ± 4.87 µm) by 56.10–66.30 µm (61.84 ± 3.06 µm). A medium-sized subterminal acetabulum, 1.23–1.29 mm (1.26 ± 0.04 mm) in diameter, was located at the posterior third of the body, and was pisum-type at the sagittal section. The tegument was covered with small papillae around the oral aperture and genital pore, and demonstrated an increased size from the oral aperture to the genital pore, being dome-shaped around the latter.

Molecular analyses

A 460 bp-length fragment of DNA was amplified in the 14 samples. The BLAST analysis output shared 100% identity with both *Paramphistomum cervi* Linnaeus, 1758 and *Calicophoron microbothrioides* Price & McIntosh, 1944 (Digenea: Paramphistomidae), as well as the distance between those species where 0.000 (Table 2). As expected, after BLAST analysis, the phylogenetic tree grouped Chilean sequences with *P. cervi* and *C. microbothrioides* (Figure 3). The tree also showed sequences of the same species grouped together but did not group together species of the same genus.

Discussion

This is the first study to examine the frequency of paramphistomids in Chile. We expected a higher frequency in districts restricted to the lowlands (Central Valley and coastal plain) than those that included mountainous terrain; however, there were no significant differences. This could be due to the fact that those districts containing mountains also contained flat terrain in both the low- and highlands; these were the territories used for beef cattle
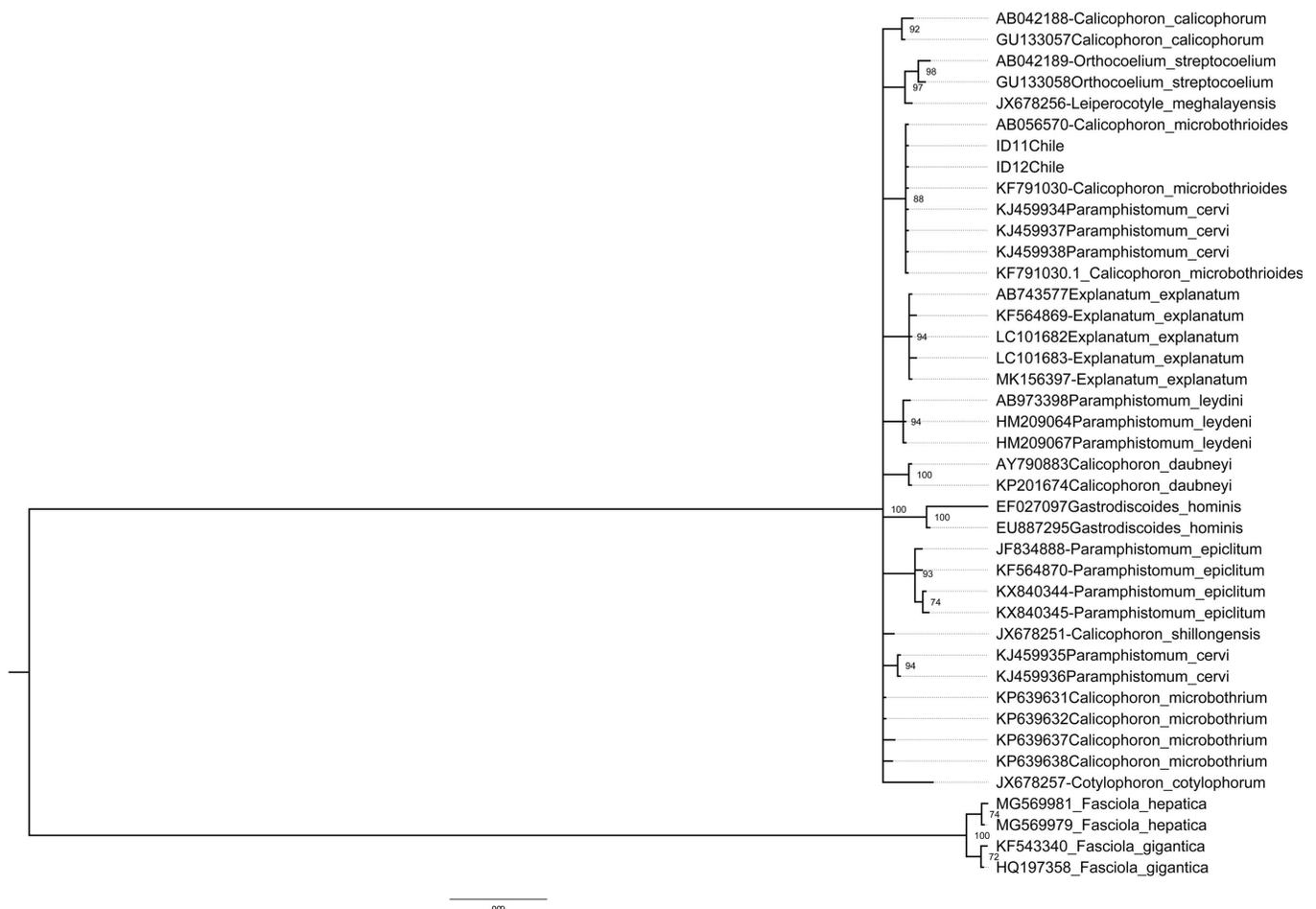

