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„Diversity of *Fascioloides magna* and *Fasciola hepatica* in Austria based on their genes and antigens"

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Statutory Declaration

I declare that I have authored this thesis independently, that I have not used other than the declared sources / resources, and that I have explicitly marked all material which has been quoted either literally or by content from the used sources.

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(Date) (Signature)

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1. Introduction

Human infestations with trematodes are an emerging problem (WHO, 1995; Keiser and Utzinger, 2005). The distribution of trematodes is facilitated when human faeces are used as fertilizers (Fried *et al.*, 2004). Among others, two, namely *Fascioloides magna* and *Fasciola hepatica*, have a great importance for the health of humans and animals and lead to a great economic loss annually (Robinson and Dalton, 2009; Králová-Hromadová *et al.*, 2011). The background of this is that *F. hepatica* has been endemic in Europe for thousands of years, while *F. magna* was introduced for the first time into Europe in the 19th century. The present thesis deals with the genetic diversity of *F. magna* and *F. hepatica* in Austria and with differences between the two species on the protein level.

1.1 Trematodes in general

1.1.1 Classification

The trematodes are a class of endoparasites and belong to the phylum Platyhelminthes. Altogether, they include 18,000 to 24,000 species and parasitise molluscs and vertebrates. The class is divided into two subclasses: Aspidogastrea (Faust and Tang, 1936) and Digenea (Van Beneden, 1858) (Fig. 1.1). The smaller group, Aspidogastrea, includes around 80 species and parasitise molluscs, fish, and turtles. Digenea represent the majority of trematodes with 8,000 species, and parasitise molluscs (intermediate hosts) and vertebrates (final hosts) (Lucius and Loos-Frank, 2008; Eckert *et al.*, 2008). To reach the adult state, most of the trematodes need several hosts and their life cycle includes alternately sexual and asexual reproduction (Keiser and Utzinger, 2009).

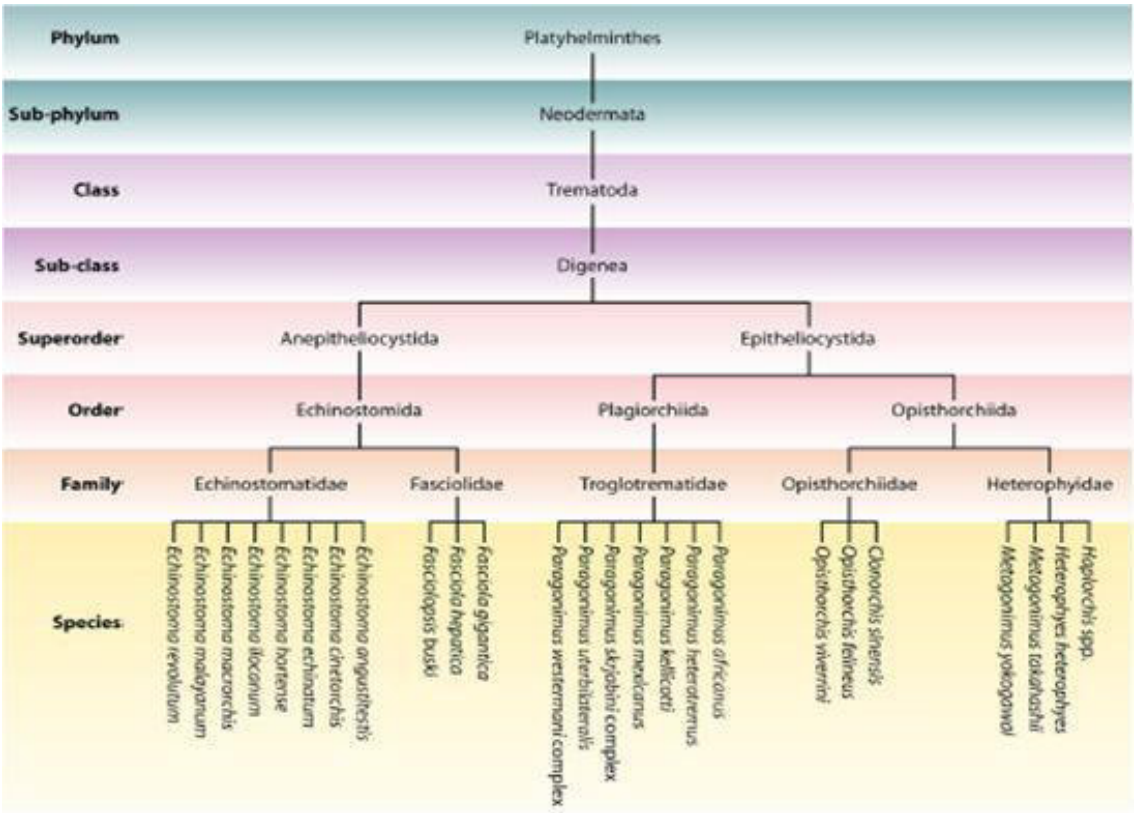

Fig. 1.1: Classification of the Platyhelminthes (from Keiser and Utzinger, 2009)**.** Both, *F. magna* and *F. hepatica* are members of the family of Fasciolidae.

1.1.2 Life cycle

When the final host excretes eggs via its faeces, and those eggs come in contact with water, miracidia hatch and infest the first intermediate host (e.g. an aquatic snail). They can localise the intermediate host through chemotaxis and chemokinesis (Keiser and Utzinger, 2009; Haider *et al.*, 2012). Several physical-chemical factors, such as temperature, humidity, light, and oxygen tension promote the hatching of the eggs (Vignoles *et al.*, 2014; Gold and Goldberg, 1976) (Fig. 1.2).

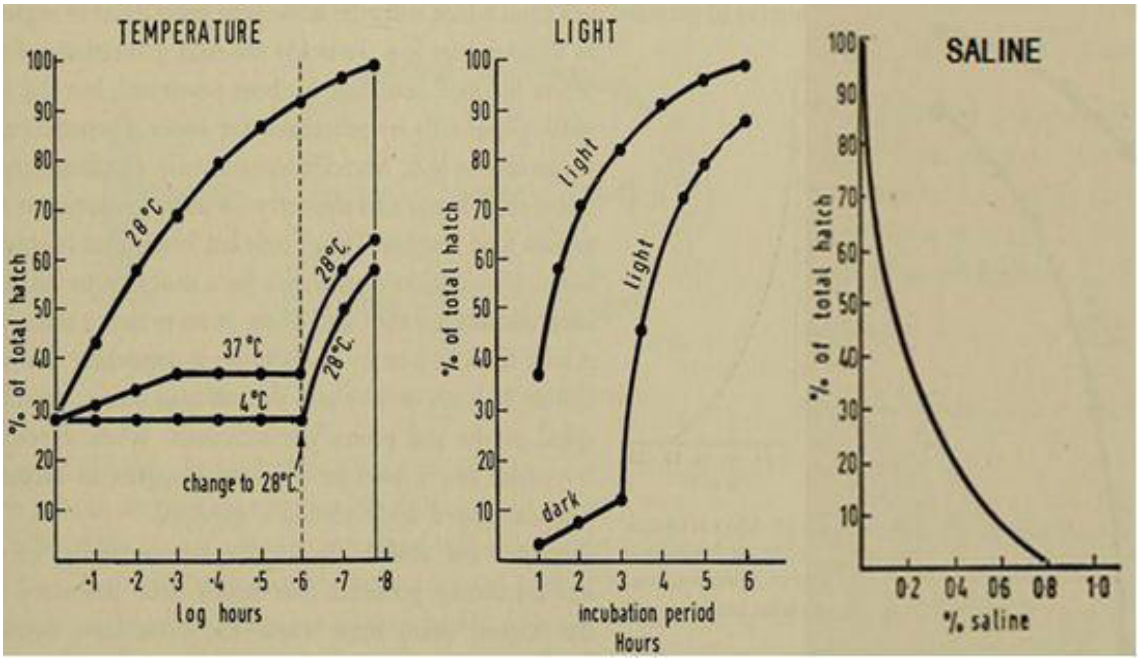

Fig. 1.2: The effect of temperature, light, and saline on hatching of trematode eggs (*Schistosoma mansoni*) (from Gold and Goldberg, 1976)**.** At high or low temperatures and in the absence of light the hatching of the eggs strongly decreases. Optimal temperature conditions and the presence of light increase the probability of hatching rapidly. Salinity has the strongest impact on the hatching. In the absence of saline 100% hatch, whereas with 0.2% saline the hatching ratio is less than 50% and at 0.8% hatching is completely inhibited.

Inside the aquatic snail, the miracidia form sporocysts via metamorphosis, which then produce either rediae or more sporocysts. This is the onset of the asexual part of the life cycle. The rediae, a larval form of trematodes with an oral sucker, produce more rediae or cercariae, the next stage in the life cycle. Based on one miracidium over days to weeks tens of thousands of cercariae may originate. The cercariae leave the aquatic snail and move through the aquatic environment until they reach and penetrate the second intermediate host, if the life-cycle acquires two intermediate hosts, or, if only one intermediate host is required, encyst on water vegetation and other structures, such as watercress,

water caltrop, water lotus, water chestnut, water lily, and stones, or directly infest the final host. In trematodes, transmitted by terrestrial intermediate hosts, modes of life cycles are altered somehow. On the water plants or within the second intermediate host the cercariae become metacercariae which are encysted in a hyaline, elastic hull (Lucius and Loos-Frank, 2008; Keiser and Utzinger, 2009). Other sources of infection are drinking water contaminated with floating metacercariae and vegetables washed in contaminated water (Robinson and Dalton, 2009). When the final host ingests the infested second intermediate host or the plants, the metacercariae excyst in the duodenum, perforate the wall of the intestine, and cross the abdominal cavity on the way to the liver. In the adult stage the flukes are fully developed and capable of sexual reproduction (Lucius and Loos-Frank, 2008; Keiser and Utzinger, 2009) (Fig. 1.3).

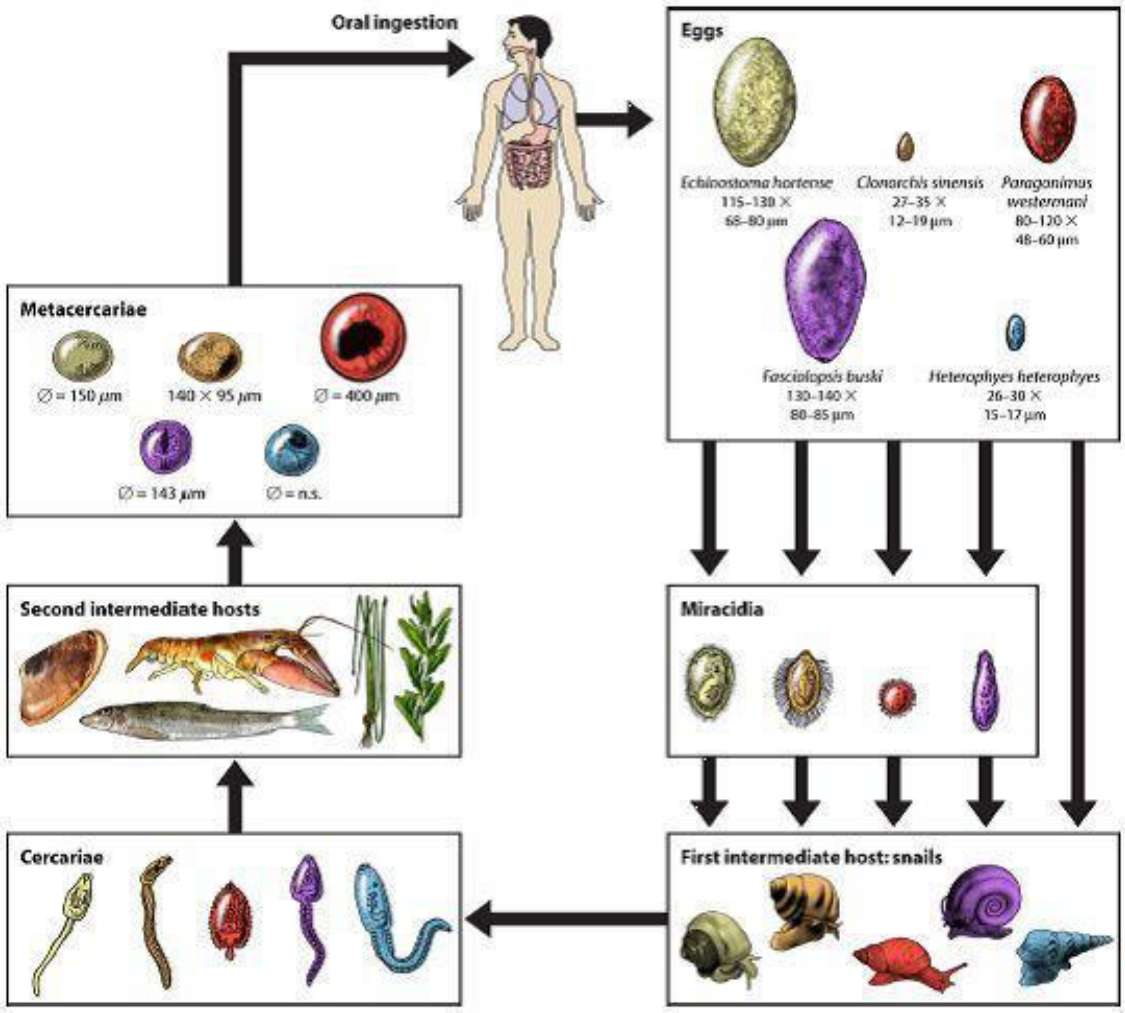

Fig. 1.3: The life cycles of different trematodes (from Keiser and Utzinger, 2009)**.**

1.1.3 Intermediate hosts

Trematodes and molluscs have a long lasting co-evolution, which has persisted for approx. 570 million years (Palaeozoic) and trematodes have invented methods to fully exploit their intermediate hosts (Lucius and Loss-Frank, 2008). The oldest fossil of the lymnaeid group is of *Galba* (between 146 and 208 million years ago), indicating that the lymnaeid group is ancient (Zilch, 1959–1960; Bargues *et al.*, 2001). *Galba truncatula* (class Gastropoda, family Lymnaeidae, genus *Galba*), was first described by Müller in 1774 and is a freshwater lymnaeid snail distributed worldwide (Fig. 1.4).

Fig. 1.4: Freshwater lymnaeid snail *Galba truncatula* (from Natural History Museum Vienna)**.**

In Europe, *G. truncatula* and *Radix peregra* have proved to act as the main intermediate host for *F. magna* and *F. hepatica* (Relf *et al.*, 2009; Haider *et al.*, 2012). Depending on the continent, other aquatic snails can also act as intermediate hosts for *F. hepatica* (Smyth, 1976; Schnieder, 2006) (Tab. 1.1). The known intermediate host spectrum for *F. magna* includes only the species *G. truncatula*, *R. peregra, Lymnaea modicella, L. caperata*, *Pseudosuccinea columella, Stagnicola palustris nuttalliana, G. bulimoides techella, and Fossaria parva* (Haider, 2010; Eckert, 2013; Králová-Hromadová *et al.*, 2016).

Tab. 1.1: Intermediate hosts for *F. hepatica* (adapted from Smyth, 1976; Schnieder, 2006)**.**

Continent/Country	Intermediate host
Europe	*L. peregra*
	L. palustris
	L. glabra
	L. stagnalis
	L. occulta
	L. turricula
Philippines	*L. philippinensis*
	L. swinhoei
	Amphipelpa cumingiana
Australia	*L. tomentosa*
	L. launcestonensis
New Zealand	*Simlimnaea tomentosa*
	L. alfredi
Japan	*L. pervia*
	L. japonica
Argentinia	*L. viatrix*
Venezuela	*L. cubensis*
Egypt	*L. cailliandi*
China	*L. pervia*
USA	*L. bulimoides*
	L. mulimoides techella
	G. ferruginea
	G. cubensis
	Fossaria modicella
	L. traskii
	P. columella
	L. modicella
Cuba	*Tarebia granifera*
Additionally in warmer regions	*L. columella*

1.2 *Fascioloides magna*

1.2.1 Distribution

F. magna is a parasitic flatworm, belonging to the Fasciolidae, and was first described in Italy by Bassi in 1875 (Leontovyč, 2014). *F. magna* originates from North America and was imported at least twice into Europe. The fluke was first introduced with game animals in La Mandria Regional Park near Turin (Italy) in the second half of the 19th century (Králová-Hromadová *et al.*, 2011; Leontovyč, 2014). The second introduction occurred in the first half of the 20th century, when *F. magna* was imported into Bohemia, Czech Republic (Ullrich, 1930). Afterwards infestations were also observed in Austria (Pfeiffer, 1983; Winkelmayer and Prosl, 2001; Ursprung *et al.*, 2006), Slovakia (Rajský *et al.*, 1994), Hungary (Majoros and Sztojkov, 1994), Croatia (Marinculić *et al.*, 2002), Serbia (Markinkovic and Nesic, 2008), Germany (Plötz *et al.*, 2015), and Poland (Demiaszkiewicz *et al.*, 2015).

1.2.2 Prevalence and distribution in Austria

In Austria, *F. magna* was detected for the first time in fallow deer in a game reserve (Pfeiffer, 1983). Today the flukes can be found in the East of Vienna and in the downstream region of the Danube, predominantly in Fischamend, Regelsbrunn, Orth, and Mannswörth (Winkelmayer and Prosl, 2001; Ursprung and Prosl, 2011; Hörweg *et al.*, 2011; Haider *et al.*, 2012) (Fig. 1.5).

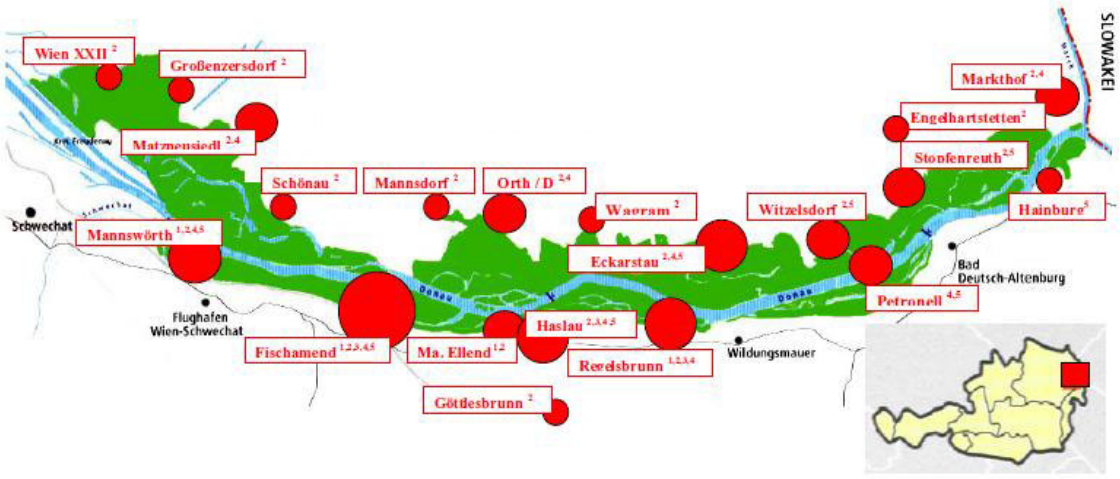

Fig. 1.5: Distribution and occurrence of *F. magna* in Austria (June 2011) (from Ursprung and Prosl, 2011)**.**

1.2.3 Morphology

F. magna has a length of 8–10 cm and an oval dorsoventrally flattened body with no anterior cone (Královál-Hromadová *et al*., 2011; Naem *et al*., 2012) (Fig. 1.6).

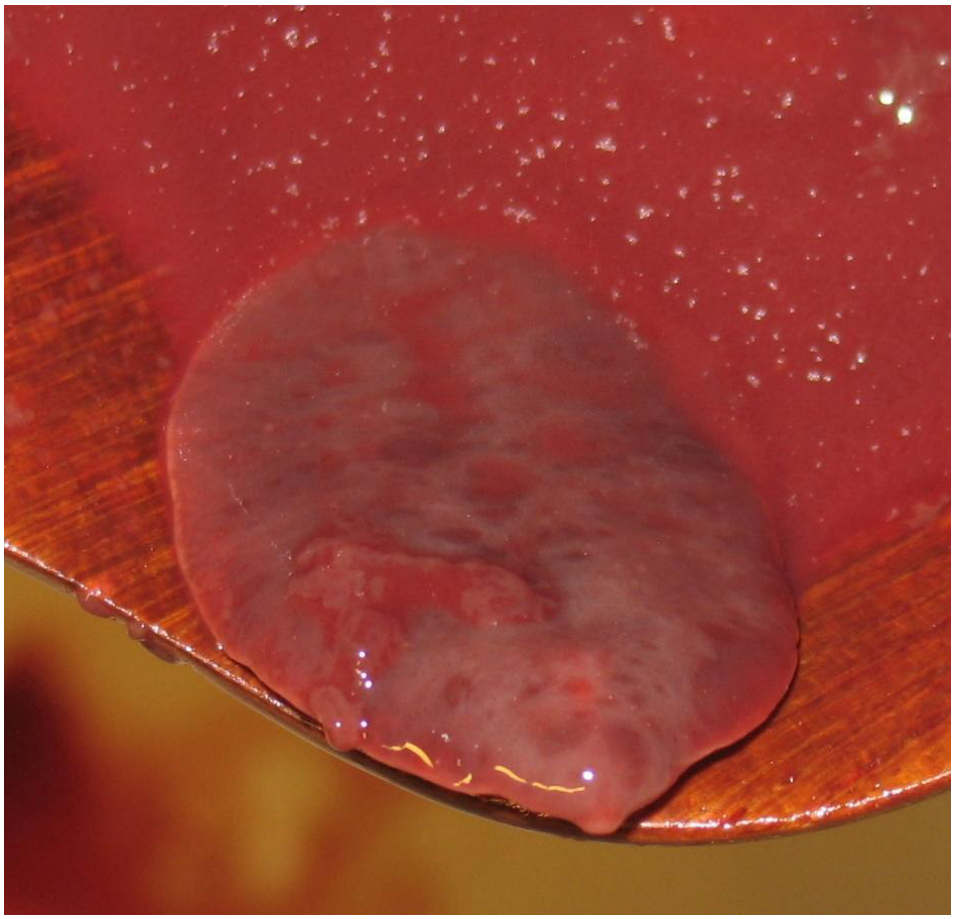

Fig. 1.6: Adult individual of *F. magna* (from Natural History Museum Vienna)**.**

Located on the tegument are sharp spines, which are small around the oral and ventral suckers but with a sharp point. Other spines contain serrated edges with 15–22 sharp points (Naem *et al*., 2012). The surface is covered by the tegument, a syncytial epithelium with sensory function and scale-like spines, implicated in nutrient absorption via membrane digestion and transport or endocytosis and secretion/excretion of endogenous material (Keiser and Utzinger, 2009; Naem *et al*., 2012). This includes components of the surface plasma membrane and the release and turnover of the glycocalyx. This plays a major role in immune evasion, because it prevents the fluke from bile acids, enzymes, and immunoglobulins of the host (Halton, 2004). Synthesis and osmoregulation are other functions of the tegument. Excretion and

osmoregulation establish a gradient for ions and water through ionic pumps and Na^+/K^+-ATPase, which reside in the surface and basal plasma membranes (Pappas, 1975; Schuster, 2002; Halton, 2004) (Fig. 1.7).

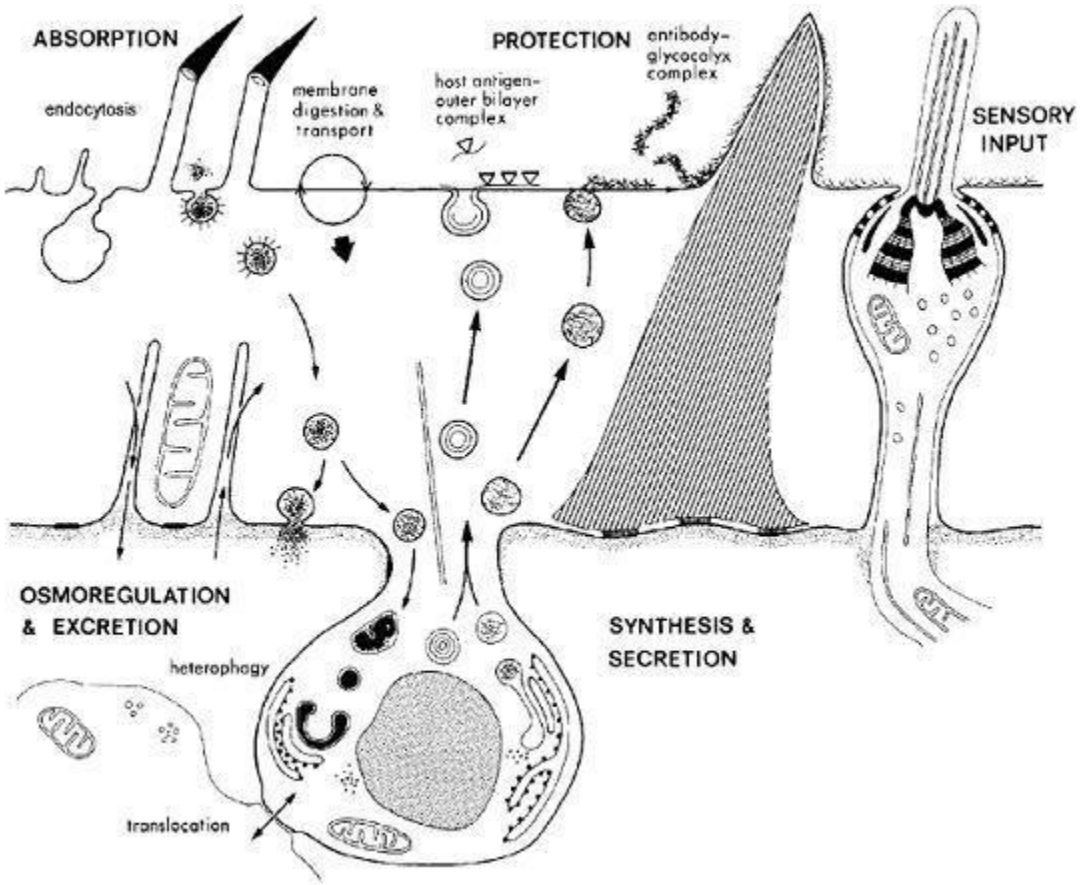

Fig. 1.7: Functions of the tegument of trematodes (from Halton, 2004)**.**

The oral sucker is covered by small dome-shaped and ciliated papillae and the ventral sucker has two spine-like structures and a smooth surface. The genital pore closely anterior to the ventral sucker contains fewer spines than the other parts of the anterior end. The fluke has well-developed spines at the anterior end of the ventral side and small spines, mostly with one or three points and blunted edges on the posterior end of the ventral side. The spines located at the posterior end of the dorsal side grow progressively fewer, smaller, and shorter. A folded tegument with no spine is around the excretory pore and small groups of tiny spines between the folds. The testes are in the middle third of the body and the uterus is a short structure folded several times in the anterior part of the body (Naem *et al.*, 2012; Špakulová *et al.*, 2003).

