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1 ABBREVIATIONS

µl	-	microlitre
A	-	adenine
BLAST	-	Basic Local Alignment Search Tool
bp	-	base pairs
C	-	cytosine
CDC	-	Centers for Disease Control and Prevention
cit. in	-	cited in
conc.	-	concentration
CTAB	-	cetyltrimethyl-ammoniumbromide
DNA	-	deoxyribonucleic acid
dNTP	-	deoxyribonucleotide
EDTA	-	ethylenediaminetetraacetic acid
EpG	-	eggs per gram
esp.	-	especially
EtBr	-	ethidium bromide
EtOH	-	ethanol
FECT	-	formalin ethyl acetate concentration technique
G	-	guanine
g	-	gram
g (force)	-	gravitational
incl.	-	including
ITS	-	internal transcribed spacer
KK	-	Kato Katz technique
LSU	-	large subunit
M	-	mol
max	-	maximum
mg	-	milligram
$MgCl_2$	-	magnesium chloride
min	-	minute(s)
min	-	minimum
ml	-	millilitre
mm	-	millimetre
NaAc	-	sodium acetate
NaCl	-	sodium chloride
NCBI	-	National Centre for Biotechnology Information

ng	-	nanogram
ÖBf	-	Österreichische Bundesforste AG (Austrian Federal Forests)
PCR	-	polymerase chain reaction
pg	-	picogram
PTEF	-	polytetrafluoroethylene (Teflon)
RAPD	-	randomly amplified polymorphic DNA
rDNA	-	ribosomal DNA
RFLP	-	restriction fragment length polymorphism
RNA	-	ribonucleic acid
rpm	-	rotations per minute
RT	-	room temperature
SAF	-	sodium acetate-acetic acid-formalin
SSU	-	small subunit
sp.	-	species
T	-	thymine
TAE	-	buffer consisting of Tris, acetic acid and EDTA
Tris	-	tris(hydroxymethyl)aminomethane
UV	-	ultra violet
V	-	volt

2 INTRODUCTION

2.1 History

The history of parasites is a very long one and a lot of research has been performed, discoveries as well as errors have been made and the interest and knowledge became larger and more intensive. In the beginnings, descriptions of a particular disease were made or the parasites causing the disease were discovered. Subsequently, hosts and life cycles were recognized and logical causal relationships between the parasites and the diseases were established.

2.1.1 History of helminths

Since some helminths are of respectable size, it is considered that already our earliest ancestors must have been aware of common worms such as *Ascaris* or tapeworms (Cox, 2002). Supporting this theory are some contemporary studies of primitive tribes in Sarawak (Malaysia) and North Borneo done by Hoeppli (1956 and 1959 [cit. in Cox, 2002]), where he found that most of the people were well aware of their own intestinal round- and tapeworms. Some alleged evidence for helminthic worms and the diseases they cause in the Bible are discussed by historians, but the text passages can be interpreted in several ways (Cox, 2002). However, there are written records in the Ebers papyrus (1500 BC) of the Egyptians referring to intestinal worms, which were confirmed by the findings of calcified helminth eggs in mummies from 1200 BC (Bryan, 1930 [cit. in Cox, 2002]). Additionally, in 1910 Ruffer provided the first record of ancient parasites, when he found calcified *Schistosoma haematobium* eggs in the kidneys of two Egyptian mummies of the twentieth dynasty (1250-1000 BC) after producing microscopic sections and staining of the tissue.

In the times of the Greeks and the Romans, Hippocrates (460-374 BC), Aristotle (384-322 BC), Celsus (25 BC-50 AD) and Galen (129-200 AD) were aware of worms from fishes, domesticated animals and also humans. As written by Foster (1965 [cit. in Sattmann, 2002]), Aristotle already knew gut worms of humans and domestic animals and fish and he described the cysticerci of pigs, which he compared to hailstones rather than believing them to be real organisms. As described by Grove (1990), they knew tapeworms (*Taenia* species), round worms (*Ascaris lumbricoides*) and threadworms (*Enterobius vermicularis*),

which were then described in detail by Paulus Aeginata (625-690 AD), who also gave clinical descriptions of the diseases they cause. Sattmann (2002) also lists Plutarch (46-120 AD), Plinius Secundus the elder (23-79 AD) and Dioskurides (40-90 AD) as important natural scientists studying parasitic worms in the ancient world. Egerton (2001) wrote about the five Roman agricultural treatises, which contain not only observations on environmental factors like soil fertility, moisture or crop pests, but also on animal parasites. Particularly the Roman author Lucius Junius Moderatus Columella, who lived at the time of the Roman Emperor Claudius, wrote in his "Res Rustica" a lot about parasites, their influences on livestock and potential therapies. After the fall of the Roman Empire, Arabic physicians such as Avicenna (Ibn Sina, 981-1037) recognized also other worms like the guinea worm *Dracunculus medinensis*, which had long been known in the Arabian world (Cox, 2002). It is also assumed that the symbol of the medical fraternity, the Aesculapian staff, originates from the old therapy of removing adult guinea worms by coiling them onto a stick and pulling them slowly out of the skin (Sattmann, 2002).

Helminthology and records of worms and the diseases they cause then stagnated a little during the Middle Ages, but experienced a boom in the 17th and 18th centuries after the Renaissance period mainly through the works of Italian, Dutch and German natural scientists (Sattmann, 2002). Francesco Redi (1626-1698) is considered to be the founder of scientific helminthology, he gave a first overview but also some detailed descriptions of parasitic worms known to that date (Redi, 1685). Nevertheless, Redi and many other famous scientists, mainly taxonomists, believed in spontaneous generation when it comes to gut worms. Bremser wrote that "parts of the intestinal slime clot into a compact mass, covering itself with an epidermis and leading its own life from that time on" in 1819 (Bremser, 1819 [cit. in Sattmann, 2002]). In 1758, Linnaeus, the founder of modern zoological nomenclature, described different helminth worms. In his "classis vermes" he characterised *Ascaris lumbricoides*, *Ascaris vermicularis* (today *Enterobius vermicularis*), *Gordius medinensis* (*Dracunculus medinensis*), *Fasciola hepatica*, *Taenia solium* and *Taenia lata* (*Diphyllobothrium latum*), classifying the latter two into the "ordo zoophyta" and the other four into the "ordo intestina". In the 19th century, numerous discoveries were made in zoology and parasitology, due to expeditions and the desire to understand characteristics, patterns and differentiation criteria of species, their habitats, hosts and evolution. Little by little, also the very complex life cycles of helminths were discovered and causal connections were revealed. While Paget recognised muscle-trichinella as a worm already in 1835, it was almost 25 years later that Leuckart and Virchow pointed out

that this muscle-trichinella is in fact the larva of the gut-trichinella by clarifying the life cycle of *Trichinella* (Enigk, 1986 [cit. in Sattmann, 2002]). Subsequently, more and more helminth species were described. While the number of described species in humans at the beginning of the 20th century was still below 30 (Cox, 2002), recent records show a ten-fold higher number, of which some were accidental or very rare parasites (Coombs & Crompton, 1991). Ashford & Crewe (1998 [cit. in Cox, 2002]) listed 280 helminth species in humans, some of these potentially arguable. Levine (1965), who included animal parasites as well, stated that by 1951, approximately 5,100 trematode species had been described. Then, in the 1950s, 2,000 cestodes and 10,000 nematodes were described. Today, approximately 8,000 trematode species are known (Aspöck, 2002). Additionally, approximately 5,000 cestodes, 500 acanthocephaleans and 15,000 parasitic nematodes have been described. Approximately 350 helminth species are potential human parasites, whereof many were only found a few times or have parasitized in humans accidentally.

2.1.2 History of trematodes

Even if helminths in general had been known and described for a long time, it was not until 1379 when Jean de Brie made the first recorded reference of liver flukes in sheep (de Brie, 1379 [cit. in Grove, 1990]). Even if he did not give any clear details on the morphology or nature of the worm, it is now believed that the fluke he found was *Fasciola hepatica*. Nevertheless, Grove (1990) assumed that there were numerous people over many years who probably found flukes independently from each other without leaving written evidence. According to Cribb et al. (2001), the earliest mention of a fluke's name, which is nowadays still recognizable, was by Garcin, *Hirudinella marina* Garcin 1730, which is now called *Hirudinella ventricosa*. He also refers to two *Fasciola* species listed by Linnaeus in 1758, but only *F. hepatica* was proven to be digenean. Wrongly described "worms" that were seen by Swammerman in 1758 while he was dissecting snails (Andrews, 1999), were first named "cercariae" by Müller in 1773. He thought that the swimming tadpole-like creatures were independent adult worms or Infusoria, but what he named *Cercaria tenax* were in fact trichomonads (*Trichomonas tenax*) (Dobell, 1939). From 1800 on, first attempts of a classification of this group were made by Dujardin, Looss and many others (Cribb et al., 2001). In 1803, Zeder described the observation of miracidia hatching out of eggs from different trematode species. A few years afterwards, in 1807, Nitzsch observed cercarial encystment when the swimming cercariae attached to the substrate, lost their tail and became covered with a gelatinous substance (Andrews, 1999).

At that point he thought that the cercariae had died and also, that cercariae and flukes were different species even if he did see similarities in the anterior end of cercariae and distome flukes. Another important step in the history of trematodes and especially in the elucidation of their life cycles was the observation of rediae transforming into cercariae by Bojanus in 1818 (Andrews, 1999). Bringing all observations of the individual stages together, it was Steenstrup who first saw the relations between them in a trematode life cycle and proposed his ideas of the alternation of generations in 1845 (Grove, 1990). As listed by Andrews (1999), further discoveries in the description of digenean life cycles were made by La Valette St. George in 1855, who observed the infection of a snail by a miracidium, by Wagener in 1857, who observed the miracidial penetration into the snail as well as the subsequent development of the rediae, or by Weinland in 1875, who established a relationship from cercariae to certain intermediate host snail species and assumed that the cercariae encyst on vegetation to be eaten by definitive hosts. The approximate life cycle of *F. hepatica* as one of the first digenean cycles was described independently by Leuckart (1881, 1882) and Thomas (1881, 1882), but it was not until 1892/93 when Lutz experimentally confirmed the ingestion of metacercariae as the mode of infection for the final hosts (Lutz, 1892 and 1893 [cit. in Andrews, 1999]). In 1914, Sinitsin described the exact migration route of young liver flukes in their final hosts and elucidated the last part of the fasciolid life cycle, which gave new impulses to studies on other trematode species.

2.1.2.1 Fasciolidae

The landmarks in the discovery of *Fasciola* and Fasciolosis were well summarized by Grove in 1990. There is no knowledge on the first discoverer of liver flukes, but, as mentioned above, the first written record was made by Jan de Brie in 1379 who probably found adult specimens of *Fasciola hepatica* in sheep and made them responsible for sheep rot and putrefaction of the liver. He also argued about possible correlations with the type of pasture and time of grazing. Other reports on worms in animal livers were made not only by scientists like Fitzherbert, Gabucinus or van Leeuwenhoek, but of course also the butchers knew about those creatures (Grove, 1990). Nevertheless, the understanding of what exactly they were was not made until considerably later. In 1670, Faber published his observations on worms in the biliary trees and 20 years later Bidloo found the eggs of liver flukes. Also, he suggested that the flukes were hermaphroditic. The first description of liver flukes in humans was made by Pallas in 1760, even if some workers reported the possible appearance in human livers before him. In 1855, Cobbold first described *Fasciola*

gigantica, which he discovered in the liver of a giraffe. As written by Grove (1990), the elucidation of the life cycle was promoted by discoveries of miracidial and cercarial development (Leuckart, 1882; Thomas, 1882), the mode of infection (Lutz, 1892) and the way of migration in the definitive host (Sinitsin, 1914). The first distinct diagnosis of human fasciolosis was made by Ward in 1911, who found eggs in faeces.

The important discoveries around *Fasciolopsis* were also summarized by Grove (1990). In 1843 George Busk discovered 14 adult flukes in the duodenum of a dead sailor. Descriptions of the worm were published by Budd in 1852, who noted that it resembled *Distoma hepaticum* in shape but *Distoma lanceolatum* in structure. In 1857 Lankester named it *Distoma buskii* after the discoverer's name. Until 1919, when Goddard published the final description and named the worm *Fasciolopsis buski*, multiple renamings and regroupings were made by several authors (Grove, 1990). In 1891, Walker found fluke eggs in faeces and consequently diagnosed fasciolopsiosis. In 1920 after 5 years of investigation, Nakagawa succeded in adjusting an experimental life cycle (Nakagawa, 1921, 1922). He hatched miracidia from eggs that he gained from pigs, he observed the miracidia swimming in the water and eventually infecting snail hosts, he observed the development in the snails and the encystation on plants. Moreover, he experimentally infected dogs and pigs and recovered flukes from them. Human self-experiments were performed by Barlow, who swallowed fluke eggs, adult worms and cysts between 1918 and 1922 (Grove, 1990). While the eggs only passed the intestine, Barlow did develop a persistent infection when ingesting the worm and a patent infection after the cysts. He also suggested that human infection can be acquired with the consumption of water plants.

Another species within the family of the Fasciolidae is *Fascioloides magna*. It was first described by Bassi in 1875, who named the worms he had found in cervids *Distomum magnum*. In 1917, it was classified as it is today by Ward.

2.1.2.2 Paramphistomidae

Trematodes belonging to the family of Paramphistomidae are non pathogenic for humans and were thus neglected by scientists for a long time. It was not until the beginning of 1900 when Fischoeder erected the family Paramphistomidae (Jones, 2005d). Even if some species of those rumen flukes cause parasitoses in wild and domestic animals, paramphistomosis is still misjudged as a serious contributor to economic losses in animal husbandry due to little awareness of the disease itself and the *Paramphistomum* species

causing it (Mage et al., 2002). The first description of *Paramphistomum daubneyi* was made by Dinnik in 1962 (Sey, 1979).

2.1.2.3 Dicrocoeliidae

Dicrocoelium dendriticum was often and long confused with *Fasciola hepatica* as was the clinical appearance of dicrocoeliosis in humans. The worm was first named *Fasciola lanceolata* and *Fasciola dendritica* by Rudolphi in 1803 and 1819, respectively. The name used today was introduced after Looss transferred the worm to the genus *Dicrocoelium* in 1899 (Grove, 1990). As Grove further describes, the first who saw cercariae of *D. dendriticum* was von Linstow in 1887. He named them *Cercaria vitrina* but did not consider them to be related to *D. dendriticum*. Almost 50 years later, several studies contributed to the understanding of the life cycle by finding intermediate host snails (Nöller, 1929, Vogel, 1929) and elucidating the mode of infection (Cameron, 1931). Krull & Mapes first described the metacercarial development in ants in 1952 and 1953, and intermediate host ant species were discovered by them and several other workers in the field.

2.1.3 Nomenclature

According to Grove (1990), the name "worm" is indefinite but widely recognized as an elongated creature that is creeping around and does not belong to any other grouping. The term worm is derived from the Latin word vermis. It has been used for centuries to describe any worm-like creature from earthworms to insect larvae or parasitic worms in animals (Moulé, 1911). Rudolphi (1808) introduced the term Trematoda in his "Entozoorum, sive vermium intestinalium historia naturalis" translating Zeder's (1800) Saugwürmer (sucking worms) into a classical term (Grove, 1990). The name Trematoda is derived from the Greek term Trematodes which means "foraminous" or "pierced with holes". On the basis of this, Rudolphi's trematodes describe creatures with a flattened or slightly rounded body, with a soft structure and sucking holes. Cribb et al. (2001) quoted the first mention of the term Digenea, which was made by Carus in 1863, with the definition of a group having a life cycle with alternation of generations, larvae in molluscan intermediate hosts and a sexual phase in their vertebrate definitive host.

The genus *Fasciola* and its most known species *Fasciola hepatica* were induced by Linnaeus in 1758 (Grove, 1990). The name is derived from the Latin word fasciola which means fillet or small bandage, hepatica indicates the location of the adult worms within the hosts liver. Both genus and species names were changed several times until the law of priority, as declared by the International Commission on Zoological Nomenclature, put Linnaeus‘ naming back into validity.

The genus *Dicrocoelium* was errected by Dujardin in 1845 (Grove, 1990). The term dicrocoelium is derived from the Greek words dikroos or dicroos for double and koilia or coelia for cavity.

2.2 Phylogeny and systematics

2.2.1 Phylogeny of helminths

In general, worms are described as multicellular invertebrate animals with an elongated shape and a creeping moving manner. In Linnaeus' "Systema Naturae" from the 18th century, he classified several multicellular organisms including insect larvae and other non-worm creatures as worms. Grove (1990) divided the "worms" into several phyla and classes: the Nematoda (roundworms), the Acanthocephala (thorny-headed worms), the Nematomorpha (hairworms or gordiid worms), the Annelida (segmented worms including earthworms and leeches) and the Platyhelminthes (flatworms), which on their part comprise the Cestoda, the Monogenea, the Trematoda and the Turbellaria (Table 2.1).

Table 2.1: Systematic classification of the Platyhelminthes with selected species. (http://www.ncbi.nlm.nih.gov/)

Superkingdom	Eukaryota			
Kingdom	Metazoa			
	Eumetazoa			
	Bilateria			
	Acoelomata			
Phylum	Platyhelminthes			
Class	Cestoda	Monogenea	Trematoda	Turbellaria
Subclass	Eucestoda		Digenea	
Superorder/Order	Cyclophyllidea	Monophistocotylea	Echinostomida	Rhapdocoela
Family	Taeniidae	Dactylogyridae	Fasciolidae	
Genus	*Taenia*	*Dactylogyrus*	*Fasciola*	*Anoplodium*
Species	*T. saginata*	*D. alatus*	*F. hepatica*	*A. stichopi*

2.2.2 Phylogeny of trematodes

The Trematoda, or flukes as commonly referred to, are a class within the phylum of Platyhelminthes (Gibson, 2002). The phylum is large and diverse and according to Blair et al. (1996) it seems to be an early diverging lineage within the Metazoa. The parasitic groups of the flatworms, Cestoda, Monogenea and Trematoda, form a derived clade and are summarized under the term Neodermata (Olson & Tkach, 2005). This term goes back to Ehlers based on the replacement of the larval epithelium by a syncitial neodermis or tegument in the adult (Ehlers, 1984 [cit. in Olson % Tkach, 2005]). In addition to this, members of the neodermatans resemble each other in structural and developmental features such as sensory receptors or flame bulbs (Blair et al., 1996). Olson & Tkach (2005) report on attempts of clarifying the phylogenetic interrelationships of the groups belonging to the Neodermata by molecular approaches but these relationships are still not fully understood.

The class Trematoda is subdivided into two subclasses, the Digenea and the Aspidogastrea. Until the late 20th century, it was believed that the Monogenea also belong to the trematodes, but nowadays the Monogenea form a distinct class with closer relationships to the cestodes rather than the trematodes (Cribb et al., 2002). The Aspidogastrea form a small but separate group with few nominal genera parasitizing in molluscs, fishes and chelonians (Gibson, 2002). The Digenea comprise more than 2,500 nominal genera almost

always using molluscs as intermediate hosts and vertebrates as definitive hosts. With their ~18,000 nominal species the Digenea are considered the largest and most specious group of internal metazoan parasites (Cribb et al., 2001). Despite a lot of work and research published in the field of classification and phylogeny dating back to the 18th century, the group remains unstable and its interrelationships at both higher and lower taxonomic levels remain unclear (Olson & Tkach, 2005). According to Gibson (1987), one of the main reasons for this is that the group of the Digenea cannot be easily split up into major subgroups by morphological features due to their non-homoplasious nature. Additionally, the Digenea's complex life-history patterns complicate a classification. Several attempts of classifications and reclassifications have been made beginning in the late 18th century. The classifications based on different morphological features such as for example sucker arrangements (Dujardin, 1845 [cit. in Cribb et al., 2001]), life cycle characteristics (van Beneden, 1858 [cit. in Grove, 1990]) or cercarial morphology (Cable, 1974 [cit. in Gibson, 1987]). In 1994, Gibson & Bray analyzed data of a host-parasite database in relation to host groups, host specificity, speciation, radiation and geographical distribution. The study comprised over 150 families of 17 groups and gave some insight in the classification and evolution of the Digenea. One conclusion was a simple classification of the Digenea, dividing the group into three orders mostly relying on the cercariae (Gibson & Bray, 1994 [cit. in Gibson, 2002]). The Strigeida (comprising blood flukes, brachylaemids, bucephalids, clinostomids, diplostomids, fellodistomids, gymnophallids, hemiuroids and strigeids), with forked-tailed cercariae that tend to penetrate the next host, the Echinostomida (comprising echinostomatids, fasciolids, notocotylids, paramphistomoids, pronocephalids and psilostomids), with cercariae encysting in the environment and the Plagiorchiida (comprising acanthocolpids, allocreadiids, lepocreadiids, opecoelids, cryptogonimids, decrocoeliids, heterophyids, lecithodendriids, microphallids, monorchiids, opisthorchiids, plagiorchiids and zoogonids), with cercariae seeking the next host but without a fork tail (Gibson, 2002). Following this three-order division, a comprehensive publication in three volumes dealing with the systematic groupings of trematodes and the keys to classify them was released by Gibson et al. (2002), Jones et al. (2005) and Bray et al. (2008).

In the past two decades molecular approaches have been increasingly applied to improve the understanding of the interrelationships and the evolution of digenean trematodes and other parasitic platyhelminths. Most of the studies were performed for groups that are of economic or medical importance such as the schistosomes. As explained by Cribb et al.

(2001), the studies can be subdivided into two broad classes. On one hand specific problems of relationships of closely related taxa within families were addressed, and on the other hand the relationships of more distantly related taxa with each other. These studies, which were manifold and numerous, used different sequences, including the 18S ribosomal DNA (SSU rDNA), 28S ribosomal DNA (LSU rDNA) and the internal transcribed spacers (ITS) 1 and 2, but also sequences from mitochondrial DNA (mtDNA) were used. Additionally, a lot of non-sequence-based works were performed as for example alloenzyme electrophoresis, thin layer chromatography, DNA hybridization, randomly amplified polymorphic DNA (RAPD) and restriction fragment length polymorphism (RFLP) (Olson & Tkach, 2005).

In 2001, Cribb et al. conducted a large scale cladistic study, in which they combined morphological features, data from all life cycle stages and molecular data from the nuclear SSU rRNA gene. Altogether, they used data from 95 different digenean and five aspidogastrean species representing approximately 36% of the ~150 families known to that time. They suggested the clade-status or the non-monophyly, respectively, for several families and concluded, that the Bivesiculidae + Transversotrematidae form a sister group to the rest of the Digenea and the Diplostomoidea + Schistosomatoidea are the next most basal taxon. This led to the awareness that the traditional division into the three orders Echinostomida, Plagiorchiida and Strigeida had to be revised, as the Echinostomida had a clear polyphyletic nature (Olson & Tkach, 2005). In 2003, a comprehensive phylogenetic study based on the complete SSU rRNA gene and partial sequences of the LSU rRNA gene was conducted by Olson et al. The study included 163 digenean taxa of 77 nominal families and seven aspidogastrean taxa as outgroups. The independently as well as combined analyses using maximum parsimony and Bayesian inference led to the conclusion that the Digenea form a dichotomy with the two major clades Diplostomida, a newly erected group, and the remainder of the Digenea, the Plagiorchiida La Rue, 1957 (Figure 2.1). The Diplostomida can be subdivided into three main lineages: the Brachylaimoidea, the Diplostomoidea and the Schistosomatoidea. Within the Plagiorchiida the newly established groups Bivesiculata and Transversotremata are the most basal lineages, the Hemiurata the next basal one. The rest of this group, the so called higher plagiorchiidans, showed inconsistent relationships due to the mostly independent lineages (13 suborders), but included also the crown clade of the Digenea, the Xiphidiata. Nevertheless, the molecular analyses supported former classifications as suggested by Gibson & Bray (1994 [cit. in Gibson 2002]) at least at the level of families and in some

cases at the level of superfamilies (Olson et al., 2003). However, the division into the orders Echinostomida, Plagiorchiida and Strigeida has now been abandoned as they reflect non-natural groupings of taxa (Olson et al., 2003). While members of the paraphyletic Strigeida were distributed among the Diplostoma and Plagiorchiida, the Echinostomatida formed a polyphyletic assemblage with its members distributed all over the Plagiorchiida. Even if this study was the largest of its kind, there are still important gaps, the filling of which could lead to the final elucidation of the phylogeny of the large and diverse group of digeneans (Olson et al., 2003).

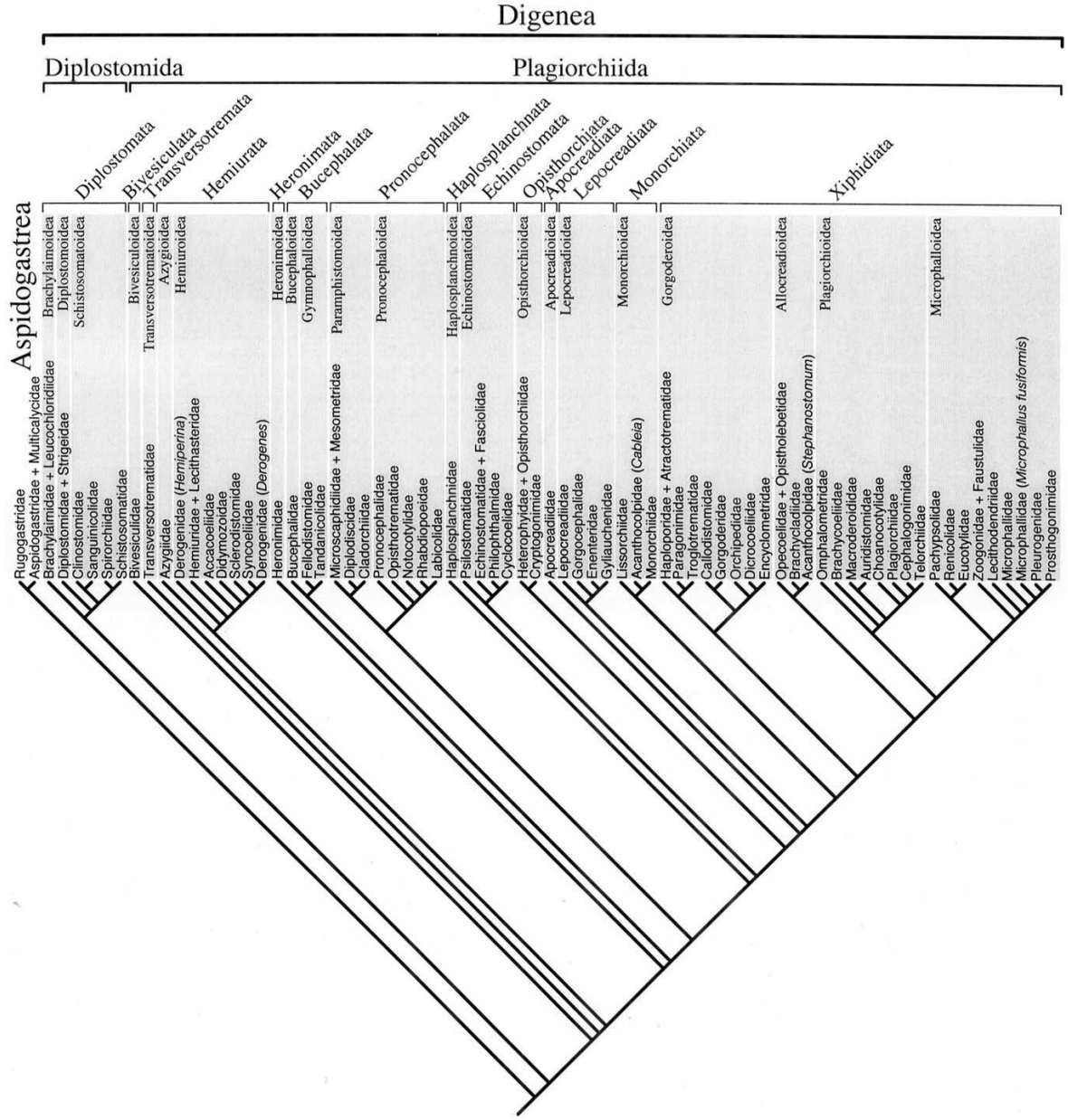

Figure 2.1: A revised classification of the Digenea based on combined analyses of SSU rDNA and LSU rDNA (from Olson et al., 2003)

2.2.2.1 Fasciolidae

The family Fasciolidae Railliet, 1895 belongs to the superfamily Echinostomatoidea Looss, 1899. The superfamily was first defined by Faust in 1929 based on simple morphological and life-history data (Kostadinova & Jones, 2005). La Rue (1957 [cit. in Kostadinova & Jones, 2005]), basing his classification of the superfamily on larval attributes, counted eight other families apart from the Fasciolidae. According to Kostadinova & Jones (2005), Cribb et al. on the contrary based their classification from 2001 on morphological, life-history and molecular data and suggested four families within the superfamily forming a strong clade: Echinostomatidae, Philophthalmidae, Fasciolidae and Cyclocoelidae. According to Olson et al. (2003) also the Psilostomatidae belong to the superfamily.

The Fasciolidae comprise the majority of genera of liver flukes of domestic animals and constitute one of the most important digenean families in veterinary but also human medicine (Jones, 2005a). The family comprises six genera: *Fasciola* Linnaeus, 1758 (type species *F. hepatica* Linnaeus, 1758), *Fasciolopsis* Looss, 1899 (type species *Distoma buski* Lankester, 1857), *Fascioloides* Ward, 1917 (type species *Fasciola magna* Bassi, 1875), *Protofasciola* Odhner, 1926 (type species *P. robusta* Lorenz, 1881), *Parafasciolopsis* Ejsmont, 1932 (type species *P. fasciolaemorpha* Ejsmont, 1932) and *Tenuifasciola* Yamaguti, 1971 (type species *Fasciola tragelaphi* Pike & Condy, 1966). In the past the family was rearranged several times, other/more genera were included, subfamilies were introduced and dismissed again, and divisions were made. According to Jones (2005a), the most recent classification of the family was published by Yamaguti in 1971, recognizing the subfamilies Fasciolinae Railliet, 1895 (*Fasciola*, *Fascioloides* and *Tenuifasciola*), Fasciolopsinae Odhner, 1910 (*Fasciolopsis* and *Parafasciolopsis*), Protofasciolinae Skrjabin, 1948 (*Protofasciola*).

2.2.2.2 Paramphistomidae

The Family Paramphistomidae Fischoeder, 1901 belongs to the superfamily Paramphistomoidea Fischoeder, 1901. According to Jones (2005b), the superfamily's name was first utilised by Stiles and Goldberger in 1910, who recognized three families within the superfamily: Paramphistomidae Fischoeder, 1901, Gastrothylacidae Stiles and Goldberger, 1910 and Gastrodiscidae Monticelli, 1892. A study based on molecular data performed by Olson et al. in 2003 indicates relationships between the superfamilies

Pronocephaloidea, Microscaphidioidea and Paramphistomoidea (Jones, 2005c). Olson et al. (2003) also suggest that the superfamily comprises the Microscaphidiidae, Mesometridae, Diplodiscidae and Cladorchiidae.

The Paramphistomidae comprise paramphistomoid digeneans that are parasitic in mammals and lack pharyngeal sacs, a cirrus-sac and the ventral pouch as explained by Jones (2005d), but the group was often used as a repository for various subfamilies or genera from different groups of vertebrates. The number of subfamilies varied between two (Fischoeder, 1901) and 15 (Näsmark, 1937). Later on, subfamilies were even denied by Skrjabin and up to 34 subfamilies were recognized by Yamaguti, respectively (Jones, 2005d). Today, the division into two subfamilies, the Paramphistominae Fischoeder, 1901 and the Orthocoeliinae Price and McIntosh, 1943, as suggested by Sey in his "Handbook of the Zoology of Amphistomes" of 1991 is widely accepted (Jones, 2005d). The Subfamily Paramphistominae comprised six genera: *Paramphistomum*, *Colylophoron*, *Calicophoron*, *Explanatum*, *Gigantocotyle* and *Ugandocycle* (Sanabria & Romero, 2008). According to Eduardo (1983), the genus *Calicophoron* Näsmark, 1937 was revised as only a few of the originally assigned species remained valid, other species, that were previously recognized as *Paramphistomum* were moved here and the genus *Bothriophoron* was synonymised with *Calicophoron*. Nevertheless, this synonymy, as pointed out by Jones (2005d) is wrong due to the older claim of *Bothriophoron* according to the ICZN (International Commission on Zoological Nomenclature), even if the name *Calicophoron* is well established and commonly used.

2.2.2.3 Dicrocoeliidae

The family Dicroceoliidae Looss, 1899 belongs to the superfamily Gorgoderoidea Looss, 1899. Members of the superfamily show a lot of variabilities concerning morphology, hosts and sites of infections. The first mentioning of the superfamily's name was by Odening in 1960, containing the single family Gorgoderidae Looss, 1899 (Bray & Blair, 2008). The comprehensive study by Olson et al. (2003 [cit. in Bray & Blair, 2008]) based on rRNA genes, suggests the division of the superfamily into the families Haploporidae Nicoll, 1914 + Atractotrematidae Yamaguti, 1939, Paragonimidae Dollfus, 1939, Troglotrematidae Odhner, 1914, Callodistomidae Odhner, 1910, Gorgoderidae Looss, 1899, Orchipedidae Skrjabin, 1913, Dicrocoeliidae Looss, 1899 and Encyclometridae Mehra, 1931.

Not only the superfamily but also the family Dicrocoeliidae is large and diverse and therefore several classifications were approached by e.g. Shtrom (1940), Skrjabin & Evranova (1952), Yamaguti (1958, 1971) or Odening (1964) with great differences concerning the subdivision with the respective genera (Pojmańska, 2008). Yamaguti (1958 [cit. in Pojmańska, 2008]) suggested a differentiation in tribes within subfamilies based on morphological features. Furthermore, Panin classified the dicrocoeliids based on life cycle types and morphological features of the cercariae (Pojmańska, 2008).

2.3 Biology

2.3.1 Biology of helminths

Plathyhelminthes or flatworms are bilaterally symmetrical and dorso-ventrally flattened with a leaf-like or tape-like shape. They are covered by a tegument, have no body cavity and the region between the internal organs is filled with spongy, mesenchymatous cells (Grove, 1990).

2.3.2 Biology of trematodes

Most of the parasitic trematodes that occur in humans have a dorsoventral flattened body and an oval to lanceolat shape (Kayser et al., 2001). Trematodes are unsegmented and occur in unisexual (schistosomes) or hermaphroditic types (Grove, 1990). A distinct and also name-giving feature are the suckers of adult trematodes (trema = hole, opening) with which they can adhere to their hosts tissues or organic surfaces (Kayer et al., 2001). They are equipped with one ventral sucker and one mouthsucker enclosing the stoma which is connected to oesophagus and the incomplete gut with one aperture only. The body surface is covered by an integument, through which environmental substrates are taken up. As described by Grove (1990), "the integument generally consists of a syncitial cytoplasmic covering continuous with nucleated portions of the cytoplasm situated in the parenchyma beneath the muscle layer".

The life cycles of trematodes are very complex and diverse. Responsible for this variability are various morphological and biological adaptations that occur in every developmental stage and can be explained by a great variety of hosts (vertebrates and invertebrates), biotopes and ecosystems that can be used by the parasites (Galaktionov & Dobrovolskij,

2003). Additionally, modifications concerning the number of hosts (one to four) or stages contribute to the diversity of life cycles. Nevertheless, some typically features such as parthenogenic and hermaphroditic generations remain with few exceptions within every digenean life cycle. Figure 2.2 shows a generalised scheme of a trematode life cycle which also indicates physiological processes that are involved (Smyth & Halton, 1983). The diagram is based mainly on the life cycle of *Fasciola hepatica* and in parts on that of *Schistosoma* spp.

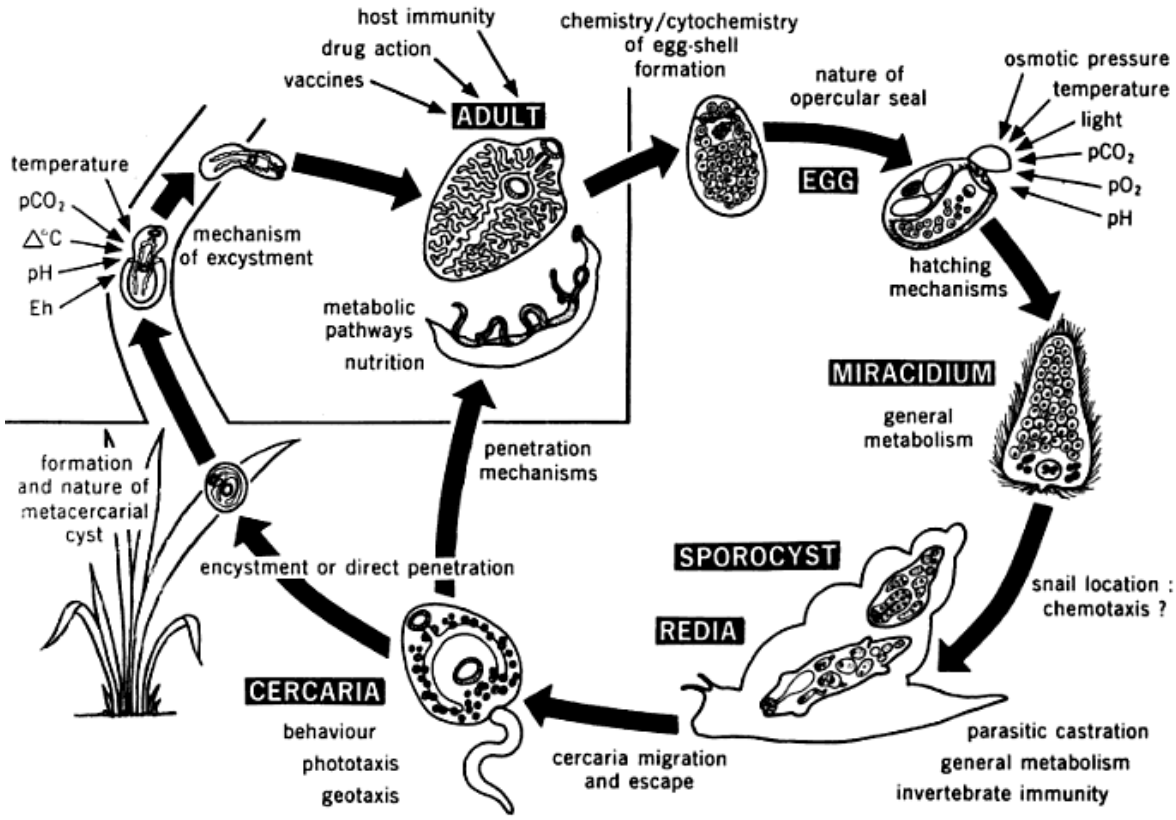

Figure 2.2: A generalised scheme of a trematode life cycle including physiological processes, mainly based on *Fasciola hepatica* and to a lesser extend on *Schistosoma* sp. (Smith & Halton, 1983)

The morphology of digenean trematodes has been reviewed in detail by Fried (1997). The following chapter is largely following his descriptions.

The typical egg of a digenean trematode is ectolecithal, oval-shaped and of a light to dark brown colour. The measurements of trematode eggs can vary greatly, but the length, which is around 100 µm in the average egg, is approximately the threefold width. Often, trematode eggs have an operculum on one pole, while the other pole is more blunt and sometimes with a knob. The contents of trematode eggs often appear as granular or homogenous masses or show the outlines of mature miracidia (Oberhauserovà et al., 2010).

The miracidium is a small, elongate larva that is of transparent colour and typically possesses eyespots. It is 100 µm long and contains large numbers of flattened ciliated epidermal plates with subjacent muscles. Most miracidia possess glandular and excretory structures, a large cephalic ganglion and a cone-shaped apical papilla with sensory receptors and gland openings. It is, despite a lot of studies, not completely understood, how miracidia find the snail intermediate hosts but it has been suggested that chemical cues from the snails are playing a role.

After the miracidial penetration of the snail host, which is usually in the snail's head-foot region or its mantle, sporocysts develop after miracidial transformation within the snail. Sporocysts are saclike structures that contain germinal cells, which will later on develop species-dependend into either daughter sporocysts or rediae. Shape, body wall structure as well as flame cell numbers and arrangements can vary greatly between species. Also the occurrence of a birth pore in contrast to sporocyst wall disruption for larvae escape is variable in different species.

The next out of (mother-) sporocysts emerging larval stage within the snail is the redia which can on its part give rise to a second or third generation of rediae. Rediae have an elongated shape and possess mouth, pharynx and a saclike gut. Dependend on the species, rediae have one or more ambulatory buds, sensory papillae and a collar at the anterior end. Rediae are generally migrating through their host snail and can therefore be located in various sites, but most often in the snail's digestive gland and gonads.

Either daughter sporocysts or second/third generation rediae give rise to cercariae, the juvenile with a muscular tail equipped trematode. The cercariae emerge from the snail host and quickly begin the search for their next host. Some species enter the host by direct penetration while others encyst on aquatic plant or likewise (metacercariae). In this context, the way of host infection is associated with penetration glands for host entry or cystogenous glands for encystment, respectively.

Digenean trematodes occur worldwide and in a great variety of habitats. Bauer et al. (1982) explained that the host is considered to be the environment of the parasite. The life of the parasite is influenced by the primary environment, meaning the host itself, and the secondary environment, meaning the environment of the host which can influence the parasite directly or indirectly through the host. According to Smyth & Halton (1983) the different habitats in which trematodes live during the different stages in their life cycles are diverse and have different physico-chemical properties and nutritional levels. The authors suggest that the parasites use these different properties in order to trigger their next developmental stage whenever a new habitat is reached. Nevertheless, certain criterias of a suitable habitat such as a connection to the outer world, sufficient nutritional levels or a surface to which the parasite can attach must be fulfilled.

2.3.2.1 Comparative features of selected trematode species

Table 2.2: Comparative characteristics of four different trematode species. (modified after Mehlhorn & Piekarski, 1995)

		Fasciola hepatica	***Fascioloides magna***	***Dicrocoelium dendriticum***	***Paramphistomum daubneyi***
Eggs	Shape/Size	oval; 130-150 x 63-90 µm	oval; 122 x 87 µm	oval; 38-49 x 22-33 µm	oval; 102-174 x 48-95 µm
	Colour	golden	yellow-brown	dark brown	greenish-yellow
	Operculum	yes	yes	yes	yes
Larval stages	Miracidium	hatches in water	hatches in water	hatches inside snail	hatches in water
	Sporocyst	mother (daughter) sporocysts	mother (daughter) sporocysts	mother and daughter sporocysts	mother (daughter) sporocysts
	Redia	instead of daughter sporocysts	instead of daughter sporocysts	no rediae	instead of daughter sporocysts
	Ceraria	naked body, undevided tail	naked body, undevided tail	spine at mouth sucker	suckers on both ends
	Metacercaria	encystation on vegetation	encystation on vegetation	accumulation in ant	enystation on vegetation

Adults	Shape/Size	bay leave shaped, anterior cone; 2-5x1 cm	leaf shaped, round anterior; 8-10x3 cm	lanceolate; 0,5-1,0x0,2 cm	conical; 0,5-1,2 cm
	Location in host	bile ducts	hepatic parenchyma	bile ducts	rumen
Life cycle	Intermediate hosts	1; freshwater snail (*Lymnaea* sp.)	1; lymnaeid freshwater snail (*Galba truncatula*)	2; land snail (*Helicella* sp., *Zebrina* sp.) and ant	1; freshwater snail (*G. truncatula*)
	Definitive hosts	ruminants, herbivorous animals	ruminants, herbivorous animals	ruminants, herbivorous animals	ruminants, herbivorous animals
Distribution		worldwide	North America, Europe	northern hemisphere	Europe, Africa, North America, South America
Human relevance		food-borne infection; fasciolosis		accidental infection; dicrocoeliosis	

2.4 Medical relevance

Trematode infections in humans are a great public health problem world-wide (Fried et al., 2004), given that according to estimates 40-50 million people are infected with food-borne trematodes (Abdussalam et al., 1995). Furthermore, over 10% of the world's population is at risk to get infected with food-borne trematodes. In their report from 2005, Keiser & Utzinger list numbers of individuals that are at risk to become infected with certain trematode species. According to them, 601.0, 293.8, 91.1 and 79.8 million people are at risk of an infection with *Clonorchis sinensis*, *Paragonimus* spp., *Fasciola* sp. and *Opisthorchis* spp., respectively. The reasons for these high numbers are among others social and agricultural practices and health education. As listed by Abdussalam et al. (1995), the major reasons for the emerging/re-emerging trematode infections are the rapidly increasing fisheries and especially the fish production in aquacultures, traditional eating and cooking habits with raw or insufficiently processed aquatic food or vegetation

or the increasing population pressure that implicates pollution of waters with sewage or excreta and the creation of water reservoirs in irrigation dams, that are suitable for snails and fish intermediate hosts. Abdussalam et al. (1995) also states that certain trematode species cause great economic damage in livestock.

The cercariae, the infective stages, enter the second intermediate hosts and encyst in the muscles or other organs or, in trematodes without a second intermediate host, cercariae either infest directly the final host or attach to water plants and form the metacercariae. These metacerariae are then ingested by humans. Abdussalam et al. (1995) investigated the viability and resistance of metacercariae and found out that the metacercariae of certain trematode species are resistant to usual habits of pickling, smoking, drying and salting of fish. Furthermore, they survive heating and freezing, respectively, up to a certain point. In another study by Ashrafi et al. (2006) potassium permanganate was investigated for its killing ability of metacercariae from fasciolid species. A lethal effect was, even if underlined by former studies, not observed. They did however suggest that potassium permanganate is useful for detaching of the metacercariae from plants similar to the effectiveness of commercial vinegar, citric acid or liquid soap.

Fried et al. (2004) report on approximately 70 species of intestinal trematodes that can infect humans worldwide. According to Keiser & Utzinger (2009) important food-borne trematodes are species belonging to liver flukes (e.g. *Clonorchis sinensis*, *Fasciola gigantica*, *Fasciola hepatica*, *Opisthorchis felineus*, *Opisthorchis viverrini*), lung flukes (e.g. *Paragonimus* spp.) and intestinal flukes (e.g. *Echinostoma* spp., *Fasciolopsis buski*, Heterophyidae). Table 2.3 gives an overview of food-borne trematodes, their distribution, reservoirs and the source of infection for humans.

