Parasitology

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Molecular typing of *Strongyloides stercoralis* in Latin America, the clinical connection

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Abstract

This study analysed *Strongyloides stercoralis* genetic variability based on a 404 bp region of the *cox*1 gene from Latin-American samples in a clinical context including epidemiological, diagnosis and follow-up variables. A prospective, descriptive, observational study was conducted to evaluate clinical and parasitological evolution after ivermectin treatment of 41 patients infected with *S. stercoralis*. Reactivation of the disease was defined both by clinical symptoms appearance and/or direct larvae detection 30 days after treatment or later. We described 10 haplotypes organized in two clusters. Most frequent variants were also described in the Asian continent in human (HP24 and HP93) and canine (HP24) samples. Clinical presentation (intestinal, severe, cutaneous and asymptomatic), immunological status and eosinophil count were not associated with specific haplotypes or clusters. Nevertheless, presence of cluster 1 haplotypes during diagnosis increased the risk of reactivation with an odds ratio (OR) of 7.51 [confidence interval (CI) 95% 1.38–44.29, $P = 0.026$]. In contrast, reactivation probability was 83 times lower if cluster 2 (I152V mutation) was detected (OR = 0.17, CI 95% 0.02–0.80, $P = 0.02$). This is the first analysis of *S. stercoralis cox*1 diversity in the clinical context. Determination of clusters during the diagnosis could facilitate and improve the design of follow-up strategies to prevent severe reactivations of this chronic disease.

Research Article

*These authors contributed equally to this work.

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Introduction

Strongyloidiasis is a soil-transmitted, intestinal parasitic disease caused by the nematode genus *Strongyloides*. It represents one of the most neglected diseases worldwide despite its wide distribution and high prevalence in tropical and subtropical regions of Africa, Southeast Asia and Latin America (300–400 million of infected people worldwide) (Schär *et al*., 2013; Savioli *et al*., 2014). *Strongyloides* infection in humans is caused by two species, *Strongyloides stercoralis* with a worldwide distribution, and *S. fuelleborni*, which is mainly limited to sporadic cases in Africa and New Guinea (Ashford *et al*., 1992). Its life cycle is more complex than most other nematodes due to its alternation between free living and parasitic cycles, and the ability to autoinfect and multiply within the host.

Larva currens is a fast-moving serpiginous eruption due to skin penetration by filariform larvae of *S. stercoralis*. Then, they travel to the bloodstream and reach the lung, and if the parasite load is high, there may be pulmonary symptoms. After ascending the tracheobronchial tree, they enter the small intestine to dwell as parthenogenetic females that begin oviposition. Rhabditoid larvae then emerge from these eggs. They may differentiate into L3 larvae in the environment or may become auto-infective filariform larvae in the host intestine, the latter being able to penetrate through the bowel mucosa or perianal skin re-infecting the host.

Strongyloidiasis is a chronic asymptomatic infection in most patients, although severe infection can be triggered in immunosuppressed people. In these cases, the autoinfection cycle accelerates, and filariform larvae migrate towards different body locations. Sepsis, bacteraemia and meningitis are often observed in such patients.

Strongyloides stercoralis genotypes based on 18S rDNA hyper-variable regions I and IV (HVR-I, HVR-IV) were previously associated with different hosts (mainly humans and dogs) but not with geographic distribution (Nagayasu *et al*., 2017; Thanchomnang *et al*., 2017).

Several nuclear and mitochondrial genomes of this parasite have been sequenced and based on phylogenetic analysis both sets of data exhibit a similar tree topology (Hu *et al*., 2003; Kikuchi *et al*., 2016). Whole genome analysis resulted in 0.6% of variant positions when comparing strains from different hosts and geographic origins showing lower diversity levels than other nematodes (Kikuchi *et al*., 2016).

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This scenario, together with the deeper information about *Strongyloides* genus mitochondrial DNA (mtDNA) genes, highlights the usefulness of these genes as candidates for molecular markers. Furthermore, mtDNA markers have been considered to be particularly applicable to population genetics and systematic research due to the low recombination levels, their high mutation rates, and proposed maternal inheritance (Ballard and Rand, 2005; Kern *et al*., 2020). Particularly in nematodes, substitution patterns have suggested that these genes can be more appropriate for identifying and differentiating cryptic species, as well as for establishing relationships of closely related species (Blouin, 1998, 2002).

Genetic variability of cytochrome c oxidase subunit 1 (*cox*1) mitochondrial gene of both *S. fuelleborni* and *S. stercoralis* strains isolated from humans, non-human primates and dogs from Asia and Africa revealed the same population structure (Nagayasu *et al*., 2017; Thanchomnang *et al*., 2017). Each species was monophyletic, with *S. fuelleborni* subclusters associated with the geographic origin of each strain and *S. stercoralis* variants, with different hosts (Hasegawa *et al*., 2010, 2016). Nevertheless, the assessment of this variability in other geographical settings such as Latin-America and the study of possible associations of genetic variants with clinical outcomes in humans are lacking (Jaleta *et al*., 2017). In this sense, a prospective, descriptive and observational study showed strongyloidiasis reactivation (parasitological and/or clinical) time after concluding ivermectin treatment (Repetto *et al*., 2018*b*). Based on these results, our aim was to study *S. stercoralis* genetic variability in Latin American human hosts and to perform the first analysis of potential relationships between specific parasite populations and the clinical parameters of patients enrolled in a post treatment follow-up study.

Materials and methods

Study design and patients

A prospective, descriptive, observational study was conducted between January 2009 and December 2020 in Buenos Aires, Argentina, to evaluate clinical and parasitological evolution of strongyloidiasis after ivermectin treatment. Patients aged >18 years attending the Instituto de Nefrología/Nephrology, and the Hospital de Clínicas José de San Martín, División Infectología (Universidad de Buenos Aires, UBA) were referred to the Clinical Parasitology Unit at the latter hospital for evaluation. Stool samples were sent to the Laboratorio de Parasitología Clínica y Molecular of the Instituto de Investigaciones en Microbiología y Parasitología Médica (IMPaM, UBA-CONICET) for parasitological diagnosis. All subjects presented the history of residence in *S. stercoralis* endemic areas and current residence outside these areas during this study. North-eastern and north-western regions of Argentina and other Latin-American tropical and subtropical regions were considered endemic areas (Repetto *et al*., 2016). All patients answered a rigorous questionnaire at each medical appointment during the follow-up, thus guaranteeing the absence of parasite re-exposure risk (i.e. travel to the endemic area). Those patients who returned to or visited endemic areas were withdrawn from this study.

