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Spatial Pattern of Genetic Diversity in the Blood Fluke *Aporocotyle argentinensis* (Digenea, Aporocotylidae) from South American Hakes (Pisces: Merluccidae)

Marcelo E. Oliva [1,2,*], Leyla Cárdenas [3,4], Isabel M. Valdivia [5], Paulina Bruning [4,6], Luis Figueroa-Fabrega [5] and Rubén Escribano [2]

1 Instituto Ciencias Naturales Alexander von Humboldt, Universidad de Antofagasta, Antofagasta 1270300, Chile
2 Millenium Institute of Oceanography, Universidad de Concepción, Concepción 4070386, Chile
3 Instituto de Ciencias Ambientales y Evolutivas, Facultad de Ciencias, Universidad Austral de Chile, Valdivia 5110566, Chile
4 Centro FONDAP de Investigación en Dinámica de Ecosistemas Marinos de Altas Latitudes (IDEAL), Valdivia 5090000, Chile
5 Laboratorio de Estudios Ecosistémicos—LECOS, Escuela de Ingeniería y Negocios, Universidad Viña del Mar, Viña del Mar 2520000, Chile
6 Ocean, Department of Biology, Université Laval, Québec, QC G1V 0A6, Canada
* Correspondence: marcelo.oliva@uantof.cl; Tel.: +56-55-263-7404

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Abstract: Distribution of blood fluke *Aporocotyle* spp. parasitizing *Merluccius* species from the coasts of South America (Peru, Chile and Argentina) constitutes an excellent opportunity to evaluate the geographical amplitude in which a parasite can exploit the same host species. Phylogenetic analyses (partial sequences of SSU rDNA, LSU rDNa, and *cox1* gene) were performed to characterize the genetic lineage of *Aporocotyle* species described from South American Hake: *Merluccius australis*, *M. gayi*, and *M. hubbsi.* The Phylogenetic analyses (SSUrDNA and LSUrDNA) revealed an absence of genetic variability in *Aporocotyle* obtained over a gradient of 6800 km, covering two oceans and three closely related hosts. Consequently, the species infecting *Merluccius* spp. in South America is *Aporocotyle argentinensis* Smith 1969, by priority law. Phylogeographic analysis suggests a pattern of spatial differentiation and genetic population structure associated with the geographical distribution of the host's species. A specimen with a haplotype found in *M. gayi* was collected from *M. australis* from Puerto Montt, and three worms (from Coquimbo, Constitución and Talcahuano, host *M. gayi*) harbored a haplotype found in *M. australis* + *M. hubbsi*, suggesting that the gene flow between different hosts and geographical distributions occurs when the distribution of adequate hosts overlaps, avoiding speciation in blood flukes from South American hakes.

Keywords: phylogeography; genetic lineage; SSU rDNA gene; LSU rDNA gene; *cox1* gene; spatial differentiation; genetic population structure; host induced variability

1. Introduction

Systematic parasitology is traditionally based on morphological traits. However, problems that potentially confound the use of morphology in parasites include the challenges of consistent specimen preservation, plasticity of features depending on hosts or other environmental factors, and morphological convergence [1]. Molecular markers can be excellent tools to show the actual level of biodiversity in parasites [2]. The use of these tools in parasite systematics revealed the existence of cryptic species, i.e., two or more distinct species that are erroneously classified (and hidden) under one species name [3]. By contrast, lineages identified as independent species can be correctly recognized as synonymous [4,5]. As a consequence, the establishment of the actual number of species in a given host-parasite

system is an urgent requirement for a theoretical framework of the study of diversity, ecology, evolution, and co-speciation in parasites.

Eighteen species are described in the genus *Aporocotyle* (Digenea: Aporocotylidae Odhner, 1912) [6] that infect the heart, bulbous arteriosus, and blood vessels of marine fishes of five teleost orders (Gadiformes, Ophidiiformes, Perciformes, Pleuronectiformes, and Scorpaeniformes) from the Atlantic, Pacific, Antarctic, and Indian oceans and the Japan and Baltic seas. Notably, five of the species are described from five species of *Merluccius*: *Aporocotyle spinosicanalis* from *M. merluccius; A. argentinensis* from *M. hubbsi*; *A. margolisi* from *M. productus*; *A. wilhelmi* from *M. gayi*; and *A. australis* from *M. australis* (Gadiformes). *Aporocotyle* species parasitizing hakes worldwide are apparently highly host specific. Recently, a possible cospeciation of members of *Aporocotyle* with their hosts was suggested, at least for members of the genus *Merluccius* [6]. In addition, of the 18 recognized species in the genus, only two are registered from more than one host species: *Aporocotyle simplex* in three species of flatfishes of the subfamily Pleuronectinae [7] and *Aporocotyle garciai* described from two Ophiididae: *Genypterus* sp. from Perú and *Hoplobrotula armata* from Japan [8,9].

