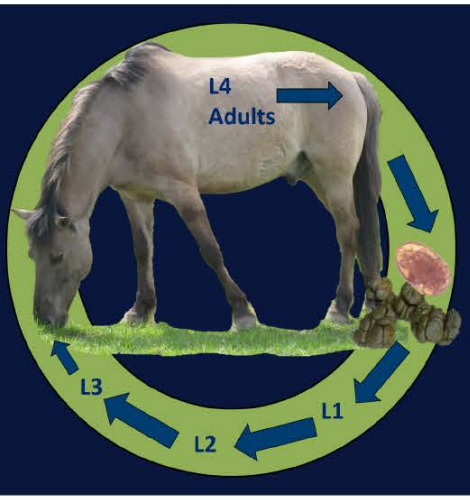
Aus dem
Institut für Parasitologie und Tropenveterinärmedizin
des Fachbereichs Veterinärmedizin
der Freien Universität Berlin

Molecular and Proteomic Species Characterisation of Cyathostomins



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PhD of Biomedical Sciences
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vorgelegt von
Christina Maria Bredtmann
Tierärztin aus Recklinghausen

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Coronocyclus coronatus, Cylicostephanus calicatus, Cylicostephanus longibursatus, Cylicostephanus minutus, MALDI-TOF, Polymerase chain reaction

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List of Abbreviations

AR	Anthelmintic Resistance
ATP	Adenosin Tri-Phosphate
AU	Arbitrary Units
BLASTN	Basic Local Alignment Search Tool for Nucleotides
BZ	Benzimidazole
Cab.	*Caballonema*
CAL	*Cylicostephanus calicatus*
CAT-score	Correlation-adjusted *t*-score
COI	Cytochrome Oxidase subunit 1
Cor.	*Coronocyclus*
COR	*Coronocyclus coronatus*
Cya.	*Cyathostomum*
Cyc.	*Cylicocyclus*
Cyd.	*Cylicodontophorus*
Cys.	*Cylicostephanus*
D	Day
Da	Dalton
DAAD	Deutscher Akademischer Austausch Service
DFG	Deutsche Forschungsgemeinschaft
DNA	Desoxyribonucleic Acid
DL3	Developing Third stage Larvae
EL3	Early Third stage Larvae
ELISA	Enzyme-Linked Immunosorbent Assay
EPG	Eggs Per Gramm (faeces)
ESCCAP	European Scientific Counsel Companion Animal Parasites
ETS	External Transcribed Spacer
FAO	Food and Agriculture Organization of the United Nations
FEC	Faecal Egg Count
FECRT	Faecal Egg Count Reduction Test
IGS	Intergenic Spacer
ITS	Internal Transcribed Spacer
L	Larvae
LL3	Late third stage Larvae

LDA	Linear Discrimant Analysis
LON	*Cylicostephanus longibursatus*
LSU	Large Subunit
MALDI-TOF MS	Matrix-Assisted Laser Desorption/Ionisation – Time Of Flight Mass Spectrometry
MIN	*Cylicostephanus minutus*
Mio.	Million (1,000,000)
ML	Macrocyclic Lactone
MSP	Main Spectra
mt	Mitochondrial
m/z	Mass by charge ratio
NCBI	National Center for Biotechnology Information
NTS	Non-transcribed Spacer
OTU	Operational Taxonomic Unit
PCR	Polymerase Chain Reaction
Pgp	P-Glycoprotein
r	Ribosomal
RLB	Reverse Line Blot
S	Svedberg
SDA	Shrinkage Discriminant Analysis
spp.	Species
SSU	Small subunit
VMP	Veterinary Medicinal Products
WAAVP	World Association for the Advancement of Veterinary Parasitology

1 Preface

With a global population of more than 59 Mio. horses, 43 Mio. asses and 9 Mio. mules (FAO, 2018a, 2018b, 2018c), equids play an important role for agriculture and for recreation. Except for arctic regions and the central African "Tsetse belt", their distribution is almost global. Including Zebras, the latter is covered as well. More than 440,000 horses were counted in Germany alone (FAO, 2018d) and the economic and cultural importance of these animals is the focus of a newly founded working group in the German parliament (Barz, 2018).

Consequently, welfare of these animals is of importance and similarly to other grazing animals, equids are at the constant risk of infestations with gastro-intestinal parasites. During the past decades, worm control in horses has, due to the essential lack of alternative effective control measures, predominantly relied on routine use of anthelmintics. However, this has led to the widespread development and spread of equine worm populations (Von Samson-Himmelstjerna, 2012).

Whereas cestodes such as *Anoplocephala* spp. and trematodes are only represented by a few species, the nematodes are represented by 83 species in 28 genera (Lichtenfels, 1975; Lichtenfels et al., 2008). Among these, the large roundworms (*Parascaris equorum* and *P. univalens)* and the Strongylinae (large strongyles) are of both veterinary and scientific interest because of their impact particularly on young horses, and their development of anthelmintic resistance or their high pathogenicity due to a visceral migration, respectively.

This dissertation however, focusses on the cyathostomins which often comprise over 95 % of the total worm burden of a horse (Nielsen, 2012). Cyathostomins are historically also referred to as cyathostomes, trichonemes, "small strongyles" or "small redworms". Although some of these terms are still commonly used, they will be avoided in the further manuscript as recommended by the Third WAAVP International Workshop on the Systematics of the Cyathostominea of Horses (Lichtenfels et al., 2002).

Cyathostomins are currently considered to be the most important equine parasites, because of their global distribution, the increasing number of reports regarding anthelmintic resistance and the potentially fatal outcome of the infection. Currently, 50 morphologically described species of cyathostomins are recognised as valid and occur in mixed infections with up to 29 species infecting one host (Chapman et al., 2003; Lichtenfels et al., 2008). Despite high research activity on the cyathostomins, species-specific knowledge is rare. Only little is known about the species-specific shares in the development of anthelmintic resistance or the species-specific pathogenicity, due to limited availability of appropriate tools to study cyathostomins on a species-specific basis.

Therefore, this thesis is structured as follows:

In **chapter 2**, an introduction into cyathostomins is given, including general and species-specific aspects of morphology, occurrence, life cycle as well as cyathostomin associated disease, anthelmintic management and resistance. Furthermore, an introduction into molecular and proteomic diagnostic methods is given.

This is followed by **chapter 3**, where in the context of a publication the current status, challenges and future perspectives for cyathostomin species identification and species-specific research are reviewed and discussed.

In **chapter 4**, a novel protocol for concurrent proteomic and molecular characterisation of cyathostomins is presented and evaluated with the example of two closely related cyathostomin species.

Two further cyathostomin species and their phylogenetic relationship are examined in **chapter 5**.

Finally, the work is discussed in **chapter 6** and summarised in English and German language in **chapters 7** and **8,** respectively.

2 Literature review

2.1 Overview on cyathostomins

2.1.1 Systematics

This thesis focusses on the parasitic group of the cyathostomins. This group currently comprises 50 species within the subfamily of the Cyathostominae that, next to the subfamily Strongylinae, belongs to the family Strongylidae in the order Strongylida (phylum Nematoda, class Chromodorea, order Rhabditida). The 50 cyathostomin species are organised into 14 genera and are summarized in **Table 2-1** (Lichtenfels et al., 2008). For improved reading of this thesis and to reduce confusion of genera, the names of the following genera are abbreviated with three letters instead of one: *Caballonema* (*Cab.*), *Coronocyclus* (*Cor.*), *Cyathostomum* (*Cya.*), *Cylicocyclus* (*Cyc.*), *Cylicodontophorus* (*Cyd.*), *Cylicostephanus* (*Cys.*), *Cylindropharynx* (*Cyp*.), *Parapoteriostomum* (*Par.*), *Petrovinema* (*Pet.*), *Poteriostomum* (*Pot.*).

Since the first descriptions of these parasites (Molin, 1861), the taxonomic organisation, based on morphological descriptions of these parasites, underwent constant changes and re-assessments, which resulted in a list of 93 species level names, including synonyms, for 51 recognised species in 1998 (Lichtenfels et al., 1998).

Back then, also the genus *Gyalocephalus* with the only species *Gyalocephalus capitatus* was grouped in its own subfamily of Gyalocephalinae. Whereas the overall taxonomy of the Strongylidae is generally accepted, it is speculated that the classification of subfamilies does not represent natural groups (Lichtenfels et al., 2008) and although research focus on these parasites is high, no comprehensive representation of a resolved molecular phylogeny of the subfamilies or different cyathostomin species is available.

