Tânia Raquel Martins dos Santos

Undergraduate in Biology

Genetic characterization of Portuguese *Fasciola hepatica* isolates

Dissertation presented to obtain the Master Degree in
Molecular, Genetics and Biomedicine

Supervisor: Prof.ª Doutora Maria Manuela Calado
Professora Auxiliar
Instituto de Higiene e Medicina Tropical, Universidade Nova de Lisboa

Co-supervisor: Doutora Ana Júlia Pinto Fonseca Sieuve Afonso
Investigadora Auxiliar
Instituto de Higiene e Medicina Tropical, Universidade Nova de Lisboa

Members of the Jury:

President: Prof.ª Doutora Paula Maria Mendes Bernardo Gonçalves
Principal Examiner: Doutora Fernanda Henriques de Jesus Rosa
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Part of the results discussed in this dissertation was presented in the following communications:

R. Santos, M. Calado, J. Sampaio, C. Ferreira, A. Afonso and S. Belo. Contribution to the genetic characterization of *Fasciola hepatica* populations in Portugal. XXXVII Portuguese Genetic Conference, Lisbon, Portugal, May 28th-30th 2012 [poster communication]

R. Santos, M. Calado, J. Sampaio, C. Ferreira, A. Afonso and S. Belo. Contribution to the genetic characterization of *Fasciola hepatica* populations in Portugal. Arquivos Portugueses das Ciências Biológicas. Tomo XXXVI *(in press)*

ACKNOWLEDGEMENTS

I want to express my gratitude to all that supported me in the realization of this Master and dedicate to them several words in my native language:

Em primeiro lugar quero agradecer à Professor Doutora Silvana Belo por tudo o que fez para permitir que este projecto fosse avante e por me ter recebido tão bem na sua equipa.

Quero também agradecer à Professora Doutora Manuela Calado por toda a ajuda prestada, pelos conhecimentos partilhados, pela paciência que teve comigo e por todo o tempo que me disponibilizou. A sua orientação e supervisão foram fundamentais para levar este trabalho a bom porto. Muito obrigada!

Agradeço à Doutora Ana Afonso por todo o apoio e ajuda que me deu, pelos ensinamentos que me transmitiu e pela disponibilidade que sempre teve comigo. A nível pessoal tenho de agradecer pelas suas palavras amigas e pelos conselhos que me deu ao longo destes anos, sem ela nada disto seria possível. Muito obrigada!

À Mestre Cátia Ferreira agradeço a ajuda prestada nas primeiras etapas deste trabalho. Os seus conhecimentos foram essencias à realização deste trabalho. Agradeço toda a disponibilidade, todas as dúvidas esclarecidas e todos os artigos emprestados.

À Técnica Especialista Isabel Clemente agradeço o carinho, os ensinamentos e as palavras amigas.

Ao Professor Doutor José Paulo Sampaio agradeço por ter coordenado o Mestrado de forma tão eficiente e dedicada e pela prontidão na ajuda prestada.

Agradeço a todos os meus professores que me abriram os olhos para o mundo da ciência e do desconhecido. Sem eles não teria percebido que o caminho da investigação é sempre o mais divertido.

Às minhas gurias, fantásticas colegas de Mestrado, agradeço o companheirismo, o carinho e a amizade que agora nos une.

Agradeço a todos os meus amigos, por estarem sempre lá, sem pedir nada em troca. Por estarem presentes, mesmo quando eu me ausentava por meses a fio. Pelas palavras de encorajamento e apoio reforço positivo. Por todos os momentos de diversão e pelas gargalhadas. Mas, principalmente, pelo carinho, respeito e confiança demonstrados ao longo dos anos. Obrigada!

Ao Timon, muito obrigada por toda a ajuda fornecida ao longo deste trabalho e pela disponibilidade e paciência demonstradas. Obrigadão! Espero estar à altura de retribuir.

Quero agradecer à minha família o amor e apoio incondicional que sempre me deram. São eles que dão sentido a todos os meus projectos de vida.

À minha mãe-tia e ao meu pai-tio agradeço todo o carinho, conforto e amor dados.

Quero também agradecer ao meu mano, à Susana e à Elsa, por serem os melhores irmãos do mundo, por cuidarem de mim, por serem seres humanos fantásticos e, especialmente, por serem a imagem do que eu queria ser quando fosse grande.

Ao meu pai agradeço por toda a confiança que sempre depositou em mim e por permitir que eu seguisse o meu caminho, com toda a liberdade.

À minha mãe quero agradecer por tudo o que sou. É o meu maior exemplo de amor, moral, coragem, preserverança, esforço e espírito de sacrifício. Agradeço por nunca me ter deixado sozinha em momento algum e por ser o meu porto de abrigo. Agradeço sobretudo por ser para mim muito mais do que o que eu mereço.

Quero ainda agradecer ao Splinter por ser extraordinário. Por cuidar de mim, dar força e por me puxar para o lado bom, sempre. Por acompanhar esta viagem como se fosse sua. Por fazer deste planeta um lugar muito mais bonito para se viver. Obrigada! Muito obrigada!

Ao Xuxu agradeço a companhia constante ao longo deste nosso percurso.

ABSTRACT

Fasciola hepatica is a parasitic trematode with debilitating and socio-economically devastating effects. At present near to 600 million animals and 2.4 million people in the entire world suffer from fascioliasis. Genetic characterization is of the utmost importance to an efficient epidemiologic control of helminth infections. In the present study we aimed to provide the first insights into the genetic variability of *F. hepatica* in Portugal. 47 isolates from different hosts (cattle and sheep) and geographical locations (Beja, Castelo Branco, Coimbra, Évora, Faro, Leiria, Lisboa, Portalegre, Santarém and Setúbal) were analyzed through Random Amplified Polymorphic DNA-Polymerase Chain Reaction (RAPD-PCR), Restriction Fragment Length Polymorphism (RFLP) and sequencing of NADH dehydrogenase subunit 1 (*nad*1) gene, cytochrome c oxidase subunit 1 (*cox*1) gene and Internal Transcribed Spacers (ITS) region. RAPD-PCR and RFLP patterns were similar for all the analyzed samples, despite their host and geographical origin. Nucleotide sequencing revealed low levels of genetic diversity within Portuguese isolates and no direct correlation was observed between haplotype and geographical location or host. Phylogenetic analysis revealed a high similarity within samples from Mediterranean countries, such as Portugal, Spain, Tunisia, Algeria and Egypt, possibly due to livestock import/export trade between these countries. Moreover, Portugal presents a low risk of fascioliasis drug-resistance.

Keywords: *Fasciola hepatica*; genetic characterization; Portugal; *cox*1; *nad*1, ITS region.

RESUMO

A *Fasciola hepatica* é um parasita tremátode com efeitos devastadores na saúde e a nível socio-económico. Actualmente, perto de 600 milhões de animais e 2.4 milhões de pessoas em todo o mundo sofrem de fasciolíase. A caracterização genética é de extrema importância para um controlo epidemiológico eficiente de infecções helmínticas. Neste trabalho pretendemos iniciar o estudo sobre a variabilidade genética de *F. hepatica* em Portugal. 47 isolados de *F. hepatica* provenientes de diferentes hospedeiros (bovinos e ovinos) e localizações geográficas (Beja, Castelo Branco, Coimbra, Évora, Faro, Leiria, Lisboa, Portalegre, Santarém e Setúbal) foram analisados através de Random Amplified Polymorphic DNA-Polymerase Chain Reaction (RAPD-PCR), Restriction Fragment Length Polymorphism (RFLP) e sequenciação do gene codificante da subunidade I da desidrogenase NADH (*nad*1), do gene codificante da subunidade I do citocromo C oxidase (*cox*1) e da região que comprime os Internal Transcribed Spacers (ITS). Os padrões de RAPD-PCR e de RFLP foram semelhantes em todas as amostras analisadas, independentemente do hospedeiro e da região de origem. A sequenciação nucleotídica revelou baixos níveis de diversidade genética nos isolados portugueses e não foi observada nenhuma correlação entre o haplótipo e a localização geográfica ou o hospedeiro. A análise filogenética revelou elevada semelhança nas amostras vindas de países mediterrânicos, como Portugal, Espanha, Tunísia, Argélia e Egipto, provavelmente devido ao comércio de animais entre estes países. Os nossos dados levam-nos ainda a crer que Portugal apresenta um baixo risco de resistência a fármacos contra a fasciolíase.

Termos-chave: *Fasciola hepatica*; caracterização genética; Portugal; *cox*1; *nad*1; região ITS.

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ABBREVIATIONS

π	Nucleotide diversity
ATP	Adenosine Triphosphate
bp	Base pairs
BSA	Bovine Serum Albumine
CTAB	Hexadecyltrimethylammonium Bromide
***cox*1**	Cytochrome c oxidase subunit 1
dATP	Deoxyadenosine Triphosphate
dCTP	Deoxycytidine Triphosphate
dGTP	Deoxyaguanosine Triphosphate
DNA	Deoxyribonucleic Acid
Dna SP™	DNA Sequence Polymorphism
dTTP	Deoxythymidine Triphosphate
EDTA	Ethylenediamine Tetraacetic Acid
ELISA	Enzyme-Linked Immunosorbent Assay
EtBr	Ethidium bromide
F. gigantica	*Fasciola gigantica*
F. hepatica	*Fasciola hepatica*
Fig	Figure
G + C	Guanine + Cytosine
G. truncatula	*Galba truncatula*
G. cubensis	*Galba cubensis*
H	Haplotype
HCL	Hydrochloric acid
Hd	Haplotype diversity
ITS	Internal Transcribed Spacers
K	Average number of nucleotide differences
KCl	Potassium chloride
MEGA™	Molecular Evolutionary Genetics Analysis
$MgCl_2$	Magnesium chloride
NaCl	Sodium chloride
***nad*1**	NADH dehydrogenase subunit 1
NADH	Nicotinamide Adenine Dinucleotide Hydride
NCBI	National Center for Biotechnology Information
PCR	Polymerase Chain Reaction
pH	Negative logarithm for hydrogen ion

RAPD	Random Amplified Polymorphic DNA
rDNA	Ribosomal DNA
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribonucleic Acid
rpm	Rotations per minute
TAE	Tris-acetate + EDTA buffer
TE	Tris-HCL + EDTA buffer
WHO	World Health Organization

Chapter I. INTRODUCTION

Chapter I. INTRODUCTION

1. General Aspects

Fascioliasis, or fasciolosis, is one of the most prevalent helminth infections of ruminants in the world causing significant morbidity and mortality and considerable socioeconomic problems (Okewole *et al*., 2000).

The disease is caused by digenean trematodes, commonly referred to as liver flukes. The two etiological agents of fascioliasis are *Fasciola hepatica* (Linnaeus, 1758) and *Fasciola gigantica* (Cobbold, 1885). The former species has a world-wide distribution, mainly in temperate climates and the latter species exists predominantly in tropical areas (Mas-Coma *et al*., 2005).

Animals become infected by ingesting encysted metacercariae attached to aquatic or semi-aquatic plants. Evidence also indicates that infection may occur by drinking water from ponds and sloughs contaminated with floating metacercariae. Once ingested, the metacercariae excyst, migrate through the intestinal wall to the body cavity and then to the liver. The young flukes migrating in the hepatic parenchyma eventually locate a bile duct and complete their development to the adult stage (Troncy, 1989). When the hermaphrodite fluke reaches sexual maturity it begins to lay eggs that reach the small intestine and are excreted with the stools to continue the life cycle in a freshwater snail from the family *Lymnaeidae*, its intermediate host in which the parasite reproduces assexually (Maher *et al*., 1999).

The fluke devastates the liver while dwelling in the bile ducts and gall bladder. The infection can result in biliary cirrhosis, sclerosing cholangitis associated with destructive jaundice, liver abscesses and other serious hepatic and ectopic clinical manifestations (Price *et al*., 1993).

Parasites from the genus *Fasciola* infect mainly sheep, goat, cattle and occasionally affect humans, thus considered as a zoonotic infection (Andrews, 1999; Okewole *et al*., 2000; Savioli *et al*., 1999).

Other animals that may be affected include horses and pigs. Recently was also reported that llamas in South America, camels in Africa and marsupials in Australia may be acting as reservoir hosts in these regions (Mas-Coma *et al.*, 2005).

Fascioliasis causes significant economic loss, as valued by animal productivity, estimated at approximately 3.2 billion US dollars per annum to the global agricultural community with 600 million animals infected (Mas-Coma, 2005; McManus and Dalton, 2006; Spithill *et al.* 1997).

The economic losses due to fascioliasis are caused by morbidity, reduced growth rate, liver condemnation, increased susceptibility to secondary infections and metabolic diseases, weight loss, anemia, lower milk and wool production, decreased reproductive efficiency and in some cases mortality of the animals (Behm and Sangster, 1999; Loyacano *et al*., 2002; Malone *et al*., 1998).

Apart from its veterinary and economic importance throughout the world, fascioliasis has recently been shown to be a widespread zoonosis affecting people from the entire world (Mas-Coma *et al*., 1999a). Accordingly to the World Health Organization (WHO), at present there are nearly 2.4 million people, from 61 countries, that suffer from fascioliasis, but the number of people subjected to the risk of infection is 180 million worldwide (Keiser and Untzinger, 2005; WHO, 2007). Therefore, fascioliasis is no longer considered only as a secondary zoonotic disease but is now recognized as an emerging or re-emerging human disease in several countries (Mas-Coma, 2004; Mas-Coma *et al*., 1999a).

Fasciola hepatica is a physically and economically devastating parasitic trematode whose rise in recent years has been attributed to climate change. Climate has an impact on the free-living stages of the parasite and its main intermediate host *Galba truncatula*, with the interactions between rainfall and temperature having the greatest influence on transmission efficacy (Fox *et al*., 2006).

Due to its zoonotic signification and economic importance the treatment and prevention of fascioliasis is of major importance.

Several control methods against *Fasciola hepatica* are available and can either be used independently or as a combination of two or more of them, for example: reduction of the number of intermediate snail hosts, water treatment against metacercariae and chemotherapy against the adult parasites (Savioli *et al*., 1999; WHO, 2007).

However, for an efficient eradication of fascioliasis it is extremely important to understand the biology of this parasite. Genetic characterization of *Fasciola hepatica* populations is of major importance for a well-organized management of this disease. Control programs should consider the genetic diversity of the parasite as being the primary cause of resistance to antihelmintics and fast adaptation to climate changes.

Fig. I.1. Adult specimen of *Fasciola hepatica.* Picture by Raquel Santos.

2. *Fasciola hepatica*

2.1. Taxonomy

According to Lofty *et al.* (2008) and the National Center for Biotechnology Information (NCBI) (2012a) the taxonomic classification of *Fasciola hepatica* is presented as follows:

- Phylum: Platyhelminthes
- Subphylum: Neodermata
- Class: Trematoda
- Subclass: Digenea
- Superorder: Anepitheliocystida
- Order: Echinostomida
- Suborder: Echiostomata
- Superfamily: Echinostomatoidea
- Family: Fasciolidae
- Genus: *Fasciola*
- Specie: *Fasciola hepatica* (Linnaeus, 1758)

2.2. Life Cycle

Fasciola hepatica was among the first digenetic trematodes to have its live cycle completely elucidated, one which represents a typical example of a digenean life cycle, with miracidial and cercarial stages. The life cycle is heteroxenic and complex involving several phases and two hosts: a mammalian definitive host and an amphibious snail acting as the intermediate host (Andrews, 1999).

There may be some variations in the *Fasciola* life cycle, particularly within different definitive hosts, but the main factors affecting the life cycle tend to be the requirement of suitable temperature and sufficient moisture (Andrews, 1999).

Like all trematodes, with the exception of the genus *Schistosoma*, *Fasciola* spp. is hermaphrodite. Self-fertilization is the most common form of sexual reproduction. However, in stress conditions cross-fertilization can occur (Fletcher *et al*., 2004). The presence of assexually reproducing diploid and triploid *Fasciola* in Asia suggests that abnormal spermatogenesis and parthenogenic reproduction can be frequent in this parasite (Terasaki *et al*., 1998; Terasaki *et al*., 2000).

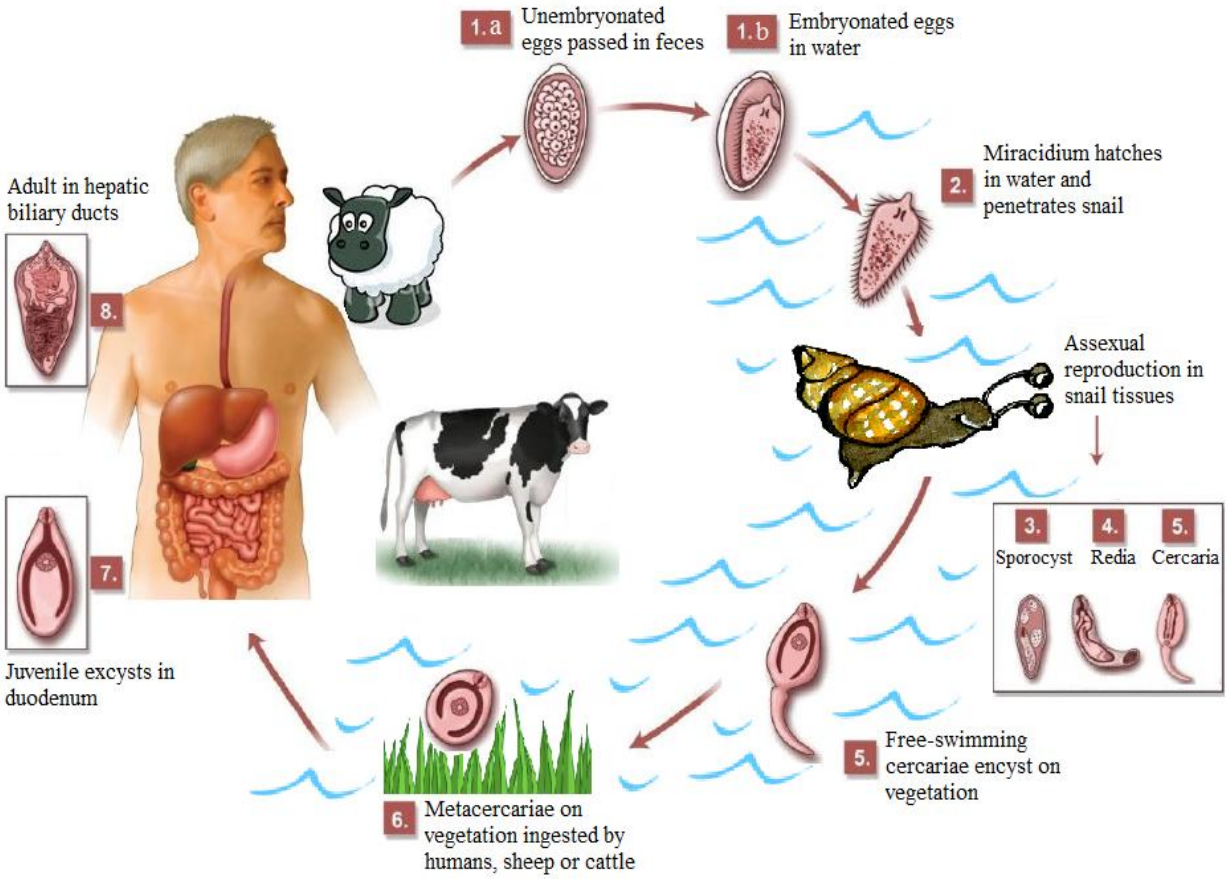

Fig. I.2. *Fasciola hepatica* life cycle including the stages egg **(1.a** and **1.b)**; miracidium **(2)**, sporocyst **(3)**, redia **(4)**, cercaria **(5)**, metaceraria **(6)**, juvenile **(7)** and adult **(8)**. Adapted from Dusak *et al*., (2012).

(1) Egg:

The oval-shaped eggs are laid by the adult parasite in the bile duct system of the definitive host and pass into the host alimentary tract, specifically the duodenum, with the bile. The eggs then leave the host through the faeces. Eggs have a thin shell and at this stage they are still not embryonated. Despite their thin shell, eggs are structurally strong (Andrews, 1999).

Embryonic maturation and development occurs when the eggs are released in fresh water and is regulated mainly by temperature, moisture and light, but also by the chemical characteristics of the water. Eggs can withstand temperatures from 0ºC to 37ºC, but the optimum temperature for egg development is between 15ºC to 25ºC and it generally takes 9 to 15 days before the eggs hatch to release the fully developed and motile miracidium (Togerson and Claxton, 1999).

(2) Miracidium:

The miracidium is the first free-living larval stage of *F. hepatica* and is a non-feeding organism, therefore its life span is determinate by the amount of energy stored (Graczyk and Fried, 1999). The miracidium is a fragile ciliated larva which swims vigorously in order to locate the intermediate host, then it actively penetrates the snail using a retractile papilliform process possibly

facilitated by the secretion of proteolytic enzymes (Simpkin *et al*., 1980; Smythe and Halton, 1983). The need to find a suitable host to penetrate is an urgent one. Miracidia that fail to do so generally die within 24 hours (Andrews 1999; Smythe and Halton, 1983).

(3) Sporocyst:

After penetrating the snail, specifically inside the muscle tissue, the miracidium loses its cilia and metamorphoses into a rounded sporocyst, which migrates into the snail's digestive gland (Smythe and Halton, 1983).

(4) Redia:

The sporocyst contains a fixed number of germ balls. Mother sporocysts reproduce by asexual mitotic divisions giving rise to the first generation of daughter rediae. The daughter rediae of the first generation free themselves by breaking through the body wall of the mother sporocyst, which subsequently dies. The daughter rediae then migrate to the snail's hepatopancreas (Graczyk and Fried, 1999). Usually every sporocyst gives rise to 5 to 8 motile rediae. Under adverse conditions, the rediae delay their development and give rise to a second generation of rediae, also by mitotic divisions. However, in adequate conditions, the germ cells in the brood chamber of the rediae develop into cercariae (Andrews, 1999; Smythe and Halton, 1983).

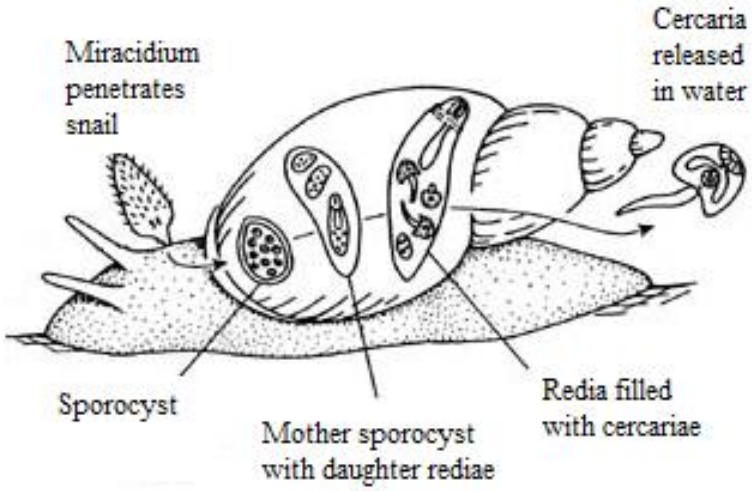

Fig. I.3. *Fasciola hepatica* life cycle. Assexual reproduction with sporocyst, redia and cercaria stages, inside intermediate host, in detail. Adapted from Roberts and Janovy (2009).

(5) Cercaria:

The mobile cercaria generally leaves the snail by migrating through the snail's tissues and become free swimming. The cercariae release happens during moist conditions when a critical temperature of 10°C is exceeded. The whole process, from snail infection by the miracidium to cercariae emergence usually lasts 40 to 80 days and is temperature-dependent: warmer temperatures reduce the number of days needed (Andrews, 1999; Smythe and Halton, 1983).

(6) Metacercaria:

After emerging from the snail, the cercaria attaches to various objects such as submerged blades of grass or other aquatic vegetation like watercress. The tail falls away and the cercarial body secretes a four-layered cyst covering from cystogenous glands located on the lateral regions of the body. The formation of the cyst wall may take up to two days. The metacercaria (the encysted and resistant form of the cercariae) is the infective form to the definitive host (Andrews, 1999).

One miracidium hatching from an egg can produce up to 4,000 infective cysts (metacercariae) due to the vegetative multiplication (mitotic division) at the sporocyst and redia stages. Metacercariae are able to survive for up to a year in appropriate conditions, like high humidity and cool temperatures, but show reduced survivability at increased temperature and in dry conditions (Andrews, 1999; Suhardono *et al.*, 2006).

(7) Juvenile:

Upon being swallowed, along with the contaminated vegetation, by the definitive host, the metacercarial cysts enter the small intestine and are stimulated to excyst, releasing the juvenile parasite. The excystement, which occurs in the duodenum, is stimulated by high carbon dioxide concentrations, reducing conditions and temperature at 39°C (Andrews, 1999).

The emergence of the parasite is also stimulated by the presence of bile, bile salts and gastric juices (Mulcahy *et al.*, 1999; Sukhdeo and Mettrick, 1986). Newly excysted juveniles penetrate the intestinal wall and enter the peritoneal cavity within 24 hours. From there, they migrate directly to the liver over a period of approximately five days. The immature flukes (also referred to as adeloscaria) then penetrate the liver tissues and migrate throught the liver parenchyma consuming liver cells and blood for about six weeks until they find the bile ducts (Andrews, 1999).