The eggs are yellowish and approximately 168 x 101 µm in size. At the pole, they have a lid, called operculum. This lid enables the passage of the miracidium out of the egg (Naem *et al.*, 2012) (Fig. 1.8).

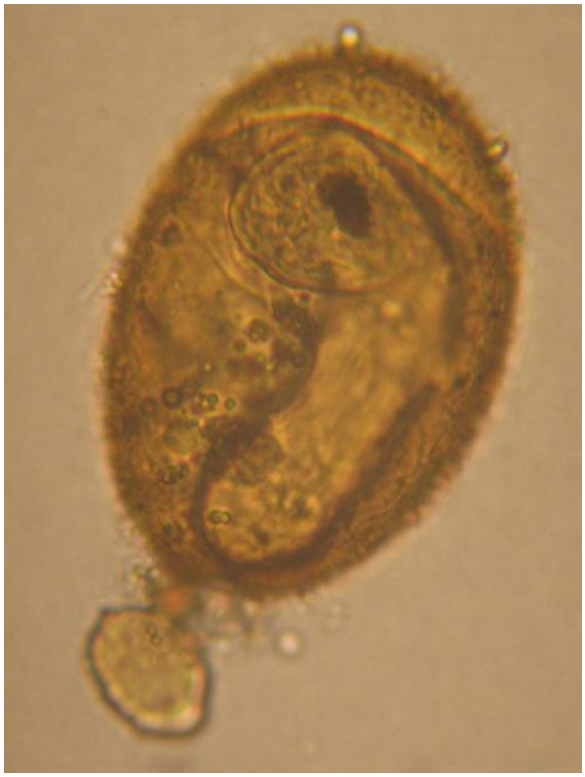

Fig. 1.8: Egg of *F. magna* containing a miracidium (from Natural History Museum Vienna)**.**

1.2.4 Life cycle

The life cycle of *F. magna* includes one final host and one intermediate host (Fig. 1.9).

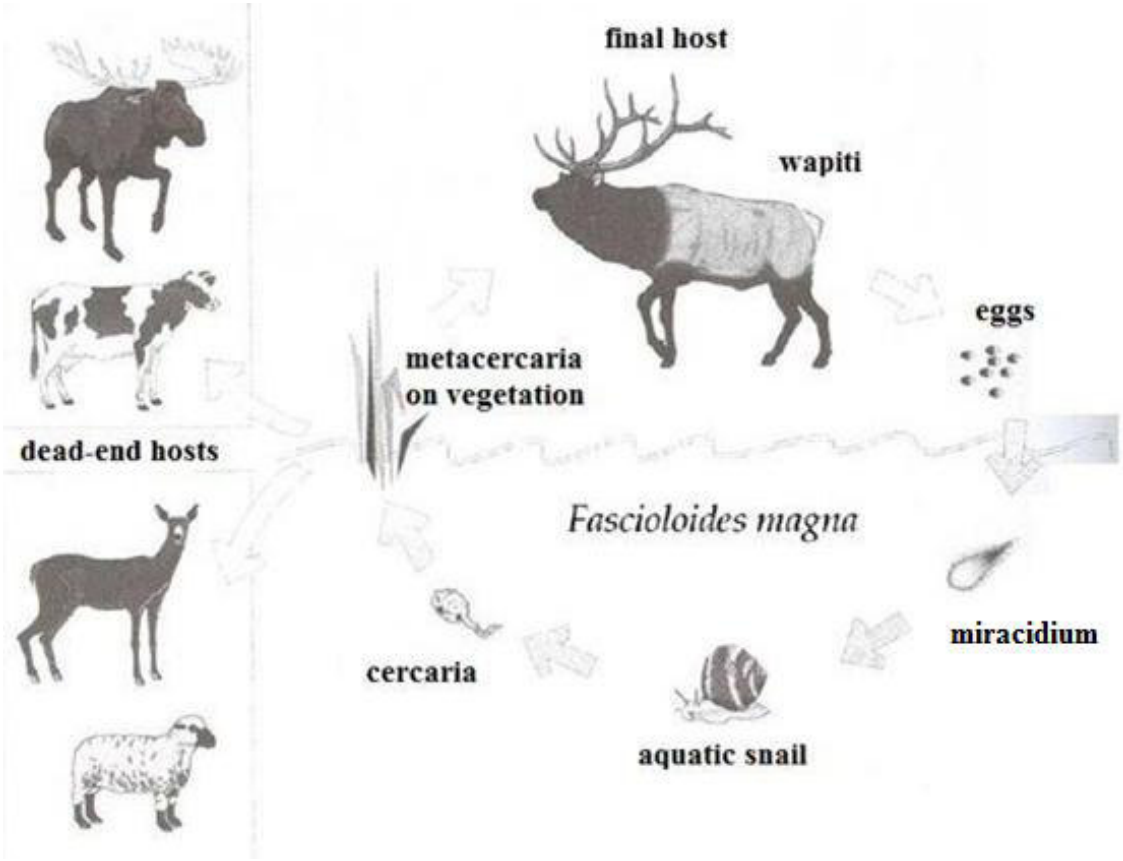

Fig. 1.9: The life cycle of *F. magna* (from Samuel *et al.*, 2001, modified)**.**

F. magna usually is situated pairwise in a common capsule in the parenchyma of the liver of the final host with a connection to the bile duct. They can survive for up to five years (Samuel *et al.,* 2001) (Fig. 1.10).

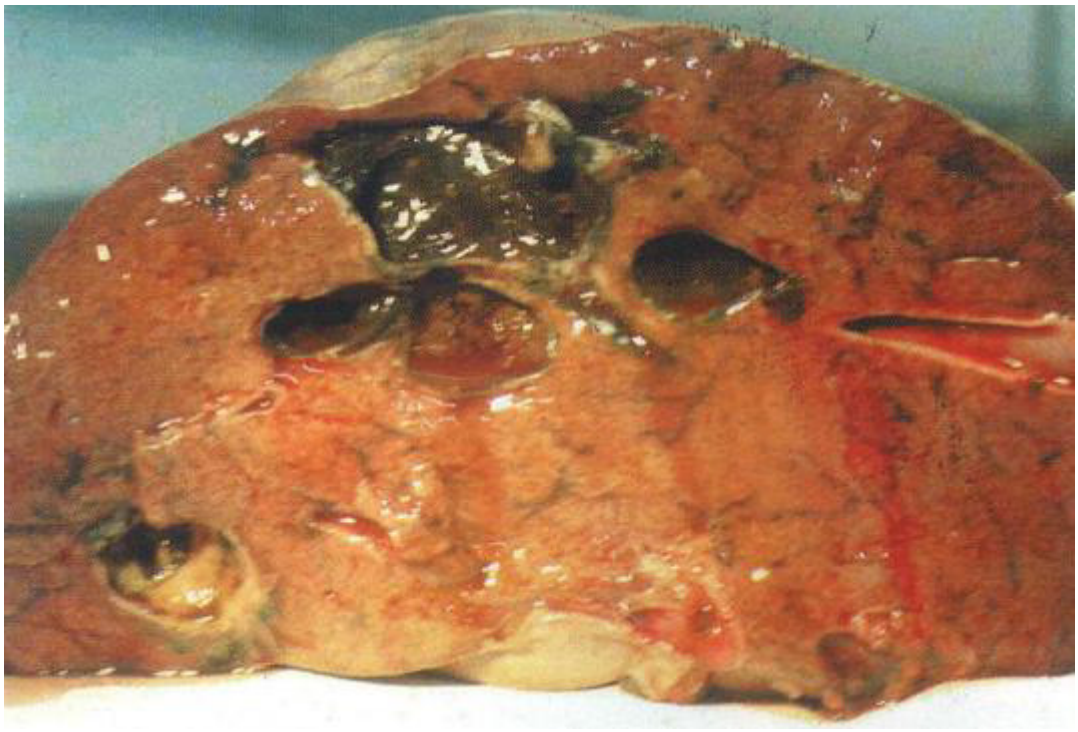

Fig. 1.10: Pseudocysts in a liver of red deer infested with *F. magna* (from Špakulová *et al*., 2003)**.**

A mature fluke can release up to 4,000 eggs per day. The eggs are first collected in the cavity of the capsule and then released through the connection. They enter the duodenum and leave the host with the faeces. (Samuel *et al*., 2001; Eckert *et al*., 2008). Outside the host, several physical-chemical factors, such as temperature, humidity, light, and oxygen tension force embryonation, which can take as long as 35 days (Samuel *et al*., 2001). The miracidia hatch from the eggs and seek a snail from the family Lymnaeidae (*Galba truncatula*), the intermediate host.

The miracidia penetrate the host and form sporocysts. The sporocysts contain germinal cells that produce up to 14 mother rediae, which migrate to the digestive glands, renal organ, reproductive organ, and pulmonary sac of the snails (Fig. 1.11).

Fig. 1.11: Redia of *F. magna* (from Natural History Museum Vienna)**.**

Up to nine daughter rediae can be produced asexually from one mother redia and each daughter redia produces nine or 10 cercariae, which mature in the digestive glands of the snail (Samuel *et al.*, 2001) (Fig. 1.12).

Fig. 1.12: Cercaria of *F. magna* (from Natural History Museum Vienna)**.**

The development within the snail takes 40 to 58 days and dependents on snail species and temperature (Haider *et al.*, 2012). The mature cercariae leave the snail and search for vegetation, where they encyst and are then called metacercariae. After ingestion, the metacercariae are stimulated in the stomach and the intestine to leave their cyst. The flukes subsequently perforate the wall of the intestine and cross the abdominal cavity to the liver. There the flukes penetrate the Glisson's capsule and continue their way across the liver, searching for other flukes.

1.2.5 Medical relevance

Fascioloidosis is a plant-borne zoonotic disease caused by *F. magna*. *F. magna* has veterinary medical importance and has an impact on domesticated and non-domesticated animals. Definitive hosts are cervids, primarily from the new world (Samuel *et al.*, 2001). In Europe, *F. magna* infests the liver of red deer (*Cervus elaphus*), fallow deer (*Dama dama*), and roe deer (*Capreolus capreolus*) (Ursprung and Prosl, 2011; Kasny *et al.*, 2012). Humans are not known to be a host (Samuel *et al.*, 2001). Horses, pigs, sheep, and goats are dead-end hosts, as the parasite cannot complete the migratory stage; in sheep and goats the infestation is fatal within six months because the flukes migrate through different tissues and cause heavy damage. (Schnieder 2006; Samuel *et al.*, 2001; Sattmann *et al.*, 2014; Novobilský *et al.*, 2007; Špakulová *et al.*, 2003). In cattle and moose, the flukes are unable to connect their cyst with the bile duct, the flukes rarely mature and the few eggs produced do not reach the intestine. The infestation is thus not patent (Eckert *et al.*, 2008; Samuel *et al.*, 2001). In roe deer, *F. magna* causes great damage to the liver and other organs, and even infestations with low numbers of flukes are fatal (Bazsalovicsová *et al.*, 2013; Ursprung and Prosl, 2011) (Fig. 1.13). Because of the heavy liver damages, *F. magna* can kill an entire roe deer population in an area (Ursprung and Prosl, 2011). In 1995, 90% of red deer and 60% of roe deer in Slovakia were infested (Ursprung and Prosl, 2011; Kasny *et al.*, 2012). In Austria, the fluke spread through roe and red deer and led to heavy damage and death of the hosts (Winkelmayer and Prosl, 2001). From 2001 to 2006 a treatment programme was organised due to a prevalence in Austrian deer of up to 100% (Sattmann *et al.*,

2014). Deer were treated with triclabendazole (Fasinex®) admixed to winter food. In the following years the number of infestations decreased, but it was not possible to eradicate the fluke completely. In 2006, a shift to the Northern side of the Danube and a resurgence in the hosts was recorded (Ursprung *et al.*, 2006; Ursprung and Prosl, 2011).

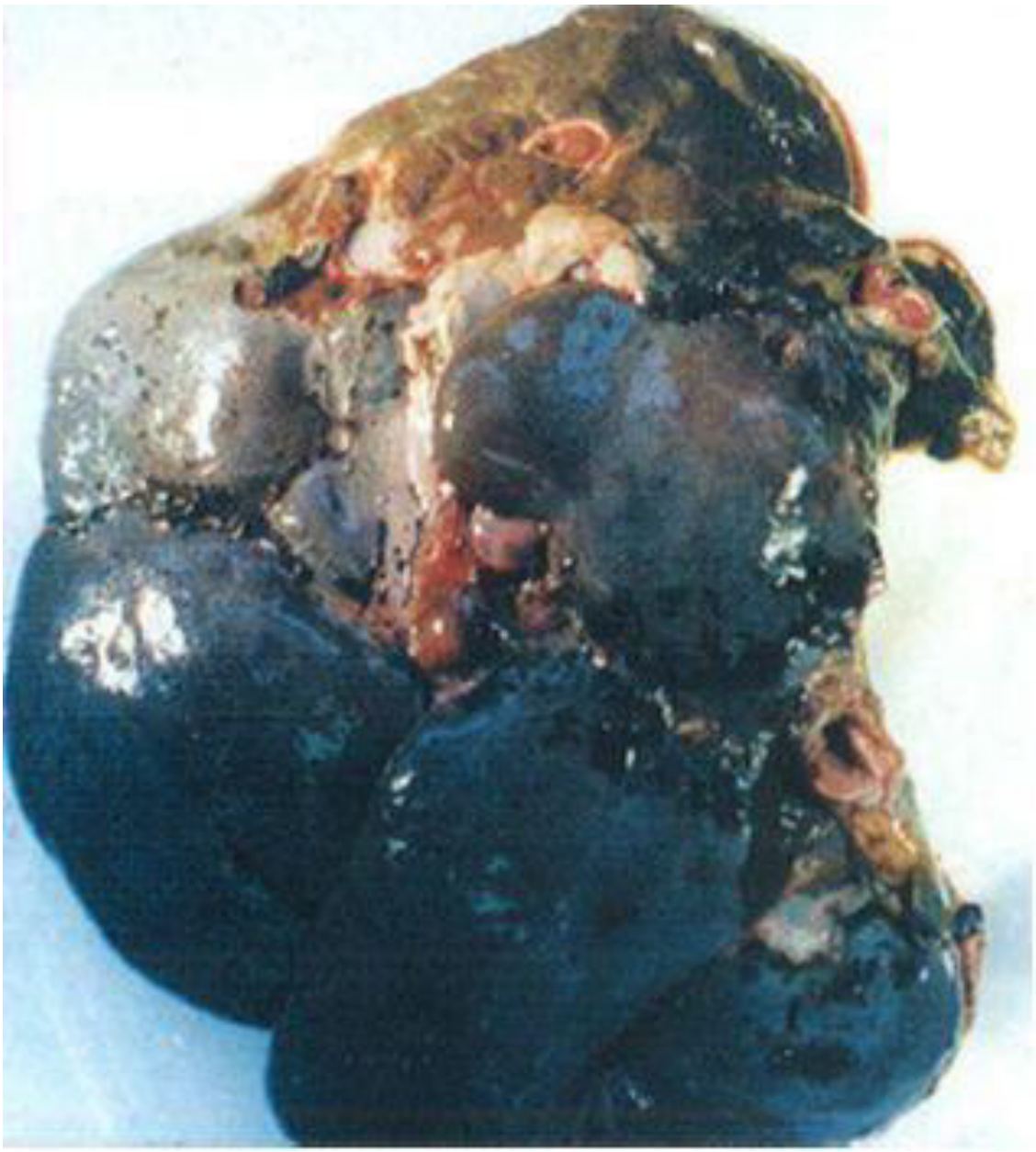

Fig. 1.13: Fascioloidosis at a liver of a three year old roe deer (from Špakulová *et al.*, 2003)**.**

1.2.6 Diagnosis

Today, diagnosis is based on immunological tests like enzyme-linked immunosorbent assay (ELISA) and immunoblotting (Severin *et al*., 2015). Previous methods for confirmation of a *F. magna* infestation have been based on examination of livers of dead animals and coprological examinations (Severin *et al*., 2015)

1.2.7 Molecular typing

For the investigation of the diversity of *F. magna* sequence analyses of mainly two genes have been established: *cytochrome c oxidase subunit 1* (*cox1*) and *nicotinamide dehydrogenase subunit 1* (*nad1*). The *cox1* gene is part of the respiratory complex IV in the mitochondrion and codes the information for an important enzyme in the aerobic metabolism (Lafontaine and Tollervey, 2001). The *nad1* gene is part of the respiratory chain and catalyses the transfer of electrons from NADH to ubiquinone (Lafontaine and Tollervey, 2001; Brandt, 2006).

Radvánský *et al*. (2011), Králová-Hromadová *et al*. (2011), and Bazsalovicsová *et al*. (2015) examined samples of *F. magna* from Slovakia, the Czech Republic, Croatia, Hungary, Italy, the United States of America (USA), and Canada. Radvánský *et al*. (2011) described seven haplotypes for *cox1* and Králová-Hromadová *et al*. (2011) described 16 haplotypes for *cox1* and 18 haplotypes for *nad1*. Bazsalovicsová *et al*. (2015) examined flukes from North America and described 32 haplotypes for *cox1* and 28 haplotypes for *nad1*. Haplotype 1 of the *cox1* gene and haplotype 3 of the *nad1* gene were shared by strains from Italy, the USA, and Canada. The most common haplotypes in Europe were haplotype 3 for *cox1* and haplotype 4 for *nad1*. These haplotypes are known to be common in Hungary, the Czech Republic, Slovakia, Croatia, and Austria (Králová-Hromadová *et al*., 2011; Sattmann *et al*., 2014). Other haplotypes known to occur in Hungary, the Czech Republic, Slovakia and Croatia are *cox1*: Ha 4, Ha 5; *nad1*: Ha 5, Ha 6, and Ha 7.

Known *cox1* and *nad1* haplotypes for *F. magna* with exemplary reference sequences are shown in tabs. 1.2 and 1.3. The mitochondrial genome of *F. magna* has a length of 14,047 bp. The position of the *cox1* gene in the mitochondrial genome is in the range of bp 6,840–bp 8,384 and the position of the *nad1* gene is in the range of bp 5,162–bp 6,064

Tab. 1.2: *cox1* haplotypes of *F. magna* (Radvánský *et al*., 2011; Královà-Hromadová *et al*., 2011; Bazsalovicsová *et al*., 2015)**.** Position in the *cox1* gene: bp 177–bp 615**.**

Haplotype	Known occurrence	Original description	Reference sequences
1	Italy Canada USA	Radvánský *et al*., 2011	GU599860 GU599861
2	Italy	Radvánský *et al*., 2011	GU599863
3	Croatia Czech Republic Hungary Slovakia	Radvánský *et al*., 2011	GU599867 GU599864
4	Czech Republic Slovakia	Radvánský *et al*., 2011	GU599868 GU599869
5	Czech Republic	Radvánský *et al*., 2011	GU599870
6	Canada	Radvánský *et al*., 2011	GU599871
7	USA	Radvánský *et al*., 2011	GU599872
8	USA	Královà-Hromadová *et al*., 2011	GU599873
9	Canada USA	Bazsalovicsová *et al*., 2015	KP635014 KP635022
10	USA	Královà-Hromadová *et al*., 2011	GU599875
11	USA	Královà-Hromadová *et al*., 2011	GU599876
12	USA	Královà-Hromadová *et al*., 2011	GU599877
13	USA	Královà-Hromadová *et al*., 2011	GU599878
14	USA	Královà-Hromadová *et al*., 2011	GU599879
15	USA	Královà-Hromadová *et al*., 2011	GU599880
16	USA	Bazsalovicsová *et al*., 2015	KP635023
17	Canada	Bazsalovicsová *et al*., 2015	KP635015

18	Canada	Bazsalovicsová *et al.*, 2015	KP635016
19	USA	Bazsalovicsová *et al.*, 2015	KP635021
20	Canada	Bazsalovicsová *et al.*, 2015	KP635012
21	Canada	Bazsalovicsová *et al.*, 2015	KP635013
22	Canada	Bazsalovicsová *et al.*, 2015	KP635019
23	USA	Bazsalovicsová *et al.*, 2015	KP635031
24	USA	Bazsalovicsová *et al.*, 2015	KP635032
25	USA	Bazsalovicsová *et al.*, 2015	KP635033
26	USA	Bazsalovicsová *et al.*, 2015	KP635034
27	USA	Bazsalovicsová *et al.*, 2015	KP635035
28	Canada	Bazsalovicsová *et al.*, 2015	KP635020
29	USA	Bazsalovicsová *et al.*, 2015	KP635024
30	USA	Bazsalovicsová *et al.*, 2015	KP635025
31	USA	Bazsalovicsová *et al.*, 2015	KP635026
32	USA	Bazsalovicsová *et al.*, 2015	KP635027
33	USA	Bazsalovicsová *et al.*, 2015	KP635028
34	USA	Bazsalovicsová *et al.*, 2015	KP635029
35	USA	Bazsalovicsová *et al.*, 2015	KP635036

Tab. 1.3: *nad1* haplotypes of *F. magna* (Králová-Hromadová *et al.*, 2011)**.**
Position in the *nad1* gene: bp 498–bp 902**.**

Haplotype	Known occurrence	Citation	Reference sequences
1	Italy	Králová-Hromadová *et al.*, 2011	GU599834
2	Italy	Králová-Hromadová *et al.*, 2011	GU599835
3	Italy Canada USA	Králová-Hromadová *et al.*, 2011	GU599836 GU599845
4	Croatia Czech Republic Hungary Slovakia USA	Králová-Hromadová *et al.*, 2011	GU599844 GU599837
5	Czech Republic Slovakia	Králová-Hromadová *et al.*, 2011	GU599838 GU599842

6	Czech Republic	Králová-Hromadová *et al*., 2011	GU599839
	USA		KP635059
7	Czech Republic	Králová-Hromadová *et al*., 2011	GU599840
8	Canada	Králová-Hromadová *et al*., 2011	GU599846
9	USA	Králová-Hromadová *et al*., 2011	GU599849
10	USA	Králová-Hromadová *et al*., 2011	GU599850
11	USA	Králová-Hromadová *et al*., 2011	GU599851
12	Canada	Králová-Hromadová *et al*., 2011	KP635041
	USA		GU599852
13	USA	Králová-Hromadová *et al*., 2011	GU599853
14	USA	Králová-Hromadová *et al*., 2011	GU599854
15	USA	Králová-Hromadová *et al*., 2011	GU599855
16	USA	Králová-Hromadová *et al*., 2011	GU599858
17	USA	Králová-Hromadová *et al*., 2011	GU599857
18	USA	Králová-Hromadová *et al*., 2011	GU599859
19	Canada	Králová-Hromadová *et al*., 2011	KP635042
	USA		KP635060
20	Canada	Králová-Hromadová *et al*., 2011	KP635043
21	Canada	Králová-Hromadová *et al*., 2011	KP635044
22	Canada	Králová-Hromadová *et al*., 2011	KP635045
23	Canada	Králová-Hromadová *et al*., 2011	KP635046
24	Canada	Králová-Hromadová *et al*., 2011	KP635038
25	Canada	Králová-Hromadová *et al*., 2011	KP635039
26	USA	Králová-Hromadová *et al*., 2011	KP635061
27	USA	Králová-Hromadová *et al*., 2011	KP635052
28	USA	Králová-Hromadová *et al*., 2011	KP635053
29	USA	Králová-Hromadová *et al*., 2011	KP635062
30	Canada	Králová-Hromadová *et al*., 2011	KP635040
31	USA	Králová-Hromadová *et al*., 2011	KP635063
32	USA	Králová-Hromadová *et al*., 2011	KP635050

1.3 *Fasciola hepatica*

1.3.1 Distribution

F. hepatica was first described by Linnaeus in 1758 and detected for the first time in humans by Pallas in 1760 when he detected it in the bile duct of a human corpse in Berlin, Germany (Pallas, 1760). *F. hepatica* has a worldwide distribution and infests a good number of mammal species, mainly cattle and sheep but also humans, and it is estimated that up to 50 million humans are affected, with more than 180 million at risk of infestation (Nyindo and Lukambagire, 2015) (Fig. 1.14).

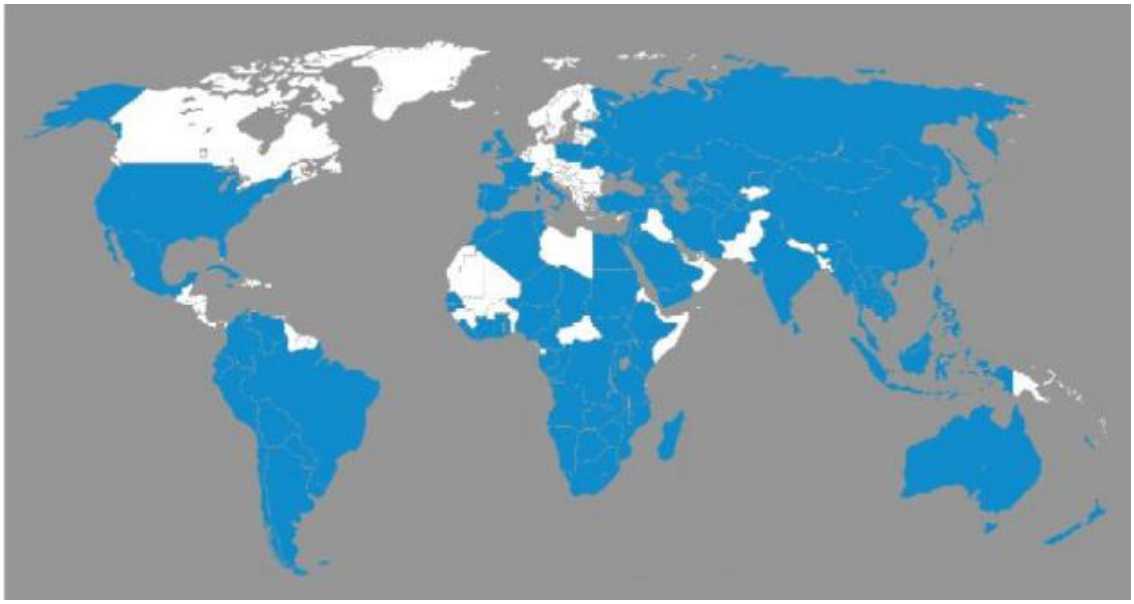

Fig. 1.14: Distribution of fasciolosis (from http://www.bvgh.org/Current-Programs/Neglected-Disease-Product-Pipelines/Global-Health-Primer/Diseases/cid/ViewDetails/ItemID/23.aspx; cited in Nyindo and Lukambagire, 2015)**.** Countries with reported cases of human fasciolosis are coloured in blue.