Table 2-1: Overview of cyathostomin species (based on Lichtenfels et al. 2008)

Genus	Species	Described by	Host species (*Equus*)								Predilection site	Distribution
			E. caballus	*E. caballus x E. asinus*	*E. asinus*	*E. przewalski*	*E. hemionus*	*E. quagga burchelli*	*E. hartmannae*	*E. zebra*		
Caballonema	*Cab. longicapsulatum*	Abuladze, 1937	x								Caecum, colon	Asia
Coronocyclus	*Cor. coronatus*	Loos, 1900	x	x	x	x	x				Caecum, colon	Cosmopolitan
	Cor. labiatus	Loos, 1902	x	x	x	x	x	x			Caecum, colon	Cosmopolitan
	Cor. labratus	Loos, 1900	x	x	x	x	x				Caecum, colon	Cosmopolitan
	Cor. sagittatus	Kotlán, 1920	x	x		x					Colon	Europe, Asia
	Cor. ulambajari	Dvojnos et al., 1994	x								Caecum, colon	Asia
Cyathostomum	*Cya. alveatum*	Loos, 1900	x	x	x			x			Caecum, colon	Cosmopolitan
	Cya. catinatum	Loos, 1900	x	x	x	x	x	x			Caecum, colon	Cosmopolitan
	Cya. montgomereyi	Boulenger, 1920	x	x				x			Caecum, colon	Africa
	Cya. pateratum	Yorke and Macfie, 1919	x	x	x	x	x	x			Caecum, colon	Cosmopolitan
	Cya. tetracanthum	Mehlis, 1831	x		x			x			Caecum, colon	Cosmopolitan
Cylicocyclus	*Cyc. adersi*	Boulenger, 1920			x			x		x	Caecum, colon	Africa
	Cyc. ashworthi	Le Roux, 1924	x	x	x	x	x				Caecum, colon	Cosmopolitan
	Cyc. asini	Matthee et al., 2002			x			x		x	Caecum, colon	Cosmopolitan
	Cyc. auriculatus	Looss, 1900	x		x			x		x	Caecum, colon	Asia, Africa

	Cyc. brevicapsulatus	Ihle, 1920	x		x					Caecum, colon	Asia, Europe, North and South America
	Cyc. elongatus	Looss, 1900	x	x	x	x	x	x		Caecum, colon	Cosmopolitan
	Cyc. gyalocephaloides	Ortlepp, 1938						x		Caecum, colon	Africa
	Cyc. insigne	Boulenger, 1917	x	x	x	x	x			Caecum, colon	Cosmopolitan
	Cyc. leptostomum	Kotlán, 1920	x	x	x	x	x	x		Caecum, colon	Cosmopolitan
	Cyc. nassatus	Looss, 1900	x	x	x	x	x	x		Caecum, colon	Cosmopolitan
	Cyc. radiatus	Looss, 1900	x	x	x	x	x	x		Caecum, colon	Cosmopolitan
	Cyc. triramosus	Yorke and Macfie, 1920						x	x	Caecum, colon	Africa
	Cyc. ultrajectinus	Ihle, 1920	x		x		x	x		Caecum, colon	Cosmopolitan
Cylicodontophorus	*Cyd. bicoronatus*	Looss, 1900	x	x	x	x	x			Caecum, colon	Cosmopolitan
	Cyd. reineckei	Scialdo-Krecek and Malan, 1984						x	x	Caecum, colon	Africa
Cylicostephanus	*Cys. asymetricus*	Theiler, 1924	x		x					Caecum, colon	Cosmopolitan
	Cys. bidentatus	Ihle, 1925	x	x	x	x	x	x		Caecum, colon	Cosmopolitan
	Cys. calicatus	Loos, 1900	x	x	x	x	x			Caecum, colon	Cosmopolitan
	Cys. goldi	Boulenger, 1917	x	x	x	x	x	x		Caecum, colon	Cosmopolitan
	Cys. hybridus	Kotlán, 1920	x		x	x				Caecum, colon	Asia, Europe, North America
	Cys. longibursatus	Yorke and Macfie, 1918	x	x	x	x	x	x		Caecum, colon	Cosmopolitan
	Cys. minutus	Yorke and Macfie, 1918	x	x	x	x	x			Caecum, colon	Cosmopolitan
Cylindropharynx	*Cyp. brevicauda*	Leiper, 1911		x	x			x		Caecum, colon	Africa
	Cyp. intermedia	Theiler, 1924						x	x	Caecum, colon	Africa
	Cyp. longicauda	Leiper, 1911		x	x			x	x	Caecum, colon	Africa
Gyalocephalus	*G. capitatus*	Looss, 1900	x	x	x	x	x	x		Caecum, colon	Cosmopolitan

Hsungia	*H. pekingensis*	K'ung and Yang, 1964	x		x						Caecum, colon	Asia
Parapoteriostomum	*Par. euproctus*	Boulenger, 1917	x	x	x	x	x	x			Caecum, colon	Cosmopolitan
	Par. mettami	Leiper, 1913	x	x	x	x	x				Caecum, colon	Cosmopolitan
	Par. mongolica	Popova, 1958	x								Caecum, colon	Asia
	Par. shuermanni	Ortlepp, 1962						x			Caecum, colon	Africa
Petrovinema	*Pet. poculatum*	Looss, 1900	x	x	x	x	x				Caecum, colon	Cosmopolitan
	Pet. skrjabini	Ershov, 1930	x		x						Caecum, colon	Asia
Poteriostomum	*Pot. imparidentatum*	Quiel, 1919	x	x	x	x	x	x			Caecum, colon	Cosmopolitan
	Pot. ratzii	Kotlán, 1919	x	x	x	x	x	x			Caecum, colon	Cosmopolitan
Skrjabinodentus	*S. caragandicus*	Funikova, 1939	x	x							Caecum, colon	Asia
	S. longiconus	Scialdo-Krecek, 1983						x	x		Caecum, colon	South Africa
	S. tshoijoi	Dvojnos and Kharchenko, 1986	x								Caecum, colon	Asia
Tridentoinfundibulum	*T. gobi*	Popova, 1958	x	x							Caecum, colon	Asia, Europe, North America

2.1.2 Morphology

2.1.2.1 General morphology

As typical nematodes, all cyathostomins are round, symmetric, and cannot be separated into different tagmata. Similar to the Strongylinae, Cyathostominae have a well-developed buccal capsule, a mouth collar with two leaf crowns and a copulatory bursa. In contrast to the globular or funnel-shaped buccal capsule of the Strongylinae, cyathostomins are described having a cylindrical buccal capsule, which is also considerably smaller than the buccal capsule of Strongylinae. The characteristics of the mouth collar, cephalic papillae, internal (ILC) and external leaf crown (ELC), extra chitinous support of the ELC, size and shape of buccal capsule and oesophageal funnel are the main characteristics experts use for species identification of adult Cyathostominae. To a minor extent, also the posterior ends, such as the copulatory bursa are used to identify and discriminate the different species (Dvojnos and Kharchenko, 1994; Lichtenfels, 1975; Lichtenfels et al., 2008). From species identification keys based on measurements, a more practical method based on descriptive features of the characteristics mentioned above was published (Tolliver, 2000). Based on this practical method, the adults of the different cyathostomin species are first clustered as small, medium, or large-sized with sizes of 4 mm up to 22 mm. Then the buccal capsule is clustered as either round, rectangular or square. After that, (1) distinguishing details of the buccal capsule, (2) extra-long length of the male bursa, (3) the shape of the female´s end of the tail or (4) an extra-long length of oesophagus end are determined and lead to a species diagnosis (Tolliver 2000).

In addition to species identification of adult cyathostomins, species-specific descriptions of fourth-stage larvae (L4) for some but not for all species were published. These descriptions are based on moulting L4, that already show some features of the adult specimens (Kharchenko and Kuzmina, 2010).

After larval culture of faeces or of extracted eggs from uterus of gravid female cyathostomins, the infective third-stage larvae (L3) can be obtained and allocated to the *Strongylus* spp. or to eight distinct *Cyathostomin* sensu lato larvae types (Types A-H). Features include different parameters, such as larval length and width (including the sheath), the oesophageal and

intestinal length and the arrangement or number of intestinal cells (Kornaś et al., 2009; Santos et al., 2018).

First stage larvae have a long pharynx and a rhabditiform oesophagus and have a size of 0.3 to 0.7 mm. Eggs are elliptic to oval, with a thin shell, have a size of 75-155 × 42-73 µm and contain 8-16 blastomeres at the point of expulsion with the host faeces (Nemeseri & Hollo 1964). Although significant differences in the size of eggs were found, the eggs of the *Strongylus* spp. and the cyathostomins are not reliably distinguishable (Kuzmina et al., 2012).

2.1.2.2 Morphological features of selected species

Globally, only few experts are able to reliably distinguish individual species morphologically and even those experts face the constant challenge to unambiguously identify some morphologically very similar species, such as *Cyc. insigne* and *Cyc. gyalocephaloides* or *Cyc. ashworthi* and *Cyc. nassatus* (Lichtenfels et al., 2005, 1997). As a consequence, the latter were analysed molecularly to finally claim them to be different species (Hung et al., 1997).

The main discriminatory features of the four cyathostomin species that are examined in this thesis are described as examples:

Coronocyclus coronatus, first described by Looss (Looss, 1900) is identified as a small (6.8 - 10.5 mm long) cyathostomin species that can be found globally in the equine caecum and colon. The walls of the rectangular buccal capsule are relatively thick and described as "boomerang"-like **(Figure 2-1)**. The external leaf crown protrudes from the buccal capsule and bows outward, giving the appearance of a crown (Lichtenfels et al., 2008; Tolliver, 2000). Hence the name "*coronatus*".

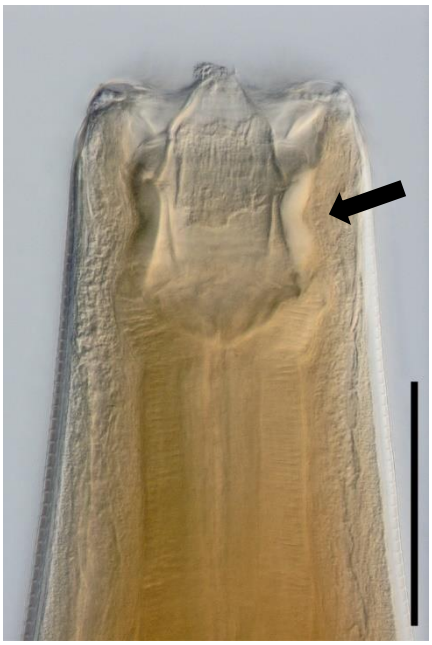

Figure 2-1: Buccal capsule and part of oesophagus of *Coronocyclus coronatus*. Arrow indicates "boomerang like" wall of buccal capsule. Protruding external leaf-crown not visible (picture by T.A. Kuzmina)

Cylicostephanus calicatus (Looss, 1900), is again a ubiquitous small (5.1 - 8.2 mm long) cyathostomin found in caecum and colon (Lichtenfels et al., 2008). The walls of the buccal capsule are flat, relatively long and straight towards the opening. Two different morphotypes of the buccal capsule are observed: A “forma major” and a “forma minor” has been described. Furthermore, the mouth can appear “open” or “shut” **(Figure 2-2 and Figure 2-3)** (Kharchenko, 1997; Tolliver, 2000). Small specimens of the species *Cys. calicatus* are easily confused with *Cylicostephanus minutus*, but the tails of the males are longer, and the female´s tails are tapered in *Cys. calicatus* in comparison to *Cys. minutus*.