(8) Adult:

After about four weeks in the bile ducts, the flukes reach sexual maturity, generally within 3 months of the initial infection. Flukes attach to the bile duct wall using their suckers, with their spines securing them in place. This abrades the host epithelia and ruptures blood vessels, providing the parasite with additional nutrition (Dawes, 1963).

Adult *F. hepatica* can survive for many years in the livers of infected hosts: one to two years in cattle or as long as 20 years in sheep (Andrews, 1999). Occasionally ectopic infections can occur, with flukes located in almost any organ (Mas-Coma *et al.*, 1999b; Nithiuthai *et al.*, 2004; Rim *et al.*, 1994). The rate of egg production can reach the astronomic value of 20 000 to 50 000 eggs per day (Andrews, 1999).

2.3. Morphology

The clade Digenea comprises a large group of species with medical and economic importance. As endoparasites of vertebrates, they present several structural adaptations for parasitism: various penetration glands or glands for cyst material production, organs for adhesion, such as suckers, and increased reproductive capacity. Digeneans also have an incomplete digestive system, with the mouth at the anterior end, and a well-developed reproductive system. The excretory and nervous systems are very simple and the sense organs are poorly developed (Hickman *et al*., 2004).

The hermaphroditic adult *Fasciola hepatica* has a flat leaf-like body and an outer tegument covered with tiny spines. They generally reach 20 to 30 mm and 8 to 15 mm in length and width, respectively, what makes them one of the largest digeneans to parasite humans (Fairweather *et al*.,1999; Valero *et al*., 2005). In heavy infections specimens are usually smaller (Muller, 2002).

The fluke has an anterior elongation, known as a cephalic cone, on which the oral and ventral suckers are located. The oral sucker has a diameter of 1.0 mm and the ventral sucker, which lies close behind it, has one of about 1.6 mm (Muller, 2002).

The intestine of the adult parasite is highly branched, with numerous diverticulae extending from the anterior to the posterior of the body (Fairweather *et al*.,1999; Mas-Coma, 2004).

Female reproductive organs are present near the ventral sucker and male reproductive organs are present near the center of the body. The pair of testes, highly branched, is located in the posterior half of the body, while the dense ovary is located just above the testes and is linked to a short convoluted uterus that opens into a genital pore above the ventral sucker. The vitellaria glands are highly dispersed and branched in the lateral and posterior region of the body (Fairweather *et al*.,1999; Mas-Coma, 2004).

Eggs of *Fasciola hepatica* (Fig. I.5.A) are broadly ellipsoidal, very large (130-150 μm long by 60-90 μm wide) and have a yellowish brown shell with an operculum. The embryonic cells are rather indistinct (Valero *et al*., 2002).

The miracidium (Fig. I.5.B) has a conical shaped body covered with cilia and may be up to 130 μm by 30 μm (Mas-Coma, 2004).

The cercaria (Fig. I.5.C) has two suckers, a rounded body measuring between 250-350 μm long and a long thin unbranched tail measuring approximately 500 μm long (Fairweather *et al*.,1999; Mas-Coma, 2004).

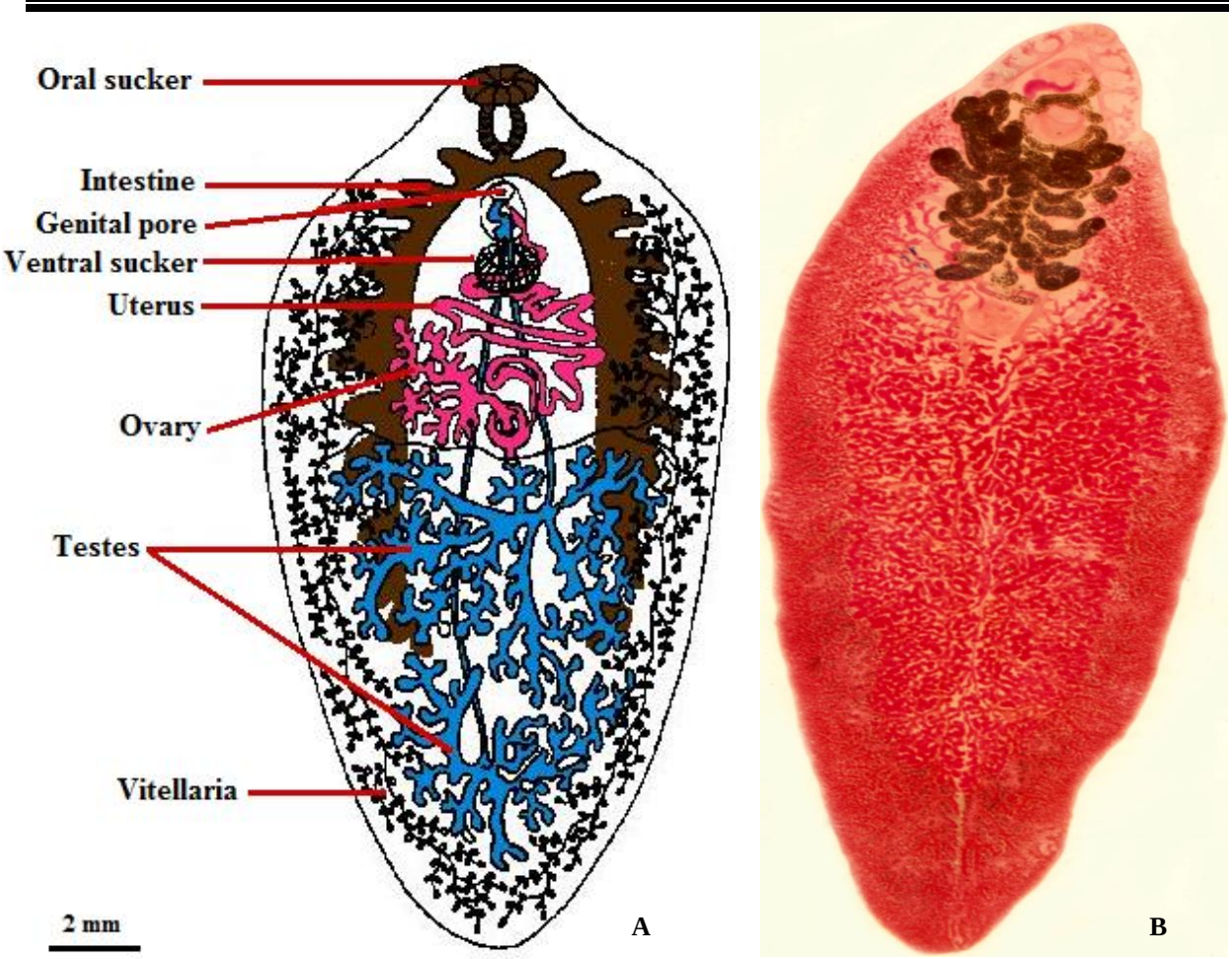

Fig. I.4. *Fasciola hepatica* adult morphology. **A**: Schematic draw of *F. hepatica* internal structures **B**: Optical microcopy image of *F. hepatica*. Picture **A** adapted from Masaba, C. (2010) and picture **B** by Raquel Santos.

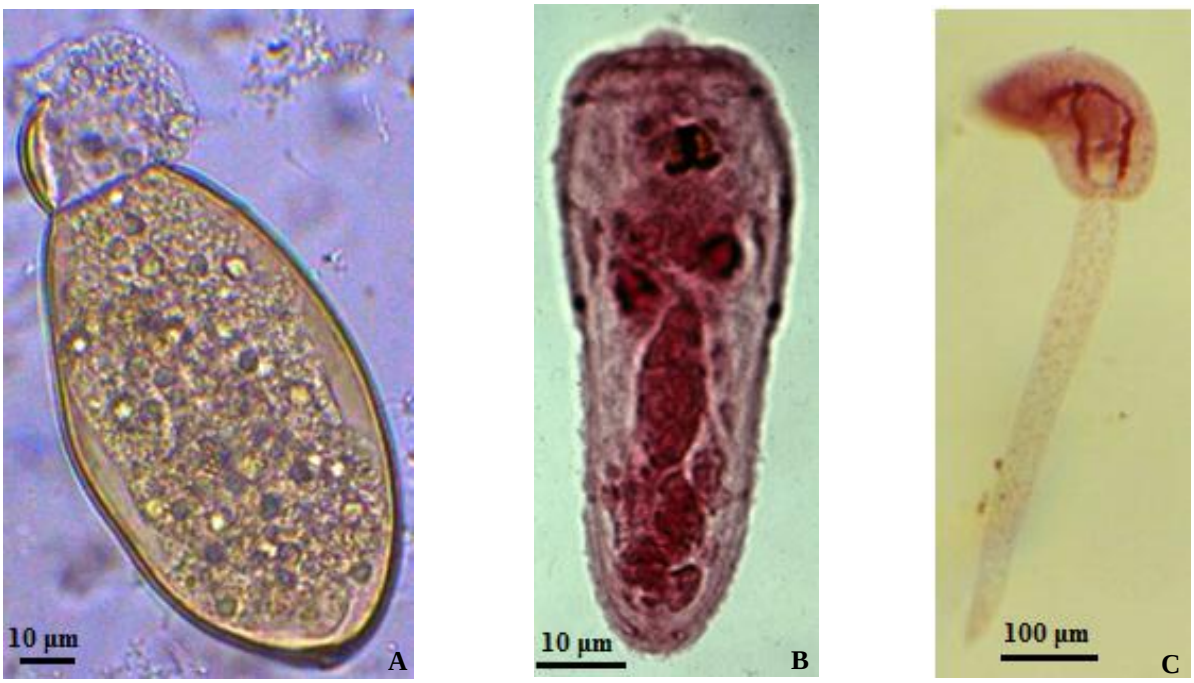

Fig. I.5. *Fasciola hepatica* egg (**A**), miracidium (**B**) and cercaria (**C**). Pictures by Buckelew, T. (2007).

2.4. Genetics

The diploid chromosome number of *Fasciola hepatica* is 20 (2n=20) consisting of five pairs of submetacentrics, four pairs of subtelocentrics and one pair of telocentric chromosomes. Observations on gametogenesis demonstrated that after the two successive meiotic divisions both sperm and ovum show haploidy (Terasaki *et al*., 2000; Yin and Ye, 1990).

However, in Asia it is not unusual to find, along with the diploids (2n=20), triploid (2n=30) and mixoploid (with diploid and triploid cells) populations of *F. hepatica* (Terasaki *et al*., 2000).

Despite the socioeconomic impact of fascioliasis, presently there is no large-scale nuclear Deoxyribonucleic Acid (DNA) sequencing project on *Fasciola hepatica* (Jefferies *et al*., 2001).

However, in 2001 the complete nucleotide sequence and gene organization of the mitochondrial genome was determined. It comprises 14 462 base pairs (bp), contains 12 protein-encoding, 2 ribosomal and 22 transfer Ribonucleic Acid (RNA) genes and is the second complete flatworm (and the first trematode) mitochondrial sequence to be described in detail. The gene arrangement resembles that of some other trematodes (Le *et al*., 2001).

In the NCBI (2012b) Gene Browser we can find several of these mitochondrial genes, like the genes encoding for Cytochrome c oxidase, Nicotinamide Adenine Dinucleotide Hydride (NADH) dehydrogenase, Cytochrome b oxidase and Adenosine Triphosphate (ATP) synthase proteins.

Although genomic sequence datasets for *Fasciola* species are scant, a recent study on the transcriptome of adult *Fasciola hepatica* was able to give many insights into the genome organization of this parasite. The average Guanine + Cytosine (G + C) content of the predicted coding sequences is 47.0%. The number of predicted genes expressed by the adult *Fasciola hepatica* is 23 447. The estimated number of expressed proteins is 44 597 and a significant proportion of the predicted proteins, 3 804 (8.5%), is conserved across the eukaryotic organisms (Young *et al*., 2010).

Based on their function, proteins can be grouped into 3 major groups: biological process, molecular function and cellular component (Young *et al*., 2010).

The predicted proteins assigned to the group “biological process”, 8 761 (19.64%), are predominantly associated with metabolic processes, cellular processes and biological regulation processes. The predicted proteins assigned to the group “molecular function”, 16 679 (37.40%), are predominantly associated with binding processes, catalytic activities and transporter activities. Finally, the predicted proteins assigned to the group cellular component, 4 264 (9.56%), are predominantly associated with the membrane, cytoplasm, organelles and macromolecular complexes (Young *et al*., 2010).

2.5. Geographical Distribution

Unlike *Fasciola gigantica* that occurs mainly in tropical areas such as Africa, the Middle East, Eastern Europe and south and eastern Asia, *Fasciola hepatica* has a worldwide distribution and has been reported in all continents except Antarctica (Andrews, 1999).

Due to the colonizing ability of the intermediate hosts and the parasites' ability to infect a large range of primary hosts, *F. hepatica* has succeeded in expanding from the European original geographical area and nowadays it is considered very cosmopolitan in its distribution and can be found in almost all temperate regions (Andrews, 1999; Mas-Coma and Bargues, 1997).

Fasciola hepatica is present in a very wide diversity of environments. This parasite is unique in being able to survive in areas from below the sea level, as in the Gilan province, besides the Caspian Sea, in Iran, up to the very high altitude, as in the Andean Altiplanos and valleys of Bolivia, Peru and Venezuela (Ashrafi *et al*., 2007; Mas-Coma *et al*., 2003).

This huge adaptability to different environments has made of *F. hepatica* the vector-borne parasite presenting the widest latitudinal, longitudinal and altitudinal distribution known (Mas-Coma *et al*., 2003).

Infections with *F. hepatica* have been reported in North America: Canada, United States of America and Mexico; Central America: Puerto Rico, Jamaica and Cuba; South America: Bolivia, Peru, Ecuador, Uruguay, Argentina, Chile, Brazil, Venezuela and Colombia; Europe: France, Spain, Portugal, the former Soviet Union, Turkey, United Kingdom, Ireland, Switzerland, Italy, Netherlands, Germany, Austria and Poland; Africa: Egypt, Kenya, Morocco, Algeria, Tunisia, Lybia, Ethiopia, Tanzania, Zimbabwe, Zambia and South Africa; Asia: Russia, Iran, Japan, Koreas, Vietnam, Thailand, Iraq, China, India, Nepal, Kazakhstan and Mongolia; Oceania: Australia and New Zealand (Esteban *et al*., 1998; Mas-Coma *et al*., 1999b; Mas-Coma *et al*., 2005; Yilmaz and Godekmerdan, 2004).

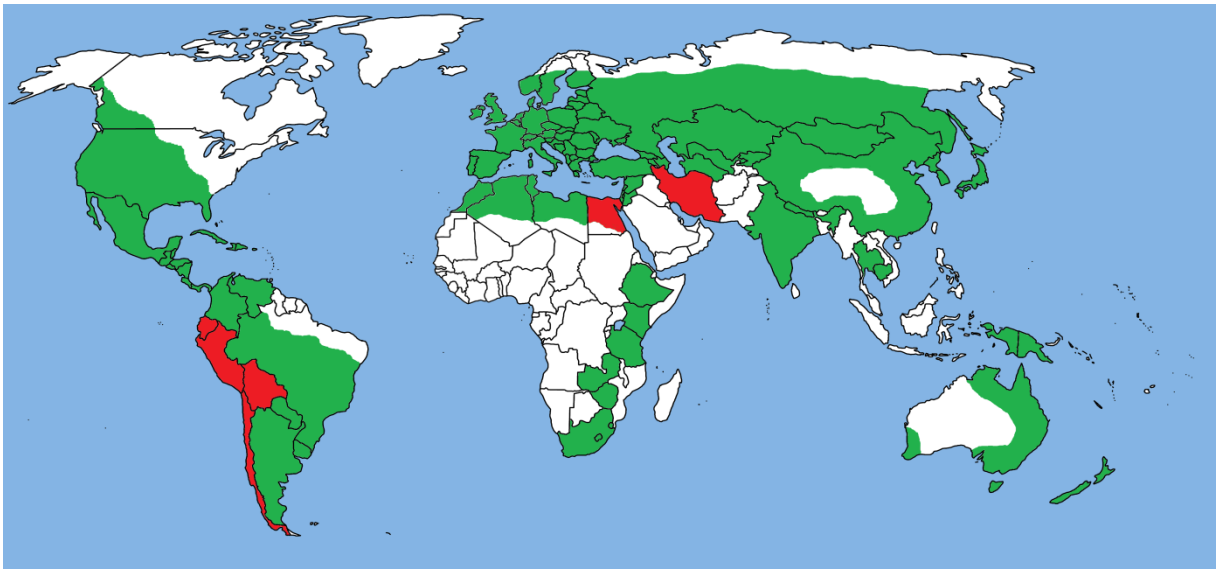

Fig. I.6. *Fasciola hepatica* worldwide distribution. Countries painted green have low to medium animal prevalence and countries painted red have high animal prevalence of fascioliasis. Image by Raquel Santos.

2.6. Intermediate Hosts

The geographical distribution of trematode species is depending on the distribution of their intermediate hosts. *Fasciola hepatica* has a wide range of intermediate hosts and usually different regions of the globe have different snail species has intermediate hosts (Graczyk and Fried, 1999).

The intermediate hosts are amphibious snails of the family *Lymnaeidae*. Since traditional morphological classification is difficult on the family *Lymnaeidae*, molecular methods are being used to study the phylogenetic relationships among this family (Bargues and Mas-Coma, 1997).

In the past, most of the species in question were classified under the genus *Lymnaea*, but a number of these have been reassigned to other genera, including *Galba*, *Fossaria*, *Pseudosuccinea*, and *Stagnicola* (Acha and Szyfres, 2003).

Lymnaeidae is a taxonomic family of small to large thin-shelled and air-breathing freshwater snails that have an elongate, dextral and ovoidal shell with a large opening and a simple lip. Lymnaieds are cosmopolitan, with members on all continents and even some islands. In common with other freshwater pulmonates they feed on algae and organic detritus and are hermaphroditic (Kohl, 2012).

Lymnaeids have a single pair of sensory tentacles on the head and an eye at the base of each tentacle. Like all pulmonates, they have a space within their mantle, lined with vascularized membrane, which is used for gas exchange. Most species maintain a bubble of air in the cavity, and occasionally refresh it at the surface of the water (Myers *et al*., 2012).

The most common intermediate host for *F. hepatica* is *Galba truncatula* (Muller, 1774), formerly known as *Lymnaea truncatula*. It is the main intermediate host in Africa, Asia and Europe (Graczyk and Fried, 1999).

Along with *G. truncatula*, it is not unusual to find *Austropeplea viridis* (Quoy and Gaimard, 1832) and *Austropeplea ollula* (Gould, 1859) as intermediate hosts in Asia, *Pseudosuccinea columella* (Say, 1817) in Africa and *Stagnicola fuscus* (Pfeiffer, 1821) and *Stagnicola palutris* (Müller,1774) in Europe (Bargues *et al*., 1997; Correa *et al*., 2010; Mas-Coma *et al*., 2001).

Fascioliasis has a European origin but succeeded in expanding to the rest of the world. In regions of the New World, where the disease was introduced, different varieties of snails, which may not be morphologically distinct from *G. truncatula*, play a very important role as *Fasciola hepatica* intermediate hosts (Mas-Coma and Bargues, 1997; Mas-Coma *et al*., 2003). These include the snails *Galba cubensis* (Pfeiffer, 1839), *Galba viatrix* (d'Orbigny, 1835), *Lymnaea diaphana* (King, 1830) and *Pseudosuccinea columella* (Say, 1817) in South America; *Fossaria bulimoides* (Lea, 1841), *Hinkleyia caperata* (Say, 1829), *Lymnaea humilis* (Say, 1822) *G. cubensis* and *P. columella*, in North America; *G. cubensis* and *P. columella* in Central America and *Lymnaea tomentosa* (Pfeiffer, 1855), *Austropeplea ollula* (Gould, 1859) and *P. columella* in Australia and New Zealand (Acha and Szyfres,

2003; Bargues and Mas-Coma, 1997; Bargues *et al*., 1997; Bargues *et al*., 2001; Bargues *et al*., 2003; Correa *et al*., 2010).

Recently, *G. truncatula* has been reported not only in the old world, but also in South America, especially in hyperendemic areas for fascioliasis, such as the Andean Altiplano (Bargues *et al*., 1997; Mas-Coma *et al*., 2001).

Interestingly, the most important intermediate snail host of *Fasciola gigantica, Radix auricularia* (Linnaeus, 1758), is resistant to *F. hepatica* while *F. gigantica* does not appear to be capable of developing in *G. truncatula* (Boray, 1985; Mas-Coma *et al*., 2005).

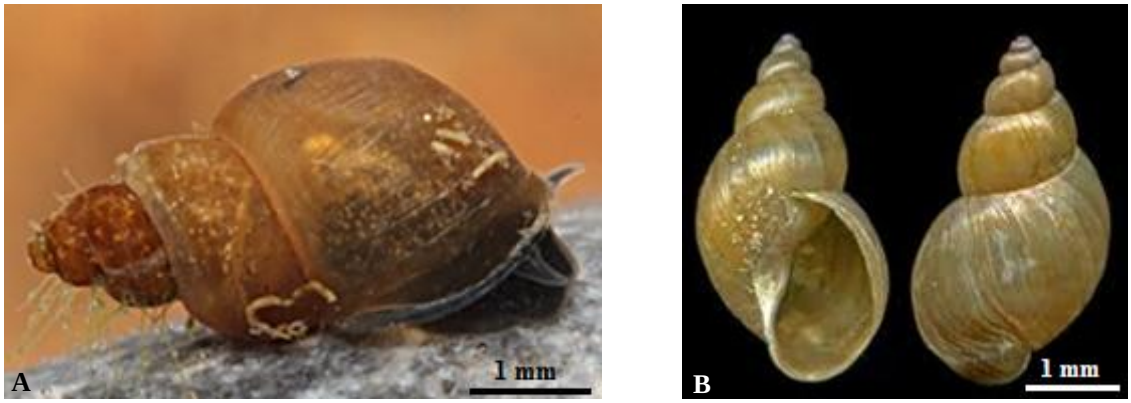

Fig. I.7. *Galba truncatula* adult specimen (**A**) and details of its sheel (**B**). Pictures **A** and **B** by Mrkvicka, A. (2002) and Biggs, J. (2008), respectively.

2.7. Primary Hosts

Fasciola hepatica has a wide range of primary, or definitive, hosts. Domestic herbivorous mammals, such as *Ovis aries* (sheep), *Capra aegagrus* (goats) and *Bos primigenius* (cattle) are considered the predominant primary hosts. Because of its prevalence in sheep, *Fasciola hepatica* is commonly known as sheep liver fluke (Mas-Coma *et al*., 2005).

It has been estimated that a sheep with a mild subclinical infection can contaminate a pasture with more than 500 000 *Fasciola* eggs a day, and one with a moderate infection can shed 2.5 to 3 million eggs a day. Sheep are followed in importance by cattle, but their release of *Fasciola* eggs is much lower (Acha and Szyfres, 2003).

F.hepatica has shown an ability to expand from infecting farm animals to parasiting wild and exotic animals all over the world (Mas-Coma, 1998; Mas-Coma, 2004).

Sometimes, even monkeys and humans can become hosts of this parasite (Mas-Coma, 2004).

Many species of domestic and wild herbivores can serve as definitive hosts. However, studies suggest that some of these latter animals are only temporary hosts and cannot maintain the cycle by

themselves for a long time. Therefore, they do not contaminate pastures to any significant extent (Acha and Szyfres, 2003).

In Europe, ruminants are very important for disease transmission, but in the rest of the world other animals may play this role. While natural parasitization by *F. hepatica* in *Sus scrofa domesticus* (domestic pigs) and *Sus scrofa* (wild boars) occurs only occasionally in Europe, pig fascioliasis is common in other geographical areas, such as Africa and South America (Mas-Coma *et al*., 2003).

In tropical countries, like Bolivia, some authors have observed that *Eqqus africanus* (donkeys) and *Eqqus ferus* (horses) can act as reservoirs of *F. hepatica* and contribute to the spread of fascioliasis (Mas-Coma *et al*., 1998).

In Australia, *Fasciola hepatica* was found in farmed *Dromaius novaehollandiae* (emus) and a study on the prevalence of *F. hepatica* infection in native mammals revealed that half of the examined marsupials were infected with this parasite (Spratt and President, 1981 and Vaughan *et al*., 1997)

Chronic *F. hepatica* infection has also been discovered in farmed and wild *Rhea americana* (greater rheas) and in *Bubalus bubalis* (buffalo), in Brazil (Marques and Scroferneker, 2003; Soares *et al*., 2007).

In 1996, a veterinary study reported the presence of *Fasciola hepatica* in Argentinian *Lama glama* (llamas). One of the animals had 62 adult parasites in its liver (Cafrune *et al*., 1996).

During an 18-year period (1981-1998) *Fasciola hepatica* was repeatedly found in Belorussian wild animals, such as *Castor fiber* (beavers), *Lepus timidus* (hares), *Capreolus capreolus* (roe deers), *Alces alces* (elks), *Lutra lutra* (otters) and *Meles meles* (badgers) (Shimalov and Shimalov, 2000).