Table 2.3: Food-borne trematodoses (Abdussalam et al., 1995)

Parasite	**Distribution (main areas)**	**Principal reservoirs (other than man)**	**Food involved in transmission to man**
Fish- and shellfish-born trematodes			
Clonorchis sinensis	Widespread in China, incl. Taiwan and Hong Kong, Macao, Japan, Korea and Vietnam. Migrants to many other countries found to be infected; cases in Hawaii attributed to consumption of fish imported from China	dogs, cats and many other species of fish-eating mammals	Many species (~110) of freshwater fish, mainly Cyprinidae (e.g. carp, roach, dace), most important being *Pseudorasbora parva*. Metacercariae in fish muscles
Opisthorchis felineus	Commonwealth of Independent States (CIS), eastern and central Europe	cats, dogs and other mammals that eat fish or fish wastes	Freshwater fishes of family Cyprinidae (e.g. whitefish, carp, tench, (*Tinca tinca*), etc.). Metacercariae in muscle and subcutaneous tissue
Opisthorchis viverrini	Laos and North-eastern Thailand (Mekong River Basin)	dogs, cats, fishing cats (*Felis viverrina*) and other mammals that feed on fish and fish wastes	Some ten species of freshwater fish incl. *Puntius orphoides* and *Hampala dispar*. Metacercariae in fish muscles.
Heterophyes heterophyes	Mediterranean basin, especially Egypt and eastern Asia.	dogs, cats, jackals, foxes, pelicans, hawks and black kite	Brackish- and freshwater fish, esp. mullet (*Mugil* spp.), *Tilapia* and others. In Japan, species of fish genera *Acanthogobius* and *Glossogobius* also involved. Metacercariae in muscle and skin
Metagonimus yokogawai and related species	Eastern and southern Asia	dogs, cats, pigs and fish-eating birds	Freshwater fishes, such as sweetfish (*Plecoglossus altivelis*), dace (*Tribolodon hakonensis*), trout and whitebait. Metacercariae in gills, fin or tail.
Nanophyetus salmincola schikhobalowi	Eastern Siberia (mountain tributaries or Amur river) and parts of Sakhalin peninsula, northwest USA	dogs, cats, rats (?) and badgers	Salmonid and other fish. Metacercariae in muscles, fins and kidneys.
Spelotrema brevicaeca	Philippines	sea birds	Crustacea, *Amphipodes*, *Isopods* and *Brachyures*

Paragonimus westermani and related species in Asia, Africa and the Americas	Siberia, west Africa (Nigeria, Cameroon), the Americas (Ecuador through USA), Japan, Korea, Thailand, Laos, China	domestic and wild carnivore which feen on crustaccea	Fresh- and brackishwater crabs (*Eriocheir*, *Potamon*, *Parathelphusa*), crayfish and shrimps. Metacercariae in muscles, gills, liver (hepatopancreas) and cardia region. (Wild boar meat suspected as a source of infection)
Haplorchis sp.	Eastern and southern Asia	cats, dogs and fish-eating birds	Fish, frogs and toads. Metacercariae in muscle.
Molluscan shellfish-borne trematodes			
Echinostoma (Euparyphium) *ilocanum*; *E. lindoese* and *E. malayanum*	Philippines, southern China, Indonesia (Java, Kalimantan) and India	dogs, cats, monkeys, murid rodents *E. lind.* + *E. mal.*: dogs, cats, pigs, mongoose	Snails: *Pila luzonica*, *P. conica*, *Viviparus javanicus* and others, when eaten raw
E. revolutum	Taiwan, Indonesia and Mexico	ducks, geese and other birds	Clam – *Corbicula fluminea*
Hypoderaeum conoideum	Northern Thailand	aquatic birds	snails (habitually consumed raw by people in endemic area)
Plant-borne trematodes			
Fasciola hepatica	worldwide in temperate climates, sporadic human cases from many countries; outbreaks reported from France, Portugal, CIS, Egypt, Iran, Cuba, Mexico, Bolivia, Ecuador, Peru and Chile	domesticated and wild ruminants; other herbivora including rabbits and hares (survival in man up to 13 years)	Watercress (*Nasturtium officinale*); other salad plants (lettuce) grown in low-lying areas; alfalfa juice, water. Metacercariae remain infective on plants for 6-8 months (semi-humid) and for more than a year in water.
Fasciola gigantica	widely distributed in Africa, Asia, Hawaii. Human infection reported from Hawaii, Vietnam and other parts of southern Asia	domesticated and wild ruminants	"KangKong" (*Ipomea obscura*) and other aquatic vegetables
Fasciolopsis buski	China, India and other parts of southern Asia (e.g. Thailand, Indonesia, etc.)	pig, wild boar	water caltrop (*Trapa notans*), water chestnut (*Eliocharis tuberosa*), lotus (*Nymphaea lotus*) and others of genera Eichhornia, Neptunia, Zizania
Gastrodiscoides hominis	Southern Asia (India, Malaysia, Indonesia, Vietnam). Kazakhstan, wild boars have been found infected	pig, wild boar	aquatic plants, probably the same as for *F. buski*

Dicrocoelium dendriticum and *D. hospes*	widely distributed	cattle, sheep, goats, and other domestic and wild herbivore	vegetable or fruits and grass (nibbling) contaminated with ants or their remains

Another important species that cause human trematode infections are the schistosomes that belong to the group of blood flukes. As described by Cox (2002), schistosomosis or bilharziosis is caused by the infection with schistosomes (genus *Schistosoma*). The five important representatives of this genus causing human disease are *S. haematobium*, *S. intercalatum*, *S. mansoni*, *S. japonicum* and *S. mekongi* (Davis, 2009). The species can be divided into three groups by their size and the morphology of the eggs. *S. haematobium* and *S. intercalatum* produce eggs with a terminal spine, *S. mansoni* eggs with a lateral spine and *S. japonicum* and *S. mekongi* have rounded or ovoid eggs with very small spines.

According to CDC's estimates, more than 200 million people are infected with schistosomes worldwide. The symptoms of an infection with *S. haematobium* are fever and bloody urine due to the adult schistosomes in the bladder-associated blood vessels of its host (Cox, 2002). Infections with the other four schistosome species cause intestinal schistosomosis and might come along with common symptoms of illness, abdominal pain, eosinophilia or swelling of liver, spleen or lymph nodes (Kayser et al., 2001). Eggs that are not passed with the urin or the faeces, respectively, accumulate in the host's tissue. This accumulation of eggs triggers an immunological reaction of the host (Cox, 2002). As a result of this reaction, a possible relationship between schistosome infection and colon and bladder carcinomas has been discussed (Gutierrez Y, 2000 [cit. in Cox, 2002]).

Moreover, flukes of the family Schistosomidae can not only cause bilharziosis, but also a worldwide occurring and often neglected skin disease, cercarial dermatitis, caused by the cercariae (Hörweg et al., 2006). In Europe, five genera are of medical importance, namely *Orientobilharzia*, *Dendritobilharzia*, *Bilharziella*, *Trichobilharzia* and *Gigantobilharzia*. Their definitive hosts are waterfowl, which is why they are also known as "bird schistosomes". The intermediate hosts are pulmonate snails such as *Lymnaea stagnalis*, *Planorbarius corneus*, *Stagnicola* sp. or *Radix* sp. Humans, who are accidental hosts, become infected while swimming or staying in shallow freshwater, where waterfowls, water plants and water snails are present. Additionally, a number of outbreaks have been observed after swimming in salt or brakish waters (Kolářová, 2007). The cercariae penetrate the skin of the swimmers, which causes itching and a strong macula-papulo-

vesicular skin eruption. The body parts that are mostly affected are torso and extremities, duration and course of the disease can vary. In rare cases other symptoms such as big red spots, severe depression etc. have been observed (Hörweg et al., 2006). Additionally, bird schistosome cercariae can migrate through visceral and nervous tissues on non-compatible mammalian hosts and cause damage in the affected tissues (Kolářová, 2007). The author also states, that the allergic reaction to the repeated cercarial infection is able to trap and eliminate the parasites which represents a protective reaction of a sensitized host against the parasites. Hörweg et al. (2006) also discuss factors that have an impact on infection, such as duration of stay in the water, rubbing dry after exposure or usage of lotions before entering the water. While rubbing dry does not have a clear effect, the probability of infection increases the longer one stays in the water. Consistent with those findings, also Verbrugge et al. (2004) state that onset and severity of the disease are affected by the interactions of people with the lake and not by people's demographic features. Additionally, normal (sun-)lotions do not seem to have any effect, but Wulff et al. (2007) developed a cream formulation consisting of a waterproof sun lotion mixed with Niclosamid, an antihelminthic drug that has a 100 % damage rate of *S. mansoni* at a 1 % formulation and that possibly offers full penetration protection.

2.5 Veterinary relevance

Animals play an important role in the life cycle of trematodes as both, intermediate and definitive hosts. Trematodes use all kinds of animals as possible hosts, ranging from insects, fish and birds to vertebrates and humans. While in some cases the animal host only functions as a reservoir for the parasite or a place for the evolution and growth of developmental stages, in other cases the host gets seriously affected or even killed by the parasite, especially if the animal host is not an original host to the parasite.

One important trematode infection of cattle and other farm livestock is fasciolosis. It is a world wide occurring disease and according to Mage et al. (2002) it results from a single helminth species, namely *Fasciola hepatica*, in European countries and has an extensive economic effect on the farming industry. Several authors report on natural and experimental infections with liver flukes in a great variety of ruminants: cattle, sheep, deer, bison, horses etc. (Rapsch et al., 2006; Keiser et al., 2010; Foreyt, 1996; Foreyt & Drew, 2010; McClanahan et al., 2005). Furthermore, *Fascioloides magna*, the giant liver fluke imported to Europe is constantly gaining relevance in especially the middle and eastern

European countries as an animal populations and livestock threatening parasite (Rehbein et al., 2002).

Another animal trematode infection, paramphistomosis, can be caused by different species belonging to the paramphistomids that parasitize in different species of wild and domestic ruminants. Mage et al. (2002) report on *Paramphistomum cervi*, *P. daubneyi*, *P. ichikawai* or *P. microbothrium* as possible causative trematodes for the disease. Furthermore, they state that *P. daubneyi* seems to be the species that affects cattle in Europe more often than any other paramphistomid species. Sanabria & Romero (2008) state, that the pathogenicity of paramphistomids is still controversial and depending on the animal's individual state of health, the species susceptibility and the amount of ingested metacercaria.

Animal helminthoses affect both, wild and domestic livestock, and can lead to serious losses in their populations. Due to an eventual common use of pastures of wild and domestic animals parasite infections can be transferred between the two kinds of livestock. As possible negative effects of trematodes on the health of the animal host, Oberhauserová et al. (2010) list for example a reduced body mass and trophy, decreased production of milk and meat and a decrease in reproduction capacity.

The animals investigated in this study, red deer, can harbour various parasites. Protozoans, arthropods as well as helminths can parasitize in deer. Böhm et al. (2006) report on a broad spectrum of nematodes that mainly parasitize in the gastro-intestinal tract and the lungs of deer species. They further report that lungworm infections are very common in red deer and that cross infections can occur between red deer and cattle. As described by Rehbein et al. (2002), deer can also be parasitized by cestodes (tapeworms) and trematodes (liver flukes and rumen flukes). The authors also state that roe deer is usually more affected by parasites than red deer. This is similarly described by Böhm et al. (2006) who state that liver flukes cause mortality in roe deer but rarely lead to clinical symptoms in red deer. The helminthic fauna of another relative of red deer, sika deer, was investigated by Rehbein & Visser in 2007. The authors describe a similar parasitic picture as can be found in red deer even though the helminth load inside the gastro-intestinal tract is much lower than it is usually in red deer. Apart from protozoan species, cestodes and nematodes, *Fasiola hepatica* and a *Dicrocoelium* species (*Dicrocoelium chinensis*?) were found. Also *Dicrocoelium dendriticum* parasitizes in deer, even if eggs of this trematode are seldomly found in coprological analyses (Rehbein et al., 2002).

2.6 Aim of the study

The current study, which was assigned by the Österreichische Bundesforste AG, was conducted in two parallel parts in the 'Nationalpark Donau-Auen' and its surrounding areas. The 'Nationalpark Donau-Auen' is a 38 kilometres long and 9,300 hectare large floodplain located between Vienna (Austria) and Bratislava (Slovakia) (www.donauauen.at). Currently, the park consists of 65% forest areas, 15% meadows and 20% water areas. It was founded in 1996 and since 1997 it is a category II reserve area, accredited by the IUCN (International Union for Conservation of Nature). The river Danube flows approximately 36 kilometres free through the floodplain and its considerable level fluctuations are reason for the dynamic and unique landscape. The park is not only a recreation area for the local residents but also represents a habitat for a multitude of various plants and animals. At present, it is the living space for more than 800 plant species, 100 birds, 30 mammals and several reptiles, amphibians, fish species and numberless invertebrates. Some of these organisms are rare and near to extinction.

The main focus of the study lay on the occurrence of *Fascioloides magna*, the large American liver fluke or giant liver fluke in its definitive and intermediate hosts in the national park. After its first finding in wild living red deer in autumn 2000 in the Danubien floodplain in the area of Fischamend (Winkelmayer & Prosl, 2001), a following investigation showed a considerable high prevalence of *F. magna* in the red deer population (Ursprung, 2002). An interdisciplinary project was set up not only to investigate potential problems caused by the introduction of the parasite into the Nationalpark but also to develop a monitoring and extermination program (Ursprung et al., 2006). Additionally, Sattmann & Hörweg (2005) investigated the infestation rates of *F. magna* in their intermediate host snails *Galba truncatula* within the 'Nationalpark Donau-Auen'.

The two parallel parts of the current study intended to investigate the prevalences of trematode infestations/infections in both the definitive and intermediate hosts of *F. magna* in the area of the nationalpark. For this purpose, a monitoring of these hosts was performed at three previously chosen sites in Orth/Donau (Entenhaufen, Märchenteich, Neubruchwiese) from April to November 2008.

In this part of the study, it was aimed to monitor the parasite burden of cervids by collecting and investigating red deer faecal samples. As the detection of faecal samples in the wild is often difficult due to vegetation, weather or other factors that could influence

the wandering habits of deer within the area of the nationalpark, no absolute numbers of faecal samples were determined at the beginning of the monitoring. Additionally, to increase the total numbers, also samples collected by third persons (hunters, ÖBf-staff) were included in the study.

First, in order to assess the overall trematode burden in red deer, the faecal samples were examined by microscopy after sedimentation. To achieve this, first an adequeate enrichment technique had to be established. For differentiation and identification, detected eggs had to be measured and photographed.

Then, as the differentiation of closely related species like *Fasciola hepatica* and *Fascioloides magna* is often not possible by morphological means only, biomolecular techniques had to be established. As the DNA isolation from trematode eggs is difficult due to their hard shell, a functioning protocol had to be set up comparing different microbead homogenisation and faeces/soil DNA extraction kits. Subsequently, new PCR primers needed to be designed that bind on one hand to all trematodes and on the other hand very specifically to a single trematode species, *F. magna*, as this was the main aim of the study. After testing the primer pairs on positive controls made from adult helminths, the PCRs needed to be adjusted to faecal samples by variation of PCR parameters (cycle number, temperature etc.).

Subsequently, to verify and specify the positive PCR results, amplicons had to be sequenced and compared to one another and to published trematode sequences available in GenBank by multiple alignments.

Finally, the results could be subjected to statistical analysis to assess prevalence, intensity, distribution and seasonality of trematodes in the investigated animals. The collected data was also used to estimate the effectiveness of previously taken measures (medication, monitoring) and to evaluate the necessity of further strategies.

3 MATERIAL & METHODS

3.1 Samples

3.1.1 Sampling sites

In agreement with the Österreichische Bundesforste AG ÖBf, three possible high-risk sites in the area Orth/Donau were chosen as sampling sites, these were "Entenhaufen", "Neubruchwiese" and "Märchenteich" (Figures 3.1 – 3.4). The selection was based on previous studies (Sattmann & Hörweg 2005, Reckendorfer & Schaefer 2003), in which suitable habitats for the parasite hosts and risk factors had been determined. All chosen sites are situated directly at a branch of the river Danube and therefore act not only as a habitat for the intermediate host snails but also as passing paths and watering places for the deer.

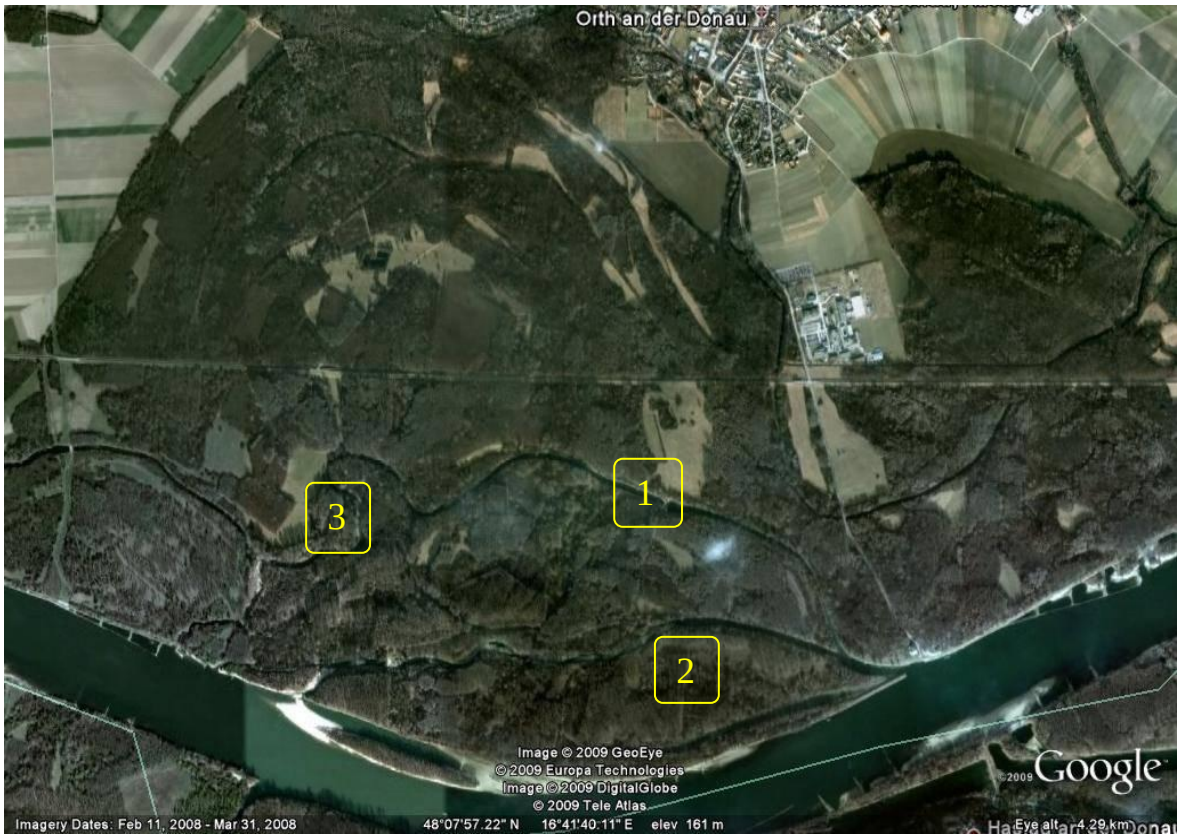

Figure 3.1: Sampling sites in Orth/Donau: 1) Märchenteich, 2) Entenhaufen, 3) Neubruchwiese (Google Earth).

Figure 3.2: Sampling site Orth Märchenteich with collected deer faeces (upper right corner).

Figure 3.3: Sampling site Orth Entenhaufen with collected deer faeces (upper right corner).

Figure 3.4: Sampling site Orth Neubruchwiese with collected deer faeces (upper right corner).

In addition, staff members of the ÖBf collected deer faeces samples from nine other sites within or in the vicinity of the Nationalpark area (sites listed from East to West: Markthof, Stopfenreuth, Witzelsdorf, Eckartsau, Mannsdorf/Donau, Fischamend, Schönau/Donau, Mühlleiten, Großenzersdorf; shown on Figure 3.5) between February and November 2008.

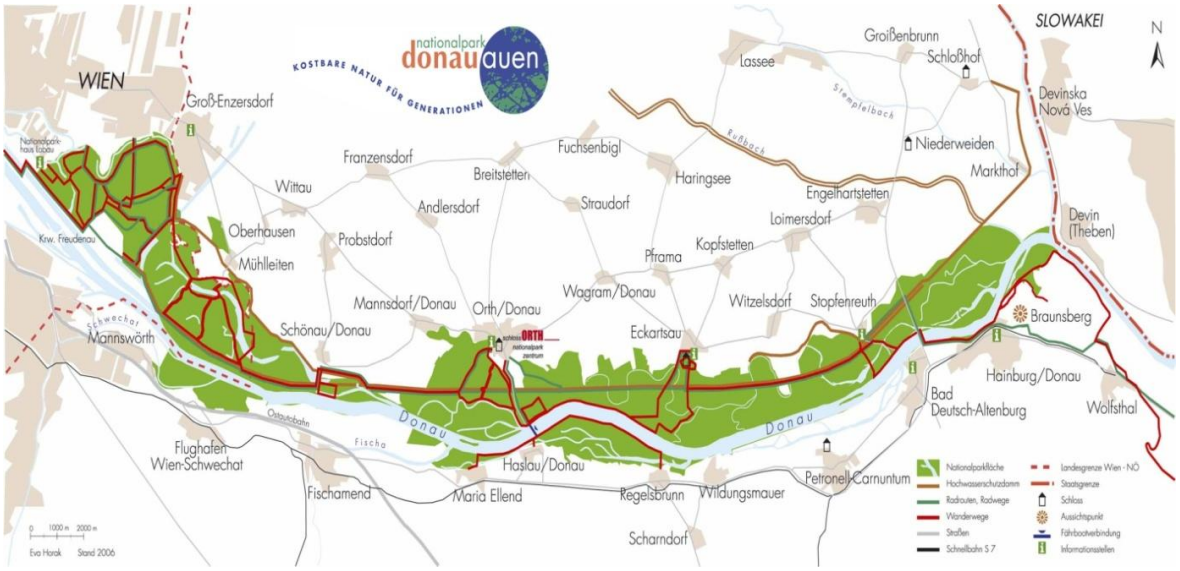

Figure 3.5: Map of the Nationalpark Donau-Auen area (www.donauauen.at/files/254_NPKarte.jpg).

One supplementary sampling round was performed at the south bank of the Danube namely in Haslau/Donau, Regelsbrunn, Bad Deutsch-Altenburg and Hainburg/Donau (Figure 3.5), but no faeces samples were obtained from any of these locations. Thus, those four locations were not taken into account for any further calculations.

3.1.2 Sample drawing

Samples were collected at the three sampling sites in Orth/Donau approximately every two weeks between May and November 2008 in collaboration with Michaela Haider, who was working on the occurrence of digenean trematodes in the intermediate host snail *Galba truncatula* (Haider, 2010). The inspection of these sites was done in pairs/groups mostly in the morning hours or early afternoon. Typical animal passages and watering holes were searched for droppings, which were, if confirmed to be red or row deer faeces, collected and bagged in labelled plastic bags. The label contained sampling site, date and time. In addition to this a sampling protocol was kept to record environmental parameters like air and water temperature, degree of cloudiness, water level and vegetation. A copy of the sampling protocol is attached in the appendix.

The collected samples were cooled, transferred to the laboratory and stored at -20°C until further processing.

The samples collected by ÖBf staff members were stored at +4°C at an administration office in Eckartsau. Later they were transferred to the laboratory for further processing. No sampling protocol was kept for those provided samples and some of them were only labelled with sampling site but without sampling dates. Samples without dates were not considered in seasonal distribution calculations.

Altogether 66 samples were collected in 17 sampling rounds (15 at Orth/Donau locations, 1 in Fischamend and 1 at Danube south bank) and 92 samples were provided by ÖBf staff members, giving a total of 158 faeces samples.

3.2 Microscopy

The samples were defrosted slowly in the refrigerator at +4°C and subsequently processed for microscopic examination. Fresh samples or samples from the ÖBf stored at +4°C were processed directly.

3.2.1 Sedimentation after Benedek (modified)

Approximately 5 g of faeces were mixed with a small amount of tab water. The mixture was filtered through a small meshed strainer and rinsed into a tapered glass with a sharp jet of water. After 3-5 min of sedimentation the supernatant containing particulate material

was drained. The tapered glass was refilled with water to allow further sedimentation and purification of the sediment. This process was repeated 2-3 times until the supernatant became clear. The supernatant was drained and the sediment transferred to a Petri dish. With a disposable pipette a few drops of sediment were mounted onto a microscope slide, covered with a cover glass (18 x 18 mm) and examined for trematode eggs at 40–100 x magnification under the light microscope. All in all, six cover glasses were examined of each sample meaning that approximately 0.4 g of sediment of every sample was examined. Possible findings were measured and photographed, morphological structures and colours were recorded. Sediments of all samples were transferred to 2 ml tubes and stored at -20°C for molecular analysis.

3.2.1.1 Calculation of eggs per gram

Eggs per gram (EpG) were calculated roughly for all positive samples. Given that approximately 0.4 g of sedimented sample was examined, the total count of trematode eggs found in the sample was extrapolated to gram using the formula: EpG = egg count / amount of examined sample (0.4).

3.2.1.2 Calculation of positivity rates

Positivity rates were determined for each location with positive findings. The number of positive samples was put into relation with the total number of collected samples. Positivity rates were not only calculated in general for all trematode findings, but also for fasciolids and paramphistomids separately.

3.2.2 SAF method

The enrichment method with sodium acetate-acetic acid-formalin (SAF) fixed faeces samples was modified to sodium acetate-acetic acid-alcohol fixation in order to be able to use the samples for PCR.

Altogether, eight samples were processed with the SAF-method and slight alterations in the processing were tested. According to the standard protocol used in the parasitology laboratory at the Medical University Vienna, an approximately hazelnut-sized faeces sample was mixed with 10 ml stock solution (15 g sodium acetate, 20 ml glacial acetic

acid, 40 ml 70% ethanol, 925 ml water). The fixed sample was vortexed and filtered through wound gauze into a centrifuge tube. After two min of centrifugation at 550 x g (at room temperature), the supernatant was carefully drained. The sediment was mixed with a few drops of physiological sodium chloride solution, and a few drops of this mixture were mounted onto a microscope slide, covered with a cover glass and scanned for trematode eggs at 40–100 x magnification under the light microscope. Found eggs were measured, photographed and identified by morphology.

Possible improvements of sensitivity were evaluated by varying sample constitution (untreated and sedimented faeces), incubation period with stock solution and sample amounts.

None of the SAF samples was further processed with molecular techniques.

3.3 Liver inspection

In collaboration with ÖBf members, organs of game animals, which were shot during the annual control hunt in autumn/winter 2008, were inspected for helminth infestations. On the 28th of October 2008, the fresh liver of a doe (red deer) was examined in the laboratory. The outer surface of the liver seemed inconspicuous; nevertheless, the hunter indicated little elevations and scars as an evidence for liver fluke infestation (Figure 3.6 left). The organ was cut into slices and washed with tap water over a large bucket. The pieces were examined carefully for channels through the tissue, migrating or encapsulated flukes and possible egg accumulations. The contents of the bucket were filtered and screened as well. Two adult *F. magna* were found, photographed, measured, fixed in alcohol and partly used for DNA extraction (Figure 3.6 right). One was kept in sodium chloride solution for a life cycle establishing experiment with their intermediate host snail *G. truncatula*, but had to be fixed in alcohol after failure. Additionally, on the 3rd of December 2008, the organs (liver, rumen) of numerous game animals were inspected right after they were shot, but no parasites were found.

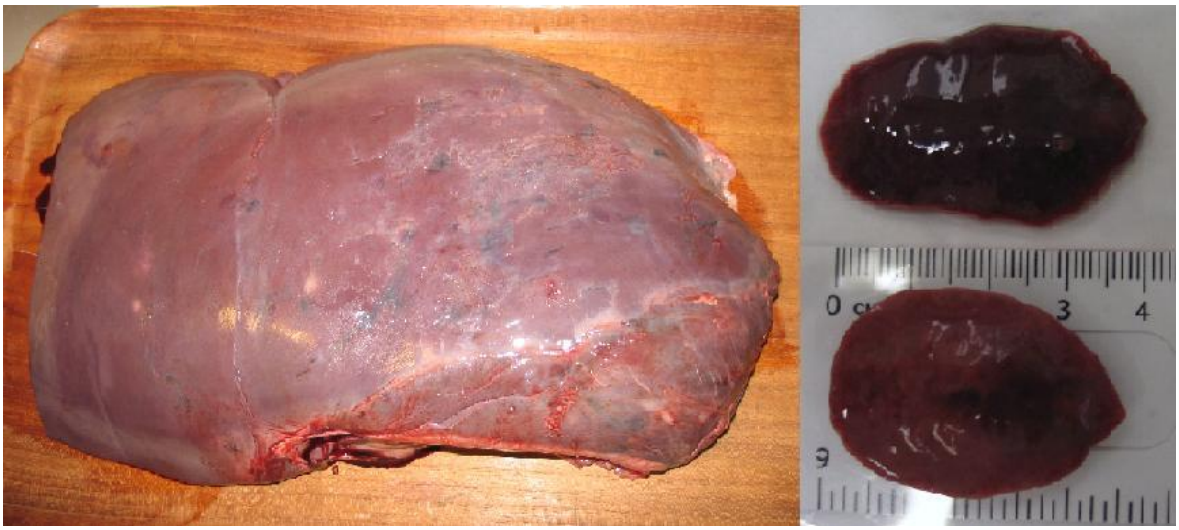

Figure 3.6: Liver of a doe with *F. magna* infestation (left) and found adults parasites (right).

3.4 Molecular biology

To complement microscopic findings, molecular techniques were used for general detection of trematodes in faeces samples and for species-specific differentiation of detected trematode eggs.

3.4.1 Sample selection

For time and financial reasons only a selection of samples was tested with molecular biological methods. The selection was made reflecting an equal distribution of all the sampling sites on one hand and on the other hand to have a well balanced assortment of microscopically positive and negative samples.

The used control samples were adult trematode specimens of *Fascioloides magna*, *Fasciola hepatica*, *Paramphistomum* sp., *Diplostomum* sp., *Aspidogaster limacoides* and adult cestode specimens of *Proteocephalus longicollis* and *Taenia saginata* from the helminth collection of the Natural History Museum of Vienna.

In addition to control samples from parasitic tissue, control samples were also obtained from purified cestode eggs of *Taenia* sp. and *Diphyllobothrium latum*. The samples were therefore homogenized as described below and DNA extraction was performed with the QIAamp® DNA Mini Kit (QIAGEN, Vienna, Austria).

3.4.2 Homogenization

In order to extract DNA, the egg shell had to be crashed first. Therefore, the faeces samples were processed with the Precellys® 24 homogeniser (PeqLab, Erlangen, Germany). Approximately 0.5 g of thawed sedimented sample (sedimentation technique after Benedek) were transferred into a homogenization tube containing glass beads with 0.5 mm diameter and filled up with sodium chloride solution (or sample solution if present). The samples were subjected to two rounds of homogenization at 6,500 rpm for 20 sec with 30 sec pause in between. Figure 3.7 shows a fasciolid egg before and after homogenization.

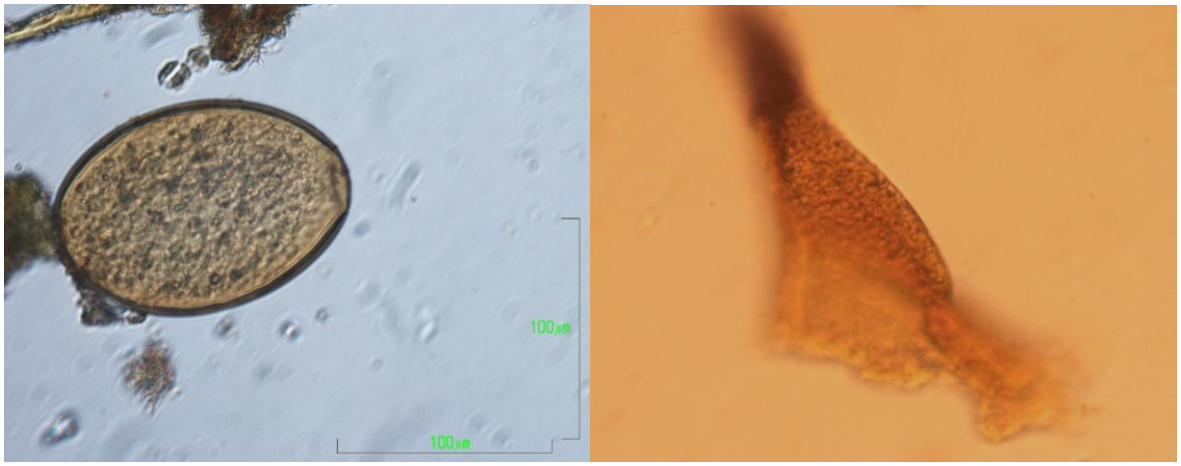

Figure 3.7: Eggs pre (left) and post (right) homogenization.

3.4.3 DNA extraction

3.4.3.1 DNA extraction with tissue kit (control samples)

For the preparation of positive control samples, adult parasites of different species were used and processed with the QIAamp® DNA Mini Kit (QIAGEN, Vienna, Austria) for tissue samples following the manufacturer's instructions. In brief, 10-20 mg of parasite tissue were cut into small pieces and transferred into a 1.5 ml microcentrifuge tube. After adding 180 µl of ATL buffer and 20 µl of proteinase K the vortexed sample was incubated at 56°C until the tissue was completely lysed. After adding additional 200 µl of AL buffer the sample was incubated at 70°C for 10 min. Afterwards 200 µl ethanol (96–100%) were added and the complete mixture was applied onto a spin column. After several centrifugation and washing steps, 200 µl AE buffer were mounted onto the spin column's membrane, incubated at RT for 1 min and centrifuged at 6,200 x g (Sigma 1-15, Sigma-

Aldrich Inc., Saint Louis, MO, USA) for 1 min in order to elute the DNA. The isolated DNA was stored at -20°C.

3.4.3.2 DNA extraction with stool kit

The DNA extraction from the collected and sedimented faeces samples possibly containing trematode eggs was performed according to the QIAamp® DNA Stool Kit manual (QIAGEN, Vienna, Austria). Briefly, 200 µl of the homogenized sample were transferred to a 2 ml microcentrifuge tube and 1.4 ml ASL buffer were added. After intensely vortexing the sample for at least 1 min, the completely homogenized sample was incubated at 70°C for 5 min. After centrifuging, the supernatant was transferred to a new tube and an InhibitEx tablet was added in order to bind any existing inhibitors. The sample needed to be vortexed immediately and continuously for at least 3 min until the whole tablet was completely suspended. After an incubation period and centrifugation steps, the supernatant was again transferred to a new tube and incubated at 70°C with 15 µl proteinase K and 200 µl AL buffer. After adding 200 µl of ethanol (96–100%), the complete lysate was applied onto a spin column. After several centrifugation and washing steps the DNA was eluted in 200 µl AE buffer and stored at -20°C.

3.4.3.3 DNA extraction with soil kit

In addition to the DNA extraction with the QIAamp® DNA Stool Kit (QIAGEN, Vienna, Austria), 45 samples were processed with the Precellys Soil DNA Kit (PeqLab, Erlangen, Germany). The selection resulted from samples with low DNA yield and negative PCR results in the first tests despite positivity in the microscopic analysis. According to the manufacturer's protocol 250 mg of thawed sediment were transferred to homogenization tubes containing three different sized glass and ceramic beads. The samples were filled up with 600 µl Soil Lysis Buffer and incubated for 20 min at 95–98°C while shaking. Afterwards the tubes were cooled down on ice, homogenized for 20 sec at 5,000 rpm and placed on ice again. After 5 min of centrifugation at full speed the supernatant was transferred to a microcentrifuge tube, Soil Binding Buffer I was added and the mixture was loaded onto a column. After washing and centrifugation steps, the column was dried by centrifugation. The DNA was eluted with 100 µl Elution Buffer and afterwards processed with a second run of loading, binding, washing and drying steps. Finally, the DNA was again eluted in 100 µl Elution buffer.

3.4.4 Spectrophotometer

To calculate the amounts of DNA needed for PCR, the DNA concentrations in the samples were measured with a NanoDrop spectrophotometer (PeqLab, Erlangen, Germany). After blanking the instrument with the elution buffer used, 2 µl of eluted DNA were dropped onto the sensor area and the concentration was measured in ng/µl at least twice. The averages of the measured concentrations were calculated.

3.4.5 Primer design

Primer design was accomplished together with my colleague Michaela Haider (Haider, 2010).

3.4.5.1 Universal primers for trematodes

The universal primers were designed to bind explicitly to trematode DNA without binding to sequences of cestodes, turbellaria, bacteria or host species. Therefore, at least one of the two primers had to bind specifically only to trematodes, whereas the other one could also be more universal. The expected amplicon was designed to be 300–800 bp long and to comprise a variable sequence section in order to have variant sequences between the primer sites for species differentiation.

A variety of different trematode sequences was downloaded from the NCBI database (http://www.ncbi.nlm.nih.gov/sites/entrez?db=nucleotide). A selection of them is shown in Table 3.1. In addition, sequences from host species (deer, snails), other platyhelminthes than trematodes (cestodes, tubellaria etc.), fishes, fungi and bacteria were downloaded. The sequences were converted into FASTA files and aligned using the computer programmes ClustalX (Thompson et al. 1997) and GeneDoc (Nicholas et al. 1997). The aligned sequences where then scanned for appropriate primer binding sites.

The primers were designed to be 18–22 bases long and to have a well-balanced AT and CG ratio. On both ends of the primers CG pairs were located and the melting temperature of both primers was aimed to be approximately the same. Wallace's rule was used to roughly calculate the melting temperature of short primer sequences: $T_m = (A+T)*2 + (G+C)*4$.

To ensure the specificity of the primers, the short sequences were compared to other sequences from the NCBI database by blasting (http://www.ncbi.nlm.nih.gov/blast) against trematodes, hosts, bacteria etc.

Table 3.1: Selection of used species and corresponding GenBank numbers.

Species	**GenBank nr.**	**Species**	**GenBank nr.**
Fascioloides magna	EF051080	*Fasciola hepatica*	X56041.1
Fasciolopsis buski	L06668.1	*Dicrocoelium dendriticum*	Y11236.1
Calicophoron calicophorum	L06566.1	Paramphistomidae sp.	FJ550131.1

The designed universal primers are located in the 18S rDNA. A region from bp 92 to bp 112 off the 18S rDNA was chosen for the forward primer, bp 498 to 519 for the reverse primer (reference sequence *F. magna* EF051080). The primer pair flanks an approximately 430 bp long sequence. Variant sequences between the two primer sites allow species differentiation (Figure 3.8).

The primers were ordered at MWG AG Biotech (http://www.mwg-biotech.com/).

Forward primer

The forward primer for trematodes binds at the beginning of the 18S rRNA gene. It consists of 21 nucleotides and has an AT/CG ratio of 52%/48%. The melting temperature lies at 62°C.

Trem_F: 5’- GGT TCC TTA GAT CGT ACA TGC - 3’

Reverse primer

The reverse trematode primer consists of 22 nucleotides with a melting temperature of 64°C. The AT/CG ratio is 55%/45%.

Trem_R: 5’- GTA CTC ATT CGA ATT ACG GAG C - 3’

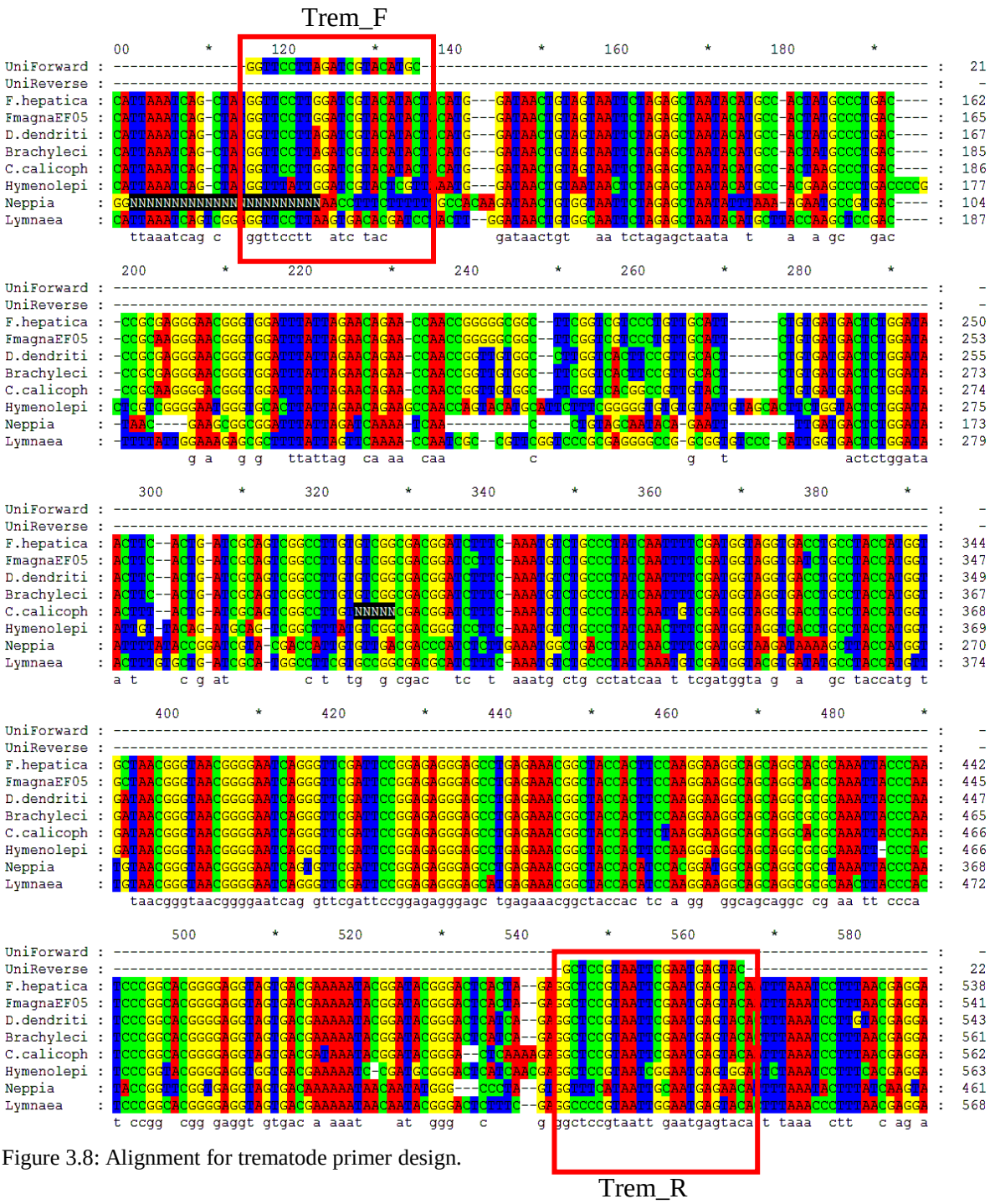

Figure 3.8: Alignment for trematode primer design.

3.4.5.1 Specific primers for *Fascioloides magna*

The primer pair used to detect *F. magna* was designed to be specific for this parasite with as little homology to other fasciolids as possible. Therefore, several sequences of *F. magna* and related species were downloaded from the NCBI database and aligned with ClustalX and GeneDoc in order to find appropriate primer binding sites (Figure 3.9). It was necessary to place the primers into highly variable regions to distinguish individual species. Since there are not many sequences of different fasciolids covering the whole transcription unit, it was difficult to find good primer binding sites. Finally, binding sites in

the internal transcribed spacers-regions (ITS) with their high degree of variability between closely related species were chosen.

The primers, located in the ITS1 and the ITS2 respectively, were designed and the appropriate AT-CG-ratio and melting temperatures were checked. The sequences were blasted against fasciolids, other trematodes, hosts and bacteria to guarantee primer specificity. The forward primer binds from bp 2256 to bp 2277 and the reverse primer from bp 2814 to 2835 (reference sequence *F. magna* EF051080). The 580 bp long amplicon produced during PCR covers parts of ITS1 and ITS2 and the whole region of the 5,8S rDNA. The primers were finally ordered at MWG AG Biotech.

Forward primer

The forward primer is located in the ITS1. It consists of 22 basepairs and has an AT/CG ratio of 50%/50%. The melting temperature is 66°C.

Magna_F: 5'- CAA GTG GCA TTG AAT GGC TTG C - 3'

Reverse primer

The reverse primer for *F. magna* is in the ITS2 and 22 bp long. The melting temperature is 66°C, the AT/CG ratio is 50%/50%.