1.3.2 Prevalence and distribution in Austria

The main endemic areas of *F. hepatica* in Austria are in Vorarlberg, Salzburg, Upper Austria, and the Tyrol (Auer *et al*., 1981; Auer and Aspöck, 2014). Samples of 28 patients were examined over the last years at the Institute for Specific Prophylaxis and Tropical Medicine of the Medical University of Vienna. Most infestations were found in Salzburg (11 patients) and the second most in Upper Austria (five patients). Four patients came from Vorarlberg, three from

Vienna, two each from Styria and Tyrol, and one patient from Burgenland. More men were infested (17 patients) than women (14 patients) (Skoll, 2015). Concerning *F. hepatica* epidemiology in livestock in Austria a survey was given by Supperer and Pfeiffer in 1986.

1.3.3 Morphology

F. hepatica reaches a size of up to 5 x 1.3 cm (Lucius and Loos-Frank, 2008). The head has the form of a cone and a length of 3–5 mm, whereas the posterior end is broadly rounded (Fig. 1.15). The body is grimy grey to brown, bilaterally flattened, and shaped like a bay leaf (Schuster, 2002; Mas-Coma, 1997).

Fig. 1.15: *F. hepatica* from ventral (orig.)**.**

The surface is covered by the tegument, respiratory and circulatory systems are missing (Keiser and Utzinger, 2009). On the ventral side, there are two suckers, an oral and a ventral sucker, and numerous spines that cover the body surface. The flukes have no external signs of segmentation and the mouth ends in the pharynx, a muscular tube that allows for sucking. The digestive system has only one opening, the oral sucker, which is not linear but branched, while it ends in several blind ducts (Lucius and Loos-Frank, 2008). Flukes use the oral and the ventral sucker to attach themselves to the wall of the bile ducts, intestine, or lung parenchyma (Keiser and Utzinger, 2009). Liver flukes are always hermaphrodites. The ovaries run laterally and disembogue, together with the cirrus, closely anterior to the ventral sucker. The testicles are located in the posterior two thirds of the body (Schuster, 2002).

The yellowish eggs of *F. hepatica* are about 130–150 x 63–90 µm in size and at the pole they have an operculum (Chen and Mott, 1990) (Fig. 1.16).

Fig. 1.16: Egg of *F. hepatica* (from Schuster, 2002).

1.3.4 Life cycle

In the life cycle, asexual phases in snails alternate with sexual phases in vertebrates (Whitfield, 1982) (Fig. 1.17).

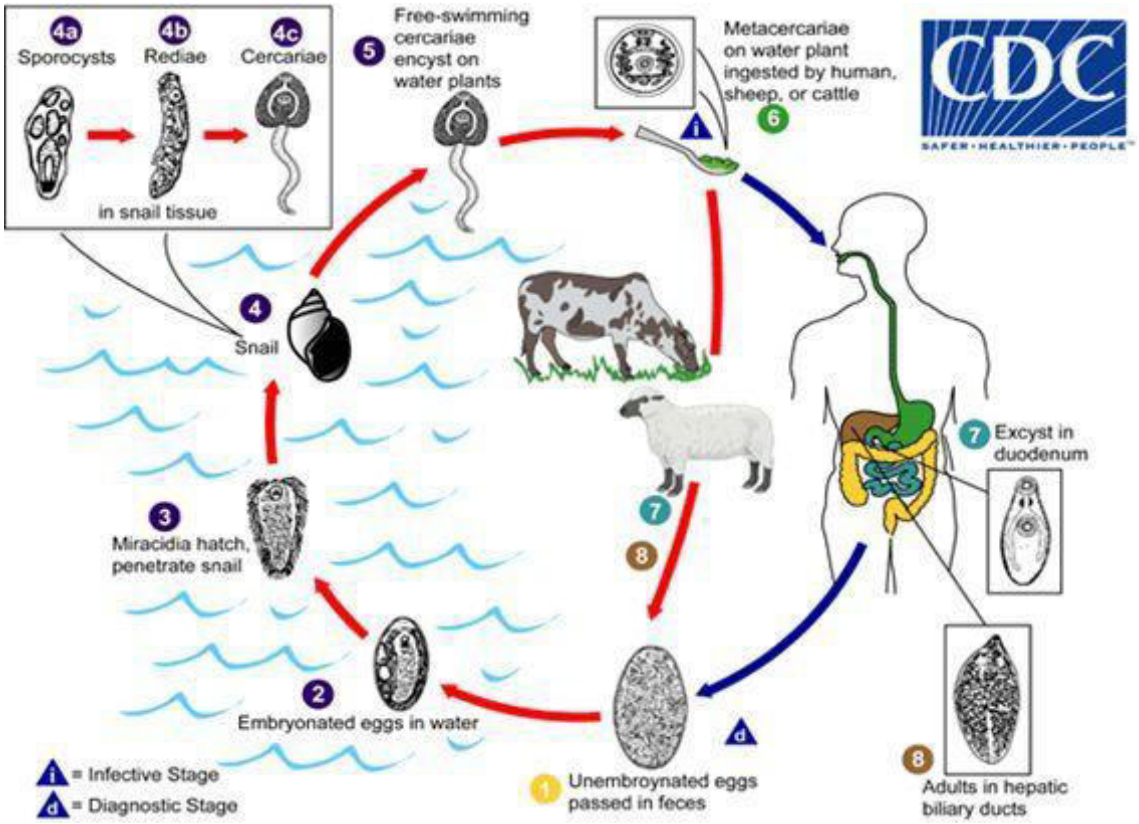

Fig. 1.17: Life cycle of *F. hepatica* (from CDC [Centers for Disease Control and Prevention]).

F. hepatica produces 20,000–50,000 eggs per day per fluke in the bile duct and the gall bladder of its hosts, which are excreted with the faeces (McManus and

Dalton, 2006). The majority of the eggs are destroyed in the gastrointestinal tract by bacterial toxins, and in water only 1/1,000,000 miracidia are able to find the intermediate host (Miliotis and Bier, 2003). In water, the miracidia complete their development and hatch in 9 to 15 days in the presence of warm weather conditions while colder temperatures retard the development. At 24 °C–28 °C the development of the eggs needs 12 days, whereas at 12 °C the development needs two months. The highest hatching rate occurs at 16 °C–20 °C whereas temperatures above 20 °C are not conducive to hatching (Gold and Goldberg, 1976; Kumar, 1999). In general, miracidia only hatch in the presence of freshwater, short-wave light, and in the broad absence of faeces (Gold and Goldberg, 1976; Vignoles *et al.*, 2014; Schnieder, 2006; Kumar, 1999). It has been reported that in darkness eggs failed to hatch, but that when these eggs were subjected to temperature changes of higher than 4 °C sometimes they could even hatch in the absence of light (Gold and Goldberg, 1976).

The miracidia are decoyed by glycoconjugates which are localised in the mucilages of the snail. The higher the concentration of the glycoconjugates is, the better the directed movement towards the snail will be. Lower concentrations lead to the contrary (Schnieder, 2006).

After contacting the intermediate host, a freshwater snail, the miracidia penetrate the tissue of the snail and form primary sporocysts. The sporocysts produce the first generation of rediae, which in turn produce the second generation of rediae that develop in the digestive gland of the snail (Robinson and Dalton, 2009). To reallocate resources of the snail to the production of parasitic stages, the snail is castrated mechanically by the rediae eating its gonads, which leads to a higher weight of the snail because of its growth due to an increase of the mass of the parasites, called gigantism. By this strategy, a miracidium in a snail with 6–8 mm of height can produce 350–500 cercariae (Lucius and Loss-Frank, 2008). After six to seven weeks, cercariae emerge from the snail either by active escape or passive extrusion and move through the aquatic environment until they reach various structures like vegetation, e.g. watercress (*Rorippa amphibian*), ramson (*Allium ursinum*), and caltrop nuts (*Trapa natam*) as well as salad, and windfalls. Water trickle and drains are also

important ways in which animals and humans become infested. (Keiser and Utzinger, 2009; Mas-Coma, 1997; Slifko *et al.*, 2000; Kühn and Schirmeister, 1989). On the vegetation, the cercariae encyst to metacercariae, and are ingested by cattle, sheep, or humans eating uncooked plants and/or drinking contaminated water (Usip *et al.*, 2014) (Fig. 1.18). Metacercarial cysts are highly resistant and can persist for a long time (Mas-Coma, 1997). Their infectivity is dependent on age: older metacercariae have a lower infectivity. They have a lifespan of a maximum of 48 weeks (Mas-Coma, 2005a).

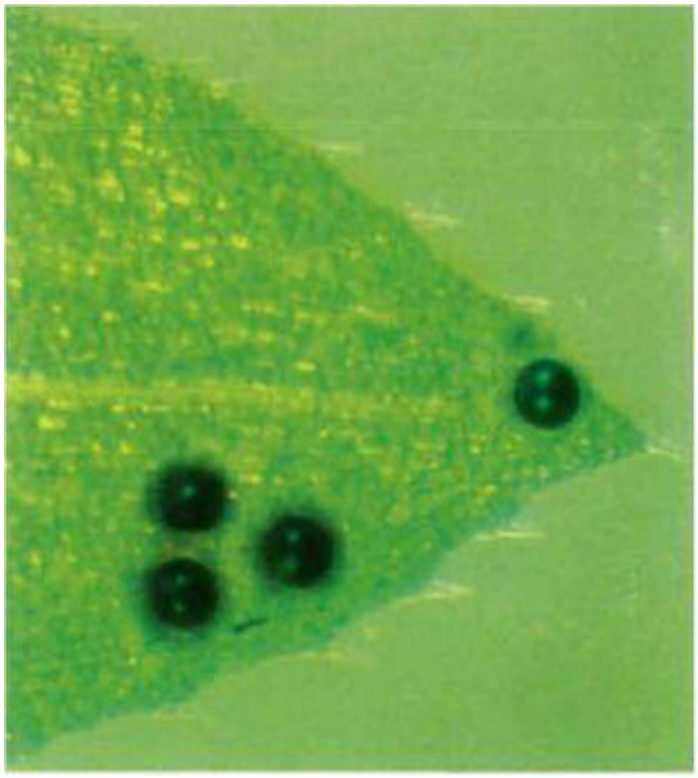

Fig. 1.18: Metacercariae located on vegetation (from Schuster, 2002)**.**

When metacercariae come into contact with low pH in the stomach, the early immature juvenile initiates the process of excystment by digesting the wall of the cyst. Having arrived in the duodenum, the liver fluke excysts, and perforates the intestinal wall to reach the peritoneal cavity (Usip *et al.*, 2014). After migration to the liver parenchyma through the glisson's capsule, the flukes start to feed for 8–12 weeks (Rokni *et al.*, 2002). This stage causes the disease fasciolosis. At the end of the migration, the parasites reach the bile ducts, where they mature into adults and begin to produce eggs (Beckham *et al.*, 2006; Dalton *et al.*, 2003; Dusak *et al.*, 2012).

In sheep, *F. hepatica* can survive as long as 11 years, whereas in cattle they survive only between nine and 12 months (Mas-Coma and Bargues, 1997; Samuel *et al.*, 2001). Depending on geographical region, there are also other definitive hosts, such as oxen, buffalos, various wild ruminants, horses, donkeys, pigs, rabbits, beavers, coypus, elephants, and camels (Kumar, 1999). Because of the long life-span of the flukes in sheep, the high egg output, and the inability of the sheep to acquire immunity against the fluke, it is assumed that today's *F. hepatica* originated mainly in sheep, especially in species of the genus *Ovis* (Mas-Coma *et al.*, 2009).

1.3.5 Medical relevance

Fasciolosis is a plant-borne zoonotic disease caused by trematodes of the genus *Fasciola*, namely *F. hepatica* and *F. gigantica* (Parkinson *et al.*, 2007). *F. hepatica and F. gigantica* have medical and veterinary importance. *Fasciola* infests the liver of many herbivorous mammals, including humans. During migration, the flukes can deviate from their path to the bile duct, thus other organs than the liver might be affected as well, which is called ectopic infestation (Dalimi and Jabarvand, 2005). While *F. hepatica* occurs in countries with a moderate climate, *F. gigantica* occurs mainly in tropical and subtropical parts of the world (Schnieder, 2006). This is explainable by the minimum temperature thresholds for their development. *F. hepatica* needs at least 10 °C and *F. gigantica* 16 °C for development (Malone *et al.*, 1998; Yilma and Malone, 1998). Because of their close relationship, hybrid forms of *F. hepatica* and *F. gigantica* also exist (Itagaki and Tsutsumi, 1998; Agatsuma *et al.*, 2000; Hoa Le *et al.*, 2008). More than 300 million cattle and 250 million sheep were infested by *F. hepatica* worldwide and fasciolosis causing an economic damage of three billion US$ globally per year through loss in productivity of milk and meat (Robinson and Dalton, 2009). For human infestations, estimations for the postdecades vary between 2.4 and 17 million in more than 70 countries with more than 180 million humans at risk of infestation or even more because of the unknown situations in many countries, primarily in Asia and Africa (Mas-Coma *et al.*, 2014; WHO, 2015). In 2015, fasciolosis affected 50 million humans with 180 million at risk of infestation with high prevalences reported from Italy, Spain,

Great Britain, France, Greece, Turkey, Bolivia, Peru, Cuba, China, Egypt (Nile Delta), Vietnam, and the Northern Iran. Hyperendemics were reported from Egypt, Ethiopia, Iran, Iraq, Syria, and Saudi Arabia (Nyindo and Lukambagire, 2015). Altogether, fasciolosis is one of the most widely disseminated zoonotic diseases in the world and is classified as an emerging human disease (WHO, 2015; Nyindo and Lukambagire, 2015).

Fasciolosis is classified into two phases: an invasive or acute phase and a latent or chronic phase. The invasive or acute phase begins when the worm perforates the intestinal wall, and it lasts for two to four months (Valero *et al.*, 2003). When the fluke reaches the liver parenchyma, it eats its way through the liver to the bile duct, where the fluke produces eggs in order to start a new life cycle. The destruction of liver parenchyma may lead to intensive internal bleeding (Fig. 1.19). Symptoms are hepatomegaly, splenomegaly, fever, nausea, abdominal pain, skin rashes, pancreatitis, ascites, marked eosinophilia, and lower milk production (McConville, 2008; Fica *et al.*, 2011; Centers for Disease Control and Prevention [CDC], 2015).

Fig. 1.19: Acute fasciolosis at a goat (from Schuster, 2002).

The latent or chronic phase starts with the egg production in the bile duct. When the gall bladder is depleted, the eggs are transported to the intestine. To start a new life cycle, the eggs must be deposited with the faeces. The flukes can survive for up to 13.5 years in the human body (Valero *et al.*, 2003). Symptoms are abdominal pain, anaemia because of internal bleeding, cholelithiasis, jaundice, enlarged gall bladder and bile duct and, because of the congested bile duct, cholangitis, and cholecystitis (Parkinson *et al.*, 2007; Valero *et al.*, 2003; Valero *et al.*, 2008; Valero *et al.*, 2006) (Fig. 1.20).

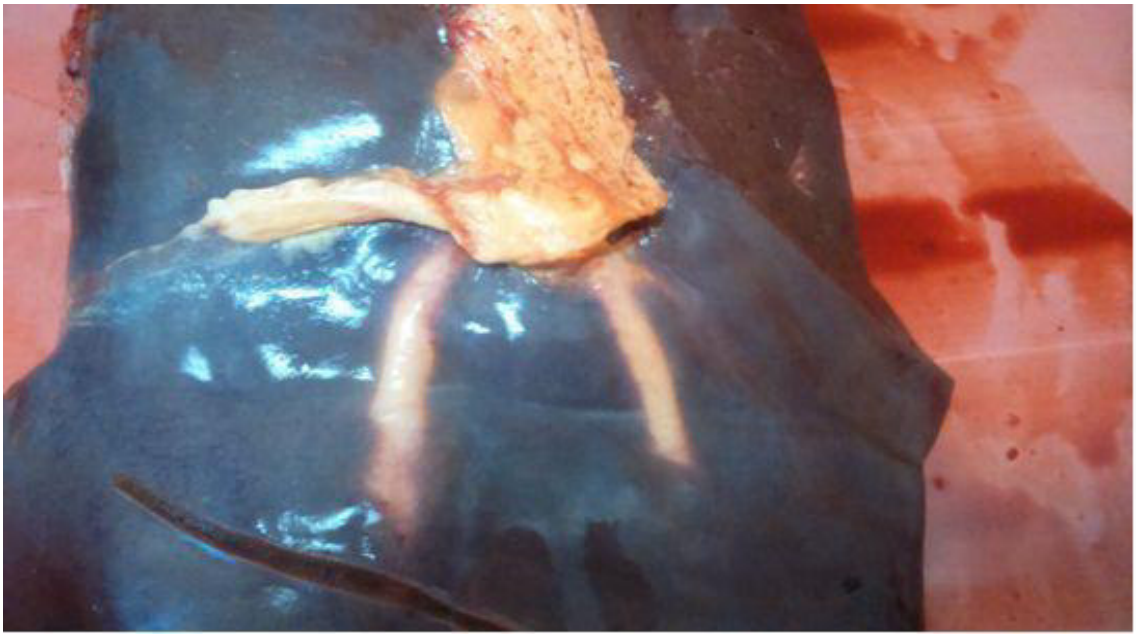

Fig. 1.20: Chronic fasciolosis at a cattle (orig)**.** Enlarged bile ducts in the chronic phase of a *F. hepatica* infestation.

Parasite fragments and/or debris obstruct the bile duct. Consequently, the gallbladder swells and an acute pancreatitis can occur. Because of the backlog of the gall, the patient suffers from jaundice and cholestatic hepatitis. A bacterial superinfection can lead to acute cholangitis and cholecystitis and increase the problems. The symptoms are the same as in the latent phase (WHO, 2006).

1.3.6 Diagnosis

The diagnosis of a *Fasciola* infestation is based on the clinical symptoms in combination with marked eosinophilia and immunological tests like ELISA and immunoblotting (Fica *et al.*, 2011; Deplazes *et al.*, 2013).

1.3.7 Treatment

Triclabendazole (6-chlor-5-(2,3-dichlorphenoxy)-2-methylthio-benzimidazol) (TCBZ, Fasinex®) is a benzimidazole derivate and was introduced in the 1980s in veterinary practice. It was not used in humans until the epidemic of fasciolosis near the Caspian Sea in 1989 (Massoud, 1990). It is the only drug against fasciolosis that is recommended and named as essential medicine by the World Health Organisation (WHO) (WHO Model List of Essential Medicines, 18th list, April 2013). It is active against both, immature and adult flukes, and can be applied in the acute and chronic phases (Millán et al, 2000; Fairweather, 2005; WHO, 2015; Keiser and Utzinger, 2009).

For treatment of humans, a single dose with 10 mg/kg body weight should suffice. In case of treatment failure, a second dose with 10 mg/kg for a total dose of 20 mg/kg is indicated. The efficacy of triclabendazole is 80% to 90% (Kappagoda *et al*., 2011). In 1999, a study from Fairweather and Boray, (1999b), showed that the effect of other drugs like niclofolan, brotianide, oxyclozanide, and rafoxanide, is much weaker than that of TCBZ. During the past years, resistance against triclabendazole has been observed (Gaasenbeek *et al*., 2001; Moll *et al*., 2000; Mitchell *et al*., 1998; Winkelhagen *et al*., 2012). Although praziquantel is the preferred drug for trematodes in general, triclabendazole is the drug of choice to treat a fasciolosis because *F. hepatica* has a poor response to praziquantel (Kappagoda *et al*., 2011).

Another benzimidazole derivate is ‘Compound Alpha’ (Cpd α) or ‘Compound 6’ (Cpd 6) (5-chloro-2-methylthio-6-(1-napthyloxy)-1H-benzimidazole) having a very similar chemical structure to TCBZ (Usip *et al*., 2014; McConville *et al*., 2009). The advantage of this drug is its high impact on adult TCBZ-susceptible flukes, while the disadvantages are its low activity against juvenile TCBZ-susceptible flukes and the lack of any demonstrable activity against TCBZ-resistant flukes (McConville *et al*., 2009).

Nitazoxanide (Alinia®, Daxon®, Colufase®) (2-acetolyloxy-N-(5-nitro-2-thiazolyl)benzamide) is a nitrothiazolyl-salicylamide derivate and was first described 1984 by Francois Rossignoll (Rossignoll and Maisonneuve, 1984).

This drug is effective against a broad range of parasites including trematodes (Fox and Saravolatz, 2005). For children up to 11 years of age, the recommended dosage is 100 or 200 mg (7.5 mg/kg) twice daily for three days, and for people older than 12 years of age this is 500 mg twice daily for seven days. With a 7 day course, the cure rates are 60% (Anderson and Curran, 2007; Kappagoda *et al*., 2011).

1.3.8 Molecular typing

For *F. hepatica* no haplotypes have been established yet, but two genes, *cox1* and *nad1*, and two spacer regions, internal transcribed spacer 1 (ITS-1) and ITS-2, have been used for phylogenetic analysis.

The ITS is located between the small and the large subunit rRNA genes. In eukaryotes two types of ITS exist. ITS1 is located between 18S and 5.8S, and ITS2 is located between 28S and 5.8S (Lafontaine and Tollervey, 2001).

One of the largest studies on the diversity of *F. hepatica* was conducted by Semyenova *et al*. (2006). They examined individuals of *F. hepatica* from Eastern Europe and Western Asia and described two main lineages for *cox1* and *nad1*. The sequences were homogenous with no variation between flukes from Russia, Belarus, Armenia, Ukraine, and Turkmenistan. In general, *cox1* and *nad1* strains from Eastern Europe are rather similar to strains from Western Asia (Semyenova *et al*., 2006), while strains from Southern Europe (Spain, Italy, and Portugal) show high accordance with strains from Northern Africa (Algeria, Tunisia, and Egypt) (Martins dos Santos, 2012; Farjallah *et al*., 2013; Martínez-Valladares and Rojo-Vázques, 2014).

In 2005, Semyenova *et al*. examined the ITS-2 region of 58 *F. hepatica* and *F. gigantica* from Eastern Europe and Western Asia. They found no sequence variation between 43 individuals of *F. hepatica* from Russia, Ukraine, Armenia, Belarus, and Turkmenistan, indicating a high homogeneity.

Martins dos Santos (2012) described 12 haplotypes for *cox1* and five haplotypes for *nad1* for strains from Portugal. She revealed a low genetic

distance to strains from Tunisia and Algeria in *cox1* and a low genetic distance to sequences from Egypt and Italy in *nad1*.

In another study from Europe, Farjallah *et al*. (2013) examined the diversity of *F. hepatica* in Sardinia (Italy) by sequence comparisons of the ITS-1 and ITS-2 regions and the *cox1* and *nad1* genes. They revealed one haplotype for ITS-1, two haplotypes for ITS-2, three haplotypes for *cox1* and five haplotypes for *nad1*. In the *cox1* gene the Sardinian strains were similar to strains from France, Tunisia, Algeria, China, Australia, Uruguay, Japan, Ukraine, Turkey, Belarus, Armenia, Turkmenistan, and Russia. In the *nad1* gene the Sardinian strains were similar to strains from Russia, Belarus, Ukraine, Bulgaria, Armenia, Georgia, Azerbaijan, Turkey, China, Korea, Japan, Egypt, and Australia. The Sardinian strains show also similarities to sequences from Ireland, Egypt, the USA, and Uruguay.

Walker *et al*. (2007) examined the diversity of *F. hepatica* from Ireland for three different mitochondrial regions (*cox3*/*nad4*, *atp6*/*nad1*, and *cox1*/–rRNA) and revealed 35 haplotypes.

Walker *et al*. (2011) examined the *cox3* gene from 422 flukes from two farms from two geographical regions in the Netherlands and revealed 92 haplotypes. Two of these haplotypes were also found in samples of *F. hepatica* from Ireland, Poland, and Great Britain.

Martínez-Valladares and Rojo-Vázques (2014) examined 56 *F. hepatica* eggs for the *cox1* gene and 51 *F. hepatica* eggs for the *nad1* gene from three geographical regions in Spain. The eggs were collected from faeces of different sheep. In total, they revealed 32 haplotypes for the *cox1* gene and the *nad1* gene each.

Amer *et al*. (2011) examined flukes from Egypt and revealed 13 haplotypes for *cox1* and 14 haplotypes for *nad1*, indicating a high diversity within the population in Egypt.

Farjallah *et al*. (2009) examined *F. hepatica* from Tunisia and Algeria. They revealed identical sequences for the ITS-1 and ITS-2 regions from both

countries and a high sequence similarity of these isolates with isolates from Niger, Turkey, Ireland, Iran, and Egypt.

1.4 Aims of the study

The main aim of the present thesis was the genetic characterisation of Austrian isolates of *F. magna* and *F. hepatica.* Samples were isolated from infested animals, but included also recent and historic material from the Natural History Museum Vienna (NHMV). The genetic diversity was examined by sequencing the mitochondrial DNA (mtDNA) encoding the *cox1* and *nad1* genes. This was achieved by DNA extraction, PCR, DNA sequencing, and computer-assisted phylogenetic analyses. It was also aimed to proof the hypothesis of a low genetic diversity of *F. magna* and a high genetic diversity of *F. hepatica* in Austria.

A further aim of this thesis was to assess differences between the genera *Fasciola* and *Fascioloides* on the protein level. It is largely unknown whether the human parasite *F. hepatica* that has been endemic in Austria for hundreds of years shares important antigens with the imported and newly established parasite *F. magna*. For this reason, protein and antigen profiles were compared by SDS-Page and Western blot analysis using pooled human serum.

2. Materials & Methods

2.1 Samples

26 samples of *F. magna* (Fm), collected from various regions in the southeast of Vienna were included in this study (Tab. 2.1).

Tab. 2.1: Samples of *F. magna* with their year of collection, host, and source.