Figure 3. Bayesian inference tree based on 460 nucleotides of the Internal Transcribed Spacer 2 segment of the ribosomal DNA, reconstructed under TPM3uf+G substitution model. Posterior probability values are indicated to each node. *Fasciola* spp. were used as outgroup control.

Table 2. Distance matrix of paramphistomids using the ITS 2 DNA region.

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29
1	AB042188- Calicophoron_ calicophorum																													
2	AB042189- Orthocoelium_ streptocoelium	0.034																												
3	AB056570- Calicophoron_ microbothrioides	0.026	0.029																											
4	AB743577 Explanatum_ explanatum	0.035	0.026	0.026																										
5	AB973398 Paramphistomum_leydini	0.038	0.040	0.035	0.020																									
6	AY790883 Calicophoron_ daubneyi	0.031	0.040	0.029	0.031	0.029																								
7	EF027097 Gastrodiscoides_hominis	0.072	0.062	0.069	0.065	0.071	0.075																							
8	EU887295 Gastrodiscoides_hominis	0.037	0.034	0.040	0.037	0.046	0.040	0.036																						
9	GU133057 Calicophoron_ calicophorum	0.006	0.037	0.020	0.029	0.032	0.026	0.072	0.037																					
10	GU133058 Orthocoelium_ streptocoelium	0.040	0.008	0.029	0.026	0.040	0.037	0.065	0.037	0.037																				
11	HM209064 Paramphistomum_leydeni	0.035	0.037	0.032	0.017	0.003	0.026	0.071	0.043	0.029	0.037																			
12	HM209067 Paramphistomum_leydeni	0.035	0.037	0.032	0.017	0.003	0.026	0.071	0.043	0.029	0.037	0.000																		
13	ID11Chile	0.026	0.029	0.000	0.026	0.035	0.029	0.069	0.040	0.020	0.029	0.032	0.032																	
14	ID12Chile	0.026	0.029	0.000	0.026	0.035	0.029	0.069	0.040	0.020	0.029	0.032	0.032	0.000																
15	JF834888- Paramphistomum_ epiclitum	0.032	0.038	0.044	0.035	0.044	0.043	0.081	0.046	0.035	0.038	0.041	0.041	0.044	0.044															
16	JX678251- Calicophoron_ shillongensis	0.020	0.029	0.023	0.020	0.023	0.023	0.069	0.034	0.014	0.029	0.020	0.020	0.023	0.023	0.026														
17	JX678256- Leiperocotyle_ meghalayensis	0.029	0.014	0.023	0.026	0.034	0.034	0.066	0.031	0.026	0.011	0.032	0.032	0.032	0.032	0.032	0.023													
