FINAL TECHNICAL REPORT – PGTF INT21K09

Technological Innovation Network in the study of parasitic diseases caused by helminths: genetic and genomic characterization with a focus on human and animal health

Parasitic helminths represent a huge burden on human development due to their significant impact on human health and animal production. Helminth parasites are the most neglected infectious agents in humans in developing countries and cause a greater global health impact than other commonly studied diseases (https://www.who.int/health-topics/). For this reason, the project focus on species of neglected studied helmiths. In our region, we have a health problem with the parasite *Dioctophyme renale* (Eiras *et al*., 2021), commonly known as the "giant kidney worm," and considered the largest parasitic nematode described in terrestrial vertebrates. *Dioctophyme renale* (Goeze, 1782) is a zoonotic parasitic nematode that infects the kidneys of mammals, mainly carnivores. This parasite has a worldwide distribution and has been observed in various species, both domestic and wild. In dogs, the infection usually involves the right kidney often causing unilateral loss of the organ, and in some cases bilateral involvement can result in total renal failure. In addition, blockage of the ureters or renal pelvis by adult worms may result in hydronephrosis. Adult worms can also be found in the abdominal cavity, subcutaneous tissue and other organs such as the uterus and ovary (Khullar et al.,2022). Humans may be incidental hosts and develop unspecific clinical symptoms including back pain, fever, weight loss, urinary retention, hematuria and pyuria. Fatalities are rare but have been reported in extreme cases due to renal failure, sepsis, or coexisting medical conditions (Li et al., 2010; Norouzi et al., 2017; Khullar et al., 2022). The life cycle described by Mace and Anderson (1975) involves an oligochaete (Lumbriculus variegatus) in which *D. renale* develops the infective stage. This invertebrate presents a Holarctic distribution, with only two reports in South America: one in Argentine Patagonia (Miserendino, 2007) and another in Minas Gerais, Brazil (Marchese et al., 2015), and no evidence of the presence of larval stages of *D. renale*. Therefore, the life cycle outside the definitive host is unknown for this region. Different biogeographical units converge in the region spanning northeastern Argentina and southern Brazil, specifically the sub-Brazilian domain, Paranaense and Chaco domains. These domains, which encompass more than 16 biogeographical districts host a variety of ecosystems including swamps and marshes, fields and scrubland, pampas, gallery forests, espinal, Chaco Forest, and Parana jungle (Pereyra, 2003). Cities, rural villages and protected natural areas, all communities with different levels of anthropic influence coexist in this area and animals affected by dioctophymosis can be observed in all these systems. With regard to urban areas the most common domestic host is the dog with prevalences ranging between 0.03 and 35.3% (Burgos et al. 2008, Radman et al., 2017). In the La Plata River riparian area, a prevalence of 42.1% is reported (Burgos et al., 2015). In addition, there are also reports of *D. renale* in cats (Pedrassani 2014, 2017, Butti 2019). Regarding wild ecosystems, we recently reported the first molecular characterization of *D. renale* in the pampas fox (*Lycalopex gymnocercus*) (Facelli et al. 2024). Also, there are reports of cases in other wild mammals including maned wolf (*Chrysocyon brachyurus*), Geoffroy's cat (*Leopardus geoffroyi*), Neotropical otter (*Lontra longicaudis*); bush dog (*Speothos venaticus*), crab-eating fox (*Cerdocyon thous*), little grison (*Galictis cuja*), coati (*Nasua nasua*), capuchin monkey (*Cebus apella*), two-toed sloth (*Choloepus hoffmanni*), among others (Khullar et al., 2022; Trindade et al., 2018; Etchenique et al.,