Magna_R: 5'- GAC GAT AAA GCG ACA GTA GTG G - 3'

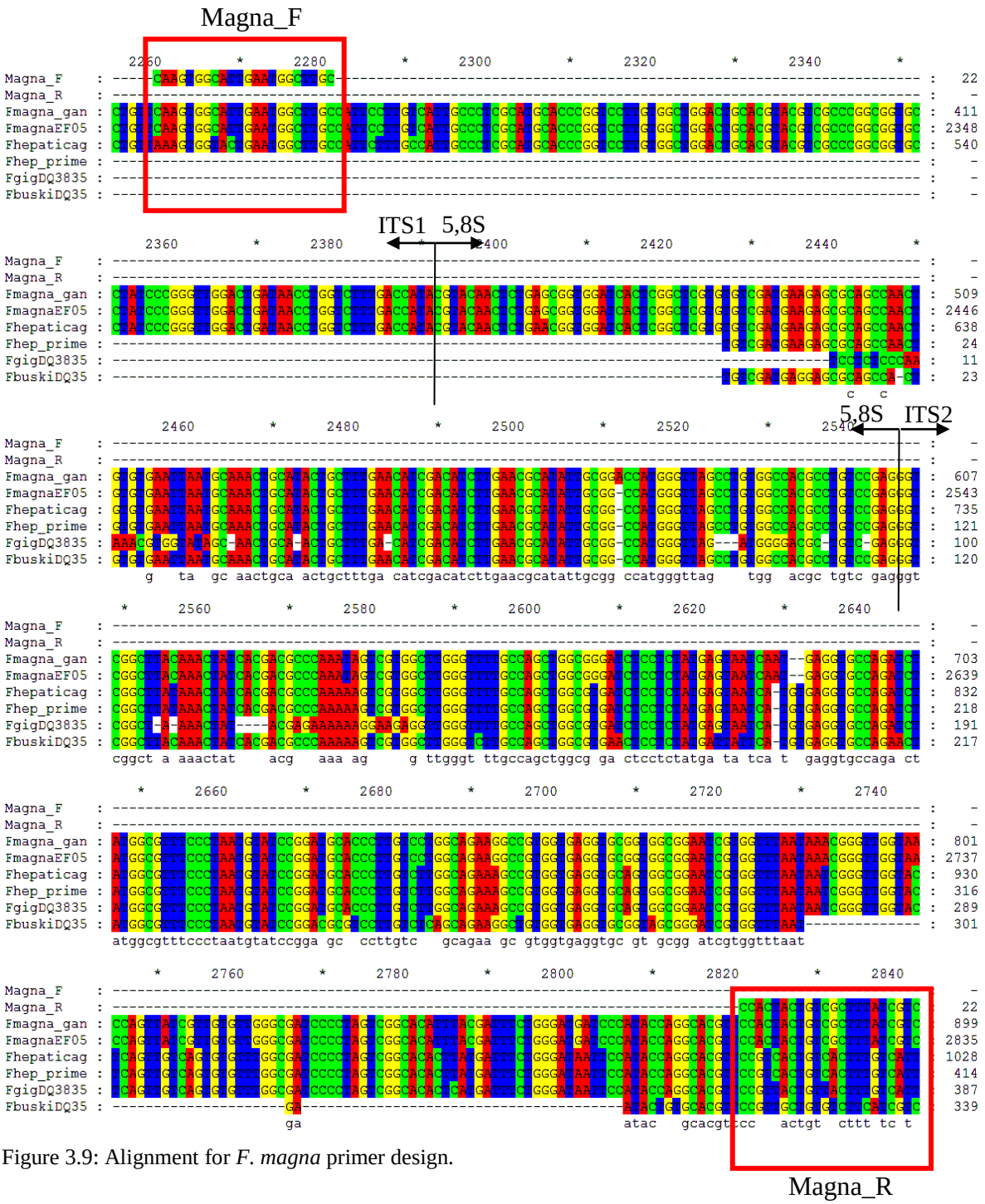

Figure 3.9: Alignment for *F. magna* primer design.

3.4.1 PCR setup

3.4.1.1 Trem_F/Trem_R

3.4.1.1.1 Specificity testing

The specificity of the designed primers was tested with DNA isolated from *F. magna*, *F. hepatica*, *Paramphistomum* sp., *Taenia saginata* and *Diphyllobothrium latum* as well as

with DNA extracted from sedimented deer faeces and host snail tissue as negative controls (Table 3.2).

Table 3.2: Control samples for specificity testing of trematode primers.

Species	**DNA conc.**	**Dilution**	**DNA amount used in PCR**
F. magna (tissue)	205.02 ng/µl	1:4	1 µl, 3 µl
F. hepatica (tissue)	294.22 ng/µl	1:5	1 µl, 3 µl
Paramphistomum sp. (tissue)	85.87 ng/µl	1:1	1 µl, 3 µl
T. saginata (tissue)	68.57 ng/µl	1:3	1 µl, 3 µl, 5 µl
T. saginata (eggs)	0.88 ng/µl	1:1	5 µl, 10 µl
D. latum (eggs)	0.74 ng/µl	1:1	5 µl, 10 µl

PCR reactions were set up in 0.2 ml soft PCR tubes with 31.25 µl master mix and distilled water to fill up the reaction volume of 50 µl. All samples were run in multiple set ups with DNA concentrations between 1 and 10 µl. The master mix consisted per sample of 5 µl PCR buffer ($MgCl_2$ free, Solis BIODYNE), 5 µl $MgCl_2$ (25 mM), 5 µl forward primer (1µM), 5 µl reverse primer (1µM), 1 µl dNTP mix (20 mM of each, 20 µmol, Solis BIODYNE), 0.25 µl polymerase (Hot Fire DNA Polymerase I, 5U/µl, Solis BIODYNE) and 11 µl distilled water.

According to the melting temperatures of the primers of around 60°C, the annealing temperatures were calculated to be approximately 55°C. Therefore, the PCR was performed with GastB (modified after Gast & Byers, 1995), a standard PCR programme operating with annealing temperatures of 52°, 54° or 56°C respectively. The highest possible temperature, namely 56°C, was chosen, since the PCR is more specific the higher the annealing temperature is.

The PCR was performed in a thermal cycler (Eppendorf) with the GastB programme: after an initial denaturation step to hot start the polymerase at 95°C for 15 min, 30 cycles of 1 min at 95°C, 2 min at 56°C and 3 min at 72°C and an elongation step at 72°C for 7 min followed.

Regardless of the applied amount of DNA, the primers produced expected amplicons of approximately 430 bp for all trematodes tested. There were no amplicons in any of the cestode samples (Figure 3.10), snail tissue or faeces (Figure 3.11).

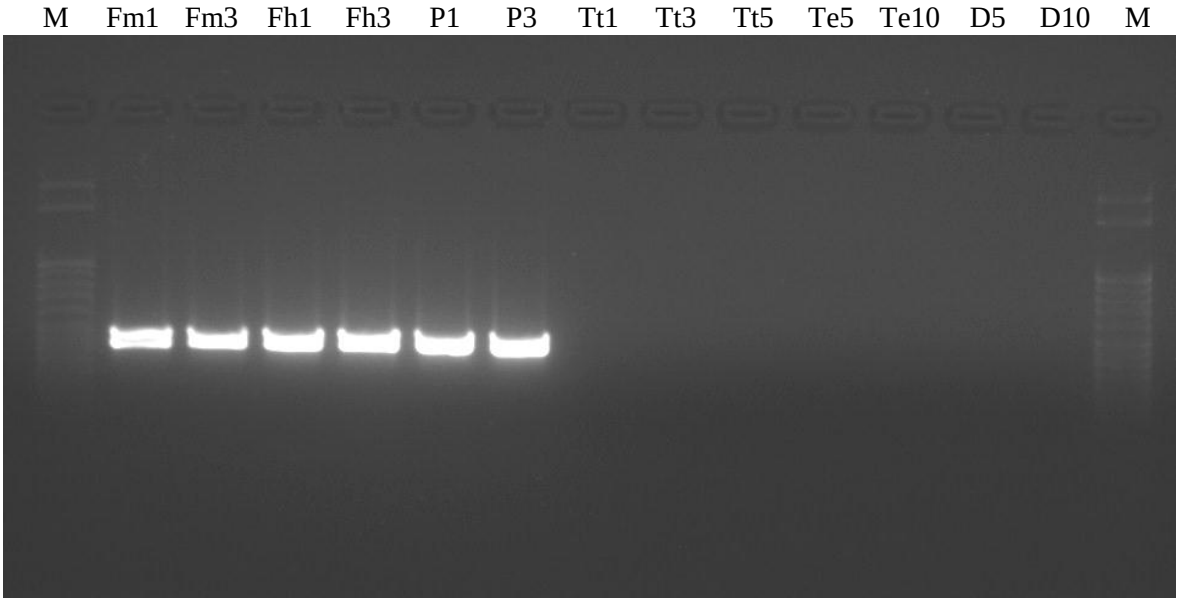

Figure 3.10: Trematode primers specificity testing, trematodes and cestodes. M=Marker, Fm=*F. magna*, Fh=*F. hepatica*, P=*Paramphistomum* sp., Tt=*T. saginata* tissue, Te=*Taenia* sp. eggs, D=*D. latum*.; numbers describe used amount of DNA.

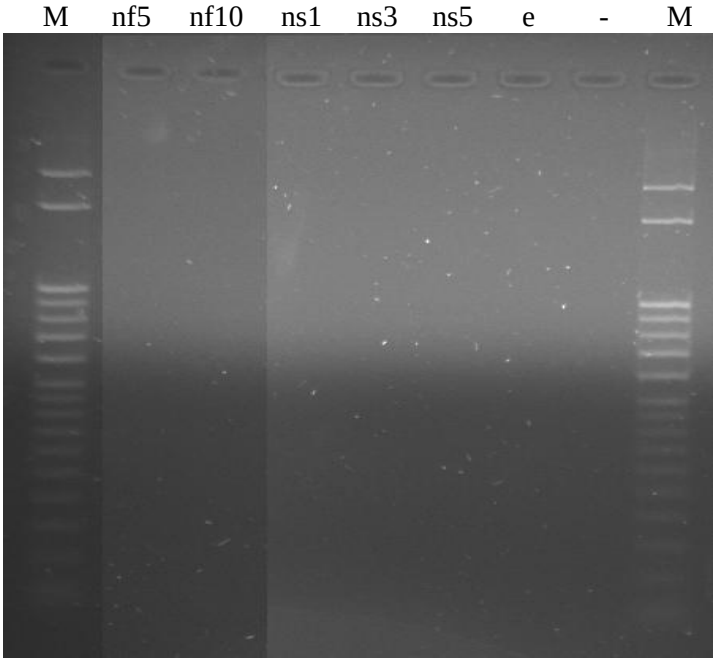

Figure 3.11: Trematode primers specificity testing, faeces and snails. M=Marker, nf=negative faeces sample, ns=negative snail tissue, e=empty, -=negative control; numbers describe used amount of DNA.

3.4.1.1.2 Sensitivity testing

The sensitivity of the universal primers was tested with a *F. magna* DNA dilution series. The average DNA concentration was calculated after spectrophotometric measurement (initial concentration 205.02 ng/µl). 1 µl DNA each of a 1:1, 1:10, 1:100, 1:1000 and

1:10000 dilution was amplified with the GastB 52°C cycling programme to find out the detection limit of the designed primers. It was demonstrated that the primers are able to detect 0.2 ng of parasitic DNA in the initial samples (Figure 3.12).

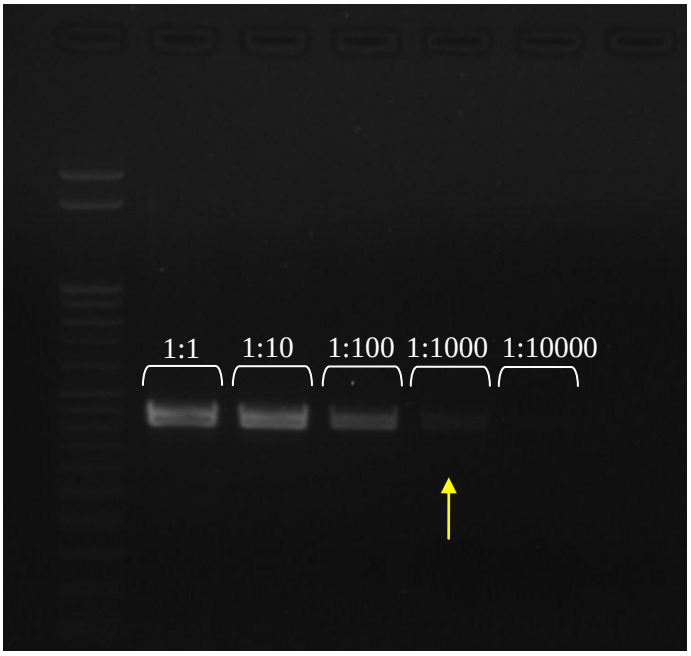

Figure 3.12: Trematode primers sensitivity testing.

3.4.1.1.3 PCR establishment

After ensuring both specificity and sensitivity of the designed primers, PCR was established for deer-soil samples with low amounts of DNA. Two microscopically positive samples (samples no. 63 and 124) were used as controls. Small amounts of sedimented faeces of both samples were homogenized with two differently sized homogenizer beads (0.5 mm glass beads and 1.4 mm ceramic beads, PeqLab, Erlangen, Germany) to evaluate possible differences in DNA isolation efficiency from eggs. After isolating the DNA with the QIAamp® DNA Stool Kit manual (QIAGEN, Vienna, Austria) following the protocol and measuring the DNA concentrations, PCRs were performed with the GastB cycling programme testing all three possible annealing temperatures (52°C, 54°C and 56°C) on the primers to find out potential differences in the detection of trematode DNA (Figure 3.13). To assure correctness of PCR and exclude possible errors, positive and negative controls were always included into the setups. The tests showed that the best annealing temperature for these samples is 52°C, where most gel bands were visible (Figure 3.13, bottom).

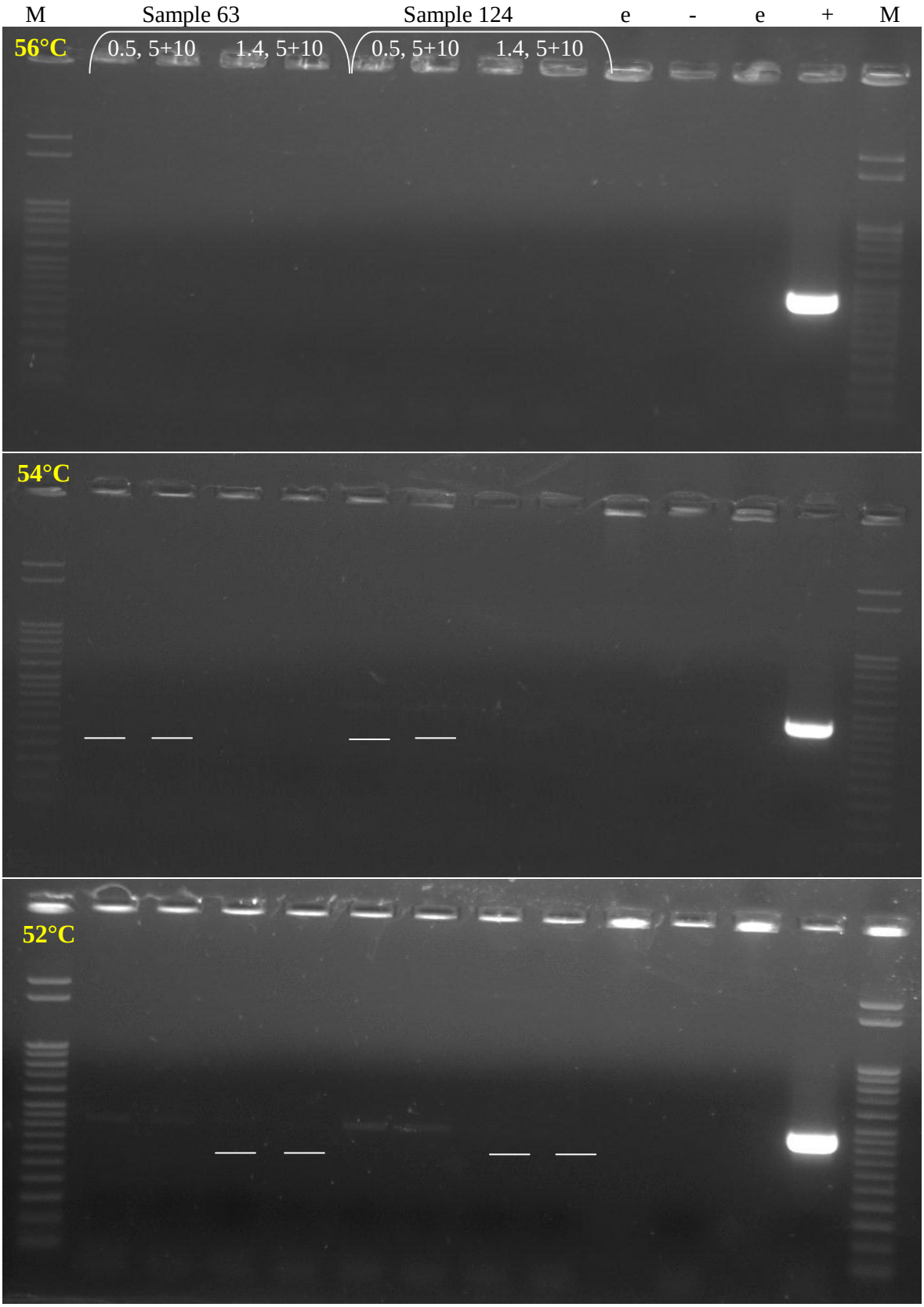

Figure 3.13: Trematode primers establishment testing at three different annealing temperatures. Top = GastB 56°C, middle = GastB 54°C, bottom = GastB 52°C. M=Marker, e=empty slot, -=negative control, +=positive control; 0.5 and 1.4 refer to diameter of homogenizer beads, 5 and 10 µl of DNA were used (faint bands were marked).

Due to very low amounts of DNA after extraction with the DNA Stool Kit and the consequential faint gel bands, a definite evaluation of PCR results was difficult. Therefore, it was aimed to enhance sensitivity by a second amplification step under the same conditions as in the first PCR with a purification step in between. For the first PCR 8 µl DNA were amplified, negative and positive controls were treated as usual. The PCR product was then purified using the Xact DNA Gel Extraction Kit (genXpress, Wiener Neudorf, Austria) following the protocol for the DNA extraction from enzymatic reactions, eluting in 30 µl elution buffer. For the second PCR, 1 µl and 3 µl respectively of three differently diluted purified PCR products were used and amplified again with the same amount of reaction mix and the same PCR cycling programme as in the first PCR. The first negative control was treated as a normal sample concerning purification and second amplification, and a second negative control was included to rule out errors in the second PCR. The positive control from the first PCR was excluded.

Figure 3.14 shows the gel of the second test PCR. It demonstrates that after 8 µl of DNA in the first PCR, 1 µl of the 1:10 diluted purified PCR product was appropriate for the second PCR. PCRs were performed with 52°C annealing temperature.

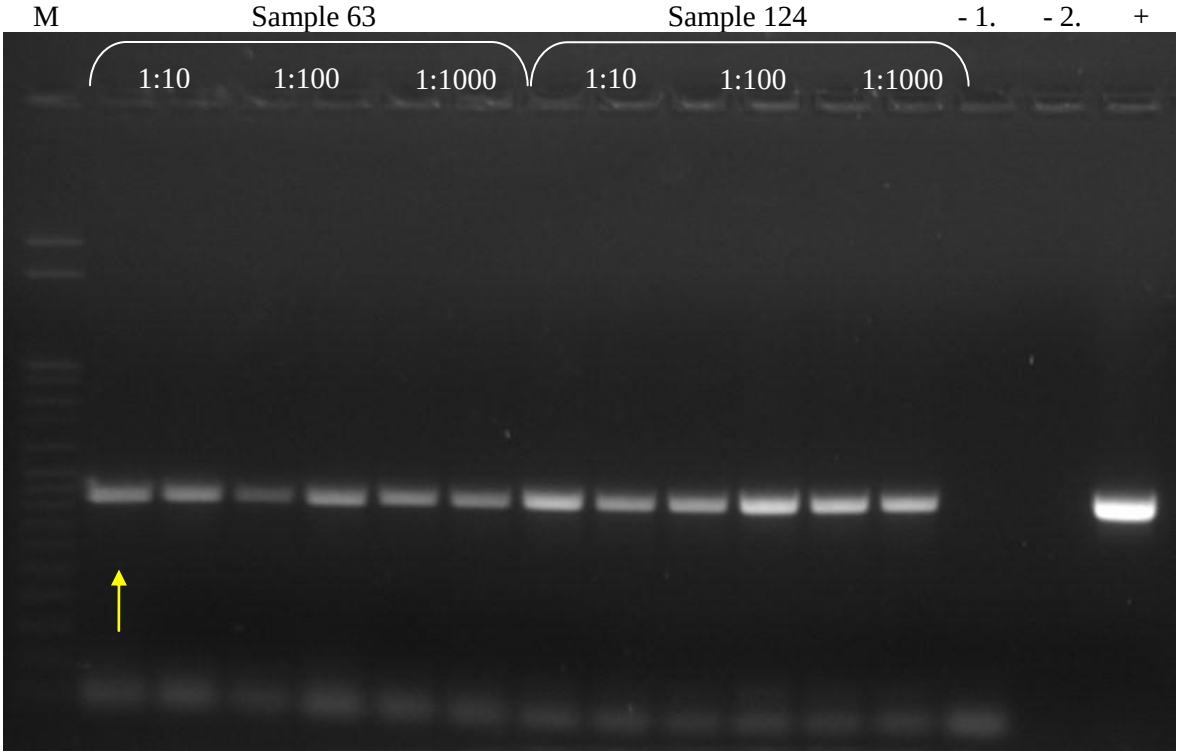

Figure 3.14: Trematode primers standardisation with positive samples in different dilutions (1:10, 1:100, 1:1000). M=Marker, - 1.=negative control of first PCR, - 2.= negative control of second PCR, +=positive control.

Best results were achieved with the cycling temperature of 52°C and the use of 1 µl of the 1:10 diluted PCR product in the second amplification step. This was then standardized for all further trematode PCRs.

In case of the samples processed with the Precellys Soil DNA Kit (PeqLab, Erlangen, Germany), further PCRs were performed in the same way as described above with two consecutive amplifications with purification in between.

3.4.1.2 Magna_F/Magna_R

3.4.1.2.1 Specificity testing

To evaluate the specificity of the *F. magna* primers, a PCR was performed with DNA extracted from *F. magna* tissue, *F. hepatica* tissue, *Paramphistomum* sp. tissue, *T. saginata* tissue, intermediate host snail tissue and deer faeces (Table 3.3).

Table 3.3: Control samples for specificity testing of *F. magna* primers.

Species	**DNA conc.**	**Dilution**	**DNA amount used in PCR**
F. magna (tissue)	205.02 ng/µl	1:4	1 µl, 3 µl
F. hepatica (tissue)	294.22 ng/µl	1:5	1 µl, 3 µl
Paramphistomum sp. (tissue)	85.87 ng/µl	1:1	1 µl, 3 µl
T. saginata (tissue)	68.57 ng/µl	1:3	1 µl, 3 µl, 5 µl
faeces sample	1.95 ng/µl	1:1	10 µl
snail sample	137.76 ng/µl	1:1	10 µl

Several preparations with diverse concentrations of DNA were included. Each reaction consisted of target DNA, 31.25 µl master mix (both primers, PCR buffer, $MgCl_2$, dNTP mix, polymerase and distilled water) and distilled water to total up the reaction volume of 50 µl. The PCR was performed with the GastB cycling programme at 56°C due to given melting temperatures of both primers around 60°C.

As displayed in Figure 3.15, there were strong gel bands with *F. magna* DNA. There were also faint bands with *F. hepatica* showing that, although much less, the primers also bind to *F. hepatica* DNA. There was no binding, neither to other trematodes or cestodes nor to negative faeces or snail samples. The positive snail samples shown on Figure 3.15 were artificially spiked with purified *F. magna* DNA (Haider, 2010), whereas the microscopically positive faeces sample did not show any amplification.

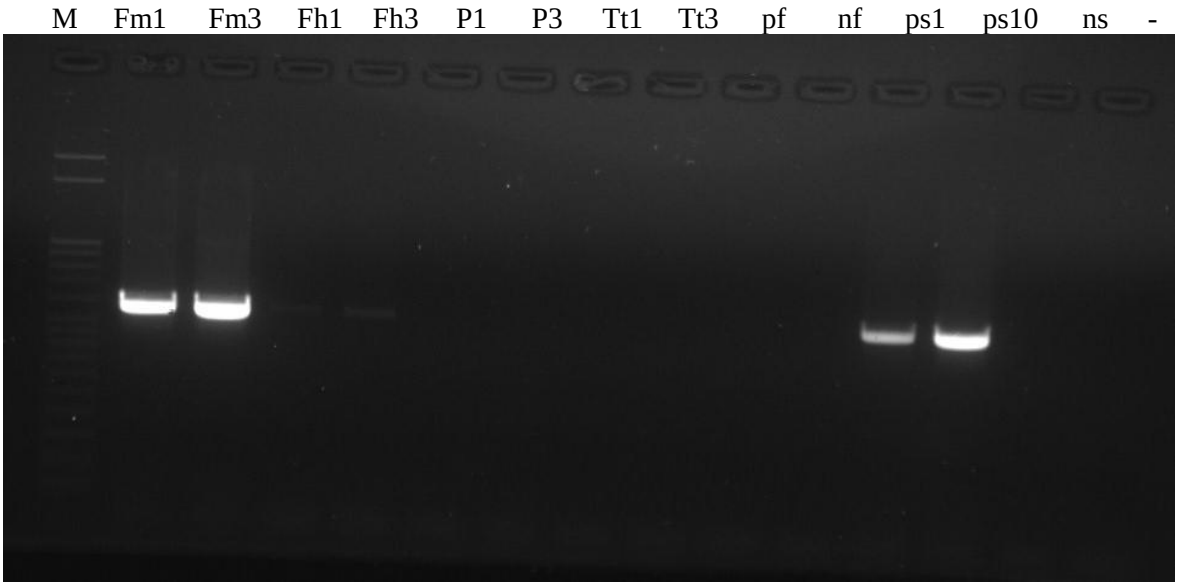

Figure 3.15: *F. magna* primers specificity testing. M=Marker, Fm=*F. magna*, Fh=*F. hepatica*, P=*Paramphistomum* sp., Tt=*T. saginata* tissue, pf=positive faeces sample, nf=negative faeces sample, ps=positive snail tissue, ns=negative snail tissue, -=negative control; numbers describe used DNA amount (in case of positive snail tissue numbers refer to artificially added parasitic DNA in ng).

3.4.1.2.2 Sensitivity testing

The sensitivity of the specific primers was tested with differently concentrated *F. magna* DNA (initial concentration 205.02 ng/µl). 1 µl of 5 different dilutions (1:1, 1:10, 1:100, 1:1000 and 1:10000) were used for PCR. The subsequently performed gel electrophoresis then showed the detection limit of the designed primer pair, which was 0.02 ng of parasitic DNA (Figure 3.16).

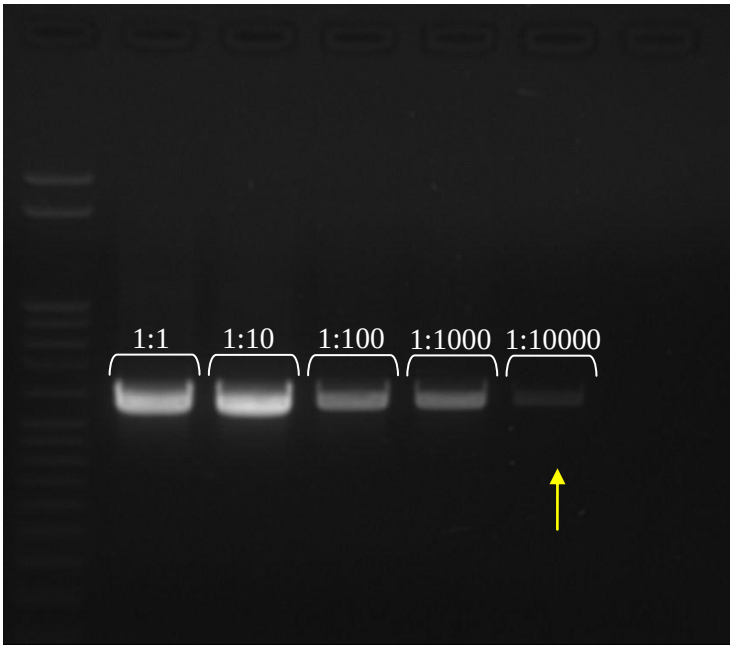

Figure 3.16: *F. magna* primers sensitivity testing.

3.4.1.2.3 PCR establishment

The establishment of the specific *F. magna* PCR was performed according to the trematode PCR establishment. Briefly, two microscopically positive samples were homogenized, the DNA was extracted using the QIAamp® DNA Stool Kit (QIAGEN, Vienna, Austria) and the DNA concentrations were measured. PCRs were performed with the GastB cycling programme with three different annealing temperatures (Figure 3.17). While there was only one band at the gel displaying the PCR results of 52°C (Figure 3.17 bottom), best results were achieved at 54°C as shown in the middle in Figure 3.17.

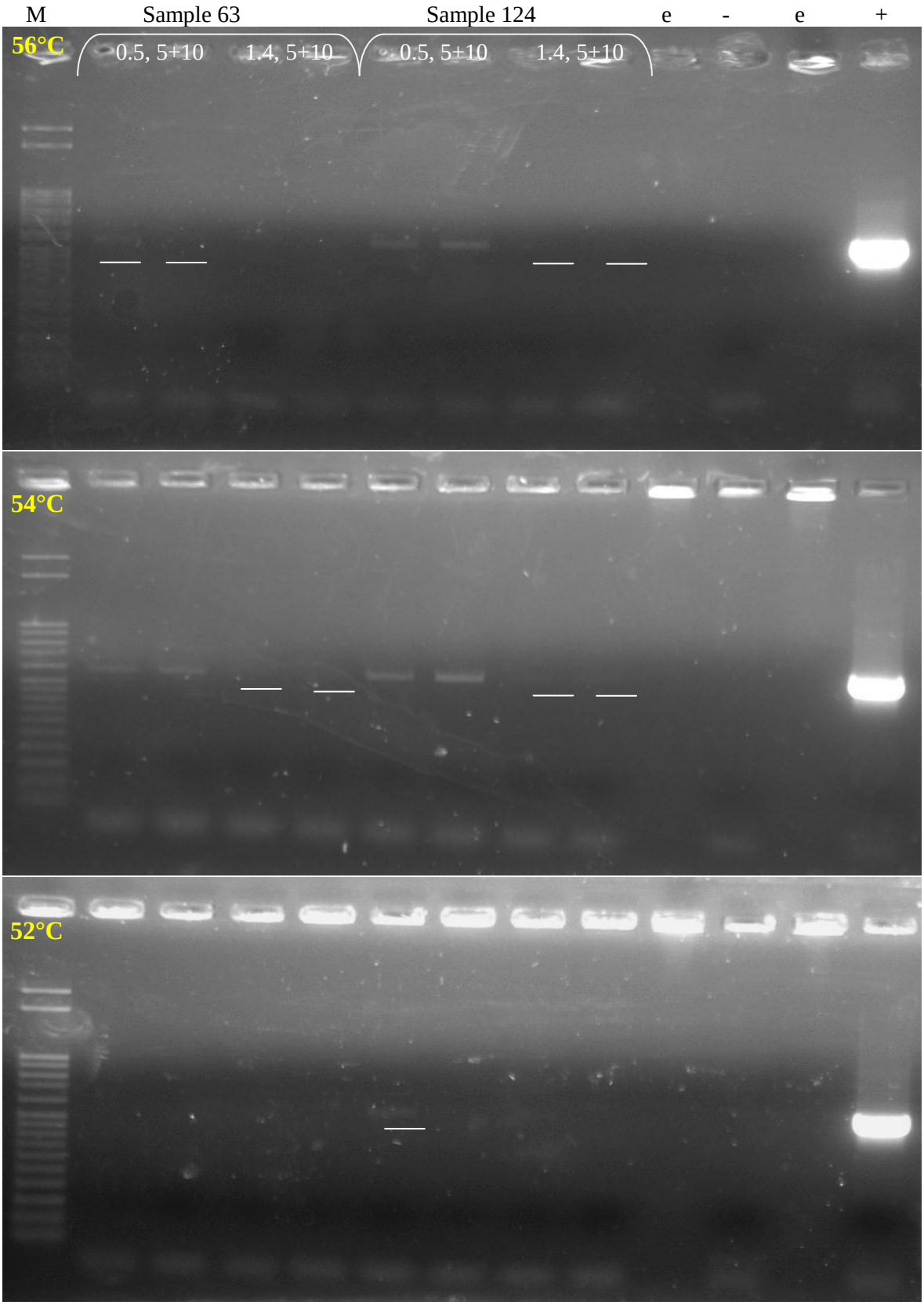

Figure 3.17: *F. magna* primers establishment testing at three different annealing temperatures. Top = GastB 56°C, middle = GastB 54°C, bottom = GastB 52°C. M=Marker, e=empty slot, -=negative control, +=positive control; 0.5 and 1.4 refer to diameter of homogenizer beads, 5 and 10 µl of DNA were used (faint bands were marked).

Again, to increase the sensitivity, a second amplification round was performed. The purified PCR product from the first amplification was diluted several times and amplified again under the same setup conditions. Figure 3.18 shows the standardisation test gel, 1 µl of the 1:10 diluted PCR product giving optimal results in the second PCR. Both PCRs were performed with GastB 54°C.

All subsequently performed *F. magna* PCRs (stool kit and soil kit) were carried out following this standard.

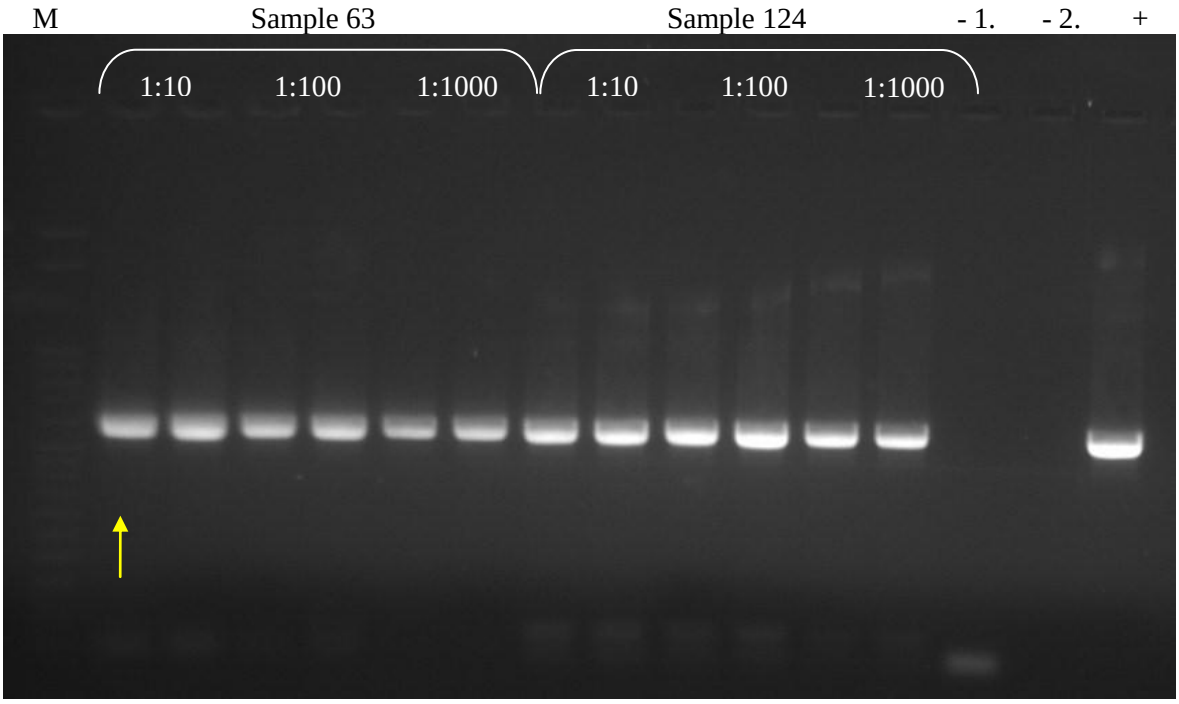

Figure 3.18: *F. magna* primers with positive samples in different dilutions (1:10, 1:100, 1:1000). M=Marker, - 1.=negative control of first PCR, - 2.= negative control of second PCR, +=positive control.

3.4.2 Primers for *F. hepatica*

For the detection of *F. hepatica* and the differentiation between *F. hepatica* and *F. magna*, a well-established PCR from the literature was chosen (Kaplan et al. 1995). The primers Fasc_F and Fasc_R amplify a repetitive 124 bp long target.

Forward primer

The forward primer consists of 22 basepairs with an AT/CG ratio of 59%/41%. The melting temperature of the primer is 62°C.

Fasc_F: 5'- ATT CAC CCA TTT CTG TTA GTC C - 3'

Reverse primer

The 20 bp long reverse primer has an AT/CG ratio of 45%/55% and a melting temperature of 62°C.

Fasc_R: 5'- ACT AGG CTT AAA CGG CGT CC - 3'

3.4.2.1.1 Specificity testing

To test the specificity of the primers, tissue-extracted DNA of *F. hepatica*, *F. magna*, *Paramphistomum* sp, *Aspidogaster* sp, *Proteocephalus* sp. and *Diplostomum* sp. was amplified with the given primers and a slightly modified cycling programme following Velusamy et al. (2004) (15 min at 95°C, 35 cycles of 1 min at 94°C, 1 min at 65°C, 1 min at 72°C and 10 min at 72°C).

The primers bind exclusively to *F. hepatica* sequences and produce a repetitive 124 bp long amplicon as shown in Figures 3.19 and 3.20.

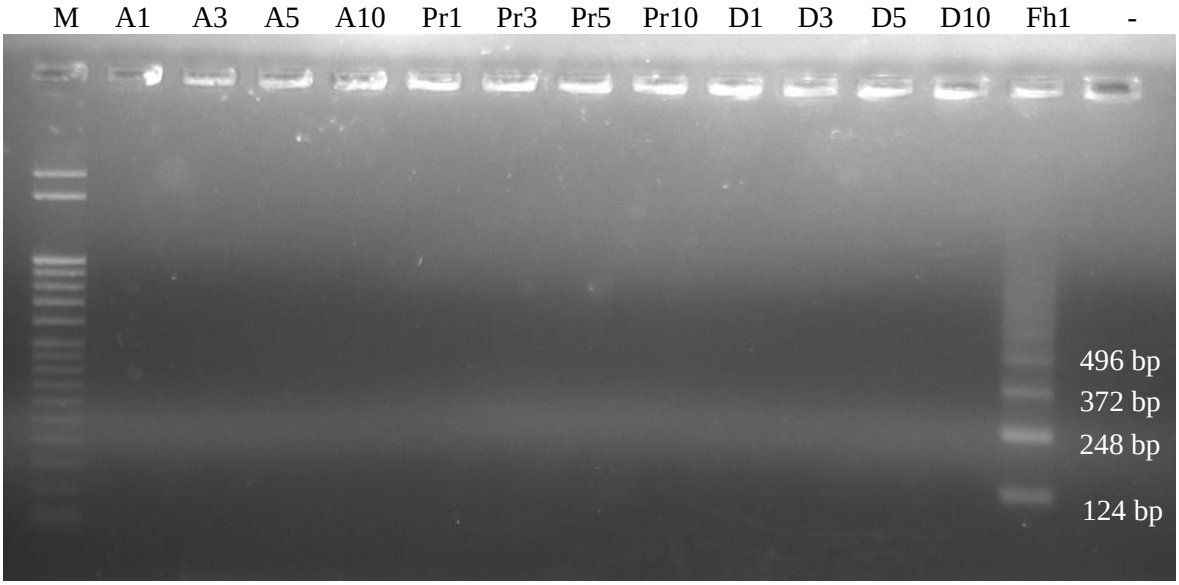

Figure 3.19: *F. hepatica* primers specificity testing, Gel 1. M=Marker, A=*Aspidogaster limacoides*, Pr=*Proteocephalus longicollis*, D=*Diplostomum* sp., Fh=*F. hepatica*, -=negative control. Numbers refer to amount of DNA used in PCR.

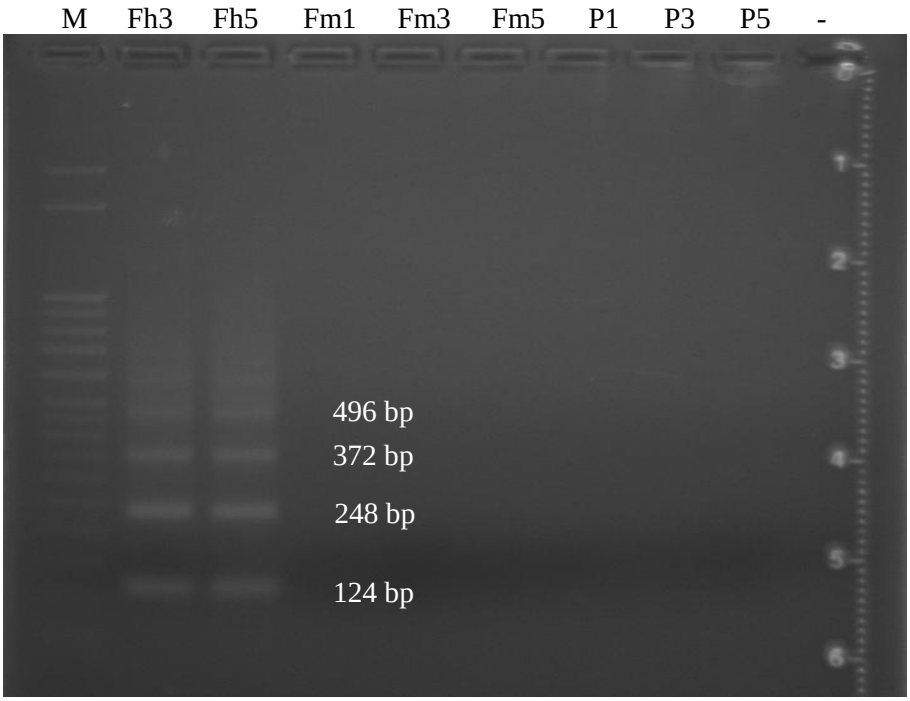

Figure 3.20: *F. hepatica* primers specificity testing, Gel 2. M=Marker, Fh=*F. hepatica*, Fm=*F. magna*, P=*Paramphistomum* sp. -=negative control. Numbers refer to amount of DNA used in PCR.

3.4.2.1.2 Sensitivity testing

The sensitivity was tested with a dilution series of DNA purified from an adult *F. hepatica* (initial concentration 294.22 ng/µl). The measured DNA concentration was diluted with distilled water 5 times in order to receive 1:1, 1:10, 1:100, 1:1000 and 1:10000 dilutions. 1 µl of each was then used for PCR, which was evaluated by gel electrophoresis to determine the detection limit of the primers. As shown in Figure 3.21 the primers bind to as little as 0.03 ng of parasitic DNA.

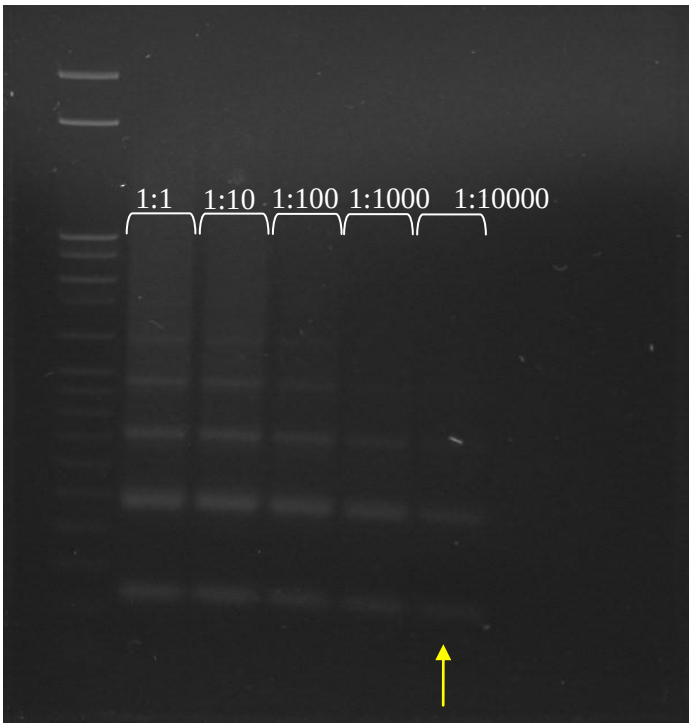

Figure 3.21: *F. hepatica* sensitivity testing.

3.4.3 Gel electrophoresis

PCR results were evaluated by an agarose gel electrophoresis. Depending on the number of samples, differently sized agarose gels were cast. To prepare a 2% agarose gel with 105 cm² size, which were made with 15 and 20 slots respectively, 1.4 g agarose (Sigma-Aldrich Inc., Saint Louis, MO, USA) and 70 ml 1x TAE buffer (0.04 M Tris-acetat, 0.001 M EDTA) were boiled on a magnetic stirrer. After cooling down the liquid gel 5.5 µl EtBr (final concentration 0.08‰) were added. After pouring the gel onto a casting plate and setting the comp, it was left to solidify for at least 45 min.

To prepare larger gels (150 cm², 20 slots) 2 g agarose, 100 ml 1x TAE buffer and 7 µl EtBr were used. For smaller gels (60 cm², 8 slots) 1 g agarose, 50 ml 1x TAE buffer and 4.5 µl EtBr were used.

After the gel had completely polymerized, the samples were loaded. The PCR products were mixed with 10x DNA loading buffer at a ratio of 1:10 (loading buffer: PCR product). The sample-loading buffer-mixes were consecutively filled into the slots. In order to find out the amplicon's lengths, 17 µl of step marker (DirectLoad Step Ladder, 50 bp, Sigma-Aldrich Inc., Saint Louis, MO, USA) were loaded into the first slot. Alternatively, another 17 µl of step marker were filled into an idle slot at the end. To check and compare if the PCR worked properly, positive and negative controls were loaded after the samples.

The loaded gel was then placed into a horizontal electrophoresis system (BIO-RAD Wide Mini-Sub® Cell GT or Mini Sub™ DNA Cell GT alternatively) and the system was refilled with 1x TAE buffer if necessary to ensure that the gel was completely covered with buffer. The applied voltages were 90 V for 105 cm² and 150 cm² gels, 65 V for 60 cm². Electrophoresis was brought to a halt when the loading buffer had reached the lower end of the gel.

The gels were evaluated under UV light, illuminating the intercalating DNA-EtBr-complexes. Positive bands were cut out for sequencing.

3.4.4 Purification of agarose gel bands

To purify DNA of bands cut out of agarose gels the Xact DNA Gel Extraction Kit (genXpress, Wiener Neudorf, Austria) was used. The manufacturer's protocol for agarose gels was followed incubating the weighed gel piece at 50–60°C with 40 µl of binding buffer per 10 mg of gel. The completely dissolved gel mixture was transferred to a spin column, centrifuged and washed. Depending on size and intensity of the band, 25–50 µl elution buffer were used to elute the DNA. The DNA was then stored at -20° C.

3.4.5 Sequencing

Sequencing was performed to identify the parasitic DNA detected in PCR on a species specific level and to verify the specificity of the designed primers.

3.4.5.1 Sequencing PCR

For this PCR only one primer of the primer pair was used at a time. Each sample was sequenced several times in different concentrations in both directions. 1 µl and 3 µl purified DNA respectively, were mixed with 1 µl primer, 2 µl AB Mix (Big Dye Terminator v1.1 cycle sequencing Kit, AB Applied Biosystems, Austria) and filled up with distilled water to a total reaction volume of 10 µl. The sequencing PCR programme begins with an initial denaturation phase at 96°C for 30 sec and continues with 30 cycles of denaturation at 96°C for 10 sec, annealing at 50°C for 5 sec and elongation at 60°C for 4 min.