Admission records of patients included past residence in endemic areas, clinical manifestations attributable to *S. stercoralis* infection, underlying illnesses and complete blood and eosinophil counts. Clinical strongyloidiasis was categorized as asymptomatic, intestinal, cutaneous or severe disease (hyperinfection and disseminated forms). Eosinophilia corresponds to ⩾450 eosinophils/μL peripheral blood. Immunological status was defined according to the presence of chronic illness, immunosuppressive or steroid therapy, haematologic malignancies, human immunodeficiency virus (HIV) infection, human T-lymphotropic virus 1 (HTLV-1) infection and transplantation or connective tissue diseases. HIV and HTLV-1-infected patients were screened by enzyme immunoassays and confirmed by Western blotting. Exclusion criteria included the risk of novel exogenous infections over the last 5 years and pregnancy.

Participants collected three fresh stool samples for diagnosis and follow-up. Fresh samples were stored at −20°C until DNA extraction. *Strongyloides stercoralis* diagnosis was performed by light microscopy, agar plate culture and PCR as previously described (Repetto *et al*., 2010, 2013). After ivermectin treatment (200 μg kg^{-1} once a day for 2 days and repeated after 2 weeks), patients were followed-up to assess parasitological reactivation. This was defined as the detection of larvae by light microscopy and/or agar plate culture 30 days after treatment or the re-emergence of clinical symptoms. Follow-up consisted in the patient' evaluation every 3 months.

DNA extraction, PCR amplification and sequencing

DNA extraction was performed from non-fixed stool samples using the method standardized in our laboratory (Repetto *et al*., 2013). Primers for nested-PCR amplification and sequencing of *cox*1 were: cox1_F443: 5′-CATCCTGGTTCTAGTGTTGATT-3′, cox1_R879: 5′-TGAGCTCAAACTACACAACCAA-3′ for the first amplification round (product of 458 bp) and cox1_F144: 5′-TAGTGTTGATTTGGCTAT-3′, cox1_R559: 5′-ATTGGTTTA ATTGGTTGTGT-3′ for the second amplification (product of 435 bp). PCR was performed in a 20 μL (first step) or 50 μL (second step) reaction mixture containing 1 μM of each primer, 0.5 mM of each dNTP, PCR buffer at 1× final concentration, 0.1 μg μL^{-1} BSA, 3 mM $MgCl_2$, 0.0625 U μL^{-1} of Maxima Hot Start Taq DNA polymerase (ThermoFisher Scientific, MA, USA) and 4 μL of purified DNA as template in the first step, or 2 μL of the first PCR product as template of the second step. Both amplification rounds were carried out in an ESCO Swift™ Maxi Thermal Cycler Block (ESCO Technologies Inc., Lab Division, MO, USA) with an initial 4 min denaturation at 95°C, followed by 35 amplification cycles (denaturation at 95 °C for 45 s, annealing at 51 °C for 1 min and elongation at 72°C for 90 s) and a final extension step at 72 °C for 10 min. Five microlitres of each amplified product were electrophoresed in a 2% agarose gel stained with GelRed® Nucleic Acid Gel Stain (Biotium, CA, USA) together with a molecular size marker (MassRuler™ Express Forward DNA Ladder Mix, ThermoFisher Scientific, MA, USA) to confirm PCR product size. Nested PCR products were directly sequenced using cox1_F144 and cox1_R559 oligonucleotides by means of capillary electrophoresis sequencing performed by Macrogen (Seoul, South Korea).

Sequences and phylogenetic analysis

Consensus sequences were obtained through Forward and Reverse strands assembly using STADEN Package software (MRC-LMB, UK). We also visually checked both strands for the detection of ambiguous sites when two peaks overlapped in both chromatograms. Consensus sequences were aligned and trimmed in frame (404 bp) using MEGA7 software (Kumar *et al*., 2016). Haplotype reconstruction was performed by DNAsp v.6 using the PHASE algorithm based on the whole haplotype population (Rozas *et al*., 2017).

Sequences obtained from the patient population were further analysed in the context of 934 worldwide *cox*1 sequences obtained from Genbank and DDBJ databases (Supplementary Table 1). These sequences correspond to eight *Strongyloides* species (*S. stercoralis*, *S. ratti*, *S. venezuelensis*, *S. fuelleborni*, *S. mirzai*,