No.	Year	Host	Location[1]	N°	E°
Fm1	2013	Red deer	Markthof, LA	48°11'34.908"	16°57'26.6754"
Fm2	2013	Red deer	Markthof, LA	48°11'34.908"	16°57'26.6754"
Fm3	2013	Red deer	Witzelsdorf, LA	48°8'58.3794"	16° 50'5.8194"
Fm4	2013	Fallow deer	Mannsdorf, LA	48°9'9.2622"	16°39'35.301"
Fm5	2013	Red deer	Mannsdorf, LA	48°9'9.2622"	16°39'35.301"
Fm6	2013	Red deer	22. district, Untere Lobau, Vie	48°14'43.2"	16°56'43.7"
Fm7	2013	Red deer	22. district, Untere Lobau, Vie	48°13'51.132"	16°28'42.3366"
Fm8	2013	Red deer	Eckartsau, LA	48°8'42.8958"	16°47'50.8308"
Fm9	2013	Red deer	Orth/Donau, LA	48°8'41.3658"	16°42'5.4498"
Fm10	2013	Red deer	Fischamend, LA	48°7'7.1646"	16°36'45.4968"
Fm11	2013	Red deer	Fischamend, LA	48°7'7.1646"	16°36'45.4968"
Fm12	2013	Red deer	Fischamend, LA	48°7'7.1646"	16°36'45.4968"
Fm13	2013	Red deer	Fischamend, LA	48°7'7.1646"	16°36'45.4968"
Fm14	2014	Red deer	Markthof, LA	48°11'34.908"	16°57'26.6754"
Fm15	2014	Red deer	Markthof, LA	48°11'34.908"	16°57'26.6754"
Fm16	2014	Deer	Markthof, LA	48°11'34.908"	16°57'26.6754"
Fm17	2014	Red deer	Markthof, LA	48°11'34.908"	16°57'26.6754"
Fm18	2014	Red deer	Eckartsau, LA	48°8'42.8958"	16°47'50.8308"
Fm19	2014	Red deer	Orth/Donau, LA	48°8'41.3658"	16°42'5.4498"
Fm20	2014	Red deer	22. district, Untere Lobau, Vie	48°13'51.132"	16°28'42.3366"

[1] Vie = Vienna, LA = Lower Austria

Fm21	2014	Red deer	Fischamend, LA	48°7'7.1646"	16°36'45.4968"
Fm22	2014	Red deer	Fischamend, LA	48°7'7.1646"	16°36'45.4968"
Fm23	2014	Red deer	Orth/Donau, LA	48°8'41.3658"	16°42'5.4498"
Fm24	2014	Red deer	Petronell, LA	48°6'39.488"	16°51'24.126"
Fm25	2014	Red deer	Witzelsdorf, LA	48°8'58.3794"	16°50'5.8194"
Fm26	2014	Red deer	Engelhartstetten, LA	48°10'2.152"	16°53´01.483"

For a better overview the regions are marked on a map (Fig. 2.1).

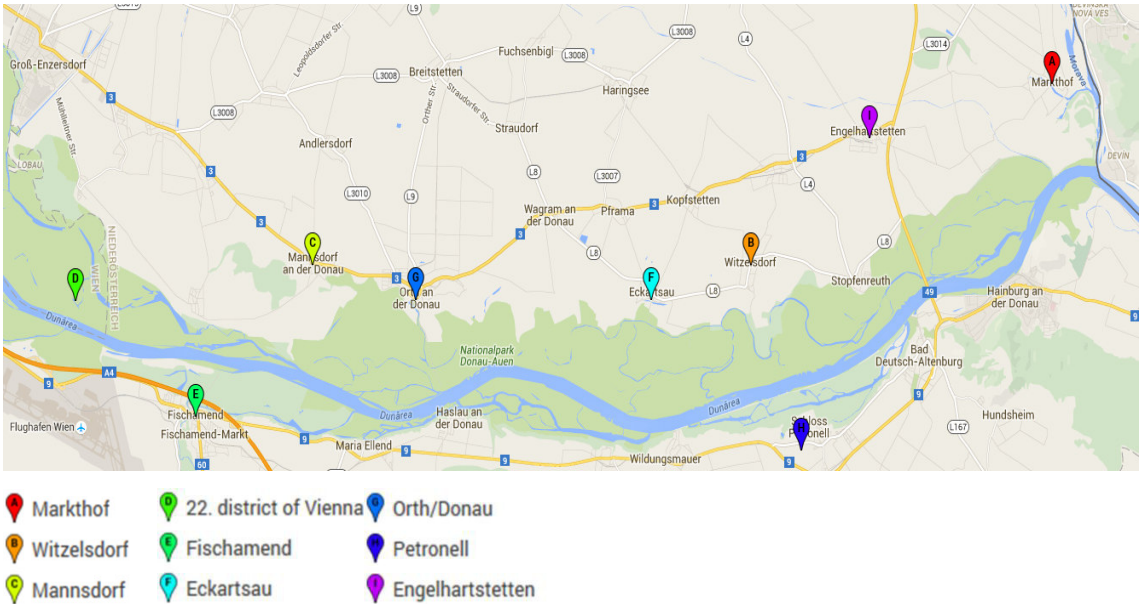

Fig. 2.1: Samples of *F. magna* from various regions in Austria.

Eight samples of *F. hepatica* (Fh) were provided by the Natural History Museum Vienna (Tab. 2.2).

Tab. 2.2: Samples of *F. hepatica* with their year of collection, host, and source.

No.	Year	host	Location[2]	N°	E°
Fh1	2004	Cattle	Mooslandl, LA	47°39'11.310"	14°45'50.985"
Fh2	2013	Cattle	Hall (Oberhall), Sty	47°34'23.613"	14°27'41.8422"
Fh3	2013	Cattle	Weissenbach/Enns, Sty	47°42'16.7688"	14°37'45.8292"
Fh4	2013	Cattle	Admont (Krumau),	47°35'0.204"	14°31'5.844"

[2] Tyr = Tyrol, Sbg = Salzburg, Ibk = Innsbruck, Car = Carinthia

			Sty		
Fh5	2013	Cattle	Johnsbach, Styr	47 31'59.2098"	14°36'16.0122"
Fh6	2013	Cattle	Weißenbach/Enns, Sty	47°42'16.7688"	14°37'45.8292"
Fh30	2015	Cattle	St. Aegyd, LA	47°51'11.47"	15°33'39.117"
Fh31	2015	Cattle	Schwarzenbach an der Pielach, LA	47°56'46.968'	15°23'0.491"

Further 13 samples of *F. hepatica* (Fh) were collected at the abattoir Alpenrind GmbH Salzburg (Tab. 2.3).

Tab. 2.3: Samples of *F. hepatica* with their year of collection, host, and source.

No.	Year	host	Location	N°	E°
Fh7	2015	Cattle	Hopfgarten im Brixental, Tyr	47°25'51.5274"	12°8'59.6034"
Fh8	2015	Cattle	Itter, Tyr	47°28'12.671"	12°8'36.472"
Fh9	2015	Cattle	Westendorf , Tyr	47°25'58.836"	12°12'55.770"
Fh10	2015	Cattle	Eugendorf, Sbg	47°52'01.679"	13°07'23.462"
Fh11	2015	Cattle	Seekirchen am Wallersee, Sbg	47°53'41.1108"	13°07'25.6542"
Fh12	2015	Cattle	Seekirchen am Wallersee, Sbg	47°53'41.1108"	13°07'25.6542"
Fh13	2015	Cattle	Eugendorf, Sbg	47°52'7.7046"	13°07'43.5828"
Fh14	2015	Cattle	Zell am Ziller, Tyr	47°14' 0.0954"	11°52'54.822"
Fh15	2015	Cattle	Feldkirchen, Car	46°43'31.136"	14°5'52.442"
Fh16	2015	Cattle	Ottenschlag, LA	48°41'55.623"	15°9'07.093"
Fh17	2015	Cattle	Ebbs, Tyr	47°37'46.2468"	12°12'53.4672"
Fh18	2015	Cattle	Söll, Tyr	47°30'13.8198"	12°11'32.5278"
Fh19	2015	Cattle	Maishofen, Sbg	47°21'39.744"	12°48'26.589"

Examined were the livers of cattle from various regions in Austria. The focus was on signs of infestations like dilated bile ducts (Fig. 1.20). To get the samples of *F. hepatica*, a section across the bile duct was performed and the flukes were drained off manually (Fig. 2.2). When the bile duct was clogged up

with calcifications and debris, the bile duct was opened in its direction to collect the samples (Fig. 2.3).

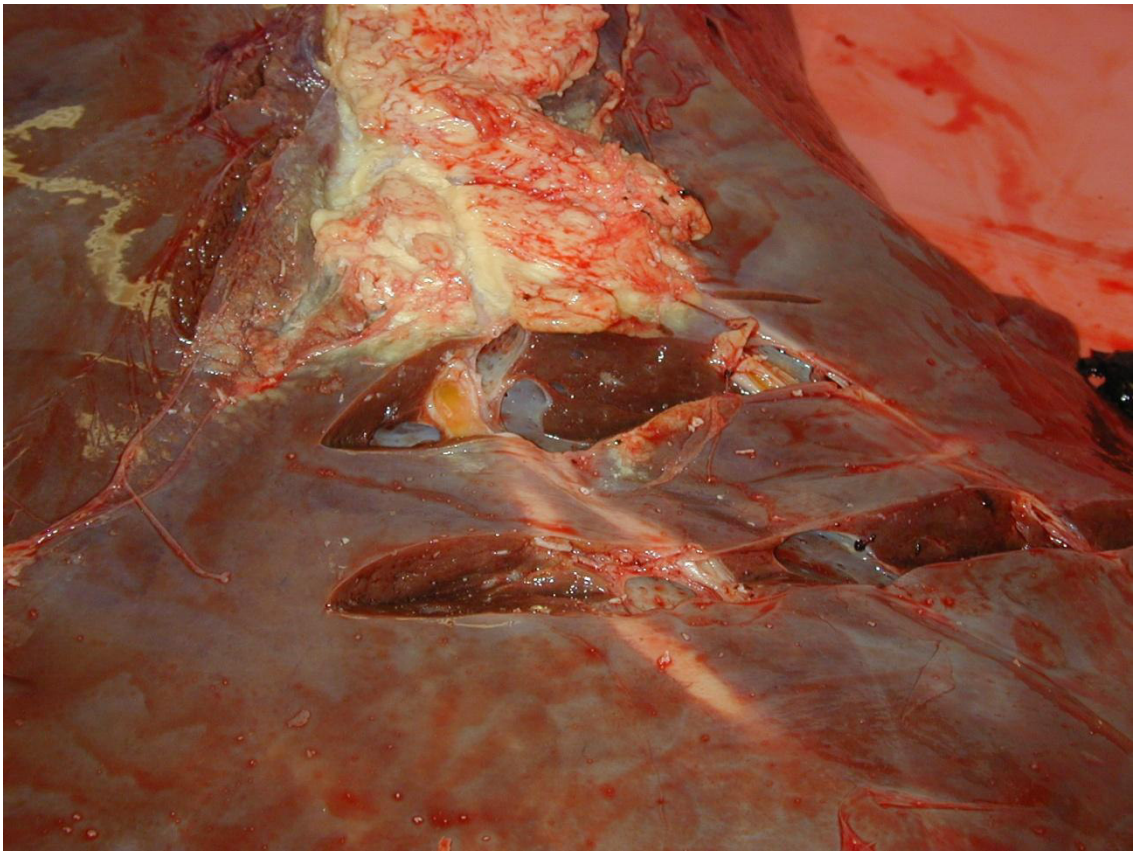

Fig. 2.2: Section of the bile ducts (orig.)**.** A cut across the bile duct was performed to get samples of *F. hepatica*.

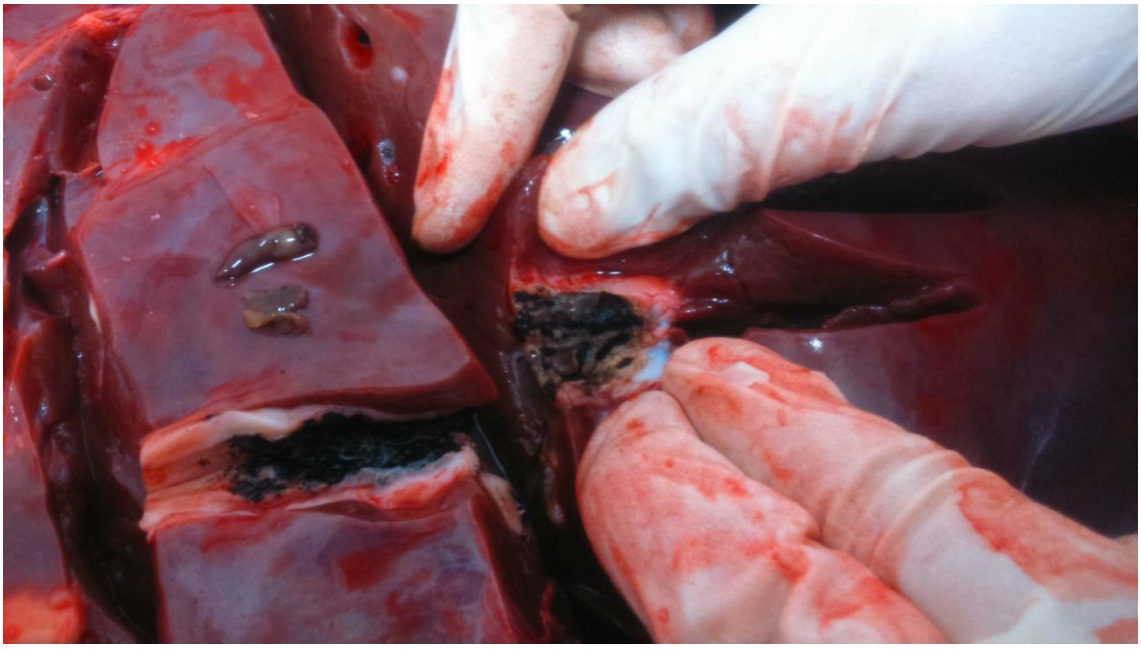

Fig. 2.3: Section of a bile duct (orig.)**.** Because the bile duct was clogged up it was opened in its direction.

Finally, also 10 samples of *F. hepatica* provided by the AGES and collected from the livers of cattle from the Tyrol were included in this study (Tab. 2.4).

Tab. 2.4: Samples of *F. hepatica* with their year of collection, host, and source.

No.	Year	host	Location	N°	E°
Fh20	2015	Cattle	Tyrol (community unknown)	47°15'13.467"	11°36'5.3532"
Fh21	2015	Cattle	Leutasch, Ibk	47°22'8.0178"	11°8'38.8458"
Fh22	2015	Cattle	Kössen, Ibk	47°40'7.251"	12°24'14.9184"
Fh23	2015	Cattle	Angerberg, Tyr	47°30'19.3392"	12°1'48.8676"
Fh24	2015	Cattle	Angerberg, Tyr	47°30'19.3392"	12°1'48.8676"
Fh25	2015	Cattle	Angerberg, Tyr	47°30'19.3392"	12°1'48.8676"
Fh26	2015	Cattle	Mühlbachl, Ibk	47°8'12.8286"	11°26'45.0852"
Fh27	2015	Cattle	Neustift im Stubaital, Tyr	47°6'32.565"	11°18'18.8856"
Fh28	2015	Cattle	Ellbögen, Tyr	47°10'3.6474"	11°27'30.639"
Fh29	2015	Cattle	Innsbruck, Tyr	47°16'9.1632"	11°24'14.7666"

For a better overview the regions are marked on a map (Fig. 2.4).

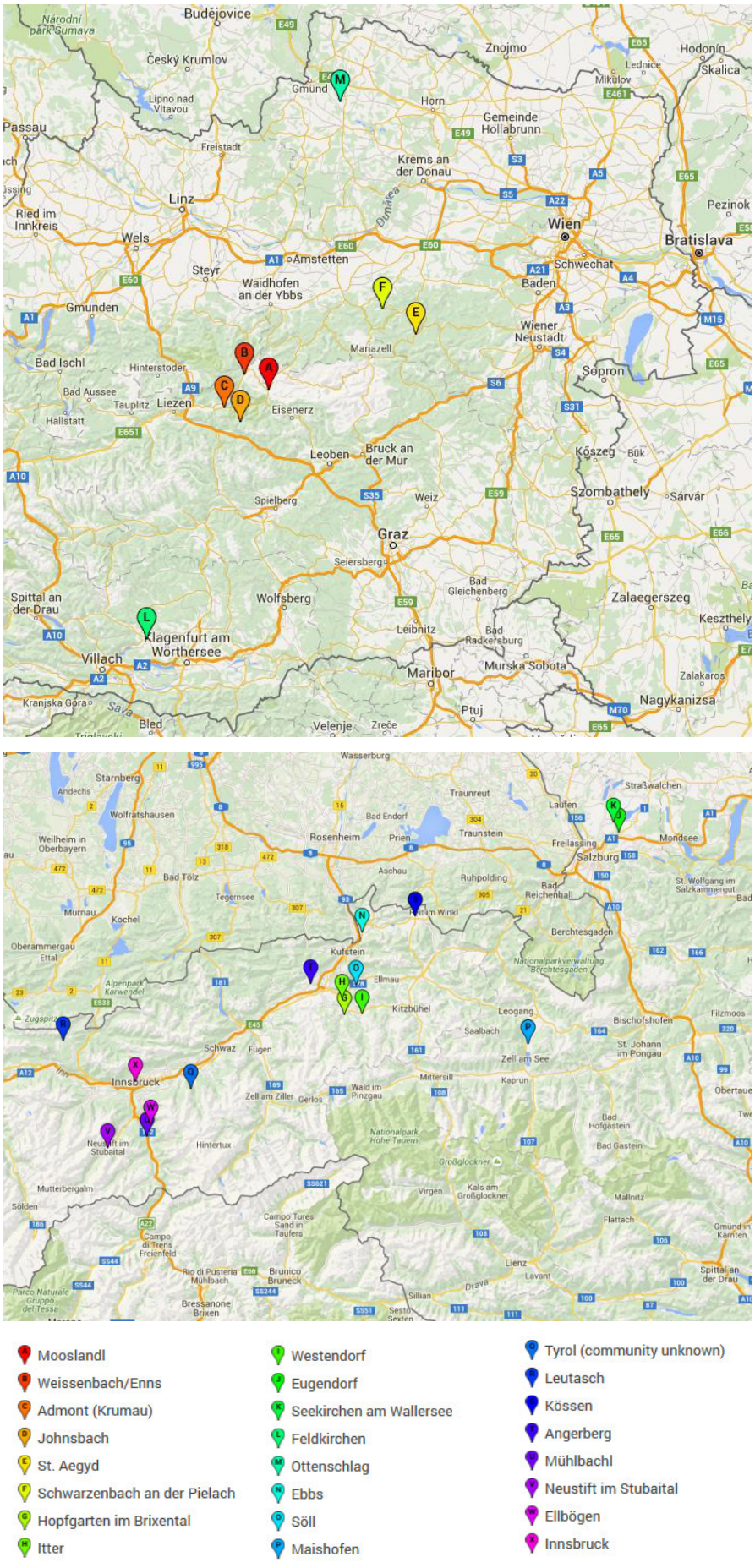

Fig. 2.4: Samples of *F. hepatica* from various regions in Austria.

2.2 Molecular biological techniques

In this study, partial sequences of the *cox1* and *nad1* genes were examined.

2.2.1 Primers

2.2.1.1 *F. magna*

To amplify a fragment of the mtDNA genes *cox1* and *nad1* the primer pairs from Królová-Hromadová *et al.* (2008), were used (Tab. 2.5):

Tab. 2.5: Primers for PCR amplification of mitochondrial genes *cox1* and *nad1* of *F. magna*. The expected size of the *cox1* amplicon is 439 bp and of the *nad1* amplicon 405 bp.

Gene	Primer	Sequence	CG%	Melting temperature (T_M) (°C)
cox1	FmCOX1 fwd *(FM_COX1_VAR1_F)*	5´-GGTCATGGGGT TATAATGA-3´	42.11	54
cox1	FmCOX1 rev *(FM_COX1_VAR1_R)*	5´-ACAGCATAGTA ATAGCCGC-3´	47.37	56
nad1	FmNAD1 fwd *(FM_NAD1_VAR1_F)*	5´-TTATGTGTGTT GTTGTTTTG-3	30.00	52
nad1	FmNAD1 rev *(FM_NAD1_VAR1_R)*	5´-CTACAACACAT AAAAAAAAG-3´	25.00	50

2.2.1.2 *F. hepatica*

The primers were chosen from the literature, the forward primer for *cox1* from Itagaki *et al.* (1998), the reverse primer for *cox1* and the primers for *nad1* from Semyenova *et al.* (2006) (Tab. 2.6).

Tab. 2.6: Primers for PCR amplification of mitochondrial genes *cox1* and *nad1* of *Fasciola hepatica*. The expected size of the *cox1* amplicon is 429 bp and of the *nad1* amplicon 316 bp.

Gene	Primer	Sequence	CG %	Melting temperature (T_M) (°C)
cox1	FhCOX1 fwd *(no orig. name)*	5´-TTGGTTTTTTGGGCATCCT-3´	42.11	54
cox1	FhCOX1 rev *(no orig. name)*	5´-AGGCCACCACCAAATAAAAGA-3´	42.86	60
nad1	FhNAD1 fwd *(no orig. name)*	5´-TATGTTTTGTACGGGATGAG-3´	40.00	56
nad1	FhNAD1 rev *(no orig. name)*	5´-AACAACCCCAACCAACACTTA-3´	42.86	60

All primers were ordered from Microsynth AG Switzerland (http://www.microsynth.ch/) and used in a dilution of 10 pmol/µl.

2.2.2 DNA isolation

In order to isolate DNA from *F. magna* and *F. hepatica*, the QIAamp DNA Mini Kit 250 (QIAGEN, Vienna) was used.

In brief, 20–25 mg tissue of the respective fluke was cut into small pieces with a scalpel. The tissue was taken from the apical zone to avoid inclusion of genitals that could contain foreign sperm (Moazeni *et al*., 2012).

180 µl buffer ATL and 20 µl Proteinase K were added to the samples and incubated on a shaker (Thermomixer comfort, Eppendorf, Hamburg, Germany) at 56 °C with 450 rpm (revolution per minute) over night. After a centrifugation step with the 1-15 Microfuge (Sigma-Aldrich, St. Louis, USA) for 1 min with 6,000 x g (times gravity), 200 µl buffer AL was added and after a 15 sec pulse-vortexing the samples were incubated at 70 °C for 10 min. After an additional centrifugation step 200 µl of 100% ethanol were added and pulse-vortexed again for 15 sec. After centrifugation, the sample was added into the QIAamp Mini spin column and centrifuged. Then the column was washed with 500 µl buffer AW1 and after a centrifugation step, the column was washed with 500 µl AW2 and centrifuged with 18,000 x g for 3 min. Following an additional centrifugation step with 16,000 x g for 1 min, the column was washed with 200 µl buffer AE, incubated at room temperature for 1 min and centrifuged with 6,000 x g for 1 min. The amount of the DNA was measured using the NanoDrop ND-1000 spectrophotometer (Peqlab, Erlangen, Germany) and then was stored at -20 °C.

2.2.3 PCR

All pipetting steps were performed in a sterile bench (Heraeus, Germany).
For every PCR, a mastermix was prepared and mixed with sterile ddH_2O and different amounts of DNA (1 µl, 3 µl, 6 µl) to a reaction volume of 50 µl. Sterile ddH_2O without DNA was used as negative control. The positive control was done by morphological identification of the flukes. For the mastermix, 11 µl ddH_2O, 5 µl PCR buffer (Solis Biodyne), 5 µl $MgCl_2$ (25 mM), 5 µl forward primer (1 µM), 5 µl reverse primer (1 µM), 1 µl dNTP´s (20 mM) and 0.25 µl polymerase (Hot Fire DNA Polymerase I, 5 U/µl, Solis Biodyne) were used. The PCR reaction was performed in 0.2 ml soft PCR tubes using a thermocycler from Eppendorf programmed for 15 min initial denaturation with 95 °C and 30 cycles with denaturation at 95 °C for 1 min, annealing at 56 °C for 2 min, extension at 72 °C for 3 min and final extension at 72 °C for 7 min. Because of good results of previous test-PCR´s (data not shown), the temperature specifications were used for all primer pairs.

2.2.4 Gel electrophoresis

DNA was separated in a 2% agarose gel. In brief, 1 g or 2 g agarose (Sigma-Aldrich Inc., Saint Louis, MO, USA) were dissolved in 50 ml or 100 ml 1x TAE (Tris-acetate-EDTA) buffer respectively (Carl Roth GmbH + Co. KG, Germany). After a short boiling period with a magnetic stirrer and a short cooling period, 3 µl or 5 µl (according to volume of 1x TAE buffer) GelRed (GeneOn GmbH, Germany) were added under stirring conditions to guarantee a uniform distribution in the liquid. After polymerisation of the agarose gel, the slots were loaded with a mixture of PCR product and 10x DNA loading buffer (Bromophenol blue), in a 1:10 ratio (loading buffer : PCR product) up to a volume of 30 µl. To validate the size of the amplicons one slot was loaded with 20 µl of a Step marker (50–3000 bp, Sigma-Aldrich Inc., Saint Louis, MO, USA). The electrophoresis was performed in an electrophoresis chamber (BIO-RAD Wide Mini-Sub Cell GT/Mini Sub Cell GT) containing 1x TAE buffer, using 100 V/300 mAmp.

In order to visualize the bands, the agarose gel was exposed to UV light, and for further usage, the gel bands were cut out using a scalpel.

2.2.5 Purification of agarose gel bands

The amplicons were purified according to the manufacture protocol of QIAquick® Gel Extraction Kit (250) (Qiagen, Vienna).
The gel bands were weighted, and 2 volumes of Buffer GQ were added to 1 volume of gel. After incubation for 10 min at 50 °C, or until the gel was dissolved, 1 gel volume of isopropanol was added. Following a centrifugation step in a column for 1 min with 16,000 x g and discarding the flow-through, the column was washed with 750 µl Buffer PE and centrifuged for 1 min with 16,000 x g. An additional centrifugation step for 1 min with 16,000 x g was performed to remove Buffer PE completely and 50 µl Buffer EB was added and centrifuged for 1 min to elute the DNA. The samples were stored at -20 °C.

2.2.6 Sequencing PCR

Sequencing PCRs were run with 3 µl of the respective PCR product mixed with 2 µl AB mix, 2 µl forward or reverse primer (10 pmol) and 1 µl AB Big Dye® Terminator 5x sequencing buffer (Applied Biosystems, Austria) and filled up with ddH_2O to a total volume of 10 µl. The PCR amplification started with an initial denaturation phase at 96 °C for 30 sec followed by 40 cycles of denaturation at 96 °C for 10 sec, annealing phase at 50 °C for 5 sec and an elongation phase at 60 °C for 4 min.

2.2.7 Purification of sequencing PCR products

Following the PCR amplification, the PCR products were transferred into 1 ml tubes and mixed with 1 µl sodium acetate buffer and 33 µl 100% ethanol for precipitation. After an incubation period of 17 min on ice, the samples were centrifuged with 13,000 x g, 4 °C, for 30 min. The supernatant was discarded and the remaining pellet was washed with 90 µl of 70% ethanol. An additional

centrifugation step with 13,000 x g, 4 °C, 10 min, followed. The supernatant was discarded and the sample tubes were air-dried for 3 min. 20 µl HiDi Formamide (Applied Biosystems, Austria) were added, the remaining alcohol was evaporated and the tubes were incubated at 95 °C for 5 min on a heat block and then incubated for 5 min on ice. The tubes were stored at 4 °C until they were used for sequencing. The sequencing was performed in an automated ABI PRISM 310 Sequencer (PE Applied Biosystems, Langen, Germany).

2.2.8 Sequence analysis

For phylogenetic investigation, all sequences obtained were first blasted against sequences of trematodes available in GenBank by using BLAST (Altschul *et al.*, 1990). For haplotype identification, multiple alignments were performed using ClustalX (Thompson *et al.*, 1997) and manually edited with GeneDoc (Nicholas *et al.*, 2007). For the comparison of the obtained sequences of *F. magna* and *F. hepatica* with sequences from GenBank wobble bases were excluded.