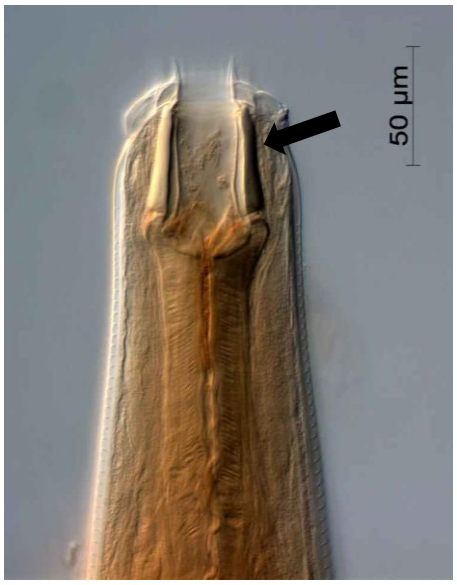

Figure 2-2: Buccal capsule (“Mouth open”) and anterior end of oesophagus of *Cylicostephanus calicatus.* Arrow indicates flat, long and straight wall of buccal capsule (picture by T. A. Kuzmina)

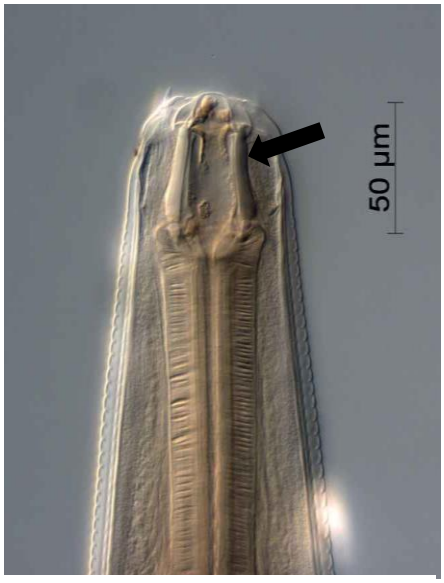

Figure 2-3: Buccal capsule (“Mouth shut”) and anterior end of oesophagus of *Cylicostephanus calicatus.* Arrow indicates flat, long and straight wall of buccal capsule (picture by T. A. Kuzmina)

Cylicostephanus longibursatus (Yorke and Macfie, 1919) has a size of 4.3 to 8.0 mm and is again listed as a small cyathostomin species. The wall of the buccal capsule is described as looking like "a set of parentheses". C*ylicostephanus longibursatus* got its name from the extremely long bursa of the males **(Figure 2-5)** (Lichtenfels et al., 2008; Tolliver, 2000).

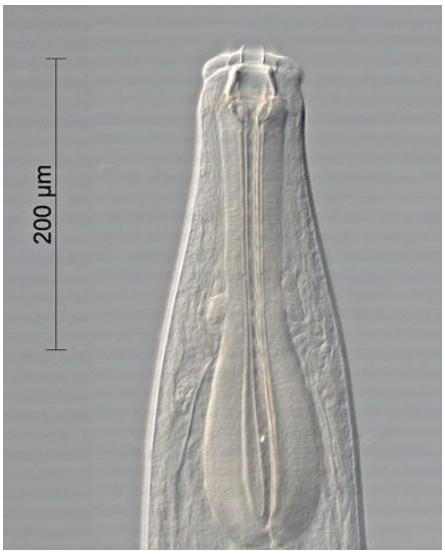

Figure 2-4: Buccal capsule and oesophagus of *Cylicostephanus calicatus* (picture by T.A. Kuzmina)

Figure 2-5: Long bursa of male *Cylicostephanus longibursatus* (picture by T.A. Kuzmina)

Cylicostephanus minutus (Yorke and Macfie, 1919) is named "*minutus",* because it is described as the smallest (4.0 - 6.8 mm) of all cyathostomin species. The cuticle is striated, and slightly constricted at the transition zone from buccal capsule to oesophagus. The leaf elements of the ELC look like antennae of snails **(Figure 2-6)**. As mentioned above it can be confused with *Cys. calicatus*. In contrast to *Cys. calicatus*, the ends of the females are "fatter" and end into a teat-like tip. The bursa of the male is not as long as in *Cys. calicatus* **(Figure 2-7)** (Lichtenfels et al., 2008; Tolliver, 2000).

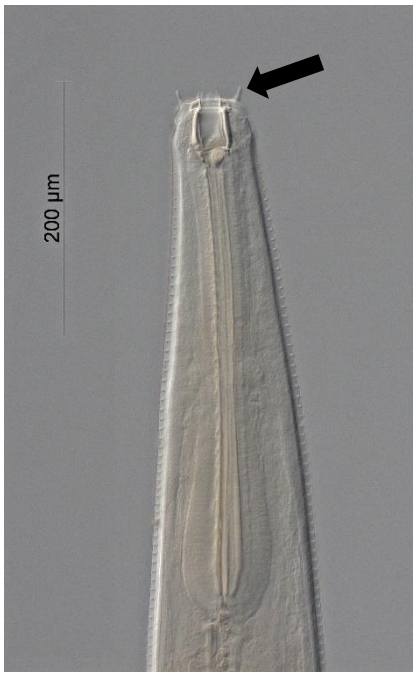

Figure 2-6: Buccal capsule and oesophagus of *Cylicostephanus minutus*. Arrow indicates "snail-like" antennae. Striated cuticle visible at outer margin of parasite (picture by T.A. Kuzmina)

Figure 2-7: Bursa of male *Cylicostephanus minutus* (picture by T.A. Kuzmina)

2.1.3 Occurrence of cyathostomins

Cyathostomins occur everywhere, where horses, donkeys and zebras occur. Of the 50 species, 13 are considered to be the most common species and represent about 99 % of the total cyathostomin burden worldwide **(Table 2-2)** (summarised by Traversa et al., 2007). In contrast, some species are found very rarely or only in specific host species **(Table 2-1)**. Co-infections of different species with up to 29 species within one host have been described (Chapman et al., 2003).

Table 2-2: Most common cyathostomin species (Traversa et al., 2007)

Genus	***Species***
Coronocyclus	*Cor. coronatus*
	Cor. labiatus
	Cor. labratus
Cyathostomum	*Cya. catinatum*
	Cya. pateratum
Cylicocyclus	*Cyc. ashworthi*
	Cyc. insigne
	Cyc. leptostomum
	Cyc. nassatus
Cylicostephanus	*Cys. calicatus*
	Cys. goldi
	Cys. longibursatus
	Cys. minutus

Most research on the species-specific occurrence of cyathostomins is based on morphological identification of adults after collection from the large intestinal content after necropsy. In the USA, the worm burden of 55 adult horses revealed, that only ten species (*Cyathostomum catinatum*, *Cyathostomum coronatum* (now *Coronocyclus coronatus*), *Cylicocyclus nassatus*, *Cylicostephanus longibursatus*, *Cys. goldi*, *Cys. calicatus*, *Cys. minutus*, *Cylicocyclus leptostomum*, *Cyc. insigne* and *Cya. pateratum*) comprised 98.9 % of the total

cyathostomin burden. Further 11 species were found occasionally (Reinemeyer et al., 1984). Similarly, the same few most prevalent species were found in studies in Great Britain, Poland and Australia (Bucknell et al., 1995; Gawor, 1995; Mfitilodze and Hutchinson, 1990; Ogbourne, 1976).

In Louisiana, two surveys of gastrointestinal helminths in ponies with limited access to anthelmintic treatment were conducted twenty years apart. In 117 ponies, 26 cyathostomin species were found in the year 2000 in comparison to 24 species in 37 ponies necropsied before 1981. *Cylicostephanus longibursatus* was the most prevalent species in both studies, followed by *Cya. catinatum*, *Cys. minutus*, *Cys. goldi*, *Cyc. nassatus* and *Cys. calicatus* in the newer study. The ranking of prevalence of the remaining species is similar in both studies, but the prevalence of the species was reduced in the newer study (Chapman et al., 2002).

In a study in central Kentucky, 13 cyathostomin species of four genera were found in four horses. *Cylicocyclus insigne* was found to be the most prevalent species, followed by *Cyc. nassatus*, *Cya. catinatum*, *Cyc. leptostomum* and *Cys. longibursatus* (Lyons et al., 2009). In a later study, cyathostomins from two horses of the same herd were identified and 11 species of three genera were found with *Cyc. leptostomum, Cys. longibursatus* and *Cys. minutus* being the most prevalent species. In contrast, *Cyc. insigne*, the most prevalent species in the study before, had a prevalence below 5 % (Lyons et al., 2010).

In a Brazilian study with 36 horses, 2 to 17 species were found within each horse and the most abundant species were *Cys. longibursatus*, *Cyc. nassatus* and *Cya. catinatum* (Silva et al., 1999).

In China, 1 to 15 cyathostomin species were found in the caeca of 34 donkeys. In total, 18 species could be identified and the most prevalent cyathostomin species were *Cyc. nassatus*, *Cor. labratus*, *Cor. labiatus*, *Cya. tetracanthum* and *Cor. coronatus* (Bu et al., 2009).