In Corsica, there are regions in which humans become contaminated but the normal definitive hosts (livestock) are not even present. In those places *Rattus rattus* (peridomestic rat) has proven to be the main reservoir host (Mas-Coma *et al*, 1988; Mas-Coma *et al*, 2003).

A similar phenomenon has been detected in France, where *Myocastor Coypus* (nutria), a recently introduced rodent, plays a major role in disease transmission to humans (Menart *et al*, 2001).

Furthermore, infection has also been reported in different species of the families Leporidae (rabbits) and Camelidae (camelids) (Mas-Coma, 2004).

2.8. Ecology

Fasciola hepatica is dependent on a consistent set of suitable environmental conditions to survive. They require acceptable moisture and temperature conditions both for the intermediate and primary hosts and for their own growth and development (Mas-Coma, 2004).

Environmental factors play a key part in the life cycle of this parasite, most importantly water and temperature.

Adequate temperature, between 10-25ºC, is fundamental for the surviving of *Fasciola* eggs, for the development of the free living stages and for the formation of metacercarial cysts. In fact, the growth of *F. hepatica* in warmer environments, between 17-25ºC, reduces the total amount of time required to complete the fluke development (Andrews, 1999).

Moisture is necessary for the development of fluke eggs in dung, for penetration of the free living stages into the intermediate snail host and to enable migration of cercariae, which is shed from snails, to form cysts on grass (Andrews, 1999).

F. hepatica is found in almost all temperate regions where sheep, goats, cattle and other ruminants are raised because, virtually, all these areas have sufficient humidity and adequate temperature conditions, at least during part of the year, to sustain a snail population (Mas-Coma, 2004).

Therefore, the ecology of *Fasciola hepatica* is strictly linked to the ecology of its intermediate host. Along with the presence of water and appropriate temperatures; physiographic characteristics, soil composition, and climatic factors are of major importance in the surviving and reproduction rate of lymnaeids and, hence, in the presence of *F. hepatica* (Myers *et al*., 2012).

Specimens of the family *Lymnaeidae* can be found in pasturelands in widely diverse settings throughout the world, from Andean valleys at elevations of over 3 700 meters to sea level flatlands (Mas-Coma *et al*., 2003).

Lymnaeids are able to adapt to very wide and extreme physical and chemical conditions and to water bodies with a large range of aquatic vegetation (Mas-Coma, 2004).

From the ecologic standpoint, the habitat of *Lymnaeidae* can be divided into two broad types: primary foci, or reservoirs, and areas of dissemination:

The primary foci are located in permanently wet environments such as streams, lakes, lagoons, or canals. Snails are usually found in stagnant water bodies or near the banks, where water flows slowly. They begin to lay their eggs in springtime when temperatures rise above 10°C and continue to do so as long as the thmperature remains above this level. Warmer temperatures allow faster growth of the eggs (Acha and Szyfres, 2003; Chapuis *et al*., 2007).

Since new snails begin to lay eggs at 3 weeks of age, they can produce up to three generations in a single season as long as they have enough water (Acha and Szyfres, 2003).

During unusually dry and hot summers, many snails may die, but a few of them estivate and resume their development when the temperature falls and moist conditions return. Many of them also may die during very cold winters, but some go into hibernation and resume their development when temperatures once again rise above 10°C. The snails that manage to survive dry conditions, heat, and cold are the seeds for the next season's crop of snails (Luzün-Peña *et al*., 1994).

Temperature above 10°C is a key factor in the epidemiology of fascioliasis because when it is any colder *Fasciola* eggs fail to develop, snails do not reproduce, sporocyst, redia and cercaria stages do not develop inside the snail, and the cercariae do not encyst (Luzün-Peña *et al*., 1994).

The adaptation on lymnaeids to permanent water bodies makes transmission throughout the year possible, as observed in southern Europe and the Mediterranean islands (Valero *et al*., 1998).

Areas of dissemination are characterized by the alternation of flooding and droughts, and have large concentrations of *Lymnaeids.* Snails may reach these areas directly from original foci carried by rising waters, or they may be reactivated after estivation during long dry spells. Seasonal foci of this kind turn pastures into enzootic areas in which serious outbreaks can occur (Acha and Szyfres, 2003; Chapuis *et al*., 2007).

As with many parasites there is a distinct seasonal pattern in fascioliasis outbreaks, with two key periods of infection, summer and winter (Altizer *et al*., 2006).

Fasciola eggs transmitted by infected animals in springtime and early summer develop inside the snails and produce cercariae and metacercariae until the end of summer. The animals that ingest them begin to show signs of the disease at the end of autumn and during winter; this is considered a summer outbreak and occurs mostly in warm temperate regions (Fox *et al*., 2011; Goodall *et al*., 1991).

Eggs excreted during unfavorable winters do not develop until suitable conditions are encountered. In early spring, the eggs commence their development and the metacercariae from this cycle appear at the end of spring or in early summer. When ingested by animals, these metacercariae produce symptoms in summer and autumn; this is a winter outbreak and occurs mostly in cool temperate regions (Fox *et al*., 2011; Goodall *et al*., 1991).

In sub-tropical and tropical regions disease outbreaks can occur all year round (Togerson and Claxton, 1999).

In conclusion, the climatic factors like rainfall and air temperature are decisive for the development of *Fasciola hepatica*, mainly because they have a very strong impact in the life cycle of its intermediate host. A temperature range of 10-25ºC and high levels of moisture are the primary determinants of transmission efficiency (Fox *et al*., 2011)

3. Fascioliasis

3.1. Historical Background

Fasciola is an ancient parasite coexisting with man and animal back to approximately 3 500 BC. *Fasciola* eggs have been found in human coprolites from populations of the Stone Age, living at the end of the Mesolithic period, 5 000 to 5 100 years ago and the Neolithic, a period marked by the domestication of animals and the development of agriculture (Aspöck *et al*., 1999; Bouchet, 1997; Dittmar and Teegen, 2003).

Interestingly, liver fluke eggs have been found in many palaeoparasitological studies performed in Europe but never in coprolites from the New World, which clearly indicates that fascioliasis in the Americas is a relatively recent introduction (Gonçalves *et al*., 2003)

The earliest reference to fascioliasis in literature is contained in the *Black Book of Chirk*, published around 1200, in which disease in sheep in mentioned (Froyd, 1969).

Fasciola hepatica was first observed in France by Jean de Brie, in 1379. De Brie made mention of the disease "liver rot" in sheep but he didn't considered the fluke as being the etiologic agent of the disease, instead he thought it was a consequence of the liver being affected by toxic substances produced by certain plants eaten by the sheep. His observations have made *F. hepatica* the first trematode to be recognized (Andrews, 1999).

In the sixteenth century, fascioliasis appeared in the form of an epidemic throughout Europe, with the worst hit areas being the Netherlands and Germany. However the first sketch of an adult *Fasciola* was made only in 1668, by Francesco Redi. By showing that parasites lay eggs, Redi destroyed the false doctrine of spontaneous generation. However it was not until the end of the 18th century that reference was made to the intermediate stages of *F. hepatica* life cycle (Mufti, 2011).

The earliest insight into the pathology of "liver rot" was made in 1755 by Frank Nicholls, a physician that reported calcification of the bile ducts in bullocks infected with *F hepatica*. In 1758 *Fasciola hepatica* was identified by Linnaeus. "*Fasciola*" in Latin means fillet or small bandage and "*hepatica*" means of the liver (Mufti, 2011).

Müller, in 1773, observed several different kind of cercariae swimming in water but could not correlate them with intermediate stages of helminthes. In 1803, Johann Zeder reported observing the hatching of eggs from a number of different species of trematode and the escape of a ciliated embryo, miracidium, into the water. Christian Nitzsch, in 1807, followed with the first account of cercariae encysting. The next observation of relevance was made in 1818 by Ludwig Bojanus who discovered the redial stages of trematodes, but unfortunately he did not work with *F. hepatica*. Unlike his predecessors, Bojanus noted the resemblance between rediae, cercariae and adult flukes (Andrews, 1999).

Despite all these advances, it was only by the middle of the 19th century that most of the individual parts of the life cycle were brought together. In 1842 Johannes Steenstrup published his work *On the Alternation of Generations* where he fitted the theory to various forms of life, including trematodes. Adolphus von La Valette St George, in 1855, demonstrated, by feeding-experiments, that certain encysted cercariae from water snails developed into sexually mature flukes in birds and Guido Wagener, two years after, observed the penetration of miracidia into snails and the subsequent development of rediae (Andrews, 1999).

In 1875 the German helminthologist David Weinland was the first person to suspect that larval stages of liver fluke occurred in *Lymnaea truncatula*. He conjectured that cercariae encysted on grass in order to be eaten by sheep and that these cercariae were in fact young liver flukes. Adolpho Lutz, in

1892, successfully infected several herbivorous animals by adding metacercariae to their food, confirming that mammals acquire adult fluke by ingestion of metacercariae. The final piece of the jigsaw was added by Dimitry Sinitsin in 1914, who proved that young flukes in the rabbit, after liberation from their cysts in the small intestine, penetrated the wall of the gut and migrated to the liver via the peritoneal cavity (Andrews, 1999).

3.2. Sources of Infection and Transmission

Mammals become infected with *Fasciola hepatica* when grazing in pastures with contaminated vegetation. Infection occurs through the ingestion of metacercarial cysts located on the plants surface. Water has also been cited as a source of infection through direct drinking of cysts on water surface (Mas-Coma *et al*., 2005).

Humans become infected through ingestion of aquatic plants contaminated with metacercariae and direct drinking or contamination of food or utensils with cysts on water surface (Mas-Coma *et al.*, 2005).

Experimental evidence in mice and pigs also suggests that people who consume raw dishes prepared from fresh livers infected with immature flukes can also become infected (Taira *et al*., 1997).

Metacercariae infectivity is dependent upon storage time, being lower when cysts are older. Moreover, metacercariae viability and infectivity do not show differences between isolates from different reservoir species. Thus, flukes from secondary reservoirs, such as pigs and monkeys, involve the same potential risk of infection as those from sheep and cattle (Valero and Mas-Coma, 2000).

There are several contamination sources for human infection:

- Ingestion of wild freshwater plants

Most human reports are related to watercress. However, the general term watercress includes different aquatic species, such as common watercress, *Nasturtium officinale* (common watercress) and *N. silveris* and *Roripa amphibian* (wild watercress). Wild watercress has been reported as the main source of human infection in areas where fascioliasis in domestic animals is highly endemic. Other wild aquatic vegetables reported as vehicles of human infection are *Taraxacum officinale* (dandelion), *Valerianella olitora* (lamb's lettuce) and *Mentha viridis* (spearmint) and *Mimulus glabratus* (roundleaf monkeyflower) (Esteban *et al*., 1997a; Mas-Coma and Bargues, 1997; Mas-Coma *et al*., 1999a).

- Ingestion of cultivated freshwater plants

Metacercariae-carrying species may be so important in the human diet of a given area as to warrant production at the commercial level, thus explaining infection of subjects living far from the endemic area. In France, where common watercress is a popular salad ingredient (10 000 tons are consumed each year) human infection is more frequent here than in other European countries (Gil-Benito *et al.*, 1991).

- Ingestion of wild and cultivated terrestrial plants

The amphibious characteristics of intermediate species, such as *Galba truncatula*, and the long survival capacity and dryness resistance of metacercariae explain the human contamination by consumption of plants collected in dry habitats with temporary water bodies and plants collected in plantations of non-aquatic vegetables frequently irrigated (Mas-Coma *et al*., 1999a).

Eruca sativa (arugula), *Lactuca sativa* (lettuce), *Allium porrum* (leek), *Portulaca oleracea* (purslane) and other contaminated plants that are eaten raw can be a dangerous source of *Fasciola hepatica* infection (El-Sayed *et al*., 1997; Motawea *et al*., 2001).

Thanks to the transport of vegetables, both aquatic and terrestrial, from rural endemic zones to cities, plants carrying metacercariae can be sold in non-controled city markets giving rise to urban infections (Mas-Coma *et al*., 1999a).

- Drinking of contaminated water

Consumption of natural water in often cited as a human infection source. In 1996, a water analysis conducted on a river crossing Tambillo, in the Bolivian Altiplano, reported that there were up to seven metacercariae in only half a litre of water. Moreover, of all the metacercariae collected in this study, about 13% were floating on water (Barges *et al*., 1996).

In the Egyptian locality of Tiba, where a prevalence of 18% was initially found, human infection has markedly decreased after the construction and utilization of so-called "washing-units", in which the water is appropriately filtered (Esteban *et al*., 2003).

- Washing of kitchen utensils or other utensils with contaminated water

Washing with metacercariae-carrying water may be the source of inadvertent infections. In Egypt, women usually wash kitchen utensils and clothes at irrigation canals where lymnaeids and livestock are present. Infection through ingestion of food containing metacercariae can be a serious health problem in the Nile Delta region (Cadel *et al*., 1996; Curtale *et al*., 2003).

- Ingestion of raw livers

In 1997, Taira and collaborators conducted a study where mice and piglets were given fresh livers infected with immature flukes. At necropsy, they recovered several live flukes and observed hemorrhagic and granulomatous lesions, typical of those caused by active migration of early immature flukes. The results suggest that humans consuming raw liver or semi-cooked liver dishes prepared from fresh livers infected with immature flukes may also become infected (Taira *et al*., 1997).

3.3. Fascioliasis in Portugal

In Portugal, *Fasciola hepatica* can be found almost across the entire country. Reports of infected livestock range from the North to the South of Portugal and also include the Azores and Madeira archipelagos (Ferreira and Oliveira, 1960; Rombert and Grácio, 1984; Rombert *et al*., 1991).

The climate in Portugal favors the spread of *F. hepatica*. The mild temperatures and humidity allow for the healthy development of the eggs, and the wetness in winter months promotes the abundance of its intermediate host, *Galba truncatula*. *F. hepatica* exhibits a preference towards the areas surrounding the great rivers such as Tejo, Douro, Minho and Vouga. It is also present in certain microclimates in the Alentejo, Beiras and Algarve regions (Grácio, 1985; Rombert *et al.*, 1991).

F. hepatica primarily affects sheep, followed by cattle, swine and goats. In a smaller scale, it also affects horses, mules, donkeys and rodents (Ferreira and Oliveira, 1960).

Studies on animal fascioliasis in Portugal are not plentiful and most data are gathered in slaughterhouses. The first studies were conducted by Silva Leitão in 1950 and showed a high presence of bovine fascioliasis in the northern regions of the country (Leitão, 1965).

Sousa studied the presence of *F. hepatica* in wild animals such as boar and deer in the Tapada Nacional de Mafra, by observing lesions and adult forms of the parasite in the animals' liver. An increase in the prevalence of the parasite between 1992 and 2000 was reported (Sousa, 2001).

In a comparative analysis based on data from the Divisions of Veterinarian Intervention of Viseu, Coimbra, Aveiro and Leiria it was shown that most cattle with livers rejected due to fascioliasis hailed from Aveiro, followed by Coimbra (Conceição, 2001).

In Portugal, man is an accidental host and reports of fascioliasis in man are somewhat scant. However, it is known that human fascioliasis is particularly present in the northern regions of Portugal (Sampaio, 1986). The first published case of human fascioliasis in Portugal was in 1948, and a total of 14 had been reported up to 1959. Until 1991, a total of over 190 cases had been diagnosed in the country, 50 of them in 1990 (Rombert *et al.*, 1991). Many of those new cases were in the central region of the country, as a consequence of a then recent outbreak in Ribatejo. The first cases started to appear in the months of February and March, and could be then backtracked to initial infections in January and February, corresponding to a development of *F. hepatica* starting in November of the previous year (Rombert and Grácio, 1990). This coincides with the typical heavy rains in this period (Rombert *et al*., 1991).

3.4. Fascioliasis in the World

Human and animal fascioliasis caused by *Fasciola hepatica* occurs worldwide. While animal fascioliasis occurs mainly in countries with high cattle and sheep production, human fascioliasis occurs mainly in developing countries (Togerson and Claxton, 1999).

A global analysis of the distribution of human cases shows that the expected correlation between animal and human fascioliasis only appears at a basic level. Although it is true that human infection is not rare in areas where infected domestic herbivorous mammals are present, high/low human prevalences are not related to high/low animal prevalences, respectively (Mas-Coma *et al.*, 2005).

The epidemiological classification of fascioliasis includes two major groups: areas where fascioliasis is endemic and areas where fascioliasis is sporadic (Mas-Coma, 2004).

Sporadic areas are those where human and/or animal reports of fascioliasis appear without any constancy (Mas-Coma *et al*., 1999a).

Endemic areas can be hypoendemic, with a prevalence of less than 1%; mesoendemic, with a prevalence of 1–10%; and hyperendemic, with a prevalence of more than 10% (Mas-Coma *et al*., 1999a).

High prevalences in humans are not necessarily found in areas where fascioliasis is a great veterinary problem. For instance, in South America human hyperendemic and mesoendemic areas are mainly found in Bolivia and Peru where the veterinary problem is less relevant than in countries such as Uruguay, Argentina and Chile, where human fasciolosis is only sporadic or hypoendemic (Esteban *et al*., 1997b). As for other example: although the infection is common in cattle in western and southeastern US, only one human case has been reported in that country and the situation is similar in China, where the infection is frequently seen in animals, but only 44 human cases were known to have occurred (Chen, 1991).

3.4.1. Human Prevalence

At present there are nearly 2.4 million people, from 61 countries, that suffer from fascioliasis, but the number of people subjected to the risk of infection is 180 million worldwide. Human fascioliasis has been reported in several countries in Europe, America, Asia, Africa and Oceania and, unfortunately, the number of affected countries, currently 61, is increasing (Keiser and Untzinger, 2005; WHO, 2007).

In Europe, human fascioliasis cases are predominantly found in Portugal, Spain, France, Turkey and the former USSR. France is considered an important human endemic area, with 3297 cases catalogued between 1950 and 1983. However, 5863 human cases were recorded from nine hospitals between 1970 and 1982, indicating that published data largely underestimate the real situation. The disease is also important in Portugal, mainly the northern region, in which 1011 cases were diagnosed in 1970-1992. In England the largest known outbreak occurred in 1972 and about 40 people were affected (Esteban *et al*., 1998). Concerning the former Soviet Union, almost all reported cases were from the Tadzhik Republic, near the Afghanistan border (Semyenova *et al*., 1996).

A serological survey of human fascioliasis performed in Turkey detected prevalences of up to 3% in the Antalya province and between 0.9 and 6.1% in the Isparta province, the Mediterranean region of Turkey (Demirci, 2003). In other European countries, fasciolosis is sporadic and the occurrence of the disease is usually combined with travelling to endemic areas (Togerson and Claxton, 1999).

In North America, human fasciolosis appears only sporadically in the United States, Canada and Mexico (Acha and Szyfres, 2003).

In Central America, fascioliasis is a human health problem in the Caribbean Islands, Puerto Rico and Cuba. In Cuba, a serious outbreak occurred in 1983 involving more than 1000 people. In the mainland, only Costa Rica and Guatemala appear in the records and in the Dominican Republic and El Salvador, fewer than 100 cases were documented (Mas-Coma *et al*., 1999b).

In South America, human fascioliasis is considered a serious problem in Bolivia, Peru, and Ecuador. Well-known human hyperendemic areas are localized predominately in the high plain called Altiplano. The Andean countries are considered to be the area with the highest prevalence of human fascioliasis in the world. In Bolivia prevalences detected in some communities were up to 72 and 100% in coprological and serological surveys, respectively. In the Northern Bolivian Altiplano a human population of around 2.5 million people is at risk (Esteban *et al*., 1997b). In Peru, human cases have been detected throughout the country, with mesoendemics and hyperendemics regions already described. The high human prevalences in Arequipa, Mantaro Valley, Cajamarca Valley and the Puno region are worth mentioning; estimations suggest that a rural population of almost 8 million people is at risk. Despite the lack of epidemiology information, the characteristics of Ecuador suggest a situation similar to that in Peru. In Chile, human fasciolosis is hypoendemic in the Valparaiso and Viña del Mar regions. In countries like Argentina, Uruguay, Brazil, Colombia and Venezuela, human fasciolosis appears to be focal in distribution and sporadic, with fewer than 100 cases reported (Mas-Coma, 2005).

In Asia, only a few cases have been described in several countries, among which Iran is worth mentioning because of the recent estimates of more than 10 000 human cases and about 6 million at risk. In eastern Asia human fascioliasis appears to be sporadic, a small number of cases were documented in Japan, the Koreas, Vietnam and Thailand (Togerson and Claxton, 1999).

Few human cases have been reported from African countries, but epidemiological surveys estimate that the population at risk may round 27.7 million and the number of infected is at least 830 000. Most human cases have been reported from Egypt, where an increasing number of cases have been diagnosed in the Nile Delta. In Egypt rural areas prevalence varies between 2 and 17%. In the Ivory Coast, Madagascar, Mali and Mozambique fewer than 100 cases are documented but in Ethiopia the number of reported human cases is almost 1000 (Mas-Coma *et al*., 2005).

Concerning Oceania, there are only 12 human reports from Australia and none from New Zealand, despite the importance of livestock production here and the high prevalence of fascioliasis in sheep and cattle (Mas-Coma *et al*., 2005).

In hypo- to hyperendemic areas of Central and South America, Europe, Africa and Asia, human fascioliasis presents a range of epidemiological characteristics related to a wide diversity of environments. Thus far well-known epidemiological patterns of fascioliasis may not always explain

the transmission characteristics in any given area and control measures should consider the results of ecoepidemiological studies undertaken in the zones concerned (Mas-Coma, 2005).

3.4.2. Animal Prevalence

Fascioliasis is considered one of the most important helminth infection of ruminants in the world. Prevalence and intensities of fascioliasis in animals are extremely high, mainly in farm animals like cattle, sheep, swine and horses, but also in wild animals (Okewole *et al*., 2000). Unfortunately, the few existent epidemiological surveys do not cover the entire geographical distribution of this disease and, therefore, the values of fascioliasis prevalence in animals are still inaccurate (Mas-Coma *et al*., 2005).

The most affected countries with animal fascioliasis are located in Asia and Africa where fascioliasis is currently the single most prevalent helminth infection of ruminants. African countries, like Morroco, Egypt, Tunisia and Ethiopia reported prevalence rates of 30-90% in Goats and Cattle. Concerning Asia, the prevalence of *F. hepatica* reaches 100% in some regions of India, up to 27-91% in Iran, up to 85% in north-east Thailand and 25-90% in Indonesia (Togerson and Claxton, 1999).

On 2007, veterinary officials in the Philippines diagnosed a level of infection of 89.5% in livestock, a sudden increase of positive cases due to erratic weather condition in the area (Sarmiento, 2007).

The Andean altiplano comprises a region strongly affected by fascioliosis. In fact, in Chile, Bolivia and Peru the incidence of *F. hepatica* can range up to 87% in sheep, 66.6% in cattle, 27.1% in pigs and 15.4% in donkeys (Grock *et al*., 1998; Mas-Coma *et al*., 1998). In the Jujuy province, Argentina, 80% of llamas were considered infected with *F. hepatica* (Cafrune *et al*., 1996).

New Zealand has no reported cases of human fascioliasis, but a prevalence of 8.5% was predicted in cattle. In Australia the prevalence of *F. hepatica* infection in humans is very low, but in native mammals this value can be as high as 59% (Spratt and Presidente, 1981).

Such high values occur worldwide, in developed countries the incidence of *F. hepatica* in livestock ranges up to 77%. In California and Florida, USA, prevalence rates of 52.7-68% were documented in cattle. In Canada, a huge outbreack of fascioliasis occurred in 1986 and affected almost 70% of all dairy cows (Togerson and Claxton, 1999).

The occurence of fascioliasis in European countries is also noteworthy. Spain (29.5%), Ireland (45%), United Kingdom (10%) and Portugal (unknown value) are countries with high frequency of *F. hepatica* infections and the main infected animals are cattle, sheep and goats (Togerson and Claxton, 1999). Curiously, in Corsica, there are habitats in which humans become contaminated but the normal primary hosts, livestock, are not present. In those places, *Rattus rattus* has proven to be the reservoir host, with very high prevalences of up to 45.1% (Mas-Coma *et al*. 2003).

3.5. Pathology

Fasciola hepatica can naturally infect sheep, cattle, goats, pigs and humans. It is also a natural pathogen of wildlife and zoo animals. In addition, experimental animals such as mice, rats, guinea pigs and rabbits have been used to explore aspects of pathology and pathogenesis of fascioliasis (Behm and Sangster, 1999).

Fascioliasis can be a very impressive disease. The appearance of a heavily infected liver from an animal with acute terminal fasciolosis is not easily forgotten. Nor are sudden deaths which can decimate flocks almost overnight. However, fascioliasis is a more complex disease than it may appear and different hosts present different pathological characteristics (Behm and Sangster, 1999).

The symptomatology depends on the parasite burden (number of ingested metacercariae), the host age and its general health condition (Arjona *et al*., 1995).

3.5.1. Clinical Manifestations in Man

The effect of fascioliasis on human health depends on the parasite burden and the duration of the infection. The following clinical periods can be distinguished: incubation phase (from the ingestion of metacercariae until the penetration of the peritoneal cavity); invasive or acute phase (fluke migration up to the bile ducts); latent phase (maturation of the parasites and start of oviposition); and obstructive or chronic phase (adult flukes localized in the bile ducts) (Chen and Mott, 1990; Mas-Coma *et al*., 1999b).