3.4.5.2 Purification of the sequencing PCR product

1 µl NaAc and 33 µl ethanol (100%) were filled into a 500 µl Eppendorf tube and placed on ice. After adding the PCR product, the mixture was incubated on ice for 17 min. The samples were then centrifuged at 4°C for 30 min at 13,800 x g (Heraeus Fresco 17 Centrifuge, Thermo Fisher Scientific Inc, Waltham, MA, USA). Subsequently, the supernatant was carefully discarded, the sample washed with 70 µl ethanol (70%) without resuspending the pellet and centrifuged again for 10 min at 13,800 x g at 4°C. Again, the supernatant was carefully discarded and the tubes where left with lids open for about 5 min for the ethanol to evaporate. 20 µl HI-DI^{TM} – 420Formamide (Applied Biosystems, Austria) were added and the tubes were left for another 5 min with open lids at room temperature. After that, the mixture was boiled in a Thermomixer (Thermomixer comfort 1.5 ml, Eppendorf AG, Hamburg, Germany) at 95°C for 5 min, the tubes were briefly spinned at 600 x g and put on ice for 5 min.

Sequencing was performed in an automated ABI PRISM 310® Sequencer (PE Applied Biosystems, Langen, Germany).

3.4.5.3 Analysis of sequences

The obtained sequences were aligned with published sequences from the NCBI database using the PC programmes ClustalX (Thompson et al. 1997) and GeneDoc (Nicholas et al. 1997) and assigned to a certain species or family on the basis of sequence similarity.

4 RESULTS

4.1 Samples

4.1.1 Overall distribution and origin of samples

Altogether, 66 deer leaflets were collected during 15 sampling rounds of the three chosen sites in Orth/Donau and one round at Fischamend. 92 samples were provided by staff members of the Österreichische Bundesforste AG ÖBf from different locations in the area of Orth/Donau and eight other sampling sites. Table 4.1 shows the exact localisations of the total 158 samples and the respective number of samples per sampling site. Figure 4.1 shows the geographical distribution of all samples and points out that almost two thirds (61%) of all samples originate from the Orth/Donau sites.

Table 4.1: Overview of sampling sites (coordinates provided by http://www.linkr.de/2009/03/24/mit-google-maps-laengen-breitengrade-ermitteln-freies-geocoding-skript/).

Sampling site	Longitude	Latitude	Number of samples
Eckartsau	16.79739	48.14526	19
Fischamend	16.61327	48.11935	9
Großenzersdorf	16.55021	48.20144	5
Mannsdorf/Donau	16.66392	48.15221	3
Markthof	16.95642	48.19356	5
Mühlleiten	16.56652	48.16963	4
Orth/Donau	16.70557	48.14615	10
Orth/Donau Entenhaufen	16.69866	48.12536	34
Orth/Donau Märchenteich (Bridge)	16.69848	48.12954	20
Orth/Donau Neubruchwiese	16.68050	48.13080	32
Schönau/Donau	16.61385	48.14140	3
Stopfenreuth	16.88027	48.14866	8
Witzelsdorf	16.83569	48.15151	6

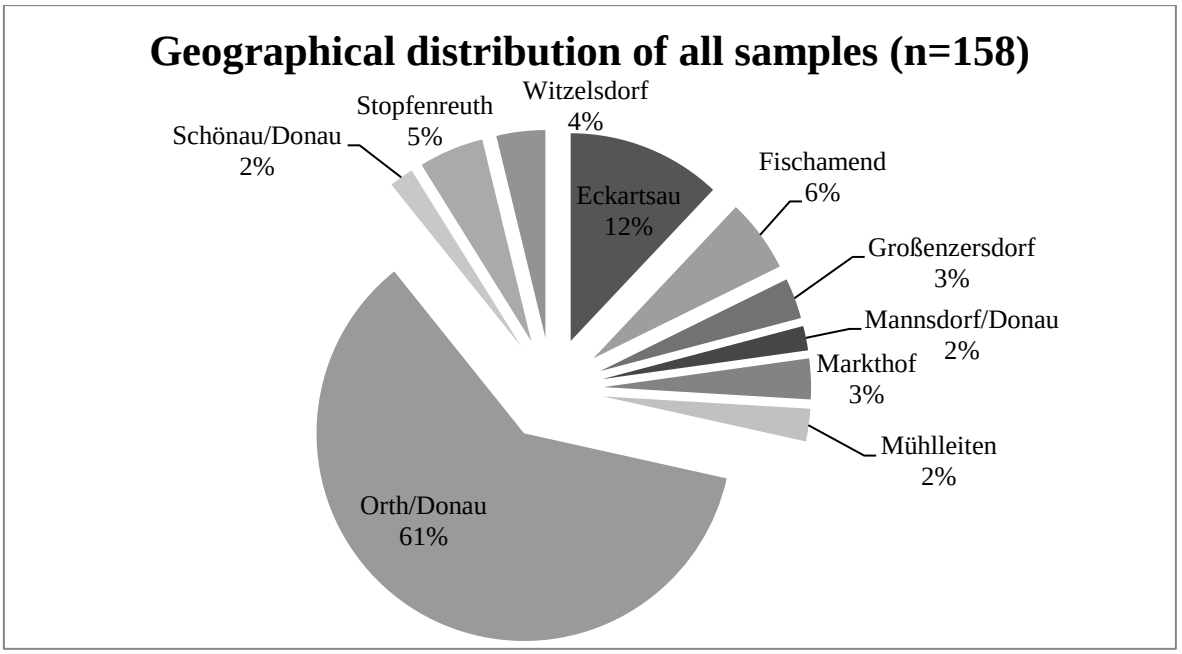

Figure 4.1: The distribution of all samples over the ten chosen sampling sites given in percentages.

4.1.2 Geographical distribution of collected samples

As shown in Figure 4.2 14% of the collected samples were found in Fischamend, the other 84% originated from the three chosen sampling sites in Orth/Donau. While there were almost equal numbers of samples from Entenhaufen (33%) and Neubruchwiese (38%), only 15% of the collected samples originated from Märchenteich, due to a lower faecal density.

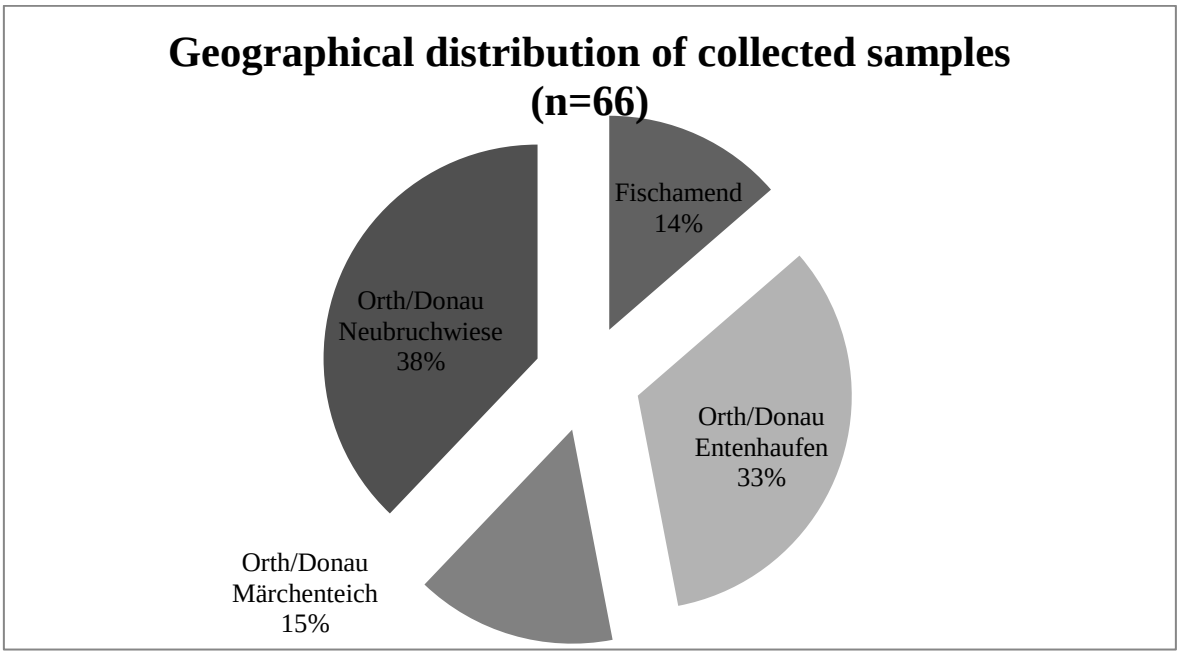

Figure 4.2: Geographical distribution of the self-collected samples over four inspected sampling sites.

4.1.3 Geographical distribution of provided samples

Of the 92 samples provided by staff members of the ÖBf, 42% originated from the area of Orth/Donau (Figure 4.3). Approximately one fifth (21%) of the samples was from Eckartsau, the percentages of samples from the other sampling sites ranged from 3% to 9%.

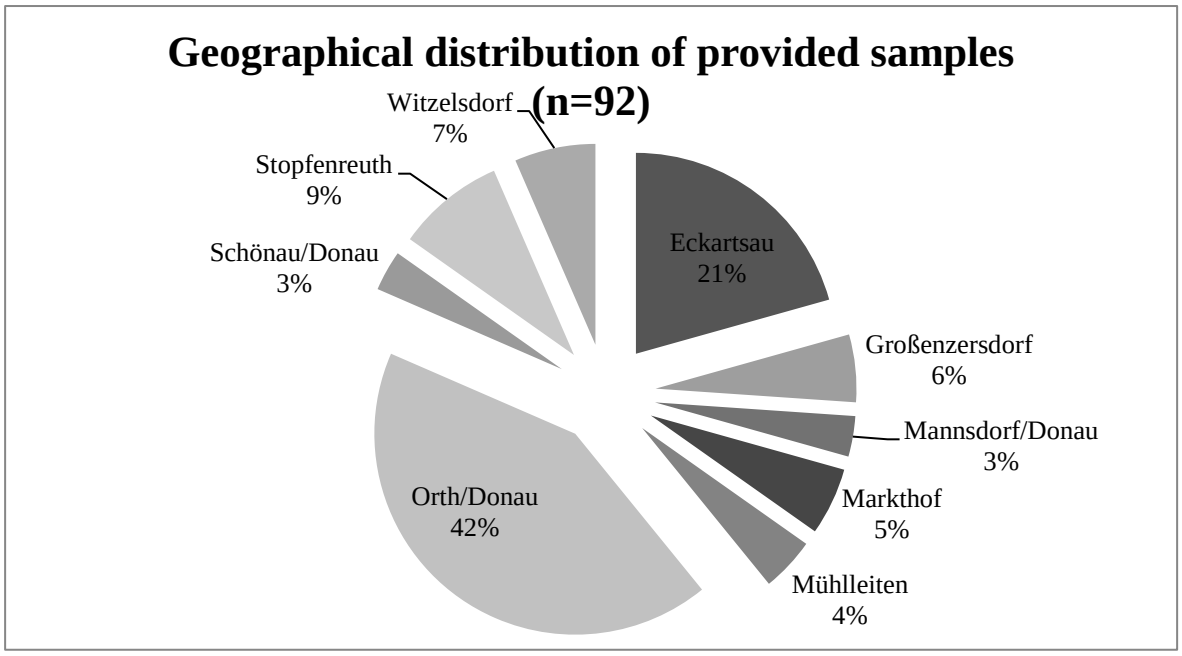

Figure 4.3: Geographical distribution of by ÖBf staff members provided samples over nine sampling sites.

4.1.4 Seasonal distribution of samples

The samples were collected between February and October 2008. 15 samples of the ÖBf provided were not dated, so the seasonal distribution was only calculated from 143 samples. Figure 4.4 shows that more than half of all samples (53%) were collected in the months August – October. In the first three months of the monitoring (February – April), when only ÖBf staff members were collecting samples, 32 samples (22%) were taken.

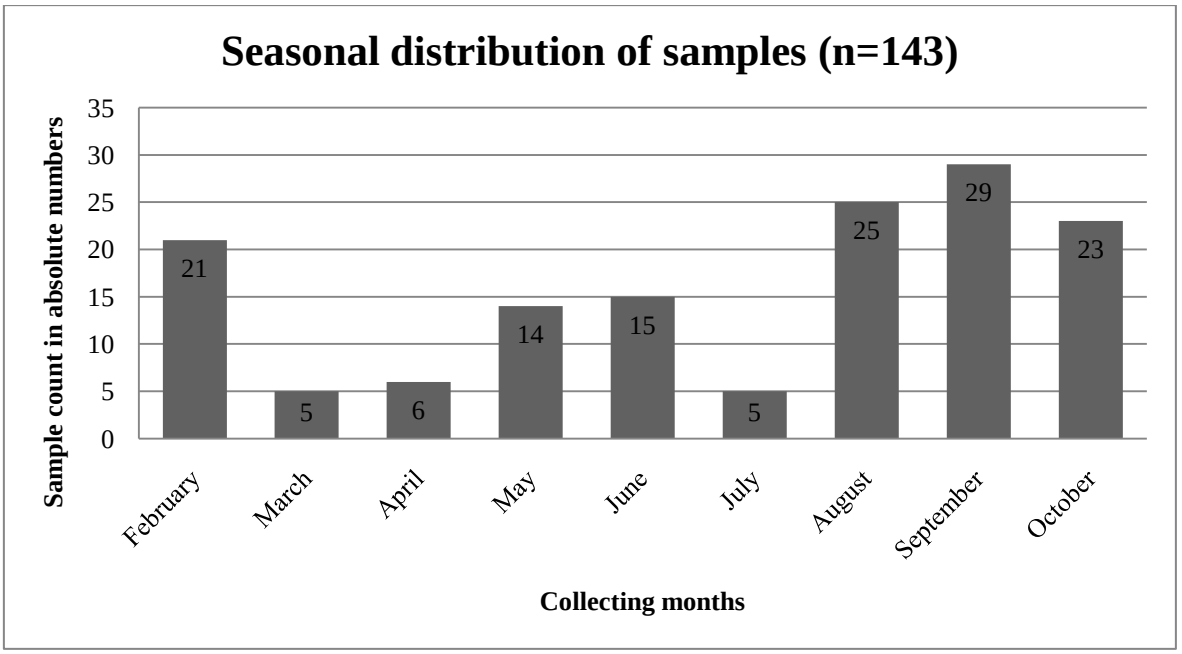

Figure 4.4: Seasonal distribution of all dated samples.

4.2 Microscopy

Altogether, 158 faeces samples were analysed by microscopy after sedimentation (modified Benedek method). Of these, 49 samples (31%) were positive for trematode eggs and in 6 samples (4%) eggs were found which could not be clearly defined as trematode eggs. In 65% of these samples no trematode eggs were found as demonstrated in Figure 4.5.

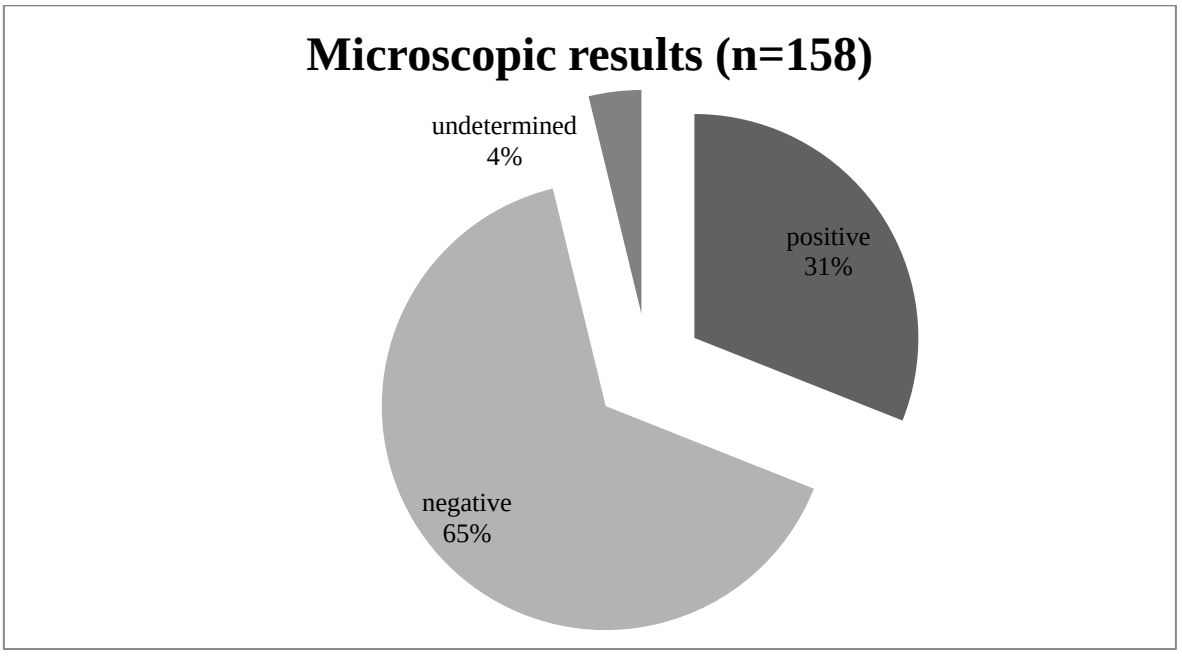

Figure 4.5: Microscopic results for trematode eggs in sedimented faeces samples.

4.2.1 Eggs per gram

Eggs per gram (EpGs) were roughly calculated from 49 positive samples (Figure 4.6). The majority of samples (25/49) contained approximately 2.5 eggs per gram. In 21 samples EpG counts ranged from 5 to 17.5. Two samples were highly positive with 40 and even 100 EpG respectively.

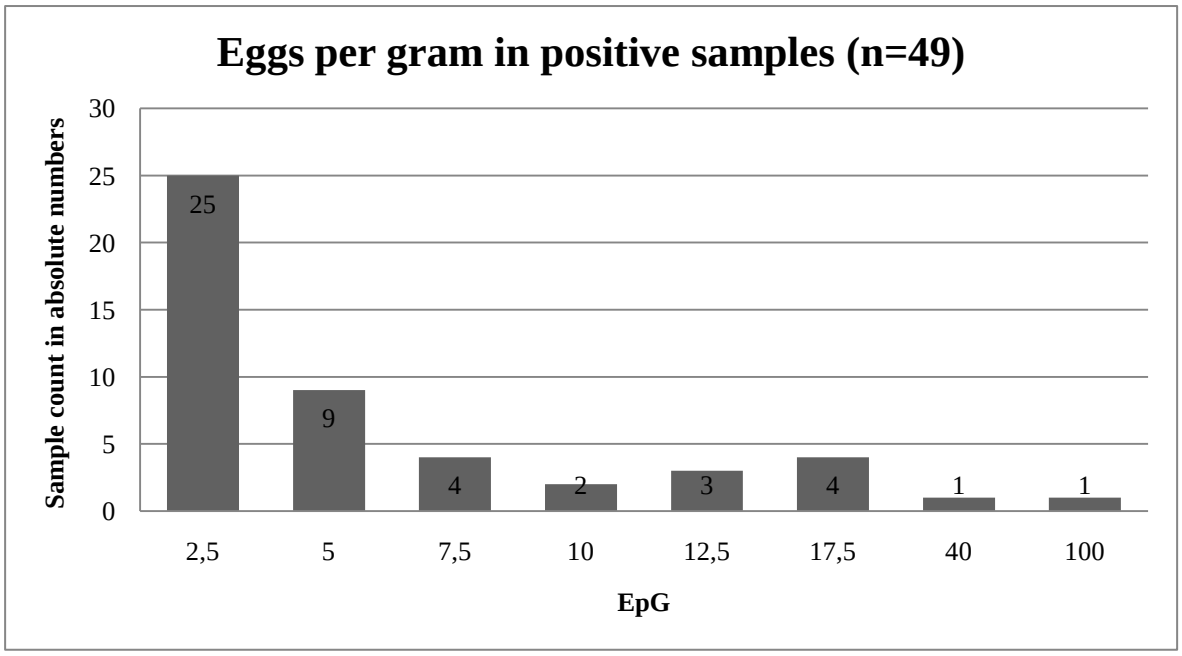

Figure 4.6: Calculated eggs per gram (EpG) in positive samples.

4.2.2 Trematode frequencies

The most abundant eggs were fasciolid eggs, i.e. in 49% of the positive samples (Figure 4.7). Eggs of Paramphistomidae were found in 27% of the samples. In four samples (8%) trematode eggs were found but could not be designated to a certain trematode family. In 16% of the samples eggs of more than one trematode family were found.

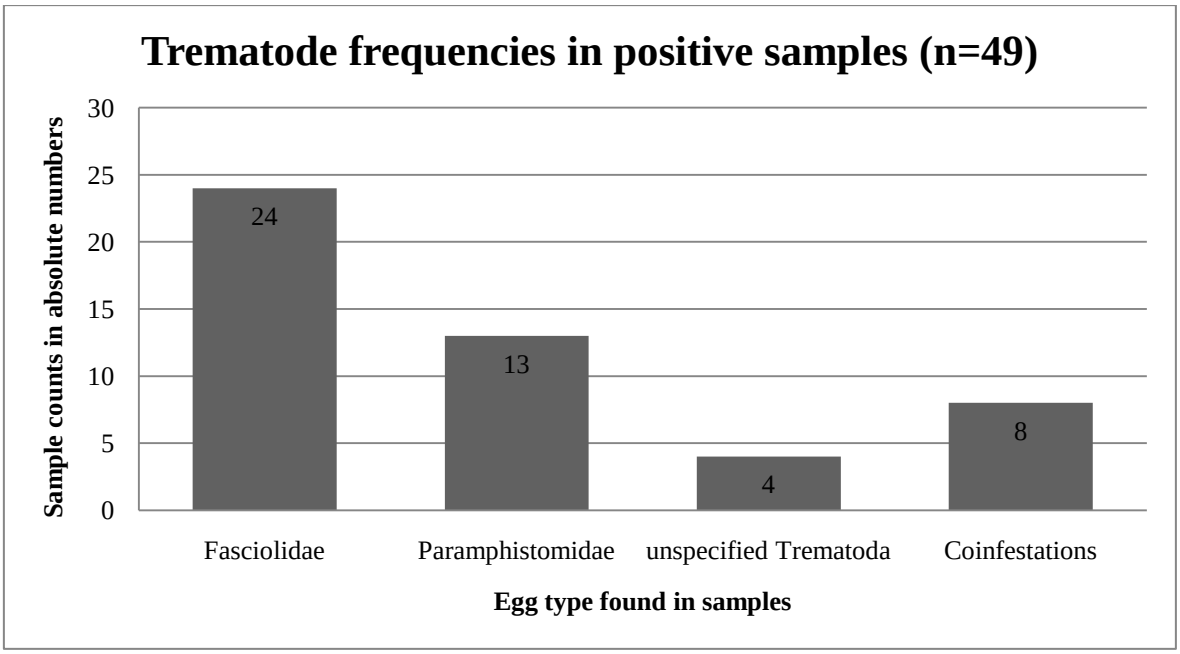

Figure 4.7: Trematode frequencies in the positive samples, coinfestations refer to samples in which more than one type of egg was found.

4.2.3 Geographical distribution

60% of the positive samples originated from the area of Orth/Donau (Figure 4.8). 12% of the positive samples came from Stopfenreuth, only small numbers of positive samples (between 2 and 8%) came from the seven other investigated locations. All samples from Witzelsdorf were negative.

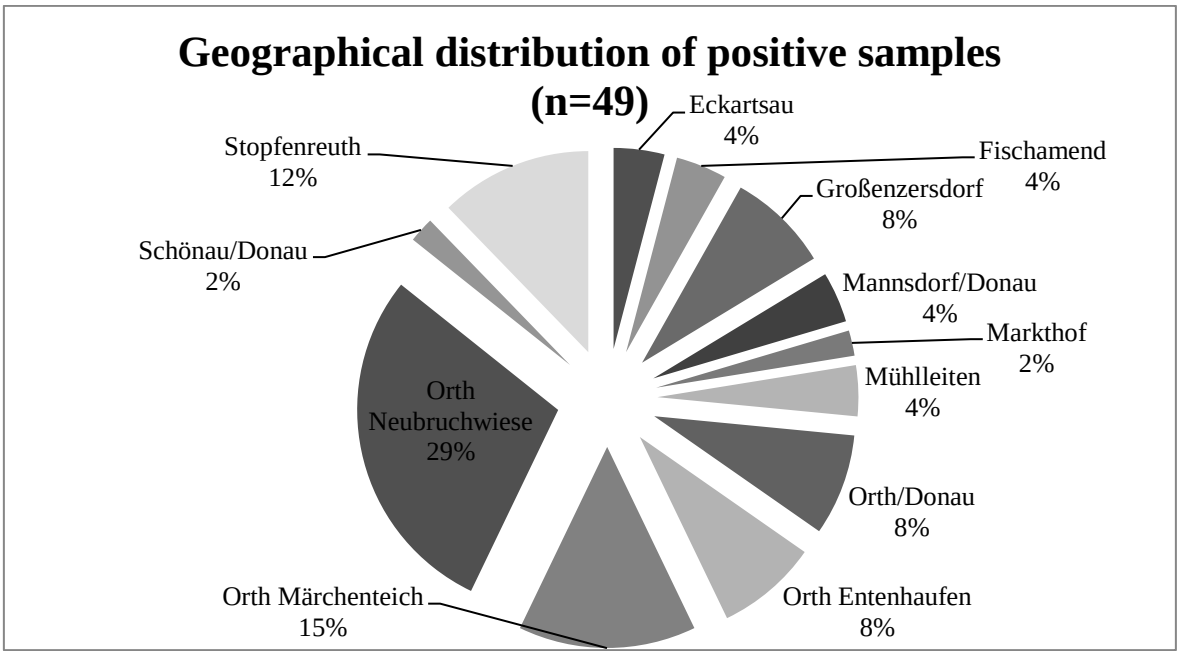

Figure 4.8: Geographical distribution of positive samples over investigated locations.

4.2.3.1 Geographical distribution of Fasciolidae

In 32 samples fasciolid eggs were found. 66% of these samples derived from the whole area around Orth/Donau, 16% in Stopfenreuth as shown in Figure 4.9. Fasciolid eggs were also found in Großenzersdorf (6%), Mannsdorf/Donau (6%), Mühlleiten (3%) and Fischamend (3%).

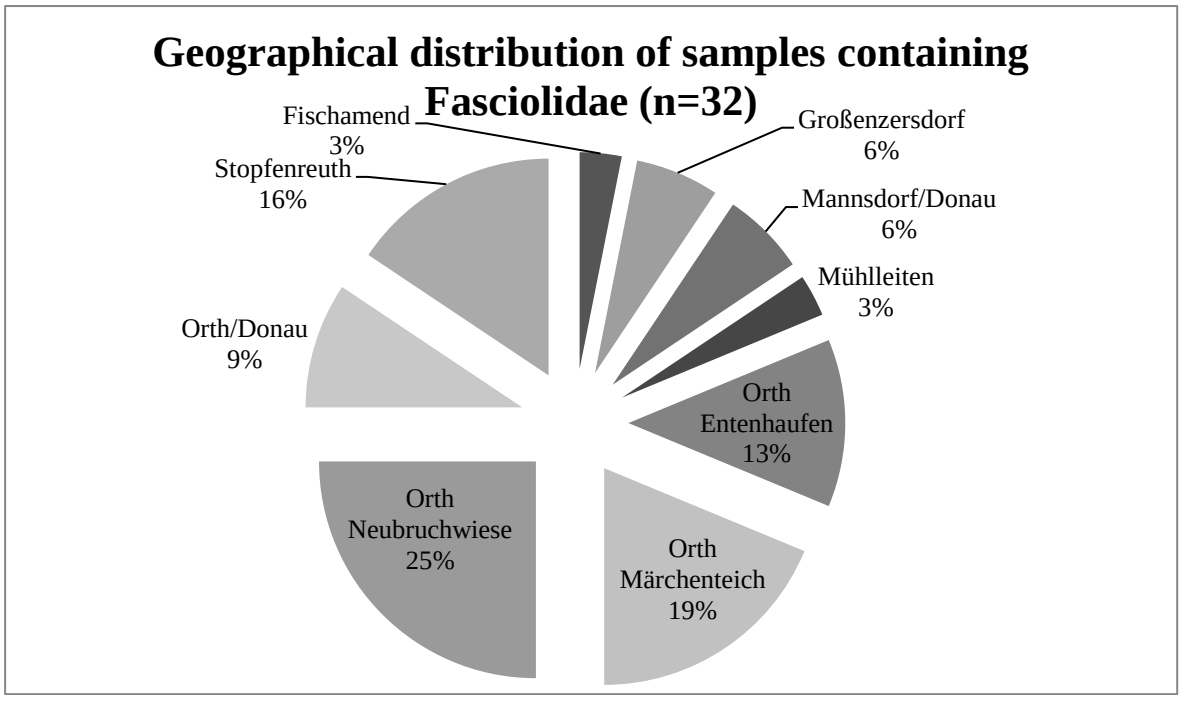

Figure 4.9: Geographical distribution of samples in which fasciolid eggs were found.

4.2.3.2 Geographical distribution of Paramphistomidae

Eggs of Paramphistomidae were found in 21 samples, of which 29% had been collected at the locations Neubruchwiese and Märchenteich in Orth/Donau (Figure 4.10). The other positive samples were distributed over the remaining eight investigated locations with percentages ranging from 5 to 14%.

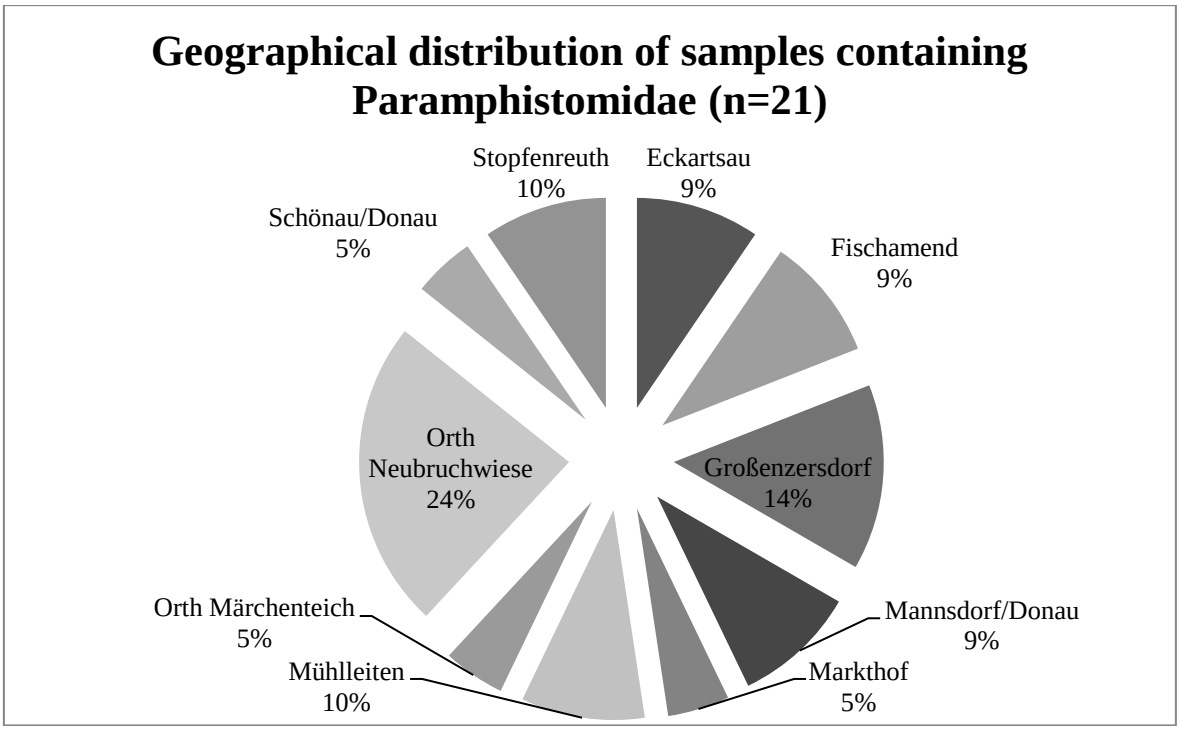

Figure 4.10: Geographical distribution of samples in which paramphistomid eggs were found.

4.2.4 Positivity rates

Positivity rates for all locations with positive samples were calculated and are shown in Figure 4.11. Highest rates were detected in Großenzersdorf (80%), Stopfenreuth (75%) and Mannsdorf/Donau (66.7%). Infestation rates between 30 and 50% were calculated for the locations Mühlleiten (50%), Orth Neubruchwiese (43.8%), Orth/Donau (40%), Orth Märchenteich (35%) and Schönau/Donau (33.3%). The rates in Eckartsau, Fischamend, Markthof and Orth Entenhaufen were below 23%.

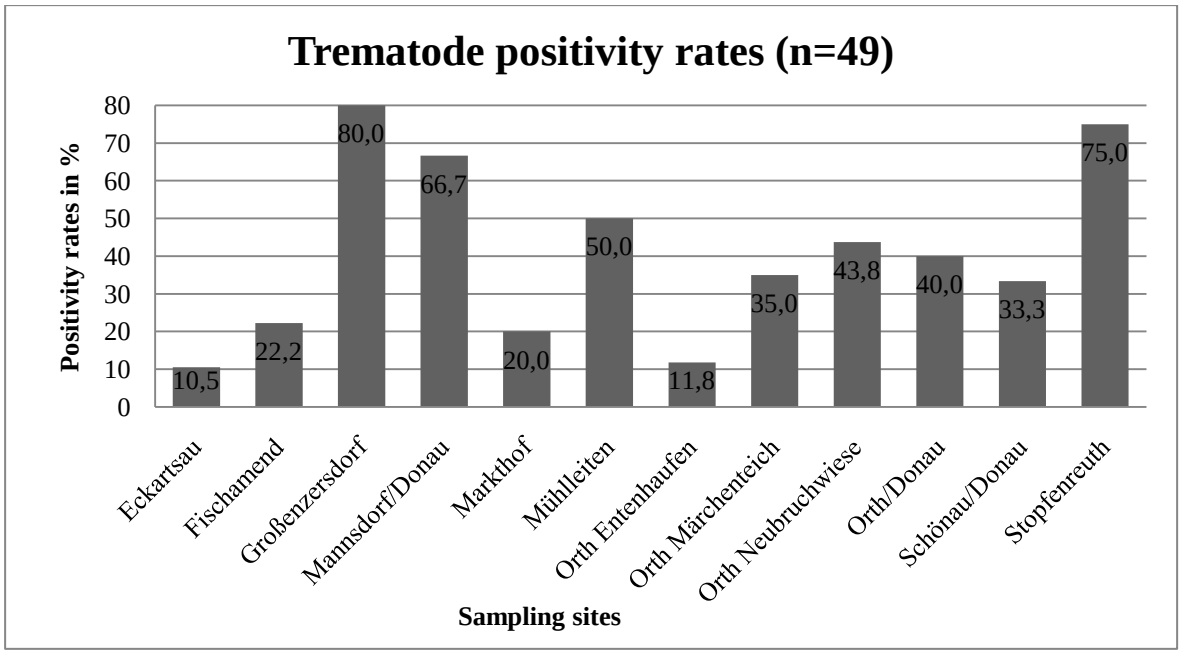

Figure 4.11: Overall distribution of trematodes on the investigated locations given in percent.

4.2.4.1 Positivity rates for Fasciolidae

Positivity rates were also calculated separately for fasciolids as shown in Figure 4.12. In this case, highest positivity rates were determined in Mannsdorf/Donau and Stopfenreuth with 66.7 and 62.5% respectively. The rates in Fischamend and Orth Entenhaufen were both approximately 11%, the other infestation rates ranged between 25 and 40%.

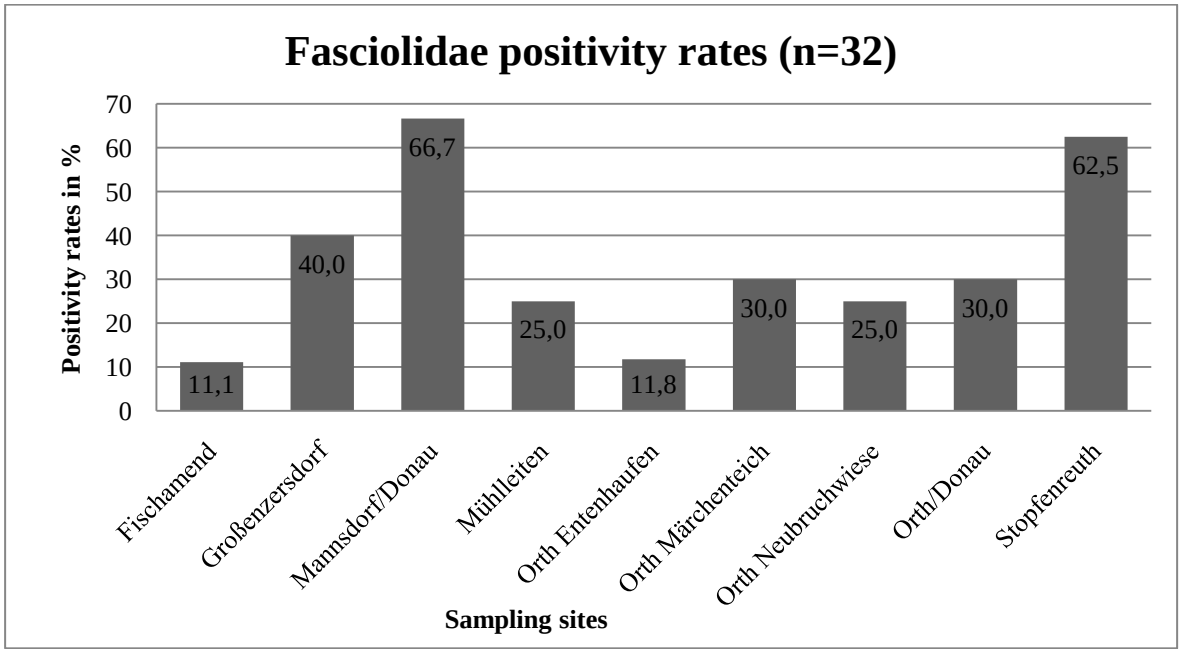

Figure 4.12: Fasciolidae positivity rates of the explored locations in percent.

4.2.4.2 Positivity rates for Paramphistomidae

The positivity rates for paramphistomids ranged from 5% at the location Orth Märchenteich to 66.7% in Mannsdorf/Donau (Figure 4.13). Großenzersdorf and Mühlleiten showed high rates too, with 60 and 50% respectively. Positivity rates along the other locations were between 10.5 and 33.3%.

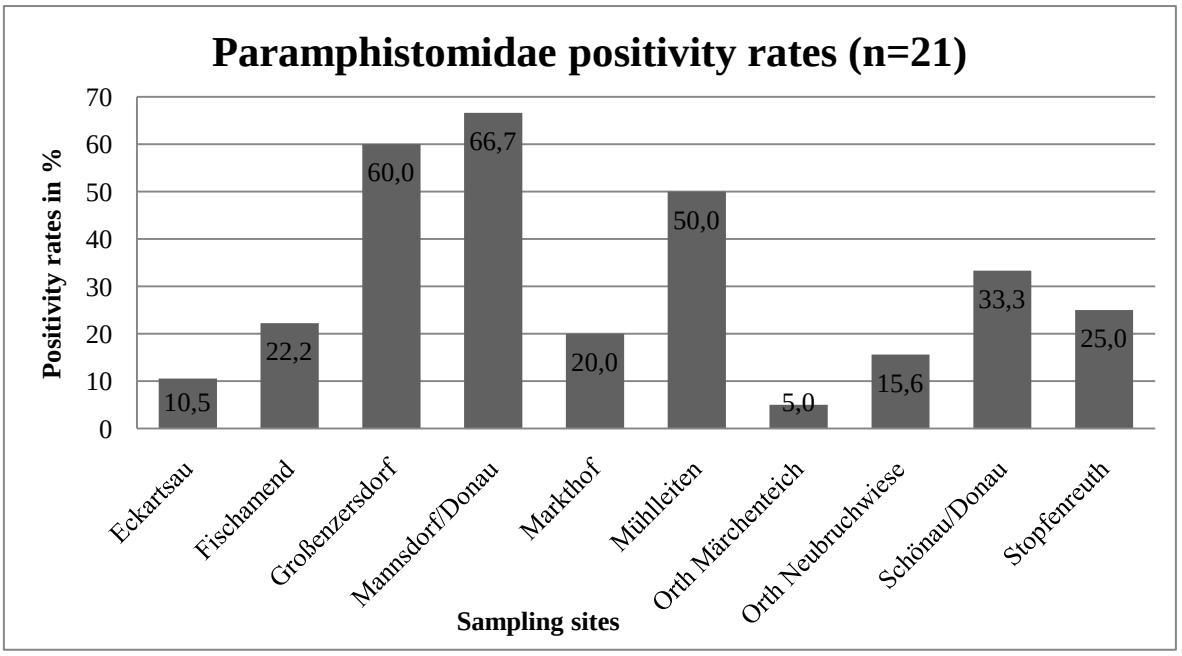

Figure 4.13: Paramphistomidae positivity rates of the investigated locations in percent.

4.2.5 Seasonal distribution of positive samples

Positive samples were found in every month during the monitoring as depicted in Figure 4.14. Nine positive samples had not been dated, so they were not considered for calculation. Highest numbers (8/40) were found in June and August, whereas only one positive sample was found in July. Positive sample counts from the other months range from 2-6/40.

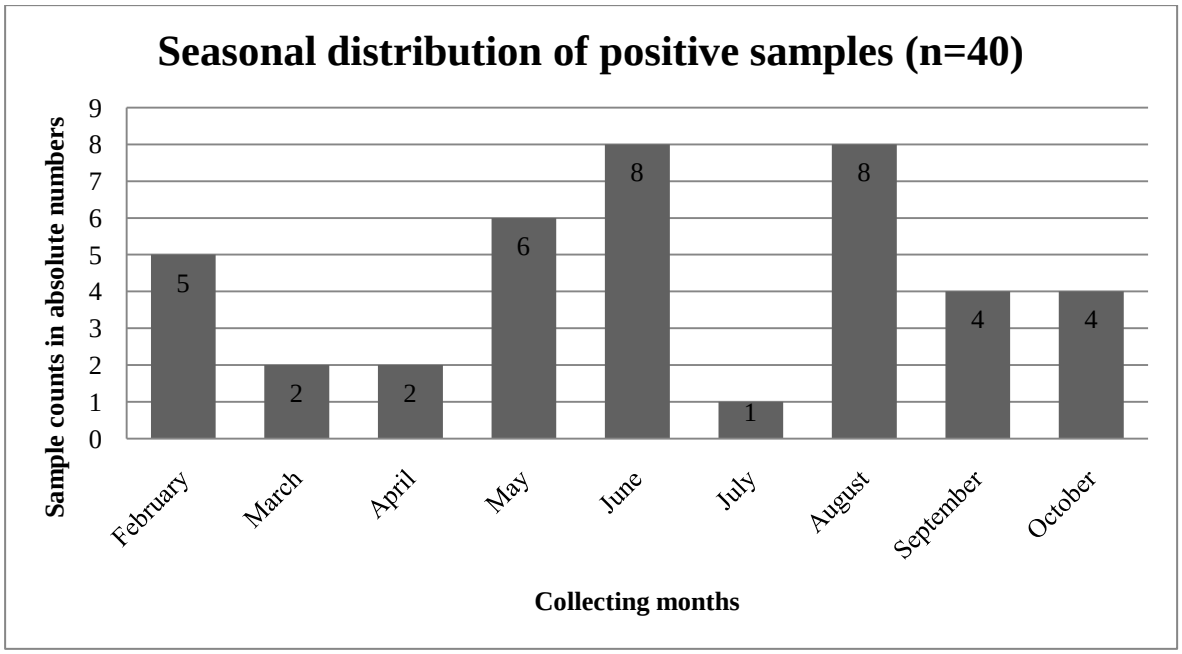

Figure 4.14: Seasonal distribution of dated positive samples.

4.2.6 Results from SAF fixed samples

Eight samples were processed with the SAF fixation method. As shown in Table 4.2, three of these samples were negative after sedimentation, the other positive for either fasciolid eggs or both Fasciolidae and Paramphistomidae. The usual protocol using untreated faeces was compared to a test using sedimented faeces (samples no. 25, 40, 63 and 71). The microscopically negative sample no. 71 also was negative after SAF fixation, no trematode eggs were found in the microscopically positive sample no. 40. Samples no. 25 and 63 were positive in both, sedimentation and SAF method, but different eggs were found.

Samples no. 78 and 79 were used to test a possible effect of prolongation of the incubation period with the stock solution. Sample no. 78 was positive in both methods with less types and numbers of eggs in SAF, sample no. 79 was negative in both, sedimentation and SAF.

Both types of faeces "constitution" (sedimented and untreated faeces) and both periods of incubations were tested with samples no. 91 and 92. Fasciolid eggs were found in all tests with sample no. 91 in almost equal numbers, sample no. 92 was negative in all tests.

Table 4.2: Overview of all samples treated with the SAF method in different variations. Fasc. = eggs of Fasciolidae, Par. = eggs of Paramphistomidae, neg. = no trematode eggs. × marks sample constitution and incubation time used with particular sample.

#	Sedimentation result	Faeces		Incubation		SAF method result
		un-treated	sedi-mented	short	over-night	
25	Fasc.	×	×	×		Fasc.+ Par. in untreated faeces
40	Fasc.	×	×	×		neg.
63	Fasc.+ Par.	×	×	×		Par. in sediment
71	neg.	×	×	×		neg.
78	Fasc.+ Par.		×		×	Par.
79	neg.		×		×	neg.
91	Fasc.	×	×	×	×	Fasc. in all tests
92	neg.	×	×	×	×	neg.

Altogether it was shown that the SAF method mostly delivered less accurate results than the sedimentation method. The different variations did not seem to alter the results or have any effects on them.

4.3 Molecular biology

4.3.1 Samples

109 samples were analyzed with molecular techniques. Beforehand, all of them were examined under the microscope. As shown in Figure 4.15, 44% of the selected samples were microscopically positive, 52% negative and 4% remained unspecified.