Species identification of adult worms collected from faeces after anthelmintic treatment with the macrocyclic lactone aversectin was performed in a Ukrainian study. In 44 examined horses, between seven and 16 cyathostomin species were found. The most prevalent species

were *Cyc. nassatus*, *Cya. catinatum*, *Cys. longibursatus*, *Cys. ashworthi*, *Cys. calicatus*, *Cys. leptostomum* and *Cys. minutus* and corresponded to the most prevalent species found in the necropsy surveys above (Kuzmina et al., 2005).

Up to 21 species-specific probes were evaluated to molecularly identify cyathostomin species in a reverse line blot (RLB) assay (Cwiklinski et al., 2012; Traversa et al., 2007). Probes for the 13 most common species were used in a broad scale study in Great Britain, Germany and Italy. Here, three to 13 species were found in each yard in the different countries and the five most prevalent species were *Cyc. nassatus*, *Cys. longibursatus*, *Cya. catinatum*, *Cyc. goldi* and *Cya. pateratum* (Traversa et al., 2010). Similarly, in the Netherlands, *Cyc. ashworthi, Cyc. nassatus, Cyc. leptostomum, Cys. goldi, Cys. longibursatus, Cya. catinatum, Cya. pateratum, Cor. coronatus* and *Cyd. bicoronatus* were identified by RLB in pooled samples which are considered to be common species in this country (Kooyman et al., 2016b).

In contrast to the common species, the rare species *Cyc. gyalocephaloides* has only been found in African zebras and *Cyc. asini* has only been described to occur in donkeys in South Africa (Lichtenfels et al., 2005; Matthee et al., 2002).

2.1.4 Life cyle and epidemiology

2.1.4.1 Life cycle in general

Cyathostomins have a simple, direct and non-migratory life cycle **(Figure 2-8)**: Adults live in the caecum and colon. Females release eggs, that are expelled with the faeces onto pastures. Depending on the environmental conditions, particularly the temperature, larvae begin to hatch after two days (Nemeseri and Hollo, 1964) and moult, in a minimum time of two weeks, up to an infective third larval stage (L3). Due to an incomplete moult, the L3 is still covered by the cuticle, which is designated “sheath” of the second stage larvae (L2). Due to its sheath, L3s are robustly protected against environmental stress and can endure on pasture for several weeks up to months, despite lack of nutritional uptake. Third stage larvae are the infectious stage, which is taken up by the equine host while feeding on grass. After exsheathing in the intestines, L3 penetrate into and encyst inside the mucosa and submucosa of the caecum,

ventral and dorsal colon (Lyons et al., 1999). Within 6-12 days L3 differentiate into fourth stage larvae (L4) and remain in a histotropic phase for usually 1-2 months. Often this phase can be considerably longer (hypobiotic stage), in case the larve undergo inhibition before L4 re-emerge into the lumen of the large intestine. There, L4 first moult to preadults, sometimes incorrectly called fifth stage larvae (L5), and subsequently complete their development to adults that finally release eggs. After infection, eggs of cyathostomins can be found in faeces not earlier than 6-12 weeks after infection (prepatent period). In contrast, the histotropic phase in hypobiotic larvae can last for at least 2.5 years (Deplazes et al., 2013a; Gibson, 1953).

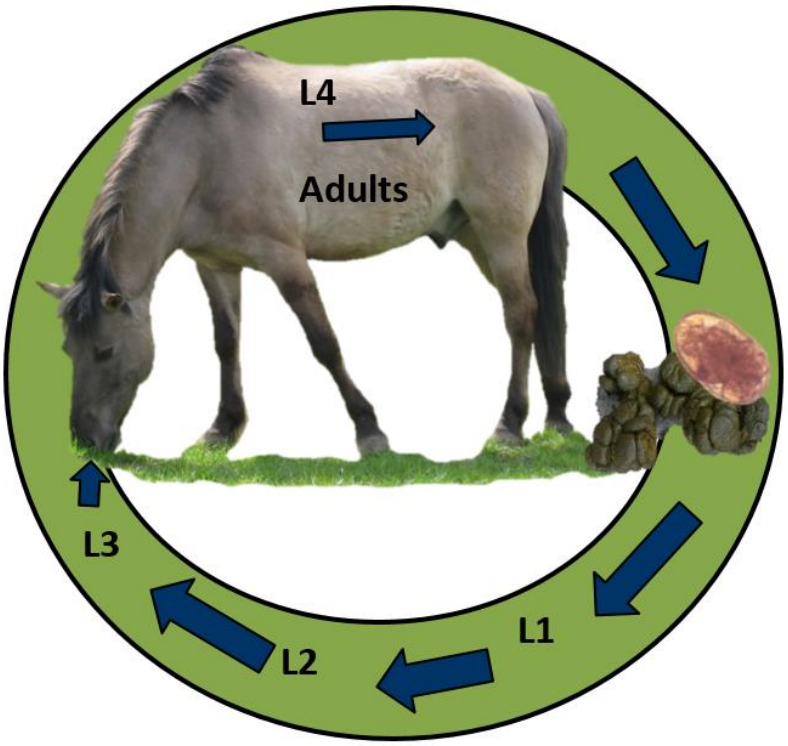

Figure 2-8: Life cycle of cyathostomins, L, Larvae

Infection occurs after uptake of infectious larvae. This usually happens on pastures, but infection in the stable after feeding fresh grass or contaminated stable equipment is possible. Previously infected horses may carry the infestation with adult cyathostomins over the winter and subsequently contaminate the pasture. In temperate climates additionally, a fraction of the free-living larvae deposited during summer or autumn survives the winter and contributes to pasture contamination in spring. It must be noted, that within an equine population typically few horses can be responsible for the biggest share of pasture contamination by

shedding high numbers of strongyle egg (Deplazes et al., 2013a). Moreover, the intensity of egg shedding has been shown to be strongly dependent on the individual horse, disregarded if the horse was treated with anthelmintic veterinary medicinal products. After reinfection these horses will most likely shed the highest egg numbers again (Scheuerle et al., 2016).

The density of infectious larvae varies with season: Whereas in temperate climates only few larvae survive the winter season and result in a low L3 density on pastures, the number of infectious larvae increases during the summer season due to the maturation of adult cyathostomins that consequently release eggs (Kuzmina et al., 2006). As a result, the highest intensity of infection will usually occur during the late summer until the end of the grazing period (Deplazes et al., 2013a). This is in accordance to other research that described that, during the grazing season, significantly higher total mucosal larval counts were observed in foals, when compared to the off season (Nielsen & Lyons 2017). However, in tropical climate zones, L3 are present during the entire year. Their survival and recovery on pastures differs between rainy and dry season: During rainy periods, the L3 are found at the grass apex, whereas during dry periods, L3 are found mainly at the base of the grass, to prevent that they dry out (Quinelato et al., 2008).

Equines of all ages are susceptible to cyathostomins. However, similar to gastro-intestinal nematode infections in ruminants, where sex related differences in the susceptibility to parasitic nematodes were described for bulls and rams (Barger 1993), male light breed foals were reported to have a higher cyathostomin worm burden in form of a higher total count of encysted cyathostomin larvae in comparison to female foals (Nielsen and Lyons, 2017).

An important feature of cyathostomins that represents the main reason of the pathogenic potential of these parasites, is the hypobiosis of larvae in the histotropic phase: The development of larvae that reside inside the mucosa or submucosa can arrest for an unspecific time (more than two years), until an unknown stimulus triggers re-emergence into the lumen (Gibson, 1953).

2.1.4.2 Species-specific knowledge on the life cycle

In an *in vitro* assay, the development of L3, previously cultured from faeces, was observed and the species composition molecularly determined on day 0 (D0) and day 21. A total of 6 species (*Cor. coronatus*, *Cys. goldi*, *Cys. longibursatus*, *Cya. catinatum*, *Cys. nassatus*, *Cyc. ashworthi* and *Petrovinema poculatum*) were found on both dates; the species *Cya. catinatum* was only found on D0 and is therefore thought not to develop under the given *in vitro* conditions. On the basis of morphological features, the larvae were classified as early L3 (EL3), developing L3 (DL3), late L3 (LL3) and L4 and the development of the species was determined. During the three weeks of the study, *Cys. goldi* and *Cys. longibursatus* developed up to LL3 or L4, whereas *Cor. coronatus*, *Cyc. ashworthi*, *Cys. nassatus* remained EL3 (Brianti et al., 2009).

With the objective to obtain data on the potential prepatent period for different species, eight naturally infected foals in the age between 31 to 92 days were necropsied and the gravid female cyathostomins identified. The earliest gravid female was *Cys. longibursatus* in a 57-day-old foal; next a foal was positive for a gravide *Cys. goldi* at 64 days, followed by foals positive for *Cya. catinatum* at 68 days, *Cor. coronatus* and *Cys. calicatus* at 70 days, *Cor. labiatus, Cyc. leptostomum, Cyc. nassatus, Cys. hybridus*, and *Cys. minutus* at 74 days, and *Cys. bidentatus* at 92 days (Lyons et al., 2011).

The potential for reproduction (fecundity) of females of 15 strongylid (including ten cyathostomin) species was studied by measuring and analysing the female nematodes and their eggs. A significant correlation was found between the number of eggs and size of the nematode species. The highest number of cyathostomin eggs was found in *Poteriostomum imparidentatum* (average of 3 225). The other cyathostomin species harbored on average egg numbers between 49 and 455 (*Cys. longibursatus* and *Cyc. insigne* respectively) (Kuzmina et al., 2012).

2.1.5 Cyathostomin associated disease

Whereas in earlier times, cyathostomins were noted as parasites with low or limited pathogenicity, cyathostomins are now recognised as important pathogens of equines, although they are not always causing clinical disease. Many hosts harbor tens of thousands of

cyathostomins without any clinical symptoms and no correlation between strongyle faecal egg count with the actual worm burden or clinical disease exists (Nielsen et al., 2010). Still, cyathostomins cause acute and chronic clinical presentations, which were described similarly in naturally acquired as well as experimentally infected horses. Apparent disease can occur in horses of all sex and age but is most common in young horses under 5 years of age (Love et al., 1999).