2018; Measures, 2008; Pinto et al., 2011). High prevalence of this parasitosis was observed in the species maned wolf, little grison and coati, with levels of 81.2%, 36.6% and 72.4%, respectively (Eiras et al., 2021). In maned wolf, as in dogs, most parasites have been found in the right kidney in (85.7% of cases), followed by the abdominal cavity (28.6%). On the other hand, in coatis the most common site where the worms were found is the abdominal cavity, accounting for 66.66% of the cases (Milanelo et al., 2009). In general, there are no clinical signs associated with this infection (Di Nucci et al., 2020; Mattos Varzone et al., 2008). However, the actual impact of the parasite on natural populations remains poorly understood. Currently, there is no information on whether domestic and wild mammals in this region are infected by the same phylogenetic lineages or whether there are host- and/or site-specific genetic variants. In addition, there is very little sequence information about this parasite in databases. *D. renale* belongs to Clade I within the Phylum Nematoda (Blaxter et al. 2015, Koehler et al. 2009), a group which is underrepresented in terms of molecular information. Clade I genome information is only available for a few species of the genera *Trichinella*, *Trichuris*, *Romanomermis* and *Sobolophyme* (Wormbase Parasite), with no genomic data for *Dioctophyme renale*. In GenBank (https://www.ncbi.nlm.nih.gov/) there are only 27 entries for *D. renale* in the nucleotide database corresponding to three genes: small subunit ribosomal RNA, Dorylipophorin (a novel lipid binding protein) and mitochondrial cytochrome c oxidase subunit I (COX1). The limited molecular knowledge about this parasite hampers its identification, genetic characterization, outbreak monitoring, and studies of host species interactions. In this work, we utilize genomic data to develop new molecular markers for investigating the population dynamics of *D. renale*, a prevalent zoonotic parasite, whose life cycle remains unknown in southern South America.

Results and Discussion

Aim 1) Sequencing of parasite genomes

In Argentina, we have established the first long-read sequencing platform applied to One Health at one of the most prestigious universities in the country, the University of Buenos Aires, operating within the Faculty of Exact Sciences (Figure 1). This platform allowed us, among other things, to implement a genome skimming strategy. Genome skimming is proposed as a low-cost and robust strategy to assemble mitogenomes from difficult to obtain specimens. Despite the potential of mitogenomics in phylogenetics, this is still an under-explored area for taxonomic groups without commercial relevance and non-model organisms. *Dioctophyme renale* is the largest parasitic nematode of terrestrial vertebrates described so far and affects domestic and wild animals — some threatened — and humans. *D. renale* belongs to Clade I, a taxonomic group underrepresented with respect to available genomic data. We have sequenced gDNA of *D. renale* with Nanopore Technologies using rapid and ligation kits. We obtained the first draft mitochondrial assembly of this pathogen worm using a low-coverage genome skimming strategy.

Figure 1. Sequencing workflow. Helminth parasites from infected animals are isolated and high-quality DNA is purified. Sequencing is performed on MinION or PromethION devices depending on the expected genome size and the number of samples. Genomes are assembled and analyzed by comparative genomics bioinformatic strategies

Domestic and wild carnivores from Argentina and Brazil are definitive host of *D. renale*

A total of 73 adult *D. renale* samples were collected from domestic and wild carnivores from different regions of Argentina and Brazil (Table 1). The most common host were (*Canis lupus familiaris),* from which 54 adult parasites were collected. Three additional samples were isolated from cats (*Felis catus*), totaling 57 adult parasites from domestic host species. Among wild carnivores, the maned wolf (*C. brachyurus* Fam. Canidae*)* was the most frequent host, with 13 samples isolated. Also, adult parasites were collected from lesser grison (*Galictis cuja*, Fam. Mustelidae) and pampas fox (*L. gymnocercus*, *Fam. Canidae*), totaling 16 adult parasites from wild species (Table 1). In Argentina, we collected samples from 4 Provinces where domestic and wild hosts of *D. renale* are present. In Brazil, we collected parasite samples from domestic animals from 3 different States but parasites from wild hosts were only collected in Santa Catarina (Figure 2). A total of 73 adults of *D. renale* were collected, of which 44% were males and 56% females. Similar proportions were observed when analyzing *C.l. familiaris* and *C. brachyurus* separately. The main anatomical site affected was the right kidney (58% of the cases) followed by the abdominal cavity (39%).

