Fasciola hepatica in Brazil: genetic diversity provides insights into its origin and geographic dispersion

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Abstract

Fasciola hepatica is a trematode parasite that affects mammals, including humans. In Brazil, fascioliasis, a disease caused by the parasite, is of great importance. The disorder affects the welfare of the Brazilian population through impairing the agricultural production of cattle, where the disease causes weight loss as a result of liver damage. This study aimed to evaluate the genetic diversity of *F. hepatica* throughout Southern Brazil to determine its geographic origin and estimate the colonization route of the parasite. To accomplish these aims, flukes were collected from slaughterhouses in three endemic areas of Rio Grande do Sul and Paraná states. DNA was isolated using the phenol–chloroform protocol from single flukes and two mitochondrial genes, cytochrome oxidase subunit I (COI) and nicotinamide dehydrogenase subunit 1 (Nad1), were amplified and sequenced. Ten haplotypes of COI were found from 75 isolated parasites and the total haplotype and nucleotide diversity observed were 0.475 and 0.002, respectively. Using the Nad1 gene, we found 24 haplotypes from 79 samples, resulting in haplotype and nucleotide diversity values of 0.756 and 0.004, respectively. An analysis of molecular variance showed that 57.4% and 77.5% of variation was within populations (F_{ST}), while 9.0 and 36.8% of variation was among groups (F_{CT}) when considering COI and Nad1 genes, respectively. For COI, the fixation index values of 0.425 and 0.368 were obtained for F_{ST} and F_{CT}, respectively, while analysis of Nad1 0.225 and 0.089 index values were obtained for F_{ST} and F_{CT}, respectively. We have determined that *F. hepatica* found in the two distinct areas originated from several geographical regions, since we found haplotypes that were shared with at least three different continents. These data are in accordance with the recent colonization of Brazil, and the recent import of cattle from South American, European and, possibly, some African countries. The observed F_{ST} and F_{CT} values for COI and Nad1 genes of *F. hepatica* may be a result of limited movement of animals within states and support the lack of geographical structure of the parasite in Brazil, which are in agreement with the observed cattle production systems in this region.

Introduction

Fascioliasis is one of the most important parasitic diseases of bovines, with approximately 700 million animals raised in areas in which there is a high level of risk of infection. *Fasciola hepatica* is a trematode parasite with a wide geographical distribution (Lotfy *et al.*, 2008). Although ruminants are the most important, and most frequently infected, livestock hosts (Dutra *et al.*, 2010), a variety of other mammals (i.e. horses, capybaras, deer and humans) can be infected and/or serve as natural reservoirs for the parasite (Mendes *et al.*, 2008; Ichikawa-Seki *et al.*, 2017).

Despite high incidence in domestic animals, very few human cases of fascioliasis have been reported in Brazil (Pritsch & Molento, 2018). The South of Brazil, which includes the states of Paraná (PR), Santa Catarina (SC) and Rio Grande do Sul (RS), is the region with the highest level of fascioliasis in ruminants in the country (Bennema *et al.*, 2014). Cattle in the state of RS are the most highly affected in the country (14.39%), with the economic impact on the region costing approximately $147 million/year due to losses in carcass weight (Molento *et al.*, 2017).