The excystment of young flukes and their migration across the intestinal wall and through the peritoneal cavity may cause focal haemorrhages and inflammation, although lesions usually are not clinically evident (Mas-Coma *et al*., 2005).

The fluke journey across the hepatic parenchyma induces major pathological changes because parasites digest the hepatic tissue and can lead to traumatic, necrotic, and inflammatory lesions, whose severity depends on the number of parasites (Behm and Sangster, 1999).

The most common clinical manifestations during acute fascioliasis, when the young parasites migrate across the hepatic parenchyma, are abdominal pain, fever, hepatomegaly, eosinophilia and mild anemia (Mas-Coma *et al*., 2005). In a study of 53 patients with eosinophilia of probable parasitic origin, 30 of the cases proved to be due to fascioliasis (El-Zawawy *et al*., 1995). Also, *F. hepatica* was the parasite most frequently associated with reduced hemoglobinemia in a group of highly parasitized individuals (Curtale *et al*., 1998). This parasite was also found in 24% of 187 patients with fever of unknown origin (Abdel-Wahab *et al*., 1996).

The latent phase can last for months or years and is generally asymptomatic. Infected subjects are often discovered during family screening after a patient is diagnosed (Chen and Mott, 1990).

In the chronic phase, which occurs after the parasite has become localized in the bile ducts, the common signs are biliary colic and cholangitis. The adult *F. hepatica* produces pericanalicular inflammation, fibrosis and adenomatous proliferation in the ductal epithelium. Massive infections can cause biliary stasis due to obstruction of the duct, atrophy of the liver, and periportal cirrhosis. Cholecystitis and cholelithiasis occur with some frequency in chronic cases (Mas-Coma, 2004).

The acute-phase eosinophilia usually persists, although sometimes the chronic infection can be asymptomatic (El-Newihi *et al*., 1995). In a study of 47 patients in Chile, the main symptoms were abdominal pain, dyspepsia, anaemia, weight loss, diarrhea, and fever. Ten of the 47 patients had jaundice and the eosinophil count was normal in 9 cases and elevated in 38 cases (Faiguenbaum *et al.*, 1962). In Spain, the most common symptoms in 6 fascioliasis patients were eosinophilia (100%, over 1000 cells/mm3), abdominal pain (100%), fever (83%), anaemia (75%) weight loss (83%), and generalized myalgia (67%) (De Gorgolas *et al*., 1992). Actually, anaemia is one of the most characteristic symptoms. Blood loss in the bile, due to the maturation of parasites, oviposition and egg movement through the bile ducts, seems to be the most important factor contributing to severe anaemia (Mas-Coma and Bargues, 1997).

During the acute phase, during the migration through the peritoneal cavity, immature flukes may deviate and enter aberrant sites in different parts of the body and cause ectopic fascioliasis. Hence it is not unusual for patients to have extra-hepatic abnormalities, such as pulmonary infiltrates, pleuropericarditis, meningitis, or lymphadenopathy caused by these parasites (Arjona *et al*., 1995). The usual pathological effects of ectopic lesions are due to the migratory tracks causing tissue damage with inflammation and fibrosis (Chen and Mott 1990; Mas-Coma and Bargues 1997).

Interestingly, the gender effect in fascioliasis is noteworthy. Prevalences and intensities in human hyperendemic areas appear to be significantly higher in females (Esteban *et al*. 1999; Esteban *et al*., 2003).

3.5.2. Clinical Manifestations in Animals

Fascioliasis is predominantly a disease of herbivores. Sheep are the most susceptible domestic species, followed by cattle and goats. In animals, unlike humans, the disease is classified according to infectious dose (amount of ingested metacercariae) and the clinical presentation is divided into four types (acute type I, acute type II, subacute and chronic). The infectious doses described below were deduced in sheep fascioliasis (Behm and Sangster, 1999).

The acute type I fascioliasis occurs when the animal ingests high doses of metacercariae at once (more than 5000 metacercariae) with consequent invasion by a multitude of young parasites in

the hepatic parenchyma. The migrating parasites destroy the hepatic tissue, causing hemorrhages, hematomas, necrotic tunnels, and peripheral inflammation (Sukhdeo *et al*., 1988). In these massive infections, the affected sheep may die suddenly without any previously clinical manifestations, or they may exhibit weakness, loss of appetite, and pain when palpated in the hepatic region and then die a few days later (Martinez-Moreno et al., 1999).

In acute type II fascioliasis the infectious dose is 1000-5000 ingested metacercariae. Animals suffer from weight loss and accumulation of fluid in the abdomen (ascites). Other frequent manifestations are eosinophilia, anemia, hypoalbuminemia, and high alanine aminotransferase and aspartate transaminase levels in serum. Death is not an unusual consequence (Martinez-Moreno *et al*., 1999).

Subacute fasciolosis is characterized by an infectious dose of 800-1000 ingested metacercariae. Animals become lethargic, anemic and may die. Weight loss is the dominant feature (Behm and Sangster, 1999).

In the chronic fasciolosis the infectious dose is lower than 800 metacercariae. Parasites are localized in the bile ducts and accumulate over time and eventually reach a pathogenic number. The symptoms are progressive anemia, weakness, loss of appetite, submandibular edema (also known as "bottle jaw"), ascites, diarrhea, weight loss and, in severe cases, death (Anderson *et al*., 1981). The animals' condition worsens when pasturage is scarce and improves when it is abundant, but they are never healed, unless treated with antihelmintics and the parasitosis has a cumulative effect over the years (Arjona *et al*., 1995).

In sheep, the invasion of juvenile fasciolae can lead to infectious necrotic hepatitis with fatal outcomes (Ross, 1965; Ross *et al*., 1966; Ross *et al*., 1967).

In cattle the manifestations of fascioliasis are usually constipation, diarrhea in extreme cases, weakness and emaciation, especially in young animals. Cattle are more resistant than sheep and can tolerate a larger parasite burden without having any significant clinical manifestations: about 1,400 parasites will cause symptoms in 60% of the animals and only a few deaths (Acha and Szyfres, 2003).

In swine and horses, fascioliasis is usually asymptomatic and becomes clinically apparent when debilitating factors, such as malnutrition or concurrent illnesses, are present (Amor *et al*., 2011).

F. gigantica, due to its size, can cause more damages than *F. hepatica*. However, the pathogenesis, pathology, and symptomatology of the infection caused by *F. gigantica* are similar to those of the parasitosis caused by *F. hepatica* (Acha and Szyfres, 2003).

3.6. Diagnosis

The diagnosis of fascioliasis is suspected on the basis of clinical manifestations (painful and febrile hepatomegaly coupled with eosinophilia and anaemia) and is confirmed by the finding of

characteristic eggs in feces (O'Neill *et al*., 1998). During the acute phase, no eggs can be seen because the parasites have not yet matured, and therefore immunologic tests are often used. However, positive reactions may not appear at such an early stage. In this phase, it is important to distinguish fascioliasis from acute hepatidis due to other causes. Epidemiologic antecedents (abundance of cases in the area, custom of eating watercress) and the presence of peripheral eosinophilia assist in the identification of fascioliasis (Arjona *et al*., 1995; Mas-Coma *et al*., 2005).

In animals, the diagnosis of acute fascioliasis is often made at autopsy based on observation of hepatic lesions and the presence of immature parasites. Ultrasound does not show the migrating parasites, but sometimes the parasite can be seen using biliary endoscopy (Fawzy *et al*.,1992).

The most effective method for detecting fascioliasis in the chronic phase is the coprologic exam. However, consumption of beef or lamb's infected livers may cause trematode eggs to appear in feces and consequently give a false positive result in the coprologic examination. A correct diagnosis can be made after excluding liver from the patient's diet for several days. If fecal observation is negative, bile can be examined by duodenal probe. In a series comparing the two approaches, coprologic observation revealed 68% of the cases, whereas the study of the bile aspirate identified 98% of the cases (De Gorgolas *et al*., 1992).

In Latin America, there have been cases of unnecessary and prolonged hospitalization of hepatic patients, and sometimes even surgical interventions have been performed, because differential diagnosis failed to take fascioliasis into account (Esteban *et al*., 1997).

A number of immunobiologic tests can be used to diagnose the infection during the prepatent period, including a skin test, complement fixation, immunofluorescence, immunoelectrophoresis, counterimmunoelectrophoresis, enzyme-linked immunosorbent assay (ELISA), and immunoelectrotransfer. The prepatent period of fascioliasis lasts for so long (more than two months in humans), that it is one of the few parasitic diseases in which immunology is useful for diagnosis. There are still cross-reactions, especially with schistosomiasis, but the search for appropriate antigens has improved the specificity and sensitivity of these tests. ELISA, used on cysteine proteinase regurgitated by the parasite, has yielded sensitivity levels of 89 to 95% and specificity levels of 98 to 100% (Carnevale *et al*., 2001; Cordova *et al*., 1999). Early diagnosis of fascioliasis makes it possible to start treatment before liver damage is too far advanced.

3.7. Treatment

At present there is no vaccine available for the prevention of fascioliasis and hence chemotherapy is the current mainstay of control. Triclabendazole is a benzimidazole derivative [6-chloro-5-(2,3-dichlorophenoxy)-2-methylthiobenzimidazole] that inhibits parasitic microtubule formation. It is the drug of choice against *F. hepatica*, since it is highly safe and effective against both

immature and adult flukes with cure rates up to 90% (McManus and Dalton, 2006; Robinson and Dalton, 2009).

Triclabendazole has been marketed since 1983 as a veterinary drug (Fasinex®) and, three years later, the human formulation of triclabendazole (Egaten®) was used for the first time in humans during an epidemic in Iran (Keiser and Utzinger, 2005). There is considerable concern of drug resistance development in humans, as drug resistance has already been reported in sheep and cattle from different parts of the world as e.g. Australia (Overend and Bowen, 1995), United Kigndom (Keiser *et al*., 2005), Argentina (Olaechea *et al*., 2011), the Netherlands (Moll *et al*., 2000; Gaasenbeek *et al*., 2001) and Spain (Alvarez-Sanchez *et al*., 2006).

Currently, the most widely used antimalarials are the two artemisinin derivatives artemether and artesunate (Haynes, 2006). Interestingly, the artemisinins also possess a broad spectrum of activity against various trematodes, such as *Schistosoma* spp., *Echinostoma caproni* Clonorchis sinensis as well as *F. hepatica* (Keiser *et al*., 2006a; Keiser *et al*., 2006b; Keiser *et al*., 2006c; Utzinger and Keiser, 2004). Hence, this could be a new line of attack again fascioliasis drug resistance.

Despite the inexistence of vaccines, the next four antigens were proposed as leading vaccine candidates for *F. hepatica* infections: leucine aminopeptidase, fatty acid-binding protein (rFh15), glutathione S-transferase and cysteine protease (FhCL1/FhCL2), with protection rates between (65 and 89.6%) (McManus and Dalton, 2006).

3.8. Prevention and Control

Individuals can prevent fascioliasis by not eating raw watercress and other metacercariae-carrying aquatic plants of wild or unknown origin. Watercress can be cultivated under controlled conditions that prevent access by animals and therefore fecal contamination, as well as infestation by snails. However, most watercress sold in markets has been gathered by people who are unaware of the sanitary conditions under which the plant was grown (Mas-Coma *et al*., 2005).

Water containing floating metacercariae is also often cited as an infection source. Contamination through water can be decreased by the utilization of the so-called washing units in which water is appropriately filtered (Mas-Coma, 2004) and by the chemical treatment of water.

Rinsing the greens for 10 minutes in running water washes away only 50% of the metacercariae, but citric acid (10 ml/L), commercial vinegar (120 ml/L), liquid soap (12 ml/L), or potassium permanganate (24 mg/L) will detach or kill all of them (El-Sayad *et al*., 1997).

A modern plan for the control of animal fascioliasis, which would ultimately forestall human infection, would include: a) preventing the consumption of metacercariae, b) strategically

administering fasciolicides to the definitive hosts and c) eliminating the intermediate hosts (Torgerson and Claxton, 1999).

Preventing the ingestion of *Fasciola* metacercariae by animals requires fencing in contaminated areas, which is difficult, expensive, and not very effective.

Step b, which corresponds to the strategic administration of fasciolicides, is meant to interrupt the life cycle of the parasite and prevent the formation of eggs and contamination of the environment. Previously, some of the chemical compounds against *F. hepatica* killed only the adult flukes, but now broad-spectrum treatments are available as well as highly sophisticated methods for calculating the best time to administer such treatments (Yilma and Malone, 1998).

Controlling snails involves ecologic, chemical, and biologic methods. The ecologic approach consists of modifying the environment to interrupt the life cycle of the snails. Drainage of the land, where this is technically and economically feasible, is the one permanent way to control or eliminate the molluscs. It is also beneficial to smooth the banks of watercourses and remove marginal vegetation to prevent the formation of backwater pools where the snails may flourish. The chemical approach consists of applying molluscicides in the field. Given the impressive capacity of Lymnaeids for reproduction and recuperation, molluscicides should be applied regularly to keep down the snail population (Crossland, 1976; Mas-Coma *et al*., 2005).

This approach is very costly and therefore cannot be applied on a large scale on most livestock-raising establishments. However, it can be used on small farms. Many traditional molluscicides are inactivated by organic materials and elevated pH levels. The biologic approach involves enlisting the "natural enemies" of the snails that serve as intermediate hosts. Although there are many known competitors, predators, and parasites of snails, this subject has not been fully studied (Torgerson and Claxton, 1999).

Vaccination against *F. hepatica* might be an appropriate control method However, as was said before, at present no vaccine is available for prevention of fascioliasis (McManus and Dalton, 2006).

4. The Importance of Genetic Characterization

Different molecular techniques and DNA markers have been applied to the study of fasciolid flukes. These molecular techniques appear to be very useful for the development of both diagnostic and epidemiological tools as well as for studies of intraspecific variability of the causal agents (Ai *et al*., 2011; Semyenova *et al*., 1996).

Most studies on fasciolid proteins have concentrated on isoenzymes. Isoelectric focusing of fluke soluble proteins can be used to confirm the presence of both *F. hepatica* and *F. gigantic* (Lotfy *et al*., 2002), although profiles of whole-body proteins and excretory/secretory products obtained with

this technique differ among worms from different hosts such as sheep and cattle (Lee *et al*., 1992). Unfortunately, very few studies have considered individual or population-level variation (Mas-Coma *et al*., 2005).

An arbitrary primer PCR is a simple and reproducible technique which uses single random oligonucleotide primers at low stringency to generate Random Amplified Polymorphic DNA (RAPD). For that reason, RAPD is a molecular technique that allows the amplification of short regions of an organism's genome without prior sequence information. This technique has proven to be a powerfull and rapid method for identifying genetic markers, tagging genes and chromosomes and detecting polymorphic genetic variation (Espinosa and Borowsky, 1998). Such variation can be used in identification of parasite strains and species in population and phylogenetic studies. The value of this technique in the study of inter- and intraspecific variation of parasites, e.g., *Schistosoma* spp. (Neto *et al*., 1993), *Echinococcus* spp. (Siles-Lucas *et al*., 1993), *Trichinella* spp. (Wu *et al*., 1998), *Echinostoma* spp. (Petrie *et al*., 1996), *Dicrocoelium* spp. (Sandoval *et al.,* 1999), *Fasciola* spp. (Semenova *et al*., 1995), *Dictyocaulus* spp. (Epe *et al*., 1995), *Metagonimus* spp. (Yu *et al*., 1997) and *Trypanosome* spp. (Basagoudanavar *et al*., 1999) is increasingly recognized.

Three possible explanations have been presented by Williams *et al*. (1990) for the observation of different RAPD-PCR profiles between or within species in RAPD assays: 1) Insertion or deletion between the primer target sites, 2) mismatches due to single base changes within the primer target site and 3) the deletion of one or both of the primer target sites.

Despite the great potential of RAPD technique, sequencing and the nucleotide analyze of genomic DNA is the ultimate basis for all biological classifications. Direct examination of the genome is the most reliable and sensitive criterion regarding the study of genetic variability (Mas-Coma *et al*., 2007).

The whole mitochondrial genome of *F. hepatica* has recently been sequenced and is suitable for studies of variation. Parts of the mitochondrial DNA of *F. hepatica* showed length heterogeneity, suggesting differences among individual mitochondrial genomes. The most important mitochondrial genes used in genetic variability studies are NADH dehydrogenase subunit 1 (*nad*1) and cytochrome c oxidase subunit 1 (*cox*1). These genes are 903 and 1533 bp respectively and are separated by the genes of several transference RNAs and NADH dehydrogenase subunit 3 (Morozova *et al*., 2004).

The first and second Internal Transcribed Spacers (ITS1 and ITS2) of nuclear ribosomal DNA, which occur between the 18S, 5.8S and 28S coding regions, have also been used for genetic variability studies. The ITS region is a particularly useful tool for molecular studies because it is a highly conserved region and, for that reason, it could be an ideal target for diagnostic purposes including detection of the DNA in clinical samples (Hillis and Dixon., 1991; Rokni *et al*., 2010).

Statistical methods for computing the variances of nucleotide and haplotype diversities within and between populations are currently being used to infer the phylogenetic relationships of the DNA sequences studied. Additionally, both mitochondrial genes *nad*1 and *cox*1 and ribosomal DNA

(rDNA) internal transcribed spacers are proper regions for investigating intraspecies differences in PCR-based formats such as PCR-RFLP (Walker *et al*., 2007).

Restriction Fragment Length Polymorphism (RFLP) is a molecular technique that uses restriction endonucleases to digest a specific fragment of DNA. Isolates with different DNA sequences usually are digested in different sites and therefore, produce singular RFLP patterns (Nei and Li, 1979). Application of this technique in mitochondrial DNA has already generated important information on the rate of nucleotide substitution in evolution and genetic structure of populations (Mas-Coma *et al*., 2005).

In recent years, an increasing number of authors have examined genetic variation of DNA by means of restriction endonucleases. Although this technique does not necessarily detect all genetic variation of DNA, it is much simpler than DNA sequencing and more accurate than RAPD, thus has a great utility for population genetics and evolutionary studies (Walker *et al*., 2007).

5. Aims

Studies on genetic variability within and between populations of *Fasciola hepatica* have important implications for epidemiology, diagnosis and effective control of fascioliasis (Mas-Coma *et al*., 2005). Recent technological advances in genomics and bioinformatics provide unique opportunities for a better understanding of the intraspecific genetic diversity of *F. hepatica*. Surprisingly, despite the widespread impact of fascioliasis, genomic datasets for *F. hepatica* are scant (Ai *et al*., 2011).

Hence, the main goal of the present project is to provide the first insights into the genetic characterization of *F. hepatica* in Portugal. Random Amplified Polymorphic DNA-Polymerase Chain Reaction (RAPD-PCR), Restriction Fragment Length Polymorphism (RFLP) and sequencing and nucleotide analysis of NADH dehydrogenase subunit 1 (*nad*1) gene, cytochrome c oxidase subunit 1 (*cox*1) gene and Internal Transcribed Spacers (ITS) region were the DNA-based molecular techniques chosen and were applied to *F. hepatica* isolates from different hosts and geographical locations in Portugal.

Specifically, our goal was to determine the polymorphisms and the genetic variability of Portuguese *F. hepatica* isolates and the genetic distances between Portuguese and worldwide isolates.

In due course, we also aimed to infer the risk of development of drug-resistance in Portugal.

Chapter II. MATERIALS AND METHODS

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1. Sample Collection

The adult liver flukes used in this study were recovered from naturally infected cattle and sheep of either gender, which were brought to two different slaughterhouses in Leiria (Mapicentro - Sociedade de Abate, Comercialização e Transformação de Carnes e Subprodutos, S. A.) and Castelo Branco (Oviger - Produção, Transformação e Comércio de Carnes e Derivados, S. A) from November 2010 until May 2011.

The livers along with gall bladders including the bile duct were examined and the infected ones were removed from the diseased animals. The bile ducts were incised longitudinally through the gall bladder and the parasites were removed with the help of fine forceps, taking all necessary precautions to avoid any damage to the parasite. The infected livers were squeezed manually to macerate the parenchyma and the flukes were carefully removed. Flukes were identified as *Fasciola hepatica* according to existing morphological keys and descriptions given by Periago *et al*., 2006.

Each parasite was thoroughly washed individually 2 to 3 times in a 0.9% saline solution to remove debris and contamination. The samples were then kept in 70% ethanol and were carried to the laboratory where they were stored at 4ºC.

2. Nuclear and Mitochondrial DNA Extraction

DNA isolation was performed according to Stothard *et al*. (1996) with some modifications. Firstly, the posterior portion of the fluke's body was cut and ground in 600 µl of lysis buffer [100 mM Tris-hydrochloric acid (Tris-HCl), pH 8.0, 1.4 M Sodium chloride (NaCl), 20 mM Ethylenediamine tetraacetic acid (EDTA), 2% Hexadecyltrimethylammonium bromide (CTAB), 0.2% 2-mercaptoethanol] previously incubated at 55ºC for 10 minutes (min). 10 µl of Proteinase K were added to the Eppendorf and the fluke's tissue was mashed and incubated at 55ºC for 90 min. Nucleic acids were extracted by adding 600 µl of Chloroform-isoamyl alcohol (24:1) and shaken for 2 min. The aqueous layer was collected by centrifugation [15 seconds at 13 000 revolutions per minute (rpm)] and transferred into a new 1.5 µl microtube containing 800 µl of ice-cold absolute ethanol.

DNA was pelleted by centrifugation during 20 min at 13 000 rpm and washed in 500 µl of 70% ethanol to precipitate the DNA and to remove excess salts. After that, DNA was pelleted again by centrifugation during 10 min at 13 000 rpm, dried in a vacuum oven during 10 min at 55ºC and then

re-dissolved in 45 µl of TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) and subsequently stored at -20°C.

DNA from all the samples was checked for shearing and concentration by 1% agarose gel electrophoresis in TAE buffer (40 mM Tris-acetate, pH 8.0, 1mM EDTA) and visualized by 0.5 µg/ml Ethidium bromide (EtBr) staining.

A molecular weight marker ranging from 100 to 1000 bp (HyperLadder™ IV, Bioline) was chosen to evaluate DNA migration. Samples were prepared with 2 µl of DNA and 1.5 µl of loading buffer (Crystal 5x DNA Loading Buffer Blue, Bioline).

Electrophoretic migration of DNA was done at 100V and the EtBr-stained agarose gel was visualized under ultraviolet light (Alphamanager™HP, Alpha Innotech).

DNA was then dilute for all samples in order to have a working concentration of 30-50 ng/µl.

3. Random Amplified Polymorphic DNA-PCR (RAPD-PCR)

DNA was submitted to random amplification with two sets of arbitrary primers: "AP" and "OSA" (Table II.1).

The PCR for the "AP" set of primers was carried out in a total volume of 25 µl containing 1 µM of primer, 1.5 µl of DNA solution (45-75 ng), 1X Green Go Taq^R Flexi Buffer (10 mM Tris-HCl [pH 9.0 at room temperature], 50 mM NaCl, 2 mM Magnesium chloride [$MgCl_2$]), 200 µM each of dATP, dCTP, dGTP and dTTP and 1.25 U Go Taq^R DNA Polymerase.

The PCR for the "OSA" set of primers was carried out in a 25 µl of total volume containing 0.8 µM of primer, 1 µl of DNA solution (30-50 ng), 1X Green Go Taq^R Flexi Buffer (10 mMTris-HCl (pH 9.0 at room temperature), 50 mM NaCl, 2 mM $MgCl_2$), 200 µM each of dATP, dCTP, dGTP and dTTP and 1.25 U Go Taq^R DNA Polymerase.

For each primer, negative controls, without DNA template, were performed concurrently to check for contamination.

The PCR was performed in a My Cycler™ Thermal Cycler (Bio-Rad).

For the "AP" set, the condition were as follows: after an initial denaturation at 94ºC for 5 minutes, samples were subjected to 45 cycles of amplification (denaturation at 94ºC for 60 seconds, primer annealing at 36ºC for 45 seconds and extension at 72ºC for 60 seconds), followed by a 5 minutes final elongation at 72ºC.

For the "OSA" set, the conditions were as follows: 75 cycles of amplification (denaturation at 94ºC for 5 seconds, primer annealing at 36ºC for 30 seconds and extension at 72ºC for 10 seconds), no initial denaturation or final elongation was required.

PCR products were checked for size and purity through 1.5% agarose gel electrophoresis in TAE buffer (40 mM Tris-acetate, pH 8.0, 1mM EDTA) and visualized by 0.5 µg/ml EtBr staining.

A molecular weight marker ranging from 100 to 1000 bp (HyperLadder™ IV, Bioline) was chosen to evaluate DNA migration. Samples were prepared with 10 µl of PCR product and 2.5 µl of loading buffer (Crystal 5x DNA Loading Buffer Blue, Bioline).

Electrophoretic migration of DNA was done at 80V and the EtBr-treated agarose gel was visualized under ultraviolet light (Alphamanager™HP, Alpha Innotech).

Table II.1.Nucleotide sequences and G + C content of the primers used in the RAPD-PCR assays.