Aporocotyle spp. parasitizing *Merluccius* species from the Pacific and Atlantic coasts of South America (Peru, Chile, and Argentina) constitute an excellent opportunity to evaluate the actual level of genetic variability in a marine parasite and the geographical amplitude in which a parasite can exploit the same host species. Along the Pacific coast of South America, two species of hake are found: *Merluccius gayi* with two populations, a northern population (Peruvian hake) from the Gulf of Guayaquil to central Peru [10] and a southern population (Chilean hake) from northern to southern Chile; this hake is the host for *Aporocotyle wilhelmi* Villalba and Fernández, 1986. The second species, *Merluccius australis*, is found in southern Chile, overlapping with *M. gayi*, and is the host for *Aporocotyle australis* Fernández and Durán, 1985 (Figure 1A). Along the Atlantic coast of South America, *M. australis* reached as north as ≈40 °S, whereas a second species from the Atlantic, *Merluccius hubbsi*, overlap in the northern limit of distribution of *M. australis* in the Atlantic (Figure 1A). *M. hubbsi* is the host for *A. argentinensis* Smith, 1969.

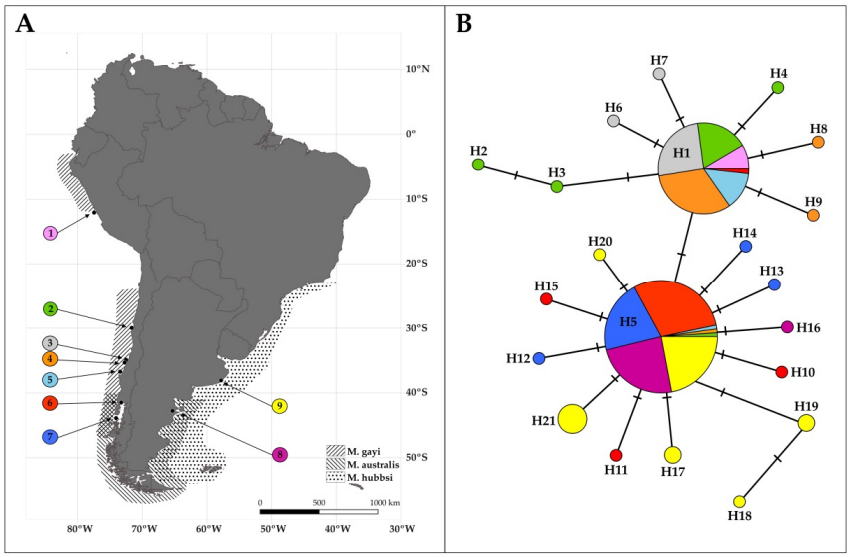

Figure 1. Host distribution in South America (dashed area) (**A**) and mitochondrial *cox1* haplotype network showing the 21 haplotypes identified in three species of hakes (**B**). Haplotypes are colored by the locality where the host were obtained. Size of circles is proportional to the number of individuals showing that haplotype. Code for localities: 1 = Callao, 2 = Coquimbo, 3 = Duao, 4 = Constitución, 5 = Talcahuano, 6 = Puerto Montt, 7 = Guaitecas, 8 = Puerto Madryn, 9 = Mar del Plata.

Our goals were to characterize the phylogenetic relationship among *Aporocotyle* species in three *Merluccius* species (*M. hubbsi*, *M. gayi*, and *M. australis*) from the Atlantic (Argentina and Falkland Islands/Islas Malvinas) and Pacific coasts (Chile and Perú) of South America, based on partial sequences of two molecular markers (SSU rDNA and LSU rDNA), and to evaluate the spatial distribution of the identified lineages of *Aporocotyle* in South American hakes, based on *cox1* gene.