Even though it is largely believed that *F. hepatica* was introduced in South America by Portuguese and Spanish settlers who zealously transported animals to the region (Mas-Coma *et al.*, 2009; Ichikawa-Seki *et al.*, 2017), Carnevale *et al.* (2017) did not find any geographic structuration within Argentinean samples, using the ITS1 and mitochondrial
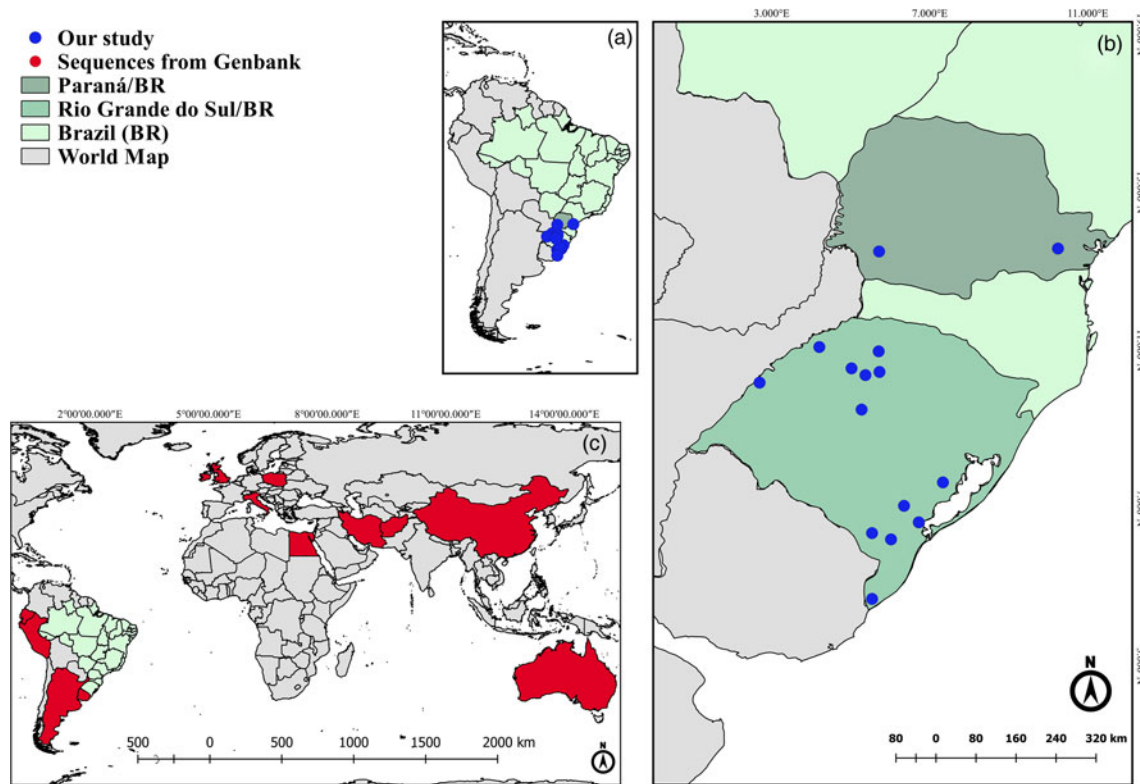
Fig. 1. (a) View of Latin America, sample points highlighted in blue; (b) geographic distribution of *Fasciola hepatica* samples in Brazil included in the study; (c) geographic origins of samples from GenBank included in our analysis (highlighted in red).

markers. *Fasciola hepatica* has been reported in Brazil since 1921, but there is little information concerning its genetic variation within local or regional populations. In addition, there is a complete lack of information regarding the geographic organization of *F. hepatica* genetic variation in Brazil, which could be useful to forecast the eventual dispersal of new, drug-resistant strains, as suggested by Beesley *et al.* (2017). This study aimed to evaluate the genetic diversity of *F. hepatica* in cattle from PR and RS and predict the spread of the parasite in the region.

Material and methods

Samples

Adult parasites were collected from cattle after liver inspection in slaughterhouses in 15 localities within RS and two in PR (supplementary table S1 and fig. 1). In total, 91 flukes were analysed in this study. After sampling, the trematodes were immediately stored in absolute ethanol at −80°C for later use, according to Itagaki *et al.* (2005). For the analysis, individual parasites collected from the same area were considered one population.

Molecular analysis

DNA extraction was performed from single flukes using phenol–chloroform, according to Green & Sambrook (2012). We amplified two mitochondrial genes, the cytochrome oxidase subunit 1 (COI) and the nicotinamide dehydrogenase subunit 1 (Nad1), using primer pairs ITA8/ITA9 and ITA2/ITA10, respectively, following the protocol described in Itagaki *et al.* (2005). After analysis using electrophoresis in an agarose gel, polymerase chain

reaction products were purified using 13% Polyethylene Glycol (PEG) precipitation and sequenced in both directions, using an ABI 3500 automated DNA sequencer (with BigDye Terminator Chemistry, Belo Horizonte, (MG), Brazil).

Statistical analysis

Base calling and sequence accuracy procedures were performed using the Staden software package (Staden, 1996), and polymorphic sites were confirmed by the visual inspection of sequence chromatograms. Indices of population diversity (number of haplotypes, haplotype diversity (Hd) and nucleotide diversity) and Tajima's D test were calculated using the DNAsp 5.0 (Librado & Rozas, 2009). Identification of haplotypes and the construction of network trees were performed using the medium joining method with Network 5.0 (Bandelt *et al.*, 1999). In addition to the samples we collected, we used sequences deposited in GenBank for the geographic comparison of haplotypes. We downloaded sequences from 14 countries (Peru, Argentina, Ecuador, Uruguay, UK, Ireland, Italy, Poland, Egypt, Afghanistan, Iran, China, Australia and Brazil), resulting in a total of 197 sequences of the COI gene (supplementary table S2) and 254 of the Nad1 gene (supplementary table S3).