18	KF564869- Explanatum_ explanatum	0.038	0.029	0.029	0.003	0.023	0.034	0.068	0.040	0.032	0.029	0.020	0.020	0.029	0.029	0.038	0.023	0.029												
19	KF564870- Paramphistomum_ epiclitum	0.032	0.037	0.044	0.035	0.044	0.043	0.081	0.046	0.035	0.038	0.041	0.041	0.044	0.044	0.006	0.026	0.032	0.038											
20	KF791030- Calicophoron_ microbothrioides	0.026	0.029	0.000	0.026	0.035	0.029	0.069	0.040	0.020	0.029	0.032	0.032	0.000	0.000	0.044	0.023	0.032	0.029	0.044										
21	KJ459934 Paramphistomum_cervi	0.026	0.029	0.000	0.026	0.035	0.029	0.069	0.040	0.020	0.029	0.032	0.032	0.000	0.000	0.044	0.023	0.032	0.029	0.044	0.000									
22	KJ459935 Paramphistomum_cervi	0.026	0.035	0.023	0.026	0.029	0.023	0.059	0.026	0.020	0.035	0.026	0.026	0.023	0.023	0.038	0.017	0.029	0.029	0.038	0.023	0.023								
23	KJ459936 Paramphistomum_cervi	0.026	0.035	0.023	0.026	0.029	0.023	0.059	0.026	0.020	0.035	0.026	0.026	0.023	0.023	0.038	0.017	0.029	0.029	0.038	0.023	0.023	0.000							
24	KJ459937 Paramphistomum_cervi	0.026	0.029	0.000	0.026	0.035	0.029	0.069	0.040	0.020	0.029	0.032	0.032	0.000	0.000	0.044	0.023	0.023	0.029	0.044	0.000	0.000	0.023	0.023						
25	KJ459938 Paramphistomum_cervi	0.026	0.029	0.000	0.026	0.035	0.029	0.069	0.040	0.020	0.029	0.032	0.032	0.000	0.000	0.044	0.023	0.023	0.029	0.044	0.000	0.000	0.023	0.023	0.000					
26	KP201674 Calicophoron_ daubneyi	0.031	0.040	0.029	0.031	0.029	0.000	0.075	0.040	0.026	0.037	0.014	0.014	0.017	0.017	0.043	0.023	0.034	0.034	0.043	0.029	0.029	0.023	0.023	0.029	0.029				
27	KP639631 Calicophoron_ microbothrium	0.020	0.029	0.017	0.020	0.017	0.017	0.069	0.034	0.014	0.029	0.014	0.014	0.017	0.017	0.032	0.011	0.017	0.023	0.032	0.017	0.017	0.017	0.017	0.017	0.017	0.017			
28	KP639632 Calicophoron_ microbothrium	0.020	0.029	0.017	0.020	0.017	0.017	0.069	0.034	0.014	0.029	0.014	0.014	0.017	0.017	0.032	0.011	0.017	0.023	0.032	0.017	0.017	0.017	0.017	0.017	0.017	0.017	0.000		
29	KP639637Calicophoron_ microbothrium	0.023	0.026	0.026	0.023	0.020	0.020	0.063	0.029	0.023	0.029	0.017	0.017	0.026	0.026	0.032	0.014	0.023	0.026	0.032	0.026	0.026	0.020	0.020	0.026	0.026	0.020	0.008	0.008	0.008

Table 2. Continued...