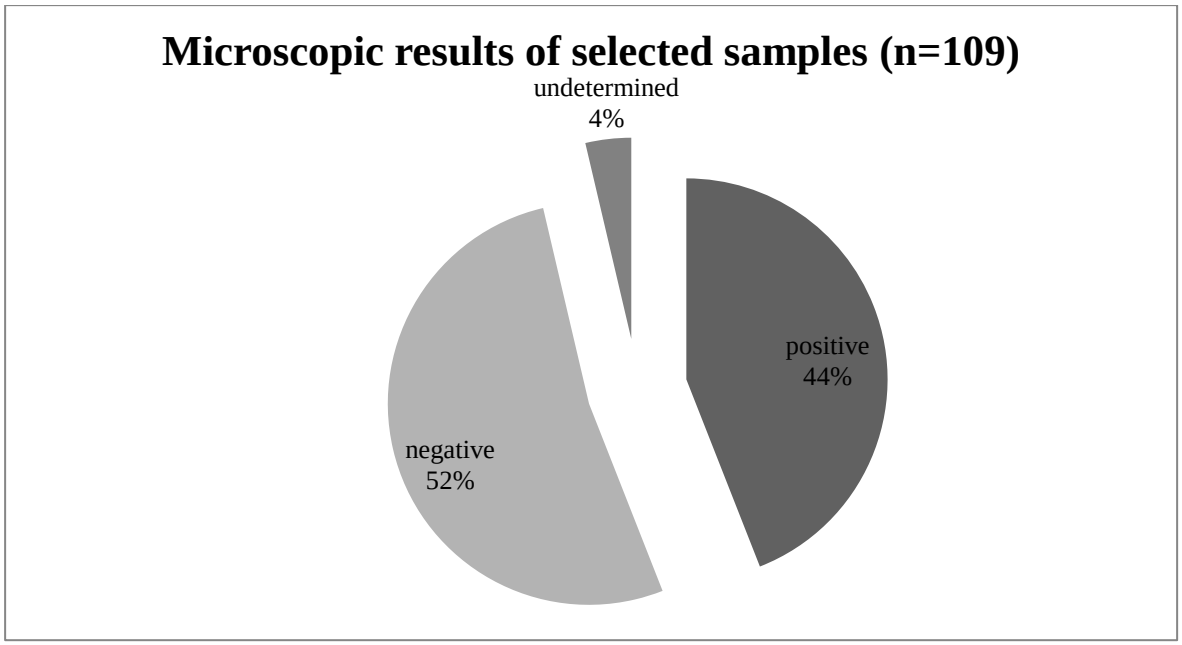

Figure 4.15: Microscopic details of samples selected for molecular methods.

Figure 4.16 displays the geographical origin of the selected samples. 47% of the samples derived from the area of Orth/Donau, the other 53% were distributed over the remaining nine sampling sites.

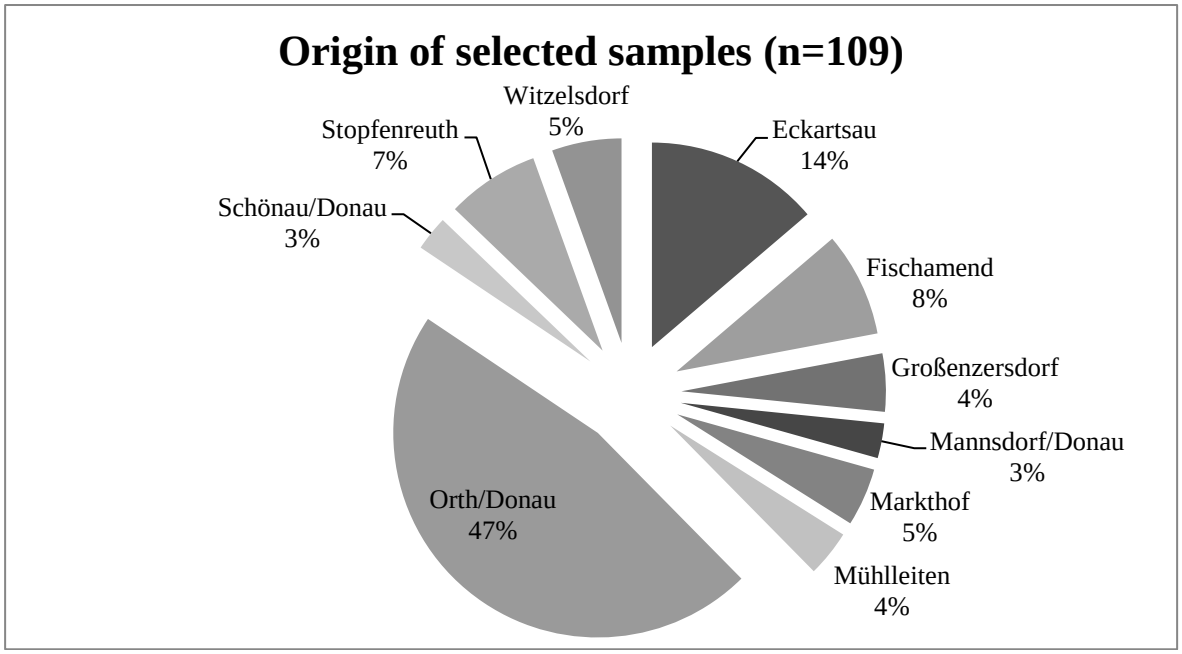

Figure 4.16: Geographical origin of samples selected for molecular techniques.

4.3.2 DNA yield

The DNA yield was calculated after homogenisation and DNA isolation with the stool and the soil kit, respectively by spectrophotometer. Table 4.3 shows the results for both kits,

whereby the average DNA amount in samples processed with the QIAamp® DNA Stool Kit (QIAGEN, Vienna, Austria) was 4.98 ng/µl, in samples processed with the Precellys Soil DNA Kit (PeqLab, Erlangen, Germany) 6.81 ng/µl. The lowest DNA amounts were 0.65 ng/µl (sample no. 21) with the stool kit and 0.14 ng/µl (sample no. 15) with the soil kit, respectively. The highest yields were 11.31 ng/µl (sample no. 8) with the stool kit and 21.07 ng/µl (sample no. 6) with the soil kit. However, while for the soil kit 250 mg of sample were used for both homogenisation and extraction, in case of the stool kit 500 mg of sample were used for homogenisation of which 200 µl where used for DNA extraction. Moreover, the total DNA yields do not reflect the actual amounts of DNA extracted from the eggs but the total DNA of all organisms in the stool-soil samples.

The DNA yields from pure parasite tissue were significantly higher. For example, both *F. magna* and *F. hepatica* extractions resulted in DNA concentrations of 205.02 ng/µl and 294.22 ng/µl, respectively.

Table 4.3: DNA yield after homogenisation and DNA extraction.

	Stool kit	**Soil kit**
Average (ng/µl)	4.98	6.81
Min (ng/µl)	0.65	0.14
Max (ng/µl)	11.31	21.07
processed amount of sample	500 mg for homogenisation thereof 200 µl for DNA extraction	250 mg for homogenisation and DNA extraction

Altogether, higher amounts of DNA were yielded with the soil kit even though the processed amount of sample was less than for the stool kit. Nevertheless, also the smallest DNA yield was achieved with the soil kit.

4.3.3 PCR results

4.3.3.1 PCR results (stool kit)

All 109 selected samples were processed with the QIAamp® DNA Stool Kit (QIAGEN, Vienna, Austria) and tested with the trematode primers (double PCR with purification step in between). As shown in Table 4.4, in 19 samples (17.4%) amplicons of the expected

length were achieved, 87 samples were negative (79.8%). Three samples (2.8%) did give amplicons, however not of the appropriate size.

Table 4.4: PCR results with three different primer pairs after DNA extraction with the stool kit.

Stool kit	Trem_F/R (n=109)	Magna_F/R (n=22)	Fasc_F/R (n=22)
positive	19	8	0
negative	87	13	22
undetermined	3	1	0

All sets of PCRs were performed in groups referring to the 13 sampling sites. The first analyzed group formed the sites Mannsdorf/Donau, Mühlleiten and Großenzersdorf. As shown on the agarose gel (Figure 4.17), 1 out of 3 samples in Mannsdorf/Donau and 2 out of 4 in Mühlleiten were positive for trematodes. In Großenzersdorf 3 out 5 samples were positive, one (sample number 158) was classified undetermined due to the fragmented gel bands. There was also a faint band in the first negative control which is possibly due to a contaminating slopover from the positive control while loading the gel.

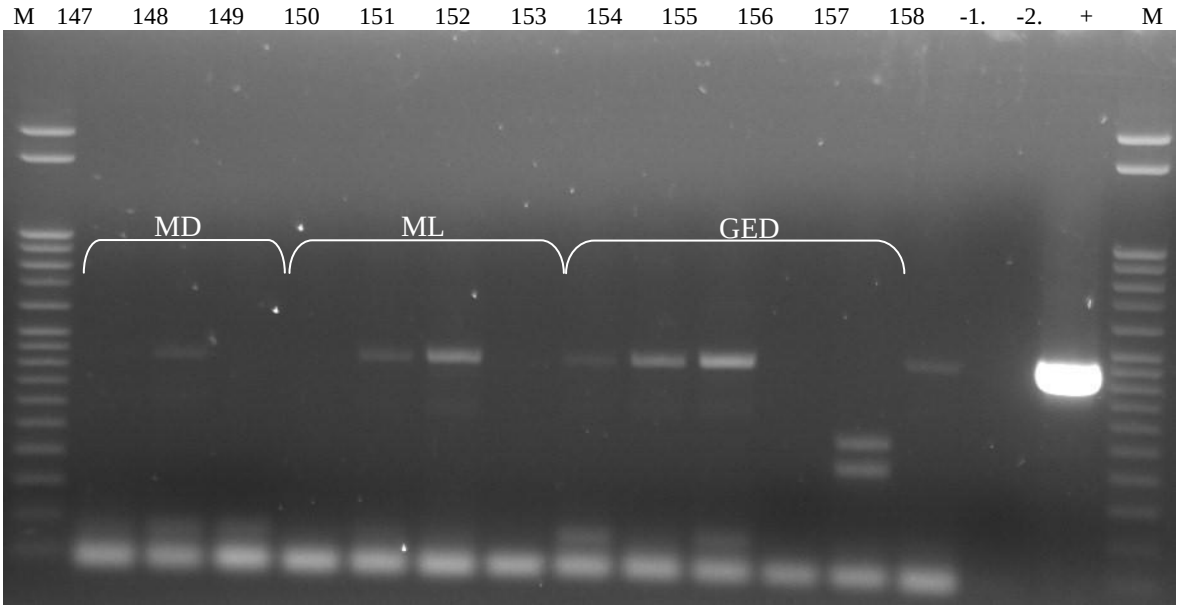

Figure 4.17: Trematodes PCR results after stool kit extraction from sampling sites Mannsdorf/Donau (MD), Mühlleiten (ML) and Großenzersdorf (GED), slots labelled with sample numbers.

The next investigated group formed the sites Witzelsdorf and Fischamend. None of the samples (six of Witzelsdorf, nine of Fischamend) was positive for trematodes (not shown).

As shown in Figure 4.18, there were no trematode positive samples in either Markthof or Schönau. Sample number 63 of Stopfenreuth was distinctly positive, sample 43 showed a fragmented amplicon and was therefore classified undetermined.

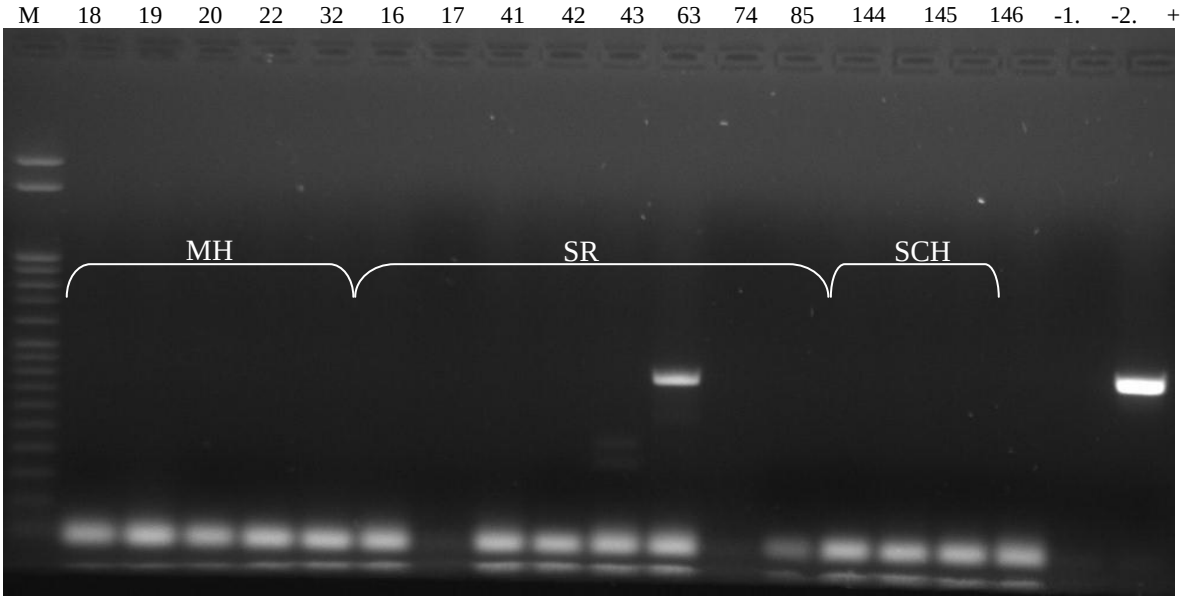

Figure 4.18: Trematodes PCR results after stool kit extraction from sampling sites Markthof (MH), Stopfenreuth (SR) and Schönau/Donau (SCH), slots labelled with sample numbers.

Of the fourth group, sampling site Orth Märchenteich (Figure 4.19), six samples were positively amplified with the trematode primers. Sample 121 showed a fragmented amplicon.

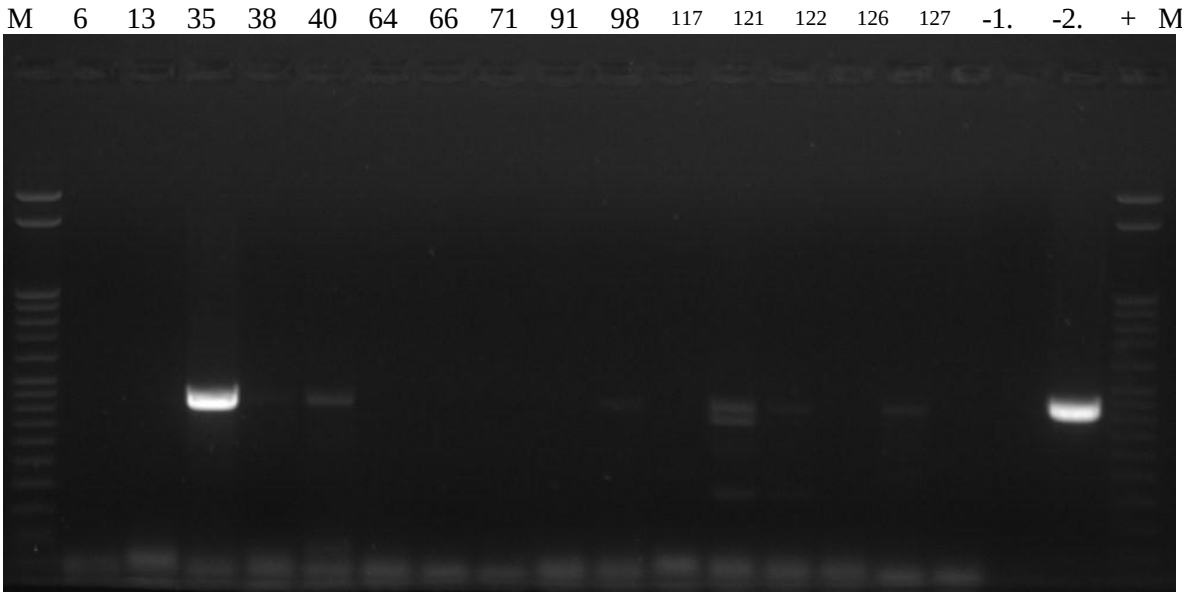

Figure 4.19: Trematodes PCR results after stool kit extraction from sampling site Orth Märchenteich, slots labelled with sample numbers.

In case of the sampling site Orth Entenhaufen, one sample showed a positive signal with the trematode primers (Figure 4.20). The other 14 samples of this group were all negative.

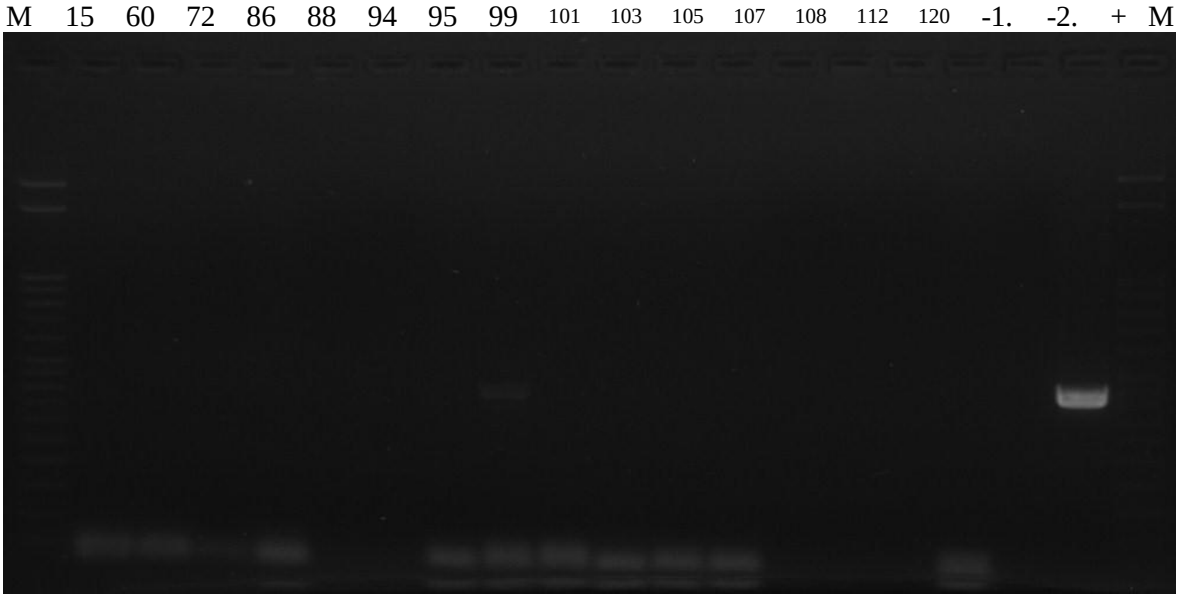

Figure 4.20: Trematodes PCR results after stool kit extraction from sampling site Orth Entenhaufen, slots labelled with sample numbers.

15 samples of the sampling site Orth Neubruchwiese were analysed with the primers binding to trematodes (Figure 4.21). One sample (124) showed a clear positive signal, sample 39 was also positive but with a fainter gel band.

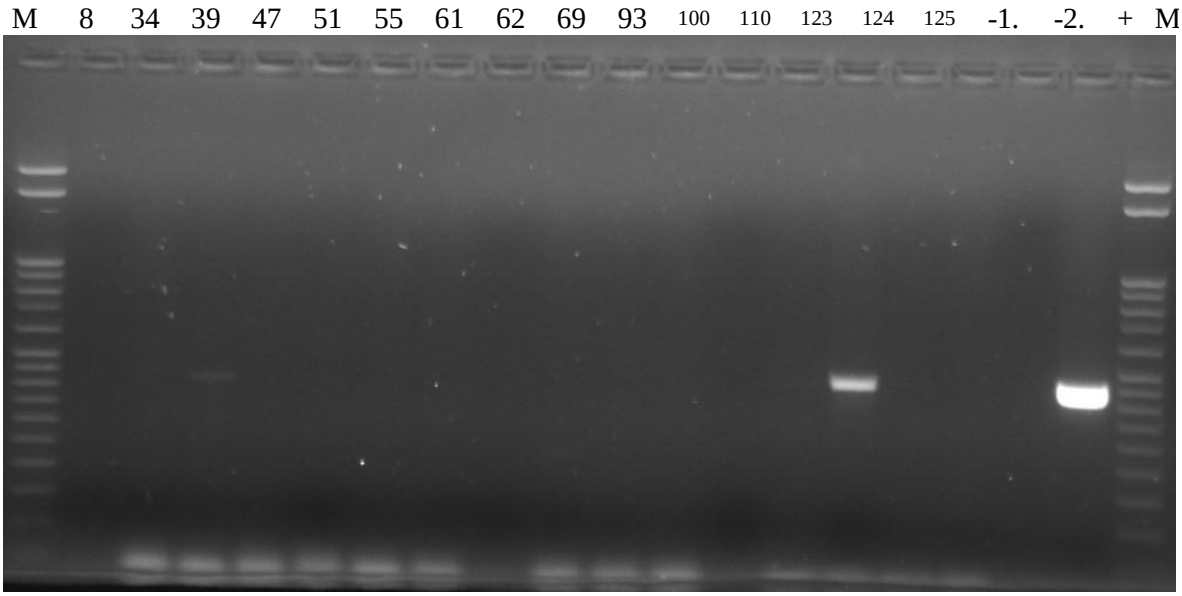

Figure 4.21: Trematodes PCR results after stool kit extraction from sampling site Orth Neubruchwiese, slots labelled with sample numbers.

The next sampling site investigated was Eckartsau. Of the 15 analysed samples, two were positive for trematodes showing very faint bands on the agarose gel (Figure 4.22).

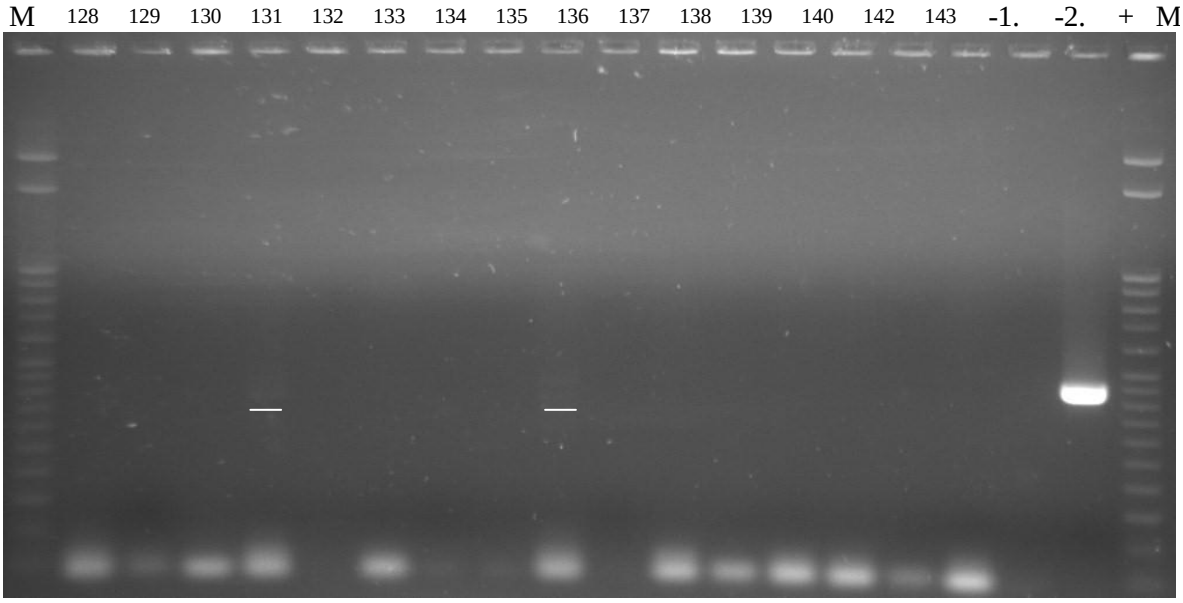

Figure 4.22: Trematodes PCR results after stool kit extraction from sampling site Eckartsau, slots labelled with sample numbers (faint bands were marked).

The last group screened for trematodes constituted the Orth/Donau samples. Three samples of this site were analysed of which one (sample no. 31) showed a positive signal on the gel (Figure 4.23).

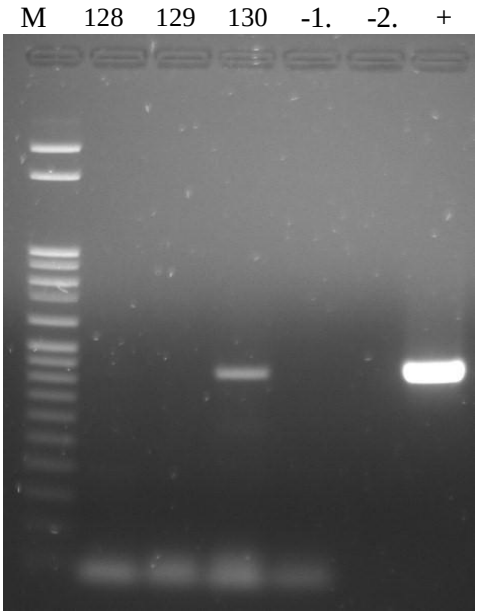

Figure 4.23: Trematodes PCR results after stool kit extraction from sampling site Orth/Donau, slots labelled with sample numbers.

The positive or undetermined (with fragmented amplicons) samples were then tested with both *F. magna* and *F. hepatica* primers (Table 4.4). Two of the samples (63 and 124) had already been used for PCR establishment and were therefore not analysed again. Another sample (86) was highly positive in microscopy but was not positive with either the trematode or any other primer pairs. None of the other samples was *F. hepatica* positive

(not shown). In case of *F. magna*, eight samples (including 63 and 124) were positive (36.4%), one sample showed a shorter fragment on the gel which could not be determined any further (Figures 4.24 and 4.25).

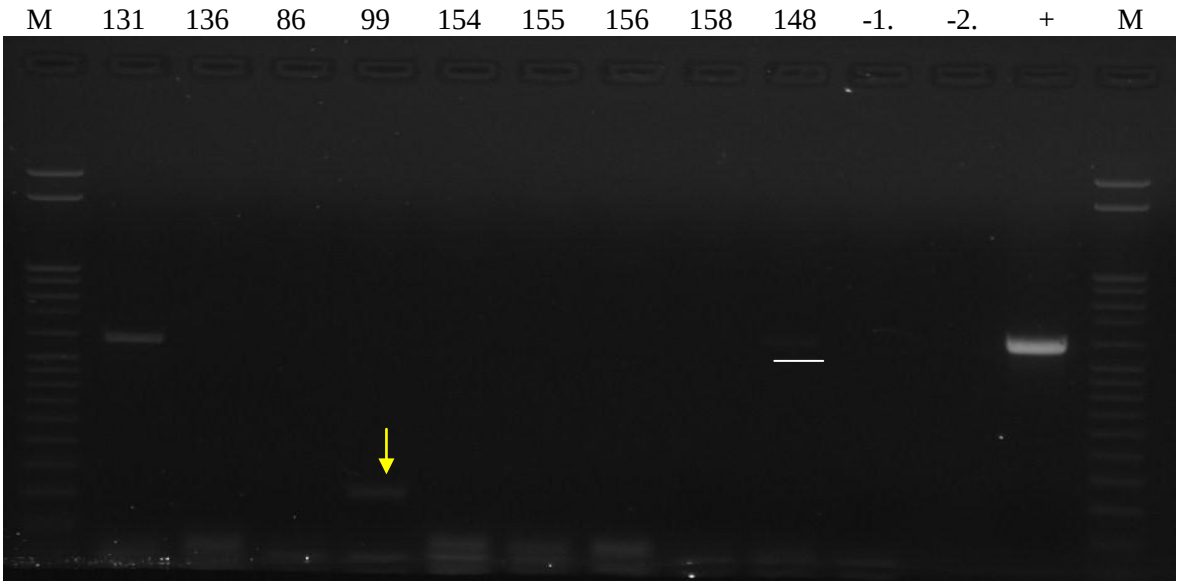

Figure 4.24: *F. magna* PCR results after stool kit extraction from trematodes positive samples, Gel 1. Slots labelled with sample numbers (faint bands were marked).

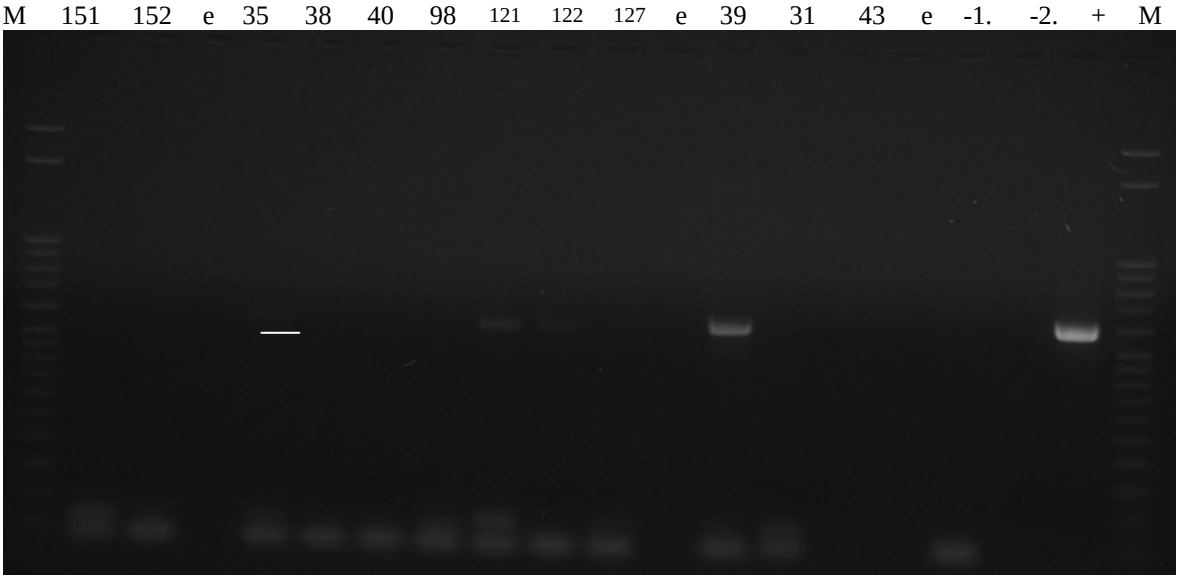

Figure 4.25: *F. magna* PCR results after stool kit extraction from trematodes positive samples, Gel 2. Slots labelled with sample numbers, e=empty slot (faint bands were marked).

4.3.3.2 PCR Results (soil kit)

Altogether, 45 samples of the 109 already processed with the stool kit were also processed with the Precellys Soil DNA Kit (PeqLab, Erlangen, Germany). One microscopically

highly positive sample (sample no. 86) was used for PCR establishment with both primer sets for trematodes and *F. magna*, which both gave positive results. The remaining 44 samples were then tested with the universal trematode primers in two consecutive rounds of PCRs (Table 4.5).

Table 4.5: PCR results with three different sets of primers after DNA extraction with the soil kit.

Soil kit	Trem_F/R (n=45)	Magna_F/R (n=15)	Fasc_F/R (n=15)
positive	14	11	0
negative	30	3	14
undetermined	1	1	1

The trematode tests were performed in groups of 15 and 14, respectively (Figures 4.26 – 4.28). 14 of the samples (31.1%, including sample no. 86) were positive for trematodes, one sample was classified undetermined as the gel showed an amplicon that was shorter than expected (sample no. 125 on Figure 4.28).

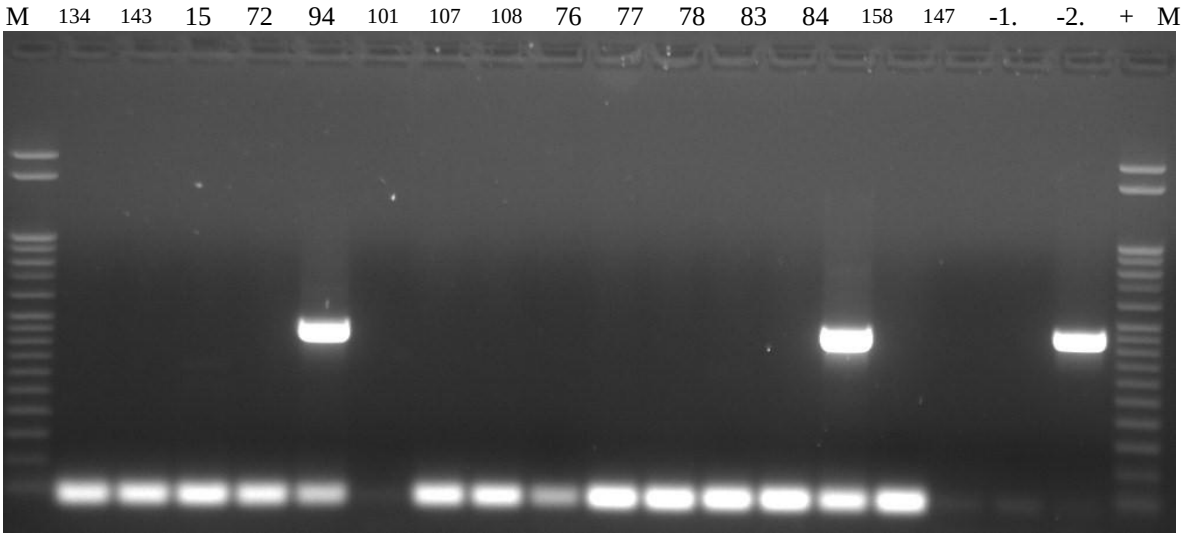

Figure 4.26: Trematode PCR results after soil kit extraction, Gel 1. Slots labelled with sample numbers.

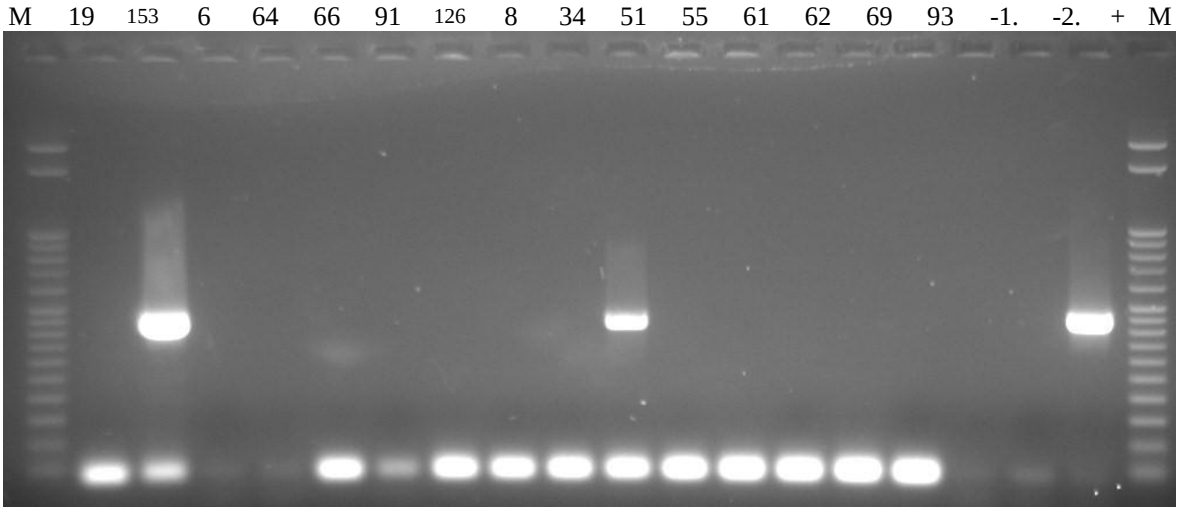

Figure 4.27 Trematode PCR results after soil kit extraction, Gel 2. Slots labelled with sample numbers.

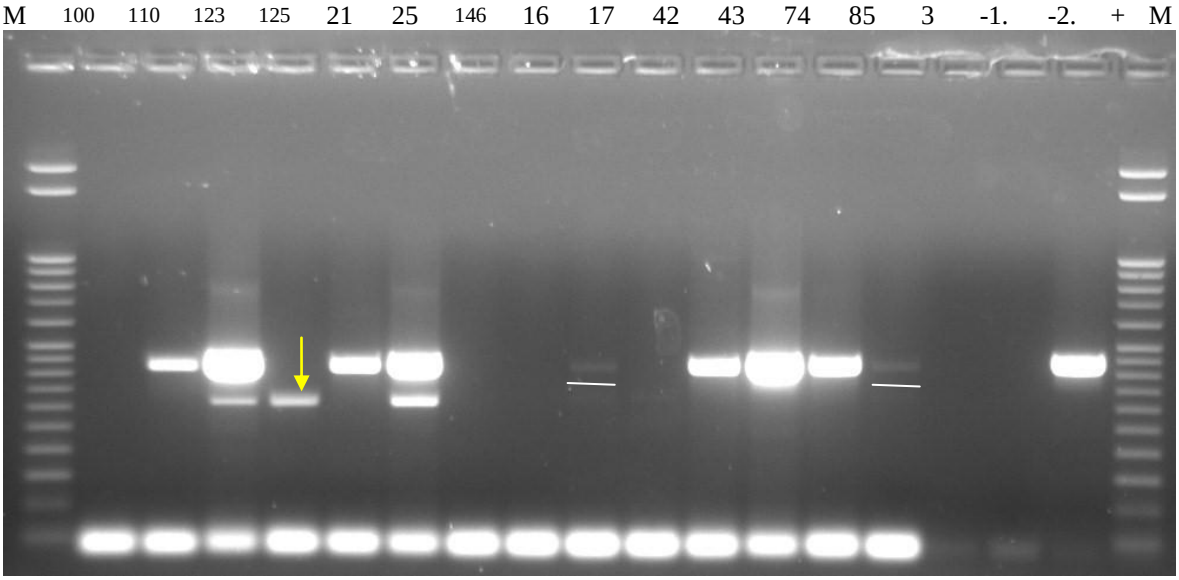

Figure 4.28: Trematode PCR results after soil kit extraction, Gel 3. Slots labelled with sample numbers (faint bands were marked).

In a next step, all positive samples including the undetermined one were tested with Magna and Fasc primers. As shown in Table 4.5, eleven samples (73.3%) showed positive gel bands for *F. magna* including the one that was unclear in the trematodes test. Three samples (20%) were negative for *F. magna*, one (6.7%) was uncertain to be positive as there was a smear of fragmented DNA on the gel (Figure 4.30). The same sample also showed a faint gel band in the *F. hepatica* test (Figure 4.29). The fragment was approximately 350-400 bp long which would fit to the 3-fold length of the 124 bp long repetitive DNA sequence that is amplified with the *F. hepatica* primers.

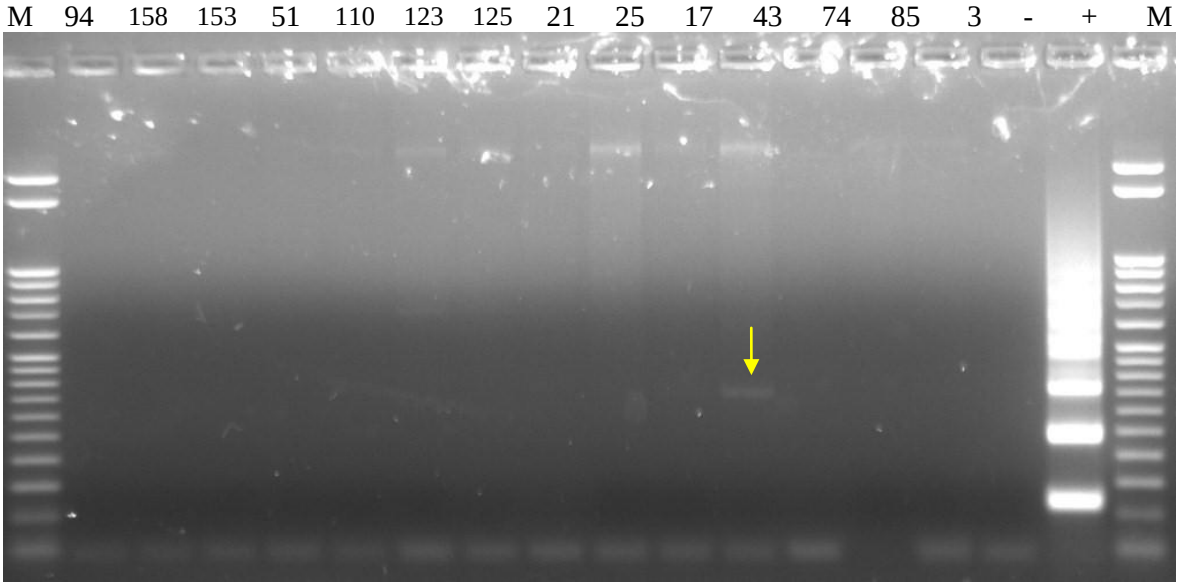

Figure 4.29: *F. hepatica* PCR results after soil kit extraction from trematodes positive samples, slots labelled with sample numbers.

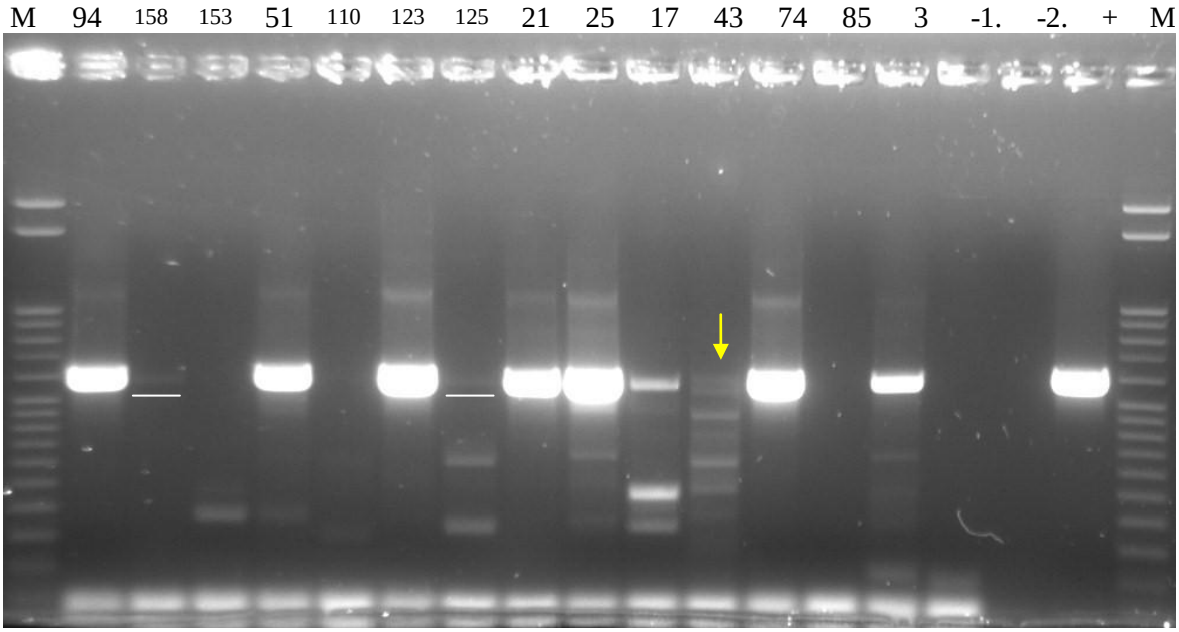

Figure 4.30: *F. magna* PCR results after soil kit extraction from trematodes positive samples, slots labelled with sample numbers (faint bands were marked).

4.3.4 Comparison of soil kit and stool kit results

The efficiencies of both kits were compared according to the PCR results they provided. Table 4.6 shows an overview of the numbers of samples processed with the particular kit, the number of positive samples and the efficiencies. The stool kit showed lower, namely 20%, efficiency in DNA extraction, while the soil kit showed 33% efficiency.

Table 4.6: Comparison of two different homogenization and DNA extraction kits.

	Stool kit	**Soil kit**
processed samples	109	45
positive samples	22	15
efficiency	20%	33%

4.3.5 Sequencing Results

The PCR amplicons of positive and undetermined samples were sequenced for further differentiation. The sequences were then aligned with trematode sequences from the NCBI database. All amplicons of *F. magna* PCR positive samples showed highest identity to *F. magna* (example shown in Figure 4.31). Seven other samples were identified as belonging to the three trematode families Fasciolidae (no picture shown), Dicrocoeliidae (Figure 4.32) and Paramphistomidae (Figure 4.33), respectively. In six cases, the sequences were too incomplete to determine the exact species, which made “trematode” the only possible classification.

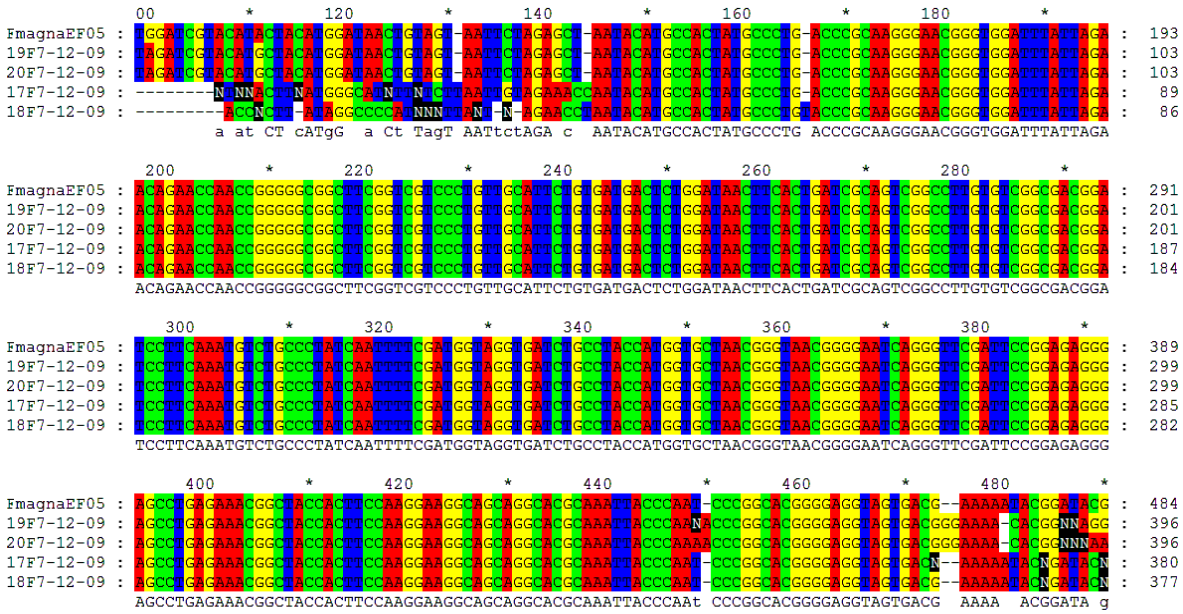

Figure 4.31: Alignment of sample no. 85 with the sequence of *F. magna* from the database.