We used the analysis of molecular variance (AMOVA) to search for the main source of genetic variability of *F. hepatica*, and F-statistics were used to estimate the proportion of genetic variability among populations (F_{ST}), among populations within groups (F_{SC}) and among groups (F_{CT}). The AMOVA was run with populations grouped according to geographical sampling (RS and PR), considering that values close to 1 indicated an

Table 1. Indexes of population diversity of *Fasciola hepatica* for the COI and Nad1 genes.

Genes									
	COI				Nad1				
State	City	N	π	h	Hd	City	N	π	h	Hd
Rio Grande do Sul	Arroio Grande	9	0.00469	3	0.556	Arroio Grande	9	0.00384	5	0.722
	Camaquã	5	0.00528	3	0.700	Camaquã	4	0.00887	2	0.500
	Canguçu	2	–	1	–	Canguçu	2	0.00177	2	1
	Herval	1	–	–	–	Herval	8	0.00602	7	0.964
	Ijuí	1	–	–	–	Ijuí	1	–	–	–
	Júlio de Castilhos	7	0.00075	2	0.286	Júlio de Castilhos	5	0.00957	3	0.800
	Palmeira	7	–	1	–	Palmeira	6	0.00059	2	0.333
	Pejuçara	6	–	1	–	Pejuçara	5	–	1	–
	Pelotas	3	0.00176	2	0.667	Pelotas	5	0.00248	3	0.800
	Santa Bárbara do Sul	3	–	1	–	Santa Bárbara do Sul	6	0.00508	5	0.933
	Santa Vitória do Palmar	6	0.00088	2	0.333	Santa Vitória do Palmar	6	0.00059	2	0.333
	Santo Cristo	4	0.00132	2	0.500	Santo Cristo	2	0.00177	2	1
	São Borja	12	0.00108	2	0.409	São Borja	10	0.00063	2	0.356
	All cities of RS	66	0.00178	8	0.375	All cities of RS	69	0.00384	23	0.756
Paraná	Curitiba	6	0.00246	3	0.733	Curitiba	4	0.00177	3	0.833
	Nova Prata do Iguaçu	3	0.00176	2	0.667	Nova prata do Iguaçu	6	–	1	–
	All cities of PR	9	0.00249	4	0.694	All cities of PR	10	0.00071	3	0.378
	All samples	75	0.00211	10	0.475	All samples	79	0.00358	24	0.756
	Tajima's D: −1.86913		$P < 0.05$			Tajima's D: −2.43824		$P < 0.01$		

N, number of samples; π, nucleotide diversity; h, number of haplotypes; Hd, haplotype diversity.

extreme differentiation between the populations, and values close to zero indicated a total genetic mix among populations. Both types of analysis were performed using the Arlequin program 3.5.2 (Excoffier & Lischer, 2010).

Results

We analysed the COI gene (379 bp) from 75 samples and obtained Hd and nucleotide diversity (π) values of 0.475 and 0.002, respectively. Among these, ten distinct haplotypes were identified. Regarding the Nad1 gene (564 bp), we identified 24 distinct haplotypes from a total of 79 samples, resulting in a Hd value of 0.756 and a nucleotide diversity value of 0.004 (table 1).

The COI haplotype network built with the samples from our study presented a star-like model, where the C_1 haplotype was the most frequently observed and consisted of 54 samples that were distributed throughout both RS and PR (fig. 2). In addition to C_1, the C_5 haplotype appeared in both areas. Six C_5 haplotypes were shared among cities in RS and two were shared within PR (fig. 2). When the 197 sequences from Genbank were included in our COI network, we observed 46 haplotypes (fig. 3). Two haplogroups were formed; the first haplogroup was the most diverse, containing haplotypes from all analysed countries. In this haplogroup, the most frequently observed haplotype was C_1, in which a total of 125 sequences were included

(including 54 from our study). The second haplogroup seemed to be more restricted, including samples mainly from Iran. In this haplogroup, the most frequent haplotype, comprising 42 sequences, was C_2, which included 36 sequences from Iran, two from Poland, one from Peru and three from Brazil (identified in our study).