The pathogenesis of larval cyathostominosis, the most important complex of clinical symptoms caused by cyathostomins, is mainly attributed to the severe mucosal lesions and inflammatory response caused by large numbers of larvae simultaneously emerging from the mucosa and submucosa of caecum, ventral or dorsal colon. It should be noted, that the larvae penetrating into the intestinal wall cause similar damage, but since this process does usually not involve large numbers of larvae simultaneously, in general no clinical diesase is associated to this phenomenon. The inflammatory reaction is characterised by a catarrhal and fibrinous inflammation of the caecum and colon with diffuse haemorrhagic foci in the mucosa. Furthermore, a characteristic “poppy seed” appearance of the mucosa is described, due to encysted cyathostomin larvae (reviewed by Love et al. 1999). Upon mucosal emergence, the permeability of the intestines increases, inducing a protein losing enteropathy and malabsorption of nutrients. As a result, wasting or weight loss is the main clinical feature of larval cyathostominosis with or without recurrent profuse diarrhoea. In 40-70 % of the cases, this may be fatal within two or three weeks, despite palliative and anthelmintic treatment (Corning, 2009). Additionally, alterations of the gastro-intestinal motility have been associated with cyathostomins (Bueno et al., 1979). These clinical presentations are accompanied by hypalbuminaemia and possibly with pyrexia, abdominal oedema and delayed shedding of coat (Love et al., 1999). Individual case reports differ in progress and clinical findings (Van Loon et al., 1995; Zakrajsek, 2017).

It is assumed, that the risk and severity of typhlocolitis and clinical manifestation of disease is increased with the number of emerging larvae (Love et al., 1999; Reinemeyer and Nielsen, 2009). Hence, most clinical cases of larval cyathostominosis in temperate zones occur in late

winter or spring when a large number of larvae simultaneously end their histotropic hypobiotic stage and re-emerge into the intestinal lumen to subsequently produce eggs as adults. A specific stimulus that induces mass-emergence is not known. Involvement of environmental and host factors has been speculated, but could not be substantiated with data at the moment (Love et al., 1999). In contrast, it is hypothesised, that the luminal presence of adult cyathostomins might have an protective effect against the re-emergence of larvae from the mucosa (Stancampiano et al., 2017).

Larvae of different species (*Cys. longibursatus*, *Cys. goldi, Cya. catinatum*, Cyc*. radiatus*, *Cyc. ashworthi, Cyc. insigne, Cyc. nassatus*) have been described to be involved in individual case reports from clinical and/or fatal cases of larval cyathostominosis and it is assumed that clinical disease is predominantly caused by mixed infections (Hodgkinson et al., 2003; reviewed by Love et al., 1999).

Colic is not seen as a prominent feature of larval cyathostominosis (Reinemeyer and Nielsen, 2009). However, the role of cyathostomins in the pathogenesis of colic is not finally elucidated, but their occurrence has been associated with several specific forms of equine colic including caecocaecal intussusception, non-strangulating infarction and caecal tympany, as well as with non-specific mild colic (reviewed by Love et al. 1999). Furthermore, spasmodic colic has been described to occur due to cyathostomins (Moudgil et al., 2017).

Histologically, eosinophilic infiltrates in the large intestinal wall are a feature of parasitic disease in equines, although they are not exclusively occurring in parasitic disease and must not be confused with other eosinophilic epitheliotropic diseases (Jones and Blikslager, 2004).

The immunological response of the mucosa during mucosal cyathostomin infection is characterised by interleukin-4 and interleukin-10, which are characteristic for a protective Th2 response as seen in other parasitic diseases. However, during emergence of larvae from the mucosa, a pro-inflammatory response, characterised by tumour necrosis factor α has been observed, but could not be statistically substantiated (Davidson et al., 2005).

Cyathostomins do not pose a zoonotic risk for the owner or veterinarian. However, an important differential diagnosis for typhlocolitis is salmonellosis caused by different serovars

of *Salmonella* spp. that may have zoonotic potential. Other differential diagnoses are equine intestinal Clostridiosis (e.g. *Clostridium perfringens* type A) as well as rhodococcocosis (caused by *Rhodococcus equi*) or equine monocytic ehrlichiosis, commonly known as Potomac horse fever (caused by *Ehrlichia risticii*) (Brown et al., 2007; Jones and Blikslager, 2004; Larsen, 1997; Manship et al., 2019).

2.1.6 Anthelmintic management

In order to prevent parasitic disease, anti-parasitic management is comprised of pasture/stable management as well as anthelmintic treatment of existing infections.

2.1.6.1 Pasture/stable management

Maintaining pasture contamination to a low number of infectious larvae leads to a lower worm burden inside of the host. Prevention or at least reduction of pasture contamination by infectious larvae and their dispersal around the faeces can be achieved by removing faeces from pasture and paddocks twice weekly. This has been shown to significantly reduce the faecal worm egg count (Corbett et al., 2014). Furthermore, moist straw bedding has been shown to be a suitable environment for larval development and should hence be considered as well to be a risk factor (Love et al., 2016). Another option is to alternate use of the grazing grounds with ruminants, which will reduce the risk of infection for both species: Infectious cyathostomin larvae will be taken up by cattle or sheep grazing on the same premises. Cyathostomins and other parasites of equines or ruminants, except for *Trichostrongylus axei* are not capable of developing in different hosts and will not become fertile adults (Deplazes et al., 2013b). Further reduction of infectious larval burdens on pastures can be achieved by cropping, for example for silage or hay (Herd, 1986). It should be noted, that these methods represent in resource-poor areas (e.g. rural African areas) the only affordable practices, since the price of chemotherapeutic treatment equals the price of a donkey used for work (Krecek and Guthrie, 1999).

2.1.6.2 Chemotherapeutic management

Usually, infections with cyathostomins cannot be prevented, and equines are treated with anthelmintic drugs. Detailed treatment strategies considering different parasite species and

age groups are published in a recent guide to the treatment and control of equine gastrointestinal parasite infections (European Scientific Counsel Companion Animal Parasites (ESCCAP), 2018). The two main strategies recommended for worm control in adult horses are briefly described in the following: The strategic and the selective treatment strategy.

The strategic treatment strategy aims to disrupt the seasonal cycle of parasite transmission by treating at strategic time-points for infection, such as a few weeks after turning horses out in spring, during the grazing season, and during autumn. Usually, all horses in a population are treated similarly, without consideration of their individual infectious burden and contribution to contamination of environment (Proudman and Matthews, 2000).

The selective therapy strategy is based on (1) a paradigm shift that accepts a certain parasite burden as physiological state in equines and (2) the awareness that the major parasite health threat is no longer a particular species but rather anthelmintic resistance (Nielsen, 2012). As a result, this strategy aims to keep the parasite burden inside the individual hosts at a level which does not lead to clinical symptoms and to delay development of further anthelmintic resistance. This goal is sought to be achieved by monitoring the infectious burden via faecal egg counts (FEC) and treating only horses with a FEC above a defined threshold in eggs per gram (EPG) faeces. This threshold is usually set between 200 or 500 EPG and is not evidence, but rather experience based by practitioners or parasitologists (Deplazes et al., 2013b; Nielsen, 2012; Nielsen et al., 2014a). Again, it has to be noted that absence of egg shedding does not imply absence of infection and that the FEC does not correlate with the worm numbers inside the host. Even horses with fewer than 100 EPG were shown to potentially harbor more than 100,000 worms (Nielsen et al., 2010).

Furthermore, this strategy aims to maintain a number of susceptible parasites in so called *refugia*. The concept of *refugia* describes the parasite stages that are not under selective pressure, e.g. due to anthelmintic treatment. Hence, parasites in *refugia* comprise of the free-living stages (eggs, L1, L2, L3), histotropic stages and parasites in untreated horses. The hope is to keep the number of susceptible parasite in *refugia* as high as possible to dilute resistant ones, that eventually evolve (Nielsen, 2012). Importantly for the scope of this thesis, with this

strategy, all cyathostomin species are evaluated and treated together, disregarding potential species-specific differences.

Apart from these two strategies above, a traditional anthelmintic treatment approach is to "treat-all at frequent interval". This is done without determination of infection intensity or without consideration of epidemiological circumstances, such as varying infectious risks with season or housing of horses. This interval dosing consequently leads to treatment at scientifically unjustified timepoints, is resource consuming and promotes development of anthelmintic resistance (Nielsen, 2012; Proudman and Matthews, 2000).

2.1.6.3 Anthelmintic compounds

Veterinary medicinal products (VMP) intended for the treatment of cyathostomin infections mainly belong to three major drug classes: Macrocyclic lactones (ML), benzimidazoles (BZ) and tetrahydropyrimidines. After oral administration, these drugs target mainly the luminal cyathostomins. Veterinary medicinal products containing the ML moxidectin have been shown to be effective against mucosal encysted cyathostomin stages as well (Zoetis UK Limited, 2018). When given on five consecutive days also fenbendazol, a compound belonging to the class of BZs, has been shown to be effective against cyathostomin larvae (Duncan et al., 1998). However, this is only the case in BZ-susceptible cyathostomin populations (Reinemeyer et al., 2015).

Piperazine does not belong to one of the drug classes named above and plays a minor but, due to the increasing resistance issues with other drugs, re-emerging role in the use in horses. However, due to its administration via drinking water or nasogastric intubation and its narrow spectrum of efficacy, it is only rarely used. It is mentioned here mainly for historical reasons, as only one VMP containing piperazine is still authorised in Germany for the treatment of "trichonemes" (Bela-Pharm GmbH & Co. KG, 2009).