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40
30	KP639638Calicophoron_ microbothrium	0.020	0.034	0.017	0.026	0.023	0.017	0.066	0.031	0.014	0.034	0.020	0.020	0.017	0.017	0.038	0.017	0.023	0.029	0.037	0.017	0.017	0.017	0.017	0.017	0.017	0.017	0.006	0.006	0.008											
31	KX840344-Paramphistomum_ epiclitum	0.032	0.038	0.044	0.035	0.044	0.043	0.081	0.046	0.035	0.038	0.041	0.041	0.044	0.044	0.006	0.026	0.032	0.038	0.006	0.044	0.038	0.038	0.038	0.044	0.044	0.043	0.032	0.032	0.032	0.038										
32	KX840345-Paramphistomum_ epiclitum	0.029	0.035	0.041	0.032	0.041	0.040	0.077	0.044	0.032	0.035	0.038	0.038	0.041	0.041	0.008	0.023	0.029	0.035	0.008	0.041	0.035	0.035	0.035	0.041	0.041	0.040	0.029	0.029	0.029	0.035	0.003									
33	LC101682Explanatum_ explanatum	0.035	0.026	0.026	0.000	0.020	0.031	0.065	0.037	0.029	0.026	0.017	0.017	0.026	0.026	0.035	0.020	0.026	0.003	0.035	0.026	0.026	0.026	0.026	0.026	0.026	0.031	0.020	0.020	0.023	0.026	0.035	0.032								
34	LC101683- Explanatum_ explanatum	0.038	0.026	0.029	0.003	0.023	0.034	0.068	0.040	0.032	0.026	0.020	0.020	0.029	0.029	0.038	0.023	0.029	0.006	0.038	0.029	0.029	0.029	0.029	0.029	0.029	0.034	0.023	0.023	0.026	0.029	0.038	0.035	0.003							
35	MK156397- Explanatum_ explanatum	0.035	0.026	0.026	0.000	0.020	0.031	0.065	0.037	0.029	0.026	0.017	0.017	0.026	0.026	0.035	0.020	0.026	0.003	0.035	0.026	0.026	0.026	0.026	0.026	0.026	0.031	0.020	0.020	0.023	0.026	0.035	0.032	0.000	0.003						
36	JX678257-Corylophoron_ corylophorum	0.047	0.050	0.038	0.047	0.050	0.038	0.086	0.049	0.041	0.050	0.047	0.047	0.038	0.038	0.053	0.038	0.044	0.050	0.053	0.038	0.038	0.038	0.038	0.038	0.038	0.038	0.038	0.041	0.041	0.038	0.053	0.050	0.047	0.050	0.047					
37	KF543340_Fasciola_ gigantica	0.438	0.459	0.456	0.460	0.454	0.464	0.494	0.482	0.451	0.458	0.449	0.449	0.456	0.456	0.467	0.469	0.472	0.454	0.466	0.456	0.456	0.468	0.468	0.456	0.456	0.464	0.455	0.455	0.455	0.461	0.467	0.467	0.460	0.459	0.460	0.480				
38	MG569981_Fasciola_ hepatica	0.450	0.471	0.456	0.460	0.455	0.464	0.507	0.494	0.450	0.464	0.449	0.449	0.456	0.456	0.461	0.468	0.478	0.454	0.460	0.456	0.456	0.468	0.468	0.456	0.456	0.464	0.455	0.455	0.467	0.461	0.461	0.461	0.460	0.459	0.460	0.480	0.014			
39	MG569979_Fasciola_ gigantica	0.450	0.470	0.456	0.460	0.454	0.463	0.507	0.494	0.450	0.464	0.449	0.449	0.456	0.456	0.461	0.468	0.478	0.454	0.460	0.456	0.456	0.468	0.468	0.456	0.456	0.463	0.455	0.455	0.467	0.461	0.461	0.461	0.460	0.459	0.460	0.480	0.011	0.003		
40	HQ197358_Fasciola_ gigantica	0.445	0.465	0.463	0.467	0.461	0.470	0.501	0.488	0.457	0.465	0.455	0.455	0.463	0.463	0.461	0.475	0.478	0.461	0.460	0.463	0.463	0.475	0.475	0.463	0.463	0.470	0.461	0.461	0.461	0.467	0.461	0.467	0.466	0.466	0.467	0.486	0.011	0.003	0.008	
41	KF791030.1_ Calicophoron_ microbothrioides	0.026	0.029	0.000	0.026	0.035	0.029	0.069	0.040	0.020	0.029	0.032	0.032	0.000	0.000	0.044	0.023	0.023	0.029	0.044	0.000	0.000	0.023	0.023	0.000	0.000	0.029	0.017	0.017	0.026	0.044	0.041	0.041	0.026	0.029	0.038	0.456	0.456	0.456	0.456	0.463