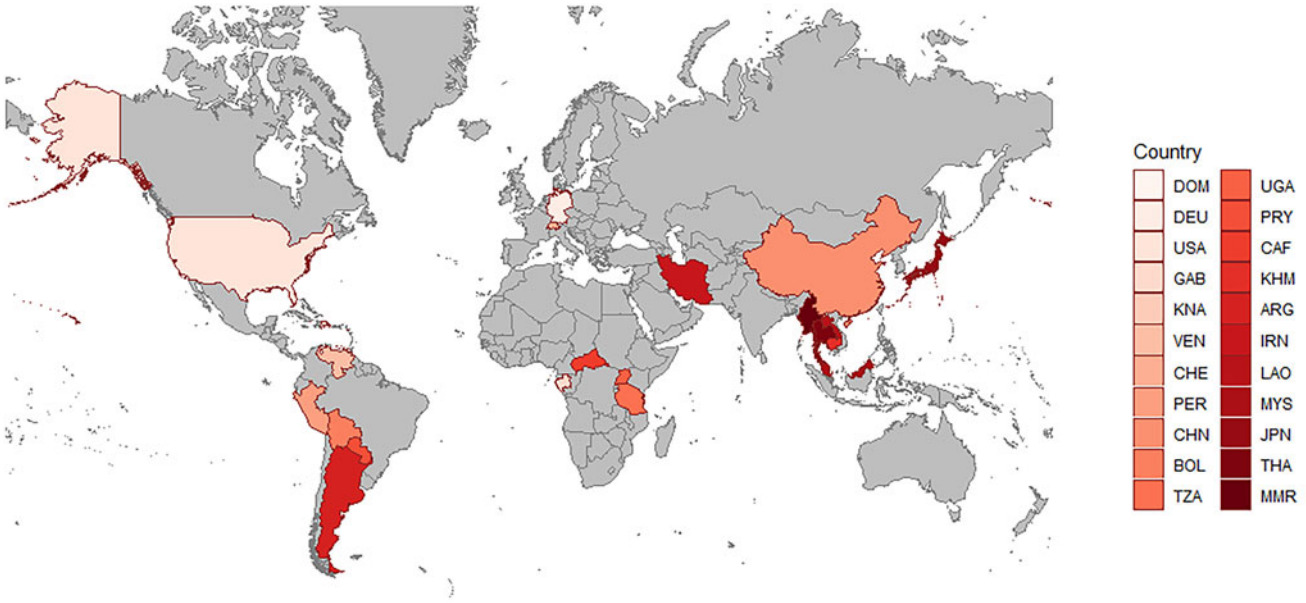

Fig. 1. Geographical distribution of *cox*1 sequences of *Strongyloides* spp. Africa: Central African Republic (CAF, 12), Gabon (GAB, 2), Tanzania (TZA, 6), Uganda (UGA, 9). America: Argentina (ARG, 26), Bolivia (BOL, 4), Dominican Republic (DOM, 1), Paraguay (PRY, 11), Peru (PER, 3), St. Kitts (KNA, 2), USA (USA, 1), Venezuela (VEN, 2). Asia: Cambodia (KHM, 19), China (CHN, 4), Iran (IRN, 38), Japan (JPN, 138), Laos (LAO, 81), Malaysia (MYS, 84), Myanmar (MMR, 327), Thailand (THA, 208). Europe: Germany (DEU, 1), Switzerland (CHE, 3). Each country is specified by a different strength of red and this intensity is not associated with number of analysed samples.

S. papillosus, *S. planiceps* and *S. vituli*) (Fig. 1). MLSTest software (http://ipe.unsa.edu.ar/software) was used for haplotype coding (Tomasini *et al.*, 2013).

Uni-dimensional nucleotide diversity measures such as the number of segregating sites (S), nucleotide diversity (π) and haplotype diversity (H_d) and selection tests based on the allele frequency (Tajima' D and Fu and Li' D) or based on comparisons of polymorphisms (synonymous and non-synonymous substitution rates, dN/dS) were also calculated using DNAsp 6 software.

Phylogenetic relationships were inferred by maximum likelihood (ML) tested with 500 bootstrap replications in MEGA 7. The selection of the nucleotide substitution model was performed through JModelTest 2 software (Darriba *et al.*, 2012). Genealogical associations of worldwide or American intraspecific genetic diversity of *S. stercoralis* was studied by haplotype network inferred by the Median-Joining algorithm (epsilon = 0) using PopART software (Leigh and Bryant, 2015).

Bioinformatic analysis was performed in a local server at IMPaM (UBA-CONICET) which is part of National System of High-Performance Computing (SNCAD) of the Ministerio de Ciencia, Tecnología e Innovación (MINCyT), Argentina.

Statistical analysis

The categorical variables were expressed in frequencies and percentages. The continuous variables are expressed as the mean (standard deviation) when they have a normal distribution.

Chi-square statistic (Chi^2) was used to evaluate association between categorical variables. The probabilities of exhibiting parasitological or clinical reactivation were estimated by odds ratio (OR).

Logistic regression models were employed to estimate the risk of developing reactivation and controlling for confounding.

Associations between categorical variables were compared by Fisher' test or χ^2. P value <0.05 was considered statistically significant. The t-test was used to contrast the eosinophil count and the haplotypes or clusters. The statistical analyses were performed using the SPSS version 23 and STATA version 13.

Results

Patient population traits

Forty-one patients with strongyloidiasis were included, treated with ivermectin and 29 (70.73%) of them were followed-up for a median of 360 days (IQ, 670 days) for the assessment of reactivation events (Table 1).

cox*1.404 marker global analysis*

Among the 41 patients, we reported 47 sequences since six of them showed one or more ambiguous sites that were resolved as two haplotypes (HP) using the PHASE algorithm. Overall, we analysed 981 *cox*1 sequences including 47 from our patient population and 934 from available databases (NCBI https://www.ncbi.nlm.nih.gov/ and DDBJ https://www.ddbj.nig.ac.jp/index-e.html). This analysis described 167 species-specific haplotypes defined by 192 SNPs and distributed in eight species, 22 countries and 18 host categories (Table 2). High haplotype diversity (H_d) was observed in most species and was higher in *S. fuelleborni* compared to *S. stercoralis* (Table 2). Hence, a low frequency of each variant was observed among the 981 sequences with 144 haplotypes (86.23%) in less than nine strains, 15 (8.98%) in 10–19 strains, 5 (2.99%) in 20–29 strains and 3 (1.80%) highly represented in the worldwide population (HP23, HP24 and HP25) (Supplementary Table 1). Also, the 404 base pair region of *cox*1 we examined (*cox*1.404 marker) was under negative selection in all datasets and neutrality tests produced an insignificant result for all populations except for worldwide *S. stercoralis* dataset for Fu and Li' D (Table 2). Phylogenetic analysis supported cluster organization according to species with low bootstrap support in intra-species branches (Fig. 2).

The median-joining network based on *S. stercoralis* worldwide variants displayed three main clusters centred in most frequent

Table 1. Patient population

Variable	*N*	%	Value
Gender			
Female	18	43.90	–
Male	23	56.10	–
Age			
Range	–	–	18–76 years old
Mean	–	–	52.6 years old
Country			
Argentina[a]	23	56.10	–
Bolivia	3	7.32	–
Paraguay	10	24.39	–
Peru	3	7.32	–
Venezuela	1	2.44	–
Dominican Republic	1	2.44	–
Eosinophil count			
⩾450 eosinophils/μL[b]	36	87.80	–
<450 eosinophils/μL	5	12.20	–
Clinical presentation[c]			
Asymptomatic	18	43.90	–
Intestinal	6	14.63	–
Cutaneous	3	7.32	–
Severe	14	34.15	–
Immunological status			
Immunocompromised	32	78.05	–
Immunocompetent	9	21.95	–
Follow-up			
Yes	29	70.73	–
No	12	29.27	–
Reactivation events (*N* = 29)			
Yes	16	55.17	–
No	13	44.83	–
Reactivation type (*N* = 16)			
Clinical reactivation[d]	2	12.50	–
Parasitological reactivation	6	37.50	–
Both	8	50.00	–