Table 1. Total *Dioctophyme renale* samples analyzed by molecular markers. Number of samples received and sequenced grouped by host and location.

Host	Province/State	Country	Samples	COX1 short	COX1-XL	ND4
Canis lupus familiaris	Buenos Aires	Argentina	28	19	17	12
Canis lupus familiaris	Santa Fe	Argentina	11	10	10	10
Canis lupus familiaris	Chaco	Argentina	1	1	1	1
Canis lupus familiaris	Rio Grande do Sul	Brazil	6	5	5	5
Canis lupus familiaris	Santa Catarina	Brazil	4	2	2	1
Canis lupus familiaris	Parana	Brazil	4	2	2	1
Felis silvestris catus	Parana	Brazil	1	1	0	0
Felis silvestris catus	Santa Catarina	Brazil	2	1	0	0
Total Domestic	**-**	**-**	**57**	**41**	**37**	**30**
Chrysocyon brachyurus	Corrientes	Argentina	7	6	4	4
Chrysocyon brachyurus	Santa Fe	Argentina	4	4	4	4
Chrysocyon brachyurus	Chaco	Argentina	2	2	2	2
Lycalopex gymnocercus	Santa Fe	Argentina	1	1*	1	1
Galictis cuja	Santa Catarina	Brazil	2	1	0	0
Total Wild	**-**	**-**	**16**	**14**	**11**	**11**
TOTAL	**-**	**-**	**73**	**55**	**48**	**41**

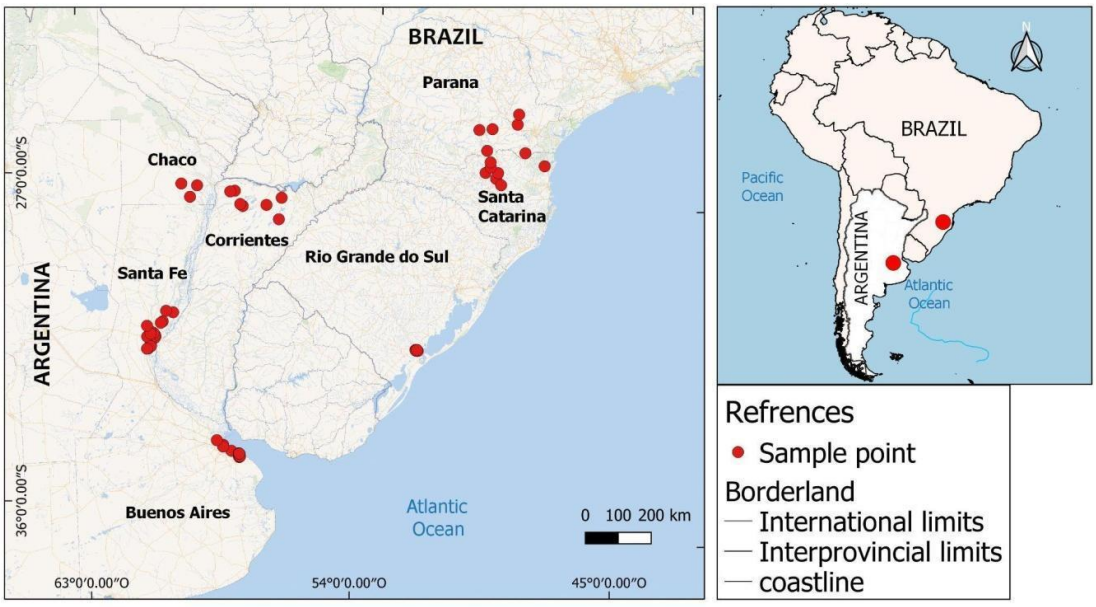

Figure 2. Geographic distribution of *Dioctophyme renale* samples Maps were plotted with Qgis version 3.16.3, OSGeo, layer (CRS) EPSG:4326 - WGS 84. Sample points (red circles)