2. Materials and Methods

Samples

In total, 331 partial sequences (98 SSU rDNA, 57 LSU rDNA, and 176 *cox1*) belonging to three nominal species of *Aporocotyle* were analyzed. The parasites were obtained for hakes *M. gayi* and *M. australis* from Chile (six localities), *M. gayi* from Peru (one locality), *M. australis* and *M. hubbsi* from Argentina (two localities), and *M. hubbsi* from Falkland Islands/Islas Malvinas (Table 1, Figure 1A). Fish were obtained from commercial catches of hakes. To extract genomic DNA, a DNA E.Z.N.A kit (Omega Bio-Tek, Inc., Atlanta, GA, USA) was used. Specimens were sequenced and amplified using described protocols for SSU rDNA, LSU rDNA, and *cox1* gene [11–13]. The PCR products were purified using a PCR E.Z.N.A. Cycle Pure Kit (Omega Bio-Tek, Atlanta, GA, USA) and sequenced in an automated capillary electrophoresis sequencer ABI 3730XL (Macrogen Inc., Seoul, Korea). To minimize sequencing errors, both strands were sequenced from all genes for each individual sample. All new sequences were deposited in Genbank (Accession codes available in Supplementary Material Table S1). Sequences were edited and aligned using Geneious 2020.2.3 (https://www.geneious.com, accessed on 2 June 2022) [14].

Table 1. The South American species of the genus *Merluccius* studied, locality, geographic coordinates, and host sample size.

Host	Locality	S	W	N
Merluccius gayi	Callao (1)	12°03′50″	77°08′58″	10
	Coquimbo (2)	29°57′37″	71°20′19″	20
	Duao (2)	34°53′58″	72°10′59″	15
	Constitución (2)	35°18′59″	72°25′43″	20
	Talcahuano (2)	36°43′52″	73°07′39″	110
Merluccius australis	Puerto Montt (2)	41°28′39″	72°56′44″	45
	Guaitecas Island (2)	43°52′43″	73°44′55″	38
	Puerto Madryn (3)	42°46′10″	65°01′18″	20
Merluccius hubbsi	Mar del Plata (3)	38°04′25″	57°30′50″	5
	Falkland Islands/Islas Malvinas (4)	51°40′58″	57°40′44″	12

1: Perú, 2: Chile; 3: Argentina, 4: UK/Argentina.

For the phylogenetic analyses, sequences available at GenBank for members of *Aporocotyle* were included, and sequences from *Psettarium nolani* (Aporocotylidae) were used as the external group (Supplementary Material Table S1).

The phylogenetic trees for nuclear genes were inferred by the maximum likelihood (ML) criteria using MEGA v. 7 [15], and the HKY model yielded the best fit for the three genes [16]. To assess the support for individual nodes, a bootstrap (1000 replicates) analysis was performed. The phylogenetic trees were inferred by Bayesian inference (BI; MrBayes v3.2) [17] and were conducted applying Markov Chains and running 10,000,000 generations, sampling each of 200 generations. Trees for ML and BI show the same topology.

Bayesian phylogenetic analyses were conducted using four simultaneous Markov Chains. The first 25% generations (burning) were discarded.

To analyze the spatial genetic structure of *Aporocotyle* from South American hakes, a phylogeographic analysis using *cox1* partial sequences was performed. Arlequin v. 3.11 [18] allows the calculation of the number of haplotypes, number of polymorphic sites, haplotype diversity, nucleotide diversity, as well as a hierarchical analysis of molecular variance (Table 2). Finally, the genealogical relationships among haplotypes were assessed with a haplotype network (Figure 1B) constructed using a median-joining algorithm,

as implemented in network 4.201 [19]. We applied a maximum parsimony algorithm to simplify the complex branching pattern and to represent all the most parsimonious intraspecific phylogenies [20].

Table 2. Mitochondrial *cox1* diversity for *Aporocotyle* by host and locality. N_p = number of parasites analyzed; N_{hap} = number of haplotypes; S = number of polymorphic sites; He = haplotype diversity; π = nucleotide diversity; k = mean number of pairwise differences. Standard deviations (SD) are also given.