Traits are expressed in absolute values (*N*), percentages (%) or range for continuous variables (age).
aArgentinean patients past residence records were distributed in Buenos Aires city, Corrientes, Entre Ríos, Formosa, Jujuy, Misiones, Santa Fe, Santiago del Estero, and Tucumán provinces.
[b]Considered eosinophilia.
[c]Clinical presentation during diagnosis.
[d]Clinical symptoms (eosinophilia).

haplotypes HP23, HP24 and HP25 (Fig. 3). HP23 was only present in Asian human samples and most related haplotypes were present in Asian dog (HP62, HP71) and human (HP21, HP48 and HP84) samples. HP25 was observed in Asian human, and dog derived isolates whereas their most related haplotypes were only present in human samples (HP35, HP37, HP49, HP56 and HP89). Wider host and geographic distributions were obtained within the HP24 cluster. As mentioned above, this haplotype was present in Asian and American continents both in dog and human samples and represents a 20.06% of the total dataset.

Table 2. *cox*1.404 marker diversity of *Strongyloides* spp. subpopulations

Population	Species	Countries (N)	Hosts (N)	*n*	*S*	*h*	H_d	$H_{d\ S.D.}$	π	dN/dS	Tajima's *D*	Fu and Li's *D*
Worldwide	[a]	22	18	981	192	167	0.934	0.005	0.07093	0.237136	−0.65871	−2.00152
Worldwide	*S. fuelleborni*	8	10	185	82	62	0.960	0.006	0.04933	0.029327	0.41266	0.25669
Worldwide	*S. stercoralis*	19	4	767	95	92	0.895	0.007	0.02382	0.042003	−0.84310	−2.71640
Africa	*S. stercoralis*	3	2	13	15	5	0.628	0.143	0.01193	0.010061	−0.01140	−0.21967
America	*S. stercoralis*	3	2	50	20	11	0.698	0.046	0.01106	0.059784	0.00191	−0.90455
Asia	*S. stercoralis*	7	3	701	89	80	0.892	0.008	0.02447	0.041569	0.68563	−2.26131
LPCM[b]	*S. stercoralis*	6	1	47	14	10	0.660	0.048	0.00943	0.061055	0.62060	−1.78938

n, number of sequences; *S*, number of polymorphic sites; *h*, number of haplotypes; H_d: haplotype diversity. $H_{d\ S.D.}$, haplotype diversity standard deviation; π, nucleotide diversity. Fu and Li' *D* test statistic: Statistical significance: Not significant, $0.10>P>0.05$. Tajima' *D*: Statistical significance: Not significant, $P>0.10$. Calculated using the total number or mutations.
[a]Includes eight species: *S. stercoralis* (767 sequences), *S. fuelleborni* (185 sequences), *S. rattí* (1 sequence), *S. planiceps* (2 sequences), *S. mirzai* (1 sequences), *S. papillosus* (2 sequences), *S. venezuelensis* (1 sequences), *S. vituli* (4 sequences) and a group of 18 sequences from undetermined species.
[b]Corresponds to 47 sequences from 41 patients from our laboratory.