Aim 2) Gene identification

COX1 is a useful molecular marker for *D. renale* population genetics analysis

The draft mitogenome of *D. renale* was compared to the mitochondrial genomes of several nematode species in order to design molecular markers for helminth identification (Arce et al., 2024). Four set of primers were designed, named COX1-S, COX1-M, COX1-L and COX1-XL (Figure3, Table 2). A total of 55 samples were amplified with COX1-S set of primers and 48 with COX1-XL (Table 1). Notably, the 10 samples that could only be amplified with COX1-S primers allowed us to obtain sequences from new domestic (*F. catus*) and wild (*G. cuja*) hosts. By performing a BLAST search against the NCBI nucleotide database, these sequences showed high similarity with homologous sequences from other *D. renale* parasites, with identities ranging from 91.78% to 100% (accession number AB854727.1 and MN304733.1, respectively). On the other hand, a remarkable difference is observed with respect to the homologous sequences of related species such as *Sobolophyme baturini* and *Trichuris muris*, with identities ranging from 81.94% to 84.93% (accession number MZ675607.1 and EU394157.1, respectively) (Supplementary Files). The COX1-XL comprises all the variable sites contained within the shorter COX1 markers, as a consequence it has the greatest haplotype richness and allows the best resolution of phylogenetic relationships. This marker will be referred to as COX1 in the following sections.

ND4, a new mitochondrial marker for *D. renale*

The ND4 gene region was selected as a result of the in-silico analysis of mitogenomic DNA variation in *Trichuris* species, a genus that shares Clade I with *D. renale* (Blaxter & Koutsovoulos, 2015). Greater variation in the proportion of polymorphic sites and mean genetic distance within a section of the ND4 gene, compared to other mitochondrial genes, was observed in these genera. Therefore, the ND4 marker could provide a better comprehension of intraspecific differences.

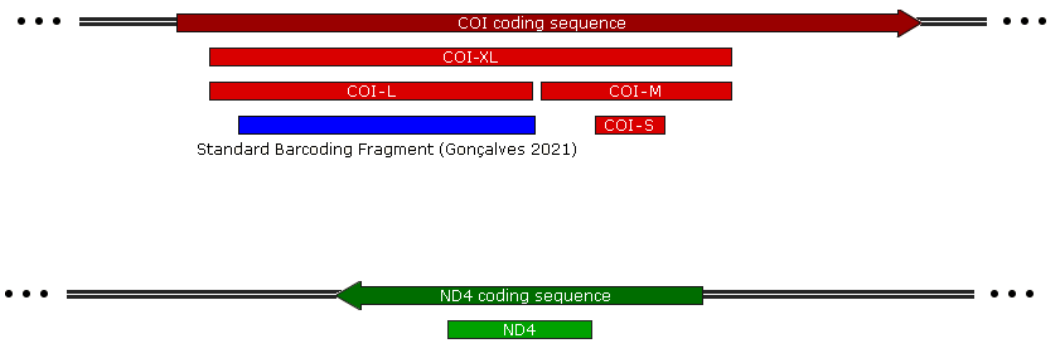

Figure 3. Genomic map showing the markers designed for this study. Arrows indicate the coding sequences and bars correspond to the expected PCR products (below) using SnapGene v1.1. A) COX1 gene. B) ND4 gene.

Table 2. Mitochondrial molecular marker amplification. Gene, primer name, sequence and expected PCR products size are shown

Gene	Primer name	Primer Sequence 5'-3'	Expected product size (pb)	Source
ND4	DRND4F	AGAAGAGGATCATATCTTAT	354	this work
	DRND4R	GCTACGAATTTTTTAATGTCG		
COX1-S	DRCOX1-SF	TGGTGTGCTTGGTTGTTTTG	116	Modified from Tokiwa et al. (2019)
	DRCOX1-SR	AACCTGCCCACCATACAAAG		
COX1-M	DRCOX1-MF	CATCCWGAGGTTTATATTYTAGC	382	Modified from Koehler et al. (2009)
	DRCOX1-MR	ASWAAGAACAWARTGRAAATGACC		
COX1-L	DRCOX1-LF	CTTCAGTTATTGGTGGGTGT	687	this work
	DRCOX1-LR	GTTGGAATAGAACAGGGTCA		
COX1-XL	DRCOX1-LF	CTTCAGTTATTGGTGGGTGT	1133	this work and modified from Koehler et al. (2009)
	COX1-MR	WAAGAACAWARTGRAAATGACC		