2.2.9 Establishment of a *cox1* and *nad1* haplotype system for *F. hepatica*

As for *F. hepatica* no widely accepted haplotype system has been established yet, all sequences available at GenBank were compared to one another and a haplotype system was established, cut off: 1 bp difference. The sequences for the *cox1* gene of *F. hepatica* can be divided into two groups (group A and B), based on the localisation of the respective fragment within the gene (group A: bp 687-bp 1,000, group B: bp 35-bp 487) and the available sequences for the *nad1* gene by one group (group C: bp 6-bp 277) (Tabs. 2.7, 2.8, 2.9). For the present study, primers were chosen for group A because group B is only represented by strains from Egypt, Iran, and Peru. The fragment lengths are 313 bp for the *cox1* gene and 271 bp for the *nad1* gene. However, as the sequences available at GenBank considerably vary in length only a fragment of 313 bp for *cox1* and 271 bp for *nad1*, being available for all sequences, could be used for set-up the typing system.

Tab. 2.7: Differences of base pairs among the *cox1* haplotypes of group A.

	A1	A3	A3	A4	A5	A6	A7	A8	A9	A10	A11	A12	A13
A1													
A2	1												
A3	4	3											
A4	4	3	6										
A5	3	2	5	1									
A6	25	24	23	22	23								
A7	4	3	6	2	1	20							
A8	3	2	5	3	2	22	3						
A9	2	1	4	4	3	23	4	1					
A10	4	3	2	6	5	23	6	5	7				
A11	4	3	2	6	5	22	6	5	4	2			
A12	4	3	2	6	5	22	6	5	4	2	2		
A13	3	2	1	5	4	21	5	4	3	1	1	1	

Tab. 2.8: Differences of base pairs between the *cox1* haplotypes of group B.

	B1	B2	B3	B4	B5	B6	B7	B8	B9	B10	B11	B12	B13
B1													
B2	8												
B3	9	2											
B4	8	1	3										
B5	9	3	4	3									
B6	5	7	9	8	9								
B7	5	5	2	1	2	7							
B8	8	6	7	6	7	9	5						
B9	4	6	5	5	7	5	4	6					
B10	9	5	6	5	6	10	4	1	7				
B11	10	6	7	6	7	11	5	2	8	1			
B12	8	6	7	6	7	9	5	2	8	1	2		
B13	8	4	5	4	5	9	3	2	6	1	2	2	

Tab. 2.9: Differences of base pairs among the *nad1* haplotypes of group C.

	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12	C13	C14	C15	C16	C17	C18	C19	C20
C1																				
C2	21																			
C3	20	1																		
C4	20	4	3																	
C5	20	4	3	2																
C6	20	4	3	2	2															
C7	20	4	3	2	2	2														
C8	19	3	2	1	1	1	1													
C9	21	3	4	5	5	5	5	3												
C10	21	3	4	5	5	5	5	4	2											
C11	20	2	3	4	4	4	4	3	1	1										
C12	19	3	2	3	3	3	3	2	2	2	1									
C13	22	4	5	6	6	6	6	5	3	1	1	3								
C14	18	3	2	2	2	2	2	1	4	4	3	2	4							
C15	20	4	3	4	4	4	4	3	3	3	2	2	3	3						
C16	20	4	3	4	4	4	4	3	5	3	2	1	4	1	2					
C17	19	2	1	2	2	2	2	1	3	5	4	3	4	3	2	2				
C18	20	3	2	3	3	3	3	2	4	4	3	2	4	2	1	1	1			
C19	19	5	4	3	3	3	3	2	6	6	5	4	7	3	5	2	2	1		
C20	18	4	3	2	2	2	2	1	5	5	4	3	6	2	1	4	2	1	2	

2.2.10 Protein extraction from tissue

For protein analysis, 300 mg of *F. magna* and *F. hepatica*, respectively, were hackled with a scalpel and homogenised with glass beads in 1.5 ml of PBS (phosphate buffered saline) in the tissue homogeniser Precellys 24 (Bertin Technologies) with 6 x 20 sec shaking and 2 min cooling at 4 °C. After a centrifugation step at 13,000 x g, 30 min, 4 °C, the supernatants were transferred into a 2 ml Eppendorf tube and precipitated according to Pumidonming *et al.* (2014) with adaptations:
In brief, the supernatants were resuspended in 10% trichloroacetic acid (w/v) (Sigma-Aldrich Inc., Saint Louis, MO, USA) in acetone (Merck KGaA, Darmstadt, Germany) in 2 ml Eppendorf tubes and incubated for 90 min at -20 °C. After a centrifugation step at 13,000 x g, 30 min, and 4 °C, the supernatant was removed and the pellet was washed twice with 90% (v/v) acetone in ddH_2O, followed by a centrifugation step at 13,000 x g, 30 min, and 4 °C. Following air drying, the proteins were resuspended at RT (room temperature) in SDS sample solubilisation buffer (1% (w/v) SDS, 100 mM Tris-HCl, pH 9.5, in ddH_2O). After another centrifugation step at 13,000 x g, 1 hour, and 4 °C, the supernatant was mixed with 4x SDS-PAGE loading buffer and heated to 95 °C for 10 min. Afterwards the mixture was loaded onto a 12.5% SDS gel as described below.

2.2.11 Isolation of membrane proteins

The extraction was performed according to an adapted protocol from Bordier, (1981). The tissues were transferred into ice cold PBS with 1 mM PMSF (phenylmethylsulfonyl fluoride) (Merck KGaA, Darmstadt, Germany), 1 mM EDTA (ethylenediaminetetraacetic acid), 1 mM DL-DTT (DL-dithiothreitol) and 1% Triton X-114 (all purchased from Sigma-Aldrich Inc., Saint Louis, MO, USA). After incubation at 4 °C for 90 min, and vortexing steps every 10 min, the samples were incubated at 30 °C for 10 min. After clouding of the solution, another centrifugation step followed at 10,000 x g, 5 min, at RT. Afterwards the

detergent phase was isolated, and the proteins were precipitated as described above.

2.2.12 SDS-polyacrylamide gel electrophoresis and immunoblotting

SDS-polyacrylamide gel electrophoresis was performed using a Mini-Protean® 3 Cell (Bio-Rad, CA, USA). Proteins were separated in 12.5% acrylamide with 20 mA, at RT, including a High Range Rainbow™ Molecular Weight Marker (RPN756E, 12–225 kDa, Amersham Biosciences Europe GmbH, Freiburg, Germany). For protein profile analysis, the SDS-PA gel was stained with Coomassie Brilliant blue R-250 (Sigma-Aldrich, Saint Louis, MO, USA) and for immunoreactivity the proteins were transferred onto a PVDF membrane (polyvinylidene difluoride) (Bio-Rad, CA, USA) via semidry blotting with 220 mA for 2 hours. For transfer, Western transfer buffer (for 10x: 250 mM Tris-HCl, 1.92 M glycine in ddH_2O) was mixed with methanol in a dilution of 1:2 (10x buffer : methanol). To verify the protein transfer the membrane was stained with Ponceau S (Sigma-Aldrich, Saint Louis, MO, USA). The unspecific binding sites were blocked with blocking buffer (PBS, 3% bovine serum albumin (BSA), 0.1% Tween 20) for 1 hour at RT on a rocking platform. The membranes were incubated with pooled human serum (10 adult individuals without worm infestations) diluted with blocking buffer at a dilution of 1:100, overnight on a rocking platform at 4 °C. After three washing steps with washing buffer (PBS, 0.1% Tween-20) the membrane was incubated with horseradish peroxidase-conjugated Affini-Pure Goat Anti-Human IgG (Jackson ImmunoResearch Laboratories, San Francisco, USA), at a dilution of 1:100 with blocking buffer, for 2 hours on a rocking platform. The reactivity was made visible by developing the membrane with 4-chloro-1-naphtole (Sigma-Aldrich, Saint Louis, MO, USA) and H_2O_2 in 1x TAE on a shaker.

3. Results

3.1 PCR

Representative PCR products from the samples Fm1-Fm13 of *F. magna* in a dilution of 1:50 is shown in figure 3.1. The amplicon size of the respective fragment of the *cox1* gene is 439 bp and of the *nad1* gene 405 bp.

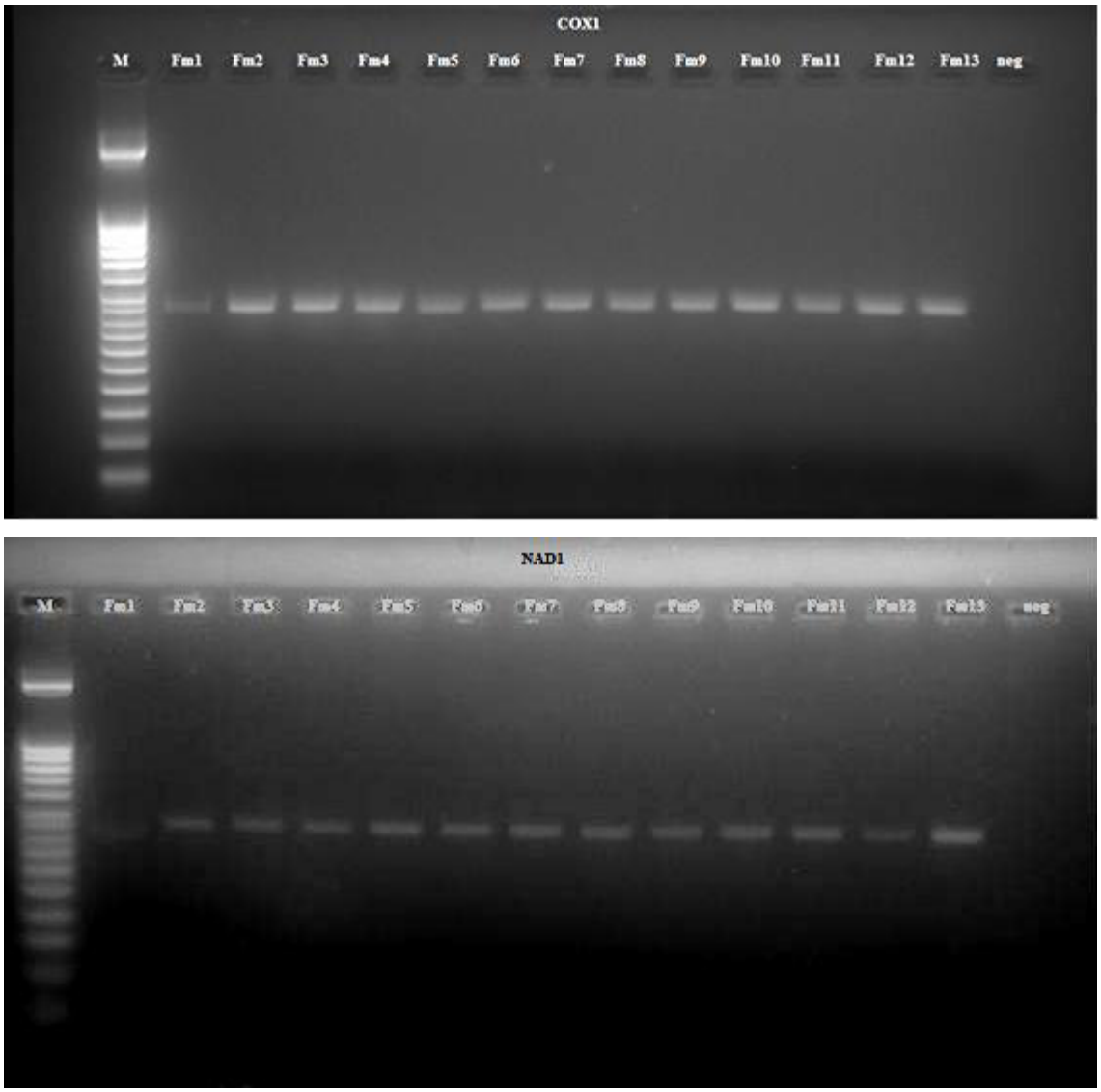

Fig. 3.1: PCR with samples of *F. magna* with a dilution of 1:50 with primers for the *cox1* and the *nad1* genes.

Representative PCR products from the samples Fh1-Fh6 of *F. hepatica* in a dilution of 1:50 is shown in figure 3.2. The amplicon size of the fragment of the *cox1* gene is 429 bp and of the *nad1* gene 316 bp.

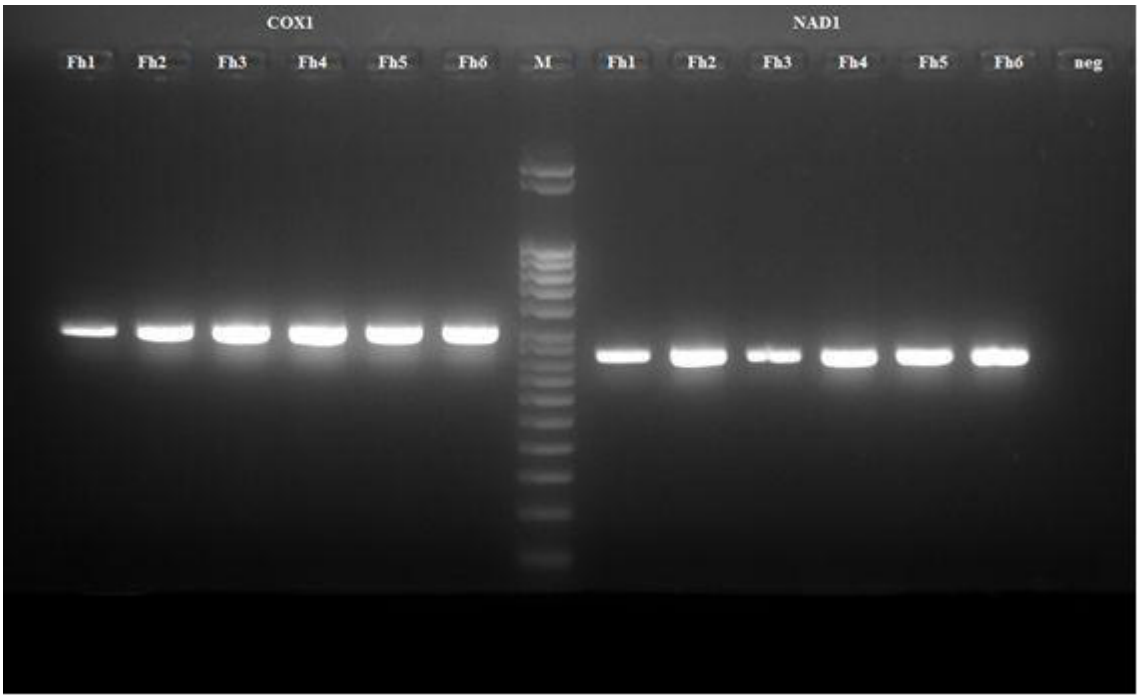

Fig. 3.2: PCR with samples of *F. hepatica* with a dilution of 1:50 with primers for the *cox1* and the *nad1* genes.

3.2 Diversity of *Fascioloides magna*

All 26 samples investigated had identical sequences (272 bp for *cox1* and 172 bp for *nad1* respectively because sequences were cut to receive a uniform length for all sequences) and are of the same haplotype, namely COX1-Ha3/NAD1-Ha4 (Fig 3.3, Fig. 3.4). The sequencing results yielded in some wobble bases in the sequences Fm3, Fm6, Fm8-Fm12, Fm15, Fm17, Fm19, Fm23–Fm26 but the wobble bases do not compromise the assignment to the haplotypes for the *cox1* gene and the *nad1* gene.

3.2.1 Alignment COX1

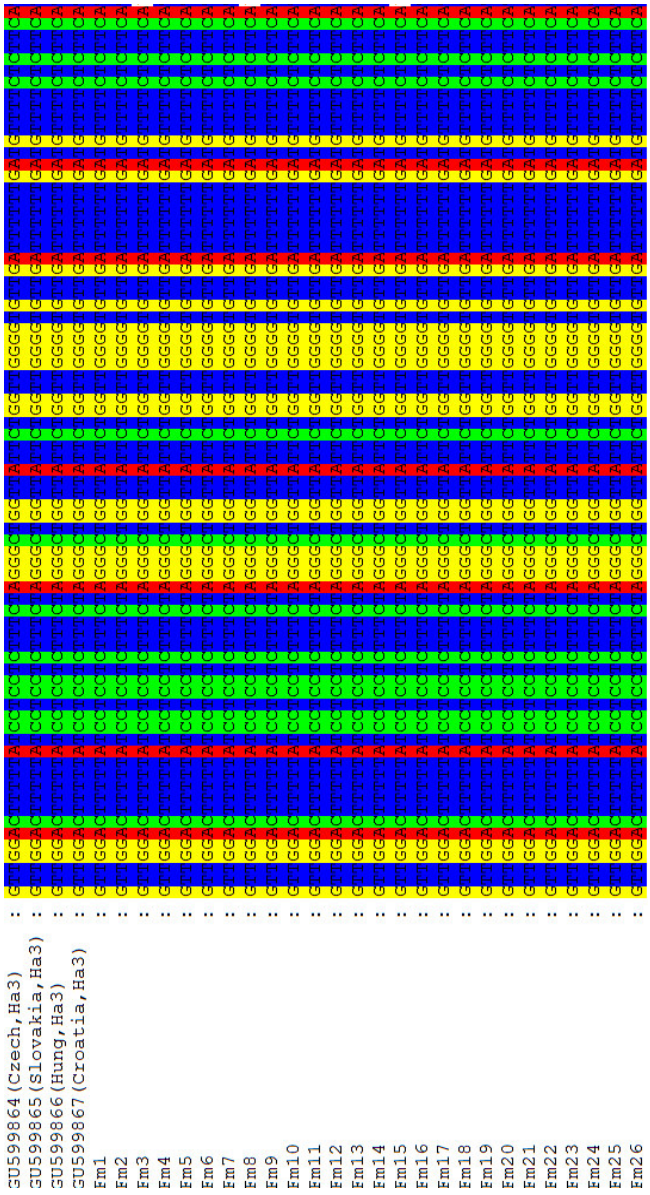

Fig. 3.3: Alignment of a fragment of the *cox1* gene of the investigated strains with reference strains for haplotype 3 of *F. magna*.
For a better overview only a part of the most similar reference strains is shown.

3.2.2 Alignment NAD1

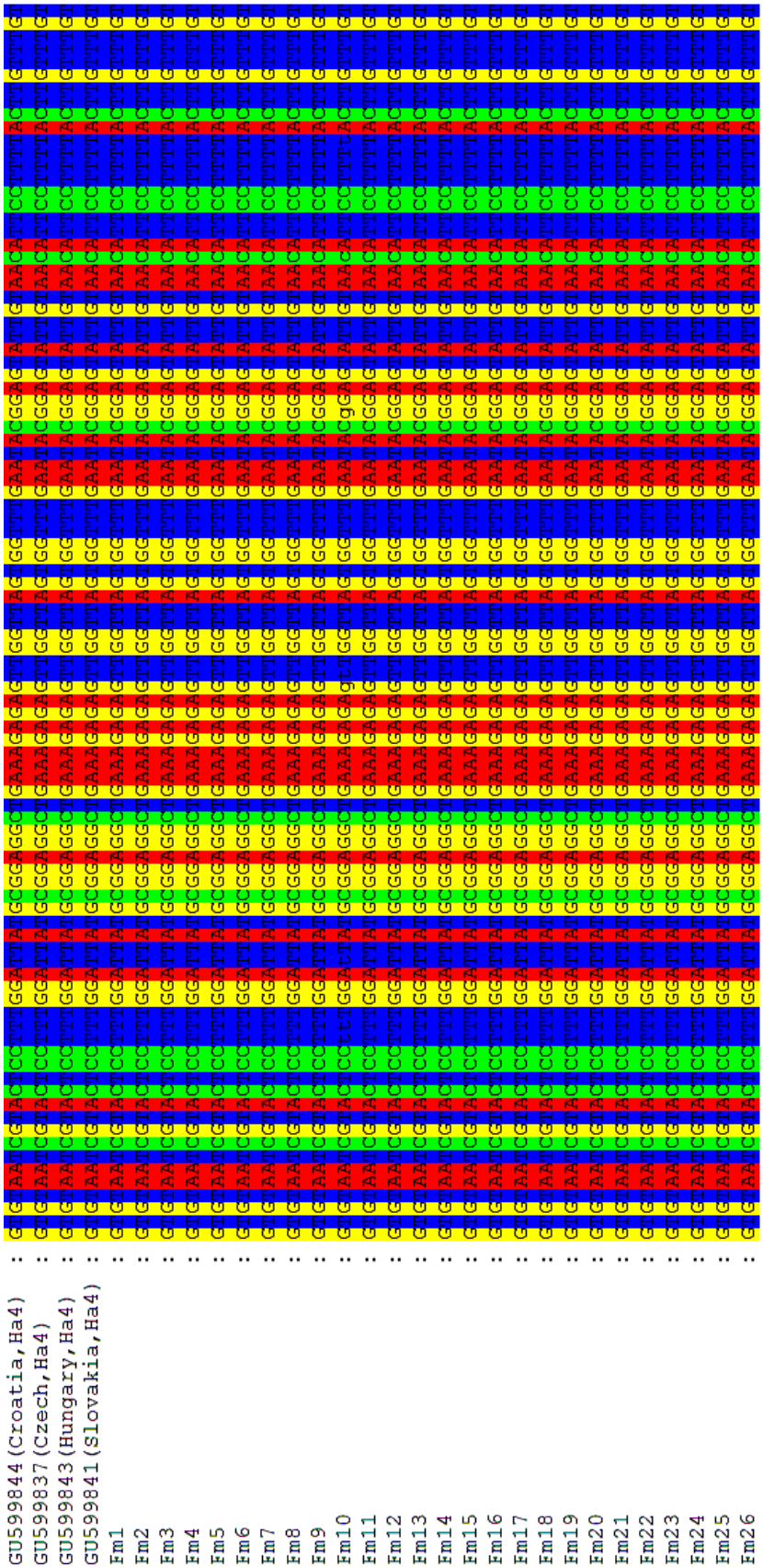

Fig. 3.4: Alignment of a fragment of the *nad1* gene of the investigated strains with reference strains for haplotype 4 of *F. magna*.
For a better overview only a part of the most similar reference strains is shown.

3.3 Diversity of *Fasciola hepatica*

3.3.1 New haplotype system

For *cox1* the available sequences were divided into two main groups, corresponding to two different regions within the *cox1* gene. The established *cox1* and *nad1* haplotypes for *F. hepatica* are shown in tabs. 3.1-3.3. The mitochondrial genome of *F. hepatica* has a length of 14,461 bp. The position of the *cox1* gene in the mitochondrial genome is from bp 6,871–bp 8,402, and the position of the *nad1* gene is in the range of bp 5,176–bp 6,078.

Tab. 3.1: *cox1* haplotypes of *F. hepatica* (Group A). Cut off: one base pair difference. Position in the *cox1* gene: bp 687–bp 1,000.

Haplotype	Isolate	Known occurrence	Citation	Exemplary sequences
A1	Ar1	Tunisia	Farjallah, *et al*., 2009	GQ231551
A2	Gf7 Ag3 FhFrG1	Tunisia Algeria France	Farjallah, *et al*., 2009 Dong *et al*., unpublished	GQ231548 GQ231549 AJ628039
A3	FhFG5 FhFG8 FspGXG3 FspGXG10 FspGXG12 FspGXG6	France	Ai *et al*., 2011	GU112488 GU112481 GU112476
A4	H27	Spain	Martínez-Valladares and Rojo-Vázques, 2014	KF111621
A5	Ar5 FhCOI-H3 FhNJG3 FhGSG2 H14	Tunisia Italy China Spain Australia	Farjallah *et al*., 2009 Farjallah *et al*., 2013 Dong *et al*., unpublished Martínez-Valladares	GQ231550 JF824674 AF216697

	H29 Geelong strain		and Rojo-Vázques, 2014 Le *at al*., 2000	
A6	FhGSG17 FhGSG18 FhGSG19	China	Ai *et al*., 2011	GU112484 GU112485 GU112486
A7	FhNJG4 FhGSG1	China	Dong *et al*., unpublished	AJ628036 AJ628037
A8	FhCOI-H1	Italy	Farjallah *et al*., 2013	JF824670
A9	FhCOI-H2	Italy	Farjallah *et al*., 2013	JF824672 JF824673
A10	FhAM1	USA	Ai *et al*., 2011	GU112482
A11	FhAM2	USA	Ai *et al*., 2011	GU112483
A12	Fh23.3	Spain	Ai *et al*., 2011	GU112457
A13	Fh2.3 Fh2.5 Fh84.4 FhCTO6 FhCTE5 FhCMA3	Spain Niger	Ai *et al*., 2011	GU112454 GU112470 FJ469984

The comparison of the sequences show that the three strains of the type A6 (China) have the lowest relationship to all other sequences with a sequence difference of up to 25 bp. Other strains from China show a much lower difference to other haplotypes (up to 6 bp).

Tab. 3.2: *cox1* haplotypes of *F. hepatica* (Group B). Cut off: one base pair difference. Position in the *cox1* gene: bp 35-bp 487.

Haplotype	Isolate	Known occurrence	Citation	Exemplary sequences
B1	Fh-Co1-Eg8	Egypt	Amer *et al.*, 2011	AB553816
B2	Sh2 Sw4	Peru	Reyna and Sanabria, unpublished	KJ716916 KJ716923
B3	Sh3	Peru	Reyna and Sanabria, unpublished	KJ716917
B4	Fh-Co1-Eg13	Egypt	Amer *et al.*, 2011	AB553812
B5	Fh-Co1-Eg9	Egypt	Amer *et al.*, 2011	AB553815
B6	Fh-Co1-Eg10	Egypt	Amer *et al.*, 2011	AB553819
B7	Ca1 Ca2 Ca3 Ca4 Sw5 Sh1 Fh-Co1-Eg1 Fh-Co1-Eg2 Geno-4 camel-B	Peru Egypt Iran	Reyna and Sanabria, unpublished Amer *et al.*, 2011 Moazeni *et al.*, 2012 Moazeni *et al.*, unpublished	KJ716910 AB553825 GQ398054
B8	Fh-Co1-Eg12 Geno-3	Egypt Iran	Amer *et al.*, 2011 Moazeni *et al.*, 2012	AB553823 GQ398053
B9	Fh-Co1-Eg7	Egypt	Amer *et al.*, 2011	AB553814
B10	Camel-A Persian-1 Ca5 Fh-Co1-Eg1 Genosheep-1 Geno-1	Iran Peru Egypt	Moazeni *et al.*, unpublished Sharifiyazdi *et al.*, 2012 Reyna and Sanabria,	FJ895605 FJ895604 KJ716914

			unpublished Amer *et al*., 2011 Moazeni *et al*., 2012	
B11	Fh-Co1-Eg5	Egypt	Amer *et al*., 2011	AB553817 AB553818
B12	Fh-Co1-Eg4	Egypt	Amer *et al*., 2011	AB553820 AB553821
B13	Gencattle-2 Geno-2 Fh-Co1-Eg	Iran Egypt	Sharifiyazdi *et al*., unpublished Moazeni *et al*., 2012 Amer *et al*., 2011	GQ398056 GQ398052 AB553826

Group B is represented only by strains from Egypt, Peru, and the Iran.