The haplotype network of the Nad1 gene was performed using only the newly identified, Brazilian samples. The analysis resulted in the identification of 24 haplotypes out of 79 total samples (fig. 2). The most frequent haplotype observed was N_1, comprising 32 individual samples, and the second was the N_7, with 23 samples. Curiously, 22 haplotypes were not shared among any city. When GenBank samples were added to the analysis, we obtained a total of 333 sequences, 88 haplotypes and four haplogroups (fig. 4). The sequences of haplogroups 1 and 2 were composed of the greatest concentration of samples from Europe, Asia and Africa, whereas the haplogroups 3 and 4 were mainly composed of a mixture of samples from South America. There were two highly shared haplotypes, N_1 and N_7, belonging to the South American haplogroups 3 and 4, respectively. Haplotype N_7 was the most frequently occurring; 90 sequences of the haplotype were identified, distributed between Afghanistan, Argentina, Ecuador, Egypt, Peru, Poland, Italy, the UK, Iran and Brazil. Of these 90 sequences, 24 were found in our study. The second most frequent haplotype
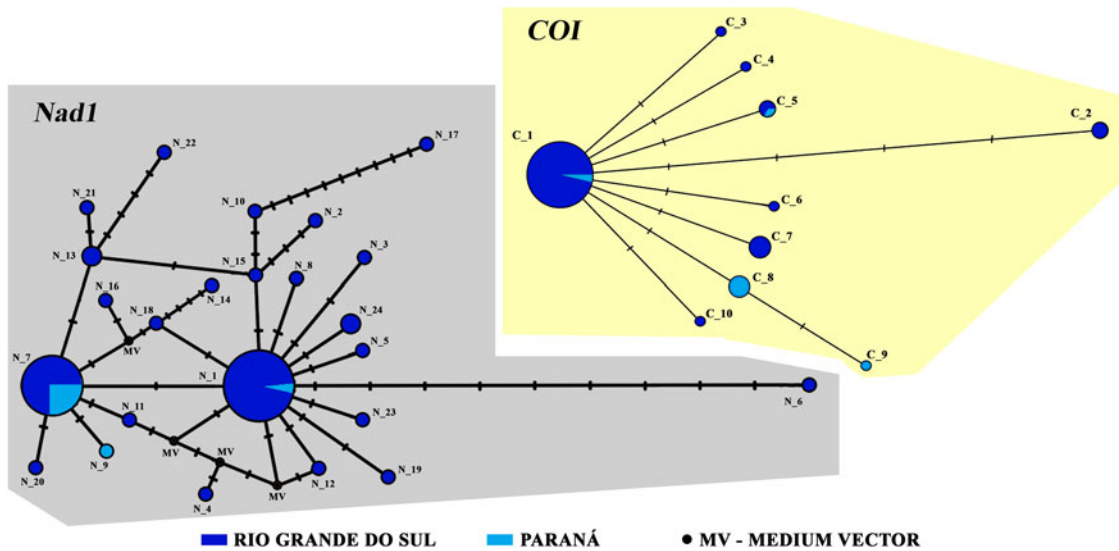
Fig. 2. Network analysis of Nad1 and COI genes of *Fasciola hepatica* samples from this study. In grey, the distribution of 24 haplotypes of the Nad1 gene are shown; in beige, the distribution ten haplotypes for the COI gene are presented.
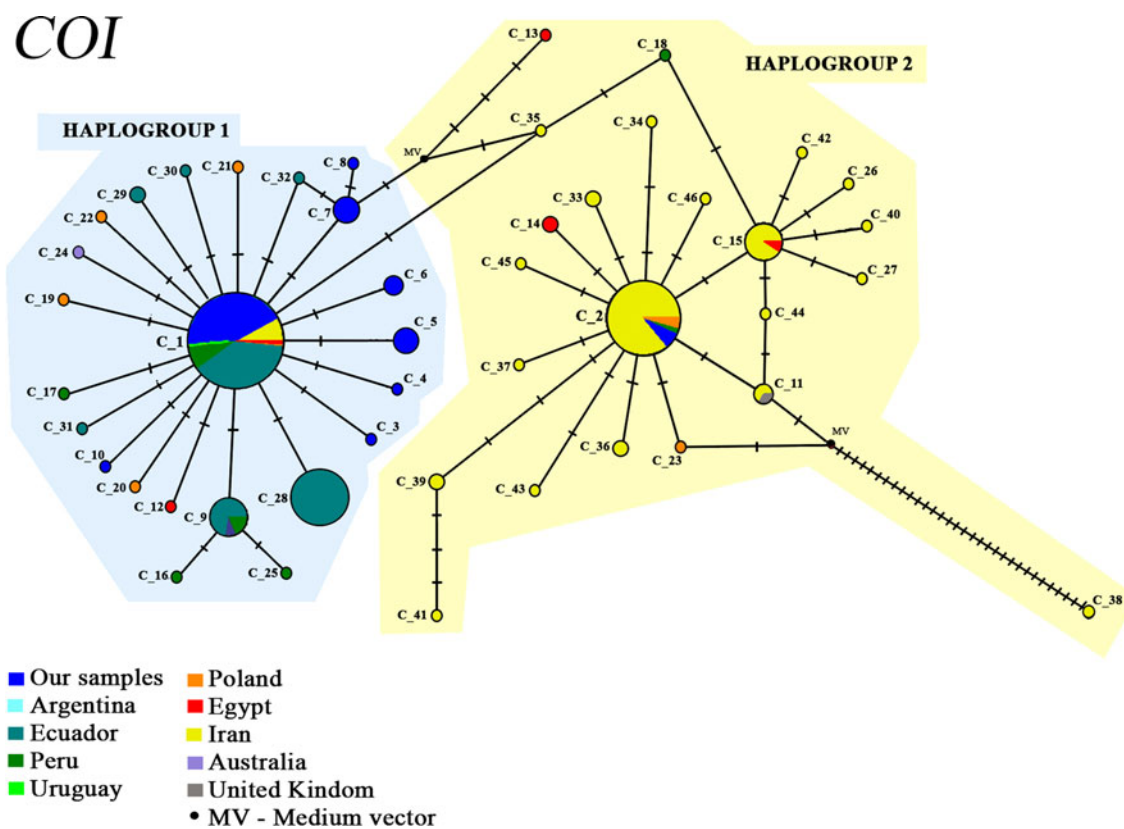