Aim 3) Parasite species determination

Phylogenetic Analysis

The **COX1-M marker** was selected in the first instance to perform phylogenetic analysis, since this region presents the highest number of homologues in the GenBank database. The phylogenetic tree topology shows that the sequences from Argentina and Brazil obtained in this work cluster with a sample from Perú (Genbank number MT246537.1) forming a South American clade. In other node are grouped those from Canada (Genbank number EU394733.1) and Iran (Genbank number MH178300.1, MH181826.1, PP326859.1, MH178400.1 and MH178401.1). The *Trichinella* substitution rate employed allows us to propose the first hypothesis of the divergence between these clades, which is estimated to have occurred approximately 3 million years ago. In this clade there is no differentiation by host species nor location. The same pattern is observed in the COX1-S phylogeny with the addition of the Japanese sequence reported by Tokiwa et al. (2019).

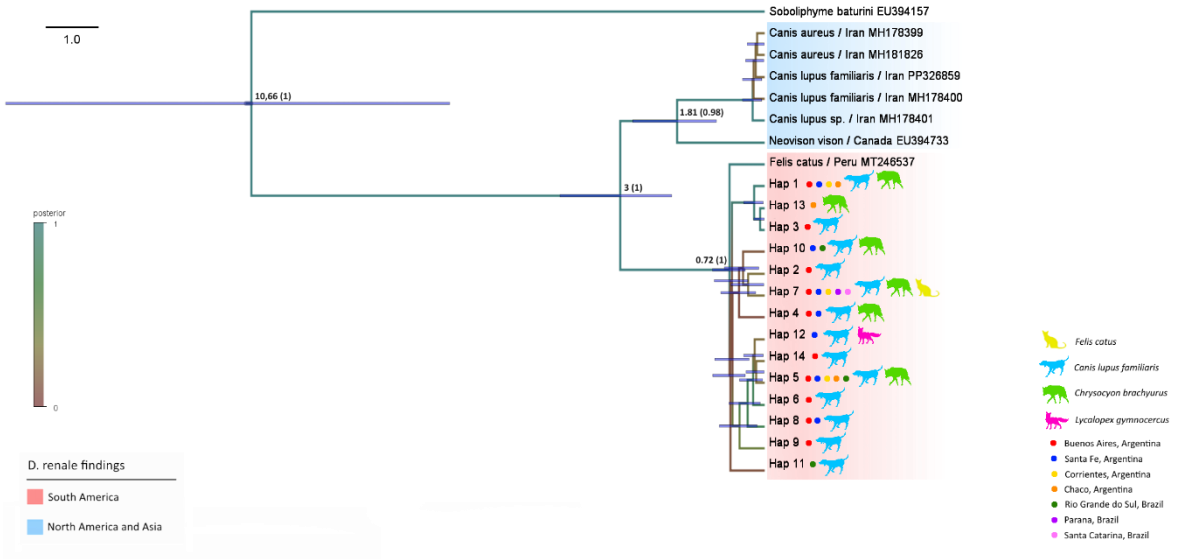

Figure 4. Bayesian inference tree obtained with BEAST v2.6.7 software and plotted with the iTOL online tool. The haplotypes found in our study for the COX1-M gene and those available in GenBank are shown. The GenBank sequence of *Sobolophyme baturini* was included as an outgroup. The nodes show the time in Mya and in brackets the posterior probability.