Tab. 3.3: *nad1* haplotypes of *F. hepatica* (Group C). Cut off: one base pair difference. Position in the *nad1* gene: bp 6-bp 277.

Haplotype	Isolate	Known occurrence	Citation	Exemplary sequences
C1	G4-3	China	Peng *et al*., 2009	AB477369
C2	LSNad7	Spain	Martínez-Valladares and Rojo-Vázques, 2014	KF111637
C3	LSNad8	Spain	Martínez-Valladares and Rojo-Vázques, 2014	KF111630
C4	U3-1 H1-1 FhND1-Eg10	China Egypt	Peng *et al*., 2009	AB477362 AB477357 AB554185
C5	Fh-C10	China	Ichikawa *et al*., unpublished	AB604929

C6	Fh-C7	China	Ichikawa *et al*., unpublished	AB604926
C7	Fh-C8	China	Ichikawa *et al*., unpublished	AB604927
C8	U1-2	China	Peng *et al*., 2009	AB477361
C9	FhN11	Poland	Norbury *et al*., unpublished	KR422390
C10	FhN1 FhN12 FhN13 FhN14 FhNDI-H3 FhND1-Eg2 IRFhN_0007 IRFhN_0030 FhNWey	Poland Italy Egypt Iran UK	Norbury *et al*., unpublished Farjallah *et al*., 2013 Akhlaghi *et al*., unpublished Norbury *et al*., unpublished	KR422392 JF824677 AB554179 KR422397
C11	complete genome unnamed FhNDI-H2	Ireland Italy	Itagaki *et al*., 2005a Farjallah *et al*., 2013	AB207156 JF824676
C12	FhND1-Eg9 ND1-P2	Egypt Peru	Amer *et al*., 2011 Ichikawa-Seki, unpublished	AB554192 LC070667
C13	FhNDI-H5	Italy	Farjallah *et al*., 2013	JF824679
C14	Ovine-B FhND1-Eg3 FhND1-Eg12 FhND1-Eg14	Iran Egypt	Amer *et al*., 2011 Sharifi Yazdi *et al*., unpublished Amer *et al*., 2011	AB554191 GQ175364 AB554187
C15	Ovine-A U1-1 FhNDI-H1a FhNDI-H1b Fh-ND1-Eg1	Iran China Italy Egypt Australia	Sharifi Yazdi *et al*., unpublished Peng *et al*., 2009 Farjallah *et al*., 2013	GQ175363 JF824675 AB207155

	Kor1 H2-1 Fh-C11 FhND1-Eg13	South Korea	Amer *et al*., 2011 Itagaki *et al*., 2005a Itagaki *et al*., 2005b	
C16	Ovine-C	Iran	Sharifi Yazdi *et al*., unpublished	GQ356033
C17	FhNDI-H4	Italy	Farjallah *et al*., 2013	JF824678
C18	H4-1	China	Peng *et al*., 2009	AB477359
C19	FhND1-Eg11	Egypt	Amer *et al*., 2011	AB554181
C20	FhND1-Eg8	Egypt	Amer *et al*., 2011	AB554186 AB554183

Haplotype C1 (China) shows the highest difference to all the other groups (up to 48 bp).

3.3.2 Assignment of the Austrian isolates to haplotypes

To determine to which haplotypes the Austrian sequences belong, 313 bp fragments for the *cox1* gene and 271 bp fragments for the *nad1* gene were examined and yielded in a 100% identity to different haplotypes (Tabs. 3.4, 3.5, 3.6).

The 31 individuals of *F. hepatica* investigated could be grouped into two different clusters for *cox1* and into three different clusters for *nad1*. The most common haplotype for *cox1* and *nad1* was represented by 21 individuals and the second most common by 10 individuals for *cox1* and nine individuals for *nad1*. One haplotype for *nad1* was represented only by one individual. Differences in the sequences were more pronounced in the *nad1* gene than in the *cox1* gene. Styria, the Tyrol, and Salzburg revealed all haplotypes except of one. Upper Austria, Lower Austria, and Carinthia only revealed one haplotype for each gene. The haplotype NAD1-C18 was found only in the Tyrol (Tab. 3.4). Thus, the Tyrol had the overall highest diversity of haplotypes.

For *cox1*, the Austrian flukes can be identified as belonging to the following haplotypes:

- COX1-A2 (21 isolates)
- COX1-A5 (10 isolates)

The sequences of COX1-A2 and COX1-A5 differ at two positions.

For *nad1*, the Austrian flukes can be identified as belonging to the following haplotypes:

- NAD1-C10 (21 isolates)
- NAD1-C15 (9 isolates)
- NAD1-C18 (1 isolate)

Altogether, the three haplotypes for *nad1* differ at 4 positions. More precisely, NAD1-C10 differs from NAD1-C15 at two positions and from NAD1-C18 at four positions. NAD1-C15 differs from NAD1-C18 at one position.

Tab. 3.4: Geographical distribution of the haplotypes in Austria.

Haplotypes	Geographical distribution
COX1-A2	Styria, Upper Austria, Salzburg, Tyrol, Lower Austria, Carinthia
COX1-A5	Styria, Tyrol, Salzburg,
NAD1-C10	Styria, Upper Austria, Salzburg, Tyrol, Lower Austria, Carinthia
NAD1-C15	Styria, Tyrol, Salzburg,
NAD1-C18	Tyrol

Tab. 3.5: Differences of base pairs among the *cox1* haplotypes and the Austrian haplotypes.

	COX1-A2	COX1-A5
A1	1	3
A2	**0**	2
A3	3	5
A4	3	1
A5	2	**0**
A6	24	23
A7	3	1
A8	2	2
A9	1	3
A10	3	5
A11	3	5
A12	3	5
A13	2	4

Tab. 3.6: Differences of base pairs among the *nad1* haplotypes and the Austrian haplotypes.

	NAD1-C10	NAD1-C15	NAD1-C18
C1	20	20	20
C2	2	3	4
C3	3	2	3
C4	3	2	2
C5	4	3	3
C6	4	3	3
C7	4	3	3
C8	4	2	3
C9	1	4	5

C10	**0**	3	4
C11	1	4	5
C12	1	2	3
C13	2	3	4
C14	2	1	2
C15	3	**0**	1
C16	3	2	2
C17	2	1	2
C18	4	1	**0**
C19	5	4	4

An overview of the haplotypes identified in Austria is shown in table 3.7.

Tab. 3.7: Haplotypes of Austrian strains of *F. hepatica*.

Haplotype	Austrian samples	Austrian provinces	Known distribution
COX1			
COX1-A2	Fh1, Fh2, Fh3, Fh6 Fh11, Fh12, Fh14, Fh15, Fh16, Fh18, Fh19, Fh20, Fh21, Fh22, Fh25, Fh26, Fh27, Fh28, Fh29, Fh30, Fh31	Styria Upper Austria Salzburg Tyrol Carinthia Lower Austria	Tunisia Algeria France
COX1-A5	Fh4, Fh5, Fh7, Fh8, Fh9, Fh10, Fh13, Fh17, Fh23, Fh24	Styria Tyrol Salzburg	Tunisia Italy China Australia Spain
NAD1			
NAD1-C10	Fh1, Fh2, Fh3, Fh6, Fh11, Fh12, Fh14, Fh15, Fh16, Fh18, Fh19, Fh20, Fh21, Fh22, Fh25, Fh26, Fh27, Fh28, Fh29, Fh30, Fh31	Styria Upper Austria Salzburg Tyrol Carinthia Lower Austria	Italy Egypt Iran United Kingdom Poland

NAD1-C15	Fh4, Fh5, Fh7, Fh8, Fh9, Fh10, Fh13, Fh23, Fh24	Styria Tyrol Salzburg	China Egypt Iran Italy Australia South Korea
NAD1-C18	Fh17	Tyrol	China Egypt

For some sequences the sequencing yielded solitary wobble bases. For the sequences of Fh7, Fh10, Fh17, Fh19, Fh20, Fh21, Fh28, Fh29, and Fh30 the assignment to the haplotypes for the *cox1* gene is nevertheless definite as the wobble bases are in highly conserved regions. For the *nad1* gene, wobble bases were found in samples Fh10, Fh14-Fh19, Fh21, Fh22, Fh24, Fh26-Fh28, Fh30, and Fh31. Also for this gene the wobble bases do not have an impact on the assignment to the haplotypes for the *nad1* gene.

3.3.3 Alignment COX1

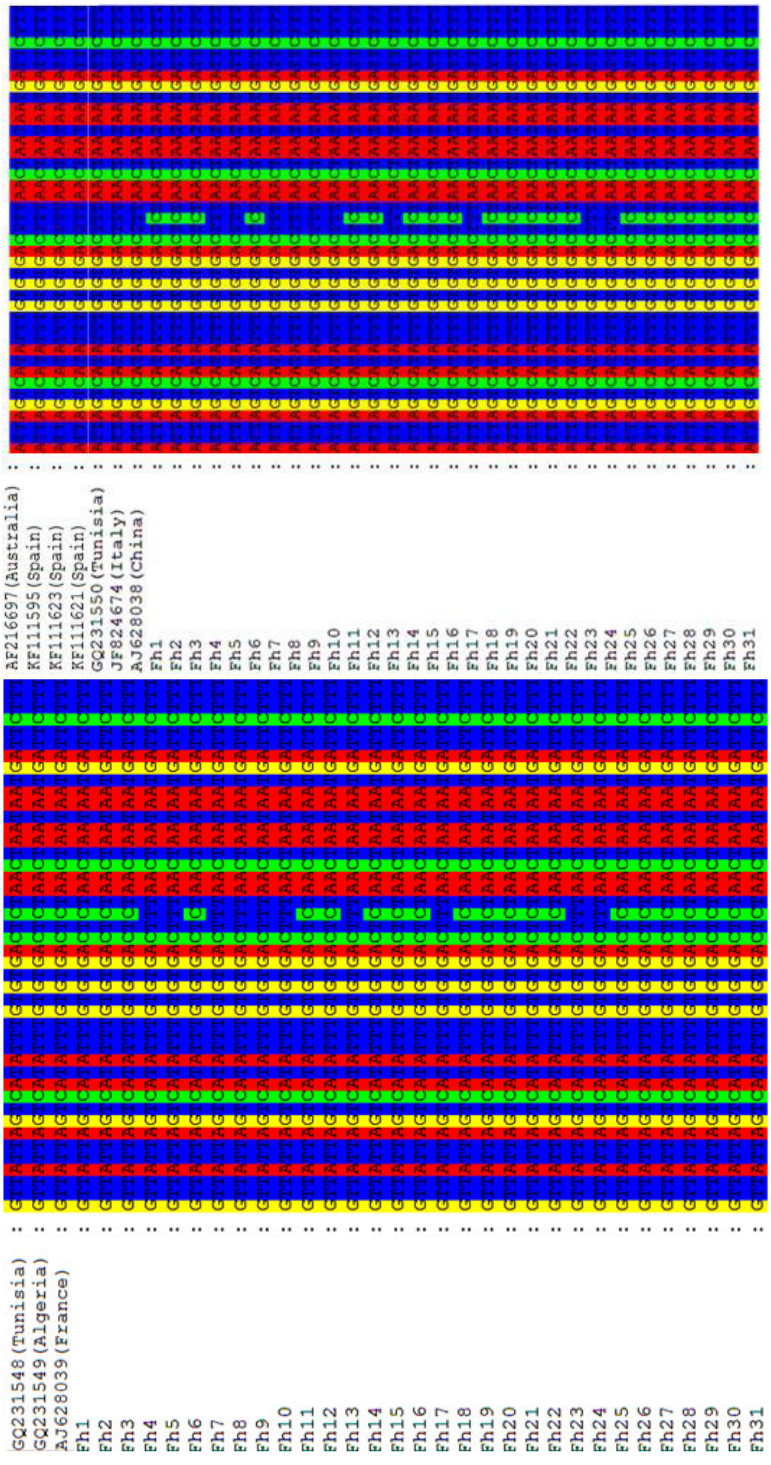

Fig. 3.5: Alignment of a fragment of the *cox1* gene of sequences of the Austrian samples Fh1–Fh31 with COX1-strains. Only a variable fragment is shown.

3.3.4 Alignment NAD1

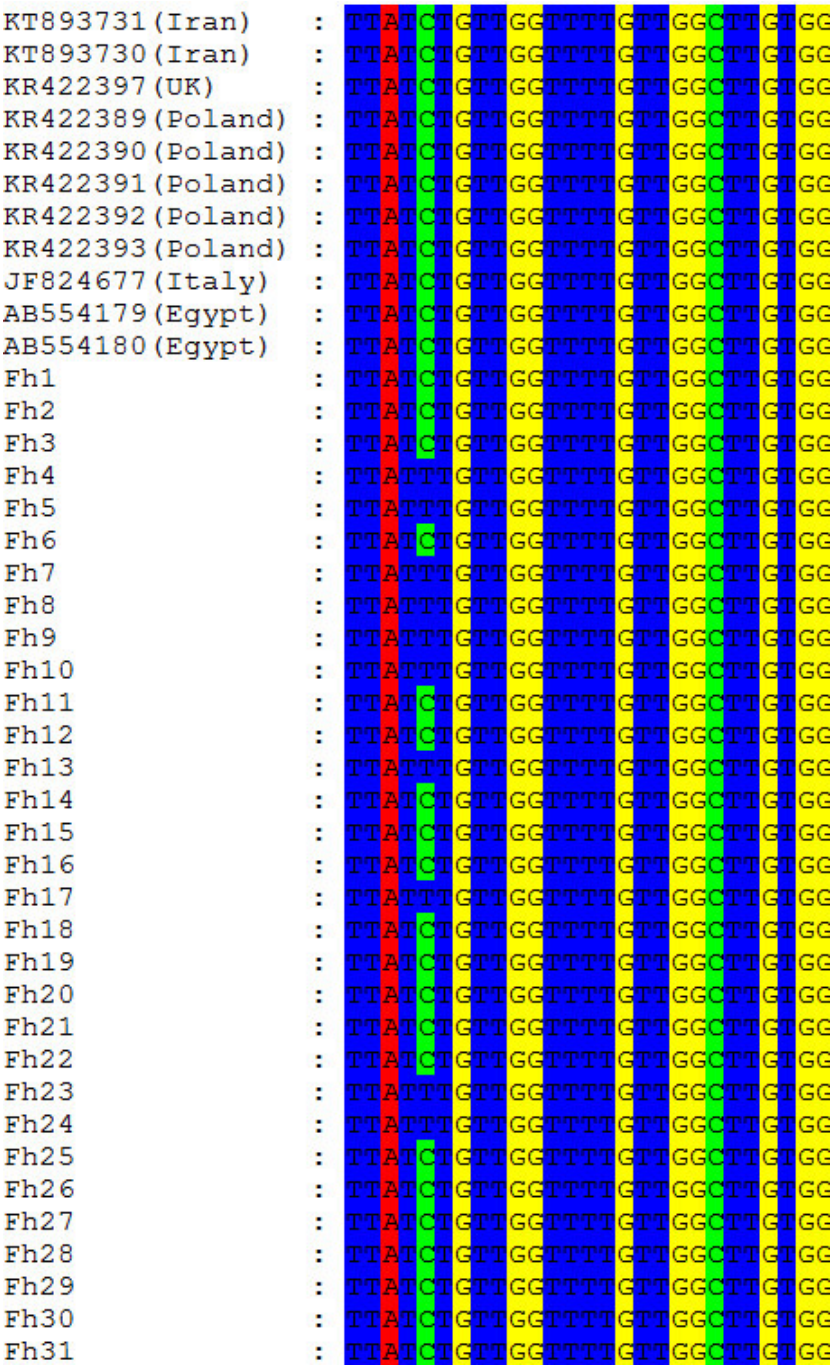

Fig. 3.6: Alignment of a fragment of the *nad1* gene of sequences of the Austrian samples Fh1–Fh31 with NAD1-strains. Only a variable fragment is shown.

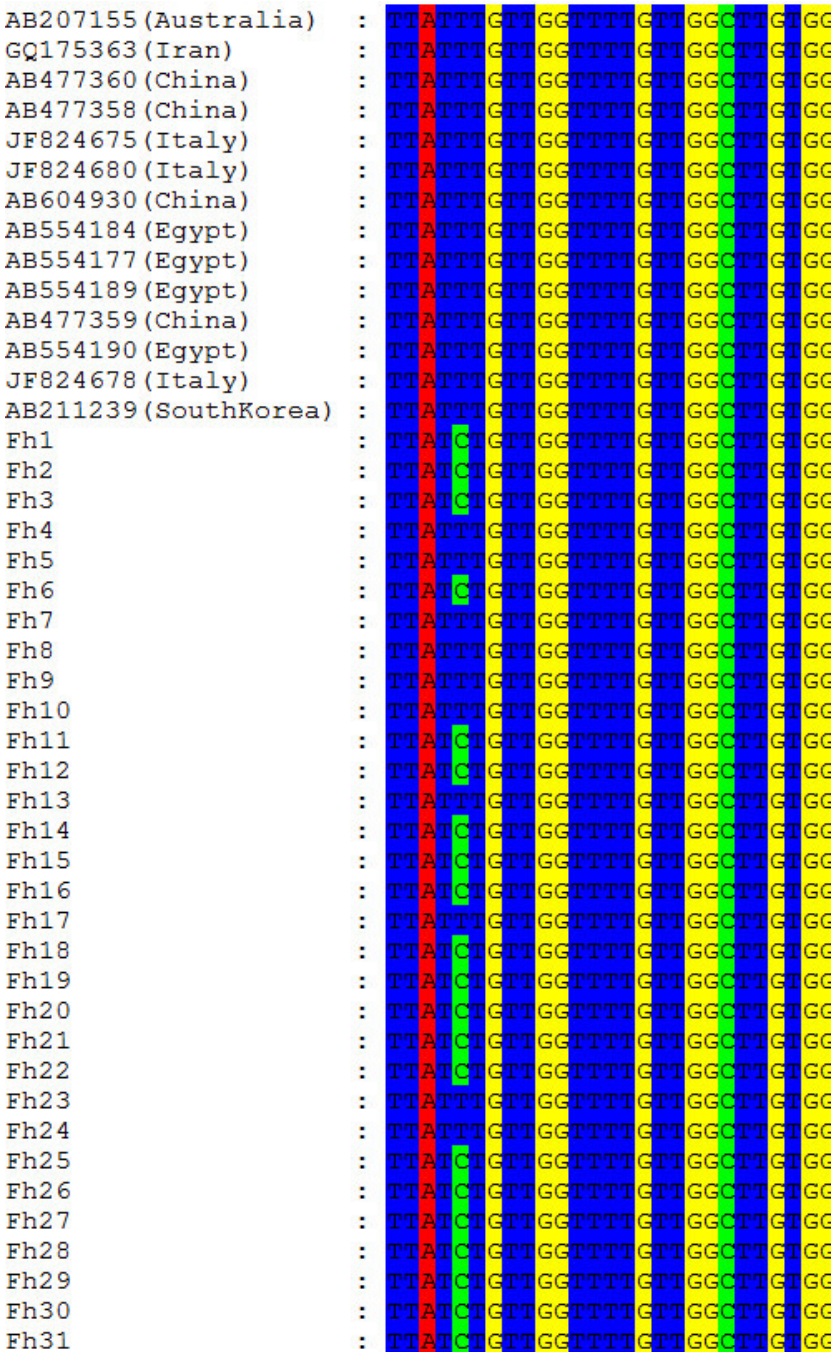

Fig. 3.7: Alignment of a fragment of the *nad1* gene of sequences of the Austrian samples Fh1–Fh31 with NAD1-strains. Only a variable fragment is shown.

3.4 Differences between the genera *Fascioloides* and *Fasciola* on the protein level

F. hepatica revealed more protein bands than *F. magna*, moreover the bands were overall stronger (Fig. 3.8). *F. magna* and *F. hepatica* share intracellular proteins at 8, 12, and 52 kDa and membrane proteins at 24 and 52 kDa. However, *F. hepatica* has additional intracellular proteins at 9, 14, 15, 24, 35, 45, 51, 70, 74, 80, 100, and 225 kDa and additional membrane proteins at 35, 45, 51, and 53 kDa. *F. magna* has an additional intracellular protein at 25 kDa and membrane protein at 10 kDa.

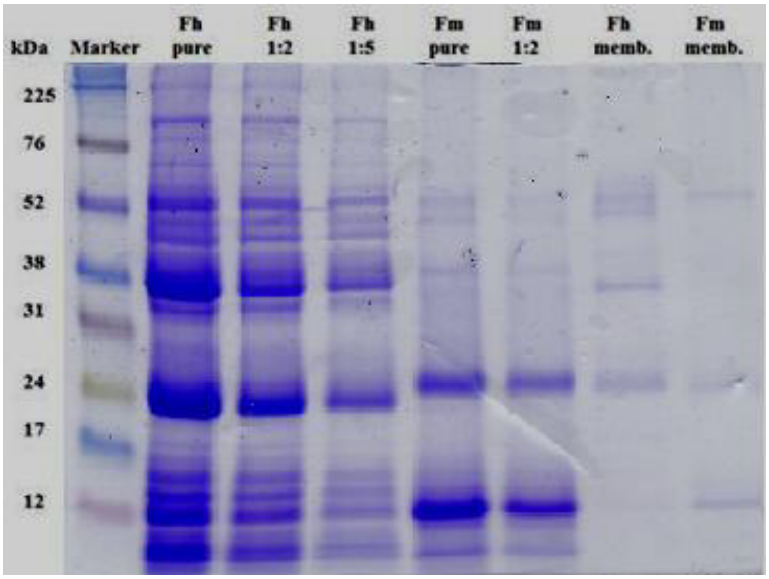

Fig. 3.8: SDS-PAGE of intracellular and membrane proteins of *F. magna* and *F. hepatica*. Comparison of the protein profiles of both flukes. Fh = *F. hepatica*, Fm = *F. magna*, memb. = membrane

3.5 Differences between the genera *Fascioloides* and *Fasciola* on the antigen-level

F. hepatica has more immunoreactive proteins with pooled human serum than *F. magna*. They share an immunoreactive intracellular protein at 52 kDa and a membrane protein at 50 kDa. However, *F. hepatica* revealed additional intracellular antigens at approx. 8, 12, 15, 16, 26, 34, 38, 40, 52, and 225 kDa and a membrane antigen at 35 kDa. *F. magna* revealed additional intracellular antigens at 9, 24, 45, and 227 kDa and a membrane antigen at 35 kDa (Fig. 3.9).

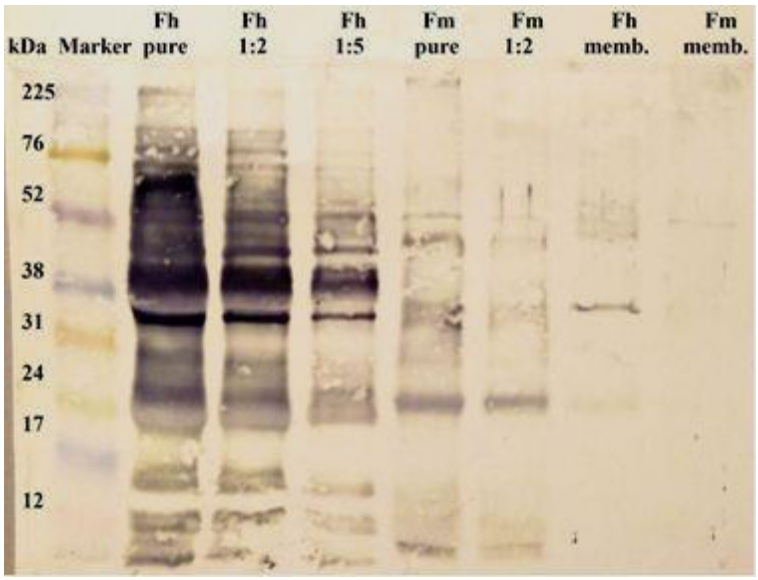

Fig. 3.9: Western blot of fractionated intracellular and membrane proteins of *F. magna* and *F. hepatica.* Comparison of the protein profiles of both flukes marked with antibodies.
Fh = *F. hepatica*, Fm = *F. magna*, memb. = membrane

4. Discussion

In the present thesis, Austrian samples of *F. magna* and *F. hepatica* were characterised based on fragments of their *cox1* and *nad1* genes. Previous studies have shown that these sequences are useful markers for identification and differentiation (Farjallah *et al*., 2009; Farjallah *et al*., 2013; Amer *et al*., 2011; Ai *et al*., 2011; Semyenova *et al*., 2006; Radvánský *et al*., 2011; Králová-Hromadová *et al*., 2011).

For *F. magna*, it was found that the Austrian population is totally homogenous. Only one *cox1* and one *nad1* haplotype was found.
For *F. hepatica*, a more diverse population was found. Altogether, two different *cox1* haplotypes and three different groups of *nad1* haplotypes were identified.

4.1 Diversity of *F. magna*

Altogether, 26 individuals of *F. magna*, isolated from Austrian red deer and fallow deer from the floodplains in the south of Vienna were examined for their *cox1* and *nad1* genes. The sequences were compared with strains published in GenBank.

It was found that all 26 samples of *F. magna* were of the same haplotype, namely COX1-Ha3/NAD1-Ha4. All 26 samples showed a 100% identity to isolates from Hungary, Slovakia, the Czech Republic, Croatia, and also to the two strains characterised in a previous study in this area, the only Austrian isolates of which sequence data are available (Sattmann *et al*., 2014). In that study, they also found a 100% sequence identity to *cox1* haplotype 3 and to *nad1* haplotype 4, known to occur in Hungary, Slovakia, the Czech Republic, and Croatia. All these data in this study indicate that there is a genetically stable population of *F. magna* in Austria.