Host	Location	N_p	N_{hap}	S	He ± SD	π ± SD	k ± SD
M. gayi	Callao	5	1	0	0	0	0
	Coquimbo	15	5	4	0.48 ± 0.15	0.00115 ± 0.00099	0.7809 ± 0.6012
	Duao	17	3	2	0.23 ± 0.13	0.00035 ± 0.00047	0.2353 ± 0.2857
	Constitución	22	4	3	0.26 ± 0.12	0.00040 ± 0.00051	0.2727 ± 0.3076
	Talcahuano	9	2	1	0.22 ± 0.17	0.000328 ± 0.00048	0.2222 ± 0.2880
Total		68	9	9	0.27 ± 0.07	0.00052 ± 0.00057	0.3443 ± 0.34692
M. australis	Puerto Montt	31	5	4	0.25 ± 0.10	0.00038 ± 0.00049	0.2581 ± 0.2948
	Guaitecas Islands	22	4	3	0.26 ± 0.12	0.0004 ± 0.00051	0.2727 ± 0.3076
	Puerto Madryn	23	2	1	0.09 ± 0.08	0.00013 ± 0.00027	0.0869± 0.1640
Total		76	9	8	0.20 ± 0.06	0.00031 ± 0.00042	0.2105 ± 0.2586
M. hubbsi	Mar del Plata	32	6	4	0.58 ± 0.09	0.00108 ± 0.00092	0.7278 ± 0.5571
Whole data set		176	21	18	0.62 ± 0.03	0.04369 ± 0.03532	0.7865 ± 0.5745

3. Results

For the SSU rDNA, a fragment of 411 bp was sequenced from each one of the 98 analyzed specimens. The tree reconstruction, including *A. spinosicanalis* (Figure 2A), a parasite of the European hake *Merluccius merluccius*, showed a unique clade that included all specimens from the three hosts and all studied localities, with a bootstrap support of 100% for ML and 1 for BI. The absence of genetic variation from worms in the three host's species strongly supports that the three described species of *Aporocotyle* from *Merluccius* spp. along the South American coast constitute a unique genetic lineage (Figure 2), but the ML support for the clade of *Aporocotyle* from South American hakes shows a weak support (ML = 70%), but it was fully supported by BI (PP = 1).

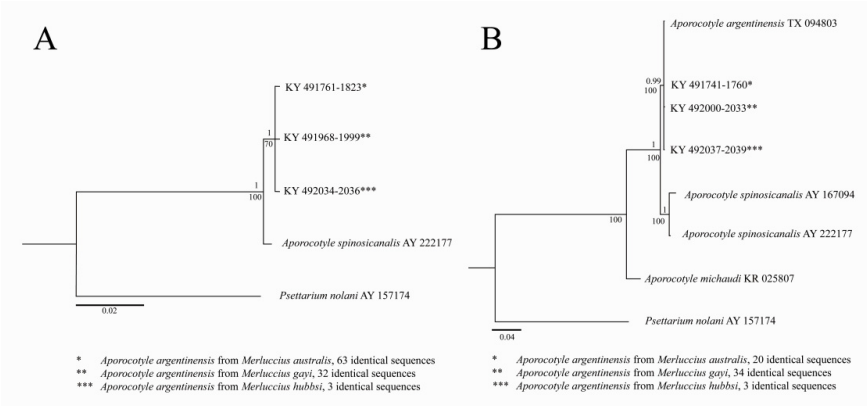

Figure 2. Molecular phylogeny of *Aporocotyle* spp. parasitizing *Merluccius* spp. from South America based on the SSU rDNA gene (**A**) and LSU rDNA gene (**B**). BI support value above and ML support below the node.

For the LSU rDNA gene, a fragment of 913 bp was sequenced from 57 specimens of *Aporocotyle* spp. Sequences of LSU rDNA genes for three *Aporocotyle* species available in Genbank were incorporated in the analysis. The topology of the phylogenetic tree reconstruction (Figure 2B) was consistent with the SSU rDNA phylogenetic tree, node support was always 100% (ML), and posterior probability (PP) = 1 (BI). The genetic distance between our samples and *A. argentinensis* (GenBank JX094803) was 0.0%.

For the *cox1* gene, a fragment of 677 bp was sequenced from 176 specimens of *Aporocotyle* spp.

The haplotype network, based on *cox1* partial sequences, showed a clear pattern in the spatial distribution of the genetic lineages of *Aporocotyle* (Figure 1B). Haplotype H1 was found primarily, at higher frequency, in worms from *M. gayi*, but one worm collected from *M. australis* at Puerto Montt also showed this haplotype. The frequency of haplotype H5 was high in worms from *M. australis* and *M. hubbsi*; however, three individuals collected from *M. gayi* showed this haplotype, one each from Coquimbo, Talcahuano, and Constitution. From each one of the primary haplotypes, an upsurge of low frequency haplotypes was observed, distant by only one mutation step. Some haplotypes from *M. hubbsi* differed in one mutational step from those from *M. australis*.

In summary, geographically, the haplotype relationships among parasites showed a concordant pattern with the current distribution of the host species but also suggested a genetic connectivity across regions (Figure 1B).