The cladogram constructed using genetic information obtained from the concatenated matrix (COX1+ND4) shows 21 haplotypes (Figure 5). Four of them were observed both in wild and domestic hosts (Hap8, Hap13, Hap14 and Hap18). Hap13 and Hap8 showed the highest geographical dispersion (four localities each) followed by Hap12 and Hap14 (two localities each). In addition, every clade with high statistical support (posterior probability > 0.9) includes samples coming from different geographical regions and host species.

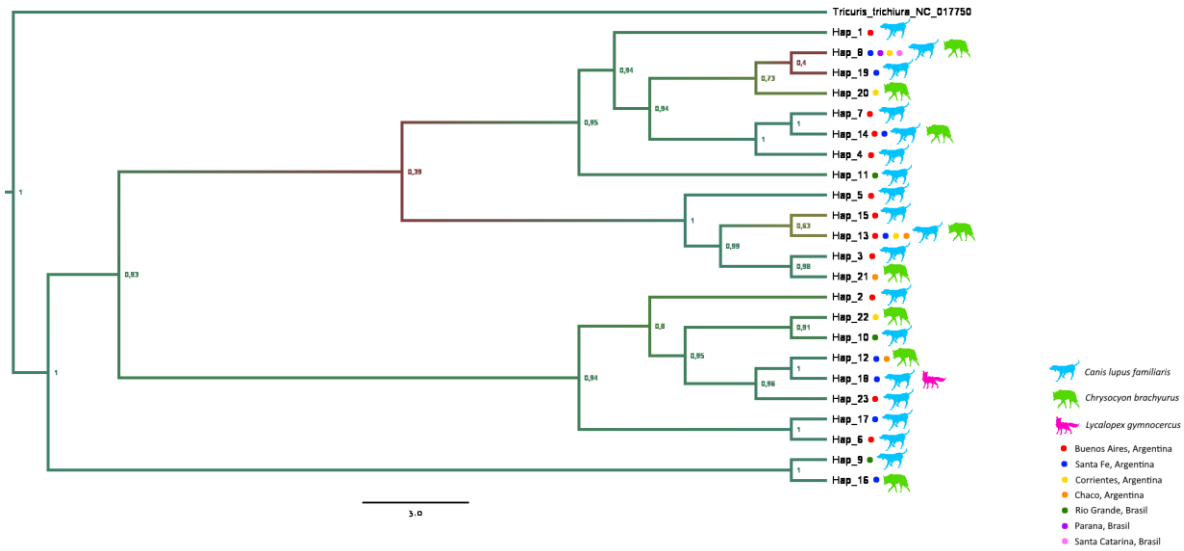

Figure 5. Proportional branch transformed phylogenetic tree from concatenated marker (COX1 + ND4) genes by using Bayesian Inference method. The GenBank sequence of *Trichuris trichiura* was included as an outgroup. Color gradient is related to branch posterior probability and values are displayed at the side of nodes. At the tips is indicated localities and host species of samples included in every haplotype. Evolutionary analyses were conducted in BEASTv10.