Research interest in the development of new anthelmintic compounds or classes is high. Three new drug classes (emodepside, monepantel and derquantel) for control of nematodes have been developed and are authorised in other animals, but the efficacy and safety in horses has

not been assessed yet (Nielsen et al., 2014b). Furthermore, alternative approaches, such as ethnoveterinary medicines derived from plants are evaluated for potential anti-parasitic efficacy that could be applied as feed additive (Peachey et al., 2015). So far, *in vitro* efficacy against cyathostomin larvae has been shown for some native Australian plants, for a supernatant from *Papaya latex* as well as methanol extracts of *Diospyros anisandra* and *Petiveria alliacea*. However, *in vivo* efficacy in parasitic stages has not been evaluated yet (Flota-Burgos et al., 2017; Payne et al., 2013; Peachey et al., 2016).

In contrast, nematophageous fungi were tested for their efficacy *in vivo*: Pelleted horse feed, enriched with spores of the fungi *Mucor circinelloides* and *Duddingtonia flagrans* were fed to horses and submitted to different grazing groups. Initial results indicate that rotational grazing and supplementation with these pellets reduce the development of strongyles in the environment. However, the results of a follow-up study are still outstanding (Hernández et al., 2018). No effect on egg-shedding has been shown when supplementing either fresh or dried garlic to horses (Buono et al., 2018)

2.1.7 Anthelmintic resistance

2.1.7.1 Anthelmintic resistance in general

Anthelmintic resistance (AR) is defined as “a heritable change in susceptibility to an anthelmintic in a population of parasitic nematodes such that a dose which normally provides ≥95 % clearance of adult worms provides ≤80 % clearance” (Geary et al., 2012). Random independent spontaneous mutations and recombination events frequently occur in the genetic code of individuals and eventually lead to occurrence of individuals with an advantage for survival and completion of the life cycle after anthelmintic treatment. Consequently, these individual worms pass alleles that lead to resistance in their offspring (reviewed by Stratford et al., 2011). With each parasite generation under anthelmintic selection, the proportion of individuals with alleles encoding resistance, increases; especially, when selection pressure is high, such as in situations when the same anthelmintic compounds are still used, despite their partial lack of efficacy. In contrast, a subsequent loss of resistant traits and reversion of AR after avoiding the specific compound classes has not been shown yet. Although limited reversion to susceptibility cannot be ruled out, based on current data, it is suggested that

clinically relevant levels will not occur and AR will remain (reviewed by Von Samson-Himmelstjerna 2012; Nielsen, Reinemeyer, et al. 2014).

In cyathostomins, AR has been described for all the drugs and drug classes described above. Resistance against benzimidazoles has been described in various reports from a total of 14 European countries, Northern America, Southern America and Australia and is hence noted to be the most common and widespread type of resistance (Kaplan 2002; reviewed by Peregrine et al. 2014). Pyrantel resistance has been reported in 12 countries with the exception of Australia, where resistance against the related compound morantel was reported (reviewed by Peregrine et al. 2014). Only recently, AR against the ML ivermectin and moxidectin was observed and thought to emerge (reviewed by Molento et al. 2012).

In the absence of fully evaluated *in vitro* assays such as the egg hatch assay, larval development assay, larval migration-inhibition assay, the only gold-standard method of diagnosing AR in cyathostomins is the faecal egg count reduction test (FECRT): Eggs of the gastro-intestinal nematode type are counted before and after anthelmintic treatment and the percentage of egg count reduction indicates the efficacy of the drug that has been administered. No universal decision on the cut-off values to declare resistance has been agreed on, but values ranging between 80 and 95 % are applied (reviewed by Nielsen, Reinemeyer, et al. 2014).

The clinical impact of cyathostomins with AR is not known: To date, there is no published case of cyathostominosis associated with AR (Von Samson-Himmelstjerna, 2012).

2.1.7.2 Species-specific knowledge on resistance

Anthelmintic resistance in cyathostomins is mostly considered with an “unispecific” approach and it is currently not possible to determine if this approach is valid. Observations on species-specific occurrences over the past twenty to forty years were “fairly consistent”, which led to the assumption that resistance had little impact on the prevalence of different species and that the various species differ little in their ability to develop resistance (Chapman et al., 2002;

Nielsen et al., 2014b). However, limited research tried to address the occurrence of AR in different species.

In the Netherlands, differences in susceptibility to MLs were assessed with a reiterative larval migration inhibition assay followed by RLB, where it was shown, that *Cya. catinatum* was less susceptible to ivermectin than the other two predominant species *Cys. longibursatus* and *Cyc. nassatus* (van Doorn et al., 2010).

In another study, it was shown, that the number of species found pre-treatment was higher than post-treatment and that eggs of different *Cylicocyclus* spp. (*Cyc. ashworthi, Cyc. leptostomum, Cyc. nassatus*) together with *Cys. longibursatus* and *Parapoteriostomum mettami* were reappearing earlier than other species after anthelmintic treatment with MLs (van Doorn et al., 2014). Similarly, *Cyc. insigne* was found to reappear earlier after anthelmintic treatment with the ML moxidectin than after treatment with pyrantel, where *Cys. longibursatus* was determined as the species reappearing first (Kooyman et al., 2016a). Species that were shown to contribute to a shortened egg reappearance period in the USA are *Cya. catinatum, Cys. longibursatus, Cyc. ashworthi* and *Cyc. nassatus* (Bellaw et al., 2017).

Not only the presence of AR, but also the mechanisms were examined in individual species: Although the mechanisms of resistance against MLs are not clear, involvement of P-glycoproteins (Pgp) of the family of the ATP-Binding-Cassette-transportproteins is speculated. These proteins might have an influence in the transmembrane transport of MLs. It was tried to identify the genetic sequence and to determine the function of different Pgps in the cyathostomin species *Cyc. elongatus*, *Cyc. insigne* und *Cys. goldi* (Kaschny, 2017). The effect of four different MLs on yeast cells, expressing Pgp-9 from *Cyc. elongatus* was determined in a growth-inhibition assay. It was shown, that the different MLs had either no effect on the yeast growth or increased the susceptibility of the yeast to the fungicide ketokonazol, which was present in the assay at a subtoxic concentration, thus confirming that the tested ML competitively interact with the *Cyc. elongatus* Pgp-9 (Kaschny et al., 2015).

Resistance to BZ has been described for the 13 most common cyathostomin species and additionally *Cyc. brevicapsulatus* and *Petrovinema poculatum* (reviewed by Čerňanská et al., 2009). Polymorphisms at codons 167, 198 and 200 in the β-tubulin isotype 1 gene have been associated with resistance to BZs in nematodes. In cyathostomins, polymorphisms at codon 167 and 200 were found. Due to its importance for the understanding of AR against BZs, the genes for up to two β-tubulin isotypes (1 and 2) were characterised for different species (Clark et al., 2005; Drogemuller et al., 2004; Hodgkinson et al., 2008; Pape et al., 2002, 1999; Von Samson-Himmelstjerna et al., 2001). To detect these polymorphisms and potentially resistant populations, a pyrosequencing assay, which is applicable for multiple cyathostomin species has been developed (Lake et al., 2009).

No species-specific, but larval-subtype specific research has been performed when larval subtypes were examined for their population structure or their sensitivity to ivermectin in a larval migration inhibition assay (Anuțescu et al., 2016; McArthur et al., 2015). No conclusion on the resistance status of individual species can be drawn from this research.

2.2 Molecular approaches

Since the helix structure of the DNA was resolved by Watson, Crick and Franklin in 1953 (Watson and Crick, 1953) and the DNA of an organism was sequenced for the first time (Sanger et al., 1977), many methods have been developed to determine the genetic structure of an organism. Mainly, these methods are applied with the aim to identify organisms and to conclude on phylogenetic relationships.

2.2.1 Molecular diagnostics

In order to identify organisms on species level, species-specific nucleotide sequences have to be identified. Assigning species-specific nucleotide sequences to a species is called DNA barcoding and mostly used for sequences of the mitochondrial marker encoding for the cytochrome oxidase subunit I (COI) (Hebert et al., 2003b), although it can also be used for other markers as well. Suitable gene loci for species identification have a reasonable interspecies variation that is higher than the rate of intraspecies variation.

2.2.1.1 Ribosomal marker

The non-coding first and second internal transcribed spacer (ITS-1 and ITS-2) of the nuclear ribosomal DNA (rDNA) sequences are commonly used for identification of strongyloid nematodes, including cyathostomins and are also applied for other helminths or insects (Adlard et al., 1993; Almeida and Stouthamer, 2015; Chilton et al., 1997; Hung et al., 1999b, 1997).

Ribosomal DNA encodes for ribosomal RNA and occurs in multiple copies within one genome of pro- and eukaryotic organisms. The 18S and 28S regions of the rDNA encode for the smaller and larger subunit (SSU and LSU) of the ribosomes. Between the 18S and 28S regions, the ITS-1, 5.8S and the ITS-2 are found. The region located between the 28S and the next 18S region is the intergenic spacer (IGS) region, which can be further divided into the nontranscribed spacer (NTS) and the external transcribed spacer (**Figure 2-9**) (Dorris et al., 1999; Zarlenga and Barta, 1990).

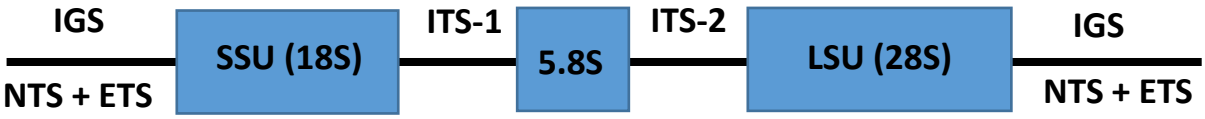

Figure 2-9: Schematic structure of a ribosomal DNA repeat (adapted from Dorris et al., 1999). ETS, external transcribed spacer; IGS, intergenic spacer; ITS, internal transcribed spacer; LSU, large (28S) subunit of the rRNA; NTS, nontranscribed spacer; SSU, small subunit (18S) of the rRNA.