The intraspecific genetic variation was analyzed using a data set of partial sequences of *cox1* and showed a clear pattern of spatial differentiation with a global FST of 0.63 (p-value < 0.0001). The pairwise FST analysis (Table 3) revealed that worms parasitizing *M. australis* from southern Chile (Puerto Montt, Guaitecas, Aysén) and Argentina (Puerto Madryn) and *M. hubbsi* from Mar del Plata do not differ significantly. However, worms parasitizing populations of *M. gayi* from Chile (Coquimbo, Duao, Constitución and Talcahuano) and those from Peru (Callao) were genetically similar. Both geographical groups were coincident with the spatial distribution of the host species, with values of pairwise FST among them ranging from 0.437 to 0.928 (Table 3).

Table 3. Pairwise FST analysis among sampled localities (below diagonal). *P*-values above the diagonal. Significant values (p < 0.001 Bonferroni corrections) after 1000 permutations. Code for localities: 1 = Callao, 2 = Coquimbo, 3 = Duao, 4 = Constitución, 5 = Talcahuano, 6 = Puerto Montt, 7 = Guaitecas, 8 = Puerto Madryn, 9 = Mar del Plata.

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
1		0.524	0.999	0.999	0.999	0.000	0.000	0.000	0.000
2	−0.014		0.394	0.418	0.762	0.000	0.000	0.000	0.000
3	−0.078	0.005		0.999	0.999	0.000	0.000	0.000	0.000
4	−0.071	−0.006	−0.025		0.999	0.000	0.000	0.000	0.000
5	−0.078	−0.039	−0.044	−0.064		0.000	0.000	0.000	0.000
6	0.792	0.638	0.754	0.730	0.727		0.999	0.389	0.006
7	0.796	0.625	0.754	0.730	0.726	−0.018		0.363	0.031
8	0.928	0.735	0.852	0.821	0.858	−0.0004	0.004		0.003
9	0.561	0.437	0.561	0.548	0.502	0.091	0.075	0.146	

The substitution saturation test [21], implemented in DAMBE V 7.3.11 software, showed no evidence of saturation substitution in any of the studied genes.

4. Discussion

The described species of *Aporocotyle* from hakes of South America are *A. argentinensis*, a parasite of *M. hubbsi* from Argentina, *A. australis* from *M. australis* in southern Chile, and *A. wilhelmi* from *M. gayi* caught in Concepcion Bay, central Chile [6]. Our results, obtained from samples along a geographical gradient of approximately 6800 km along the Pacific and Atlantic coast of South America, including the Falkland Islands/Islas Malvinas, were

highly consistent and supported the presence of a unique genetic lineage, that by priority law should correspond to *A. argentinensis*. No variation was found in the phylogenetic analysis of both nuclear genes for samples of *Aporocotyle* parasitizing the three hake species, *M. gayi*, *M. australis*, and *M. hubbsi*. Recently [22], the analysis of 252 studies, published between 2011 to 2015 and regarding the molecular approach to trematode systematics, showed that ribosomal RNA (rRNA) genes (LSU, SSU, ITS 1, and ITS 2) were widely used in taxonomy, life cycle studies, and species diagnosis.

The existence of cryptic species in Digenea, i.e., species morphologically indistinguishable but genetically different, is well documented [5], mainly due the development of molecular tools. The report of host-induced variability, impact of the site of infection in the host, as well as the effect of intensity of infection are also well documented [23], but proof of genetic identity are rare, and our results support the argument that apparent host specificity is not a reliable criterion to delineate species [5].

The pattern of genetic similarity on a wide geographic scale was reported and linked to marine species that have a high dispersal potential [24]. The absence of genetic difference in the Aporocotylid *Cardicola forsteri* parasitizing two related tuna from Australia and Mexico (*Thunnus maccoyi* from a wild population at Cabbage Patch, South Australia and *Thunnus thynus* from a farm in Spain (Mediterranean sea)) was demonstrated [25], and in a similar way, the absence of genetic and morphological variability in some species of Digenea from the Barrier Coral Reef (Australia) and French Polynesia, 6000 km apart, have been described [26]. Along the Southeastern Pacific, no genetic variability was found in *Proctoeces humboldti* (as *Proctoeces* cf. *lintoni*) from two localities 2000 km apart [27].