Primer code	Sequence (5' → 3')	G + C Content	Reference
AP1	GCCGTCCGAG	80%	Gunasekar *et al*., 2008
AP2	GTGGTGGTGG	70%	Gunasekar *et al*., 2008
AP3	GCGAGCGTCCC	81.8%	Gunasekar *et al*., 2008
AP5	CAGCCTGGGC	80%	Gunasekar *et al*., 2008
AP7	CGGTGGCGAA	70%	Gunasekar *et al*., 2008
AP8	CCGGTGTGGG	80%	Gunasekar *et al*., 2008
AP9	TCGTAGCCAA	50%	Gunasekar *et al*., 2008
AP11	CGCCCCACGT	80%	Gunasekar *et al*., 2008
AP12	TGCATCGTAC	50%	Gunasekar *et al*., 2008
AP13	CGGACGTCGC	80%	Gunasekar *et al*., 2008
AP15	CCCGCCATCT	70%	Gunasekar *et al*., 2008
AP16	TGGTCAGTGA	50%	Gunasekar *et al*., 2008
OSA9	TCGTCGCATT	50%	Aldemir, 2006
OSA10	AGCAGCAGGC	70%	Aldemir, 2006
OSA11	GGGTAACGCC	70%	Aldemir, 2006

4. Polymerase Chain Reaction Amplification of *cox*1 Gene

A 461 bp portion of mitochondrial cytochrome c oxidase subunit 1 (*cox*1) gene was amplified by polymerase chain reaction (PCR) using primers Cox1F (forward: 5'-TTGGTTTTTTGGGCATCCT-3') and Cox1R (reverse: 5'-AGGCCACCACCAAATAAAAGA-3') (Semyenova *et al*., 2006).

The PCR was carried out in a total volume of 25 µl of using Illustra™ puReTaq Ready-To-Go PCR Beads (GE Healthcare), 0.25 µM of each primer and 2 µl of DNA solution (60-100 ng).

When a bead is reconstituted to a final volume of 25 µl, the PCR reaction consisted of: 10 mM Tris-HCl (pH 9.0 at room temperature), 50 mM Potassium chloride (KCl), 1.5 mM $MgCl_2$, 200 µM each of dATP, dCTP, dGTP and dTTP and 2.5 U puReTaq DNA polymerase.

A negative control, without DNA template, was performed concurrently to check for contamination.

The PCR was performed in a My Cycler™ Thermal Cycler (Bio-Rad) and the conditions were as follows: after an initial denaturation at 94ºC for 2 minutes, samples were subjected to 30 cycles of amplification (denaturation at 94ºC for 90 seconds, primer annealing at 53ºC for 60 seconds and extension at 72ºC for 120 seconds), followed by a 2 minutes final elongation at 72ºC.

PCR products were checked for size and purity through 1.5% agarose gel electrophoresis in TAE buffer (40 mM Tris-acetate, pH 8.0, 1mM EDTA) and visualized by 0.5 µg/ml EtBr staining.

A molecular weight marker ranging from 100 to 1000 bp (HyperLadder™ IV, Bioline) was chosen to evaluate DNA migration. Samples were prepared with 2 µl of PCR product and 1.5 µl of loading buffer (Crystal 5x DNA Loading Buffer Blue, Bioline).

Electrophoretic migration of DNA was done at 100V and the EtBr-treated agarose gel was visualized under ultraviolet light (Alphamanager™HP, Alpha Innotech).

5. Polymerase Chain Reaction Amplification of *nad*1 Gene

A 379 bp portion of the mitochondrial NADH dehydrogenase subunit 1 (*nad*1) gene was amplified by polymerase chain reaction (PCR) using primers Nad1F (forward: 5'-TATGTTTTGTACGGGATGAG-3') and Nad1R (reverse: 5'-AACAACCCCAACCAACACTTA-3') (Semyenova *et al*., 2006).

The PCR was carried out in a total volume of 25 µl using Illustra™ puReTaq Ready-To-Go PCR Beads (GE Healthcare), 0.25 µM of each primer and 2 µl of DNA solution (60-100 ng).

When a bead is reconstituted to a final volume of 25 µl, the PCR reaction consisted of: 10 mM Tris-HCl (pH 9.0 at room temperature), 50 mM KCl, 1.5 mM $MgCl_2$, 200 µM each of dATP, dCTP, dGTP and dTTP and 2.5 U puReTaq DNA polymerase.

A negative control, without DNA template, was performed concurrently to check for contamination.

The PCR was performed in a My Cycler™ Thermal Cycler (Bio-Rad) and the conditions were as follows: after an initial denaturation at 94ºC for 2 minutes, samples were subjected to 30 cycles of amplification (denaturation at 94ºC for 90 seconds, primer annealing at 53ºC for 60 seconds and extension at 72ºC for 120 seconds), followed by a final elongation of 2 minutes at 72ºC.

PCR products were checked for size and purity through 1.5% agarose gel electrophoresis in TAE buffer (40 mM Tris-acetate, pH 8.0, 1mM EDTA) and visualized by 0.5 µg/ml EtBr staining.

A molecular weight marker ranging from 100 to 1000 bp (HyperLadder™ IV, Bioline) was chosen to evaluate DNA migration. Samples were prepared with 2 µl of PCR product and 1.5 µl of loading buffer (Crystal 5x DNA Loading Buffer Blue, Bioline).

Electrophoretic migration of DNA was done at 100V and the EtBr-treated agarose gel was visualized under ultraviolet light (Alphamanager™HP, Alpha Innotech).

6. Polymerase Chain Reaction Amplification of the ITS Region

The DNA region comprising the 1st (ITS-1) and 2nd (ITS-2) Internal Transcribed Spacers (ITS) and the 5.8 S gene of nuclear ribosomal DNA (rDNA) (Fig. II.1) sequences was amplified by polymerase chain reaction (PCR) using primers BD1 (forward: 5'-GTCGTAACAAGGTTTCCGTA-3') and BD2 (reverse: 5'-TATGCTTAAATTCAGCGGGT-3') (Ali *et al*., 2008).

The PCR was carried out in a total volume of 25 µl using Illustra™ puReTaq Ready-To-Go PCR Beads (GE Healthcare), 0.4 µM of each primer and 2 µl of DNA solution (60-100 ng).

When a bead is reconstituted to a final volume of 25 µl, the PCR reaction consisted of: 10 mM Tris-HCl (pH 9.0 at room temperature), 50 mM KCl, 1.5 mM $MgCl_2$, 200 µM each of dATP, dCTP, dGTP and dTTP and 2.5 U puReTaq DNA polymerase.

A negative control, without DNA template, was performed concurrently to check for contamination.

The PCR was performed in a My Cycler™ Thermal Cycler (Bio-Rad) and the conditions were as follows: after an initial denaturation at 95ºC for 5 minutes, samples were subjected to 30 cycles of amplification (denaturation at 95ºC for 30 seconds, primer annealing at 55ºC for 30 seconds and extension at 72ºC for 30 seconds), followed by a 7 minutes final elongation at 72ºC.

PCR products were checked for size and purity through 1% agarose gel electrophoresis in TAE buffer (40 mM Tris-acetate, pH 8.0, 1mM EDTA) and visualized by 0.5 µg/ml EtBr staining.

A molecular weight marker ranging from 100 to 1000 bp (HyperLadder™ IV, Bioline) was chosen to evaluate DNA migration. Samples were prepared with 2 µl of PCR product and 1.5 µl of loading buffer (Crystal 5x DNA Loading Buffer Blue, Bioline).

Electrophoretic migration of DNA was done at 100V and the EtBr-treated agarose gel was visualized under ultraviolet light (Alphamanager™HP, Alpha Innotech).

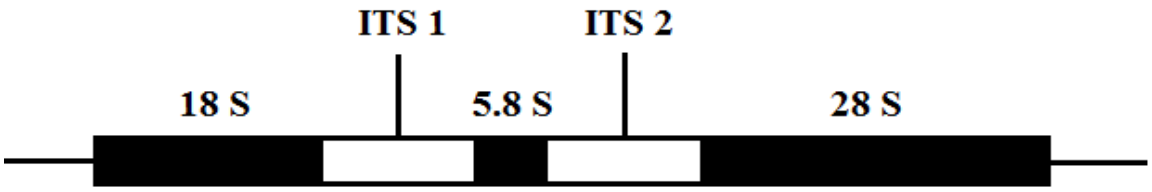

Fig. II.1. Gene segment of ribosomal DNA that contains 18S, 5.8S, and 28S tracts and the internal transcribed spacers (ITS) 1 and 2. Image by Raquel Santos.

7. Restriction Fragment Length Polymorphism (RFLP)

Three restriction endonucleases were chosen: RsaI and AluI (4 base cutters) and HinfI (5 base cutters) (Table II.2). All the enzymes were used to digest the PCR products from the amplification of the ITS region and the *nad*1 and *cox*1 genes.

Table II.2. Recognition sites of the restriction endonucleases used in the RFLP assays.

Enzyme	AluI	HinfI	RsaI
Recognition site	5' … A G▾C T … 3'	5' … G▾A N T C … 3'	5' …G T▾A C … 3'

AluI digestions were performed at 37°C for four hours in a 15 µl reaction volume containing 8.0 µl of PCR product, 10 mM Tris-HCl (pH 7.9 at room temperature), 10 mM MgCl2, 1 mM dithiothreitol, 100 µg/ml Bovine Serum Albumine (BSA) and 2 U restriction enzyme (BIORON).

HinfI digestions were performed at 37°C for four hours in a 15 µl reaction volume containing 8.0 µl of PCR product, 100 mM NaCl, 50 mM Tris-HCl (pH 7.9 at room temperature), 10 mM MgCl2, 1 mM dithiothreitol, 100 µg/ml BSA and 8 U restriction enzyme (BIORON).

RsaI digestions were performed at 37°C for four hours in a 15 µl reaction volume containing 8.0 µl of PCR product, 50 mM NaCl, 10 mM Tris-HCl (pH 7.9 at room temperature), 10 mM MgCl2, 1 mM dithiothreitol, 100 µg/ml BSA and 16 U restriction enzyme (BIORON).

A negative control, without DNA template, was performed concurrently to check for contamination.

After the digestion procedure, samples were incubated at 75ºC for 20 minutes in order to heat-inactivate the restriction endonucleases.

Restriction fragments were checked for size through 2.5% agarose gel electrophoresis in TAE buffer (40 mM Tris-acetate, pH 8.0, 1mM EDTA) and visualized by 0.5 µg/ml EtBr staining.

A molecular weight marker ranging from 100 to 1000 bp (HyperLadder^{TM} IV, Bioline) was chosen to evaluate DNA migration. Samples were prepared with 14 µl of digestion product and 4 µl of loading buffer (Crystal 5x DNA Loading Buffer Blue, Bioline).

Electrophoretic migration of DNA was done at 80V and the EtBr-treated agarose gel was visualized under ultraviolet light (Alphamanager^{TM}HP, Alpha Innotech).

8. Purification of Polymerase Chain Reaction Products

PCR products from the amplification of the ITS region and the *nad*1 and *cox*1 genes were purified using the QIAquick^{R} PCR Purification kit.

100 µl of PB buffer was added to 20 µl of the PCR reaction and mixed. To bind DNA, the sample was applied to the QIAquick column, previously placed in a 1.5 µl microtube, and centrifuged for 60 seconds at 14 000 rpm. The aqueous layer was discarded and the QIAquick column were placed back in the same microtube. 750 µl of PE buffer was added in order to wash the sample. After 60 seconds of centrifugation at 14 000 rpm, the aqueous layer was discarded and the QIAquick column was placed back in the same microtube. Another centrifugation (60 seconds at 14 000 rpm) was made to assure that all the residual ethanol from the PE buffer was removed. The QIAquick column was then placed in a new Eppendorf. To elute DNA, 50 µl of Buffer EB (10 mM Tris-HCl, pH 8.5) was added to the center of the QIAquick membrane and centrifuged for 60 seconds at 14 000 rpm. The eluted DNA was stored at -20ºC.

Purified PCR products were checked for size and purity through 1.5% agarose gel electrophoresis in TAE buffer (40 mM Tris-acetate, pH 8.0, 1mM EDTA) and visualized by 0.5 µg/ml EtBr staining.

A molecular weight marker ranging from 100 to 1000 bp (HyperLadder^{TM} IV, Bioline) was chosen to evaluate DNA migration. Samples were prepared with 2 µl of PCR product and 1.5 µl of loading buffer (Crystal 5x DNA Loading Buffer Blue, Bioline).

Electrophoretic migration of DNA was done at 100V and the EtBr-treated agarose gel was visualized under ultraviolet light (Alphamanager^{TM}HP, Alpha Innotech).

9. Sequencing

Sequencing of the purified PCR products from the amplification of the ITS region and the *nad*1 and *cox*1 genes was performed by STABVIDA using ABI PRISM^{R} BigDye^{TM} cycle-sequencing kit version 1.1 (Perkin Elmer). Only the sense strands were sequenced.

10. Analysis of Nucleotide Sequence Data and Phylogenetic Inference

Sequences from each genomic region were aligned with the use of CLUSTAL W^{TM}, version 2.0 (Larkin *et al*., 2007). MEGA^{TM}, version 5.05 (Tamura *et al*., 2001), which stands for Molecular Evolutionary Genetics Analysis and Dna SP^{TM}, version 5.10.01(Librado and Rozas, 2009), which

stands for DNA Sequence Polymorphism were the chosen softwares for processing and analysis of the nucleotide sequence data.

The average number of nucleotide differences (K), nucleotide diversity (π), number of existent haplotypes (H) and the haplotype diversity (Hd) were calculated with the use of Kimura's 2 parameter model (Kimura, 1980) with Dna SP^{TM} 5.10.01. Significance was based on 1000 random permutations of the data set.

Pairwise distances, nucleotide frequencies and phylogenetic trees for each genomic region were generated with MEGA^{TM}, version 5.05 by means of Kimura's 2 parameter substitution model and neighbor-joining statistical method (Saitou and Nei, 1987) with 1000 bootstrap replications.

A total of 37 sequences (22 *cox*1 gene fragment, 12 *nad*1 gene fragment and 3 ITS region fragment) were used in the phylogenetic analyses. In addition, the following published sequences deposited in the GenBank were used (GenBank accession numbers):

AB207154.1 (*nad*1, Uruguay), AB207155.1 (*nad*1, *cox*1, Australia), AB207156.1 (*nad*1, Ireland), AB207169.1 (*nad*1, Japan), AB211239.1 (*nad*1, South Korea), AB300704.1 (*cox*1, Japan), AB477357.1 (*nad*1, China), AB477362.1 (*nad*1, China), AB554179.1 (*nad*1, Egypt), AB554187.1 (*nad*1, Egypt), AB554188.1 (*nad*1, Egypt), AB554191.1 (*nad*1, Egypt), AB554192.1 (*nad*1, Egypt), AB554193.1 (*nad*1, Egypt), AB604926.1 (*nad*1, China), AB604927.1 (*nad*1, China), AB604929.1 (*nad*1, China), AF216697.1 (*nad*1, Australia), AJ630399.1 (*nad*1, *F. gigantica*), AM709622.1 (ITS, Spain), AM709649.1 (ITS, Spain), AM850107.1 (ITS, Niger), FJ469984.1 (*cox*1, Niger), GQ121276.1 (*cox*1, Turkey), GQ175362.1 (*nad*1, Iran), GQ175363.1 (*nad*1, Iran), GQ175364.1 (*nad*1, Iran), GQ231547.1 (ITS, Tunisia), GQ231548.1 (*cox*1, Tunisia), GQ231549.1 (*cox*1, Algeria), GQ231550.1 (*cox*1, Tunisia), GQ231551.1 (*cox*1, Tunisia), GQ356033.1 (*nad*1, Iran), GU112454.1 (*cox*1, Spain), GU112455.1 (*cox*1, Spain), GU112456.1 (*cox*1, Spain), GU112457.1 (*cox*1, Spain), GU112469.1 (*cox*1, Niger), GU112470.1 (*cox*1, Niger), GU112471.1 (*cox*1, Niger), GU112476.1 (*cox*1, France), GU112477.1 (*cox*1, France), GU112478.1 (*cox*1, France), GU112479.1 (*cox*1, France), GU112480.1 (*cox*1, France), GU112481.1 (*cox*1, France), GU112482.1 (*cox*1, USA), GU112483.1 (*cox*1, USA), HQ856045 (*cox*1, *F. gigantica*), JF432072.1 (ITS, Iran), JF496715.1 (ITS, *F. gigantica*), JF496716.1 (ITS, China), JF824676.1 (*nad*1, Italy), JF824677.1 (*nad*1, Italy), JF824678.1 (*nad*1, Italy), JF824679.1 (*nad*1, Italy), JN828957.1 (ITS, Iran) and M93388.1 (*nad*1, *cox*1, USA).

Chapter III. RESULTS

Chapter III. RESULTS

1. Sample Collection

A total of 68 adult *F. hepatica* specimens were collected in the Portuguese slaughterhouses.

Of the collected samples, 83.8% (n = 57) were originated from cattle and 16.2% (n = 11) from sheep. 20 infected cattle and 3 infected sheep contributed to the sample collection.

For this work, four flukes, recovered from infected cattle, were selected from each region, except Faro, that only contributed with three flukes. From infected sheep it was only possible to select four flukes from two regions, Leiria and Santarém (Table III.1).

Table III.1. *Fasciola hepatica* isolates according to their geographical and host origin.

Isolate	Region	Host	Isolate	Region	Host
1	Lisboa	Cattle	25	Beja	Cattle
2	Lisboa	Cattle	26	Beja	Cattle
3	Lisboa	Cattle	27	Beja	Cattle
4	Lisboa	Cattle	28	Beja	Cattle
5	Leiria	Cattle	29	Évora	Cattle
6	Leiria	Cattle	30	Évora	Cattle
7	Leiria	Cattle	31	Évora	Cattle
8	Leiria	Cattle	32	Évora	Cattle
9	Setúbal	Cattle	33	Portalegre	Cattle
10	Setúbal	Cattle	34	Portalegre	Cattle
11	Setúbal	Cattle	35	Portalegre	Cattle
12	Setúbal	Cattle	36	Portalegre	Cattle
13	Santarém	Cattle	37	Faro	Cattle
14	Santarém	Cattle	38	Faro	Cattle
15	Santarém	Cattle	39	Faro	Cattle
16	Santarém	Cattle	40	Leiria	Sheep
17	Castelo Branco	Cattle	41	Leiria	Sheep
18	Castelo Branco	Cattle	42	Leiria	Sheep
19	Castelo Branco	Cattle	43	Leiria	Sheep
20	Castelo Branco	Cattle	44	Santarém	Sheep
21	Coimbra	Cattle	45	Santarém	Sheep
22	Coimbra	Cattle	46	Santarém	Sheep
23	Coimbra	Cattle	47	Santarém	Sheep
24	Coimbra	Cattle			

2. Nuclear and Mitochondrial DNA Extraction

DNA was successfully extracted from all the forty-seven *Fasciola hepatica* isolates.

In the agarose gel electrophoresis (Fig. III.1) a smear was visible in almost all lanes, meaning that DNA was too concentrated. Therefore, it was dilute for all samples in order to have a working concentration of approximately 30-50 ng/µl.

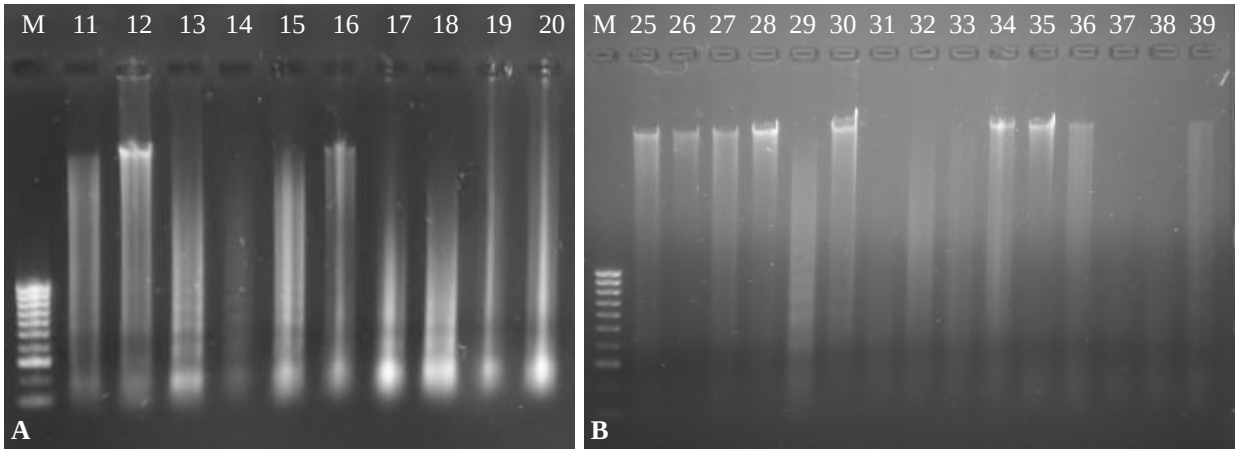

Fig. III.1. Agarose gel electrophoresis of DNA extraction from isolates 11 - 20 (**A**) and 25 - 39 (**B**).
M- 100bp molecular weight marker.

3. Random Amplified Polymorphic DNA-PCR (RAPD-PCR)

DNA from isolates from different regions was submitted to RAPD-PCR with two different sets of primers: "AP" and "OSA". The figures III.2 and III.3 represent RAPD-PCR with the "AP" set of primers and figures III.4, III.5 and III.6 represent the RAPD-PCR with the "OSA" set of primers.

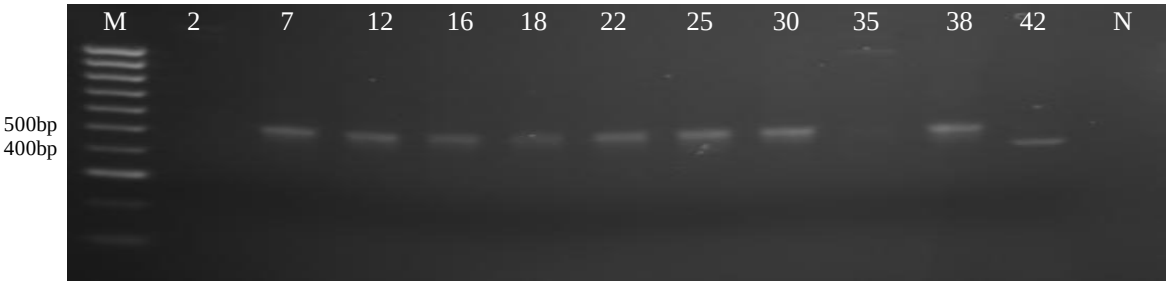

Fig. III.2. Agarose gel electrophoresis of primer AP9 RAPD-PCR products from isolates 2, 7, 12, 16, 18, 22, 25, 30, 35, 38 and 42. M- 100bp molecular weight marker. N- Negative control.

In figure III.2 a single band of approximately 500 bp was visible in lanes 7, 12, 16, 18, 22, 25, 30 and 38. Lane 42 presented a smaller band, of approximately 400 bp. For samples 2 and 35 primer AP9 was unable to produce any amplification fragment.

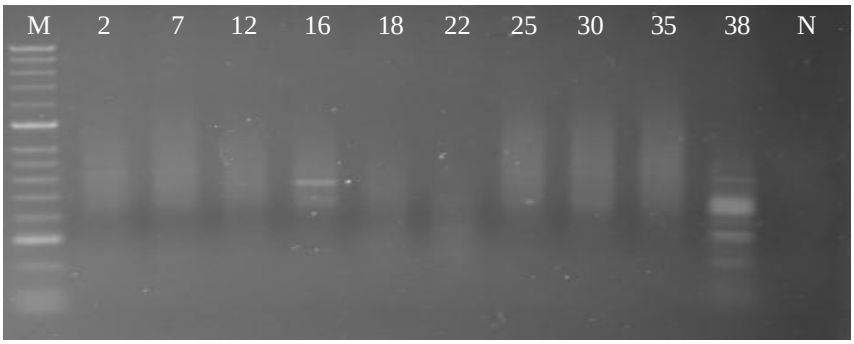

Fig. III.3. Agarose gel electrophoresis of primer AP2 RAPD-PCR products from isolates 2, 7, 12, 16, 18, 22, 25, 30, 35 and 38. M- 100 bp molecular weight marker, N- Negative control.

For primer AP2, a 2000 bp molecular weight marker (HyperLadder^{TM} II, Bioline) was used.

We made several RAPD-PCR duplicates with increasing concentrations of both primer, DNA template, Go Taq^{R} DNA Polymerase and $MgCl_2$. PCR conditions were also changed several times, mainly the temperature for primer annealing. However the RAPD-PCR pattern produced by the majority of samples presented no defined DNA bands (Fig. III.3).