Whereas the 5.8S, 18S and 28S regions are usually highly conserved, the ITS-1 and ITS-2 regions were shown to have low intraspecific variation (<1 %) and enough inter-specific variation (>1.5 %) to allow species differentiation and are acknowledged as an “excellent diagnostic tool” (Blouin, 2002; Dorris et al., 1999; Gasser and Newton, 2000).

Another ribosomal marker that has been previously applied is the IGS region: This region has been shown to have a high rate of intra-specific variation in cyathostomins with up to 23 % and inter-specific variation between 3 to 62 % and was used to develop species-specific probes for a reverse line blot (RLB) for the identification of cyathostomins (Cwiklinski et al., 2012; Traversa et al., 2007).

2.2.1.2 Mitochondrial marker

The mitochondrial genome is a usually circular DNA region which is only inherited maternally in eukaryotic organisms including nematodes (Anderson et al., 1995; Wolstenholme, 1992). It evolves more quickly than the ITS regions in nematodes and reaches a higher level of substitutions (Blouin, 2002). The variability of mitochondrial DNA sequences within a species ranges up to 2 % and sequence differences between closely related species are usually 10-20 % (Blouin, 2002). A mitochondrial marker commonly used is the sequence of the cytochrome c oxidase subunit I (COI) locus (Duscher et al., 2015; Hebert et al., 2003b; Lv et al., 2016). Due to its high intra- and inter-species variability, it is advocated to be used for identification of new and/or cryptic species (Blouin, 2002; Hebert et al., 2003b, 2003a).

2.2.2 Molecular phylogenetics

Phylogeny describes the evolutionary relationship of different species, organisms or genes and is based on the assumption that organisms derive from common ancestors and that organisms with similar features are more closely related than organisms with differing features. The science of phylogeny (= phylogenetics) aims to elucidate this evolutionary history of all types of organisms (Lerner and Lerner, 2014). Before molecular methods were available, phylogenetic considerations were mainly based on morphology or other phenotypic traits. Nowadays, molecular phylogeny compares the nucleotide sequences of single or multiple loci or even larger data sets, such as whole mitochondrial genomes (Bu et al., 2013; Glaeser and Kämpfer, 2015; Qiu et al., 2018; Yang and Rannala, 2012).

A phylogenetic tree is a visualisation of phylogeny (Lerner and Lerner, 2014). Each branch represents a common feature and each node a separation event. For molecular phylogenetic trees of species, this would mean that a branch represents genetical similarity and a node a speciation event. However, trees can also be set up for genes or individuals and the meanings of branches and nodes consequently differ (Yang and Rannala, 2012). Furthermore, phylogenetic trees can be rooted or unrooted: A root can for example be set by an outgroup which is more distantly related to the other species examined or by a more ancient species (Yang and Rannala, 2012).

Phylogenetic trees are mathematically constructed based on different methods and models that are either distance-based or character-based (Yang and Rannala, 2012). To statistically support the construction of phylogenetic trees, the calculation procedure is often iterated (typically 1 000 times), which is named “bootstrapping” (Efron, 1979).

In distance-based methods, such as the widely used neighbour joining method, the distance between every set of sequences is calculated and the resulting distance matrix used for tree construction. This and other distance-based methods are computationally efficient, but perform poorly for very variable sequences (Yang and Rannala, 2012).

Character-based method, such as maximum parsimony, maximum likelihood and Bayesian inference method, simultaneously compare all sequences in an alignment with a focus on one character. As a result, many possible trees are constructed and “the best tree” identified. Parsimony methods are mostly simple and intuitive and do not account for multiple nucleotide substitutions over time (Yang and Rannala, 2012). They are therefore especially prone for the problematic phenomenon called “long-branch attraction”: Long-branch attraction occurs when multiple nucleotide changes over time lead to nucleotide similarities of distantly related organisms and are therefore clustered more closely together than they actually do (Bergsten, 2005). Furthermore, the use of outgroups to root the trees and too few taxa included can lead to this phenomenon. To overcome the problem of long-branch attraction, most other methods include assumptions or models on the transition and transversion rate of nucleotides. To further reduce the risk for this phenomenon, the third codon position, which evolves more quickly than the other codon positions can be analysed separately or omitted in phylogenetic analysis.

2.3 Proteomics

Proteomics is the study of the protein properties, such as protein expression, post-translational modifications and interactions with the ultimate goal to obtain an understanding of processes and interactions at protein level (Anderson and Anderson, 1998; Blackstock and Weir, 1999). Proteomic approaches are applied to diagnose diseases and to gain knowledge on the pathogenesis of disease, but also to identify organisms based on their protein profile (Bizzini and Greub, 2010; Ceciliani et al., 2014; Clark et al., 2013).

2.3.1 Matrix-Assisted Laser Desorption/Ionisation Time-Of-Flight Mass Spectrometry (MALDI-TOF MS)

To characterise the protein expression or protein profile of an organism, mass spectrometry (MS) is performed. One widely used MS system for protein profiling is the matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS).

2.3.1.1 Species identification with MALDI-TOF MS

This method is routinely applied for identification of bacteria and fungi (Bizzini and Greub, 2010). Commercial MS devices contain master spectra libraries with reference spectra of many bacterial pathogens. In the past years, research was forwarded to extend the existing libraries with in-house databases for more species, as well subspecies or resistant strains to discriminate closely related bacterial subspecies, e.g. of *Mycobacterium kansasii* genotypes (Murugaiyan et al., 2018).

The first report of the application of MALDI-TOF MS to multicellular organisms describes the use for species identification of sibling species of the fruit fly *Drosophila* (Campbell, 2005). From then on, MALDI-TOF MS was applied to identify other insect species as well as ticks (Diarra et al., 2017; Hoppenheit et al., 2013; Karger et al., 2012; Yssouf et al., 2015, 2014). Furthermore, identification of pathogens within the blood meal or haemolymph of vectors was shown to be possible (Diarra et al., 2017; Mediannikov and Fenollar, 2014; Yssouf et al., 2015).

Consequently, MALDI-TOF MS was also evaluated to differentiate nematodes: The seed-gall nematodes *Anguina tritici* and *A. funesta* and the root-knot nematode, *Meloidogyne javanica* were differentiated and diagnostic species- specific peaks were identified (Perera et al., 2005). A publication on the species differentiation of different *Trichinella* spp. was the only report on nematodes of veterinary concern, until just recently another report on the species differentiation of different *Ascaris* spp. and *Dirofilaria* spp. was published (Mayer-Scholl et al., 2016; Nagorny et al., 2019).

2.3.1.2 The principle of MALDI-TOF MS

The principle of MALDI-TOF MS is that a sample is mixed or covered by an UV-absorbing matrix, spotted on a target plate and introduced into a MS device. There, a pulsed laser beam ablates and ionises material of the sample/matrix layer which then forms a plume of ionised proteins. Within an electrical field in a vacuum tube, the ionised proteins are accelerated towards an ion detector, which records the time of flight (TOF) according to the mass/charge (*m/z*) ratio and the intensity of the ionised proteins (Hillenkamp and Karas, 2007; Karas and Hillenkamp, 1988; Murugaiyan and Roesler, 2017). The visualisation of this recording is a MALDI spectrum, where the x-axis represents the *m/z* ratio in Dalton (Da) and the y-axis the relative intensity in arbitrary units (AU) **(Figure 2-10)**.

After recording, MALDI spectra are used for species identification through matching of the location and relative intensity of spectral peaks with reference spectra that are stored in a master spectra library, usually in a range of 2 000 to 20,000 Da. Knowledge of the specific identity of peaks or the protein sequences is not necessary (Campbell, 2005; Yssouf et al., 2016).

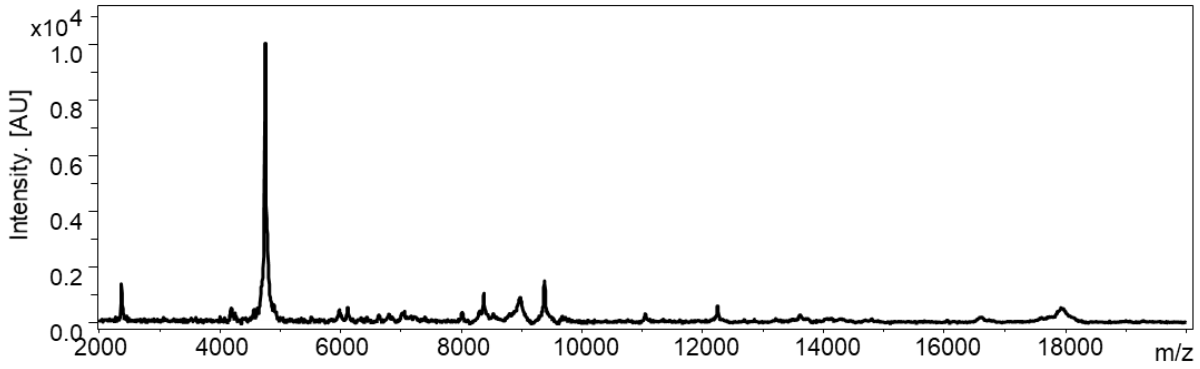

Figure 2-10: Exemplary MALDI spectrum: X-axis represents *m/z* ratio in Da, y-axis represents relative intensity in arbitrary units (AU)

Whereas the overall principle is the same for all organisms, the procedures for sample collection and preparation differ according to the type of organism. To allow the generation of reliable and repeatable results, a standardized procedure for sample preparation including the protein extraction over spotting of proteins and recording spectra up to bioinformatic analysis is necessary. A protocol, universally applicable for bacteria, was previously published containing information on bacteria culturing conditions as well as protocols for protein extraction (Freiwald and Sauer, 2009).