For the mitochondrial *cox1* gene, we recovered two central haplotypes separated by one mutational step. These haplotypes were more frequently founded in a spatial scale associated with the geographical distribution of hakes, but it is important to note that some *Aporocotyle* specimens collected from *M. gayi* shared the same haplotype with those specimens collected from *M. australis* in Puerto Montt (see Figure 1B) where the two host species overlap. As suggested [6], body size, length of the esophagus, anterior and posterior caeca, size of the cirrus sac, and size of the ovary among other metrics are affected by the host distribution and, consequently, are of doubtful value in the taxonomy of *Aporocotyle*. The ratio between esophagus length/total length and testis number was defined among the taxonomic characters considered in the description of the Chilean species of *Aporocotyle* and in the definition of their evolutionary series [28]. However, it was demonstrated that this ratio changes allometrically during the life span, at least for *Aporocotyle simplex*, and the number of testis is also a taxonomic character of questionable relevance because the number is highly variable and the degeneration of testis in larger specimens is well documented [29]. Our results strongly suggest that the described species of *Aporocotyle* from South American hakes belong to a single species, *Aporocotyle argentinensis*, and are a new evidence of the usefulness of molecular tools to obtain the correct species diagnosis in digenean parasites [30].

The distribution of each host species is definitively narrower than the parasite distribution. This does not explain the absence of genetic variability for SSU rDNA and LSU rDNA, but the gene flow can be explained for the overlap of the geographic distribution of *M. hubbsi* and *M. australis* in the South Atlantic Ocean and between *M. australis* and *Merluccius gayi* in the South Pacific Ocean. It is important to note that the nuclear markers used here may not be variable enough, but the addition of the mitochondrial gene *cox1* to the analysis supported the results of similarity. Although, it also showed evidence of a trend of an incipient speciation process that is not backed up statistically.

Parasites normally have shorter generational times than their hosts; therefore, genetic differentiation and demographic changes may be detected sooner in parasites because more mutations are fixed over time, leading to a more rapid lineage sorting [31].

Although the studied hosts are well resolved species, members of *Aporocotyle* from South American hake did not follow a similar pattern, suggesting that genetic diversification in parasites responds not only to the evolutionary history of their definitive hosts

but also intermediate hosts [32]. Additional factors such as complexity in the life cycle and environmental factors are also important. An interesting result was given by the significant differences caused by samples from Mar del Plata (Table 3).This zone corresponds to a transitional biogeographical area that responds to the particular oceanographic patterns where the oceanographic front of Peninsula Valdes influences the diversity of fish species [33,34], which could be reflected in the phylogeographic patter we found. In other words, specimens from Mar del Plata have a spatial genetic structure different from the other localities.

At the population level, the mitochondrial *cox1* revealed the occurrence of low gene flow, supported by high values of FST, between parasite populations of the three host's species, probably preventing the generation of isolated new lineages. In addition, for the *cox1* gene, we recovered two shared haplotypes, separated by one mutational step. These haplotypes are more frequently found in a spatial scale associated with the geographical distribution of hakes, but it is important to note that some *Aporocotyle* specimens collected from *M. gayi* shared the same haplotype with those specimens collected in *M. australis*.

A highly mobile host is a potential explanation for the maintenance of gene flow among parasite populations, in addition to a wide geographical distribution for the definitive and intermediate hosts. Moreover, in members of *Aporocotyle*, the absence of a second intermediate host and the direct infection of the definitive host by the cercarial stage [35,36] eliminate the trophic link between the second intermediate host and its definitive host. The intermediate host for *Aporocotyle* spp. parasitizing hakes in South America are unknown, but terebellid polychaetes has been considered the major host group for marine aporocotylids [35].

Our results emphasize the importance of overlap in geographic host distributions, as a force that can explain the spatial distribution of the genetic diversity in a blood parasite (*Aporocotyle* spp.) in different biogeographical regions. We showed the importance of studying genetic identity for morphologically different morphotypes associated with parasites of different but closely related host species. Consequently, apparent host specificity, in some cases, is not a reliable criterion to delineate species [5].

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/d14090772/s1, Table S1: Sequences for gene SSU rDNA, LSU r DNA and *cox 1* included in this study. References [6,37–41] are cited in supplementary material.

Author Contributions: M.E.O., L.C. and I.M.V. conceived and designed the study; M.E.O., I.M.V., L.F.-F. and R.E. carried out the field work; L.C., I.M.V. and P.B. performed molecular analyses. Additional analyses were performed by R.E. and M.E.O.; M.E.O., L.C. and I.M.V. wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: This study did not consider experiments with live animals. All fishes were obtained from commercial catches and none of the species are subject to conservation measures. Commercial fishermen follow national regulations concerning these fisheries.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data available as supplementary material.

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