Fig. 3. Network analysis for the COI gene. In this analysis, we grouped sequences identified in the study with samples from other regions of the world. The colours correspond to their respective geographical locations.

was N_1, consisting of 78 sequences. This group was formed by individuals from Ecuador, Peru, Egypt, Uruguay, Argentina and our newly identified samples from Brazil (34 sequences). When we compared all the existing haplotypes of this analysis, we had a total of 15 haplotypes found exclusively in the RS and PR states.

The results of the analysis of population structure showed that most of the genetic diversity observed was within populations (COI: 57.4%; Nad1: 77.5%). The F_{ST} index value for the COI and Nad1 genes were 0.425 and 0.225, respectively. F_{CT} index values for COI and Nad1 were 0.368 and 0.089, respectively. When the COI gene was considered, there was 36.8% similarity
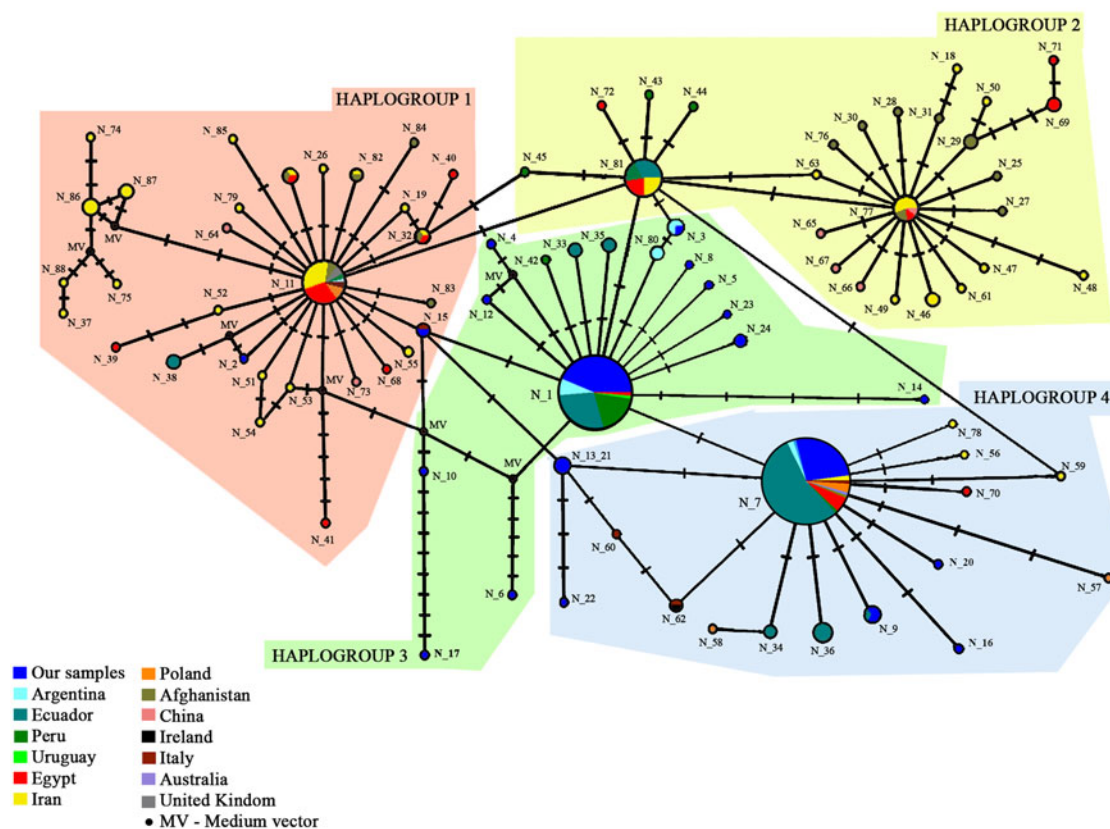
Nad1

Fig. 4. Network analysis for the Nad1 gene. In this analysis, we grouped sequences identified in the study with samples from other regions of the world. The colours correspond to their respective geographical locations.