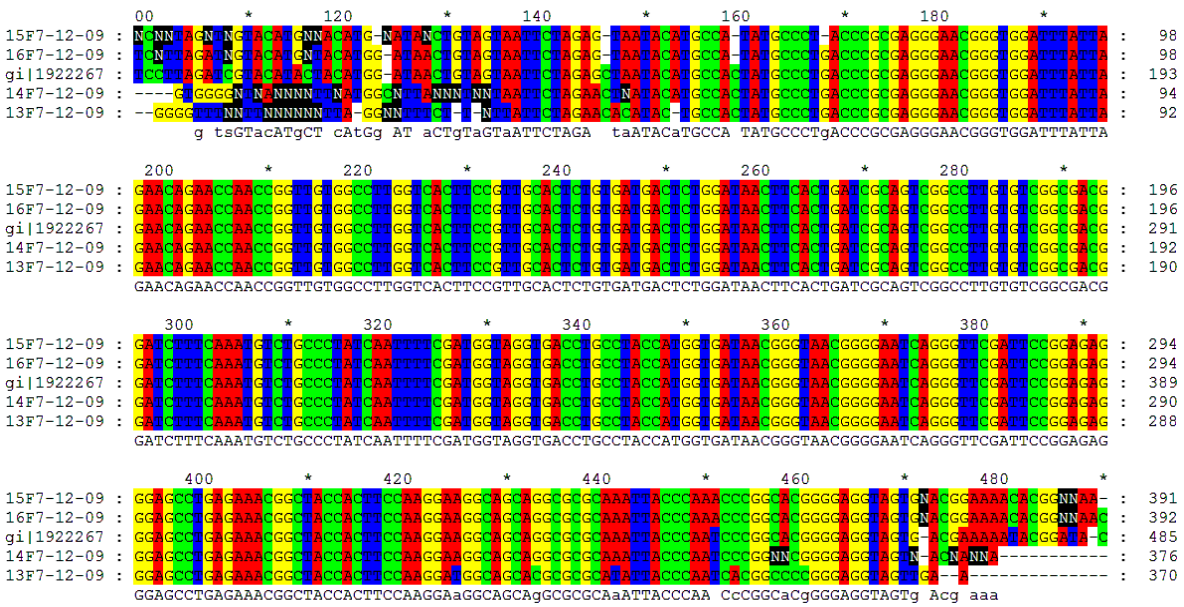

Figure 4.32: Alignment of sample no. 43 with the sequence of *D. dendriticum* from the database.

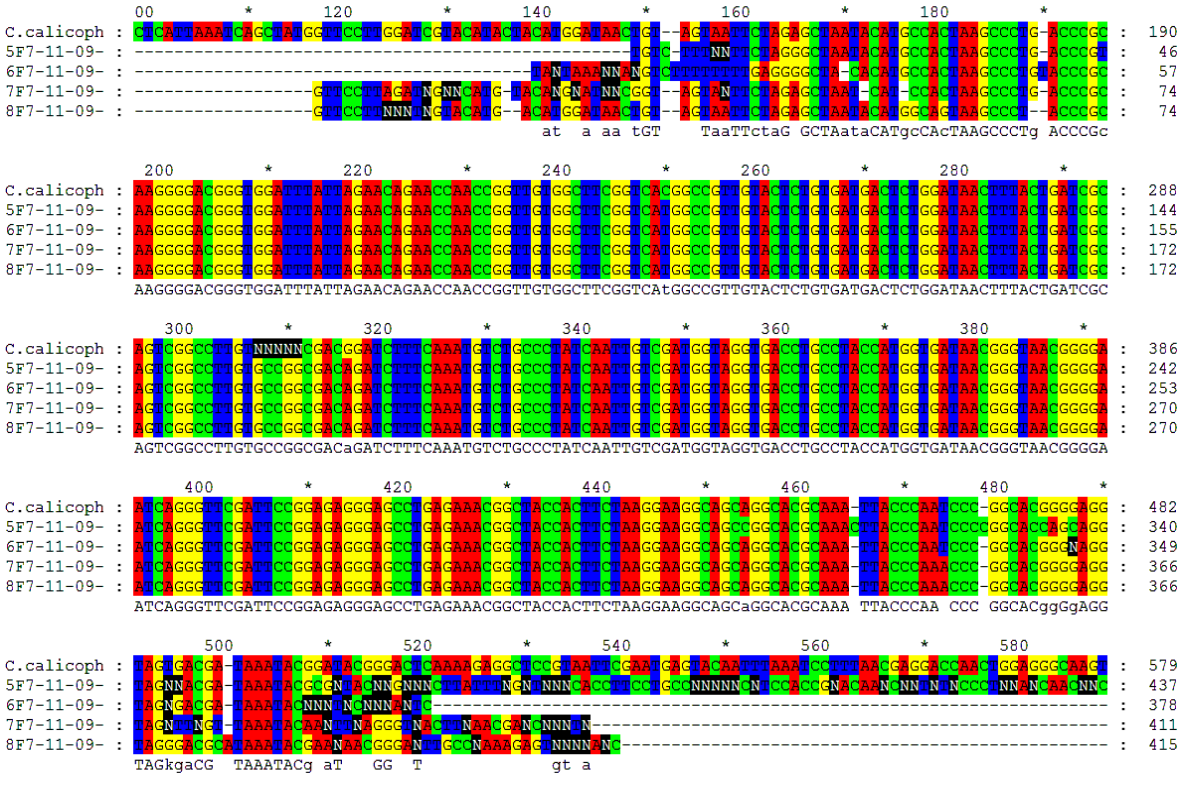

Figure 4.33: Alignment of sample no. 110 with the sequence of *C. calicophorum* from the database.

4.3.6 Combined molecular results

Altogether, 109 samples were processed with biomolecular techniques. As shown in Figure 4.34, trematodes were found in 35 samples (32%). The majority of samples (68%) was negative for trematodes.

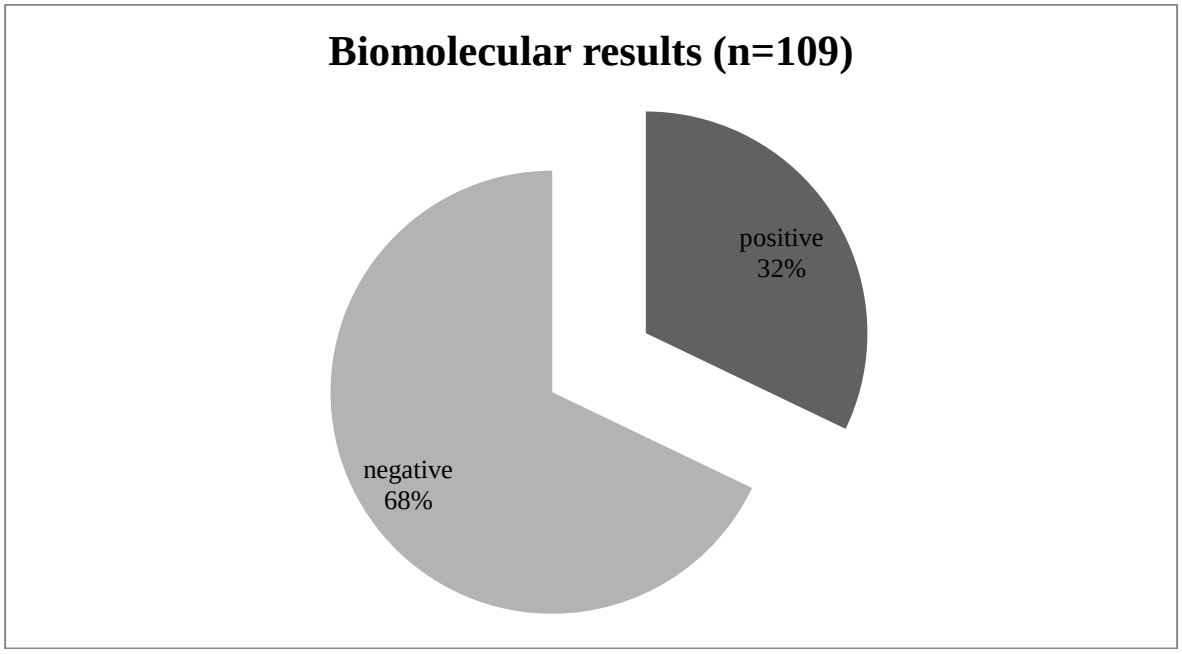

Figure 4.34: Biomolecular results for trematode DNA in processed faeces samples.

4.3.7 Trematode frequencies

Altogether, 23 samples were identified as members of the Fasciolidae as demonstrated in Figure 4.35, of which 22 samples were classified as *Fascioloides magna* and one could not be identified to the species level. In four samples, DNA of Paramphistomidae was detected and two samples were assigned to the family Dicrocoeliidae. Six samples could not be differentiated any further and are therefore only classified as "trematodes".

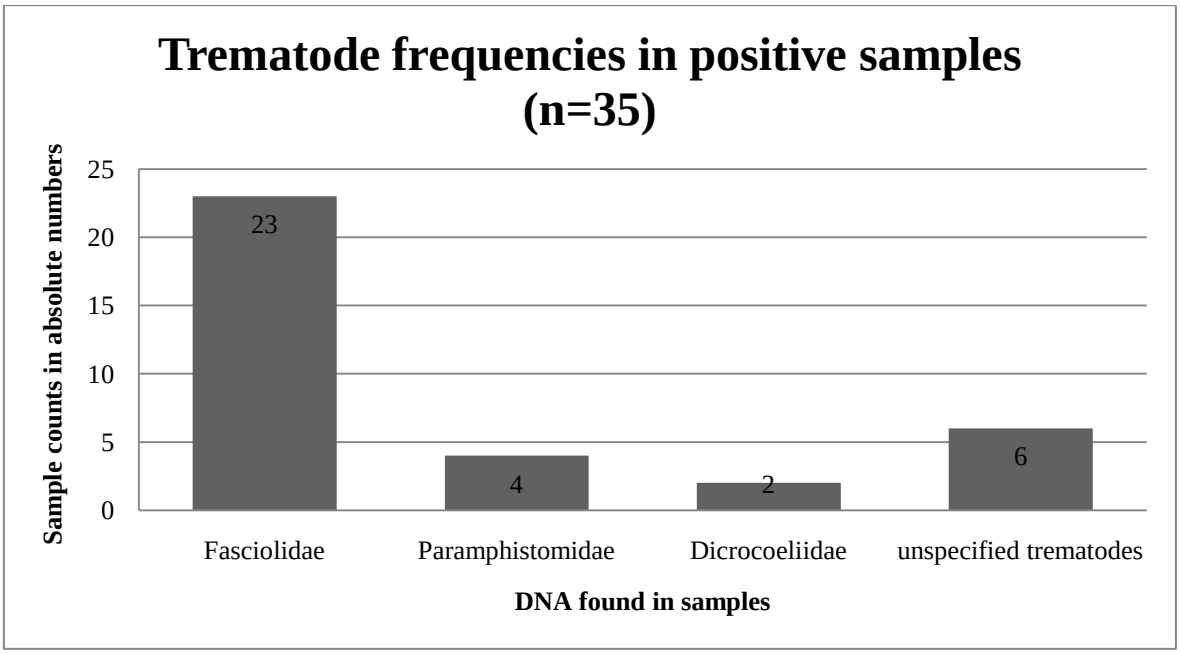

Figure 4.35: Trematode frequencies in positive samples.

4.3.8 Geographical distribution

Figure 4.36 shows the geographical distribution of the PCR positive samples. 55% of all positive samples originated from one of the Orth/Donau sampling sites. 14% of the positive samples were from Stopfenreuth, 11% from Großenzersdorf. The percentages of the other sites range from 3 to 8%.

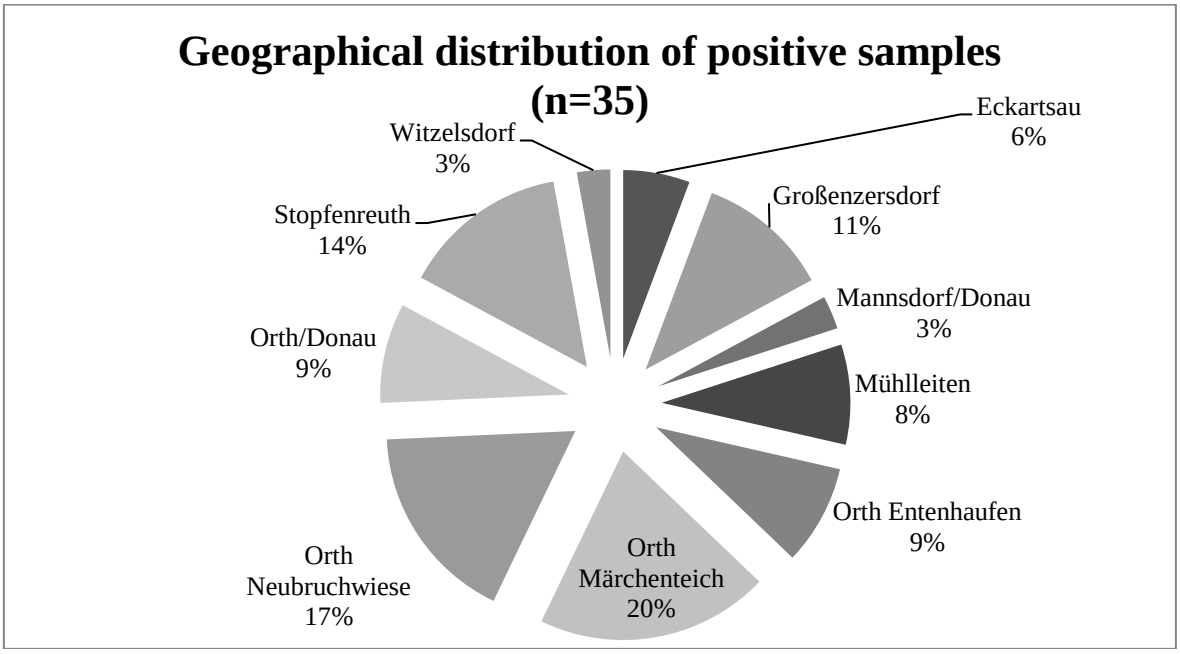

Figure 4.36: Geographical distribution of positive samples.

4.3.8.1 Geographical distribution of Fasciolidae

Fasciolid DNA was detected in 23 samples. The geographical distribution of these samples is shown in Figure 4.37. As demonstrated, almost two thirds (62%) of all fasciolid positive samples were from the sampling area of Orth/Donau. Besides Stopfenreuth, where 18% of the Fasciolidae findings are from, there was only one positive sample (4%) from each of the other locations.

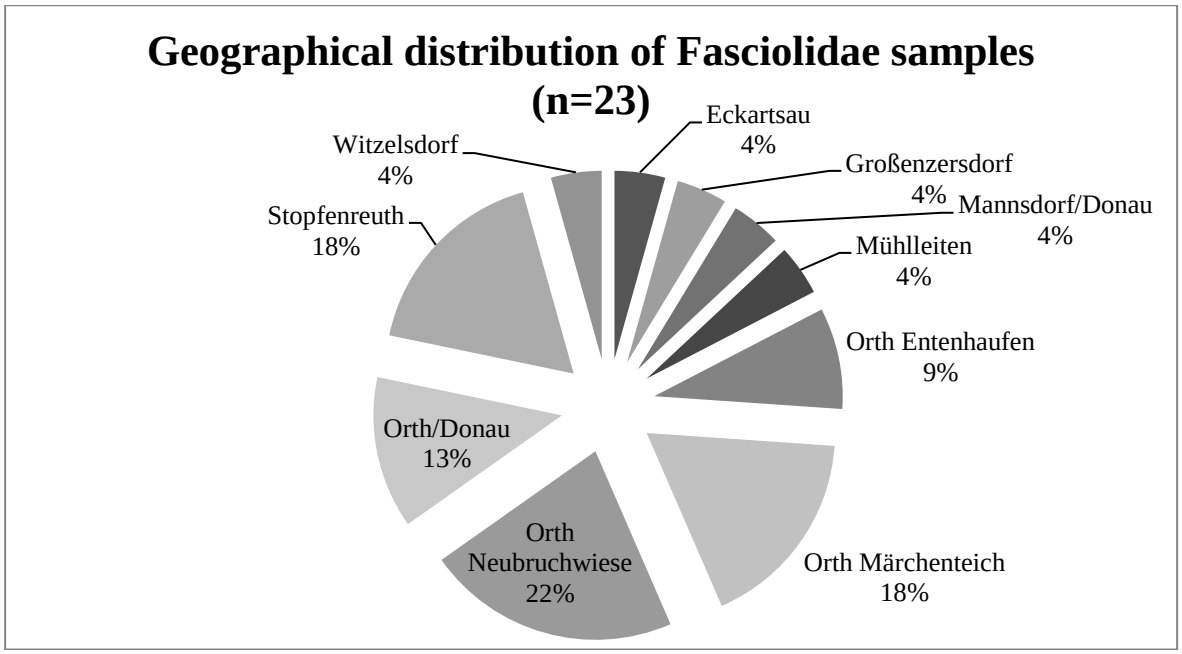

Figure 4.37: Geographical distribution of samples in which fasciolid DNA was found.

4.3.9 Positivity rates

Overall positivity rates were calculated for all trematode PCR positive samples (Figure 4.38). High rates were calculated for Großenzersdorf (80%), Mühlleiten (75%), Orth/Donau (75%) and Stopfenreuth (62.5%). Moderate positivity rates were found in Mannsdorf/Donau (33.3%), Orth Märchenteich (46.7%) and Orth Neubruchwiese (35.3%). The positivity rates in Eckartsau (13.3%), Orth Entenhaufen (20%) and Witzelsdorf (16.7%) were the smallest from all sampling sites investigated.

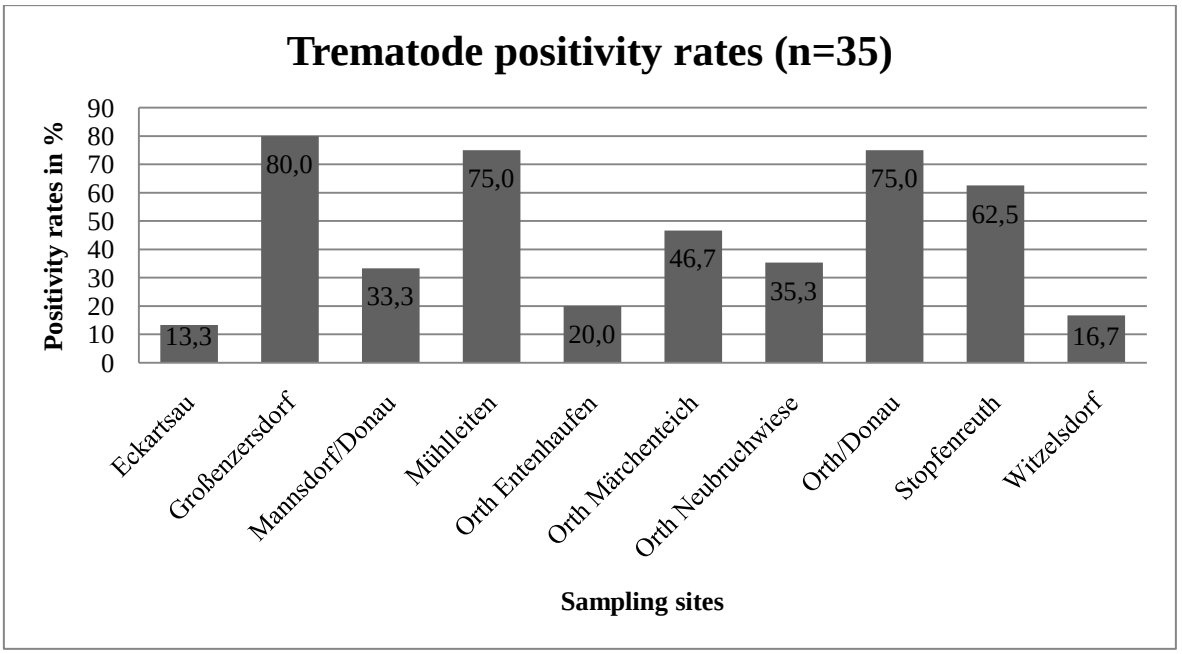

Figure 4.38: Overall distribution of trematodes on the investigated sites.

4.3.9.1 Positivity rates for Fasciolidae

The positivity rate calculations for the Fasciolidae findings are as depicted in Figure 4.39. There was one site with a very high positivity rate of 75%, which was Orth/Donau. For Stopfenreuth a positivity rate of 50% was calculated, the rates of all the other sites were below 35% (6.7% - 33.3%).

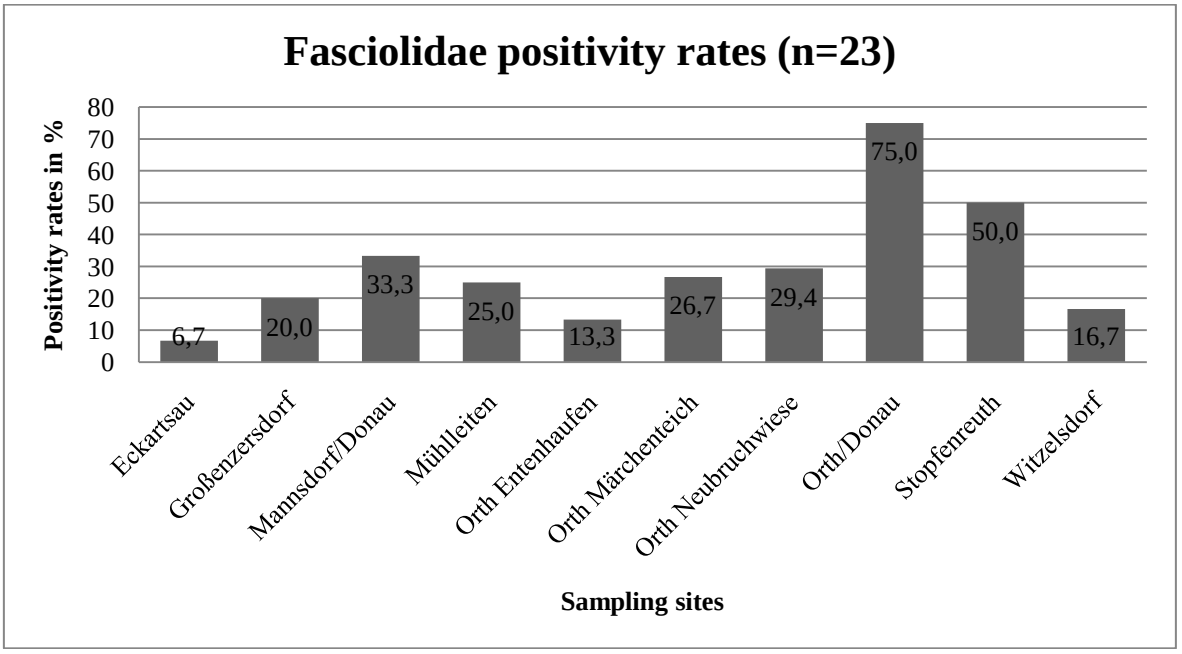

Figure 4.39: Fasciolidae positivity rates of the analysed sampling sites.

4.3.10 Seasonal distribution

A seasonal distribution of the PCR positive samples was calculated excluding the samples of which no collecting date was available. The highest number of positive samples derived from October with seven samples (Figure 4.40). Four and five samples were from May and August, respectively. During the remaining months between one and three positive samples were found.

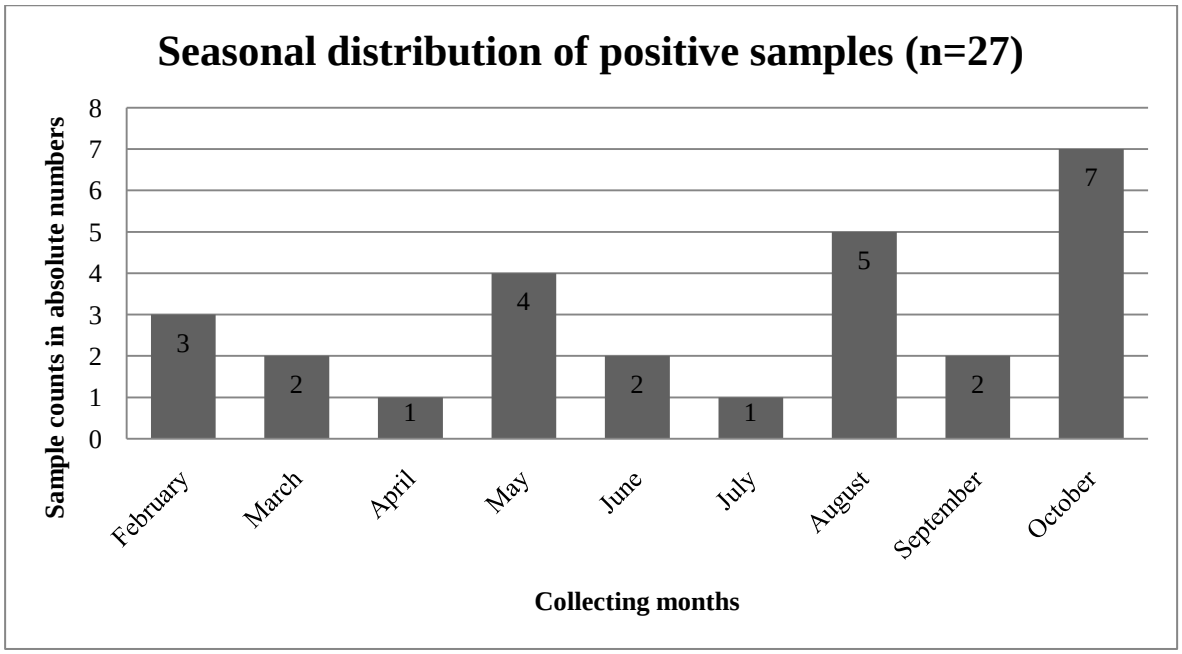

Figure 4.40: Seasonal distribution of PCR positive samples with recorded collecting dates.

4.4 Combined results of microscopy and molecular biology

158 deer faeces samples were examined under the microscope, of which 31% (49 samples) were positive for trematode eggs. Different types of eggs were found, but a distinct differentiation through microscopy only was not possible in many cases, as for example the eggs of *F. hepatica* and *F. magna* have very similar morphological features.

109 samples were analysed with biomolecular techniques, of which 32% (35 samples) gave positive signals for trematode DNA. Using different sets of primers and sequence analyses, the differentiation of species became possible in most of the samples.

After merging the results of both techniques, 59 of the investigated 158 samples were positive for trematodes (37%) and another three revealed helminth eggs, which, however, could not be specified (Figure 4.41).

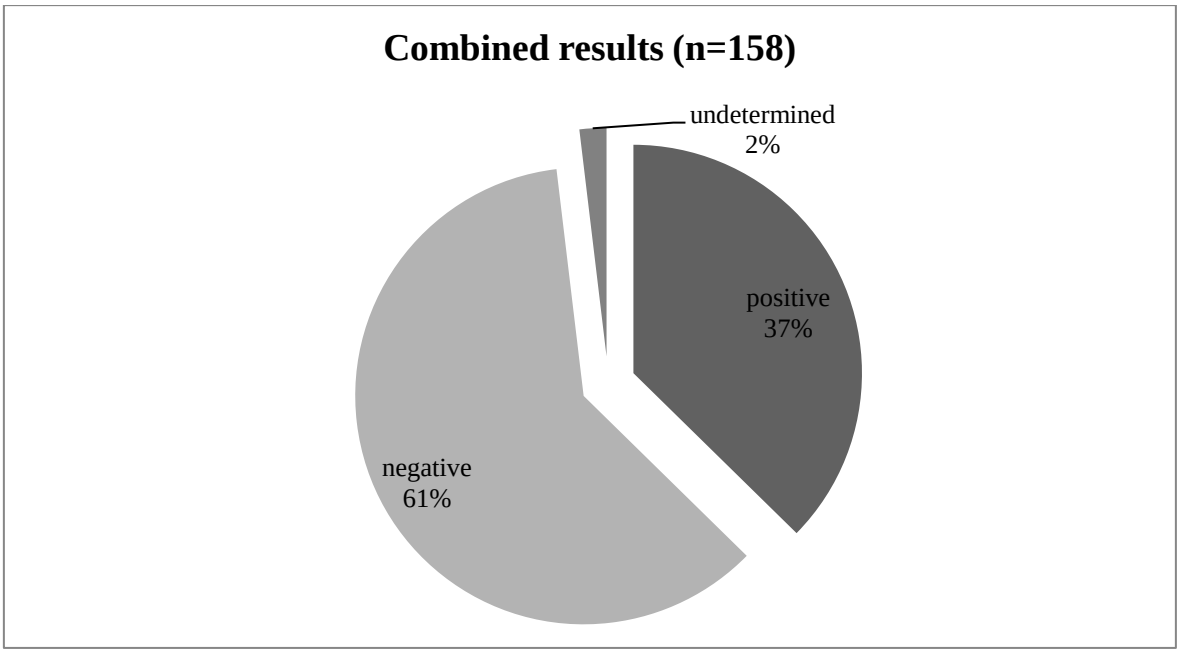

Figure 4.41: Combined results of all 158 samples.

4.4.1 Trematode families

As demonstrated in Figure 4.42, the majority of the 59 positive samples (29 samples) contained eggs or DNA of Fasciolidae. Paramphistomidae were found in eleven samples, Dicrocoeliidae in one sample. Seven samples were classified as unspecified trematodes and in eleven samples more than one trematode species was detected.

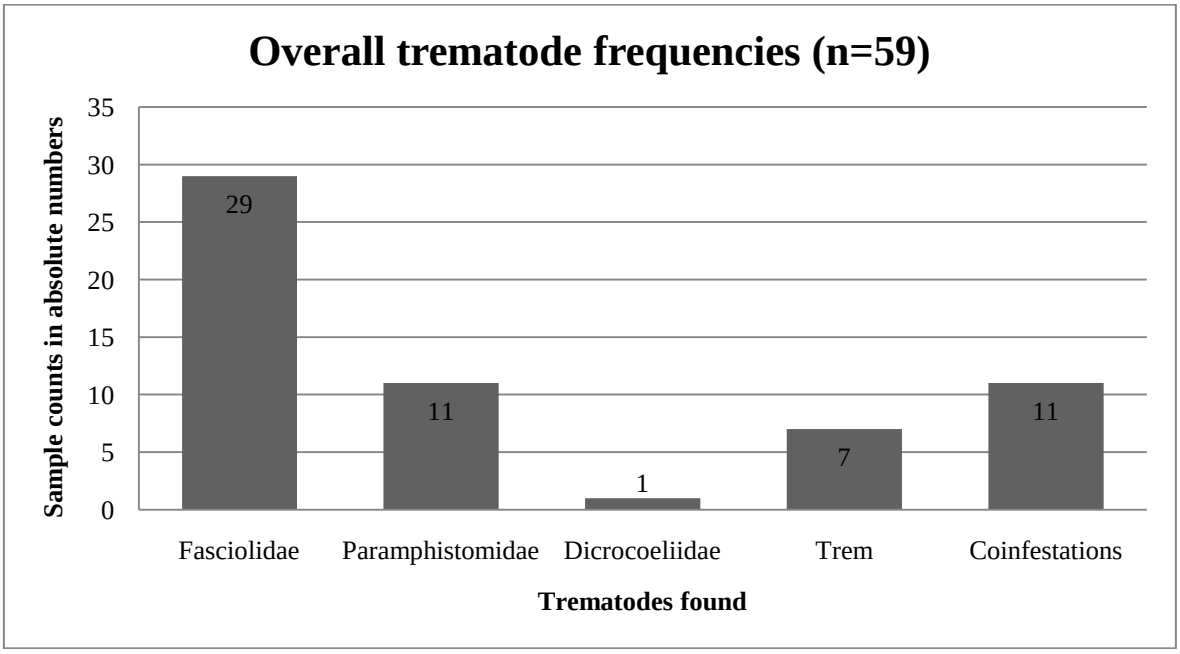

Figure 4.42: Trematode families found in all samples, coinfestations refer to samples in which more than one type of trematode egg/DNA was found, Trem refers to unspecified trematodes.

4.4.2 Overall geographical distribution of positive samples

The 59 positive samples were distributed over all investigated sampling sites (Figure 4.43). The majority of positive samples (57%) originated from the sampling sites where also most samples had been collected, the Orth/Donau locations. 12% of the positive samples were from Stopfenreuth, 7% each from Eckartsau and Großenzersdorf. There were only 1-3 positive samples from the sites Fischamend, Mannsdorf/Donau, Markthof, Mühlleiten, Schönau/Donau and Witzelsdorf.

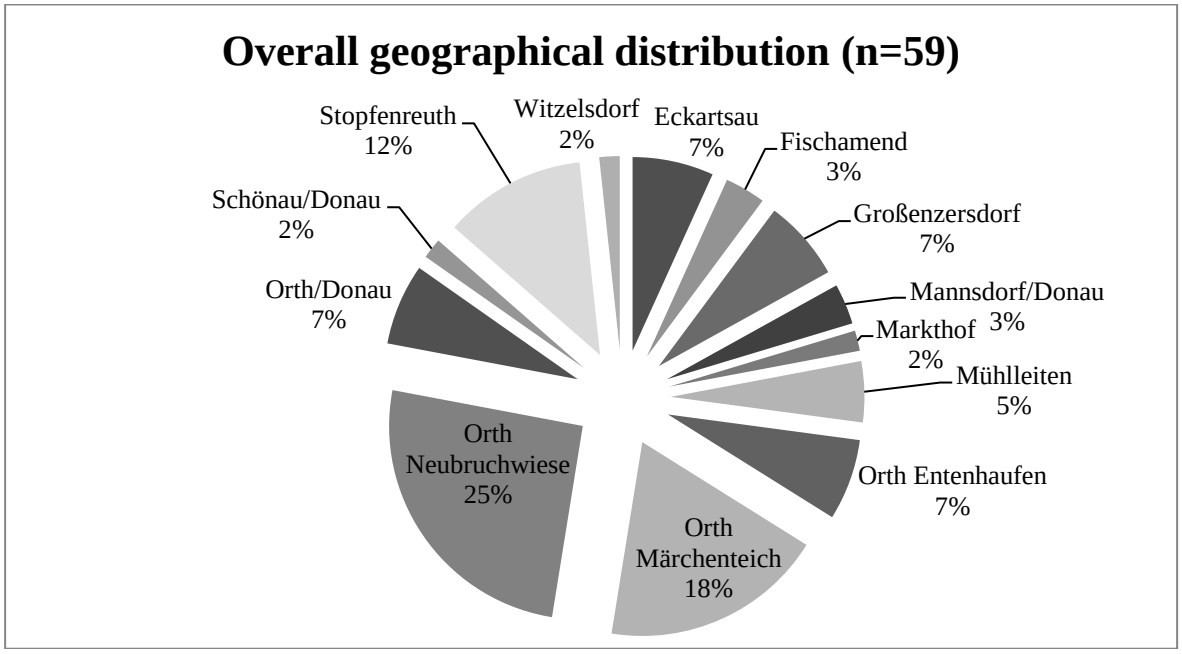

Figure 4.43: Combined geographical distribution of all positive samples.

4.4.3 Overall trematode positivity rates

The overall trematode positivity rates were calculated for each sampling site plotted against the number of collected samples at each location. As shown in Figure 4.44, low positivity rates (less than a third positive) were found in Eckartsau (21.1%), Fischamend (22.2%), Markthof (20%), Orth Entenhaufen (11.8), Schönau/Donau (33.3%) and Witzelsdorf (16.7%). Moderate positivity rates (between 33 and 66%) were found in Mannsdorf/Donau (66.7%), Orth Märchenteich (55%), Orth Neubruchwiese (46.9%) and Orth/Donau (40%). The sampling sites Großenzersdorf, Mühlleiten and Stopfenreuth showed high infestation rates, 80%, 75% and 87.5% respectively.

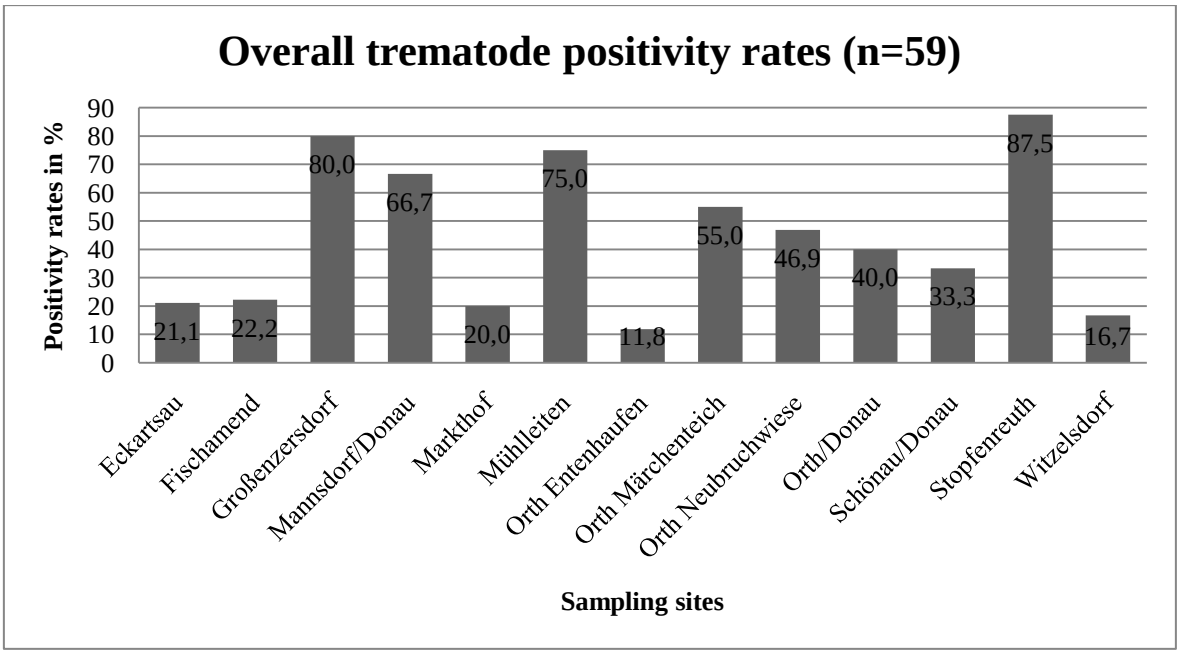

Figure 4.44: Combined trematode distribution on all investigated sampling sites.

4.4.4 Overall seasonal distribution of positive samples

The seasonal distribution of the combined positive samples from microscopy and molecular biology was calculated from 49 instead of 59 samples, because of missing collection dates on ten samples. Figure 4.45 shows that most positive samples derived from June, August and October with eight samples each. Seven positive samples were collected in May, six in September, five in February and three each in March and April. In July only one sample was positive for trematodes.

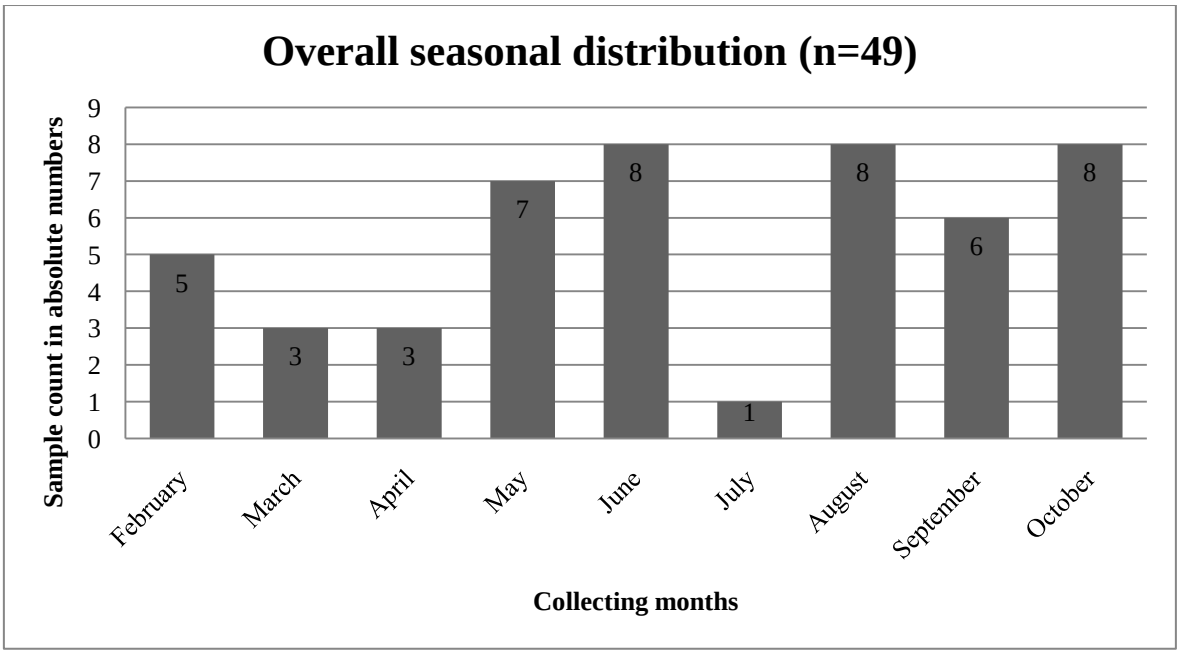

Figure 4.45: Combined seasonal distribution of all dated positive samples.

4.5 Comparison of microscopy and molecular biology

Of the 109 samples investigated with both techniques, 59 samples were positive in either one or both techniques. Nevertheless as shown in Figure 4.46, only 28 of these 59 samples (47%) were positive for trematodes in both techniques. 24 samples were only positive in microscopy and seven microscopically negative samples were positive by molecular biological means only.

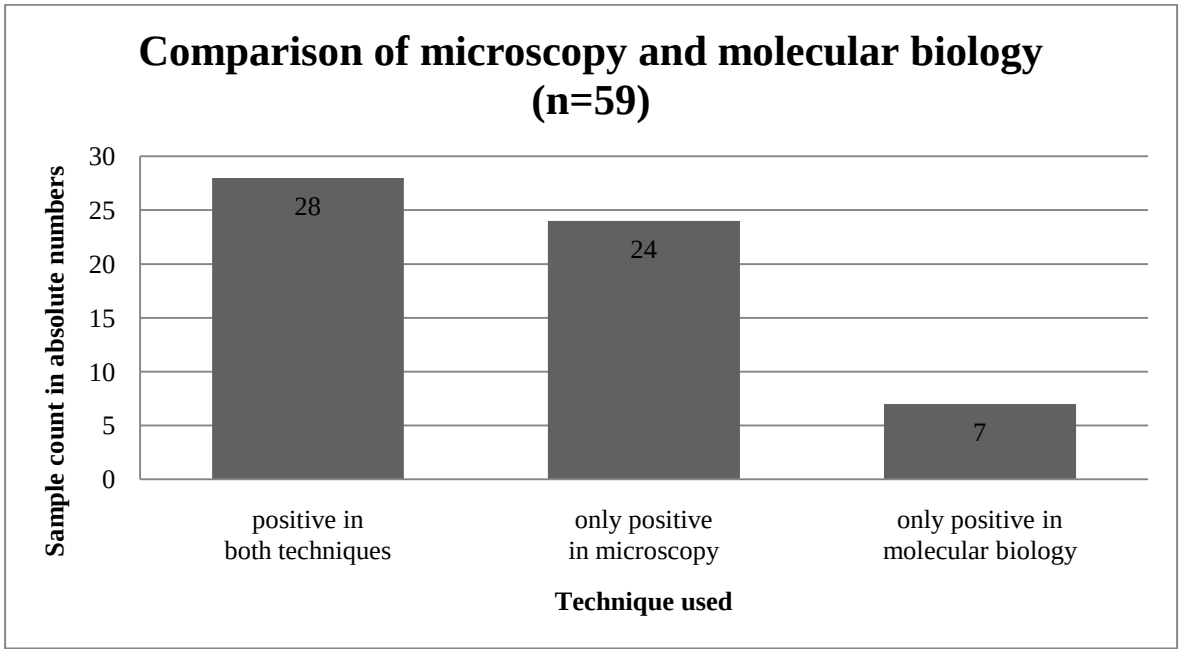

Figure 4.46: Comparison of two different analysis techniques executed on 109 samples of which 59 were positive in either or both analyses.

5 DISCUSSION

In the current study the prevalences of trematode infestations in red deer living in the 'Nationalpark Donau-Auen' and its surrounding areas were investigated. Particular emphasis was given to the introduced American liver fluke *Fascioloides magna* and the species-specific differentiation from other related trematode species. For both, identification and differentiation, microscopic and molecular methods were applied in parallel.

The central findings of this study were the relatively high overall positivity rates considering previous medication of deer in parts of the investigated area, as well as the different trematode species found in cervid faecal samples. Most surprising were the very high numbers of *F. magna* positive samples and the unexpected absence of *Fasciola hepatica*, particularly when compared to the simultaneously performed study on intermediate host snails in the same area by Haider (2010). Furthermore, difficulties were encountered in the processing of faecal samples for biomolecular analysis, which resulted in considerable discrepancies between microscopy and molecular biology.

5.1 Overall positivity rates

Altogether, 37% of all investigated faecal samples (59 of 158) were positive for trematodes. In 2% (3 of 158) of the samples, helminth eggs, that could not be specified any further, were found. As a reservoir of trematodes in wild animals such as red deer always exists naturally, these percentages are not surprising. However, due to the fact that the sampling sites are situated within or in the surroundings of the 'Nationalpark Donau-Auen' and not within a fenced deer park, the investigated animals are more or less free-living in the area. Thus, it was not possible to connect a certain faecal sample to one specific animal living in a specific area. Therefore the calculation of infection/infestation rates within the deer population was impossible. The positivity rates, meaning how many samples collected at a certain sampling site were positive for trematodes, were calculated and then used for the identification of possible high-risk sites.