livestock. Conversely, the most sloping lands were used for sheep or goat grazing. Thus, the availability of stagnant water for the cycle of flukes was also present in those districts.

The higher frequency of parasites in the adult category were in agreement with the findings of previous reports (GONZÁLEZ-WARLETA et al., 2013; SANCHÍS et al., 2013), and can be explained by longer exposure to the infections, to the amount of time the parasite takes to arrive to the forestomach and the fluke's longevity. Given the protocols followed at the slaughterhouse, and the practical difficulties associated with them, it was not possible to analyze the other segments of the digestive tract when searching for the presence of larvae. Thus, our results are narrowed to adult worms.

Taxonomically, our samples can be distinguished from the *Paramphistomum* Fischoeder, 1901 genus, as the testes are slightly lobulated or smooth in *Paramphistomum*, which contrasts with our samples, which were deeply lobulated. *Paramphistomum* also lacks an esophageal bulb, a calicophoron-type pharynx, and a pisum-type acetabulum (EDUARDO, 1982, 1983; JONES, 2005). *Paramphistomum* also has a liorchis-type pharynx and paramphistomum-type acetabulum

The present species can be distinctive from other ruminal fluke genus, *Cotylophoron* Stiles & Goldberger, 1910, because *Cotylophoron* has a true genital sucker and a cotylophoron-type acetabulum. Regarding species of *Calicophoron*, flukes herein reported can be differentiated from: *C. calicophorum* because the latter species has a tegument devoid of papillae around genital pore; *C. bothriophoron* because it has a deeply depression around genital pore and lacks of esophageal bulb; *C. microbothrium* because it has a paramphistomum-type acetabulum and a non-prominent swelling around genital pore, and lacks of esophageal bulb; *C. papillosum* because it has a calicophoron-type acetabulum and tegumental papillae only around oral opening and wall of genital atrium, *C. papillosum* also lacks of esophageal bulb; *C. papilligerum* because its caeca reach about middle section of acetabulum and is devoid of tegumental papillae; *C. raja* because it has smaller papillae around genital pore than oral opening and its esophagus lacks of bulb; *C. clavula* because it has a paramphistomum-type acetabulum and a reduced number of smaller papillae around genital pore compared to oral opening, and lacks of esophageal bulb; *C. sukari* because it has a non-prominent swelling around genital pore, calicophoron-type acetabulum, oesophagus with no bulb and vitellaria confluent in their anterior limits; *C. phillerouxi* because it has papillae only on surface around oral opening, non-prominent swelling around genital pore, paramphistomum-type acetabulum, and lacks of esophageal bulb; *C. daubneyi* because it has papillae around oral opening and no papillae around genital pore, paramphistomum-type acetabulum, and lacks of esophageal bulb; and, finally, from *C. sukumum* because it has a small swelling around genital pore devoid of papillae and lacks of esophageal bulb (EDUARDO, 1982, 1983; JONES, 2005)

The phylogenetic tree allowed us to verify that the ITS-2 segment was a good marker for the species (isolates of the same species tend to be grouped together), but not for genera (species of the same genus were not grouped together in the same branch). In a closer view, as mentioned, our sequences were 100% identical to three isolates of *P. cervi* and four isolates of *C. microbothrioides* available in GenBank.

Therefore, following morphological analysis, and in concert with the molecular analysis, the flukes were classified as *C. microbothrioides*.