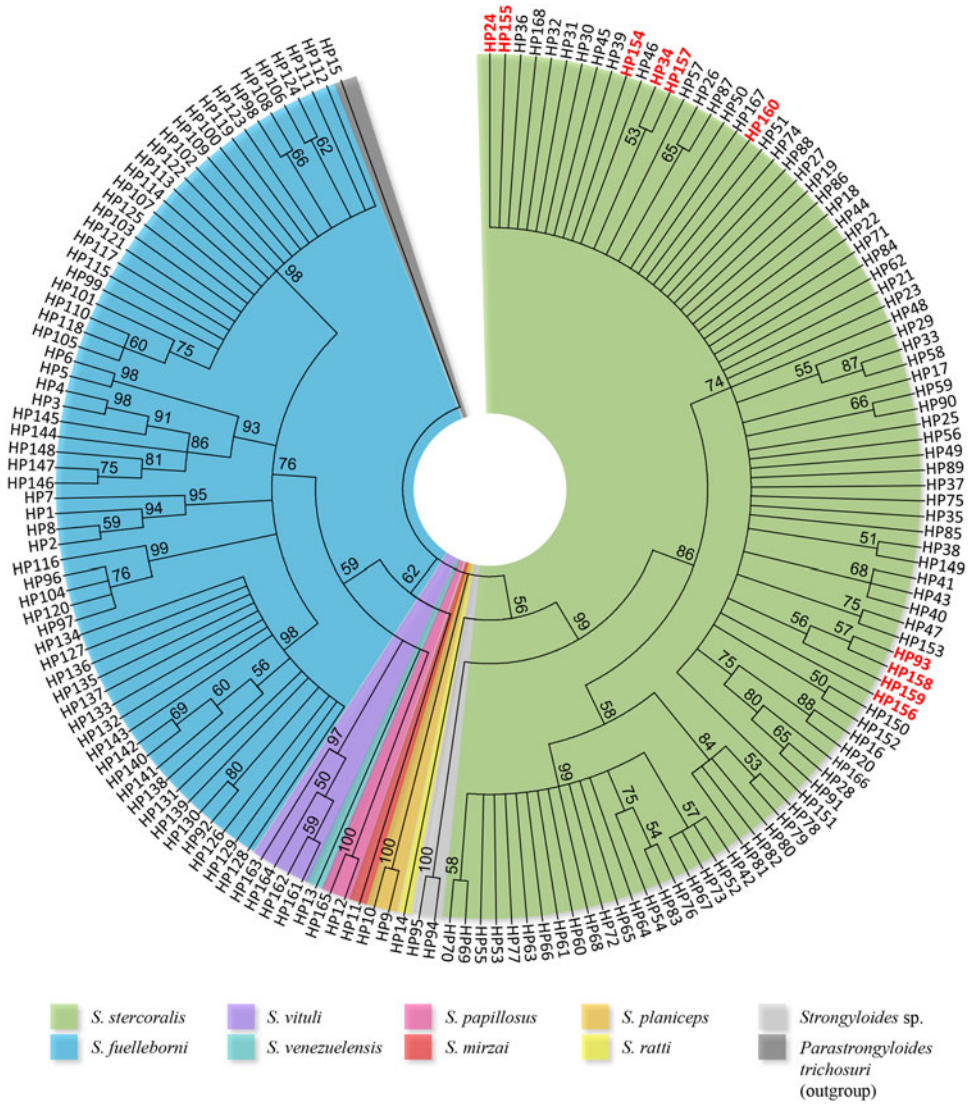

Fig. 2. Phylogenetic analysis of *Strongyloides* spp. haplotypes. HP15 corresponds to *Parastrongyloides trichosuri* (outgroup). The evolutionary history was inferred by using the ML method based on the General Time Reversible model, a discrete Gamma distribution was used to model evolutionary rate differences among sites (five categories (+G, parameter = 200.0000)), the rate variation model allowed for some sites to be evolutionarily invariable ([+ I], 51.25% sites) and 500 bootstrap replicates. The analysis involved 168 haplotypes, codon positions included were 1st and there was a total of 135 positions in the final dataset. Evolutionary analyses were conducted in MEGA7. Haplotypes found in our patient' population are shown in red.

However, HP24 most related haplotypes were identified only in human samples except for HP27 which was observed in dogs too. Concerning geographical distribution only HP34 was observed in both continents as HP24 (Fig. 3).

cox*1.404 marker in the clinical practice*

Ten haplotypes were described in our patient' population (Table 3). Sequences without ambiguous sites were HP24, HP93, HP158 and HP160, whereas HP34, HP154 and HP159 were defined after resolving a unique double signal either with HP24 (HP34 and HP154) or HP93 (HP159). On the other hand, HP155, HP156 and HP157 were defined by haplotype phasing based on population data (Stephens and Donnelly, 2003). Only HP24, HP34 and HP93 were observed in American and Asian samples while HP154–HP160 were described only in our patient' population.

Haplotype network analysis displayed two different clusters (C) centred on most frequent variants, HP24 (C1) and HP93 (C2) (Fig. 4) and associated specific amino acid variant in position 152 (isoleucine in C1 and valine in C2).

Considering the 47 sequences, median eosinophils count of HP24 (2328.50 ± 1641.88 Eo mm^{-3}), C1 (1744.85 ± 1109.44 Eo mm^{-3}) and C2 (2305.59 ± 1633.30 Eo mm^{-3}) was not associated with haplotypes/clusters [$P = 0.057$, confidence interval (CI) 95% −1655.50 to 26.77; $P = 0.091$, CI 95% −1583.79 to 122.30 and $P = 0.091$ CI 95% −122.30 to 1583.79, respectively]. No association was found between the presence of molecular markers, clinical forms and immunosuppressed status ($P > 0.05$).

Reactivation events were observed in 12 (66.67%) of HP24 cases, two of them coinfected with either HP154 or HP155 (Table 4). These events displayed only clinical symptoms ($N = 2$), microscopic larvae detection ($N = 5$) or both ($N = 5$). In contrast, both clinical and parasitological reactivation were only observed in three (33.33%) HP93 cases (Table 4). Fifteen reactivation events (68.18%) were identified in C1 (HP24, $N = 12$; HP154 $N = 1$; HP155, $N = 1$ and HP160, $N = 1$) and three events in C2 (HP93, $N = 3$). Interestingly, infections with C1 had a greater reactivation risk (OR 5.71, CI 95% 1.25–28.33, $P = 0.028$) and, after adjusting for confounding factor (immunocompromised status), this risk was significantly increased (OR = 7.51, CI 95% 1.38–44.29, $P = 0.026$). Immunocompromised status did not reveal any effect on the reactivation (OR 0.23, CI

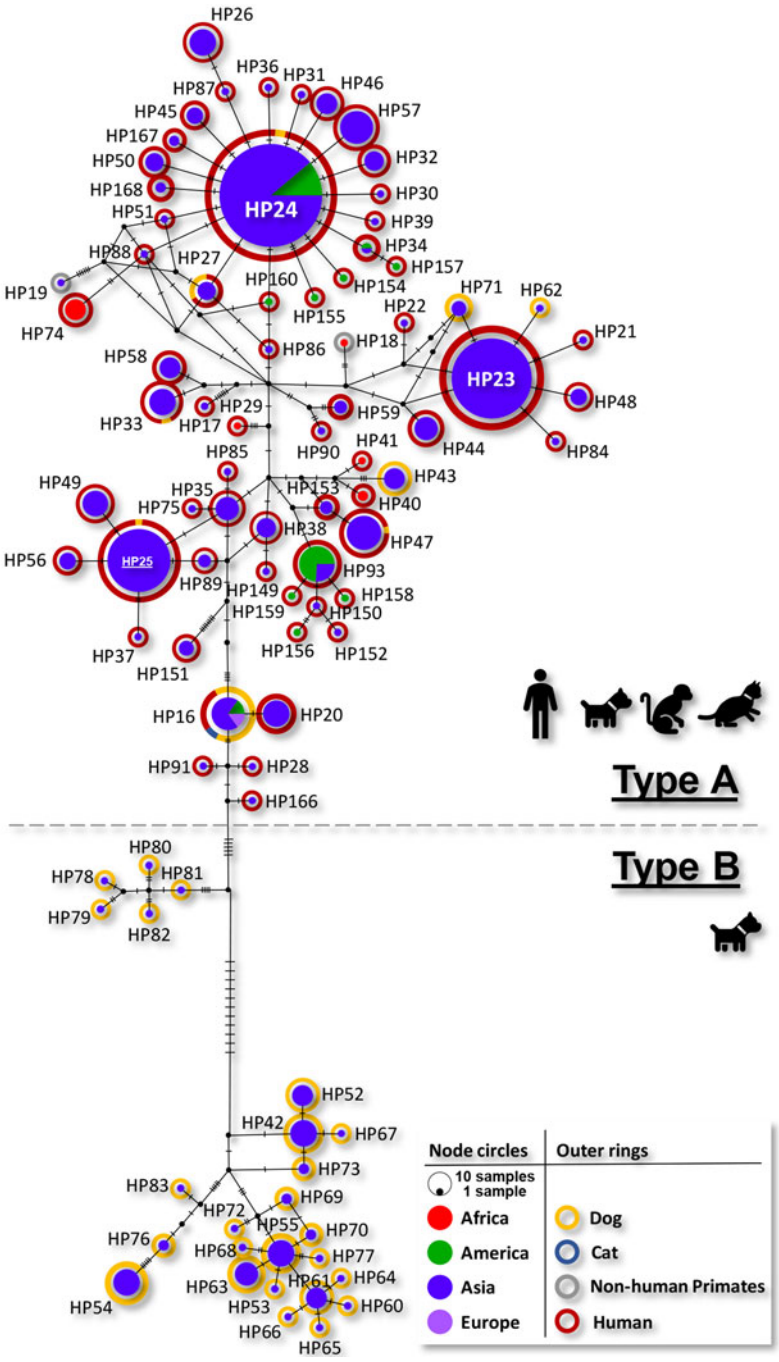

Fig. 3. Median-joining network based on worldwide *S. stercoralis* haplotype dataset.