Although other haplotypes were also found in Slovakia, Hungary, the Czech Republic and Croatia (*cox1*: Ha4, Ha5; *nad1*: Ha5, Ha6, Ha7) to date individuals with these haplotypes have obviously not been able to expand to Austria. However, these haplotypes were found much less frequently also in the other countries (Králová-Hromadová *et al*., 2011). The low frequency might be the result of competitive pressure between the flukes. The lower genetic diversity in Austria indicates a recent introduction of *F. magna* and might also be a result of the impact of treatment programmes (Ursprung and Prosl, 2011). In contrast, the findings of more haplotypes in the neighbouring countries of Austria indicate a longer residence time of *F. magna* in these countries, particularly in the Czech Republic, where *F. magna* is known to occur since the 1940ies (Ullrich, 1930; Rajský *et al*., 1994; Majoros and Sztojkov, 1994; Marinculić *et al*., 2002). The population of *F. magna* in Italy is genetically distinguishable from the rest of Europe due to import of infested animals from various regions of the USA and due to the fact that the Italian individuals of *F. magna* only exist in an enclosed area (Králová-Hromadová *et al*., 2011). The haplotypes Ha1 and Ha2 (*cox1*) and Ha1, Ha2, and Ha3 (*nad1*) which are in Europe only found in Italy are identical to haplotypes in the west of the USA and Canada (Bazsalovicsová et al, 2013; Králová-Hromadová *et al*., 2011). The haplotypes from the other European regions are identical to haplotypes in the southeast of the USA (Králová-Hromadová *et al*., 2011). All these data indicate that the ancestors of the Austrian individuals of *F. magna* originated in the southeast of the USA and were introduced into Austria via the Czech Republic. An introduction via Slovakia and Hungary might also be possible. *F. magna* was found for the first time in Slovakia in 1988 (Rajský *et al*., 1994) and in Hungary in 1994 (Majoros and Sztojkov, 1994).

Studies from Hörweg *et al*. (2011), Liesinger (2011), and Haider *et al*. (2012) demonstrated that the prevalence in intermediate and final hosts depends on the season. Hörweg *et al*. (2011) examined the prevalence of *F. magna* in Austrian samples of *G. truncatula* from 38 locations collected from July 2004 until September 2005. Snails infested with *F. magna* were found only in the summer and at one location with a prevalence of 0.03%. Haider *et al.* (2012) examined the prevalence of *F. magna* in Austrian samples of *G. truncatula*. A

peak of snails infested with *F. magna* was found in July with decreasing rates in August and September. In other months no infestation was detected. In 2011, Liesinger examined faeces from red deer on eggs of *F. magna*. The data show a peak in August, a decreasing rate in September and the highest peak in October which correlates with the data from Haider *et al*. (2012). When deer are infested with *F. magna*, the egg production starts approx. 12 weeks after infestation. In spite of an ongoing treatment programme, it was not possible to eradicate the population of *F. magna*. This could be seen in snails from their study, where the prevalence increased from 0.03% to 0.23%. Nevertheless *F. magna* to date seems to be restricted to an area in the southeast of Vienna and no further introduction of other haplotypes has occurred (Winkelmayer and Prosl, 2001; Ursprung and Prosl, 2011; Haider *et al.*, 2012, Sattmann *et al*., 2014). Samples of *F. magna* used in the present study had been isolated mainly from red deer, and only in Lower Austria and Vienna, which correlates with the studies mentioned above.

Finally, although treatment programmes were and are not able to eradicate the population of *F. magna,* they have prevented a further spread into other parts of Austria. *F. magna* has a restricted final host range. The genetic diversity of *F. magna* in Austria was shown to be stable because for *cox1* and *nad1* only one haplotype was identified. Continuing studies are needed to control the distribution of *F. magna* with a special consideration of seasonal peaks.

To understand the distribution of *F. magna* in Europe and the importance of final hosts, a closer look into the past is needed:
F. magna was introduced into Europe for the first time when Savoy King Vittorio Emanuelle II ordered 60 wapiti from Wyoming, USA. 47 of them reached the La Mandria Park, Italy, and were hunted until 1865 (Apostolo, 1996; Bazsalovicsová *et al.*, 2015). However, with these animals, *F. magna* was also introduced and led to high deer mortality in 1875–1876 and 1977–1978, as the population of deer within the enclosed park exceeded 300 (Balbo *et al.*, 1989). Since the Royal Park is an enclosed region, Italy is the only country that shares haplotype 1 and haplotype 3 with North America (Oregon) and Canada (British

Columbia and Alberta) (Králová-Hromadová *et al.*, 2011; Bazsalovicsová *et al.*, 2015).

F. magna is of North American origin and evolved with ancestral *Odocoileus* spp. This is evidenced by apparent co-evolution and benign infestations in white-tailed and black-tailed deer (Samuel *et al.*, 2001). In North America and Canada, the fluke is enzootic in five major areas: the northern Pacific Coast, the Rocky Mountain trench, northern Quebec and Labrador, the Great Lakes region, the Gulf coast, lower Mississippi and the southern Atlantic seaboard. For *cox1* the haplotypes 1, 3, 6–35 and for *nad1* the haplotypes 3, 4, 6, 8–32 are found in the USA and Canada (Bazsalovicsová *et al.*, 2015).

Because *F. magna* is the only liver fluke, that has its origin in the Nearctic Region and due the fact, that other fasciolids were absent in the Neotropical Region before Columbus, it is assumed, that they originated after Gondwana was fragmented into South America and Africa 90–100 million years ago (Pitman *et al.*, 1993).

Red deer (*Cervus elaphus*), one of the main final hosts for *F. magna*, is the most widespread deer species worldwide. The species is of Eurasian origin and entered North America 11,000–70,000 years ago during the Pleistocene. They are mixed or intermediate feeders, alternating between browsing and grazing depending on the available habitat. This makes red deer resistant to changing environments and landscape conditions through glaciations, deglaciations, faunal extinctions, and human disturbances (Samuel *et al.*, 2001; Skog *et al.*, 2009). The colonisation routes of red deer based on cytochrome *b* sequences, published by Ludt *et al.* (2004), are shown in figure 4.1. Cervoids first appeared 25 million years ago, during the transition from the Oligocene to the Miocene, near what is now the Hindukush (Afghanistan and Pakistan) (Agustí and Antón, 2002).

Fig. 4.1: Colonisation routes of red deer (*Cervus elaphus*) (modified from Ludt *et al.*, 2004)**.** The dotted lines show the migration routes of deer. The bold lines are natural barriers.

Today, the Western red deer can be classified into four groups: Western European, Balkanese, Middle Eastern, and African. Western Europe is separated from the Balkan group by the Alps and the Carpathians and from the Middle East by the Bosporus and the Caucasus (Ludt *et al.*, 2004).

Because most of the eastern and southern parts of Austria are within the Balkan group, only deer from this group might be responsible for the distribution of *F. magna* in the southeast of Vienna.

4.2 Diversity of *F. hepatica*

In this study, the 31 individuals of *F. hepatica* obtained from cattle from various regions in Austria could be grouped into two different clusters for *cox1* and into three different clusters for *nad1*. Differences were more pronounced in the *nad1* gene than in the *cox1* gene. The classification into haplotypes was not easy because the sequences available at GenBank have different lengths and are of different positions within the gene. Thus, the sequences were brought to the same length and the groups A and B for the *cox1* gene and group C for the *nad1* gene were established. Of course, this is an artificial system, but it is an attempt to establish a uniform haplotype system. If more sequences will be available the haplotype system will certainly become more heterogeneous. In general, the assignment of the Austrian samples is under reservation for several sequences because isolated bases could not be identified unambiguously.

This is the first study on the genetic diversity of *F. hepatica* in Austria. The data show a minor genetic diversity of Austrian isolates of *F. hepatica*, and high identities with isolates from other countries.

This chapter deals with the diversity of *F. hepatica* in Austria in comparison with results from other countries. Furthermore, the distribution of *F. hepatica* in the present and especially in the past will be discussed, with a focus on the anthropological impact.

4.2.1 COX1

In general, based on currently available sequences for the *cox1* gene, *F. hepatica* can be divided into two groups. Group A consists of strains from Italy, Spain, France, Algeria, Tunisia, China, Niger, Australia, and the USA. Group B consists of strains from Peru, Egypt, and Iran. The sequences of the two groups represent different locations within the *cox1* gene.

Haplotypes are usually based on SNPs. The diversity of *F. hepatica* has been examined for several European and non-European countries. The identity between strains from different continents is high (Alasaad *et al*., 2007; Semyenova *et al*., 2006).

The Austrian isolates revealed two different haplotypes which differ from one another by two bases:
Haplotype COX1-A2 is known to occur in Tunisia (GQ231548), Algeria (GQ231549), and France (AJ628039) with a 100% bp identity to the Austrian isolates.

COX1-A5 is known to occur in Tunisia (GQ231550), Italy (JF824674), China (AJ628035, AJ628038), Australia (AF216697), and Spain (KF111623, KF111621, KF111595) with a 100% bp identity to the Austrian isolates.

Thus, these haplotypes are wide-spread in both, European and non-European countries.

In Austria, both haplotypes were only found in Styria, the Tyrol, and Salzburg. One of these haplotypes was found additionally in Upper Austria, Lower Austria, and Carinthia. Between 1980 and 2013, 41 human cases of fasciolosis were registered and most of them were diagnosed in the Tyrol, Salzburg, Vorarlberg, and Upper Austria (Auer and Aspöck, 2014). Skoll (2015) reported that recent human cases of fasciolosis in Austria were found in Salzburg, Upper Austria and Vorarlberg. Salzburg, the Tyrol, and Upper Austria are known as endemic areas which correlate to the current findings. Unfortunately, no isolates from Vorarlberg were available for this study.

The current findings indicate that there is a diversity of *F. hepatica* in Austria, although *cox1* is a comparably conserved gene and although only 31 individuals, partly from the same farm, were analysed. Farjallah *et al*. (2013) examined 66 isolates from cattle and sheep from the north and the middle of Sardinia, and found three haplotypes, whereby two of them differed at one site (312 bp/313 bp) and the third was represented by only one strain (JF824674) and differed from the other types at two (311 bp/313 bp) and three sites (310 bp/313 bp) respectively. Strains from China, obtained from two geographical regions, have been divided into three groups, group 1 represented by three strains (GU112484, GU112485, GU112486), group 2 by two strains (AJ628036, AJ628037), and group 3 by two strains (AJ628035, AJ628038). In that study, however the distance between the groups was higher, group 1 differed from group two at 23 sites (290 bp/313 bp) and from group 3 at 21 sites (292 bp/313 bp). However, Ai *et al*. (2011) examined 11 individuals of *F. hepatica* from three locations in China and found no differences. Semyenova *et al.* (2006) examined 220 flukes from 33 hosts and identified 10 *cox1* types. They found two main lineages with a little genetic structuring between eastern European and western European populations of *F. hepatica*. One lineage was found in Europe, Armenia, America, and Uruguay, while the other lineage was found in Europe, Caucasus, Asia, China, Australia, and Japan. The Austrian isolates differ only at two positions.

In general, the *cox1* gene seems to have a low genetic diversity in *F. hepatica*. Moreover, only a low number of individuals of *F. hepatica* from few geographical

regions and few final hosts have been examined worldwide. A higher number of hosts, geographical regions, and samples of *F. hepatica* may increase the diversity.

Walker *et al*. (2011) examined the *cox3* gene of 422 Dutch flukes from 20 cattle from two farms and revealed 92 haplotypes. This gene shows a much higher variability. Moreover, these farms harboured also sheep, introduced from elsewhere in the Netherlands. Also a high genetic diversity was found in a study from Walker *et al*. (2007). They examined 221 individuals of *F. hepatica* and found 18 composite haplotypes for *cox3*/*nad4* and 11 composite haplotypes for *cox1*/*rrna.*

In conclusion, the Austrian isolates revealed diversity, compared to the few individuals analysed and to the broad distribution of *F. hepatica* haplotypes throughout the world.

4.2.2 NAD1

In this study the Austrian isolates revealed three haplotypes which differed in up to four bases. The differences between NAD1-C10 and NAD1-C18 were more pronounced (4 bases) than between NAD1-C10 and NAD1-C15 (3 bases), and NAD1-C15 and NAD1-C18 (one base). All haplotypes are known to have a worldwide distribution.

NAD1-C10 is known to occur in Italy (JF824677), Egypt (AB554179, AB554180), Iran (KT893730, KT893731), United Kingdom (UK) (KR422397), and Poland (KR422389, KR422390, KR422391, KR422392, KR422393).

NAD1-C15 is known to occur in China (AB477358, AB477360, AB604930), Egypt (AB554177, AB554184, AB554189), Iran (GQ175363), Italy (JF824675, JF824680), Australia (AB207155), and South Korea (AB211239).

NAD1-C18 is known to occur in China (AB477359) and Egypt (AB554190).

In Austria the co-occurrence of all three haplotypes was found only in the Tyrol. In Styria and Salzburg, two of the haplotypes were found. In Upper Austria, Lower Austria, and Carinthia only one haplotype was found.

Haplotypes NAD1-C15 is known in China, Egypt, Iran, Italy, South Korea, and Australia. Thus, this haplotype group (collected in Tyrol, Styria, and Salzburg) is widely distributed in Eurasia and Australia.

Farjallah *et al*. (2013) investigated 66 samples of *F. hepatica* from cattle and sheep from the north and the middle of Sardinia and found five *nad1* haplotypes. Another study that has relevance for the present findings was published by Semyenova *et al*. (2006). They found two main lineages and have demonstrated that eastern European and western Asian populations of *F. hepatica* have little genetic structuring, probably due to a high gene flow between them and due to the movement of final hosts. These findings indicate that also for the *nad1* gene the diversity in *F. hepatica* is rather low. Interestingly, the haplotypes from Eastern Europe were not found in Austria. Walker *et al*. (2011), Walker *et al*. (2007), and Elliot *et al.* (2014) found that environment conditions and antihelmintics have a great impact on the genetic diversity of flukes. From 2002–2007 a drought period occurred in Australia, which might have resulted in a bottleneck-effect for the fluke populations and thus in fewer fluke genotypes in Australia (Elliot *et al*., 2014).

In conclusion, the haplotypes of *F. hepatica* found in Austria are also found in other countries and continents. The results also show that *F. hepatica* in Austria is genetically more diverse than *F. magna*.

The level of diversity was higher in the *nad1* gene than in the *cox1* gene as seen by others (Elliot *et al*., 2014; Semyenova *et al*., 2006; Farjallah *et al*., 2013). With the higher diversity, the *nad1* gene allows for a better distinction between strains. In general, the phylogenetic data for *F. hepatica* is poor. In most studies, only few isolates and only from a small number of locations and few animals were investigated.

4.3 Hypothesis on the origin of *F. hepatica*

To understand the worldwide distribution of *F. hepatica*, a closer look into the past is needed. In this chapter, a short overview on the origin, the distribution routes, and the anthropological impact is given.

The results from the *cox1* and the *nad1* genes illustrate that almost all known haplotypes have a worldwide distribution.

It is generally assumed, that the ancient form of *F. hepatica* originated in Africa. One theory suggests that an ancient fasciolid form of *F. hepatica* was introduced to the Near East with African ruminants in the early Miocene (20.2–16.9 million years ago) when the sea level was low (Tassy, 1990; van der Made, 1999). Another theory is based on findings of basal fasciolids in African elephants and suggests that fasciolids originated in Africa in proboscideans that themselves originated approximately 50 million years ago. The radiation of the flukes in Eurasia occurred with this host, as proboscideans migrated and dispersed within Eurasia from approx. 18.5 to 0.8 million years ago (Lotfy *et al.*, 2008). A prehistoric invasion of *F. hepatica* into Europe is supported by findings of eggs of *F. hepatica* in human faeces collected in various regions in Europe from the Stone Age. Further findings of eggs of *F. hepatica* in human faeces are recorded from the Mesolithic period, the Neolithic, the Bronze Age, the Gallo-Roman period, and the Middle Ages (Mas-Coma *et al.*, 2005; Bouchet *et al.*, 2003).

4.4 Distribution routes of *F. hepatica*

F. hepatica is a parasite that is heavily dependent on its intermediate and final hosts. The wide distribution of *F. hepatica* has been caused firstly by natural movements of the intermediate hosts (longer lasting co-evolution between trematodes and molluscs) and secondly by natural movements of final hosts and translocations of these hosts by humans over hundreds of years all over the world and thirdly by the ability of the fluke to acquire a wide range of new hosts. Import and export of animals today and in the past seem to be major factors in

increasing the populations and the genetic structure of *F. hepatica* (Semyenova *et al.*, 2011).

For the distribution of the flukes, different scenarios are possible. The first ruminants evolved around 50 million years ago and were mainly feeding on fruits. The first global grass spreading period starting around 25 million years ago favoured the evolution of ruminants specialised on grass (Walochnik *et al.*, 2010). Subsequently, the Miocene drop of the sea level caused by a high-latitude cooling event with an increase of the Eastern Antarctic ice sheets (Flower and Kennet, 1994; Miller *et al.*, 1991). This led to the desiccation of the Tethys and the formation of vast grassland around 15 million years ago, which promoted the world wide spread and the enormous evolutionary success of modern ruminants (Walochnik *et al.*, 2010; Agustí and Antón, 2002; Haq *et al.*, 1987). The next grass-spreading period was seven to eight million years ago, when the Arabian plate stopped the circulation of warm deep-water between the Indo-Pacific and the Mediterranean Sea (Axelrod and Raven, 1978). The climate changed to cooler winters and decreased summer rainfalls and caused grass to spread over large areas of Europe and Asia (Axelrod, 1975; Cerling *et al.*, 1997). This was advantageous for fast moving grazers, because they had an oversupply of food, which led to an expansion of the population (Ludt *et al.*, 2004).

After the ancient flukes were introduced with these grazers into Eurasia, the adaption to new intermediate hosts was necessary. In the Near East, *Radix auricularia* (Linnaeus 1758) and *Radix gedrosiana* (Annandale and Prashad, 1919) were present, and the fasciolid ancestor was able to adapt to these hosts (Mas-Coma *et al.*, 2009). Due to its adaption to *G. truncatula* and Euro-Asiatic ovicaprine ancestors, the fasciolid ancestor was able to colonise colder regions (Hernandez Fernandez and Vrba, 2005). This is why *F. gigantica* and *F. hepatica* are found in different regions: *F. gigantica* needs higher temperatures for its development. Because *F. hepatica* changed to smaller lymnaeids such as *Galba* and smaller ruminants, which colonised colder regions, *F. hepatica* could adapt to colder environments. The geographical spread of *F. hepatica* in Europe, the Near East, and Asia could have taken place between 14.7 and 14.5

million years ago, when the diversification of the Caprinae occurred, and may also be the result of the greater mobility of the smaller ruminants and the wide distribution of *G. truncatula*. The adaption to other host species, such as *Bos*, *Bison*, and cervines, and the split of *Capra* and *Ovis* around 11.3 million years ago and following species 5.1–6.8 million years ago, intensified the distribution (Mas-Coma *et al.*, 2009).

For the distribution of the flukes within Eurasia and on other continents, humans were the main factor and domestication played an important role for the dispersal of the flukes. Domestication of grazers started in 15,000 BC (before Christ) and the first domesticated grazers were the wild herbivore progenitors of goats. Other species such as sheep and cattle followed (Aspöck and Walochnik, 2007; Pedrosa *et al.*, 2005). In Central Europe, domesticated cattle and sheep, as two of the main final hosts, might have played a major role for the distribution of *F. hepatica*. The oldest known specimens of *F. hepatica* date back to 7,000 years ago in Central Europe and cattle were already domesticated 6,000 BC in Greece and Anatolia. However, at that time domesticated goats and sheep played a major role in Central Europe because they were already domesticated 7,000 BC (goats) and 8,200 BC (sheep) in Southwest Asia, Persia, and Anatolia (Aspöck, 2000). After 6,400 BC, the expansion of the Neolithic culture out of the Near East into Greece, the Balkans, and Central Europe occurred. The culture spread westwards to Europe, eastwards to Asia, and southwards to Africa (Mas-Coma *et al.*, 2009) (Fig. 4.2).

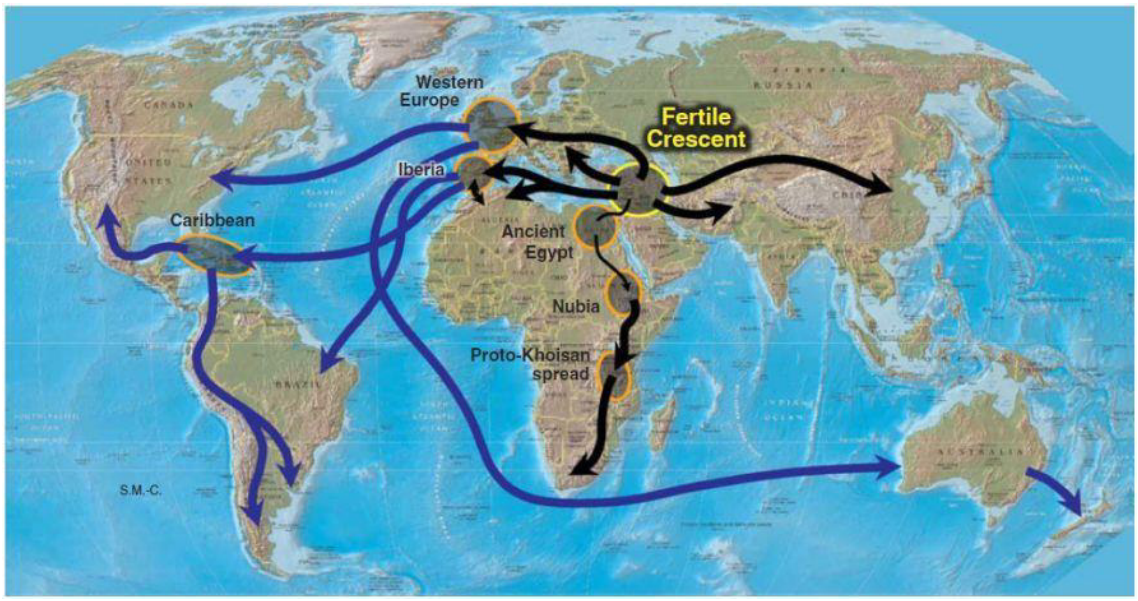

Fig. 4.2: Spreading of *F. hepatica* in the post-domestication period (from Mas-Coma *et al.*, 2009)**.** The yellow cycle illustrates the initial dispersal in the Near East with *Galba truncatula* and ovicaprines in the pre-domesticated period. The orange circles indicate regions with key roles in dispersal. The black arrows reveal the spread throughout the Near East in the post-domestication time, which originated in the Fertile Crescent, approximately 6,000 BC. Through the Balkans and Turkey-Greece, perhaps in the fourth and third millennium BC, a westward spread occurred to Central and Northern Europe. In the last 500 years the Americas and Oceania were colonised, denoted by blue arrows. The thin black lines indicate the spread to Africa through Egypt. Other colonisation routes in the Palearctic region, the Americas, the Indian subcontinent, and routes in Southeast Asia and the Far East are not included.

F. hepatica was probably spread into Europe by a southern and a northern route, whereby the southern route could include a sea passage through the Mediterranean Sea. Sheep, goats, and cattle played a major role for spreading. In the 11th and 12th centuries, the marine transport of infested sheep by Phoenicians, Greeks, and Romans can be assumed (Mas-Coma *et al.*, 2005; Bouchet *et al.*, 2003).

For Asia, two main routes, separated by the Himalaya chain, were possible: the first route through the Caspian Sea in northern and eastern directions, including the regions of Turkmenistan, Tajikistan, Iran, Uzbekistan, and Kirgizstan, and at least up to China and Mongolia to the Far East between the second and the 15th century AD (*anno domini*). An alternative southern route was also possible, but due to the absence of *G. truncatula* and appropriate lymnaeids in India and Southeast Asia, further spreading eastwards of *F. hepatica* in Southern Asia was not possible (Mas-Coma *et al.*, 2009).

Northwestern Africa, including Morocco, Algeria, and Tunisia, has a seasonally mild temperature and is optimal for the development of *G. truncatula*. At the beginning of the 20th century, in Egypt *F. hepatica* was absent, so the introduction of *F. hepatica* in northwestern Africa through northeastern Africa was not possible and probably occurred from the Levant, possibly through the Mediterranean Sea with the import of animals by the Phoenicians, Greeks, and/or the Romans, or with the medieval common Mediterranean market of the 11th the 12th century (Mas-Coma *et al.*, 2009). The Mediterranean Sea connected the northeast of Africa with Iberia, the Near East, and southern Europe and acted as a natural corridor for import and export of animals (Pereira *et al.*, 2006).

Because of the dominating presence of *Radix natalensis* (Kraus, 1848), *F. hepatica* was unable to distribute in the sub-Saharan, large Western, and Central African region (Brown, 1994). This lymnaeid is resistant to *F. hepatica* infestation, thus preventing a successful colonisation by this fluke. Due to the dry climate and the high temperatures, *G. truncatula* and *F. hepatica* were not endemic in Egypt in the past since the areas were desert (Mas-Coma *et al.*, 2009). However, today, *G. truncatula* is endemic in Eastern Africa, especially in the flat lowlands of the Nile Delta, and in the mid-1990ies, *F. hepatica* reappeared in imported animals (El-Azazy and Schillhorn van Veen, 1983). The reappearance of both genera today was made possible by the construction of the Aswan Dam, which provided suitable environmental conditions (Mas-Coma *et al.*, 2009). *Pseudosuccinea columella* (Say 1817), which is endemic in South Africa and the eastern equatorial countries, may also play an important role in transmission (Van Damme, 1984; Brown, 1994; Appleton, 2003). In the first millennium BC *F. hepatica* expanded southwards with Proto-Khoisan people, the oldest existing human group, and in the third millennium *F. hepatica* expanded to today's Kenya and Tanzania (Schlebusch *et al.*, 2012; Mas-Coma *et al.*, 2009).

The colonisation of Oceania and the Americas started between the 15th and 19th centuries, mainly from the western part of Europe. The spread of *F. hepatica* into these continents was driven by human migration and import of sheep,

cattle, and goats. Equids and other animals imported by Europeans played an important role for diffusion of the flukes into South America and the Caribbean (Apt *et al.*, 1993; Mas-Coma *et al.*, 1997).

For thousands of years, animals have been involved in different human activities, such as transportation, transhumance and trade. Especially sheep, goats, cattle, zebu cattle, taurine, buffaloes, donkeys, and pigs played and play an important role in the dissemination of the flukes. The transportation to other countries or continents and the seasonal movement of livestock between mountains and the lowlands, or the movement from one place to another due to the availability of grass or other plants enabled a wide expansion of *F. hepatica* to other areas (Mas-Coma *et al.*, 2009).

The distribution routes of humans and *F. hepatica* into Europe and Asia, seen in figures 4.2 and 4.3, show high similarities, with a beginning in the Fertile Crescent. After humans migrated out of Africa, they settled in the Fertile Crescent. In the post-domestication period, during migration to Asia, Africa, and the southern and northern parts of Europe, domesticated animals played an important role because of the need for milk, flesh, and transportation of people and goods.