Especially in the past 50 years, several studies on the parasite fauna of deer and other ruminants were conducted, which clearly support the natural reservoir function of wild

animals. In 2002, Rehbein et al. published a large-scale work on the endoparasites of red deer and other deer species in Nordrhein-Westfalen/Germany. Apart from protozoa, arthropods and helminths other than trematodes, they listed the liver flukes *Fasciola hepatica*, *Fascioloides magna* and *Dicrocoelium dendriticum* and the rumen fluke *Paramphistomum cervi* as possible parasitizing trematodes in red deer, which were also mentioned in previous studies on red deer parasites from different German regions or neighboring contries. However, the investigation of 85 red deer in that implied study did not deliver any positive results for trematode burden in these animals, while the infection rates with nematodes were in some cases up to 100%.

A similar study that dealt with a relative of red deer, namely sika deer (*Cervus nippon*), was conducted by Rehbein & Visser in 2007. They investigated the endoparasites of 108 free-living sika deer in Austria. Apart from different protozoa, cestodes and many nematodes, two trematode species were found. These were *Fasciola hepatica* and a *Dicrocoelium* species, which was probably *D. chinensis*. In the course of that study, 87 deer livers were investigated, the prevalences for the located flukes were 27.6% and 4.6% for *D. chinensis* and *F. hepatica*, respectively. Remarkably, the intensity of infestation ranged from three to 326 flukes per liver in case of *D. chinensis*.

In 1990, Pybus conducted a study on the hepatic and pulmonary helminths of wild cervids in Canada. During the liver and lung inspection of 702 animals of four different cervid species (mule deer, moose, white-tailed deer and wapiti) the Giant liver fluke F. *magna* and the lancet liver fluke *D. dendriticum* were found. While the second was only found in two wapiti, one white-tailed deer and one mule deer, *F. magna* was found in the livers of 29 animals excluding a highly infected adult wapiti, which carried 608(!) adult parasites. In wapiti, the natural definitive host of *F. magna*, the prevalence was 29.2% in adult animals, which resembles the numbers in our current study.

5.2 Trematode species

The intention of the current study was to investigate not only the trematode burden in red deer itself, but also to differentiate the trematode species that occur in these ruminants. Thus, trematode eggs found during microscopy were identified by morphological characters, such as size, colour and texture. Additionally, trematode DNA was amplified using a universal primer pair that binds to different trematode species. To discriminate the morphologically very similar eggs of *F. hepatica* and *F. magna*, specific PCRs were

established. Positive PCR results were sequenced to confirm and determine trematode species.

The analyses revealed that 49% of the positive samples (29 of 59) contained either eggs or DNA of Fasciolidae, 19% (11 of 59) were Paramphistomidae, and one sample contained only DNA of Dicrocoeliidae. 12% of the samples (7 of 59) contained unspecified trematodes. The remaining 19% of the samples (11 of 59) showed coinfestations with more than one 'sort' of trematode egg or DNA. Interestingly, the coinfestations were consistently mixes of paramphistomides and fasciolides. Only one sample contained the mentioned two trematode families plus DNA of *Dicrocoelium dendriticum*. Furthermore, both *D. dendriticum* infestations were only detected by PCR and subsequent sequencing, but no eggs could be found by microscopy.

Surprisingly, the prevalence of the introduced parasite *F. magna* was high (17.7%). It was not only the most frequently found trematode in the investigated samples, but, apart from one Fasciolidae sample which could not be determined to the species level, also the only representative of this trematode family. On the other hand, the naturally in this area occurring liver fluke *Fasciola hepatica* was not found in any of the investigated samples. This was even more surprising considering previous anti-*Fascioloides* treatments in this area (Ursprung et al., 2006).

Fascioloides magna, the Giant or American liver fluke is a natural parasite of wapiti (*Cervus elaphus nelsoni*), white-tailed deer (*Odocoileus virginianus*), caribou (*Rangifer tarandus*) and other deer species in North America (Pybus, 2001). For enrichment and blood refreshment reasons of the European deer fauna, North American wapiti and white-tailed deer were imported to Europe. At the end of the 19th century some individuals were brought to several zoos or animal enclosures in Austria, Germany, Italy or England (Erhardová-Kotralá & Kotrlý, 1968). Together with the living game animals also their parasites were introduced to Europe. After decades of adaptations to the new environmental circumstances and locally occurring intermediate host snails on the part of *F. magna* as well as the escape of several of the introduced cervid specimens and the subsequent mixing with free-living animals, the parasite is nowadays occurring in partly very high prevalences in different European countries. After the first European description of *F. magna* in red deer held in the Royal National Park La Mandria in Turin/Italy by Bassi (1875) and several following discoveries in game animal in for example the former

Czechoslovakia, Croatia and Hungary (Erhadová-Kotrlá & Kotrlý, 1968, Rajković-Janje et al., 2007, Majoros & Sztojkov, 1994), the parasite was also discovered in Austria.

The first description of *F. magna* in Austria was made by Pfeiffer in 1983, who found the parasite in previously from the Netherlands imported fallow deer in a game reserve in Lower Austria. In 2000, *F. magna* was also found in wild deer in Fischamend, east of Vienna (Winkelmayer & Prosl, 2001). The hunters in authority noticed shabby looking red deer with shaggy coats. Adults of *F. magna* were recoverd from the severely altered livers of those animals. This was surprising due to the fact that liver fluke infestations were hardly occurring in wild animals of that area until then. As reported by Ursprung et al. (2006), the studies following the first occurrence in wild animals showed a considerably high prevalence in the red deer population living in this area, which implicated the necessity of more intensive studies on prevalence, distribution and consequential problems of this parasite. In their study that lasted from 2000-2005, they investigated faeces samples for *F. magna* eggs and found an average prevalence of 6.3%, but they had more than 50% positive faeces samples in some of the investigated areas along the river Danube. They also investigated livers of shot or dead red and roe deer, which were positive in 15.8%. Additionally, starting in 2001, they put out fodder with the anthelminthic drug triclabendazol on spots with known fluke occurrence, which resulted in significant reduction of prevalence, egg counts in faecal samples and fluke numbers in affected livers.

Similar studies on the prevalence of *F. magna* were also conducted in neighbouring countries of Austria. In Croatia, faeces samples and livers of red deer were collected from October 2002 to April 2003 (Rajković-Janje et al., 2007). In their study, 34.53% of the faeces samples were positive for *F. magna* eggs, and in 28.57% of the investigated liver samples they found adult parasites. An almost identical study on faecal samples and livers of red and fallow deer (*Dama dama*) was conducted by Novobilský et al. in 2007 in the Czech Republic from September 2003 to December 2005. They investigated 20 different sampling sites and obtained highly varying prevalences from 4% up to 95%.

In 2005, Sattmann & Hörweg conducted a study to investigate the prevalence of *F. magna* in *Galba truncatula* in the ‘Nationalpark Donau-Auen’. Snails of the family Lymnaeidae can host developmental stages of flukes such as *Fascioloides magna* (Sattmann & Hörweg, 2005). In Europe, mainly the snail species *G. truncatula* is a known intermediate host for this parasite and related trematode species. *G. truncatula* and other possible intermediate host snails like *Stagnicola* sp. and *Radix* sp. occur in the area of the ‘Nationalpark Donau-

Auen'. Over 100 sampling sites within or near the national park were chosen after a previous risk analysis by Reckendorfer & Groiss (2006), and these sites were not only obvious snail environments like river benches or river-near sites, but also back waters, floodplain waters, ponds, temporarily dry falling ditches, tractor tracks or flooded grasslands (Sattmann & Hörweg, 2005). They collected over 10,000 *G. truncatula* specimens and examined them for developmental stages of digenean trematodes. The overall trematode prevalence was 2.43% (244 of 10,059); the most frequent trematode found was the rumen fluke *Paramphistomum* sp. with a prevalence of 1.56% (157 of 10,059). *F. magna* stages, mostly rediae and in two cases cercariae, were found in seven *Galba* specimens (prevalence 0.07%) and verified by PCR due to the morphological resemblance to *F. hepatica*. The relatively low prevalence of *F. magna* was discussed by the authors as a possible consequence of previous medication of the deer population in this area.

In 2008, a monitoring of both intermediate and definitive hosts was initiated. In parallel with the current study on the parasites of red deer, Haider (2010) did a study on the parasites of the intermediate host snail *G. truncatula* at the same three sampling sites in Orth/Donau. 3871 snail specimens were collected and microscopically examined for developmental stages of *F. magna* and related trematode species. Species determination was achieved by microscopy and additional biomolecular techniques like PCR and sequencing. The overall prevalence of digenean trematodes was 2.48 (96 of 3871) and the trematodes found most frequently were representatives of the superfamily Paramphistomoidea with 74 positives. Further trematode species found in the snails were 18 Echinostomatoidea, two Plagiorchiida and four Strigeida. The most interesting result in comparison with the current study was the overall low *F. magna* prevalence in intermediate host snails of 0.26%, only 10 individual snails were infected with this parasite. However, there was also one snail infected with the naturally in this area occurring liver fluke *Fasciola hepatica*, which could not be detected in any of the faecal deer samples in the current study.

Faciola hepatica, the common liver fluke, occurs naturally in wild and domesticated animals in Austria. In the current study, no faecal sample was positive for *F. hepatica*. As stated by Winkelmayer & Prosl (2001), there was little occurrence of liver flukes in deer in Fischamend on the south bank of the Danube before the introduction of *Fascioloides magna*. This would also concur with the minor infection of snails with *F. hepatica*

(prevalence of 0.03%) (Haider, 2010). However, multiple studies from Austria and other European countries show the frequent occurrence of *F. hepatica* in different host species with partly very high prevalences. Rehbein & Visser (2007) showed a prevalence of 4.6% of sika deer infected with *F. hepatica* in Austria. In 2005, Dreyfuss et al. investigated natural watercress beds in Central France from 1990-2004 for occurrence of metacercariae of *Fasciola hepatica* and its intermediate host snails. The annual prevalences of *G. truncatula* infections were between 1.2 and 2.4%. In 2008, Rapsch et al. investigated the infection risk of *F. hepatica* in *Lymnaea truncatula* in Switzerland. Their results showed prevalences of bovine fasciolosis from 8.4 to 21.4%. In an Austrian study conducted by Duscher et al. (2011), lactating cows were investigated for *Fasciola hepatica* by screenings of milk, blood and faecal samples. They found eggs of *F. hepatica* passed by 17.8% of the investigated animals. Furthermore, antibody screenings showed almost 50% positive samples. A similar study from England and Wales found seroprevalences of up to 84% in dairy cattle (McCann et al., 2010). These findings are important due to the fact that pastures are sometimes commonly used by domesticated and wild ruminants (Prosl & Kutzer, 2006). Consequentially, if other necessary circumstances for the successful establishment of a parasite's life cycle such as the appearance of intermediate hosts in which the parasite can undergo multiplication and suitable environments such as river benches or flooded pastures are given, parasites can easily be transmitted between wild and domesticated animals. This is especially important because of the unequal adaptations of the different host species to the various parasites.

5.3 Geographical distribution and risk sites

The current study had the intention to investigate the prevalences of trematodes and especially of the introduced American liver fluke *Fascioloides magna* in the national park 'Donau-Auen'. In the course of the current study, 158 faecal deer samples were collected at ten different sampling sites with emphasis on one of these sites, namely Orth/Donau, where most samples originate from and where also the most positive samples (57%) were collected.

To find out whether a certain sampling site might have a higher risk potential for deer infection than other sites in this area, the trematode positivity rates were calculated for each site. It was shown that the sampling sites Großenzersdorf (80%), Mühlleiten (75%) and Stopfenreuth (87.5%) are potentially high-risk sites. However, the mentioned high

percentages need some reconsideration as there were only few samples collected at the sites Großenzersdorf (five), Mühlleiten (four) and Stopfenreuth (eight). Additionally, it is – at least theoretically - even possible that all positive faecal samples from one of these potentially high-risk sites, originated from one single deer. This is mainly due to the fact that faecal samples were collected from the ground without knowing the specific deer that left it, and also because collections were performed over a long period of time.

From 2000-2005, Ursprung et al. (2006) investigated the same area with main focus on the prevalence of *F. magna* in red and roe deer. In the beginning, they found positive livers mainly in Fischamend. In the middle of 2001, there were also positive findings in Mannswörth, Maria Ellend, Haslau and Regelsbrunn (all south bank of Danube) and by the end of the same year the parasite was also found in different sampling sites at the north bank of the river (Großenzersdorf, Schönau, Mannsdorf, Markthof and Orth/Donau). This clearly pointed to the rapid spread of the parasite and the important role of the river Danube in its distribution.

The in the current study more intensely investigated sites in Orth/Donau (Entenhaufen, Märchenteich and Neubruchwiese), which are in close vicinity to each other, showed highly different positivity rates but with more samples collected. Märchenteich showed a positivity of 55% in 20 collected samples. The positivity rates of Neubruchwiese (46.9% of 32 samples) and Entenhaufen (11.8% of 34 samples) have even more significance.

These percentages stand in contrast to the trematode prevalences in the intermediate host snails collected in the same area and time period (Haider, 2010). The highest prevalence was, as in the current study, found at Märchenteich with 4.38% positives in 1347 examined snail samples. The sampling site Entenhaufen showed a trematode prevalence of 1.22% (1975 samples). Surprisingly, at the site Neubruchwiese, where almost every second faecal sample was positive in the current study, there was no trematode-positive snail in Haider's study. Admittedly, this was also the site where the fewest snail numbers (122) had been collected. In addition to the Orth sampling sites, Haider (2010) also did one sampling round each at the Danube-near locations Fischamend, Haslau and Regelsbrunn. At these sites, which were summed up as one site for calculation reasons, the trematode prevalence was 3.04% in 427 investigated snails.

This was interesting regarding the distribution of the investigated parasites, particularly *F. magna*. In 2005, Sattmann & Hörweg confirmed the wide distribution of *Galba truncatula*,

the snail species *F. magna* uses as intermediate hosts, within the whole area of the national park and its surrounding areas with exceptional high densities in Orth/Donau on the northern riverside and Fischamend on the southern riverside of the Danube. At exactly those two sites, the authors found snails infected with *F. magna*. This was especially important as it was the first description of infected intermediate hosts north of the river Danube, where beforehand only infected definitive hosts had been found. Thus, also Sattmann & Hörweg clearly point to the spread of the introduced trematode *F. magna* in Austria.

5.4 Seasonality

According to the trematode life cycle with alternation of generations and hosts, a seasonality of trematode infestation/infection within the hosts was expected. In the current study, no significant seasonal distribution was recorded for the 49 positive samples that were properly labelled with collection dates and time. The most positive faecal samples were found in June, August and October (eight each), in July only one positive sample was collected.

Obviously, there is no seasonality in the faeces disposal of the deer, but the opportunities to find these samples depend on several factors such as vegetation, time of the day, temperature, humidity, water levels and so on. During the collection period in 2008, several samplings were hindered by very high water levels. In two cases, the water levels were extremely high and one time the bridge, which represents sampling site Märchenteich and which needed to be crossed to reach sampling site Entenhaufen, was entirely under water, which made sampling impossible during those days. Furthermore, sampling was more difficult on wet days with high water levels due to slippery grass, harder detectable samples and altered wandering behaviour of deer.

Also, the temperature on and shortly before the sampling days was important. As the intention of the study was to collect mainly fresh faecal samples and therefore obviously old samples were not even collected in the first place, high temperature made the differentiation between fresh and old samples difficult due to accelerated decomposition and desiccation.

Additionally, other animals such as wild boars, insects and also bacteria occurring in the environment contribute their part to decompose the faeces, which was, apart from the rising temperatures, a reason for sampling mainly in the morning and forenoon hours.

Nevertheless, a true seasonality of trematode infestation in deer could not be detected, also given to the small numbers of positive samples.

However, in the parallel study conducted by Haider (2010), a seasonality of snail infection was detected. Prevalence was highest in July with 10.11% positive samples in 524 collected in this month. This seasonality was attributed to a seasonal size distribution of the snails' shells and the seasonally changing snail densities. The author explained that the shell size might be crucial, as slightly more than half of the infected snails had shells larger than 5 mm.

While Haider (2010) was able to detect a two-peak reproduction cycle in the intermediate host snail *Galba truncatula*, Sattmann & Hörweg could not do so in 2005 due to little infection quantities. Nevertheless, they found young rediae equipped with typically large suckers at the end of July and the beginning of August as well as cercariae in October. This is also concordant with their assumption, that a certain seasonality with peaks in the summer months occurs naturally and also temperature-dependend. They assume that the snail infection by miracidia mostly takes place in spring and early summer due to high water levels and the consequential egg water contact opportunities. The infection of the definitive hosts by metacercariae occurs mainly during summer and autumn months.

The risk periods of infection were also investigated by Díaz et al. (2007). From March 2001 to February 2004, they collected faecal samples of cattle in a Spanish area with oceanic climate and searched for *Paramphistomum daubneyi* eggs. They found more cattle passing trematode eggs in September and March with highest numbers of eggs in May. Considering climatic data from the sampling period, they suggested that high risk periods in this kind of climatic areas are months with preceding periods of elevated rainfall, namely May-June and November-December. Furthermore they recommended the administration of anthelmintics in June and November.

Water is a crucial point in the establishment of the trematode life cycle, not only in terms of rainfall but also the vicinity to bodies of flowing or standing waters and their water level fluctuations. All the main sampling sites in the current study in Orth/Donau lay near or directly at backwaters of the Danube. Not only sampling itself was influenced by

fluctuating water levels as mentioned above, but also the parasite community is. As explained by Gérard (2001), a drought has a high impact on snail communities and consequentially on their trematode parasites as well, where a certain period of time is necessary for recolonialization of snails and the subsequent new or re-infection by the trematodes. This delay in the life cycle will then also infect the definitive hosts. Further influencing factors on the incidence of definitive host infection are air temperature, rainfall and/or potential evapotranspiration (Mas-Coma et al., 2009).

5.5 Microscopy versus Molecular biology

The investigation of deer faecal samples was performed in parallels with two different types of analyses, microscopy and molecular biology. In direct comparison, both microscopy and molecular biology showed similar results. With microscopy, 31% (49 of 158) of the samples showed trematode eggs. With biomolecular means, namely PCR and sequencing, 32% (35 of 109) of the samples were positive for trematodes.

Parasitological methods to estimate and identify trematode eggs in faeces samples are traditionally based on microscopical techniques such as sedimentation, concentration or staining methods. Common used techniques are the Kato Katz (KK) technique or the formalin ethyl acetate concentration technique (FECT) on which numerable prevalence studies were based (Stensvold et al., 2006). Nevertheless, the diagnostic accuracy of the mentioned techniques is argued, given that it is often not possible to distinguish between closely related trematode species by morphology only, and the sensitivity of detection might be low particularly in samples with low egg numbers. Thus, biomolecular techniques were considered and expected to increase diagnostic sensitivity and specificity. In 1997, Kaplan et al. published the field-use of a DNA-based technique to determine the prevalence of *F. hepatica* in intermediate snail hosts and compared their results with results achieved by microscopical means. Their DNA probe showed a sensitivity of 100% with the abilitiy to detect a single miracidia in the snail sample, as well as a specificity of almost 100% depending on the infection prevalence. Furthermore, the DNA probe method was significantly more sensitive than both crushing and dissection of the snails.

Similar results were shown by Cucher et al. (2006), who established a PCR for the detection of *F. hepatica* in intermediate host snails. The published PCR worked with a detection limit of 10 pg of genomic DNA and showed no cross-reactions to related trematode species or snail species. The comparison of the newly established system and

direct examination was significant to that effect that the infection rates of the snails were considerably higher with PCR.

The same trend was observed by Haider (2010) in the parallel analysis of *G. truncatula* by microscopical and biomolecular means. With microscopy, 2.32% of the investigated snails showed positive results for digenean trematodes. On the other hand, 10.29% of the samples analysed with PCR were positive for trematodes.

So according to the exemplarily shown studies, the greater sensitivity of molecular techniques is obviously true for snail samples or in-vitro studies. Nevertheless, results with molecular techniques applied on faeces samples are inconsistent. In the current study, only 28 of the 59 trematode positive samples (47%) were positive in both techniques. With the standard technique in coprological analyses, microscopy, another 24 of these 59 samples were positive but showed no signal in any PCR. On the other hand, only seven samples, that were microscopically negative, were positive during the biomolecular analysis. These results clearly favour a higher sensitivity of microscopical techniques rather than biomolecular ones.

This is concordant with the findings of Stensvold et al. in 2006. The authors evaluated microscopy and PCR based methods to detect *Opisthorchis viverrini* in human faecal samples. The parasitological methods investigated, Kato Katz and FECT, showed similar sensitivities in the detection of the eggs up to 85% depending of the degree of infection. PCR on the other hand only showed positive signals in samples where microscopical methods had shown trematode eggs beforehand. Furthermore, PCR results were varying greatly (average PCR sensitivity 50%) depending on the egg counts in the faeces samples although high EpGs (Eggs per gram) did not guarantee a success of the established PCR. On the contrary, PCR could not detect *O. viverrini* DNA in three of the five samples that had the highest EpGs of 6,000 or more in the study.

Umesha et al. (2008) on the other hand demonstrated higher sensitivity of a PCR system compared to microscopy for the detection of *O. viverrini* in human stool samples. In their assay, all microscopically positive samples were as well positive with PCR, but additionally also 23 of 30 microscopically negative samples showed positive signals with the new technique. They showed that in this case PCR was significantly more accurate than microscopy with a sensitivity of 5 eggs/g of stool.

The discrepancies between the applied techniques might have several reasons. To assure a high sensitivity of microscopical techniques, microscopy experience of the researcher is often needed. Furthermore, low infections might easily be missed when sedimentation rather than concentration techniques are applied. In the current study, all faeces samples were sedimented, examined, transferred to Eppendorf tubes for storage and then transferred to new tubes for biomolecular processing. Filtering, water draining or the transfer steps are very likely reasons to miss some trematode eggs in the faeces sample, particularly in samples which contained only few eggs in the first place.

Reasons for low detection sensitivity with polymerase chain reaction were explained by Stensvold et al. (2006) who listed unsuccessful DNA extraction, inappropriate sample volume, low primer affinity, polymerase inhibiting and DNA degrading substances in the faeces sample etc. In their study they ruled out several of these reasons as for example the unsuccessful DNA extraction by checking that the eggs in the sample were broken open or the possibility of inbitory substances in the faeces by using a special DNA extraction kit for stool samples.

Also in the current study it was aimed to minimize possible influences that could decrease the PCR sensitivity starting with the crushing of trematode eggs. The disintegration of trematode egg shells is difficult and is more or less well achieved by different techniques like repeated heating and cooling with boiling water and liquid nitrogen, respectively, chemical reagents (NaOH) and autoclaving or the Teflon (PTFE) method. In the current study, a homogenization technique with glass or ceramic beads was applied to mechanically destroy the eggshells in order to extract the DNA. The general success of the egg disruption was checked by microscopy after homogenization. As noted by Oberhauserová et al. (2010), the DNA extraction past bead-homogenization as in the current study did not provide fine results in their study. Stensvold et al. (2006) on the other hand observed that even samples without broken eggs showed positive PCR signals in a few cases assuming other factors as main contributors to the low PCR sensitivity.

The next step in outruling PCR limiting factors was the selection of a DNA extraction protocol adjusted to faecal samples. In the current study as well as in the one of Stensvold et al. (2006), the QIAamp® DNA Stool Mini Kit involving a special InhibitEX tablet to bind inhibitors was used. The same kit was also used by Li et al. (2003) on bacterial cells. With this kind of material, the method seems appropriate, as it provided with 95% approximately the same amount of lysed cells as a bead beating method. Unfortunately in

our current study, working with faeces-soil-samples containing trematode eggs, the kit did not show this high efficiency particularly when compared to a specialized kit for soil samples. Here, the soil kit efficiency was 33% compared to only 20% with the stool kit. Furthermore, the existing kits for DNA extraction from faecal samples often result in relatively low DNA yields (Yu & Morrison, 2004). In the current study, the average DNA yields achieved with the QIAamp® DNA Stool Kit and the Precellys Soil DNA Kit were 4.98 ng/µl and 6.81 ng/µl, respectively, both kits with wide ranges between minimum and maximum DNA yield. Similarly unsatisfactory DNA yields were achieved by Tang et al. (2008) who compared different methods for DNA extraction from pig faeces. Consequently, they established a more effective method that involved a pretreatment with polyformaldehyde, subsequent DNA lysis in the presence of cetyltrimethyl-ammoniumbromide (CTAB), salt, PVP and β-mercaptoethanol and the DNA isolation with chloroform rather than phenol. The authors achieved a 1.3- to 11-fold higher DNA yield with their new method in comparison with existing methods such as QIAamp® DNA Stool Mini Kit.

Regarding the establishment of the PCRs, a balance between satisfying specificity and sensitivity was aimed. As it was intended to determine trematodes and particularly fasciolides in red deer samples on species level, the primer pairs Trem_F/Trem_R and Magna_F/Magna_R were tested with a standard PCR from our lab at different annealing temperatures to achieve the best possible results. In general, higher annealing temperature increases PCR specificity but decreases the sensitivity of it. The specificity of both primer pairs was tested with DNA of different trematode species, cestodes, deer faeces and snail tissue and proved high specificity for the universal primers. The *F. magna* primer pair did amplify specifically *F. magna* DNA but also the related *F. hepatica*. To assure species differentiation *F. hepatica* specific primers (Kaplan et al., 1995) were used additionally. When the sensitivity was tested with pure trematode DNA, both primer pairs showed high sensitivity at the lowest annealing temperature, namely 52°C. However, in the course of the PCR establishment with faeces samples little adjustments were needed. The sensitivity of the universal primers was highest with 52°C. In case of the *F. magna* specific primers, this temperature was not appropriate, but the sensitivity increased at 54°C. Furthermore, to receive even greater sensitivity, a double PCR was performed. Thus, as explained by Mayer & Palmer (1996) who increased the detection sensitivity in a *Cryptosporidium parvum* PCR assay by double PCR, the chance of obtaining positive results was increased and the signals stronger. The authors suggested inhibitory factors like humic and fulvic

acids interfering during PCR, which made one round of amplification too less. They also found that some samples showed positive signals at higher dilutions rather than lower ones, which might point to the necessity of diluting the inhibitors before amplification. The presence of inhibitory substances was also attested by Stensvold et al. (2006) by artificially spiking PCR negative but FECT positive samples with genomic DNA of *O. viverrini*, which then still resulted in no positive PCR result. Organic samples like faeces contain inhibitory substances that can decrease the sensitivity of a PCR by 1,000-fold (Tang et al., 2008). Ruminant faeces in particular contain high levels of PCR-inhibitors which are difficult to remove (Thornton & Passen, 2004). The authors suggested that very little amounts of faeces (40 µg faeces in a 100 µl PCR) can affect the efficiency of amplification greatly. They found that phytic acid was a strongly interfering substance in PCR. Thornton & Passen (2004) explain that phytic acid is the main form of stored phosphorous in plants and thus uptaken with fodder plants by ruminants that cannot completely digest it. Their study showed that below 10 µM phytate the PCR worked efficiently but was completely inhibited above 0.4mM. The phytic acid can inhibit PCR reactions if it enters in solution and thus can possibly interfere with the Mg2+ concentration, or if large amounts of phytic acid salts alter the ionic compositions of the reaction. While Thornton & Passen (2004) treated the faecal samples with phytase in order to reduce inhibitors, Duenngai et al. (2008) used cetyltrimethyl-ammoniumbromide to remove amplification inhibitors. Stensvold et al. (2006) suggested that snap freezing the samples to -20°C immediately after disposal might prevent the development of inhibitors and downregulate the effect of already present ones.

Concluding, molecular biological methods are a promising device in prevalence studies on digenean trematodes. Although there is a lot of research going on already, there are still some barriers to overcome when working with faecal samples.

5.6 Medical and veterinary relevance

The current study was conducted mainly to investigate the *Fascioloides magna* burden in red deer which was obviously surprisingly high. Moreover, the detection and differentiation of both, human and animal pathogen trematodes was aimed.

Fascioloides magna, the introduced liver fluke of ruminants, naturally uses wapiti and white-tailed deer in North America as their definitive hosts. As described by Ursprung et al. (2006), these original hosts are very well adaptated to the parasite and suffer hardly from even severe fluke infestations. Through the increase and spread of the deer in North

America as well as the export to other continents like Europe, the deers' parasites were introduced to other wild and domestic ruminants. But other than the original hosts, some of the new hosts suffer from severe damages in the liver and often die after intense infestations. As further described by Ursprung et al. (2006), other than in the natural definitive host, where adult flukes get encapsulated within the liver parenchyma with open connections to the bile ducts for egg disposal, *F. magna* does not reproduce in many dead-end or aberrant hosts. One reason for this is that some hosts like sheep, goats or roe deer suffer so much from the liver migration of the parasite that they die too early for the trematode to reach reproductive age. Other hosts like elk or cattle encapsulate the trematodes in very solid capsules without any connections to the environment, which prohibits egg disposal. Furthermore, these animals seem to suffer much less from *F. magna* infestations, even if severe changes in the liver through heavy parasite load can occur. In red deer on the other hand, *F. magna* is able to reproduce with disposal of eggs. Nevertheless, livers of affected red deer individuals show pathological alterations such as scars and channels in the parenchyma (Erhardová-Kotrlá & Kotrlý, 1968). Furthermore, general weakening, decrease of trophy or sudden death can come along with *F. magna* infection.

So far, there are no reports on human infections with *Fascioloides magna*. However, *F. magna* and the human pathogen *F. hepatica* are very alike considering life cycle, intermediate host snail species and the wide range of possible definitive host species. Therefore, it could still be speculated on a possible risk through *F. magna* on humans. Considering the high prevalence of the parasite in deer faecal samples indicated in the current study as well as the increasing prevalence in snails (Haider, 2010), a potential risk in connection with certain alimentary habits has to be kept in mind.

Human infections with *F. hepatica* on the other hand occur more or less frequently in some European and overseas countries (Mas-Coma et al., 2005). Most infections can be traced back to the consumption of common watercress, *Nasturtium officinale*, or other water plants (Mas-Coma et al., 1998). In Austria, human infections are not very frequent given that the incidence of fasciolosis is 1.1 cases per year (Auer, 2009). Between 1955 and 1993 only 13 cases of human fasciolosis were reported in Austria (Haiabi et al., 1997). This concurs with the findings of the current study, where no *F. hepatica* could be detected, as well as Haider's study (2010) where only one snail was positive for *F. hepatica*. *Dicrocoelium dendriticum*, which was found in two faecal samples in the current study, is

an accidental parasite in humans with only few reported cases. To acquire dicrocoeliosis, the consumption of ants adhering to plants carriing infectious larval stages is essential. Additionally, trematodes belonging to the family Paramphistomidae were found in faecal samples. Nevertheless, these species do not occur in humans, but can be responsible of considerable economical losses in livestock (Reyes et al., 2008).

Summing up, different digenean trematode species occur in red deer in the 'Nationalpark Donau-Auen', pathogenic for humans and animals. Particularly the introduced American liver fluke *F. magna* shows a high potential for rapid spreading and adapting to new environments. Due to its impact on the local fauna and the consequential economical losses, further monitoring of the reported high-risk sites is advisable.

6 GLOSSARY

abiogenesis: spontaneous generation; The origin of living from nonliving matter, as by biopoiesis.

acetic acid: ethanoic acid; A carboxylic acid CH_3COOH, that is used as a carbon source by certain green algae. Combined with coenzyme A, it plays a crucial role in the energy metabolism of all organisms.

acoelomate: Describing any bilaterally symmetrical animal of the subkingdom Eumetazoa that does not possess a coelom. Examples of acoelomate animals are the platyhelminthes.

adenine: A purine derivative. It is one of the major component bases of nucleotides and the nucleic acids DNA and RNA.

agarose: A polysaccharide gained from agar that is used for various applications in life sciences (e.g. gel electrophoresis).

alignment: Arrangement of DNA sequences.

alternation of generations: The occurrence within the life cycle of an organism of two or more distinct forms (generations), which differ from each other in appearance, habit, and method of reproduction. The phenomenom occurs in some protoctists, certain lower animals (e.g. cnidarians and parasitic flatworms), and in plants. The malaria parasite (Plasmodium), for example, has a complex life cycle involving the alternation of sexually and asexually reproducing generations.

amplicon: A piece of DNA that was formed as a product of natural or artificial amplification (PCR).

amplification: A way in which a gene can be overexpressed. Genetic amplification can happen naturally or artificially as in polymerase chain reaction, where short strands of DNA are amplified with the aid of enzymes.

base pairing: The chemical linking of two complementary nitrogenous bases in DNA and in certain types of RNA molecules. Of the four such bases in DNA, adenine pairs with thymine and cytosine with guanine. In RNA, thymine is replaced by uracil. Base pairing is responsible for holding together the two strands of DNA molecule to form a double helix and for faithful reproduction and reading of the genetic code. The links between bases take the form of hydrogen bonds.

cercariae: Larval stages of trematodes that develop from rediae. They leave the intermediate host in order to enter the definitive host via active penetration of the host or passive ingestion of encysted cercariae (metacercariae) by the host.

Cestoda: cestodes, tapeworms; A class of flatworms comprising the tapeworms – ribbon-like parasites within the gut of vertebrates. Tapeworms are surrounded by partially digested food in the host gut so they are able to absorb nutrients through their whole body surface. The body consists of a scolex (head), bearing suckers and hooks for attachment, and a series of proglottids, which contain male and female reproductive systems. The life cycle of a tapeworm requires two hosts, the primary host usually being a predator of the secondary host. Taenia solium has humans for its primary hosts and the pig as its secondary host. Mature proglottids, containing thousands of fertilized eggs, leave the primary host with its faeces and develop into embryos and then larvae that continue the life cycle in the gut and other tissues of a secondary host.

chronic: A persistent and long-lasting disease or medical condition.

clade: A group of organism that share a common ancestor.

cladistics: A method of classification in which animals and plants are placed into taxonomic groups called clades strictly according to their evolutionary relationships. These relationships are deduced on the basis of certain shared homologous characters (known as synapomorphies) that are

thought to indicate common ancestry (monophyletic). Implicit in this is the assumption that two new species are formed suddenly, by splitting from a common ancestor and not by gradual evolutionary change. Also, it requires that truly homologous characters are distinguished from homoplasic features, i.e. ones resulting from convergent evolution (homoplasy). A diagram indicating these relationships (cladogramm) therefore consists of a system of dichotomous branches: each point of branching represents divergence from a common ancestor.

class: A category used in the classification of organisms that consists of similar or closely related orders. Similar classes are grouped into a phylum. Examples include Mammalia (mammals), Aves (birds), and Dicotyledoneae (dicots).

classification: The arrangement of organisms into a series of groups based on physiological, biochemical, anatomical, or other relationships. The smallest group commonly used is the species. Species are grouped into genera (see genus), the hierarchy continuing up trough tribes, families, orders, classes, and phyla (see phylum) to kingdoms and, in some systems, to domains.

cysticercus: bladderworm; A larval stage of some tapeworms, consisting of a fluid-filled sac containing an inverted scolex. It develops in the muscle of the secondary host and matures into an adult worm in a primary host that eats this infected tissue.

cytoplasm: The material surrounding the nucleus of a cell. It consists of a matrix (cytosol) in which the cell's organelles are suspended. The cytoplasm may be differentiated into dense outer ectoplasm, which is concerned primarily with cell movement, and less dense endoplasm, which contains most of the cell's structures.

cytosine: A pyrimidine derivative. It is one of the principal component bases of nucleotides and the nucleic acids DNA and RNA.

Digenea (digenean trematodes): Subclass of the Trematoda with alternation of generations and hosts.

DNA (deoxyribonucleic acid): The genetic material of most living organisms, which is a major constituent of the chromosomes within the cell nucleus and plays a central role in the determination of hereditary characteristics by controlling protein synthesis in cells. It is also found in chloroplasts and mitochondria. DNA is a nucleic acid composed of two chains of nucleotides in which the sugar is deoxyribose and the bases are adenine, cytosine, guanine and thymine. The two chains are wound round each other and linked together by hydrogen bonds between specific complementary bases to form a spiral ladder-shaped molecule (double helix). When the cell divides, its DNA also replicates in such a way that each of the two daughter molecules is identical to the parent molecule.

electrophoresis (cataphoresis): A technique for the analysis and separation of nucleic acids, proteins and other compounds, based on the movement of charged colloidal particles in an electric field. There are various experimental methods. In one the sample is placed in a U-tube and a buffer solution added to each arm, so that there are sharp boundaries between buffer and sample. An electrode is placed in each arm, a voltage applied, and the motion of the boundaries under the influence of the field is observed. The rate of migration of the particles depends on the field, the charge on the particles, and on other factors, such as the size and shape of the particles. More simply, electrophoresis can be carried out using an adsorbent, such as a strip of filter paper, soaked in a buffer with two electrodes making contact. The sample is placed between the electrodes and a voltage applied. Different components of the mixture migrate at different rates, so the sample separates into zones. The components can be identified by the rate at which they move. In gel electrophoresis the medium is a gel, typically made of polyacrylamide, agarose or starch. Electrophoresis, which has also been called electrochromatography, is used extensively in studying mixtures of proteins (PAGE), nucleic acids, carbohydrates, enzymes, etc. In clinical medicine it is used for determining the protein content of body fluids.

encystation: The formation of cysts. In certain trematodes when metacercariae encyst on plants to get ingested by definitive hosts.

enzyme: A protein that acts as a catalyst in biochemical reactions. Each enzyme is specific to a particular reaction or group of similar reactions.

eosinophil (eosinophilia): A type of white blood cell (leucocyte) that has a granular cytoplasm. Its function involves the regulation of allergic responses and it also produces an enzyme capable of destroying parasites.

epidemiology: The study of diseases that affect large numbers of people. Traditionally, epidemiologists have been concerned primarily with infectious diseases that arise and spread rapidly among the population as epidemics. However, today the discipline also covers non-infectious disorders. Typically the distribution of a disease is charted in order to discover patterns that might yield clues about its mode of transmission or the susceptibility of certain groups of people. This in turn may reveal insights about the causes of the disease and possible preventive measures.

epithelium: A tissue in vertebrates consisting of closely packed cells in a sheet with little intercellular material. It covers the outer surfaces of the body and walls of the internal cavities (coeloms) and is often underlain by a basement membrane. It also forms glands and parts of sense organs. Its functions are protective, absorptive, secretory, and sensory.

eukaryote: eucaryote; An organism consisting of cells in which the genetic material is contained within a distinct nucleus.

evolution: The gradual process by which the present diversity of plant and animal life arose from the earliest and most primitive organisms, which is believed to have been continuing for at least the past 3000 million years.

flame bulbs (flame cells): Ciliated cells that form part of the excretory and osmoregulatory system of platyhelminths, rotifers, and nemertine worms. This system, known as a protonephridium, consists of branching tubules that open to the exterior trough excretory pores; flame cells occur at the ends nitrogenous waste products, diffuses into the flame cells and is directed trough the tubules to the exterior by movements of the cilia, which resemble the flickering of flames.

flukes: see Trematoda

GenBank: A database provided by the National Library of Medicine (USA) that contains nucleic acid and protein sequences.

gene: A unit of heredity composed of DNA. In classical genetics a gene is visualized as a discrete particle, forming part of a chromosome that determines a particular characteristic. It can exist in different forms called alleles, which determine which aspect of the characteristic is shown (e.g. tallness or shortness for the characteristic of height). A gene occupies a specific position (locus) on a chromosome. In view of the discoveries of molecular genetics, it may be defined as the sequence of nucleotides of DNA (or RNA) concerned with a specific function, such as the synthesis of a single polypeptide chain or of a messenger RNA molecule, corresponding to a particular sequence of the genetic code. One or more of these structural genes, coding for protein, may be associated with other genes controlling their expression.

genome: The whole of genes within a eukaryotic cell that also includes the genes of DNA containing organelles and nuclear genes.

guanine: A purine derivative. It is one of the major component bases of nucleotides and the nucleic acids DNA and RNA.

habitat: The place in which an organism lives, which is characterized by its physical features or by the dominant plant types. Freshwater habitats, for example, include streams, ponds, rivers, and lakes.

helminthology: The study of worms.

hermaphrodite (bisexual): An animal, such as the earthworm, that has both female and male reproductive organs.

homology (homologous): Describing a character that is shared by a group of species because it is inherited from a common ancestor. Such characters, called homologies, are used in cladistics to determine the evolutionary relationships of species or higher taxa. They are divided into two types:

a shared derived homology (apomorphy) is unique to a particular group and may be used to define a monophyletic group; a shared ancestral homology (plesiomorphy) is not unique to the group, or may not be exhibited by all descendants of the ancestor in which it arose (paraphyletic)

host: An organism whose body provides nourishment and shelter for a parasite. A definitive (or primary) host is one in which an animal parasite becomes sexually mature; an intermediate (or secondary) host is one in which the parasite passes the larval or asexual stages of its life cycle.

DNA hybridization: A method of determining the similarity of DNA from different sources. Single strands of DNA from two sources, e.g. different bacterial species, are put together and the extent to which double hybrid strands are formed is estimated. The greater the tendency to form these hybrid molecules, the greater the extent of complementary base sequences, i.e. gene similarity. The method is one way of determining the genetic relationship of species.

infection: The invasion of any living organism by disease-causing microorganisms (see pathogen), which proceed to establish themselves, multiply, and produce various symptoms in their host. Pathogens may invade via a wound or (in animals) trough the mucous membranes lining the alimentary, respiratory, and reproductive tracts, and may be transmitted by an infected individual, a carrier, or an arthropod vector.

infestation: A type of parasitism where one of the three criteria for infection (invasion, reproduction, immune response) is not fulfilled.

integument: The outermost body layer of an animal, characteristically comprising a layer of living cells – the epidermis – together with a superficial protective coat, which may be a secreted hardened cuticle, as in arthropods, or dead keratinized cells, as in vertebrates.

internal transcribed spacers-regions (ITS): non coding gene locus between two genes that code for rRNAs.

larva: (pl. larvae): The juvenile stage in the life cycle of most invertebrates, amphibians, and fish, which hatches from the egg, is unlike the adult in form, and is normally incapable in sexual reproduction.

life cycle: The complete sequence of events undergone by organisms of a particular species from the fusion of gametes in one generation to the same stage in the following generation. In most animals gametes are formed by meiosis of germ cells in the reproductive organs of the parents. The zygote, formed by the fusion of two gametes, eventually develops into an organism essentially similar to the parents.

metacercaria (pl. metacercariae): Developmental stage of digeneans.

Metazoa (Eumetazoa): A subkingdom comprising all multicellular animals. It excludes the Porifera (sponges) and Placozoa, which are placed in a separate subkingdom, Parazoa.

miracidium: The first larval stage of trematode worms (flukes), which hatches from eggs excreted in the faeces of the primary host. Its leaflike body is covered with cilia, enabling the larva to swim towards a secondary host, in which it continues its development.

mitochondrial DNA (mtDNA): A circular ring of DNA found in mitochondria. In mammals mtDNA makes up less than 1% of the total cellular DNA, but in plants the amount is variable. It codes for ribosomal and transfer RNA but only some mitochondrial proteins (up to 30 proteins in animals), the nuclear DNA being required for encoding most of these. Human mtDNA codes for 13 proteins and some RNA. Mitochondrial DNA is generally inherited via the female line only, although there are some exceptions to this.

mitochondrion: Cell organelles that function as energy sources and contain DNA.

molecular systematics (biochemical taxonomy): The use of amino-acid or nucleotide-sequence data in determining the evolutionary relationships of different organisms. Essentially it involves comparing the sequences of functionally homologous molecules from each organism being studies, and determining the number of differences between them. The greater the number of differences, the more distantly related the organisms are likely to be.

Mollusca: A phylum of soft-bodied invertebrates characterized by an unsegmented body differentiated into a head, a ventral muscular foot used in locomotion and a dorsal visceral hump covered by a fold of skin – the mantle – which secretes a protective shell in many species. Respiration is by means of gills (ctenidia) or a lunglike organ and the feeding organ is a radula. Molluscs occur in marine, freshwater and terrestrial habitats and there are six classes, including the Gastropoda (snails, slugs, limpets, etc.), Bivalvia (bivalves, e.g. mussels, oysters) and Cephalopoda (squids and octopuses).

Monogenea: Subclass of the Trematoda that is characterised by the absence of alternation of hosts or generations.

monophyly (monophyletic): In systematic, describing a group of organisms that contains all the descendants of a particular single common ancestor. In cladistics such a grouping is called a clade and is the only type of group regarded as valid when constructing classification schemes.

monostome: Certain type of trematode cercariae that lack an abdominal sucker.

morphology: The study of the form and structure of organisms, especially their external form.

Nematoda: nematodes, roundworms; A phylum of pseudocoelomate invertebrates comprising the roundworms. They are characterizes by a smooth narrow cylindrical unsegmented body tapered at both ends. They shed their tough outer cuticle four times during life to allow growth. The microscopic free-living forms are found in all parts of the world, where they play an important role in the destruction and recycling of organic matter. The many parasitic nematodes are much larger; they include the filarial (*Wuchereria*) and Guinea worm (*Dracunculus*), which cause serious diseases in humans.

nucleic acid: A complex organic compound in living cells that consists of a chain of nucleotides. There are two types: DNA (deoxyribonucleic acid) and RNA (ribonucleic acid).

nucleotide: base; An organic compound consisting of a nitrogen-containing purine or pyrimidine base linked to sugar (ribose or deoxyribose) and a phosphate group. DNA and RNA are made up of long chains of nucleotides.

oesophagus: The section of the alimentary canal that lies between the pharynx and the stomach.

operculum: A lid or flap of skin covering an aperture, such as the gill slit cover of fish and larval amphibians and the horny calcareous operculum secreted by many gastropod molluscs, which closes the opening of the shell when the animal is inside. The term is also used for anatomical features of other animals or plants.

paraphyly (paraphyletic): In systematics, describing a group of organisms that excludes one or more descendants of a particular single common ancestor.

parasitism: An association in which one organism (the parasite) lives on (ectoparasitism) or in (endoparasitism) the body of another (the host), from which it obtains its nutrients. Some parasites inflict comparatively little damage on their host, but many cause characteristic diseases (these are, however, never immediately fatal, as killing the host would destroy the parasite's source of food). Parasites are usually highly specialized for their way of life, which may involve one host or several (if the life cycle requires it). They typically produce vast numbers of eggs, very few of which survive to find their way to another suitable host. Obligate parasites can only survive and reproduce as parasites; facultative parasites can also live as saprotrophs. The parasites of humans include fleas and lice (which are ectoparasites), various bacteria, protozoans, and fungi (endoparasites causing characteristic diseases), and tapeworms.

parasitology: The study of parasites.

parthenogenic (parthenogenesis): The development of an organism from an unfertilized egg. This occurs sporadically in many plants (e.g. dandelions and hawkweeds) and in a few animals, but in some species it is the main and sometimes only method of reproduction.

pathogen: Any disease-causing microorganism. Pathogens include viruses and many bacteria, fungi and protozoans.

phylogeny: The evolutionary history of an organism or group of related organisms.

phylum: (pl. phyla); A category used in the classification of organisms that consists of one or several similar or closely related classes. Phyla are grouped into kingdoms.