95% 0.24–2.33, $P = 0.21$). The presence of C2 supported that the infection was 83 times less likely to reactivate following treatment (OR = 0.17, CI 95% 0.02–0.80, $P = 0.02$).

Finally, HP24, C1 and C2 were not associated with eosinophil count when only followed-up population was analysed ($P = 0.231$, CI 95% −1207.39 to 303.32; $P = 0.061$, CI 95% −1506.67 to 35.48 and $P = 0.061$ CI 95% −35.48 to 1506.67, respectively). Like the previous analysis of the 47 sequences, no association was found between haplotypes, the different clinical forms and immunosuppressed status ($P > 0.05$).

Discussion

Our study was based on three main aspects: first, the probable maternal mitochondrial inheritance already probed in a free-living nematode, *Caenorhabditis elegans* (Al Rawi *et al.*, 2011; Zhou *et al.*, 2011; Sato and Sato, 2013) combined with the role of female larvae in auto-infective cycle outside endemic areas; second, the worldwide information about the mitochondrial marker (*cox*1) but the lack of information of genetic variability of *S. stercoralis* in Latin-America and third, the role of this variability in the clinical outcome of the disease.

Previous studies described *cox*1 variability based on different gene regions (Fig. 5) (Nagayasu *et al.*, 2017; Thanchomnang *et al.*, 2017; Barratt *et al.*, 2019; Basso *et al.*, 2019; Fadaei Tehrani *et al.*, 2019; Spotin *et al.*, 2019; Sanpool *et al.*, 2020). This marker was firstly proposed to differentiate imported *Strongyloides* spp. in Japan, allowing the identification of different *S. fuelleborni* and *S. stercoralis* groups according to host species or geographic distribution, respectively (Hasegawa *et al.*, 2010). Particularly in *S. stercoralis*, this marker is associated with the host species and not with the geographic distribution of the samples (Thanchomnang *et al.*, 2017). Other studies suggested a two clade organization of *cox*1 haplotypes from Asian and African

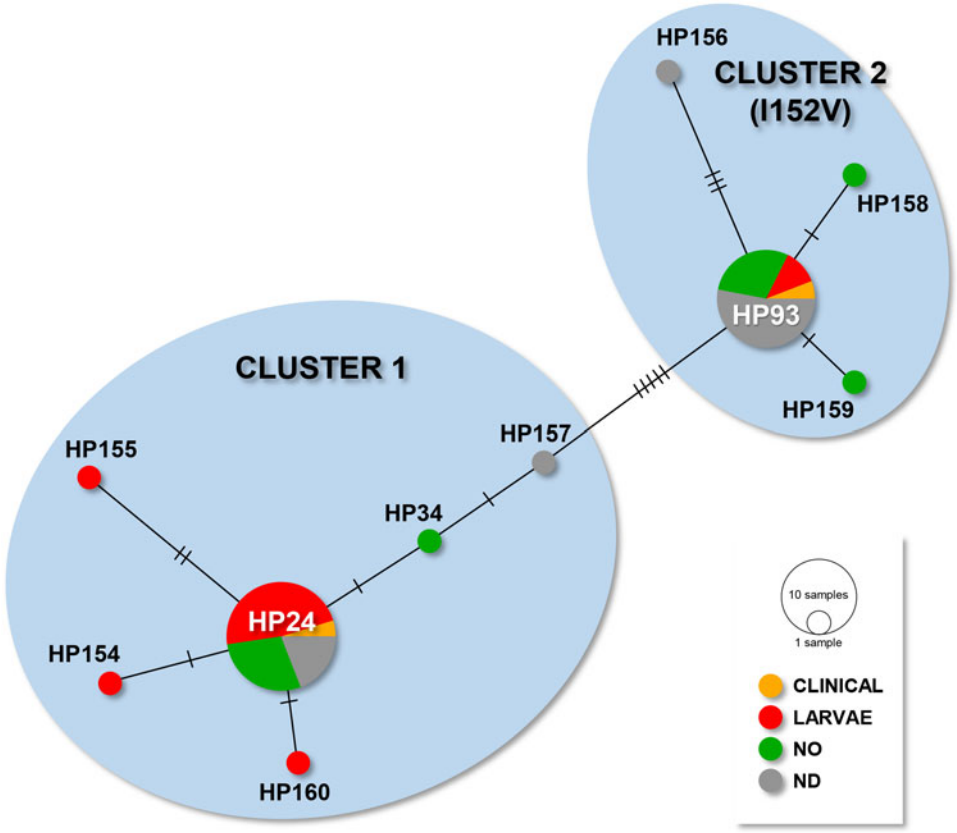

Fig. 4. Median-joining network based on patient' population *S. stercoralis* haplotype dataset.
Abbreviations: Clinical: only clinical reactivation; Larvae: parasitological reactivation; NO: no reactivation events; ND: patients were not followed-up.