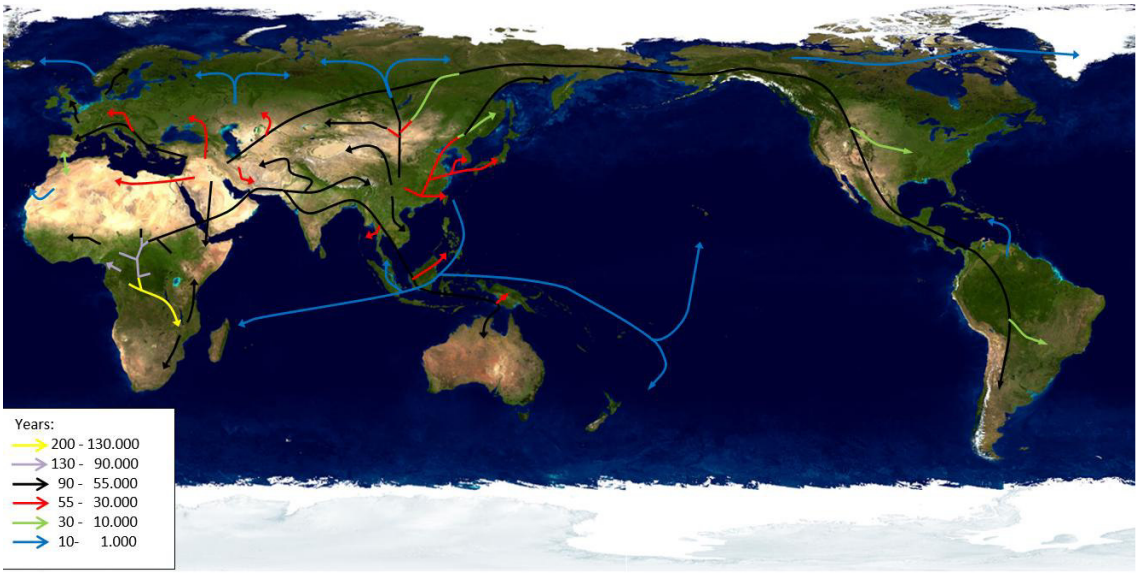

Fig. 4.3: Distribution routes of humans (modified from Kivisild *et al.*, 2003, original picture from Mauricio Lucioni, Lima, Peru)**.**

4.5 Other impacts on the distribution of *F. hepatica*

4.5.1 The Roman Empire

Archaeological findings indicate that the Romans were infested with trematodes (*Fasciola hepatica*), nematodes (*Ascaris* sp.), cestodes (*Taenia* sp.), and adenophorea (*Capillaria* sp.) (Scarre, 1995; Dittmar and Teegen, 2003; Bakker, 2001). Romans may have spread parasites by themselves and with the transport of animals on their ships. The poor hygienic conditions in the Roman forts, the absence of veterinary investigations, and the expansion range of the Romans all enabled the unimpeded spread of parasites.

4.5.2 Middle Ages

Because of the poor hygienic conditions in the Middle Ages, many people were infested by parasites. Various parasite eggs have been found in latrines: *F. hepatica*, *Ascaris lumbricoides*, *Trichuris trichura* and *Diphyllobothricum latum* (Sczech, 1993). In cities, due to the lack of sanitary facilities and sewers, often a fatal cycle occurred: fountain – earth – latrine – earth – fountain (Sczech, 1993). This water and faecal cycle, with the presence of lymnaeid snails and water plants further amplified the distribution and the occurrence of infestations with *F. hepatica*.

4.6 Protein profiles of *F. magna* and *F. hepatica*

Proteins are important for the classification of parasite species. Especially the tegument, acting as an interface between the flukes and the host is covered by important proteins. For this thesis, proteins were extracted from the body and the tegument to test whether *F. magna* and *F. hepatica* share important proteins.

4.6.1 Intracellular proteins

It was found that *F. magna* and *F. hepatica* share intracellular proteins with 8, 12, and 52 kDa respectively. For *F. magna* there was an intracellular protein at 25 kDa while only for *F. hepatica* intracellular proteins with 9, 14, 15, 24, 35, 45, 51, 70, 74, 80, 100, and 225 kDa were found.

F. magna and *F. hepatica* are two different species of two different genera. Interestingly, they share three intracellular proteins with the same mass.

One of the most prominent bands was a band at approx. 12 kDa and it was present in the profile of both flukes. Figueroa-Santiago *et al.* (2011) described a FhSAP2 fusion protein (11.5 kDa), a member of the *F. hepatica* saposin like-2 protein family. This protein is thought to play a role in pore formation, membrane binding, cell lysis, and to interact with lipids (Cabán-Hernandez and Espino, 2013).

Kim *et al.* (2003) examined a low molecular weight protein (8 kDa), which can be used as diagnostic parameter in fasciolosis because it has no cross-reactions with other human trematodes. However, *F. magna* also has a band of this size, indicating that this protein is shared by both flukes and is essential in the mature stage. It is probably needed to attach on the bile duct and for nutrition.

Gaudier *et al*. (2012) described a protein FhTP16.5 with approx. 17 kDa that is poorly expressed in miracidiae, but highly expressed in juvenile and adult stages. This protein is detectable in humans with chronic fasciolosis and is a useful marker in serodiagnosis of fasciolosis (Gaudier *et al*., 2012). Hillyer and Soler de Galanes (1988) revealed multiple proteins of 17 kDa to 26 kDa, a protein with 63 kDa, and a 17 kDa protein after they had incubated live *F. hepatica* in phosphate buffered saline (PBS). The multiple proteins in the range of 19 to 26 kDa were not seen in the present study. Only one protein with 24 kDa was observed. However, Gaudier *et al*. (2012) used RPMI 1640 medium for the transportation of the flukes. RPMI 1640 contains the reducing agent

glutathione and high concentrations of vitamins such as biotin and vitamin B_{12}, which might have increased the production of the 17 kDa protein.

Another study that has relevance for the present findings is from Collins *et al*. (2004). They describe a procathepsin L1 with approx. 37 kDa and a cathepsin L with 24.5 kDa. They are located in the gut of the parasites. Cathepsins are important for tissue penetration, feeding, and immunomodulation, which allow the establishment of the parasite in the host (Collins *et al.*, 2004). In order to overcome the intestinal wall, *F. hepatica* uses the proteases cathepsin B and cathepsin L. Every hour, the fluke secretes up to 1 µg of cathepsin L, which degrades proteins associated with the extracellular matrix. While cathepsin L is essential for juvenile flukes for excystment, gut penetration, and the following migration to the liver, the lower expressed cathepsin B is necessary for migration and manifestation in the host, and has intracellular household functions. Therefore, cathepsin L is most decisive during the liver-based migration and adulthood, while cathepsin B is most decisive in newly excysted flukes and the early migratory phase (McGonigle *et al*., 2008).

4.6.2 Membrane proteins

F. magna and *F. hepatica* shared two membrane proteins with a mass of 24 and 52 kDa, respectively. For *F. magna* there was an additional membrane protein at 10 kDa while for *F. hepatica* at 35, 45, 51, and 53 kDa.

It is possible that these proteins are produced in the membrane, or that they are produced in the cells of the body and then transported to the membrane.

Proteins between 25 and 48 kDa and of 50 kDa have also been identified in the tegumental syncytium but to date no characterisation of these proteins has been performed (Abdel-Raman *et al*., 1999; Hanna and Trudgett, 1983)

Cantacessi *et al*. (2012) published a general overview of the proteome of *F. magna*. In total they described 80 proteins in the excretory/secretory products. The largest group consists of antioxidant proteins, followed by proteases,

structural proteins, and proteins involved in carbohydrate metabolism, protease inhibitors, and finally, unknown and miscellaneous proteins. In total 27 excretory/secretory proteins are shared by *F. magna* and *F. hepatica* and these might also have the same function. Further investigations are needed to examine whether these proteins are located in vesicles/excreted in the body for transportation to the membrane (Abdel-Rahman *et al*., 1999).

In general, the higher number of intracellular and membrane proteins of *F. hepatica* might indicate a more complex proteome in *F. hepatica*, possibly in correlation to the wider and more complex host spectrum, but may also reflect technical shortcomings. The low number of identities between these proteins correlates with the rather distant relationship of *F. magna* and *F. hepatica*.

4.7 Antigen profiles

The results of this study indicate that *F. magna* and *F. hepatica* share one intracellular antigen with a mass of 52 kDa and a membrane antigen with 50 kDa. Several antigens were found only in *F. magna* or *F. hepatica*. Overall, less common antigens than common proteins were detected, indicating that not all proteins are recognised as antigens by human antibodies.

Hillyer and Soler de Galanes (1988) described a 17 and a 63 kDa antigen after immunoblot with human sera. They tested several closely related trematodes, but the 17 kDa antigen was only found in *F. hepatica*. A 63 kDa antigen showed cross-reactivity with *Schistosoma mansoni* and *Trichinella spiralis*. They hypothesised, that the 17 kDa antigen may prove to be a useful marker for fasciolosis. In this thesis the 17 kDa antigen couldn't be observed, neither in *F. magna* nor in *F. hepatica*. The 63 kDa antigen was visible as a weak band. This indicates that the 63 kDa antigen persists for a longer time than the 17 kDa antigen. In cured patients, the 17 kDa antigen was absent one year post cure and the 63 kDa antigen was diminished one year post cure. Because in the present study only sera from humans who were not infested with trematodes or *T. spiralis* were used, these bands were not seen in the antigen profile.

Abdel-Rahman *et al*. (1999) described a coproantigen with 26 kDa. It is secreted by the gut and excreted by the tegument of the fluke, and its antigenicity is enhanced by proteolytic enzymes found in the digestive tract. This antigen is stable under various conditions and is therefore useful in diagnostic tests for a *F. hepatica* infestation. It is possible that this is a cysteine proteinase, located in Fas1 and Fas2 antigens. Fas1 and Fas2 are specific and sensitive antigens for the diagnosis of fasciolosis (Cordova *et al*., 1997; Kamel *et al*., 2013). In the present study, this antigen was only found in *F. hepatica* in the body and not in the tegument. Because this antigen can only be found in the presence of proteolytic enzymes, the antigen was missing in the antigen profile for the membrane.

All of these described antigens are specific for *F. hepatica*, while antigens with bands of approx. 19–20, 37, 50, 60-63, and 85 kDa have shown cross-reactivity when patients were infested with other parasites (visceral leishmaniosis, Chagas disease, *Hymenolepsis nana* and strongyloidosis). In the present thesis, the band with 225 kDa has only been found in *F. hepatica*, but a band higher than 225 kDa has been found in *F. magna*. It is possible that these two antigens have the same function in both flukes.

In conclusion, some of the blotted proteins act as antigens and in general, *F. hepatica* revealed more antigens than *F. magna* in this study. The low number of identities between these antigens show that *F. magna* and *F. hepatica* share few antigens.

5. Conclusion

In this study, the genetic diversity of Austrian isolates of *F. magna* and *F. hepatica* was investigated. It was shown that all Austrian isolates of *F. magna* investigated so far belong to one and the same haplotype. As the isolates derived from various geographic locations and also from different years, it can be assumed that, until now, only one haplotype exists in Austria. This indicates a single introduction of *F. magna* into Austria, possibly directly from the Czech Republic or via Slovakia and Hungary, where the same haplotype is known to occur. The isolates of *F. hepatica* revealed two different haplotypes for *cox1* and three haplotypes for *nad1*. Both *cox1* haplotypes were found in Styria, Tyrol, and Salzburg. One haplotype was only found in Upper Austria, Lower Austria, and Carinthia. All three *nad1* haplotypes were found in Tyrol, two haplotypes were found in Styria, Tyrol, and Salzburg and one haplotype was found only in Upper Austria, Lower Austria, and Carinthia. All these data indicate that Tyrol, Salzburg, and Styria are the most important endemic areas. Generally, *F. hepatica* is more diverse due to its long persistence in Eurasia and its broad host range.

The distribution of parasites is strongly coupled with the distribution and the possibility of migration of the respective hosts. *F. magna* occurs in restricted areas in Europe because of its recent introduction, its limited animal host range, and its hosts typically not leaving their territories. *F. hepatica* has been able to expand worldwide due to its broad host range and the anthropological impact. Humans with their ability to migrate long distances, to overcome natural barriers, and to import/export animals, have been significantly involved in the dissemination of *F. hepatica*.

The protein profile revealed three intracellular and two membrane proteins that were shared by both flukes. Overall, *F. hepatica* revealed a more complex protein and particularly antigen pattern, than *F. magna*.

6. Abbreviations

°C	-	Degree Celsius
µl	-	Microliter
A	-	Adenine
ALT, ALAT, old: GPT	-	Alanine transaminase
AST, ASAT, old: GOT	-	Aspartate transaminase
AT	-	Alkaline phosphatase
bp	-	Base pairs
C	-	Cytosine
Car	-	Carinthia
CDC	-	Centers for Disease Control and Prevention
COX1	-	Cytochrome oxidase subunit 1

Cpd α, Cpd 6	-	Compound alpha
ddH_2O	-	Double-distilled water
DNA	-	Deoxyribonucleic acid
dNTP	-	Deoxyribose nucleoside triphosphate
EDTA	-	Ethylenediaminetetraacetic acid
e.g.	-	exempli gratia
ERCP	-	Endoscopic retrograde cholangiopancreatography
et al.	-	*et alii, et aliae, et alia*
EtOH	-	Ethanol
Fh	-	*Fasciola hepatica*
Fig.	-	Figure
Fm	-	*Fascioloides magna*

fwd	-	forward
G	-	Guanine
g	-	gravitational
g	-	Gram
Gamma GT	-	Gamma-glutamyl transpeptidase
GLDH	-	Glutamate dehydrogenase
Ibk	-	Innsbruck
IgE	-	Immunoglobulin E
IgG	-	Immunoglobulin G
LA	-	Lower Austria
M	-	Mol
m	-	Molar

mA	-	Miliampere
max	-	Maximum
mg	-	Milligram
$MgCl_2$	-	Magnesium chloride
min	-	Minimum
ml	-	Millilitre
NaAc	-	Sodium acetate
NAD1	-	Nicotinamide dehydrogenase subunit 1
NCBI	-	National Centre for Biotechnology Information
OH	-	Hydroxy
PCR	-	Polymerase chain reaction
pmol	-	Picomol

rev	-	Reverse
rpm	-	Revolutions per minute
RT	-	Room temperature
Sbg	-	Salzburg
SDS-PAGE	-	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SNP	-	Single-nucleotide polymorphism
spp	-	Species
T	-	Thymine
Tab	-	Table
TAE	-	Buffer with Tris, acetic acid and EDTA
TCBZ	-	Triclabendazole
T_M	-	Melting temperature

Tyr	-	Tyrol
UV	-	Ultraviolet
V	-	Volt
Vie	-	Vienna
x g	-	times gravity

7. Glossary

Acute	A disease or medical condition that lasts for less than 6 months.
Adenine	A purine derivate and one component of nucleotides, DNA and RNA.
Adult	Phase in that an organism completed the juvenile phase and/or reach the sexual maturity.
Agarose	A polysaccharide, consisting of D-Galactose and 3,4-Anhydro-L-Galactose. Used for gel electrophoresis.
Alignment	Arrangement of DNA sequences.
Alinia	A synthetic antiprotozoal agent, containing the active ingredient nitazoxanide. It is also effective against a broad range of parasites including *F. hepatica.*
Alternation of generation	Changing of sexually and asexually reproduction and forms/generations.
Amplicon	DNA-sequence, a product of amplification.
Amplification	Overexpression of genes. Can happen in an optimised environment like PCR or naturally.
Anterior	Pertains the front or the lower surface of animals.
Cercariae	Free swimming larval stage of trematodes. Appears in the asexual part of molluscan intermediate hosts.
Chronic	A disease or medical condition that lasts for over 6 months.
Class	Used for classification of organisms. A class consist of orders.

Classification	Hierarchical categorisation and determination of relationships of organisms. The basis of the hierarchy is the species, followed by genus, family, suborder, order, subclass, class, phylum, and at last kingdom.
Compound Alpha	A fasciolicide with a very similar chemical structure to triclabendazole.
COX1	A highly conserved mitochondrial gene, used for genetic typing and barcoding of organisms.
Cytosine	A pyrimidine derivate and one component of nucleotides, DNA and RNA.
Dead-end host	A host preventing the parasite from completing its development.
Dorsal	Pertains the back or the upper surface of animals.
Electrophoresis	Motion of dispersed particles in an electric field.
Encystment	Development of an environment resistant wall to help the organism to survive unfavourable environment conditions.
Endemic	Occurrence of species in a specific area.
Enzootic	Occurrence of species in specific animals.
Enzyme	A protein catalyzing biochemical reactions.
Eosinophilia	Increased number of eosinophils.
Eosinophils	White blood cells, part of the immune system. Important for combating helminths.
Epidemiology	Study of patterns, effects and causes of diseases.

Epithelium	Cells covering body surfaces or lining the internal surfaces of body cavities, tubes and hollow organs.
Excretion	Waste products of the metabolism, eliminated with faeces and/or through the skin.
Excystment	Parasite escapes from the protecting wall for further development.
Faeces	Waste products of the metabolism and non digestible components.
Family	A taxonomic unit which contains genera and species.
Fascioloidosis	The disease caused by *F. magna.*
Fasciolosis	The disease caused by *F. hepatica.*
Final host	A host in which a parasite attains sexual maturity.
Gel electrophoresis	A method to separate DNA or proteins by length.
GenBank	Sequence database with open access.
Gene	A protein or RNA coding unit of the DNA.
Genome	The whole of genes of an organism.
Glycocalyx	Polysaccharides, secreted by liver flukes to evade from the immune system of the host.
Guanine	A purine derivate and one component of nucleotides, DNA and RNA.
Habitat	A place where a population normally lives.
Haplotype	A variation of a gene sequence on the same chromosome. Can be specific for a population,

	individuals or a species.
Helminths	Parasitic worms, including nematodes, trematodes and cestodes.
Hermaphroditism	An individual contains male and female reproductive systems.
HiDi	Highly deionised
Homology	Characteristics of organisms, derived from a common ancestor.
Infection	Invasion and multiplication of body invading organisms. These organisms can cause disease.
Infestation	Settlement of parasites in a body, which are unable to breed in the host.
Intermediate host	A host in which the larval stages of a parasite occur. The life cycle of parasites can include more than one intermediate host.
Juvenile	Immature form of an organism. Phase before organisms reach adulthood and/or sexual maturity.
Larva	Immature form of organisms. To reach maturity, further development is required.
Life cycle	The course of the development of organisms.
Metacercariae	Part of the larval stage of parasites. The infective stage for the final host.
Miracidium	Ciliated embryo, infective for the intermediate host.
Mitochondrion	A double membrane-bound organelle in eukaryotic cells.
Mollusca	Phylum of invertebrates.

Morphology	Description of form and structure of organisms but not their function.
NAD1	A highly conserved mitochondrial gene, used for genetic typing and barcoding of organisms.
Nucleic acid	Chain of nucleotides. Two forms of nucleic acids: DNA and RNA.
Nucleotide	Organic molecule. Consists of pyrimidine or purine base, ribose or desoxyribose and a phosphate group. They can form nucleic acids like DNA and RNA.
Operculum	A lid on parasite eggs. Enables the escape from the egg.
Oral sucker	The opening to the digestive gland. Flukes use the oral and the ventral sucker in order to attach on the wall of bile ducts and the intestine.
Parasite	Organism, living in or on other organisms and use them to get nourishment from it and for reproduction.
Pathogen	An organism which can cause a disease.
PCR	Technique to amplify DNA and to generate many copies of a DNA fragment.
Phylum	Category for classification. Consists of classes.
Phylogeny	The evolutionary history, development and relationship of groups of organisms.
Plant-borne disease	Diseases transmitted with plants.
Platyhelminthes	A phylum of acoelomate invertebrate organisms.
Polymerase	Enzyme which catalyzes the formation of polynucleotides. Needs a template strand of

	DNA or RNA.
Posterior	Pertains the back or the upper surface of animals.
Prevalence	A term in epidemiology. Describes the percentage of infected individuals in a population at a certain point of time.
Primer	A short single-stranded DNA-fragment which marks the starting point for the DNA synthesis in a PCR reaction. Can be used as forward and as reverse primer.
Proteinase	Enzymes able to destruct proteins.
Rediae	One part of the larval stage in the development of trematodes.
SDS-PAGE	A method to separate proteins by length.
Sequencing	A method to determine the nucleotide order in a DNA molecule.
SNP	A DNA sequence variation within a population. Between members of a species or paired chromosomes a nucleotide in a genome differs.
Species	For classification of organisms. It describes a group of organisms which usually breed among themselves to produce fertile offspring.
Syncytial epithelium	An epithelium, consisting of a group of cells, resulting from multiple cell fusions and interconnected with gap junctions.
Systematic	The study and classification of organisms. The goal is the reconstruction of their evolutionary history and relationships.
Taxon	Part of the classification. Consists of a group of one or more populations.

Taxonomy	The specification of groups of biological organisms based on the anatomical and morphological characteristics.
Tegument	Cells covering the external body surface. The function is, among others, nutrient absorption and immune evasion.
Thymine	A pyrimidine derivate and one component of nucleotides, DNA and RNA.
Trematoda	Part of the class within the phylum Platyhelminthes.
Triclabendazole	An antihelminthic drug and belongs to the benzimidazole family. Triclabendazole prevents polymerization of microtubules.
Vector	Carrier which is important for the transmission of parasites between hosts.
Ventral sucker	Opening, located near the oral sucker. Important for the movement together with the oral sucker, also called acetabulum.
Ventral	Pertains the front or the lower surface of animals.
Western-Blot	Technique to transfer proteins from a gel to a membrane to detect them with staining methods.
Zoonosis	A disease, transmitted from animals to humans.

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9. Appendix

9.1 Abstract

9.1.1 English

Diversity of *Fascioloides magna* and *Fasciola hepatica* in Austria based on their genes and antigens.

The aim of this study was to investigate the genetic diversity of *F. magna*, and for the first time, the diversity of *F. hepatica* in Austria. *Fascioloides magna* was introduced into Europe in the second half of the 19th century with game animals from North America and it mainly infests the liver of deer. Roe deer, sheep and goats are dead-end hosts and they usually die within six months after infestation. Human infestations are to date unknown. The common liver fluke *Fasciola hepatica* also is a parasitic flatworm, which however, occurs in more than 70 countries and infests the liver of various mammals including humans. It is estimated that around 50 million humans worldwide are infested by *F. hepatica*. As the main hosts are sheep and cattle, *F. hepatica* also has a great impact on meat and milk production and leads to significant economic loss annually.

Altogether, 26 individuals of *F. magna* isolated from deer, collected from the floodplains in the South of Vienna, which is the only known endemic region in Austria, and 31 individuals of *F. hepatica*, isolated from cattle, from various regions in Austria, were analysed by molecular biological methods. In order to distinguish the different haplotypes of the flukes, the sequences of the mitochondrial genes *cox1* and *nad1* were investigated and compared with reference strains. Moreover, as for *F. hepatica* no widely accepted haplotype system has been established yet, a new typing system was set up.

It was shown that all 26 individuals of *F. magna* are of the same haplotype, namely COX1-Ha3/NAD1-Ha4, respectively. Austria has this haplotype in common with Hungary, Slovakia, the Czech Republic and Croatia. The 31 individuals of *F. hepatica* could be grouped in two clusters for *cox1* and into three different clusters for *nad1*, whereby the most common *cox1* and *nad1*

haplotype was shared by 21 individuals and the second most common by 10 individuals for *cox1* and nine individuals for *nad1*. One *nad1* haplotype was represented only by one individual. Differences were more pronounced in the *nad1* gene than in the *cox1* gene.

This study indicates that the Austrian *F. magna* population is rather homogenous and might even be the result of a single introduction into Austria, while the Austrian *F. hepatica* population is more diverse. Moreover, it was shown that the protein profiles and even more so the antigen profiles of *F. magna* and *F. hepatica* are highly diverse and that *F. hepatica* reveals a more complex protein and antigen pattern than *F. magna*. In total, only three intracellular proteins and two membrane proteins are shared by both flukes. The antigen profile revealed one common intracellular and one common membrane antigen.

9.1.2 German

Zusammenfassung:

Molekularbiologische Charakterisierung von *F. magna* und *F. hepatica*-Isolaten aus Österreich.

Ziel der Studie war die Untersuchung der genetischen Diversität von *F. magna* und *F. hepatica* in Österreich. *Fascioloides magna* wurde in der zweiten Hälfte des 19. Jahrhunderts mit Jagdtieren aus Nordamerika importiert und befällt hauptsächlich die Leber von Wildtieren. Rehe, Schafe und Ziegen sind Fehlendwirte und sterben etwa 6 Monate nach dem Befall. Infektionen beim Menschen sind nicht bekannt.

Der gemeine Leberegel *Fasciola hepatica* ist in über 70 Ländern heimisch und befällt die Leber von zahlreichen Säugetieren, darunter auch die des Menschen. Schätzungen zufolge sind rund 50 Millionen Menschen weltweit mit *F. hepatica* infestiert. Da Schafe und Rinder Hauptendwirte darstellen, hat *F. hepatica* einen sehr großen Einfluss auf die Fleischproduktion und verursacht jährlich erhebliche wirtschaftliche Verluste.

Insgesamt wurden 26 Individuen von *F. magna* aus Lebern von Wild aus den Überschwemmungsgebieten südlich von Wien, dem einzig bekannten Endemiegebiet Österreichs, und 31 Individuen von *F. hepatica* aus Lebern von Rindern aus verschiedenen Regionen in Österreich, mit molekularbiologischen Methoden untersucht. Für die Bestimmung der Haplotypen wurden die Nukleotidsequenzen der mitochondrialen Gene *cox1* und *nad1* untersucht und mit Referenzstämmen verglichen.

Alle 26 Exemplare von *F. magna* sind vom selben Haplotyp, nämlich COX1-Ha3/NAD1-Ha4. Diesen Haplotyp hat Österreich mit Ungarn, der Slowakei, der Tschechischen Republik und Kroatien gemein. Die 31 Individuen von *F. hepatica* können in 3 Gruppen eingeteilt werden, wobei der häufigste Haplotyp für *cox1* und *nad1* bei 21 Individuen, der zweithäufigste *cox1*-Haplotyp bei 10 Individuen, der zweihäufigste *nad1*-Haplotyp bei 9 Individuen und 1 *nad1*-Haplotyp bei einem Individuum gefunden wurde. Weiters wurde deutlich, dass die Unterschiede im *nad1*-Gen ausgeprägter waren als im *cox1*-Gen.

Die Studie zeigt, dass die österreichische *F. magna*-Population eine größere Homogenität aufweist, was auf eine einzige Einschleppung schließen lässt, während hingegen *F. hepatica* eine höhere Diversität besitzt, was bereits von anderen Ländern bekannt ist. Es hat sich gezeigt, dass die Proteinprofile und insbesondere die Antigenprofile der beiden Leberegel sehr unterschiedlich sind. *F. hepatica* lieferte ein komplexeres Protein-Profil als *F. magna* und folglich mehr Antigene. Insgesamt waren 3 intrazelluläre und 2 Membranproteine den beiden Egeln gemeinsam. Das Antigenprofil zeigte, dass nur ein intrazelluläres und ein Membran-Antigen den beiden Egeln gemeinsam war.

9.2 Index of Figures

9.3 Index of Tables