Conclusions

Nematode parasites affecting wild animals are under-studied in South America. Frequently, the identification of these parasites is based on morphological determinations. Mitochondrial DNA-based markers employed in diagnosis offer the advantage of having thousands of mitochondria per cell and several mitochondrial genome copies per organelle, allowing the detection of parasites even when there is a limiting amount of DNA. Amplification and sequencing of COX1 gene is the barcoding approach currently and extensively used for high-throughput species delimitation and discovery (Hebert et al., 2003). However, the amplification of large fragments of COX1 and obtaining a good quality sequence is not always possible. Parasites collected from hosts that were road-killed and have spent several days decomposing, are often poorly preserved. In this case, PCRs with smaller targets are more sensitive than those with larger amplicons. Since both, specificity and sensitivity are required for species determination, we designed primers to amplify different sections of the COX1 gene. This approach allowed us to evaluate different alternatives. The COX1-L primers were designed to amplified the COX1 region standardized for barcoding (Hebert et al., 2003). This marker showed a lower proportion of polymorphic sites than other markers evaluated. Nevertheless, it has still proven useful for the molecular analysis of *D. renale* (this work) and other nematode species (Poon et al., 2017). The COX1-M marker showed the highest genetic diversity and could be the marker of choice when sample status prevents sequencing of the larger markers. However, extreme difficult samples with degraded DNA, poor quality and/or low mass could be analyzed by COX1-S marker since has high sensitivity and is still retain sufficient genetic information. Based on these results the molecular markers COX1-S and COX1-M developed in this work has the best performance, particularly when morphological determinations are challenging due to small or poorly preserved samples and accurate species identification is required. The higher mutation rate due to mitochondrial oxidative stress, which is about 10 times higher than that of nuclear DNA, is an advantage for using mtDNA to study diversity. This increased mutation rate increases its potential for population studies without the bias of recombination events. The greatest variation is observed in the COX1-M section of the gene which, together with the ND4 marker, would be the best candidates for population genetics and phylogeography studies of this species. In order to evaluate the genetic diversity found in this work against genetic information from parasites sampled in other regions of the world, COX1-S was chosen as the marker since the available sequences from other countries are short. The phylogenetic trees obtained with COX1-S marker indicates that the South American samples, including the sample from Peru, are highly divergent from those found in other regions of the world (Canada and Iran). Furthermore, if this segregation would have occurred 3 millons years ago, according to *Trichinella* mtDNA substitution rate, the variants found would have originated well before the arrival of domestic fauna in the region. This divergence time is consistent with Great American Biotic Interchange, a massive exchange of flora and fauna species between the North and South American landmasses resulting from the formation of the Isthmus of Panama. Then *D. renale* may have dispersed with its hosts between the north and south of the continent following the formation of the isthmus. The co-divergence of parasitic nematodes with the mastofauna they infect has already been proposed by Jimenez et al. (2017). Based on the limited sequence information available for this parasite, these preliminary results suggest that *D. renale* would have colonized the area in a gradual process dispersed with wildlife and would not be the result of an anthropogenic introduction. No genetic structuring by locality or host was observed in the studied area, as evidenced

by data from Bayesian phylogenies and haplotype networks. *D. renale* does not seem to encounter geographical barriers that prevent its dispersal throughout the study area. However, the statistical analysis performed shows that geographical distribution is better at explaining genetic diversity than host species. This may indicate a small contribution of geographical distribution, which increases when the Chaco and Corrientes populations are considered as one, probably due to shared water resources from the Parana River but still less than 6% of the genetic diversity (Supplementary file S6). Furthermore, the Mantel test revealed no evidence of isolation by distance. Establishing a source/sink relationship between the studied localities is challenging, but it is worth noting that Buenos Aires Province exhibited higher haplotype diversity, but lower nucleotide diversity compared to Santa Fe Province in Argentina. On the other hand, Santa Fe has haplotypes absent in Buenos Aires Province and in some cases shares haplotypes with Brazil. This suggests that Santa Fe Province population is a contact zone between two regions, Buenos Aires Province (more temperate areas) and northern Argentina/southern Brazil (more tropical areas). This would also explain the greater nucleotide diversity that is observed when populations are structured. No differences in haplotype diversities were observed between Brazil and Argentina, making it difficult to establish a clear direction of transmission between the two. This may be attributed to the distant evolutionary time during which this process occurred and the large scale of migrations. The observed pattern supports the idea of multiple transmissions of parasites between domestic and wild animals, with several haplotypes being shared among hosts. Since no significant differences were found in the haplotype diversities between domestic and wild species, it cannot be assumed that there is unidirectional transmission. Instead, this pattern is indicative of a high rate of transmission occurring in both directions between domestic and wild species. Spillover of parasites at the domestic animal - wildlife interface is a pervasive threat to animal and human health. This information is crucial for future studies aiming to understand the ecology of the parasite in South America and its impact on wild populations, as well as risk of transmission to humans. Population expansion was examined in this study using two commonly used marker genes in nematode population studies. Our results showed no significant evidence of population growth in the studied area. Considering the number of informative sites studied, results suggest that *D. renale* has been present in the region for a considerable period of time, enough to lose the genetic imprint of their colonization.