Table 3. Haplotype/cluster distribution in the patient' population

Haplotype/Cluster	*N*[a]	%	Cluster	N-FU[b]	%-FU
HP24	22	46.81	C1	18	62.07
HP34	1	2.13	C1	1	2.13
HP154	1	2.13	C1	1	2.13
HP155	1	2.13	C1	1	2.13
HP157	1	2.13	C1	-	-
HP160	1	2.13	C1	1	2.13
HP93	17	36.17	C2	9	31.03
HP156	1	2.13	C2	-	-
HP158	1	2.13	C2	1	2.13
HP159	1	2.13	C2	1	2.13
C1	27	57.45	-	19	65.52
C2	20	42.55	-	10	34.48

C1, cluster 1; C2, cluster 2; FU, follow-up.
Values are expressed in absolute values (*N*) and percentages (%).
[a]Total number of sequences was 47.
[b]Total number of sequences in followed-up population was 33.

human and canine samples (Nagayasu *et al.*, 2017). Clade II or type B involves canine hypothetically non-zoonotic ancestral haplotypes and clade I or type A includes both human and dog hosts organized in five subclades (Ia, Ib, Ic, Id and Ie) with neither host nor geographic distribution associations. Also, *S. stercoralis* clusters according to 18S HVR-I and HVR-IV variability were described concluding that nuclear and mitochondrial genomes do not coevolve (Jaleta *et al.*, 2017). Nevertheless, none of these studies consider the clinical context. The 404 bp region of *cox*1 we examined (cox1.404) includes the largest number of SNPs to obtain an adequate discriminatory power when detecting intra-species subpopulations.

Ten *cox*1.404 haplotypes in 47 *de novo* sequences from 41 stool samples were analysed with 934 available sequences from eight species (167 haplotypes). This marker was under negative selection and based on neutrality tests, only worldwide *S. stercoralis*

Table 4. Patients population

Patient	Gender	Age	Country	CF	IC	Eo	FU	CR	PR	HP	C
1	M	64	Paraguay	Intestinal	Yes	2209	Yes	No	No	93	2
2	M	57	Argentina	Severe	Yes	300	Yes	No	No	93	2
3	F	67	Argentina	Asymptomatic	Yes	1575	Yes	No	No	93–159	2–2
4	M	ND	Argentina	Intestinal	No	1236	Yes	No	No	93	2
5	F	55	Bolivia	Asymptomatic	Yes	2550	Yes	No	No	24–34	1–1
6	M	31	Argentina	Asymptomatic	Yes	3690	Yes	No	No	24	1
7	F	31	Peru	Severe	Yes	600	Yes	No	No	24	1
8	M	62	Argentina	Severe	Yes	1504	Yes	No	No	93	2
9	M	64	Bolivia	Severe	Yes	3250	Yes	No	No	24	1
10	M	36	Argentina	Asymptomatic	Yes	1573	Yes	No	No	24	1
11	F	68	Argentina	Asymptomatic	Yes	568	Yes	No	No	158	2
12	F	65	Argentina	Asymptomatic	No	3000	Yes	No	No	24	1
13	M	50	Argentina	Intestinal	Yes	3214	Yes	No	Yes	24–155	1–1
14	M	45	Dominican Rep.	Intestinal	Yes	2090	Yes	No	Yes	24	1
15	M	46	Argentina	Severe	Yes	1474	Yes	No	Yes	24	1
16	F	55	Argentina	Asymptomatic	Yes	3310	Yes	No	Yes	24–154	1–1
17	F	67	Argentina	Asymptomatic	No	3916	Yes	No	Yes	24	1
18	F	73	Argentina	Asymptomatic	Yes	930	Yes	No	Yes	160	1
19	M	30	Bolivia	Asymptomatic	Yes	1880	Yes	Yes	No	24	1
20	M	49	Peru	Severe	Yes	2486	Yes	Yes	No	24	1
21	F	59	Argentina	Severe	Yes	30	Yes	Yes	Yes	93	2
22	F	63	Argentina	Severe	Yes	1812	Yes	Yes	Yes	24	1
23	M	47	Argentina	Asymptomatic	No	1200	Yes	Yes	Yes	24	1
24	F	54	Paraguay	Asymptomatic	No	2360	Yes	Yes	Yes	24	1
25	F	42	Argentina	Cutaneous	Yes	3950	Yes	Yes	Yes	93	2
26	F	53	Paraguay	Asymptomatic	Yes	740	Yes	Yes	Yes	24	1
27	F	76	Paraguay	Intestinal	Yes	1940	Yes	Yes	Yes	24	1
28	M	73	Argentina	Asymptomatic	No	2688	Yes	Yes	Yes	93	2
29	F	48	Paraguay	Cutaneous	No	1818	Yes	Yes	Yes	93	2
30	M	72	Argentina	Severe	Yes	550	No	–	–	24	1
31	M	59	Argentina	Severe	Yes	8466	No	–	–	24	1
32	M	30	Argentina	Severe	Yes	2000	No	–	–	93	2
33	M	46	Peru	Asymptomatic	No	1287	No	–	–	93	2
34	F	48	Paraguay	Asymptomatic	Yes	3364	No	–	–	93	2
35	M	42	Argentina	Severe	Yes	1	No	–	–	93	2
36	M	25	Argentina	Intestinal	Yes	20	No	–	–	24	1
37	M	58	Paraguay	Cutaneous	Yes	2550	No	–	–	93	2
38	F	74	Paraguay	Severe	Yes	12	No	–	–	93	2
39	M	18	Paraguay	Asymptomatic	Yes	1100	No	–	–	93–156	2–2
40	F	58	Venezuela	Severe	No	1063	No	–	–	24–157	1–1
41	M	44	Paraguay	Asymptomatic	Yes	2630	No	–	–	93	2

Gender (M: Male, F: Female), age (years), geographic origin (country), clinical form (CF), immune system commitment (IC) and eosinophils/μL peripheral blood (Eo) were recorded in each case (Eo > 450 eosinophils/μL peripheral blood was considered eosinophilia). Twenty-nine patients were followed in time (FU) and clinical and/or parasitological reactivation was evaluated (CR and PR, respectively). In all patients *cox*1.404 marker was established at the time of diagnosis (HP: haplotype/s and C: Cluster).
Forty-one patients were included in the study.