We conducted an additional exploration to determine additional taxonomical commentaries in Zheng et al. (2014), the study that published on *P. cervi* and whose sequences were identical to those in the present study. Upon further exploration, we did not find information about their identification. The authors also published *P. cervi* sequences that diverged from our samples. The supposition that observed distances are real distances means that a lack of mutations in the studied segment may explain the presence of two genera in the same clade. However, the real distance could be longer than the observed distance, in such a way that divergent mutations could take place first, and with convergent ones taking place later; thus, identical sequences are observed. In addition, misclassifications of paramphistomids have been reported previously in the literature (SANABRIA et al., 2009). All above underline the need of including molecular and morphological approach together in order to have a more precise classification of these parasites.

There were no published articles (with an associated PMID code) for the sequence of *C. microbothrioides* in the record of this sequence. Therefore, the morphological analysis allowed us to finally determine the species.

It is difficult to establish the economic importance of *C. microbothrioides* since studies in this species are scarce. Other species of *Calicophoron* as *C. daubneyi* are more studied. For instance, animals with single infection of adult *C. daubneyi* showed lower cold carcass weight and lower fat class than helminth-free animals (BELLET et al., 2016). The higher abundance of *C. daubneyi* has been also associated with higher frequency of thin or watery fecal consistence and lower frequency of normal feces (MALRAIT et al., 2015). On the other hand, the intensity of infection with another *Calicophoron*, *C. microbothrium*, was associated with severity of lesions in the abomasal folds, comprising edema, as well as in the duodenum, comprising duodenal thickening, corrugation, hyperemia, petechiation, ulceration, callous atrophy, hyperplasia of Brunner's glands and Peyer's patches, and infiltration of eosinophils, mast cells, basophils and lymphocytes in the lamina propria (MAVENYENGWA et al., 2005). Although there is no knowledge about the economic impact of *C. microbothrioides*, the mentioned damage of other *Calicophoron* species suggest that it is important to develop control strategies. The lack of knowledge also underlines the need of developing new research about the pathology and the production and economic loses because *C. microbothrioides* in Chile.

This research represents the first report of *C. microbothrioides* in Chile. As mentioned, only one case of paramphistomid infection was reported in Chile previously and was documented in a bull that was diagnosed shortly after the arrival. The species *Cotylophoron cotylophorum* was identified in that case (GONZÁLEZ & PLAZA, 1966). It is interesting that in Chile, to the best of authors' knowledge, there is no report of eggs belonging to Paramphistomidae. It is possible that a misidentification could have happened, confusing them with *F. hepatica*. Worldwide reports of *C. microbothrioides* are scarce. For instance, this species was previously reported in Malaysia (LEE et al., 1987), the United

States (HERD & HULL, 1981; ZUKOWSKI et al., 1993), and Canada (BOUVRY & RAU, 1984). The literature also mentions the presence of this species in Europe, encompassing Bulgaria, the Czech Republic, and Hungary (FAUNA-EUROPAEA, 2019). There are no reports of *C. microbothrioides* in published papers in South America; the only precedents correspond to two records of this species from Peru in GenBank.

Future studies must be focused on exploring the economic importance of this species, and on determining the pathological damage to the host, since these consequences seem to differ in various regions (FOSTER et al., 2008; DORNY et al., 2011; ZINTL et al., 2014) and in the different paramphistomid species (See literature reviewed in ZINTL et al., 2014). An important intermediate host for ruminal flukes (*C. daubneyi*) in Europa, *Galba truncatula* (AUGOT et al., 1996; DREYFUSS et al., 2014), is also present in Chile (ARTIGAS et al., 2011), and one of the localities reported to harbor this snail, Chillán, is in the region where this study was performed, in such a way that *G. truncatula* can be an important intermediate host for this fluke. Future studies must also focus on the cycle of this parasite species, particularly to confirm *G. truncatula* as an intermediate host and to search for other possible intermediate hosts. A deeper knowledge of the intermediate host could guide control strategies.

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