Table 2. AMOVA results based on the COI and Nad1 genes of *Fasciola hepatica* from Southern Brazil.

	Source of variation	Degrees of freedom	Percentage of variation (%)
COI	Among groups	1	36.81
	Among groups within population	13	5.75
	Within populations	60	57.45
	F-statistic	F_{ST}: 0.425	
		F_{CT}: 0.368	
		F_{SC}: 0.091	
Nad1	Among populations	1	8.99
	Among groups within population	13	13.51
	Within populations	64	77.51
	F-statistic	F_{ST}: 0.225	
		F_{CT}: 0.089	
		F_{SC}: 0.148	

among groups (table 2). The Tajima's D test of neutrality produced negative values, which were significant for both COI (−1869, $P < 0.05$) and Nad1 (−2438, $P < 0.01$) genes (table 1).

Discussion

This is the first report of the genetic characterization of *F. hepatica* from infected cattle isolated from different regions of Brazil. Diversity indices, evaluated using two mitochondrial genes for analysis, produced findings similar to others that were carried out in different countries. For example, a study in Peru analysed the Nad1 fragment from 78 individual parasites and found eight haplotypes (Hd = 0.685 and π = 0.00175) (Ichikawa-Seki *et al.*, 2016). A study conducted in Argentina examining 22 individuals, identified seven haplotypes for the COI gene. When two other mitochondrial genes were analysed – Nad4 and Nad5 – four and three haplotypes were identified, respectively (Carnevale *et al.*, 2017). Elliott *et al.* (2014) analysed 208 specimens in a study that yielded only six COI haplotypes (Hd = 0.482 and π = 0.003), and 18 Nad1 haplotypes (Hd = 0.832 and π = 0.005) in Australia.

A possible explanation for both high Hd and low nucleotide diversity could be related to the arrival of *F. hepatica* in Peru, Argentina, Australia and Southern Brazil, with a very small number of individuals, each from a much larger parental population, creating a Founder's effect. To better explain the large number of haplotypes observed, we suggest that the introduction of *F. hepatica* in Brazil occurred in several separate human/cattle immigration waves. A similar scenario was pointed out to explain the findings of Semyenova *et al.* (2006), in which researchers analysed populations from eastern Europe and western Asia with two different lineages. Lineage 1 was shared with Europe, Caucasus, Asia and Oceania, and lineage 2 was shared with European, Armenian and American populations (the USA and Uruguay).

We hypothesize that the introduction of *F. hepatica* to Brazil could have happened in accordance with two different scenarios. First, it could be due to land migration of wild animals by the Great American Interchange (i.e. wild ruminants from Peru). Second, effects could be due to Portuguese and Spanish colonization (i.e. movement of Catholic settlements and commerce). As nucleotide substitutions are rare, we suppose that there has not been enough time to generate many nucleotide substitutions with regard to ancestral haplotypes (C_1 and N_7). The same pattern has been observed in other helminth parasites after the introduction to new areas, including with *Echinococcus granulosus* in South America (Sharma *et al.*, 2013). Also, the exclusive haplotypes found in our samples generally contained only one substitution compared to the more frequent haplotypes. The neutrality test (table 1) and all the networks calculated for both genes obtaining a star-like model, indicating population expansion or lineage sorting (Avise, 2000).

The large number of haplotypes identified in our study may be associated with the optimal conditions for the intermediate host, since the landscape is formed by lowland areas with a large number of water sources (Dutra *et al.*, 2010; Bennema *et al.*, 2017). Epidemiological studies show that the dynamics of ruminant diseases should be combined with the understanding of climatological and environmental data, since these factors directly influence the continuity of the parasite cycle (Charlier *et al.*, 2016). Thus, we believe that, once brought into Americas, parasites faced numerous challenges (different climate and host adaptation). Accordingly, some local hosts may have offered ideal environments for parasite establishment. The lowlands of the Pampa region in the South of RS represents a complete habitat to the intermediate host, as well as being used to sustain large cattle herds.