Prior to this project, very little information was available on the diversity of *D. renale*, a zoonotic parasite that is widespread in riparian regions of Argentina and southern Brazil. Here we provide the first descriptions of the genetic diversity of the parasite in the region by complementing molecular methods and classical and probabilistic phylogeography. Our results strongly suggest that this parasite has been present on the continent long enough to develop local genetic variants, rather than being the product of an introduction from the countries where it is reported. Although it is widely distributed in the molecular data, there is no significant evidence of population expansion. Also, the phylogenies show transmission between localities and bidirectional transmission between domestic and wild species. We now have new tools to understand the ecological dynamics of this parasite such as molecular markers to study its genetic diversity as well as for identification and reporting in cryptic cases.

Publications

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- Kamenetzky L., Nanopore Community Meeting Boston. Lighting talk “Consolidation of a long-read sequencing platform in southern South America”. 16 de septiembre 2024, Estados Unidos
- Kamenetzky, L. Genómica aplicada al estudio de parásitos helmintos. V Congreso Colombiano de Bioquímica y Biología Molecular C2B2, 8-10 noviembre 2023, Colombia
- Kamenetzky, L. Descentralización de la Secuenciación. Presentación de la Plataforma Nanopore de Exactas, FCEN UBA, 29 de agosto de 2023
- Kamenetzky L. Estrategias de análisis de genomas completos: implementación en parásitos helmintos. INGEBI, 7 de junio de 2023

- Kamenetzky L. Bioinformatic tools for whole genome analysis of helminth parasites, Argentine Congress of Bioinformatics and Computational Biology, 24-25 de Noviembre, 2022

Workshops and congress presentations

Natalia Macchiaroli, Inés Sananez, Lucas Arce, Gisela Franchini, Laura Kamenetzky. Análisis del Mitogenoma del párasito nematodo *Dioctophyme renale* obtenido por tecnología de secuenciación de tercera generación Oxford Nanopore. V Congreso Colombiano de Bioquímica y Biología Molecular C2B2, 8-10 noviembre 2023, Colombia

Natalia Macchiaroli, Inés Sananez, Lucas Arce, Gisela Franchini, Laura Kamenetzky. Abordajes genomicos en Una Salud en SudAmerica: aplicación de plataforma Oxford Nanopore (ONT) en Argentina. XXXIV Reunion SAP, 1-3 de noviembre 2023, Argentina.

Aldana Claps , Emilio Kolomenski , Franco Fernández , Natalia Macchiaroli, Marisol Delea, Cecilia Fernández , Tania Castro , Julieta Laiseca , Laura Kamenetzky , Melisa Taboas , Liliana Dain, High precision characterization of rccx rearrangements in 21- Hydroxylase argentine patients using oxford nanopore long Read sequencing SAIC, Mar del Plata 15-17 denNoviembre 2023, Argentina

Fainstein, Lola, Alvarez, Florencia, Alba Posse, Ezequiel, Macchiaroli, Natalia, Kamenetzky, Laura, Lozano, Verónica, Figuerola, Eva, Gasulla, Javier. Characterization of cyanobacterial blooms using metagenomic long reads analysis and microbial ecology. SAIB, 14-17, noviembre 2023, Argentina

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Missions

Two missions could be carried out successfully, one at the prestigious workshops of International Parasitology, where we presented the advances in sequencing parasite genomes. In this prestigious workshop, knowledge and protocols were exchanged with specialists in genomics and parasitology in order to develop the next steps of the project. The second mission was from the collaborating laboratory in Uruguay to Argentina. These missions involved one research stay in the Center of Genomics at Buenos Aires University, the research was trained in Bioinformatics tools to analyze sequencing data. Also, part of the results and conclusions obtained have been published in the prestigious journal Parasitology (Arce *et al*., 2024. Despite the critical economic situation in Argentina, we could start with this seed project thanks to the synergistic collaboration established among the different groups and the PGTF grant that allowed to carry out the missions.

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