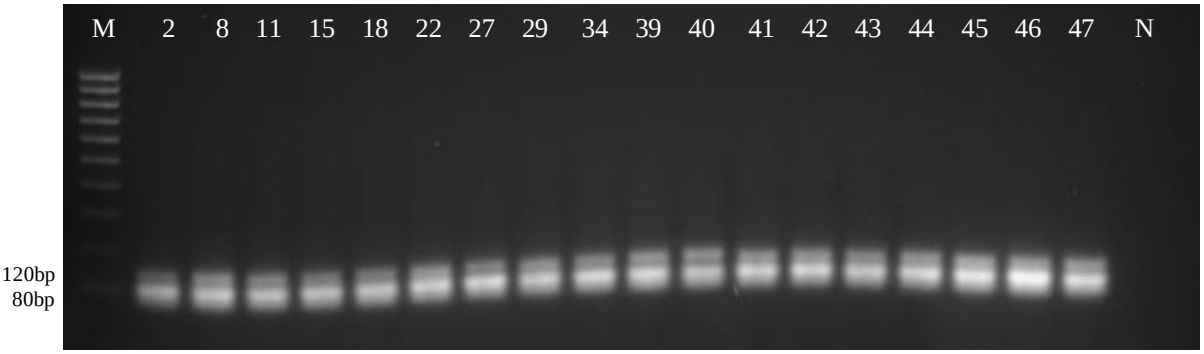

Fig III.4. Agarose gel electrophoresis of primer OSA9 RAPD-PCR products from isolates 2, 8, 11, 15, 18, 22, 27, 29, 34, 39, 40, 41, 42, 43, 44, 45, 46 and 47. M- 100 bp molecular weight marker. N- Negative control.

The arbitrary primer OSA9 allowed the PCR amplification of two fragments with approximately 80 and 120 bp. All samples presented the same amplification products (Fig. III.4).

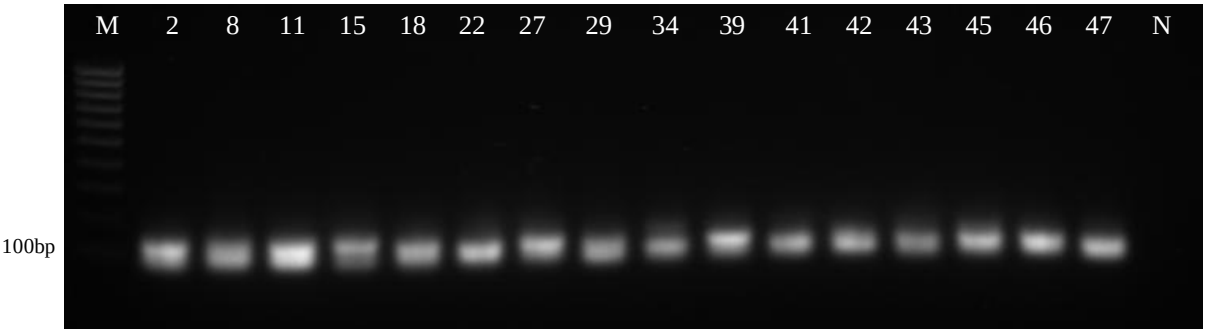

Fig III.5. Agarose gel electrophoresis of primer OSA10 RAPD-PCR products from isolates 2, 8, 11, 15, 18, 22, 27, 29, 34, 39, 41, 42, 43, 45, 46 and 47. M- 100 bp molecular weight marker. N- Negative control.

The arbitrary primer OSA10 allowed the amplification of a single DNA fragment with approximately 100 bp. All isolates presented the same pattern (Fig. III.5).

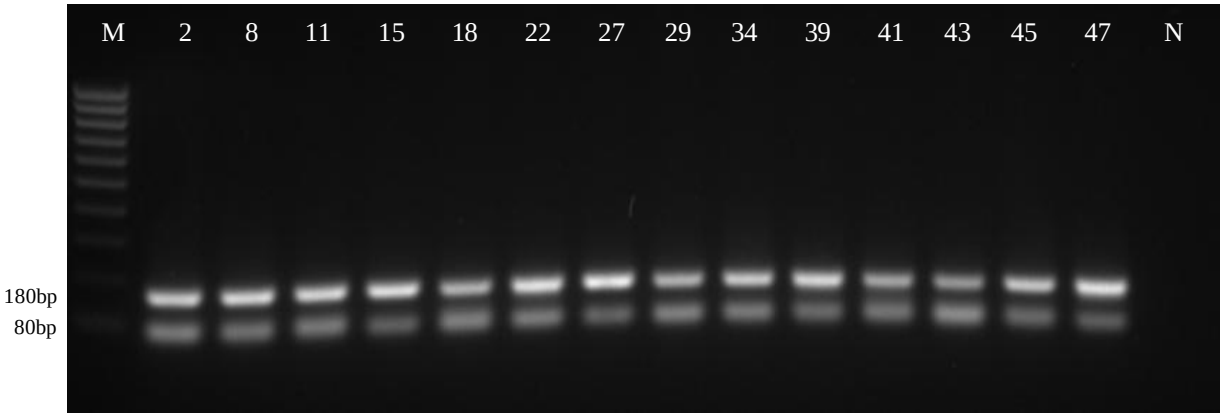

Fig III.6. Agarose gel electrophoresis of primer OSA11 RAPD-PCR products from isolates 2, 8, 11, 15, 18, 22, 27, 29, 34, 39, 41, 43, 45 and 47. M- 100 bp molecular weight marker. N- Negative control.

The arbitrary primer OSA11 allowed the PCR amplification of two polymorphic fragments with approximately 80 and 180 bp. All lanes displayed the same amplification products (Fig. III.6).

4. PCR Amplification of the Cytochrome C Oxidase Subunit 1 (*cox*1) Gene

DNA from all isolates was submitted to PCR amplification with specific primers in order to amplify a region of the mithocondrial *cox*1 gene. A monomorphic DNA segment with approximately 500 bp was visible for *F. hepatica* isolates of each host (Figures III.7 and III.8).

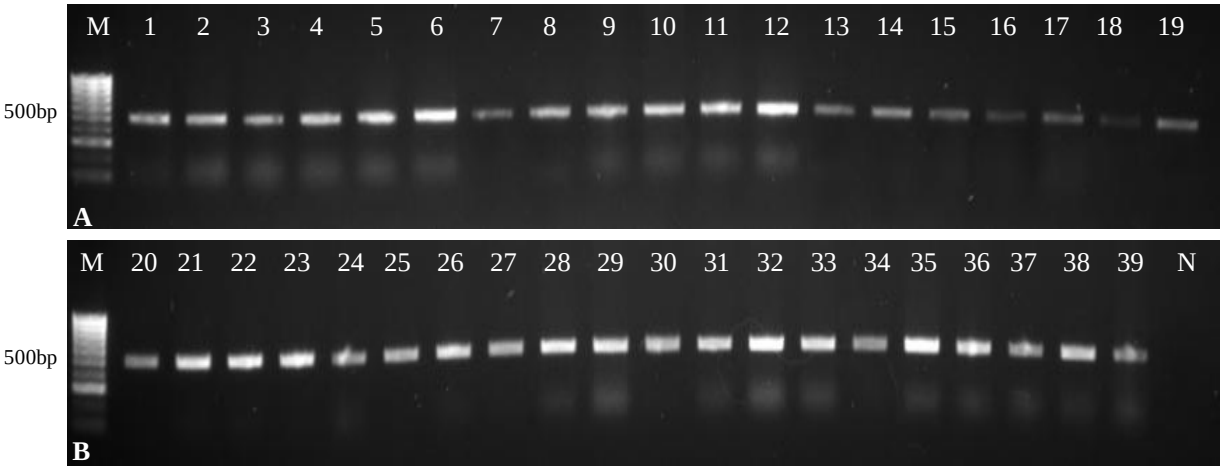

Fig III.7. Agarose gel electrophoresis of the *cox*1 gene PCR product from isolates 1 - 19 (**A**) and 20 - 39 (**B**). M- 100 bp molecular weight marker. N- Negative control.

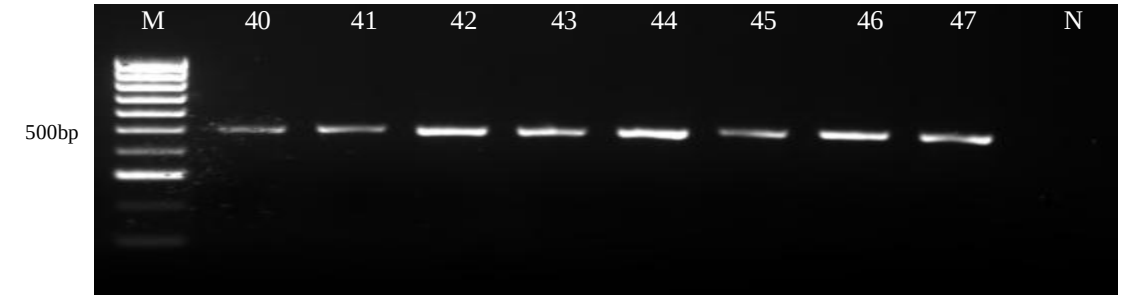

Fig III.8. Agarose gel electrophoresis of the *cox*1 gene PCR product from isolates 40 - 47. M- 100 bp molecular weight marker. N- Negative control.

5. PCR Amplification of NADH Dehydrogenase Subunit 1 (*nad*1) Gene

DNA from all isolates was submitted to PCR with specific primers in order to amplify a region of the mithocondrial *nad*1 gene. A monomorphic DNA segment with approximately 400 bp was visible for all *F. hepatica* isolates of each geographical and host origin (Figures III.9. and III.10).

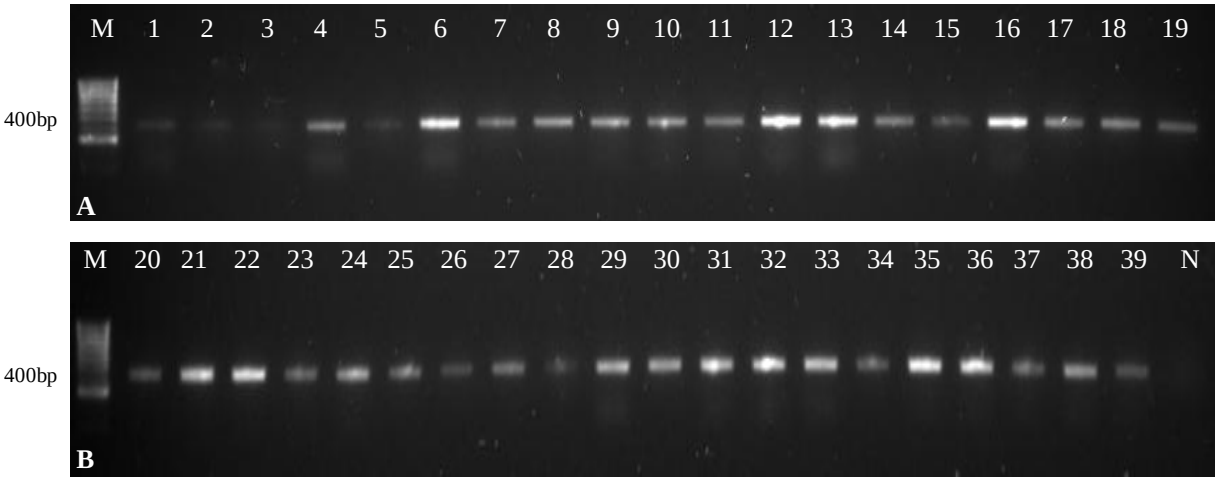

Fig III.9. Agarose gel electrophoresis of the *nad*1 gene PCR product from isolates 1 - 19 (**A**) and 20 - 39 (**B**). M- 100 molecular weight marker. N- Negative control.

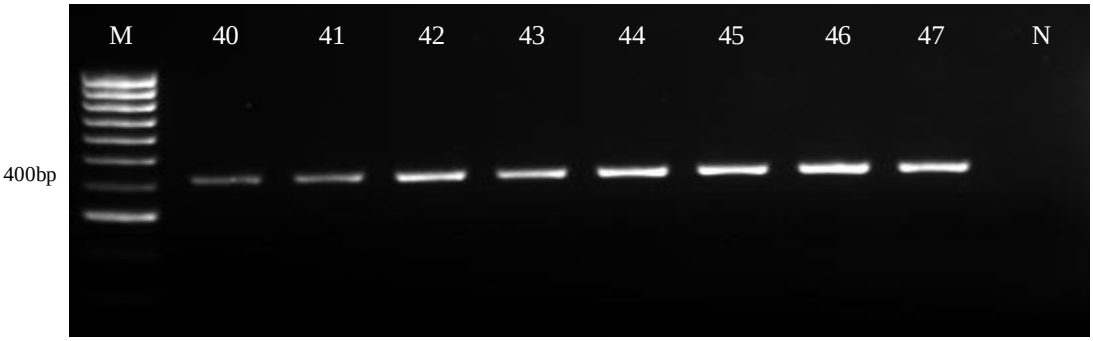

Fig III.10. Agarose gel electrophoresis of the *nad*1 gene PCR product from isolates 40 - 47. M-100 bp molecular weight marker. N- Negative control.

6. PCR Amplification of the Internal Transcribed Spacers (ITS) Region

DNA from all isolates was submitted to PCR with specific primers in order to amplify the ITS region. A monomorphic DNA segment with approximately 1000 bp was visible for all *F. hepatica* isolates of each geographical and host origin (Figures III.11 and III.12).

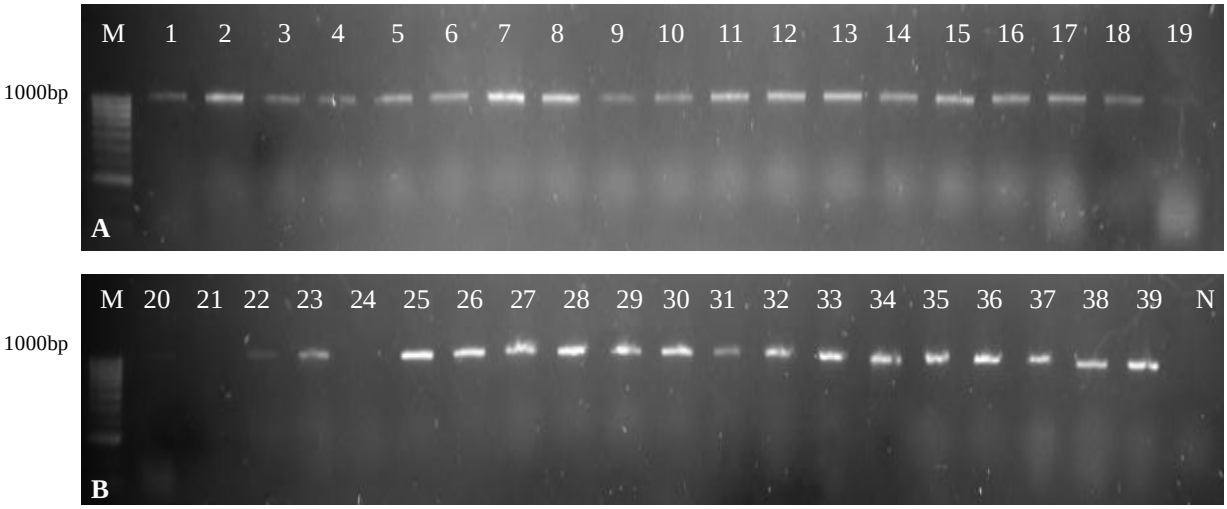

Fig III.11. Agarose gel electrophoresis of the ITS region PCR product from isolates 1 - 19 (**A**) and 20 - 39 (**B**). M- 100 bp molecular weight marker. N- Negative control.

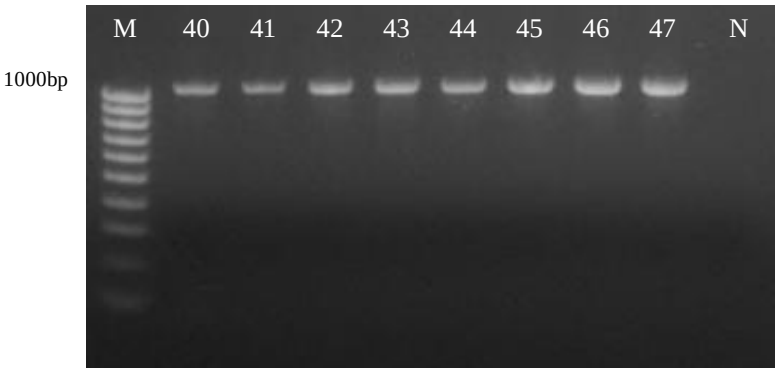

Fig III.12. Agarose gel electrophoresis of the ITS region PCR product from isolates 40 - 47. M- 100 bp molecular weight marker. N- Negative control.

7. Restriction Fragment Length Polymorphism (RFLP)

The PCR products from the amplification of the ITS region and the *nad*1 and *cox*1 genes were submitted to the endonuclease activity of three different enzymes: RsaI, AluI and HinfI (Figures III.13 - III.18).

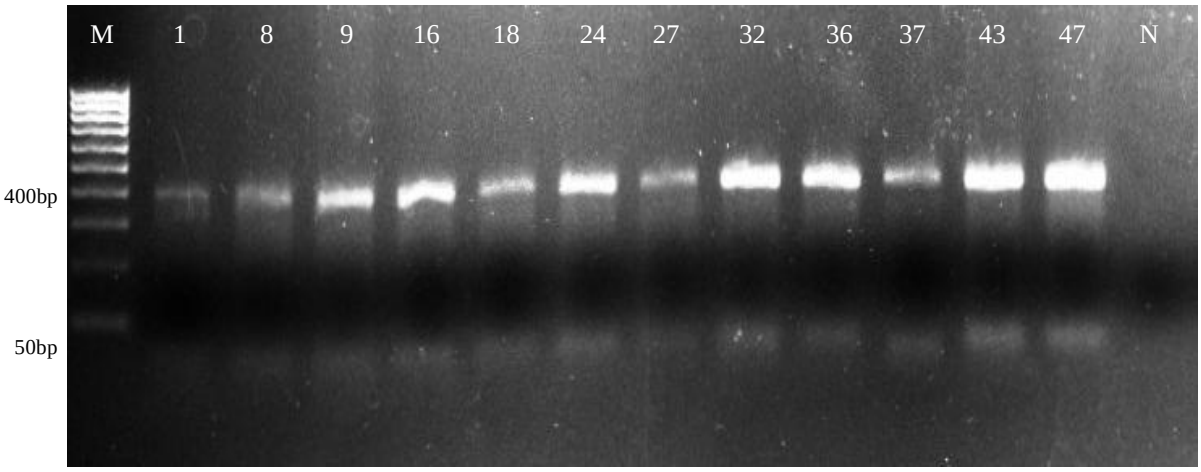

Fig III.13. Agarose gel electrophoresis of the *cox*1 gene PCR product after digestion with HinfI. Isolates: 1, 8, 9, 16, 18, 24, 27, 32, 36, 37, 43 and 47. M- 100 bp molecular weight marker. N- Negative control.

The result of the digestion of the *cox*1 gene with HinfI enzyme was a first band, clearly visible, with approximately 400 bp and a second dimly visible band with less than 50 bp. The RFLP pattern was the same for all the analyzed isolates (Fig. III.13).

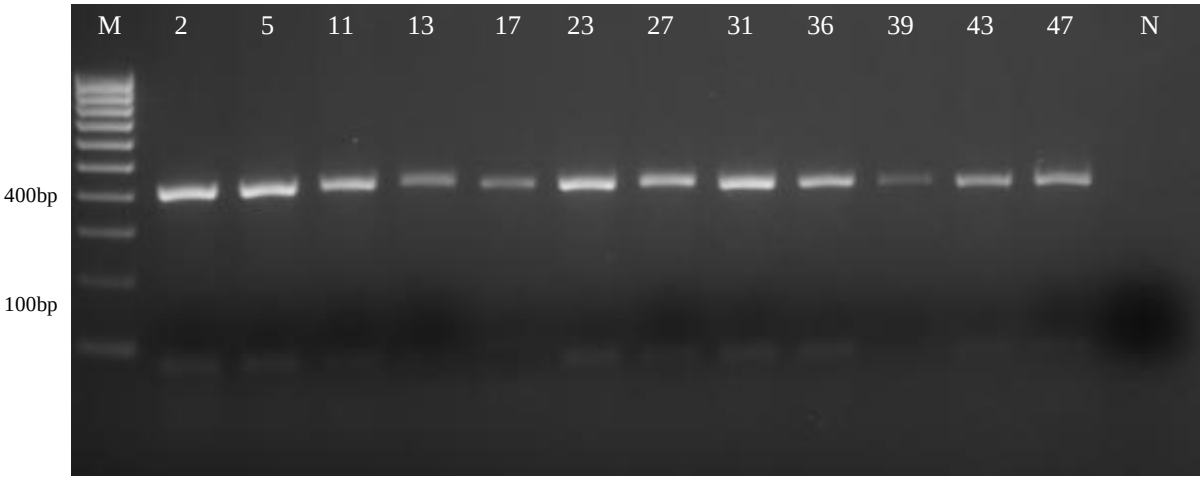

Fig III.14. Agarose gel electrophoresis of the c*ox*1 gene PCR product after digestion with AluI. Isolates: 2, 5, 11, 13, 17, 23, 27, 31, 36, 39, 43 and 47. M- 100 bp molecular weight marker. N- Negative control.

A segment of the *cox*1 gene was digested by the restriction endonuclease AluI and the result was a polymorphic pattern composed of a first, clearly visible, band with approximately 400 bp and a second dimly visible band with less than 100 bp. All the isolates presented identical RFLP profile (Fig. III.14).

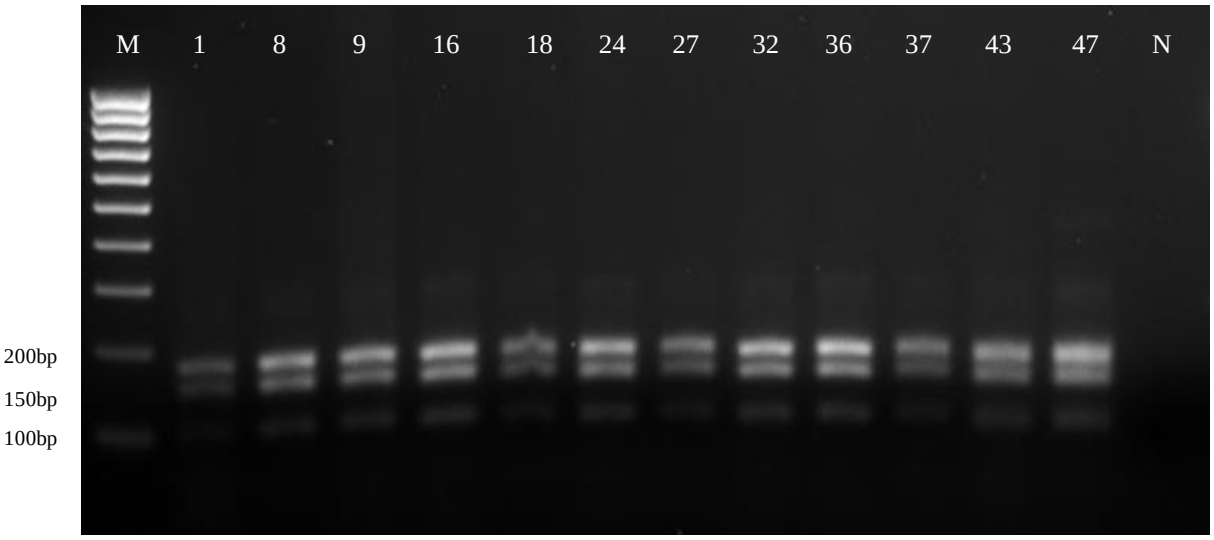

Fig III.15. Agarose gel electrophoresis of the *nad*1 gene PCR product after digestion with HinfI. Isolates: 1, 8, 9, 16, 18, 24, 27, 32, 36, 37, 43 and 47. M- 100 bp molecular weight marker. N- Negative control.

A segment of the *nad*1 gene was digested by the restriction endonuclease HinfI and the result was a polymorphic pattern composed of three bands with approximately 200, 150 and 100 bp.

Despite the differences in geographical and host origin, all the isolates presented the same RFLP pattern (Fig. III.15).

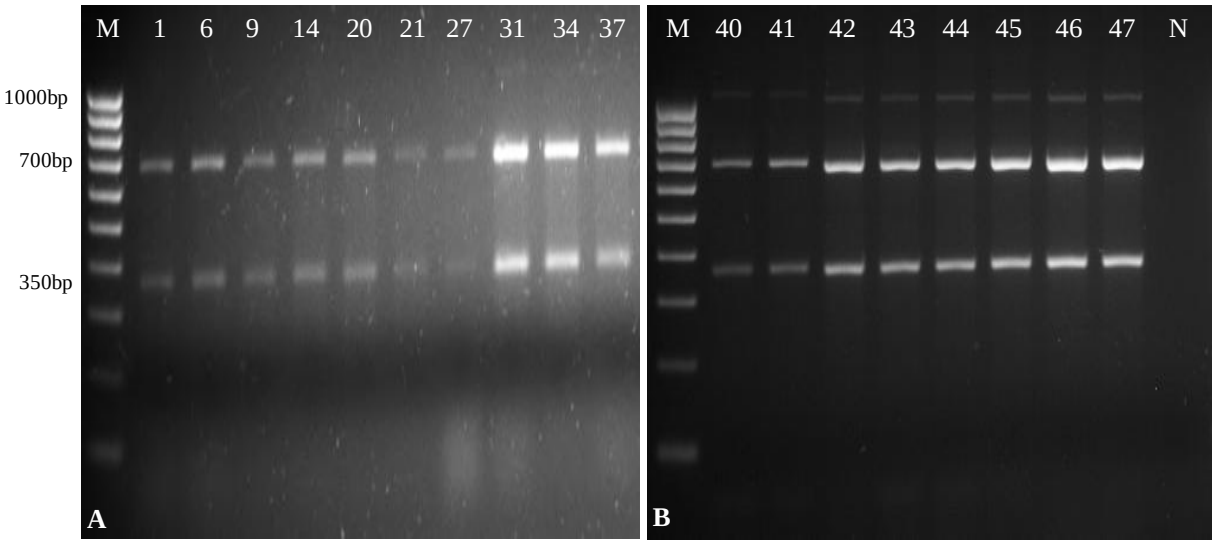

Fig III.16. Agarose gel electrophoresis of the ITS region PCR product after digestion with AluI. **A**: Isolates 1, 6, 9, 14, 20, 21, 27, 31, 34 and 37. **B**: Isolates 40 - 47. M- 100 bp molecular weight marker. N- Negative control.