The highest portion of the genetic diversity was found within populations (table 2), in accordance with population dynamics of the usual, definitive cattle host in this region, and could be due to cattle movement that contributes to the mixture of populations of *F. hepatica* within areas observed. However, an important proportion of genetic diversity of the species was found among groups (flukes sampled in each Brazilian state comprised a different group). This observation can be explained by the limited cattle movement occurring between these two states, while the cattle movement within each state was considerably high. These findings are supported with calculated F_{CT} index (COI: 0.368; Nad1: 0.089) and F_{ST} (COI: 0.425; Nad1: 0.225) values, showing a geographic structuring among and within RS and PR samples.

In a study analysing flukes from the UK, Beesley *et al.* (2017) found that the widespread movement of definitive hosts could significantly contribute to the dispersal of *F. hepatica* variants, leading to the low F_{ST} values. Walker *et al.* (2011) reported low levels of genetic structure in fluke populations from the Netherlands. The aforementioned study contrasts with our data; differences that are probably due to our wide geographical sampling area, differences in cattle migration/commerce and the timing of the establishment of fluke populations from South America, which were established more recently than those in Europe (supplementary tables S4 and S5).

Taken together, this comparison of nucleotide and Hd indicates that the colonization of Southern Brazil was made by several *F. hepatica* haplotypes. This agrees with a statement made by Ichikawa-Seki *et al.* (2016), arguing that the *F. hepatica* population in Peru was originated by numerous haplotypes, from multiple regions, but mainly originating from Europe.

Analysing the network tree constructed using the whole set of sequences, we observed a tree topology consisting of two main groups, with neither seeming to be characteristic of any specific region of the world. The great mixture among the samples suggests a high level of parasite circulation among populations from Europe, Asia and Africa. Moreover, this tree topology shows that the south Brazilian populations of *F. hepatica* were originated by at least three haplotypes shared by different areas of the world. The high dispersion capacity of the definitive hosts, i.e. dispersion caused by the transport of animals for breeding, demonstrates an increase in the dispersion of some parasite genotypes, which occasionally became more frequent, increasing opportunities for parasite adaptation and causing problems in the management of disease (Auld & Tinsley, 2015).

Our data regarding the genetic diversity of *F. hepatica* demonstrated that the parasite possesses a relatively high level of Hd, and presents a tree network topology with a great mixture in both lines of ancestral sequences mainly from Europe, Asia and Africa, and derivate population sequences from South America. This may be explained by the carriage of different variants of *F. hepatica* to the Americas through the introduction of infected animals. As *F. hepatica* faced intense circulation in Europe, Asia and Africa before the American colonization, our data cannot determine the exact centre of origin of our samples. The partition of the AMOVA and the value of F_{ST} support the lack of geographical structure in Brazil, which are in agreement with the observed cattle production systems in this region.

The molecular characterization of *F. hepatica* from Brazil can be used as a key factor to understand epidemiological aspects of the disease. In addition, understanding the geographical structuration observed in different regions, related to the fact that flukes can infect many mammals (including humans), may provide insights to aid local management and regional health programs designed to combat the parasites. Furthermore, nuclear markers, such as microsatellites or genes associated with parasite adaptation, should be used for future studies.

References

Auld SK and Tinsley MC (2015) The evolutionary ecology of complex lifecycle parasites: linking phenomena with mechanisms. *Heredity* **114**, 125–132.

Avise J (2000) *Phylogeography: the history and formation of species.* Cambridge, MA, Harvard University Press.

Bandelt H, Forster P and Rohl A (1999) Median-joining network for inferring intraspecific phylogenies. *Molecular Biology and Evolution* **161**, 37–48.

Beesley NJ, Williams DJ, Paterson S and Hodgkinson J (2017) *Fasciola hepatica* demonstrates high levels of genetic diversity, a lack of population structure and high gene flow: Possible implications for drug resistance. *International Journal for Parasitology* **47**, 11–20.

Bennema SC, Scholte RGC, Molento MB, Medeiros C and Carvalho ODS (2014) *Fasciola hepatica* in bovines in Brazil: data availability and spatial

distribution. *Revista do Instituto de Medicina Tropical de São Paulo* **56**, 35–41.

Bennema SC, Molento MB, Scholte RG, Carvalho OS and Pritsch I (2017) Modelling the spatial distribution of *Fasciola hepatica* in bovines using decision tree, logistic regression and GIS query approaches for Brazil. *Parasitology* **14**, 1–9.

Carnevale S, Malandrini JB, Pantano ML, Soria CC, Rodrigues-Silva R, Machado-Silva JR, Velásquez JN and Kamenetzky L (2017) First genetic characterization of *Fasciola hepatica* in Argentina by nuclear and mitochondrial gene markers. *Veterinary Parasitology* **245**, 34–38.