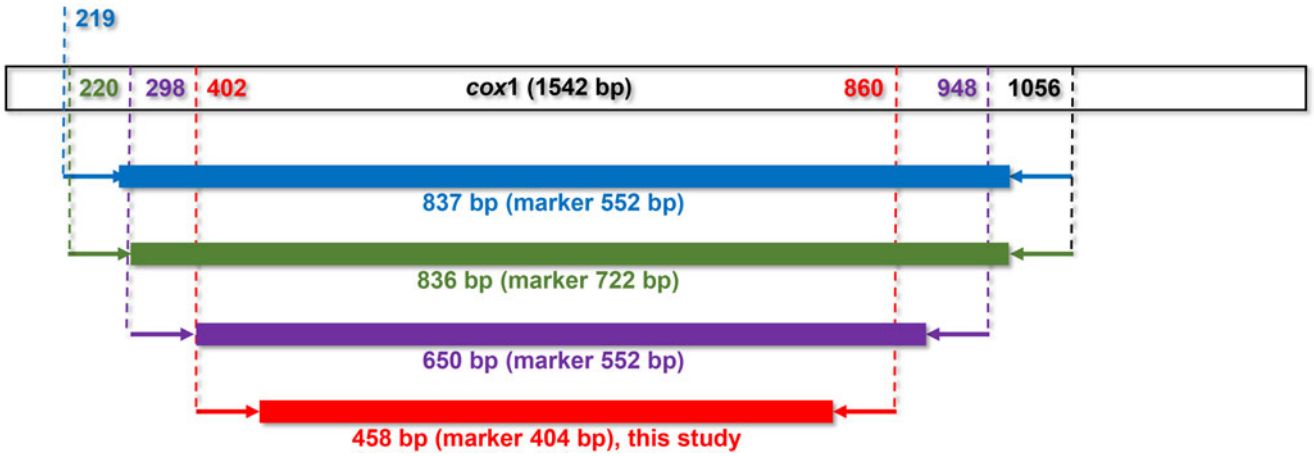

Fig. 5. Reported *cox*1 regions. Primer' locations are specified within the *cox*1 locus. Hasegawa and colleagues (green) initially proposed a marker of 722 bp that was also studied by Laymanivong (Hasegawa *et al.*, 2016; Laymanivong *et al.*, 2016). On the other hand, both Jaleta (purple) and Zhou (blue) studies proposed a 552 marker (Jaleta *et al.*, 2017; Zhou *et al.*, 2019).

exhibited significant demographic expansion based on Fu and Li' D statistics (Table 2).

High H_d in most representative species (*S. fuelleborni* and *S. stercoralis*) was consistent with the low frequency of each variant in the whole population despite us examining only a 404 bp fragment of *cox*1 (Fig. 5) (Hasegawa *et al.*, 2010; Laymanivong *et al.*, 2016; Jaleta *et al.*, 2017; Zhou *et al.*, 2019).

Global phylogenetic analysis of *cox*1.404 showed cluster organization according to the genus taxonomy (Fig. 2) while *S. stercoralis* tree topology showed Type A and B cluster organization according to previous studies (Jaleta *et al.*, 2017; Nagayasu *et al.*, 2017).

The presence of more than one haplotype in stool samples could be due to coinfection by different parasite populations or existence of two or more mitochondrial haplotypes per worm by heteroplasmic mitochondrial mutation as observed in *C. elegans* (Wernick *et al.*, 2016; Konrad *et al.*, 2017). Since DNA extraction was performed directly from stool or pooled larvae, we could not differentiate between these hypotheses.

We previously suggested that the parasitological cure after ivermectin administration is unlikely (Repetto *et al.*, 2018*a*, 2018*b*). As the followed-up patients stayed in non-endemic regions during the study, the auto-infective cycle became the most relevant mechanism of disease recurrence. Hypothetically, reactivation could be associated with some changes in the nematode environment that modify the host–parasite equilibrium. Regardless of this stimulus, the stage switch and asexual reproduction of larvae involved in reactivation events require the upregulation of its energy-production machinery. In this regard, a single non-synonymous mutation in *C. elegans cox*1 catalytic subunit (p.A12S) affected the mitochondrial membrane potential then modulating energy metabolism and allowing the nematode to adapt to new environments (Dingley *et al.*, 2014). Four non-synonymous mutations were described in *cox*1.404 from follow-up patients (I152V, F232L, H148P, G201S). Among them I152V, located in the fourth transmembrane domain, was the most frequent and was associated with cluster organization of haplotypes.

HP24 and HP93 were the most frequent haplotypes in our patient' population, distributed in American and Asian continents (Fig. 3) and became cluster founders in median-joining network analysis (C1 and C2, respectively). Despite their wide distribution and frequency that suggest their high fitness, C1 (HP24 founder) but not C2 (HP93 founder) was statistically associated with disease reactivation. Hence, I152V mutation, associated with C2, could be related with a lower mitochondrial energy metabolism in *S. stercoralis* reducing the odds of intra-host cycle reactivation.

Overall, *cox*1.404 haplotype/cluster determination could assist in the management of strongyloidiasis clinical evolution. C1 identification during diagnosis suggests the need to increase the frequency of medical examination compared to other genetic variants. In contrast, C2 (I152V mutation) and its protective role would avoid the irrational use of ivermectin as prophylaxis in immunocompromised patients. Also, C1 was an independent variable of reactivation, regardless of immunological status and no differences were found between immunological status, clinical forms and haplotype/cluster classification.

Coinfection and heteroplasmy theories should be further analysed, but in the context of clinical practice, PCR and sequencing are more realistic in low-income countries to generate information about the odds of future disease reactivations. *cox*1.404 variability should also be analysed during the follow-up process to better understand haplotype dynamics in time.

Other molecular markers should be studied to expand the knowledge of the epidemiology and clinical evolution of *S. stercoralis* infection. However, future analysis of nuclear and mitochondrial markers should consider that coevolution of both genomes is still unclear (Kikuchi *et al.*, 2016; Jaleta *et al.*, 2017).

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0031182021001517

Data. The 404 bp sequences (*cox*1.404 marker) from this study were deposited in GenBank under accession numbers MW680430-MW680476.

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Author contributions.

S.A.R. and P.R. conceived and designed the study protocol. D.R.S., M.F.S., M.V.R. and S.A.R. performed patient' evaluation. J.Q.B., L.B.A and E.I.B. carried out laboratory analyses. J.Q.B., M.G.R., S.A.R. and P.R. analysed and interpreted the data. M.G.R., S.A.R and P.R. supervised laboratory and data analyses and wrote the draft of the manuscript. J.M.B. and S.M.G.C. assisted in the manuscript review and editing.

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Conflict of interest. The authors declare there are no conflicts of interest.

Ethical standards. The use of samples from different collections was approved by the Ethics Committee of the Alberto C. Taquini Institute for Translational Medicine Research (Universidad de Buenos Aires). Informed consents were signed by all participants before sample collection.

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