The RFLP pattern produced after the digestion of the ITS region with AluI enzyme is composed of two bands with sizes around 700 and 350 bp for all isolates (Fig. III.16).

Additionally, sheep isolates (Fig. III.16.B) presented a third dimly band with approximately 1000 bp.

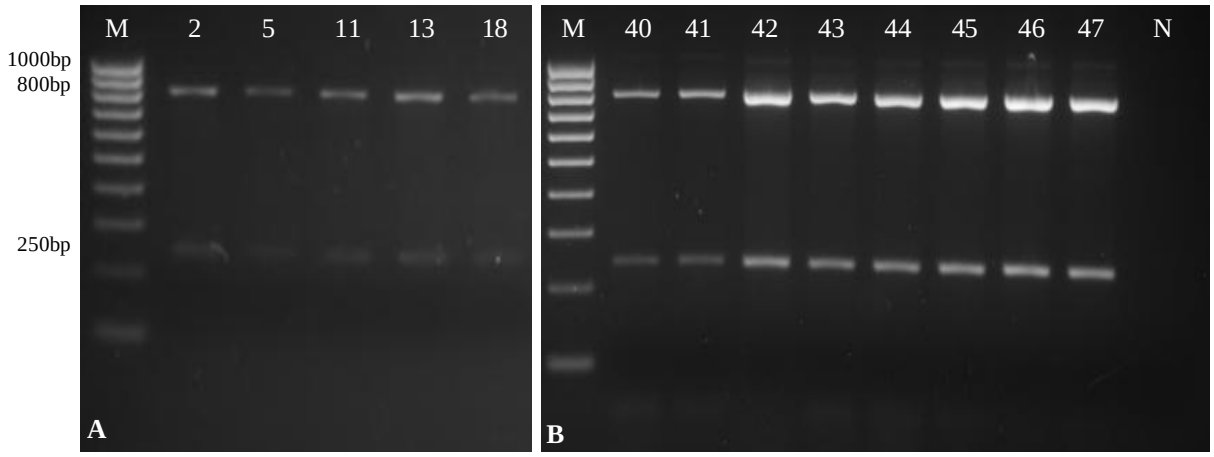

Fig III.17. Agarose gel electrophoresis of the ITS region PCR product after digestion with HinfI. **A**: Isolates 2, 5, 11, 13 and 18. **B**: Isolates 40 - 47. M- 100 bp molecular weight marker, N- Negative control.

A segment of the ITS region was digested by the restriction endonuclease HinfI and the result for all isolates was a polymorphic pattern composed of two bands with approximately 800 and 250 bp.

Additionally, sheep isolates (Fig.III.17.B) presented a third dimly band with approximately 1000 bp.

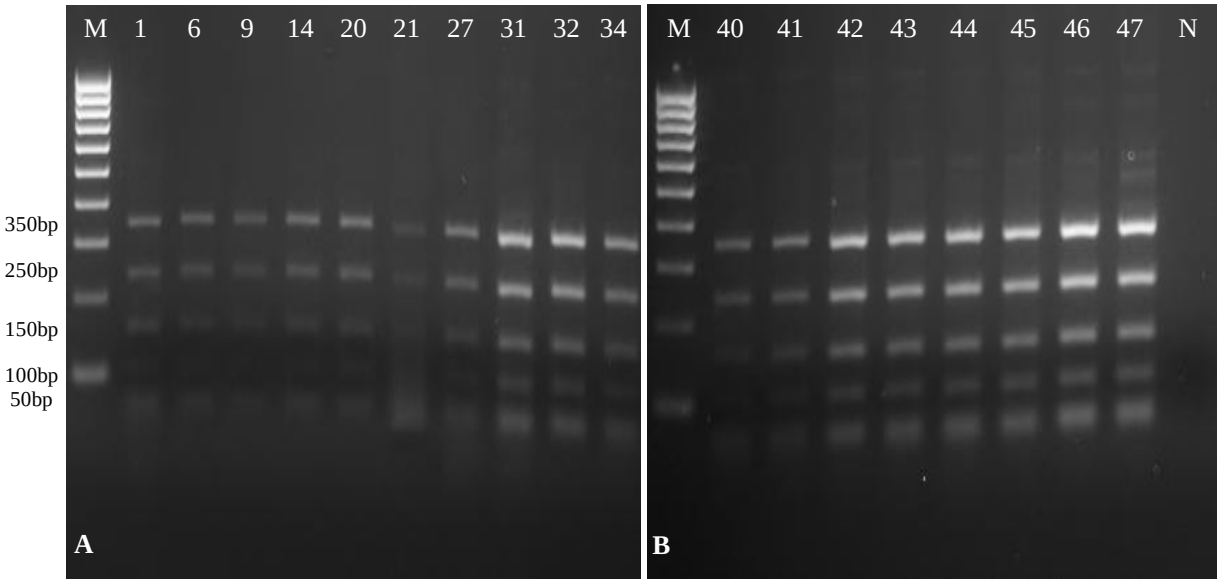

Fig III.18. Agarose gel electrophoresis of the ITS region PCR product after digestion with RsaI. **A**: Isolates 1, 6, 9, 14, 20, 21, 27, 31, 34 and 37. **B**: Isolates 40 - 47. M- 100 bp molecular weight marker. N- Negative control.

The RFLP pattern produced by the digestion of the ITS region with the RsaI enzyme is composed of 5 bands with sizes around 350, 250, 150, 100 and 50 bp.

Both cattle (Fig. III.17.A) and sheep isolates (Fig. III.17.B) displayed the same RFLP pattern.

Table III.2. Representative diagram of the PCR and RFLP patterns obtained for all *Fasciola hepatica* isolates before and after digestion of *cox*1, *nad*1 and the ITS region with endonucleases HinfI, AluI and RsaI.

	bp	No digestion	HinfI	AluI	RsaI
*cox*1	1000 800 700 600 500 400 300 200 100 50		 	 	
*nad*1	1000 800 700 600 500 400 300 200 100 50		 		
ITS region	1000 800 700 600 500 400 300 200 100 50		 	 	

8. Purification of the PCR Products

The *cox*1 gene PCR product from isolates 1, 3, 6, 8, 10, 12, 14, 15, 19, 20, 21, 22, 26, 28, 29, 32, 33, 35, 37, 38, 42 and 46, the *nad*1 gene PCR product from isolates 4, 6, 12, 13, 17, 21, 26, 30, 35, 38, 42 and 44 and the ITS region PCR product from isolates 7, 12 and 25 were successfully purified (Fig. III.19).

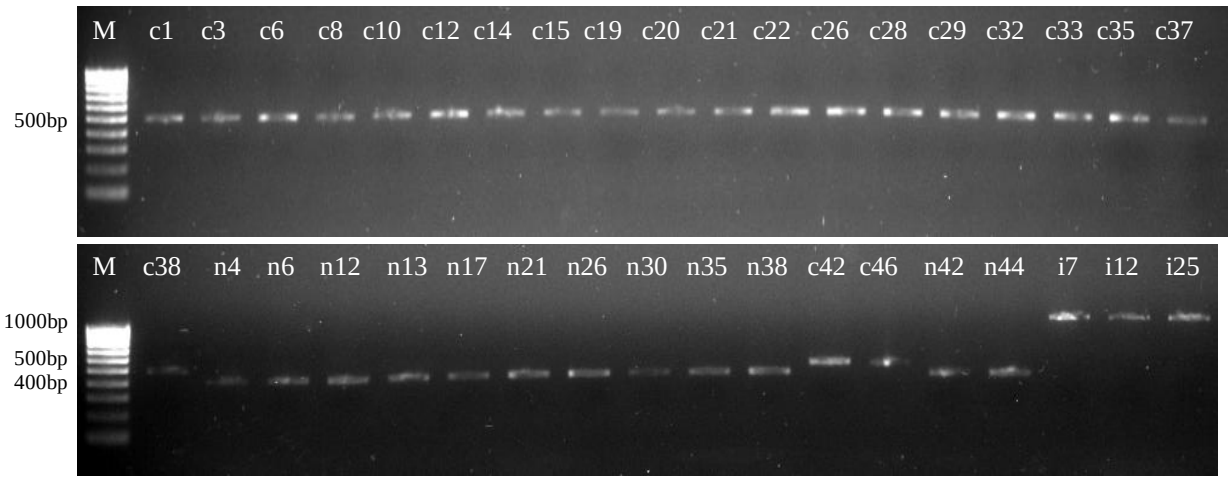

Fig III.19. Agarose gel electrophoresis of the ITS region, *cox*1 gene and *nad*1 gene PCR product after purification. **A**: Isolates 1, 3, 6, 8, 10, 12, 14, 15, 19, 20, 21, 22, 26, 28, 29, 32, 33, 35 and 37 *cox*1 gene PCR products. **B**: Isolates 38, 42 and 46 *cox*1 gene PCR products, isolates 4, 6, 12, 13, 17, 21, 26, 30, 35, 38, 42 and 44 *nad*1 gene PCR product and isolates 7, 12 and 25 ITS region PCR products. M- 100 bp molecular weight marker.

9. Sequencing

The sence strands of all the purified PCR products were successfully sequenced resulting in a 461 bp fragment for *cox*1 gene, a 379 bp fragment for *nad*1 gene and a 992 bp fragment for the ITS region.

10. Analysis of Nucleotide Sequence Data

The nucleotide sequence data was processed with MEGA™ 5.05 and DNA Sequence Polymorphism ™ 5.10.01 softwares.

The sequences were aligned and the undefined nucleotides were deleted. Therefore, we ended up with a 440 bp fragment for *cox*1 gene, a 374 bp fragment for *nad*1 gene and a 972 bp fragment for the ITS region.

For the *cox*1 gene, the average nucleotide frequencies were 16.67% (A), 48.00% (T), 24.96% (C), and 10.37% (G). The analysis of the nucleotide sequences of the *nad*1 gene presented nucleotide frequencies of 16.04% (A), 49.06% (T), 30.01% (C), and 4.88% (G). Finally, the ITS region had nucleotide frequencies of 20.40% (A), 28.60% (T), 26.10% (C), and 24.90% (G).

We found 11 polymorphic sites (at nucleotides 28, 30, 64, 114, 195, 282, 309, 405, 413, 438 and 439) in the *cox*1 gene fragment, 5 polymorphic sites (at nucleotides 1, 114, 232, 282 and 338) in the *nad*1 gene fragment and 13 polymorphic sites (at nucleotides 6, 7, 8, 843, 945, 946, 947, 948, 949, 952, 956, 962 and 963) in the ITS region fragment. For each different sequence a different haplotype was attributed (Table III.3 - III.5).

Table III.3. Comparison of the *cox*1 gene sequences of *Fasciola hepatica* isolates from different hosts and geographical regions in Portugal and respective haplotypes.

	Variable sites in the *cox*1 gene sequence													
Haplotype	**28**	**30**	**64**	**114**	**195**	**282**	**309**	**405**	**413**	**438**	**439**	Region	Host	Isolate
Hap_1	T	A	C	T	A	G	G	G	G	G	T	Faro	Cattle	37
Hap_1	T	A	C	T	A	G	G	G	G	G	T	Faro	Cattle	38
Hap_2	T	A	A	T	A	G	G	G	G	G	T	Castelo Branco	Cattle	19
Hap_2	T	A	A	T	A	G	G	G	G	G	T	Castelo Branco	Cattle	20
Hap_3	T	A	C	T	A	G	G	G	G	T	G	Setúbal	Cattle	12
Hap_4	C	A	C	T	G	G	G	A	A	G	T	Leiria	Cattle	8
Hap_5	C	A	C	C	G	G	G	A	G	G	T	Évora	Cattle	29
Hap_6	C	A	C	T	G	G	G	A	G	G	T	Lisboa	Cattle	3
Hap_6	C	A	C	T	G	G	G	A	G	G	T	Beja	Cattle	26
Hap_6	C	A	C	T	G	G	G	A	G	G	T	Portalegre	Cattle	35
Hap_6	C	A	C	T	G	G	G	A	G	G	T	Leiria	Sheep	42
Hap_6	C	A	C	T	G	G	G	A	G	G	T	Santarém	Sheep	47
Hap_6	C	A	C	T	G	G	G	A	G	G	T	Beja	Cattle	28
Hap_6	C	A	C	T	G	G	G	A	G	G	T	Évora	Cattle	32
Hap_7	C	G	C	T	G	G	G	A	G	G	T	Leiria	Cattle	6
Hap_8	C	A	C	T	A	G	G	A	G	G	T	Coimbra	Cattle	21
Hap_8	C	A	C	T	A	G	G	A	G	G	T	Coimbra	Cattle	22
Hap_9	C	A	A	T	G	G	G	A	G	G	T	Santarém	Cattle	14
Hap_9	C	A	A	T	G	G	G	A	G	G	T	Santarém	Cattle	15
Hap_10	C	A	C	T	G	A	G	A	G	G	T	Portalegre	Cattle	33
Hap_11	C	A	C	T	G	G	A	A	G	G	T	Setúbal	Cattle	10
Hap_12	C	A	C	T	G	G	G	A	G	T	G	Lisboa	Cattle	1

Table III.4. Comparison of the *nad*1 gene sequences of *Fasciola hepatica* isolates from different hosts and geographical regions in Portugal and respective haplotypes.

Variable sites in the *nad*1 gene sequence								
Haplotype	**1**	**114**	**232**	**282**	**338**	Region	Host	Isolate
Hap_A	G	T	T	C	C	Setúbal	Cattle	12
Hap_A	G	T	T	C	C	Faro	Cattle	38
Hap_A	G	T	T	C	C	Castelo Branco	Cattle	17
Hap_B	C	T	T	T	C	Leiria	Cattle	6
Hap_B	C	T	T	T	C	Lisboa	Cattle	4
Hap_B	C	T	T	T	C	Évora	Cattle	30
Hap_B	C	T	T	T	C	Santarém	Cattle	13
Hap_B	C	T	T	T	C	Coimbra	Cattle	21
Hap_C	C	T	T	T	T	Leiria	Sheep	42
Hap_D	C	C	C	T	C	Beja	Cattle	26
Hap_E	C	T	C	T	C	Santarém	Sheep	44
Hap_E	C	T	C	T	C	Portalegre	Cattle	35

Table III.5. Comparison of the ITS region sequences of *Fasciola hepatica* isolates from different hosts and geographical regions in Portugal and respective haplotypes.

Variable sites in the ITS region sequence															
Haplotype	**6**	**7**	**8**	**843**	**945**	**946**	**947**	**948**	**949**	**952**	**956**	**962**	**963**	Region	Host	Isolate
Hap_α	A	T	G	A	C	T	G	A	C	T	A	A	T	Setúbal	Cattle	12
Hap_β	T	G	T	C	C	T	G	A	C	T	G	A	T	Leiria	Cattle	7
Hap_γ	T	G	A	G	T	G	A	C	T	G	G	G	A	Beja	Cattle	25

The haplotype geographical distributions can be observed in figure III.20.

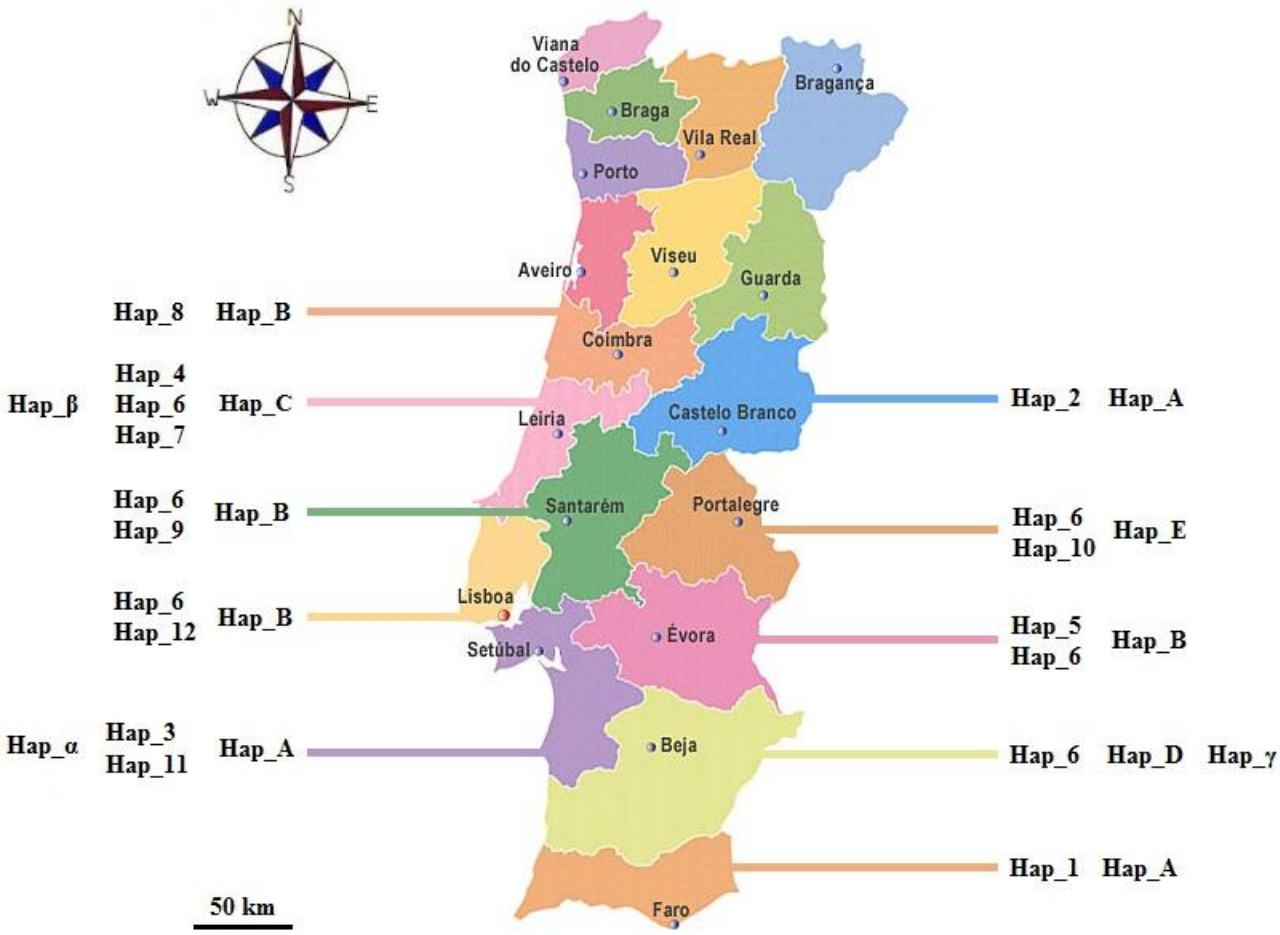

Fig III.20. Map of Portugal with correspondence between regions and haplotypes from *cox*1 gene (Hap_1 - Hap_12), *nad*1 gene (Hap_A - Hap_E) and ITS region (Hap_α - Hap_γ). Figure by Raquel Santos.

Pairwise distances between all isolates were calculated with MEGA™ accordingly to Kimura's 2 parameter model. For the *cox*1 gene, the values ranged between 0.000 and 0.014 and the overall average was 0.005. For the *nad*1 gene, the values ranged between 0.000 and 0.011 and the overall average was 0.004. Finally, for the ITS region the values ranged between 0.005 and 0.013 and the overall average was 0.010.

DNA Sequence Polymorphism ™ 5.10.01 software was used to study the genetic variability of *F. hepatica* isolates from different hosts and regions of Portugal.

The sequencing data of *cox*1 gene, *nad*1 gene and the ITS region were analyzed in order to infer the average number of nucleotide differences (K), the nucleotide diversity (π), the number of existent haplotypes (H) and the haplotype diversity (Hd). N corresponds to the number of isolates examined for each genomic region (Table III.6).

Table III.6. Number of analyzed isolates (N), average number of nucleotide differences (K), nucleotide diversity (π), number of existent haplotypes (H) and haplotype diversity (Hd) for *cox*1 gene, *nad*1 gene and ITS region.

	N	K	π	H	Hd
*cox*1	22	2.30303	0.00523	12	0.8918
*nad*1	12	1.56061	0.00417	5	0.7879
ITS	3	9.33333	0.00960	3	1.0000

The *cox*1 gene, the *nad*1 gene and the ITS region presented 12, 5 and 3 different haplotypes, respectively. The haplotype frequencies are described on Table III.7.

Table III.7. The *cox*1 gene, the *nad*1 gene and theITS region haplotype frequencies.

*cox*1		*nad*1		ITS	
Hap_1	9.2 %	Hap_A	25 %	Hap_α	33.3 %
Hap_2	9.2 %	Hap_B	41.7 %	Hap_β	33.3 %
Hap_3	4.5 %	Hap_C	8.3 %	Hap_γ	33.3 %
Hap_4	4.5 %	Hap_D	8.3 %		
Hap_5	4.5 %	Hap_E	16.7 %		
Hap_6	31.8 %				
Hap_7	4.5 %				
Hap_8	9.2 %				
Hap_9	9.2 %				
Hap_10	4.5 %				
Hap_11	4.5 %				
Hap_12	4.5 %				

11. Phylogenetic Analysis

MEGA ™ 5.10.01 software was used to study the phylogenetic relation between Portuguese *F. hepatica* sequences and published sequences of isolates from all over the world. *Fasciola gigantica* was used as an out-group.

Using partial sequences of *cox*1 gene, *nad*1 gene and the ITS region, three phylogenetic trees were built (Fig. III.21-III.23). Genetic distances were calculated from multiple sequence alignments with the neighbor-joining distance-matrix method and the Kimura's 2 parameter model. Values below the branches represent the bootstrap confidence intervals based on 1000 replications.

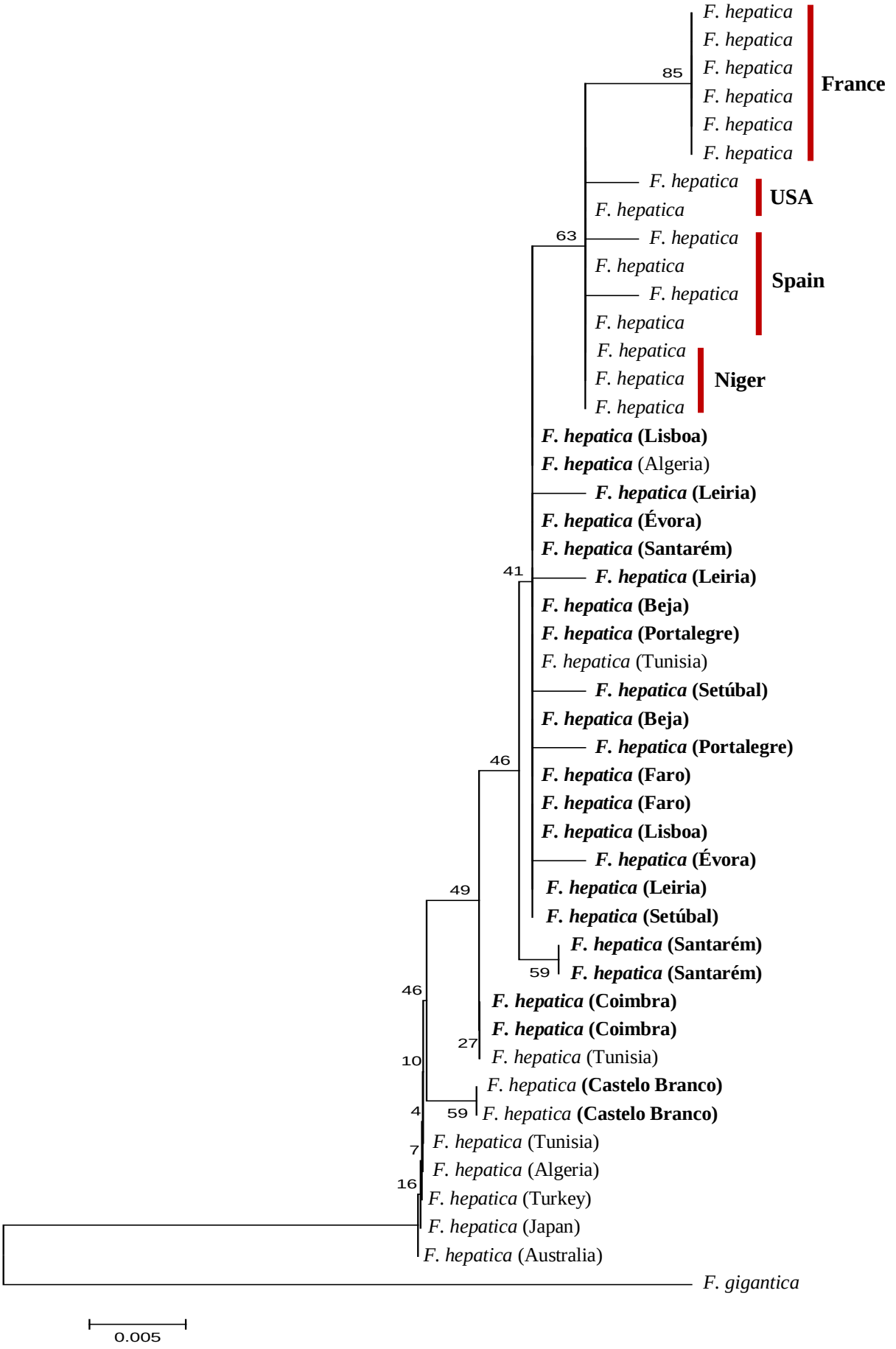

Fig III.21. *cox*1 gene phylogenetic tree representing the relationships among *Fasciola hepatica* isolates from differente geographical locations.