Charlier J, Ghebretinsae AH, Levecke B, Ducheyne E, Claerebout E and Vercruysse J (2016) Climate-driven longitudinal trends in pasture-borne helminth infections of dairy cattle. *International Journal for Parasitology* **46**, 881–888.

Dutra LH, Molento MB, Naumann CRC, Biondo AW, Fortes FS, Savio D and Malone JB (2010) Mapping risk of bovine fasciolosis in the south of Brazil using Geographic Information Systems. *Veterinary Parasitology* **169**, 76–81.

Elliott T, Muller A, Brockwell Y, Murphy N, Grillo V, Toet HM, Anderson G, Sangster N and Spithill TW (2014) Evidence for high genetic diversity of *NAD1* and *COX1* mitochondrial haplotypes among triclabendazole resistant and susceptible populations and field isolates of *Fasciola hepatica* (liver fluke) in Australia. *Veterinary Parasitology* **200**, 90–96.

Excoffier L and Lischer HEL (2010) Arlequin suite ver 3.5: a new series of programs to perform population genetics analyses under Linux and Windows. *Molecular Ecology Resources* **10**, 564–567.

Green MR and Sambrook J (2012) *Molecular cloning – a laboratory manual*. 4th edn. New York, Cold Spring Harbor Laboratory Press.

Ichikawa-Seki M, Ortiz P, Cabrera M, Hobán C and Itagaki T (2016) Molecular characterization and phylogenetic analysis of *Fasciola hepatica* from Peru. *Parasitology International* **65**, 171–174.

Ichikawa-Seki M, Shiroma T, Kariya T, Nakao R, Ohari Y, Hayashi K and Fukumoto S (2017) Molecular characterization of *Fasciola* flukes obtained from wild sika deer and domestic cattle in Hokkaido, Japan. *Parasitology International* **66**, 519–521.

Itagaki T, Kikawa M, Sakaguchi K, Shimo J, Terasaki K, Shibahara T and Fukuda K (2005) Genetic characterization of parthenogenic *Fasciola* sp.

in Japan on the basis of the sequences of ribosomal and mitochondrial DNA. *Parasitology* **131**, 679–685.

Librado P and Rozas J (2009) DnaSP v5, a software for comprehensive analysis of DNA polymorphism data. *Bioinformatics* **25**, 2496–2597.

Lotfy WM, Brant SV, DeJong RJ, Le TH, Demiaszkiewicz A, Rajapakse RP, Perera VB, Laursen JR and Loker ES (2008) Evolutionary origins, diversification, and biogeography of Liver Flukes. *American Journal of Tropical Medicine and Hygiene* **79**, 248–255.

Mas-Coma S, Valero MA and Bargues MD (2009) Fasciola, Lymnaeids and human fascioliasis, with a global overview on disease transmission, epidemiology, evolutionary genetics, molecular epidemiology and control. *Advances in Parasitology* **69**, 141–146.

Mendes EA, Lima WS and De Melo AL (2008) Development of *Fasciola hepatica* in *Lymnaea columella* infected with miracidia derived from cattle and marmoset infections. *Journal of Helminthology* **82**, 81–84.

Molento MB, Bennema S, Bertot J, Pritsch IC and Arenal A (2017) Bovine fasciliasis in Brazil: Economic impact and forecasting. *Veterinary Parasitology: Regional Studies and Reports* **12**, 1–3.

Pritsch I and Molento MB (2018) Recount of reported cases of human fascioliasis in Brazil over the last 60 years. *Journal of Tropical Pathology* **47**, 75–85.

Semyenova SK, Morozova EV, Chrisanfova GG, Gorokhov VV, Arkhipov IA, Moskvin AS, Movsessyan SO and Ryskov AP (2006) Genetic differentiation in eastern European and western Asian populations of the liver fluke, *Fasciola hepatica*, as revealed by mitochondrial *Nad1* and cox1 genes. *Journal of Parasitology* **92**, 525–530.

Sharma M, Fomda BA, Mazta S, Sehgal R, Singh BB and Malla N (2013) Genetic diversity and population genetic structure analysis of *Echinococcus granulosus* sensu stricto complex based on mitochondrial DNA signature. *PLoS One* **8**, e82904.

Staden R (1996) The Staden sequence analysis package. *Molecular Biotechnology* **5**, 233–241.

Walker SM, Johnston C, Hoey EM, Fairweather I, Borgsteede F, Gaasenbeek C, Prodöhl PA and Trudgett A (2011) Population dynamics of the liver fluke, *Fasciola hepatica*: the effect of time and spatial separation on the genetic diversity of fluke populations in the Netherlands. *Parasitology* **138**, 215–223.