Platyhelminthes: A phylum of acoelomate invertebrates comprising the flatworms, characterized by a flattened unsegmented body. The simple nervous system shows some concentration of cells at the head end. The mouth leads to a simple branched gut without an anus. Flatworms are hermaphrodite but self-fertilization is unusual. Many species are parasitic. The phylum contains the classes Turbellaria (planarians), Trematoda (flukes) and Cestoda (tapeworms).

polymerase: Any enzyme that catalyses the elongation of a polymeric molecule. FNA polymerases (types I to III) catalyse the synthesis of RNA using as a template either an existing DNA strand (DNA-dependent RNA polymerase) or an RNA strand. Type I is responsible for the synthesis of ribosomal RNA, type II for messenger RNA synthesis and type III for transfer RNA synthesis. DNA polymerases catalyse the elongation of a new DNA strand during DNA replication, using an existing DNA strand as template. RNA-directed DNA polymerase is more usually known as reverse transcriptase.

polymerase chain reaction: PCR; A technique used to replicate a fragment of DNA so as to produce many copies of a particular DNA sequence. PCR is commonly employed as an alternative to gene cloning as a means of amplifying genetic material for DNA sequencing. The technique has also proved invaluable in forensic science, enabling amplification of minute traces of genetic material for DNA fingerprinting or for detecting microsatellite DNA. The two strands of the DNA are separated by heating (denaturation) and short sequences of a single DNA strand (primers) are added (annealing), together with a supply of free nucleotides and DNA polymerase obtained from a bacterium that can withstand extreme heat. In a series of heating and cooling cycles, the DNA sequence flanked by the primers doubles with each cycle and is thus rapidly amplified.

polyphyly (polyphyletic): Describing a group of organisms that contains the descendants of two or more different ancestors, while excluding other descendants of a single ultimate common ancestor. Such a group may be constructed on the basis of certain shared traits, which may have evolved convergently (see convergent evolution), but it does not necessarily reflect any close evolutionary relationship, and is therefore rejected as a basis for phylogenetic classification.

prevalence: Percentage of infected or sick individuals in a population at a certain point of time.

primer: Short single-strand DNA fragment, that binds to one end of a certain DNA sequence (template) in a PCR system.

Protozoa: A group of unicellular or acellular, usually microscopic organisms some of them parasites, including the agents causing malaria (Plasmodium) and sleeping sickness (Trypanosoma).

redia (pl. rediae): Larval stage of certain trematodes with mouth, gut, central nervous system, salivary gland and birth pore

ribosomal DNA (rDNA): DNA-segments that contain the genes for ribosomal RNA.

RNA: ribonucleic acid; A complex organic compound (a nucleic acid) in living cells that is concerned with protein synthesis. In some viruses, RNA is also the hereditary material. Most RNA is synthesized in the nucleus and then distributed to various parts of the cytoplasm. An RNA molecule consists of a long chain of nucleotides in which the sugar is ribose and the bases are adenine, cytosine, guanine and uracil. Messenger RNA (mRNA) is responsible for carrying the genetic code transcribed from DNA to specialized sites within the cell (ribosomes), where the information is translated into protein composition (transcription, translation). Ribosomal RNA (rRNA) is present in ribosomes; it is single-stranded but helical regions are formed by base pairing within the strand. Transfer RNA (tRNA, soluble RNA, sRNA) is involved in the assembly of amino acids in a protein chain being synthesized at a ribosome. Each tRNA is specific for an amino acid and bears a triplet of bases complementary with a triplet on mRNA. RNA can associate with proteins to form complexes calles ribonucleoproteins.

roundworms: see Nematoda

rumen: The second of four chambers that form the stomach of ruminants. Ruminantia are a suborder of hooved animals comprising the sheep, cattle, goats, deer and antelopes.

sequencing (DNA sequencing, gene sequencing): The process of elucidating the nucleotide sequence of a DNA fragment. Two techniques are used, the Maxam-Gilbert method and the Sanger or dideoxy method. DNA sequencing is now employed on a major scale, for example in determining the nucleotide sequence of entire genomes.

species: 1. A group of organisms that resemble each other more than they resemble members of other groups and cannot be subdivided into two or more species. The precise definition of what constitutes a species differs depending on which species concept is applied. According to the biological species concept, a species comprises a group of individuals that can usually breed among themselves and produce fertile offspring. However, many other species concepts have been proposed, including the phylogenetic species concept and various typological species concepts. Typically, a species consists of numerous local populations distributed over a geographical range. Within a species, groups of individuals become reproductively isolated because of geographical or behavioural factors, and over time may evolve different characteristics and form a new and distinct species. 2. A rank or category used in the classification of organism. Similar species are grouped into a genus, and a single species may be subdivided into subspecies or races.

spectrophotometer: An instrument to measure concentrations of DNA, RNA or proteins by means of light emittance.

stoma (pl. stomata): A pore, large numbers of which are present in the epidermis of leaves (especially on the undersurface) and young shoots. Stomata function in gas exchange between the plant and the atmosphere. Each stoma is bordered by two semicircular guard cells, whose movements (due to changes in water content) control the size of the aperture. The term stoma is also used to mean both the pore and its associated guard cells.

syncitium (pl. syncytia): A group of animal cells in which cytoplasmic continuity is maintained. In some syncytia the cells remain discrete but are joined together by cytoplasmic bridges.

systematics: The study of the diversity of organisms and their natural relationships. It is sometimes used as a synonym for taxonomy. The term biosystematics (or experimental taxonomy) describes the experimental study of diversity, especially at the species level. Biosystematic methods include breeding experiments, field work, biochemical work (known as chemosystematics) and cytotaxonomy.

tapeworms: see Cestoda

taxon (pl. taxa): Any named taxonomic group of any rank in the hierarchical classification of organisms. Thus the taxa Papilionidae, Lepidoptera, Hexapoda, and Uniramia are named examples of a family, order, and phylum, respectively.

taxonomy: The study the theory, practice and rules of classification of living and extinct organisms. The naming, description and classification of a given organism draws on evidence from a number of fields. Classical taxonomy is based on morphology and anatomy. Cytotaxonomy compares the size, shape and number of chromosomes of different organisms. Numerical taxonomy uses mathematical procedures to assess similarities and differences and establish taxonomic groups.

tegument: External body border of trematodes and cestodes with a complicated structure, which functions as a mechanical protection and a nutrient absorption organ.

thymine: A pyrimidine derivative and one of the major component bases of nucleotides and the nucleic acid DNA.

Trematoda (trematodes, flukes): A class of parasitic flatworms (Plathyhelminthes) comprising the flukes, such as Fasciola (liver fluke). Flukes have suckers and hooks to anchor themselves to the host and their body surface is covered by a protective cuticle. The whole life cycle may either occur within one host or require one or more intermediate hosts to transmit the infective eggs or larvae (cercaria, miracidium). Fasciola hepatica, for example, undergoes larval development in a

land snail (the intermediate host) and infects sheep (the primary host) when contaminated grass containing the larvae is swallowed.

Turbellaria: A class of free-living flatworms comprising the planarians, which occur in wet soils, fresh water and marine environments. Their undersurface is covered with cilia, used for gliding over stones and weeds. Planarians can also swim by means of undulations of the body.

Vertebrata: Any one of a large group of animals comprising all those members of the phylum Chordata that have backbones (vertebral column). Vertebrates include the fishes, amphibians, reptiles, birds, and mammals.

Definitions largely following:

Hine RS, Martin E (Editors) (2005). Dictionary of Biology. Constable & Robinson Ltd, and Grange Books, London, UK. pp. 698.

Walochnik J, Aescht E (2002). Glosssar medizinischer und biologischer Fachbegriffe. Denisia 6(184):573-596.

7 REFERENCES

Abdussalam M, Käferstein FK, Mott KE (1995). Food safety measures for the control of foodborne trematode infections. Food Control 6:71-79.

Andrews SJ (1999). The Life Cycle of *Fasciola hepatica*. In: Fasciolosis, ed. Dalton JP, Wallingford, Oxon, UK; CABI Publishing 1-527.

Ashford RW, Crewe W (1998). The parasites of *Homo sapiens*. Liverpool School of Tropical Medicine, Liverpool, United Kingdom.

Ashrafi K, Valero MA, Massoud J, Sobhani A, Solaymani-Mohammadi S, Conde P, Khoubbane M, Bargues MD, Mas-Coma S (2006). Plant-borne Human Contamination by Fascioliasis. American Journal of Tropical Medicine and Hygiene 75 (2): 295-302.

Aspöck H (2002). Amöben, Bandwürmer, Zecken. Parasiten und parasitäre Erkrankungen des Menschen in Mitteleuropa. Denisia 6: 1-600.

Auer H (2009). Helminthozoonosen in Mitteleuropa: Epidemiologie – Klinik – Diagnostik – Therapie. Lecture slides, Department of Medical Parasitology, Institute of Specific Prophylaxis and Tropical Medicine, Medical University of Vienna.

Bassi R (1875). Sulla cachessia ittero-vermiosa, o marciaia dei cervi, causata dal *Distomum magnum*. Il Medico Veterinario 4: 497-515.

Bauer O, Polianski Yu, Willmott S (1982). V. A. Dogiel (1882-1953). Systematic Parasitology 4:256.

Bidloo G (1698). Brief van G Bidloo aan Antony van Leeuwenhoek, wegens de dieren, welke men zomtyds in de lever der schaapen en andere beesten vind. Delft, pp 34. In: The select works of Anthony van Leeuwenhoek containing his microscopical discoveries in many of the works of nature, translated by S Hoole from the Dutch and Latin editions, Henry Fry, London, 1798

Blair D, Campos A, Cummings MP, Laclette JP (1996). Evolutionary Biology of Parasitic Platyhelminths: The Role of Molecular Phylogenetics. Parasitology Today 12(2):66-71.

Böhm M, White PCL, Daniels MJ, Allcroft DJ, Munro R, Hutchings MR (2006). The health of wild red and sika deer in Scotland: An analysis of key endoparasites and recommendations for monitoring disease. The Veterinary Journal 171: 287-294.

Bray RA, Blair D (2008). Chapter 9 Superfamily Gorgoderoidea Looss, 1899. In: Keys to the Trematoda, Vol. 3, eds. Bray RA, Gibson DI, Jones A, CAB International and The Natural History Museum, London, 2008.

Bray RA, Gibson DI, Jones A (Editors) (2008). Keys to the Trematoda, Vol. 3. CAB International and The Natural History Museum, London. pp 824.

Bremser JG (1819). Lebende Würmer im lebenden Menschen; ein Buch für ausübende Ärzte. Schaumburg, Wien: 1-248, 4 Tafeln.

Bryan CP (1930). The Papyrus Ebers. Geoffrey Bles, London, United Kingdom.

Budd G (1852). Diseases of the liver. John Churchill, London, second edition, pp 186.

Cameron TW (1931). Experimental infection of sheep with *Dicrocoelium dendriticum*. Journal of Helminthology 9: 41-44.

Cobbold TS (1855). Description of a new trematode worm (*Fasciola gigantica*). Edinburgh New Philosophical Journal, new series, 2: 262-267.

Coombs I, Crompton DWT (1991). A guide to human helminthology. Taylor & Francis, London, United Kingdom.

Cox FEG (2002). History of Human Parasitology. Clinical Microbiology Reviews 15(4):595-612.

Cribb TH, Bray RA, Littlewood DTJ, Pichelin SP, Herniou EA (2001). Chapter 16 The Digenea. In: Interrelationships of the Platyhelminthes, eds. Littlewood DTJ, Bray RA, pp 168-185.

Cribb TH, Chisholm LA, Bray RA (2002). Diversity in the Monogenea and Digenea: does lifestyle matter? International Journal for Parasitology 32: 321-328.

Cucher MA, Carnevale S, Prepelitchi L, Labbé JH, Wisnivesky-Colli C (2006). PCR diagnosis of *Fasciola hepatica* in field-collected *Lymnaea columella* and *Lymnaea viatrix* snails. Veterinary Parasitology 137:74-82.

Davis A (2009). Chapter 82 Schistosomiasis. In: Manson's Tropical Diseases, eds. Cook GC, Zumla AI, Saunders Elsevier, 22nd edition, 2009.

de Brie J. (1379). Le bon berger ou le vray régime et gouvernement des bergers et bergères. Composé par le rustique Jehan de Brie le bon berger, Isidor Liseux, Paris, pp 160, 1879 (reprinted from the edition of 1541).

Díaz P, Pedreira J, Sánchez-Andrade R, Suárez JL, Arias MS, Francisco I, Fernández G, Díez-Baños P, Morrondo P, Paz-Silva A (2007). Risk periods of infection by *Calicophoron daubneyi* (Digenea: Paramphistomidae) in cattle from oceanic climate areas. Parasitology Research 101:339-342.

Dobell C (1939). The common flagellate of the human mouth, *Trichomonas tenax* (O.F.M.): Its discovery and its nomenclature. Parasitology 31, 138-146.

Dreyfuss G, Vignoles EP, Rondelaud ED (2005). *Fasciola hepatica*: epidemiological surveillance of natural watercress beds in central France. Parasitology Research 95:278-282.

Duenngai K, Sithithaworn P, Rudrappa UK, Iddya K, Laha T, Stensvold CR, Strandgaard H, Johansen MC (2008). Improvement of PCR for Detection of *Opisthorchis viverrini* DNA in Human Stool Samples. Journal of Clinical Microbiology 46(1):366-368.

Duscher R, Duscher G, Hofer J, Tichy A, Prosl H, Joachim A (2011). Fasciola hepatica – Monitoring the milky way? The use of tank milk for liver fluke monitoring in dairy herds as base for treatment strategies. Veterinary Parasitology 2011, Jan. 26 (Epub ahead of print).

Eduardo SL (1983). The taxonomy of the family Paramphistomidae Fischoeder, 1901 with special reference to the morphology of species occurring in ruminants. III. Revision of the genus *Calicophoron* Näsmark, 1937. Systematic Parasitology 5: 25-79.

Egerton FN (2001). A History of the Ecological Sciences, Part 4. Roman Natural History. Bulletin of the Ecological Society of America 2001, 243-246.

Ehlers U (1984). Phylogenetisches System der Platyhelminthes. Verhandlungen des Naturwissenschaftlichen Vereins in Hamburg 27:291-294.

Enigk K (1986). Geschichte der Helminthologie im deutschsprachigen Raum. Gustav Fischer Verlag, Stuttgart, New York. pp. 356.

Erhardová-Kotralá B, Kotrlý A (1968). Einschleppen eines Parasiten der Gattung *Fascioloides* beim Import lebenden Wildes aus anderen Kontinenten. Zeitschrift für Jagdwissenschaft 14(4):170-176.

Faber J (1670). Note in GJ Sach's Scholium to T. Bartholin's "Sanguis verminosus", Lipsiae, p 147.

Foreyt WJ (1996). Mule deer (*Odocoileus hemionus*) and Elk (*Cervus elaphus*) as experimental definitive hosts for *Fascioloides magna*. Journal of Wildlife Diseases 32(4):603-606.

Foreyt WJ, Drew ML (2010). Experimental Infection of Liver Flukes, *Fasciola hepatica* and *Fascioloides magna*, in Bison (*Bison bison*). Journal of Wildlife Diseases 46(1):283-286.

Foster WD (1965). A History of Parasitology. Livingsstone, Edinburgh & London: 1-201.

Fried (1997). Chapter 1 An overview of the biology of trematodes. In: Advances in trematode biology, eds. Fried B, Graczyk TK.

Fried B, Graczyk TK, Tamang L (2004). Food-borne intestinal trematodiases in humans. Parasitology Research 93:159-70.

Galaktionov KV, Dobrovolskij AA (2003). Chapter 3 The main types of trematode life cycles. In: The biology and evolution of trematodes: An Essay on the Biology, Morphology, Life Cycles, Transmissions, and Evolution of Digenetic Trematodes, eds. Galaktionov KV, Dobrovolskij AA, Kluwer, Boston, MA, 620 pp.

Gast RJ, Byers TJ (1995). Genus- and subgenus-specific oligonucleotide probes for *Acanthamoeba*. Molecular and Biochemical Parasitology 71:255-260.

Gérard C (2001). Consequences of a drought on freshwater gastropod and trematode communities. Hydrobiologica 459:9-18.

Gibson DI (1987). Questions in digenean systematic and evolution. Parasitology 95:429-460.

Gibson DI (2002). Chapter 1 Class Trematoda Rudophi 1808. In: Keys to the Trematoda, Vol. 1, eds. Gibson DI, Jones A, Bray RA, pp 1-3.

Gibson DI, Bray RA (1994). The evolutionary expansion and host-parasite relationships of the Digenea. International Journal for Parasitology 24(8):1213-1226.

Gibson DI, Jones A, Bray RA (Editors) (2002). Keys to the Trematoda, Vol. 1. CAB International and The Natural History Museum, London. pp 521.

Goddard FW (1919). *Fasciolopsis buski*, a parasite of man as seen in Shaohsing, China. Journal of Parasitology 5:141-163.

Grove DI (1990). A history of human helminthology. CAB International 1-848, Wallingford, United Kingdom.

Gutierrez Y (2000). Diagnostic pathology of parasitic infections with clinical correlations. 2nd ed. Oxford University Press, New York.

Haiabi M, Pernegger Ch, Brinninger G, Auer H (1997). *Fasciola hepatica* in Österreich – Ein Fallbericht. Mitteilung der Österreichischen Gesellschaft für Tropenmedizin und Parasitologie 19:137-142.

Haider M (2010). Bestimmung der Prävalenz von *Fascioloides magna* (BASSI 1875) und anderen digenen Trematoden in *Galba truncatula* (O.F. MÜLLER 1774) an drei ausgewählten Standorten im Bereich Orth/Donau (Niederösterreich). Diplomarbeit, Universität Wien.

Hoeppli R (1956). The knowledge of parasites and parasitic infections from ancient times to the 17th century. Experimental Parasitology 5:398-419.

Hoeppli R (1959). Parasites and parasitic infections in early medicine and science. University of Malaya Press, Singapore, Singapore.

Hörweg C., Sattmann H., Auer H. (2006). Cercarial dermatitis in Austria: Questionaires as useful tools to estimate risk factors? Wiener Klinische Wochenschrift 118[suppl 3]:77-80.

Jones A (2005a). Chapter 6 Family Fasciolidae Railliet, 1895. In: Keys to the Trematoda, Vol. 2, eds. Jones A, Bray RA, Gibson DI, CAB International and The Natural History Museum, London, 2005.

Jones A (2005b). Chapter 19 Superfamily Paramphistomoidea Fischoeder, 1901. In: Keys to the Trematoda, Vol. 2, eds. Jones A, Bray RA, Gibson DI, CAB International and The Natural History Museum, London, 2005.

Jones A (2005c). Chapter 1 Introduction and Key to Superfamilies. In: Keys to the Trematoda, Vol. 2, eds. Jones A, Bray RA, Gibson DI, CAB International and The Natural History Museum, London, 2005.

Jones A (2005d). Chapter 20 Family Paramphistomidae Fischoeder, 1901. In: Keys to the Trematoda, Vol. 2, eds. Jones A, Bray RA, Gibson DI, CAB International and The Natural History Museum, London, 2005.

Jones A, Bray RA, Gibson DI (Editors) (2005). Keys to the Trematoda, Vol. 2. CAB International and The Natural History Museum, London. pp 768.

Kaplan RM, Dame JB, Reddy GR, Courtney CH (1995). A Repetitive DNA Probe for the Sensitive Detection of *Fasciola hepatica* Infected Snails. International Journal for Parasitology 25(5):601-610.

Kaplan RM, Dame JB, Reddy GR, Courtney CH (1997). The prevalence of Fasciola hepatica in its Snail Intermediate Host Determined by DNa Probe Assay. International Journal for Parasitology 27(12):1585-1593.

Kayser FH, Bienz KA, Eckert J, Zinkernagel RM (2001). Medizinische Mikrobiologie. 10., komplett überarbeitete Auflage, Thieme Verlag.

Keiser J, Utzinger J (2005). Emerging Foodborne Trematodiasis. Emerging Infectious Diseases 11(10):1507-1514.

Keiser J, Utzinger J (2009). Food-Borne Trematodiases. Clinical Microbiology Reviews 22(3):466-483.

Keiser J, Veneziano V, Rinaldi L, Mezzino L, Duthalter U, Cringoli G (2010). Anthelmintic activity of artesunate against Fasciola hepatica in naturally infected sheep. Research in Veterinary Science 88(1):107-110.

Kolářová L (2007). Schistosomes causing cercarial dermatitis: a mini-review of current trends in systematic and of host specificity and pathogenicity. Folia Parasitologica 54:81-87.

Kostadinova A, Jones A (2005). Chapter 2 Superfamily Echinostomatoidea Looss, 1899. In: Keys to the Trematoda, Vol. 2, eds. Jones A, Bray RA, Gibson DI, CAB International and The Natural History Museum, London, 2005

Krull WH, Mapes CR (1952). Studies on the biology of *Dicrocoelium dendriticum* (Rudolphi 1819) Looss, 1899 (Trematoda: Dicroceoliidae) including its relation to the intermediate host, *Cionella lubrica* (Müller). V. Notes on infections of *Dicrocoelium dendriticum* in *Cionella lubrica*. Cornell Veterinarian 42:339-351.

Krull WH, Mapes CR (1953). Studies on the biology of *Dicrocoelium dendriticum* (Rudolphi 1819) Looss, 1899 (Trematoda: Dicrocoeliidae) including its relation to the intermediate host, *Cionella lubrica* (Müller). IX. Notes on the cyst, metacercaria, and infection in the ant, *Formica fusca*. Cornell Veterinarian 43:389-409.

La Rue GR (1957). The classification of digenetic Trematoda: a review and a new system. Experimental Parasitology 6(3):306-349.

Lankester ER (1857). Appendix B. In: F. Küchenmeister's "On animal and vegetable parasites of the human body. A manual of their natural history, diagnosis and treatment. Volume 1. Animal parasites belonging to the group entozoa", translated by E Lankester, The Sydenham Society, London, pp 452.

Leuckart KGFR (1881). Zur Entwicklungsgeschichte des Leberegels. Zoologisccher Anzeiger 4:641-646.

Leuckart KGFR (1882). Zur Entwicklungsgeschichte des Leberegels. Zweiter Mittheilung. Zoologischer Anzeiger 5:524-528.

Levine ND (1965). Refresher course on recent advances in parasitology: introductory remarks. American Zoologist 5(1):73-77.

Li M, Gong J, Cottrill M, Yu H, de Lange C, Burton J, Topp E (2003). Evaluation of QIAamp® DNA Stool Mini Kit for ecological studies of gut microbiota. Journal of Microbiological Methods 54:13-20.

Linnaeus C (1758). Systema Naturae, sive regina tria naturae systematice proposita por classes, ordines, genera, species cum characteribus differentiis synonymis, locis, 10th ed. L. Salvi, Holmiae, Sweden.

Lutz A (1892). Zur Lebensgeschichte des *Distoma hepaticum*. Zentralblatt für Bakteriologie, Parasitenkunde und Infektionskrankheiten Abteilung 11:783-796.

Lutz A (1893). Weiteres zur Lebensgeschichte des *Distoma hepaticum*. Zentralblatt für Bakteriologie, Parasitenkunde und Infektionskrankheiten Abteilung 13:320-328.

Mage C, Bourgne H, Toullieu JM, Rondelaud D, Dreyfuss G (2002). Fasciola hepatica and Paramphistomum daubneyi: changes in prevalences of natural infections in cattle and in Lymnaea truncatula fro central France over the past 12 years. Veterinary Research 33:439-447.

Majoros G, Sztojkov V (1994). Apperance of the large American liver fluke *Fascioloides magna* (Bassi, 1875) (Trematoda: Fasciolata) in Hungary. Parasitologia Hungarica 27:27-38.

Mas-Coma S, Bargues MD, Esteban JG (1998). Human fasciolosis. In: Fasciolosis. Dalton JP (ed.), CAB International, Oxon, pp. 411-434.

Mas-Coma S, Bargues MD, Valero MA (2005). Fasioliasis and other plant-borne trematode zoonoses. International Journal for Parasitology 35:1255-1278.

Mas-Coma S, Valero MA, Bargues MD (2009). Climate change effects on trematodiases, with emphasis on zoonotic fascioliasis and schistosomiasis. Veterinary Parasitology 163:264-280.

Mayer CL, Palmer CJ (1996). Evaluation of PCR, Nested PCR, and Fluorescent Antibodies for Detection of Giardia and Cryptosporidium Species in Wastewater. Applied and Environmental Microbiology 62(6):2081-2085.

McCann CM, Baylis M, Williams DJ (2010). Seroprevalence and spatial distribution of Fasciola hepatica-infected dairy herds in England and Wales. Veterinary Record 166(20):612-617.

McClanahan SL, Stromberg BE, Hayden DW, Averbeck GA, Wilson JH (2005). Natural infection of a horse with *Fascioloides magna*. Journal of Veterinary Diagnostic Investigation 17:382-385.

Mehlhorn H, Piekarski G (1995). Grundriss der Parasitenkunde: Parasiten des Menschen und der Nutztiere. 4. Auflage. Gustav Fischer Verlag, Stuttgart, Jena.

Moulé L (1911). La parasitologie dans la littérature antique. II Les parasite du tube digestif. Archives de Parasitologie 14:353-383.

Müller JF (1773). Vermium terrestrium et fluviatilium, seu animalium infusoriorum, helminthicorum et testaceorum, non marinorum succincta historia. 1:1-135. Havniae et Lipsiae.

Nakagawa K (1921). On the life cycle of *Fasciolopsis buski*, Lankester. Kitasato Archives of Experimental Medicine 4:159-167.

Nakagawa K (1922). The development of *Fasciolopsis buski* (Lankester). Journal of Parasitology 8:161-165.

Nicholas KB, Nicholas HB Jr., Deerfield DW (1997). GeneDoc: Analysis and Visualization of Genetic Variation. EMBnet News 4:14.

Nöller W (1929). Befunde bei Schnecken von Thüringer Schafweiden in einem Lanzettegelgebiete. Tierärztliche Rundschau 35:485-489.

Novobilský A, Horáčková E, Hirtová L, Modrý D, Koudela B (2007). The giant liver fluke *Fascioloides magna* (Bassi 1875) in cervids in the Czech Republic and potential of its spreading to Germany. Parasitology research 100:549-553.

Oberhauserová K, Bazsalovicsová E, Králová-Hromadová I, Major P, Reblánová M (2010). Molecular discrimination of eggs of cervid trematodes using the Teflon (PTFE) technique for eggshell disruption. Helminthologia 47(3):147-151.

Olson PD, Cribb TH, Tkach VV, Bray RA, Littlewood DTJ (2003). Phylogeny and classification of the Digenea (Platyhelminthes: Trematoda). International Journal for Parasitology 33:733-755.

Olson PD, Tkach VV (2005). Advances and Trends in the Molecular Systematics of the Parasitic Platyhelminthes. In: Advances in Parasitology, Vol. 60, eds. Baker JR, Muller R, Rollinson D, pp. 165-243.

Pallas PS (1760). Dissertatio medica inauguralis de infestis viventibus intraviventia, Lugduni Batavorum.

Pfeiffer, H., 1983. *Fascioloides magna*: Erster Fund in Österreich. Wiener Tierärztliche Monatsschrift 70:168-170.

Pojmańska T (2008). Chapter 16 Family Dicrocoeliidae Looss, 1899. In: Keys to the Trematoda, Vol. 3, eds. Bray RA, Gibson DI, Jones A, CAB International and The Natural History Museum, London, 2008.

Prosl H, Kutzer E (2006). Bedeutende Parasitosen beim Schalenwild. In: Parasiten bei Wildtieren und deren jagdwirtschaftliche Bedeutung, Duscher G (ed.). Fachtagung der Veterinärmedizinischen Universität Wien, Österreichischen Gesellschaft der Tierärzte und NÖ Landesjagdverband am 10.11.2006, Veterinärmedizinische Universität Wien.

Pybus MJ (1990). Survey of hepatic and pulmonary helminths of wild cervid in Alberta, Canada. Journal of Wildlife Diseases 26(4):453-459.

Pybus MJ (2001). Chapter 6 Liver flukes. In: Parasitic diseases of wild mammals, eds. Samuel WM, Kocan AA, Pybus MJ.

Rajković-Janje R, Bosnić S, Rimac D, Gojmerac T (2007). The prevalence of American liver fluke *Fascioloides magna* (Bassi 1875) in red deer from Croation hunting grounds. European Journal of Wildlife Research 54:525-528.

Rapsch C, Dahinden T, Heinzmann D, Torgerson PR, Braun U, Deplazes P, Hurni L, Bär H, Knubben-Schweizer G (2008). An interactive map to assess the potential spread of Lymnaea truncatula and the free-living stages of Fasciola hepatica in Switzerland. Veterinary Parasitology 154:242-249.

Rapsch C, Schweizer G, Grimm F, Kohler L, Bauer C, Deplazes P, Braun U, Torgerson PR (2006). Estimating the true prevalence of Fasciola hepatica in cattle slaughtered in Switzerland in the absence of an absolute diagnostic test. International Journal for Parasitology 36:1153-1158.

Reckendorfer W, Groiss M (2006). Der Amerikanische Riesenleberegel in den Donauauen östlich von Wien. Eine GIS-basierte Risikoanalyse. Bericht, pp. 39.

Reckendorfer W, Schaefer K (2003). Ökologische Grundlagen für das Vorkommen des Amerikanischen Riesenleberegels in den Donau-Auen östlich von Wien. Habitatcharakterisierung des Zwischenwirtes (*Galba truncatula*). (= nicht publizierte Voruntersuchung).

Redi F. (1685). Osservazioni intorno agli animali viventi che si trovano negli animali viventi. Piero Matini, Firenze: 1-243.

Rehbein S, Lutz W, Visser M, Winter R (2002). Beiträge zur Kenntnis der Parasitenfauna des Wildes in Nordrhein-Westfalen. 3. Der Endoparasitenbefall des Rotwildes. Zeitschrift für Jagdwissenschaft 48:69-93.

Rehbein S, Visser M (2007). Die Endoparasiten des Sikawildes (*Cervus nippon*) in Österreich. Wiener Klinische Wochenschrift 119 (Suppl 3):96-101.

Reyes PM, Ibarra VF, Vera MY, Cantó AG, Hernández A, Hernández CA, Castillo R, Villa MA (2008). Paramphistomicidal efficacy of an experimental compound in sheep. Parasitology Research 102:705-708.

Rudolphi CA (1803). Neue Beobachtungen über die Eingeweidewürmer. Archiv für Zoologie und Zootomie 3:1-32.

Rudolphi CA (1808). Entozoorum, sive vermium intestinalium historia naturalis, Treuttel & Würtz, Paris, three volumes, pp 1370.

Rudolphi CA (1819). Entozoorum synopsis cui accedunt mantissima duplex et indices locupletissima, Sumtibus Augusti Rücker, Berolini, pp 811.

Ruffer MA (1910). Note on the presence of *Bilharzia haematobia* in Egyptian mummies of the Twentieth Dinasty (1250-1000 BC). British Medical Journal 1:16.

Sanabria REF, Romero JR (2008). Review and update of paramphistomosis. Helminthologia 45(2):64-68.

Sattmann H (2002). Anfänge der systematischen Helminthologie in Österreich. Denisia 6(184):271-290.

Sattmann H, Hörweg C (2005). Untersuchung zur Messung der Befallsrate der Leberegelschnecke *Galba truncatula* mit dem Amerikanischen Riesenleberegel *Fascioloides magna* im Nationalpark Donau-Auen. pp 39 (donauauen.at)

Sey O (1979). Life-cycle and geographical distribution of *Paramphistomum daubneyi* Dinnik, 1962 (Trematoda: Paramphistomata). Acta Veterinaria Academiae Scientiarum Hungaricae, Tomus 27(1-2):115-130.

Sey O (1991). CRC Handbook of the Zoology of Amphistomes. CRC press Inc. Boca Ratón, Florida.

Sinitsin DF (1914). Neue Tatsachen über die Biologie der *Fasciola hepatica* L. Centralblatt für Bakteriologie, Parasitenkunde und Infektionskrankheiten 74:280-285.

Smyth JD, Halton DW (1983). Chapter 1 Digenetic trematodes: general considerations. In: The physiology of trematodes.

Steenstrup JJS (1845). On the alternation of generations; or, the propagation and development of animals through alternate generations (etc.). Translated from the German version of 1842. Ray Society, London, 132 pp.

Stensvold CR, Saijuntha W, Sithithaworn P, Wongratanacheewin S, Strandgaard H, Ørnbjerg N, Johansen MV (2006). Evaluation of PCR based coprodiagnosis of human opisthorchiasis. Acta Tropica 97:26-30.

Tang JN, Zeng ZG, Wang HN, Yang T, Zhang PJ, Li YL, Zhang AY, Fan WQ, Zhang Y, Yang X, Zhao SJ, Tian GB, Zou LK (2008). An effective method for isolation of DNA from pig faeces and comparison of five different methods. Journal of Microbiological Methods 75:432-436.

Thomas AP (1881). Report of experiments on the development of the liver-fluke (Fasciola hepatica). Journal of the Royal Agricultural Society of England 17:1-29.

Thomas AP (1882). Second report of experiments on the development of the liver-fluke (Fasciola hepatica). Journal of the Royal Agricultural Society of England 18:438-455.

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997). The Clustal_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Research 25(24):4876-4882.

Thornton CG, Passen S (2004). Inhibition of PCR amplification by phytic acid, and treatment of bovine fecal specimens with phytase to reduce inhibition. Journal of Microbiological Methods 59:43-52.

Umesha KR, Kumar S, Parvathi A, Duenngai K, Sithithaworn P, Karunasagar I, Karunasagar I (2008). *Opisthorchis viverrini*: Detection by polymerase chain reaction (PCR) in human stool samples. Experimental Parasitology 120:353-356.

Ursprung J (2002). Der Amerikanische Riesenleberegel *Fascioloides magna* (Digenea, Trematoda). In: Neobiota in Österreich, eds. Essl F, Rabitsch W, Umweltbundesamt, Wien, pp. 225-227.

Ursprung J, Joachim A, Prosl H (2006). Vorkommen und Bekämpfung des Amerikanischen Riesenleberegels, Fascioloides magna, in einer Schalenwildpopulation in den Donauauen östlich von Wien. Berliner und Müncher Tierärztliche Wochenschrift 119(7/8):316-323.

van Beneden PJ (1858). Mémoires sur les vers intestinaux. Supplement to Comptes Rendus Hebdomadaires des Séances de l'Académie des Sciences, Tome II, Paris, pp 376.

Velusamy R, Singh BP, Raina OK (2004). Detection of *Fasciola gigantica* infection in snails by polymerase chain reaction. Veterinary Parasitology 120:85-90.

Verbrugge LM, Rainey JJ, Reimink RL, Blankespoor HD (2004). Prospective study of swimmer's itch incidence and severity. Journal of Parasitology 90:697-704.

Vogel H (1929). Beobachtungen über *Cercaria vitrina* und deren Beziehung zum Lanzettegelproblem. Archiv für Schiffs- und Tropen-Hygiene 33:474-489.

Walker JH (1891). Two cases of beri-beri associated with *Distomum crassum*, *Anchylostoma duodenale* and other parasites. British Medical Journal ii: 1205.

Ward GR (1911). Hepatic distomiasis (sheep rot) in man. British Medical Journal i: 931-935.

Ward HB (1917). On the structure and classification of North American parasitic worms. Journal of Parasitology 4:1-11.

Winkelmayer R, Prosl H (2001). Riesenleberegel - jetzt auch bei uns? Weidwerk 3/2001:42-44.

Wulff C, Haeberlein S, Haas W (2007). Cream formulations protecting against cercarial dermatitis by Trichobilharzia. Parasitology Research 101:91-97.

Yu Z, Morrison M (2004). Improved extraction of PCR-quality community DNA from digesta and fecal samples. BioTechniques 36:808-812.

Zeder JGH (1800). JAE Goeze's Erster Nachtrag zur Naturgeschichte der Eingeweidewürmer mit Zusätzen und Anmerkungen herausgegeben von Johan Georg Heinrich Zeder, Siegfried Lebrecht Crusius, Leipzig, pp 320.

Zeder JGH (1803). Anleitung zur Naturgeschichte der Eingeweidewürmer. Bamberg, 432 pp.

http://www.donauauen.at

http://www.donauauen.at/files/254_NPKarte.jpg

http://www.ncbi.nlm.nih.gov/

http://www.ncbi.nlm.nih.gov/blast

http://www.ncbi.nlm.nih.gov/sites/entrez?db=nucleotide)

http://www.linkr.de/2009/03/24/mit-google-maps-laengen-breitengrade-ermitteln-freies-geocoding-skript/

8 APPENDIX

8.1 Abstract

8.1.1 German abstract

Mikroskopische und molekularbiologische Untersuchung von Rotwild-losungen auf digene Trematoden.

Digene Trematoden sind parasitische Plattwürmer aus der Gruppe der Metazoa, die sich durch obligatorischen Wirts- und Generationswechsel auszeichnen. In der Regel ist der Zwischenwirt eine Schnecke und der Endwirt ein Wirbeltier. Ziel dieser Studie war, die Prävalenz von Trematoden in Rotwild (*Cervus elaphus*) im Gebiet des Nationalparks Donau-Auen festzustellen.

Besonderes Augenmerk wurde auf den natürlich vorkommenden Großen Leberegel *Fasciola hepatica* und den eingeschleppten Amerikanischen Riesenleberegel *Fascioloides magna* sowie die Unterscheidung dieser zwei Spezies gelegt. Während *F. magna* nur für tierische Populationen eine bedeutende Rolle spielt, stellt *F. hepatica* auch für den Menschen eine Bedrohung dar.

Von Mai bis November 2008 wurde ein Monitoring der Trematodeninfektionen von Rotwild im Gebiet des Nationalparks Donau-Auen durchgeführt. Im Zuge dessen wurden 158 Rot- und Rehwildlosungen von 13 verschiedenen Standorten gesammelt, mittels Sedimentation aufbereitet und mikroskopisch untersucht. Außerdem wurden 109 ausgewählte Proben mit molekularbiologischen Methoden untersucht. Die Proben wurden homogenisiert, die DNA mittels zwei verschiedener Extrahierungskits isoliert und anschließend mit drei verschiedenen PCRs getestet. Die Proben wurden anschließend zur Artbestimmung sequenziert.

Bei der mikroskopischen Untersuchung der 158 Proben konnten in 49 Fällen (31%) Trematoden-Eier nachgewiesen werden, in 6 Proben (4%) wurden nicht eindeutig bestimmbare Eier gefunden. Bei der molekularbiologischen Untersuchung der ausgewählten 109 Proben konnten in 35 Proben (32%) Trematoden nachgewiesen werden. Die am häufigsten gefundene Trematodenart waren Vertreter der Familie der Fasciolide

(29 Proben) und Paramphistomidae (elf Proben). Bei einer Probe handelte es sich um Dicrocoeliidae und elf Proben enthielten mehr als eine Art von Trematodenspezies.

Die gesamte Trematodenlast im untersuchten Gebiet war 37%. Da eine gewisse Parasitenlast in Wildtieren durchaus üblich ist, war diese relative hohe Durchseuchungsrate nicht überraschend. Allerdings zeigt der hohe Prozentsatz der *Fascioloides magna* Infestationen das gefährliche Potential dieses eingeschleppten Parasiten. *F. hepatica* konnte in keiner Probe nachgewiesen werden.

8.1.2 English abstract

Microscopic and molecular analyses on digenean trematodes in red deer (*Cervus elaphus*).

Digenean trematodes are a large group of internal metazoan parasites that are characterized by a complex life cycle including mollusc and vertebrate hosts. This study focuses on trematodes of the red deer (*Cervus elaphus*) in the Danube backwater forests southeast of Vienna.

Particular emphasis was given to the naturally occurring liver fluke *Fasciola hepatica* and the introduced American liver fluke *Fascioloides magna* and the species specific differentiation between these two species. The former is a pathogen of medical and veterinary, the latter only of veterinary relevance.

Between May and November 2008, a *F. magna*-monitoring was performed within the area of the 'Nationalpark Donau-Auen'. Altogether, 158 faecal samples of deer were collected from 13 sites. All samples were processed for microscopy with a modified Benedek sedimentation method and subsequently screened for trematode eggs. 109 samples were chosen for molecular biological analysis, which were homogenized, the DNA was extracted by two different types of DNA-extraction kits and subjected to three different kinds of PCR (trematodes, *F. magna*, *F. hepatica*).Species-specific differentiation was achieved by sequencing.

In 49 of 158 samples (31%) trematode eggs were found by microscopical means. Additionally, in six samples eggs were found that could not be specified. With molecular

methods, 35 of 109 samples (32%) were positive in PCRs. The most frequent trematode species found were members of the family Fasciolidae (29 samples), followed by Paramphistomidae (eleven samples). One sample contained eggs of Dicrocoeliidae and eleven samples showed eggs/DNA of more than one trematode species.

The overall trematode burden in the investigated area was 37%. However, this relatively high percentage is not surprising concidering that a low constant parasite load is normal in wild living ruminants. However, the remarkably high percentage of positive *F. magna* samples points out the hazardous potential of an introduced parasite. No sample was positive with *F. hepatica*.

8.2 Index of Figures

8.3 Index of Tables

8.4 Protocol sheets

8.4.1 Sampling protocol

Protokoll-Blatt Aufsammlung

Datum

Uhrzeit

Standort

Wetter sonnig/regnerisch

bewölkt (Bewölkungsgrad in %)

Temperatur Lufttemp.

Wassertemp

Wasserstände (Wildungsmauer?)

Vegetation: Zusammensetzung? Höhe?

Kotproben

Wildspuren

Schnecken

Fotodokumentation

8.4.2 Microscopy protocol

Protokoll-Blatt Stuhlprobenuntersuchung

Probennummer

Untersuchungsdatum

Sammeldatum & Uhrzeit

Fundort

Menge (Lorbeeren & Gewicht)

Ergebnisse (wie viele Sedimenttropfen untersucht & wie viele Eier gefunden)

Anmerkungen

8.5 Acknowledgements

Special thanks I owe to Univ.-Doz. Mag. Dr. Julia Walochnik who gave me the opportunity to work on my diploma thesis in a scientifically professional but also very social and cheerful environment and for initiating the contact with HR Dr. Helmut Sattmann. Furthermore, I want to thank her for spending numerous hours of her time on my work, for her support and for the possibility to deepen my parasitological skills during employments at the Medical University.

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I am very grateful to Univ.-Prof. MMag. Dr. Sylvia Kirchengast for being my mentor and reviewing my diploma thesis, kindly spending her time and knowledge on my work.

Furthermore, I thank my colleague Mag. Michaela Haider, who worked on the second part of this study, for the company, conversation and laughs during the sampling.

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My greatest thanks I owe to my parents who supported me in every situation of my life. They encouraged me to find my own way and always showed me how proud of me they are.

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8.6 Curriculum Vitae

Personal information

Name:	Kerstin Liesinger
Residence:	1120 Wien
Date and Place of Birth:	15th of November 1982 in Villach (Austria)
Nationality:	Austria

Education

1989 – 1993:	Elementary school, Villach (Austria)
1993 – 1997:	Secondary school with focus on creativity and fine arts, Villach (Austria)
1997 – 2002:	Tourism academy, Villach (Austria) – A-level examinations with honours
2003 – 2006:	Study of general biology at the University of Vienna (Austria)
since 2006:	Study of anthropology (focus on Human Genetics) at the University of Vienna (Austria)
2007 – 2008:	Exchange semester via Erasmus Programme at the Vrije Universiteit Brussel (Belgium)
since May 2008:	Diploma thesis at the Dep. of Medical Parasitology, Inst. of Specific Prophylaxis & Tropical Medicine, Medical University of Vienna in collaboration with the Natural History Museum Vienna, 3rd Zoological Dep. Topic: Microscopic and molecular analyses on digenean trematodes in red deer (*Cervus elaphus*)

Further Activities

1998 – 2001:	Work experiences in the tourism sector in Austria, Germany and France

2002 – 2003:	Exchange year via European Voluntary Service in Aberystwyth (Wales, GB)
Summer 2005:	Work experience at the Department of Bacteriology, Institute of Pathology, Villach Regional Hospital
Since 2008:	Secretary of the Austrian Society of Tropical Medicine and Parasitology (ÖGTP)
July 2009:	Lecturer work for a workshop at the "Kinderuniversität Wien"
2009/10 + 2010/11:	Tutor at the Department of Medical Parasitology, Institute of Specific Prophylaxis and Tropical Medicine, Medical University of Vienna
2010-2011:	Biomedical technician (parasitological diagnostics) at the Institute of Specific Prophylaxis and Tropical Medicine, Medical University of Vienna

Attendance at national congresses

18. Oct. 2008:	Expert discussion of the Entomological Society of Austria in Vienna (Austria)
13. Nov. 2008:	Symposium on wild animal health in Vienna (Austria)
20.-22. Nov. 2008:	42nd annual conference of the ÖGTP in Innsbruck (Austria) +
2009 - 2010:	Advanced training events for Parasitology, Tropical Medicine and Travel Medicine in Vienna (Austria)
5. Jun. 2009:	Parasitological expert discussions in Vienna (Austria)
19.-21. Nov. 2009:	43rd annual conference of the ÖGTP in Vienna (Austria) *
02.-03. Dez. 2010:	4th annual meeting of the NOBIS Austria in Graz (Austria) +

*with oral presentation of current studies +with poster presentation of current studies

Awards

November 2009:	3rd Place Junior Award for best oral presentation at the ÖGTP meeting in Vienna