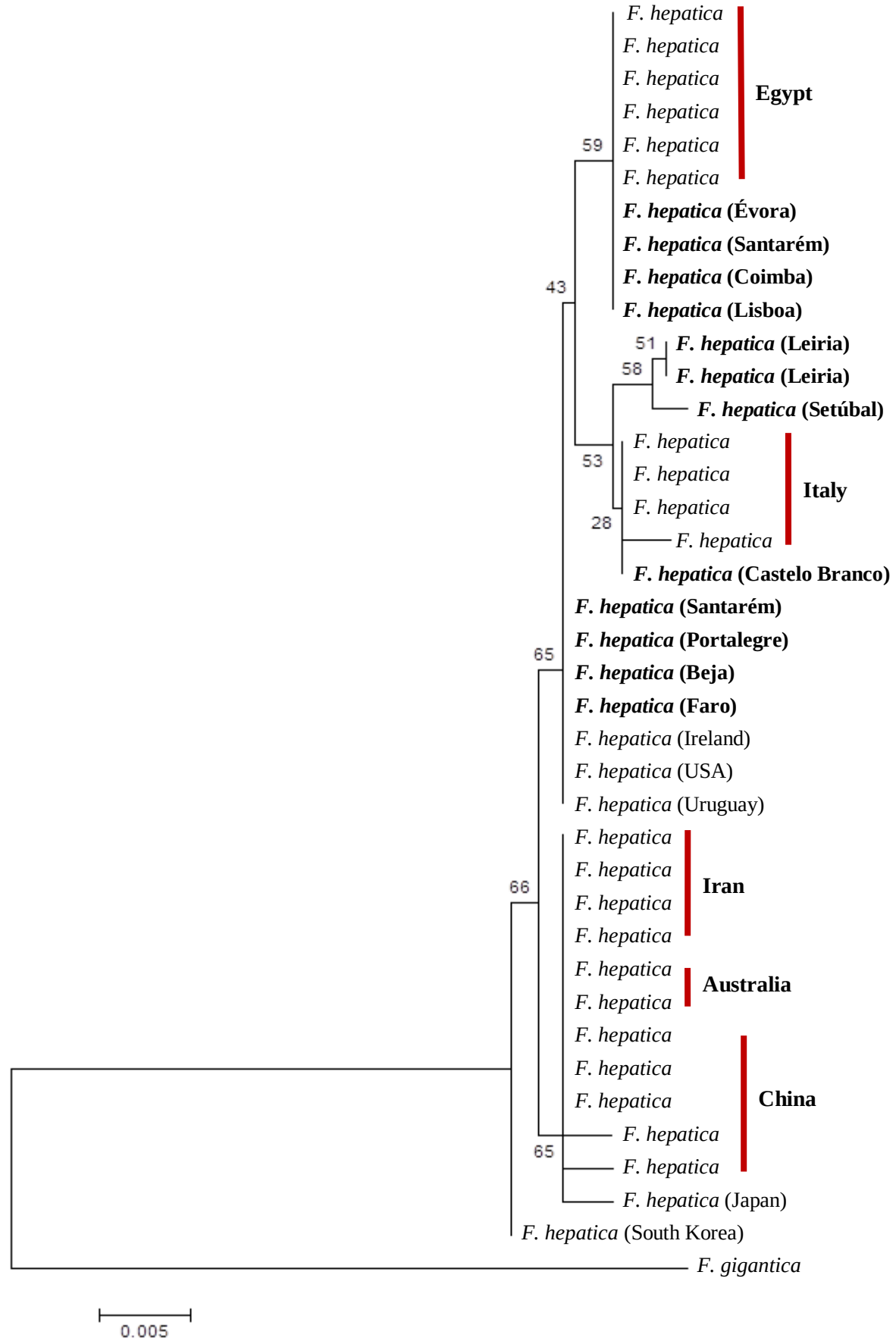

Fig III.22. n*ad*1 gene phylogenetic tree representing the relationships among *Fasciola hepatica* isolates from different geographical locations.

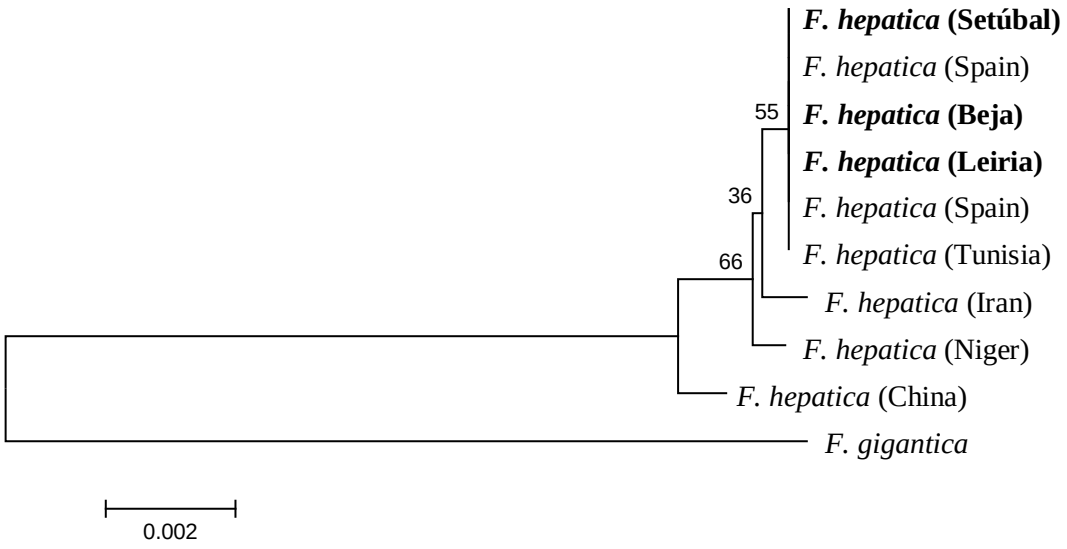

Fig III.23. The ITS region phylogenetic tree representing the relationships among *Fasciola hepatica* isolates from different geographical locations.

The phylogenetic tree of *cox*1 gene (Fig. III.21) presented several branches supported by medium bootstrap values. The genetic distance between the analyzed isolates from different countries is quite small. The constructed phylogenetic tree revealed a very close relationship between Portuguese *F. hepatica* isolates and isolates from Algeria and Tunisia. Curiously, France, the USA, Spain and Niger isolates are all grouped in the same sub-branch. Japan and Australia isolates present a low similarity with Portuguese isolates.

The phylogenetic tree of *nad*1 gene (Fig. III.22) presented a topology very similar to that of *cox*1 gene, with several branches and medium bootstrap values. Portuguese *F. hepatica* isolates are dispersed in several sub-branches with low genetic distances between them. Interestingly, isolates from Évora, Santarém, Coimbra and Lisboa are located in a single sub-branch, with a bootstrap of 59%, only with Egyptian isolates, revealing their high genetic similarity despite the geographic distance. Isolates from Leiria, Setúbal and Castelo Branco also share a sub-branch, with a bootstrap of 53%, only with the Italian isolates. The rest of the Portuguese isolates are located near the samples from Ireland, the USA and Uruguay. Isolates from Iran, China, Australia, Japan and South Korea constitute a different sub-branch which is distant from Portuguese isolates.

The phylogenetic tree of the ITS region revealed a very low genetic distance between the Mediterranean isolates since Portuguese isolates are all in the same sub branch together with isolates from Spain and Tunisia. Isolates from Iran, Niger and China constitute the other sub-branches.

Chapter IV. DISCUSSION

Chapter IV. DISCUSSION

Studies on genetic characterization of *Fasciola hepatica* populations have important implications for epidemiology, control and diagnosis of fascioliasis (Mas-Coma *et al*., 2005). Different molecular techniques and DNA markers have been applied to fasciolid flukes (Ai *et al*., 2011). However, there have been no data regarding genetic characterization of *F. hepatica* in Portugal. In the present study we aimed to provide the first insights into the genetic variability of *F. hepatica* in Portugal and for that we analyzed 47 isolates from two different hosts (cattle and sheep) and geographical locations (Beja, Castelo Branco, Coimbra, Évora, Faro, Leiria, Lisboa, Portalegre, Santarém and Setúbal). The molecular methods chosen were Random Amplified Polymorphic DNA-Polymerase Chain Reaction (RAPD-PCR), Restriction Fragment Length Polymorphism (RFLP) and sequencing of NADH dehydrogenase subunit 1 (*nad*1) gene, cytochrome c oxidase subunit 1 (*cox*1) gene and Internal Transcribed Spacers (ITS) region.

Sample Collection: From a total of 68 flukes recovered autopsy, 83.8% (57) were originated from cattle and 16.2% (11) from sheep. 20 infected cattle and 3 infected sheep contributed to the sample collection. These results are in contrast with the idea that fascioliasis is more often seen in sheep than cattle (Robinson and Dalton, 2009) but can be explained by the fact that the slaughterhouses that we visited in order to collect the infected livers and gall bladders usually receive more cattle than sheep.

Random Amplified Polymorphic DNA: Fifteen oligonucleotides of varying G + C content (50 - 81.8%) were used as arbitrary primers in RAPD assays to discern the variability, if any, between the *F. hepatica* isolates. Gunasekar *et al*. (2008) designed the AP set of random primers and used them in order to elucidate the genetic variability among isolates of *F. gigantica* from cattle, buffalo and goat. Accordingly to Aldemir (2006), the OSA set of primers were able to distinguish between different host origins (cattle or sheep) of *F. hepatica* isolates, *i. e.*, different RAPD-PCR patterns were obtained from parasites of these two hosts. Out of the 15 random primers used in the RAPD-PCR assays, only five (AP2, AP9, OSA9, OSA10 and OSA11) were able to direct the amplification of the DNA fragments from the genome of *F. hepatica*. It is interesting to note that all these five primers have a G + C content of 50 and 70%. Generally, a higher G + C content of a primer is known to be associated with increased primer stability and generation of multiple DNA fragments in RAPD assays (MacPherson and Gajadhar, 1993). However, contrarily to what was expected, the primers with higher G + C content (80 – 81.8%) were unable to amplify any DNA fragment. Primers AP9 and OSA10 produced a monomorphic DNA fragment and primer AP2 produced no defined DNA bands, instead, a smear was seen along the lanes. Therefore, these three random primers are not appropriate to differentiate *F. hepatica* isolates. In terms of polymorphic DNA fragments generated, out of a total of

15 primers, only 2 (OSA9 and OSA11) were potentially informative. However, all the lanes produced the same RAPD-PCR pattern, meaning that no genetic differences were evident between the cattle and sheep isolates from different regions of Portugal.

Another feature of the RAPD-PCR profiles generated with these primers was the variation of the band intensity. In some assays, one band was amplified more efficiently than the other in the same reaction. For example, OPA 9 and OPA11 were found to produce high intense bands of 80 and 180 bp, respectively, which were more pronounced than the other co-amplified DNA fragment. The more intense bands were probably due to priming with repeated regions of the *F. hepatica* genome, which could result in more copies being produced during PCR. Initial experiments to optimize the RAPD reaction conditions for *F. hepatica* templates involved the empirical variation of annealing temperature and DNA, primer, $MgCl_2$ and Taq DNA Polymerase concentrations. We made several duplicates, all with different conditions, however, no improvements were seen in the RAPD-PCR patterns produced by the majority of primers. This is in agreement with Kantanen *et al*. (1995) who found that some primers, due to their random nature, fail to amplify, while others produce too complex banding patterns. It also falls in line with the works of Hadrys *et al*. (1992) and Bardakei and Skibinski (1994) who concluded that the choice of a primer is of major importance towards the discriminating power of the technique. Therefore, the best strategy when using RAPD assays may be to screen as many primers as possible and to select only those that give highly reproducible bands rather trying to optimize every primer template combination.

Unfortunately, from all the tested primers only 2 (OSA9 and OSA11) were adequate for the study of genetic variability among *F. hepatica* isolates from Portugal.

PCR Amplifications: For both *cox*1 gene, *nad*1 gene and the ITS region all isolates presented DNA fragments with similar sizes. Concerning PCR amplification of these genomic regions, no differences were observed between isolates from different host and geographical origin.

Restriction Fragment Length Polymorphism: Three restriction endonucleases, AluI, HinfI and RsaI, were used in the RFLP assays. Clone Manager™, version 7.11, (Scientific & Educational Software, Cary, USA) was the software chosen to determine the expected sizes of the digestion fragments.

The *cox*1 nucleotide sequence was analyzed with Clone Manager™ and presented two recognition sites for HinfI on nucleotides 36 and 52. Therefore, in the agarose gel electrophoresis of the *cox*1 gene PCR product after digestion with HinfI we were expecting DNA bands with 16, 36 and 409 bp. However, in all lanes only two bands were visible with approximately 400 and 50 bp. It is probable that the 16 and the 36 bp bands were too small and the 2.5% agarose gel was unable to retain these fragments. The 50 bp band may be the consequence of an incomplete digestion on nucleotide 36. For AluI, Clone Manager™ identified 1 recognition site on nucleotide 84. We were them expecting two DNA fragments with 84 and 377 bp and, actually, two bands with approximately 50 and 400 bp

were observed. No RsaI recognition site was identified in the *cox*1 sequence and that is why we did not use this enzyme in any RFLP assay.

When we analyzed the *nad*1 nucleotide sequence with Clone Manager^{TM}, from the above three enzymes, only HinfI had restriction sites (nucleotides 104 and 203). Accordingly to the software used, we were expecting three DNA fragments with 99, 104 and 176 bp. In the agarose gel we observed bands with approximately 100, 150 and 200 bp. The 200 bp band may be the result of an incomplete digestion on nucleotide 104 and, as a consequence, the 99 and the 104 bp fragments remained coupled.

The ITS region, contrarily to the mitochondrial genes, presented restriction sites for all the restriction enzymes used. AluI and HinfI had only one restriction site, on nucleotides 630 and 746, respectively. For this restriction endonuclease the RFLP assay was perfect since the agarose gel displayed two bands with approximately 350 and 650 bp in AluI assay and 250 and 750 bp in HinfI assay. Curiously, in the sheep isolate lanes, a third dimly visible band of approximately 1000 bp was present. Probably these samples were more concentrated than the cattle isolate samples and this heavier band corresponds to a fraction of the PCR product from the amplification of the ITS region that was not digested. For RsaI, Clone Manager^{TM} indicated 6 restriction sites on nucleotide 194, 298, 366, 425, 771 and 925. Consequently, DNA fragments with 59, 67, 68, 104, 154, 194 and 346 bp were expected. In the agarose gel bands of approximately 350, 250, 150, 100 and 50 bp were visible. The 250 bp band may be the result of some incomplete digestions on nucleotides 298 and 366, which would also explain why the 100 bp and the 50 bp bands are so dim.

For all of the mentioned RFLP assays, the sum of the sizes of the restriction fragments equaled that of the PCR product, thus verifying its purity. Isolates of *F. hepatica* from different animal hosts and regions showed a uniform RFLP pattern indicating a highly conserved nature of both mitochondrial genes *cox*1 and *nad*1 and rDNA internal transcribed spacers.

Analysis of nucleotide sequence data: Young *et al*. (2010) defended that the average G + C content of *F. hepatica* coding sequences should be around 47.0%. However, when the *cox*1 and *nad*1 genes of Portuguese *F. hepatica* isolates were sequenced, the average G + C content were 35.33 and 34.89%, respectively. In the other hand, the rDNA internal transcribed spacers, which are not a coding region, presented an average G + C content of 51.0%. These are very interesting values, but for an accurate explanation we would need a complete genome sequencing of the studies isolates.

Only eleven (2.5%) *cox*1 sites, five (1.3%) *nad*1 sites and thirteen (1.3%) ITS region sites were variable among isolates. These low values prove the high genetic similarity between *F. hepatica* isolates from different animal hosts and regions observed in the RFLP experiments.

Pairwise distances ranged between 0.000 and 0.014, and the overall average was 0.005, 0.004 and 0.010 for *cox*1, *nad*1 and ITS region, respectively. Pairwise values can range from 0 (perfect similarity between samples) to 1 (no similarity between samples) (Tajima, 1983). The low pairwise values obtained for Portuguese *F. hepatica* isolates confirmed their low genetic variability.

K is defined as the average number of nucleotide differences between any two DNA sequences chosen randomly from the sample population (Tajima, 1983). Nucleotide diversity (π) illustrates the average number of nucleotide differences *per* site between any two DNA sequences chosen randomly from the sample population and describes the probability that two homologous nucleotides drawn uniformly at random from the sample population are not the same (Nei and Jin, 1989). H is defined as the number of different haplotypes present in the sample population and Haplotype diversity (Hd) describes the probability that two haplotypes drawn uniformly at random from the sample population are not the same (Nei and Li, 1979; Nei and Tajima, 1981). The haplotype diversity value (Hd) ranged between 0.7879 (*nad*1) and 1.0000 (ITS). The lowest (0.00417) and highest (0.00960) values of nucleotide diversity (π) were also detected in these genomic regions. This is in agreement with the general idea that coding sequences, like *nad*1 and *cox*1 are more conserved than non-coding sequences like the ITS region (Morgan and Blair, 1998). The low intraspecific variation observed in the pooled samples is in accordance with several other studies in which *F. hepatica* populations demonstrated very little or no intraspecific variation in mitochondrial genes *nad*1 and *cox*1 and in the ribosomal ITS region (Mas-Coma *et al*., 2005). A low ($0 < \pi < 0.005$) nucleotide diversity in the *cox*1 and *nad*1 genes has also been reported in trematodes *Schistosoma japonicum*, *S. mansoni* (Rollinson *et al*., 1997) and *Echinostoma caproni* (Morgan and Blair, 1998).

Out of the 11 mutations detected on *cox*1 gene, 4 nucleotide substitutions, at positions 64, 413, 438 and 439, led to amino acid change. All the other mutations were synonymous changes. The *nad*1 gene presented 3 synonymous changes and 2 non-synonymous, at positions 1 and 338. Interestingly, these non-synonymous substitutions were rather common, occurring in half of the studied isolates. The frequency of replacement changes was so high that we believe that despite the amino acid change, the protein function was not affected (Gaucher *et al*., 2002).

Although only 37 sequences were examinated, the frequency distributions of all the haplotypes attest to the genetic diversity of *F. hepatica*. For the mitochondrial genes, a main haplotype was observed: Hap_6 (31.8%) for *cox*1 gene and Hap_B (41.7%) for *nad*1 gene. The other haplotypes varied in frequency from 4.5 to 9.1% for *cox*1 gene and 8.3 to 25% for *nad*1 gene. For the ITS region we were only able to sequence 3 isolates and the result was 3 different haplotypes. However, the examined sample is so small (n = 3) that we cannot assume this haplotype frequency (33%) as a true degree of diversity.

Interestingly, some isolates from the same region as well as some isolates from adjacent regions presented the same haplotype, revealing its genetic similarity. In the other hand, some isolates from adjacent regions presented different haplotypes and isolates from distant regions presented the same. Hence, there was no obvious correlation between the haplotype and the geographical location.

Concerning the host origin, curiously, for the same region cattle and sheep isolates presented different haplotypes. This is a very interesting fact that can be the result of different mechanisms and capacities of adaptation to different hosts. However, the level of nucleotide variance between sheep

and cattle isolates is so low that we cannot infer a direct correlation between the haplotype and the host origin of the isolates. For a more accurate conclusion we would need to collect and sequence a larger number of sheep isolates.

The haplotype frequencies have a leptokurtic distribution (a few very frequent haplotypes and many rare haplotypes), and thus, they are particularly sensitive to errors arising from a limited sample size. The results from another study in Ireland provided evidence that the level of fluke diversity seen in the Portuguese isolates was typical of fluke populations (Walker *et al*., 2007).

To sum up, our analysis of nucleotide sequence data of *F. hepatica* populations revealed low levels of genetic variability either between hosts or geographic locations.

Phylogenetic analysis: For an accurate phylogenetic analysis of *F. hepatica* isolates, we decided to compare the Portuguese sequences with published sequences from different regions of the world. *Fasciola gigantica* was the species that was chosen to assume the role of the out-group. The phylogenetic trees of *cox*1, *nad*1 and the ITS region confirmed our previous results of a low genetic distance between Portuguese isolates

Bootstrap confidence intervals represent the percentage of replicate trees in which the associated taxa clustered together in a bootstrap test with 1000 replications. Our results have medium bootstrap values, which is typical of a phylogenetic tree with high similarity within the analyzed samples (Felsenstein, 1985).

The phylogenetic tree of the *cox*1 gene presented all Portuguese *F. hepatica* isolates in the same sub-branch closely related to isolates from Tunisia and Algeria; the phylogenetic tree of the *nad*1 gene presented a sub branch with only isolates from Évora, Santarém, Coimbra, Lisboa and Egypt and a sub-branch constituted only by Italian and Portuguese (Leiria, Setúbal and Castelo Branco) isolates. Last but not least, the phylogenetic tree of the ITS region revealed a very low genetic distance between the Iberian and the Tunisian isolates, since they are all in the same sub branch. This result probably reflects a high gene flow between these countries, which can be a result of migration of the parasite's definite hosts. Besides affecting a wide range of wild host species, *F. hepatica* is a common parasite of livestock and the genetic structure of this parasite is apparently strongly influenced by the importing and exporting of farm animals over hundreds of years. Actually, livestock transportation seems to be the major factor determining the mixing of the parasite's populations (Semyenova *et al*., 2006).

Italy, Portugal and Spain (Southern Europe) and Tunisia, Algeria and Egypt (North Africa) are countries located in the Mediterranean coast, which led us to suppose that the import-export of livestock across the Mediterranean sea may be the leading cause for the high genetic proximity between isolates from these countries (Pica-Ciamarra and Morgan, 2011). This is a very important observation, especially for epidemiological control. We must heighten our attention to outbreaks of fascioliasis and cases of drug-resistance in these Mediterranean countries in order to prevent similar situations in Portugal. Also, we advise a higher control of livestock during import and export since sometimes the animals are infected but do not yet present any clinical signals.

Interestingly, isolates from Japan, China, Iran, Australia and South Korea were constantly located in branches that were not closely related with those of Portuguese isolates. These results make sense in light of the geographical distance and the low importing and exporting of livestock between these countries and Europe (Mas-Coma *et al.*, 2007).

Main Conclusions

Collectively, our results demonstrate that Portuguese *F. hepatica* isolates present very low levels of genetic variability. The geographical distribution of *F. hepatica* haplotypes indicated that sequence variation was not directly related to particular geographical origins of the sample and comparison between bovine and ovine strains revealed low or inexistent genetic polymorphism concerning the host origin.

Moreover, the phylogenetic trees of *cox*1, *nad*1 and the ITS region exhibit a consistent grouping of Portuguese isolates and a high genetic similarity between Portuguese isolates and isolates from other Mediterranean countries, such as Spain, Italy, Algeria, Tunisia and Egypt.

The genetic distances observed between Portuguese and world-wide isolates allowed us to conclude that there is no distinctive genetic structure of Portuguese isolates.

Ultimately, the low level of diversity in Portuguese isolates seen in this study combined with the population dynamics of parasite infections may result in a slow and infrequent selection of resistance to antihelminthics.

Future Perspectives

It is important to continue the genetic characterization of *F. hepatica* in Portugal. Our work focused on the genetic variability of cattle and ovine strains, and so it would be interesting to expand the project in order to include goat and pig strains. Additionally we should collect isolates from wild animals and study their migration routes in order to infer a correlation, if any, between their haplotype and geographical location.

In our work we detected a high similarity between Portuguese isolates and isolates from countries located in the Mediterranean coast. A more exhaustive analysis, including samples from more locations would be very useful to identify a pattern, if any, between the importing and exporting of livestock and their genetic variability.

Ultimately, we believe it is extremely important to carry on with the molecular investigation of fascioliasis in order to identify possible genetic markers of drug-resistance.

Chapter V. REFERENCES

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