The most convenient method for sample preparation is the direct transfer method, where bacteria from colonies are directly smeared on a target plate. Other chemical sample preparation methods involve ethanol and formic acid or trifluoroacetic acid which allow on the one hand protein extraction and on the other hand inactivation of pathogenic bacteria (Freiwald and Sauer, 2009).

For multicellular organisms such as arthropods and nematodes, many factors such as sample origin (fresh, frozen, fixed), sex, developmental stage and feeding status are known to influence the protein patterns (Hoppenheit et al., 2013; Karger et al., 2012; Yssouf et al., 2016). As a result, a variety of specific protocols has been published, depending on the history of the individual samples.

For nematodes, previous reports relied on fresh specimens that were either picked from culture or directly taken from root galls and were used either washed in sterile distilled water or unwashed. The protocols involve manual grinding or homogenisation with an ultrasonic device with glass beads in different solvents or without any chemical addition (Ahmad et al., 2012; Mayer-Scholl et al., 2016; Perera et al., 2005).

2.4 Study Objectives

As outlined above, species-specific knowledge on the different cyathostomin species is very limited but highly needed: Species-specific knowledge on the length of the life cycle, pathogenicity or on the development of anthelmintic resistance would be of immense benefit to prevent clinical disease and to better understand the development and spread of anthelmintic resistance.

To achieve these ambitious goals, species-specific research needs to be facilitated first. Therefore, this thesis aims to cover the following aspects:

(1) Critical evaluation of the different methods for species identification to allow species-specific research on cyathostomins:

Which methods can be employed to reliably and effectively differentiate cyathostomin species? What are the advantages and disadvantages? Which new methods can be explored?

(2) Exploring MALDI-TOF MS as a potential new method for species identification:

Is MALDI-TOF MS a feasible tool to study cyathostomins? Is it possible to establish a protocol applicable for nematodes and how do the results obtained using MALDI-TOF MS correspond with data obtained by molecular approaches?

(3) Examination of closely related cyathostomin species:

Which information can be deducted after using different molecular markers for molecular characterisation of cyathostomin species? Are the morphological species and genera similarly represented on a molecular basis? Are there different genospecies? Is there evidence of hybridisation of different cyathostomin species?

3 Nematode Species Identification - Current Status, Challenges and Future Perspectives for Cyathostomins

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Human and animal health is globally affected by a variety of parasitic helminths. The impact of co-infections and development of anthelmintic resistance requires improved diagnostic tools, especially for parasitic nematodes e.g., to identify resistant species or attribute pathological effects to individual species or particular species combinations. In horses, co-infection with cyathostomins is rather a rule than an exception with typically 5 to 15 species (out of more than 40 described) per individual host. In cyathostomins, reliable morphological species differentiation is currently limited to adults and requires highly specialized expertize while precise morphological identification of eggs and early stage larvae is impossible. The situation is further complicated by a questionable validity of some cyathostomins while others might actually represent cryptic species complexes. Several molecular methods using different target sequences were established to overcome these limitations. For adult worms, PCR followed by sequencing of mitochondrial genes or external or internal ribosomal RNA spacers is suitable to genetically confirm morphological identifications. The most commonly used method to differentiate eggs or larvae is the reverse-line-blot hybridization assay. However, both methods suffer from the fact that target sequences are not available for many species or even that GenBank™ entries are unreliable regarding the cyathostomin species. Recent advances in proteomic tools for identification of metazoans including insects and nematodes of the genus *Trichinella* will be evaluated for suitability to diagnose cyathostomins. Future research should focus on the comparative analysis of morphological, molecular and proteomic data from the same cyathostomin specimen to optimize tools for species-specific identification.

INTRODUCTION

Parasitic helminths globally affect human and animal health and can be of zoonotic relevance (e.g., *Ascaris* spp.). In equines, the most important intestinal nematodes belong to the family Strongylidae and are comprised of two subfamilies: The Strongylinae encompassing 14 species in 5 genera (*Strongylus*, *Oesophagodontus*, *Triodontophorus*, *Bidentostomum,* and *Craterostomum*), and the Cyathostominae encompassing currently 50 valid species in 14 genera (*Caballonema*, *Coronocyclus*, *Cyathostomum*, *Cylicocyclus*, *Cylicodontophorus*, *Cylicostephanus*, *Cylindropharynx*, *Gyalocephalus*, *Hsiungia*, *Parapoteriostomum*, *Petrovinema*, *Poteriostomum*, *Scrjabinodentus*, *Tridentoinfundibulum*) (Lichtenfels et al., 2008), in contrast to previous publications listing 51 or 52 species in 13 genera (Lichtenfels, 1975; Lichtenfels et al., 2002). In the literature, the term “small strongyles” has either been coined to include only the Cyathostominae or all equine strongylidae except the genus *Strongylus*, which were designated “large strongyles” (Lyons et al., 1999). Although, still widely used, it is now recommended to avoid the terms small and large strongyles (Lichtenfels et al., 2002).

Since prevalence of the highly pathogenic *Strongylus* species declined after introduction of the macrocyclic lactones (Herd, 1990), the cyathostomins are currently recognized as the most important equine parasites because of (i) their up to 100 % prevalence in equids (Lyons et al., 1999), (ii) numerous reports of anthelmintic resistance and (iii) their pathogenicity which becomes particularly manifest in cases of sometimes fatal larval cyathostominosis (Love et al., 1999). Anthelmintic resistance against benzimidazoles is highly prevalent worldwide and pyrantel resistance is also frequently observed whereas reduced efficacy of macrocylic lactones has rarely been reported (Kaplan, 2002; Kuzmina and Kharchenko, 2008; Von SamsonHimmelstjerna, 2012; Matthews, 2014; Nielsen et al., 2014).

Cyathostomins have a direct life cycle with adults located in the lumen of caecum and colon, shedding eggs with the feces. First larvae (L1) hatch in the feces, molt twice to infectious third larvae (L3) which are ingested by equids. In the large intestine, L3 encyst inside the intestinal wall and may also undergo hypobiosis for months, before molting to fourth larvae (L4) (Corning, 2009). Synchronous excystation of large numbers of hypobiotic larvae potentially

causes larval cyathostominosis characterized by severe inflammation leading to weight-loss, diarrhea, colic, or even death (Love et al., 1999).

Although, Cyathostomins are a threat to equine welfare and scientific efforts to address this problem are frequently undertaken, research is impaired by the lack of sufficient identification methods (Lichtenfels, 2008). This perspective addresses the different methods, their advantages and limitations and gives an outlook on possible future methods for nematode identification using the cyathostomins as paradigm.

SAMPLES AND SAMPLING

The first challenge for species identification is the availability of suitable specimens. While strongylid eggs can be easily collected from feces, they have virtually no diagnostically useful morphological features. Strongylid L3 can be obtained from eggs using different fecal cultures methods (Smyth, 1990). However, only some L3 can be identified to the genus level, and this requires a high experience level. Only for a few species *in vitro* culture to the L4 (Chapman et al., 1994; Brianti et al., 2009) has been described. Therefore, adult parasites must be collected from naturally infected hosts. In horses, only a few adult strongyles are occasionally shed with the feces but collection of adult nematodes from feces after anthelmintic treatment is possible (Osterman Lind et al., 2003; Kuzmina et al., 2005; Kuzmina and Kharchenko, 2008). However, the complete worm burden representing all species in the living horse will only be documented by examination of all feces over several days, which may be associated with degradation of worms leading to distorted results. A more exact and meaningful method is the collection of adult nematodes from the content of the horse intestine (Drudge and Lyons, 1977). The critical test method, which is described in detail by Drudge et al. (1963), is a combination of both, the fecal collection over a week and collection during necropsy. This method is widely used to study the effectiveness of anthelmintic compounds (e.g., Lyons et al., 2007, 2010). Due to the need of sacrificed or slaughtered horses, these methods are restricted to research. Thus, there is a great need to develop alternatives for precise nematode diagnosis for living horses. The

immediate research aim is therefore the development of effective and specific non-invasive cyathostomin identification methods.

MORPHOLOGICAL IDENTIFICATION

For more than 100 years (Molin, 1861; Loos, 1900), a large number of cyathostomin species has been morphologically described using 93 different names. In the meantime, several previously described species are considered synonyms (Lichtenfels et al., 1998) and currently 50 species are recognized as valid. Comprehensive identification keys summing up the descriptions were published (Lichtenfels, 1975; Tolliver, 2000; Lichtenfels et al., 2008).

Morphological identification of adult strongyles relies on careful examination of faint characters at the anterior end of the adult nematodes or of the reproductive system. These traits include the size and shape of buccal capsules, internal and external leaf crowns and its extra-chitinous support as illustrated in **Figure 3-1** to point out that differences are very faint. Fine morphological structures of posterior end such as size and shape of the bursa, genital cone, gubernaculum, and spicules in males and shape of the tail, size and proportion of different parts of the reproductive system in females are also valuable for species differentiation (Lichtenfels, 1975; Dvojnos and Kharchenko, 1994; Lichtenfels et al., 2008). However, reliable morphological identification of adult cyathostomins can only be achieved following several years of intensive training and currently only few experts are available worldwide (Lichtenfels et al., 2008).

Whereas, adult cyathostomins can be discriminated, eggs, L1 and L2 cannot be differentiated from other nematodes of the family Strongylidae. Identification of L3 is possible for some genera such as *Strongylus*, *Triodontophorus*, *Gyalocephalus,* or *Poteriostomum* while most others can only be assigned to several cyathostomin larval types (Bevilaqua et al., 1993; Santos et al., 2016). The morphological features include qualitative and quantitative traits such as the number, arrangement and shape of intestinal/midgut cells, the length of the intestine and the length of the sheath tail.

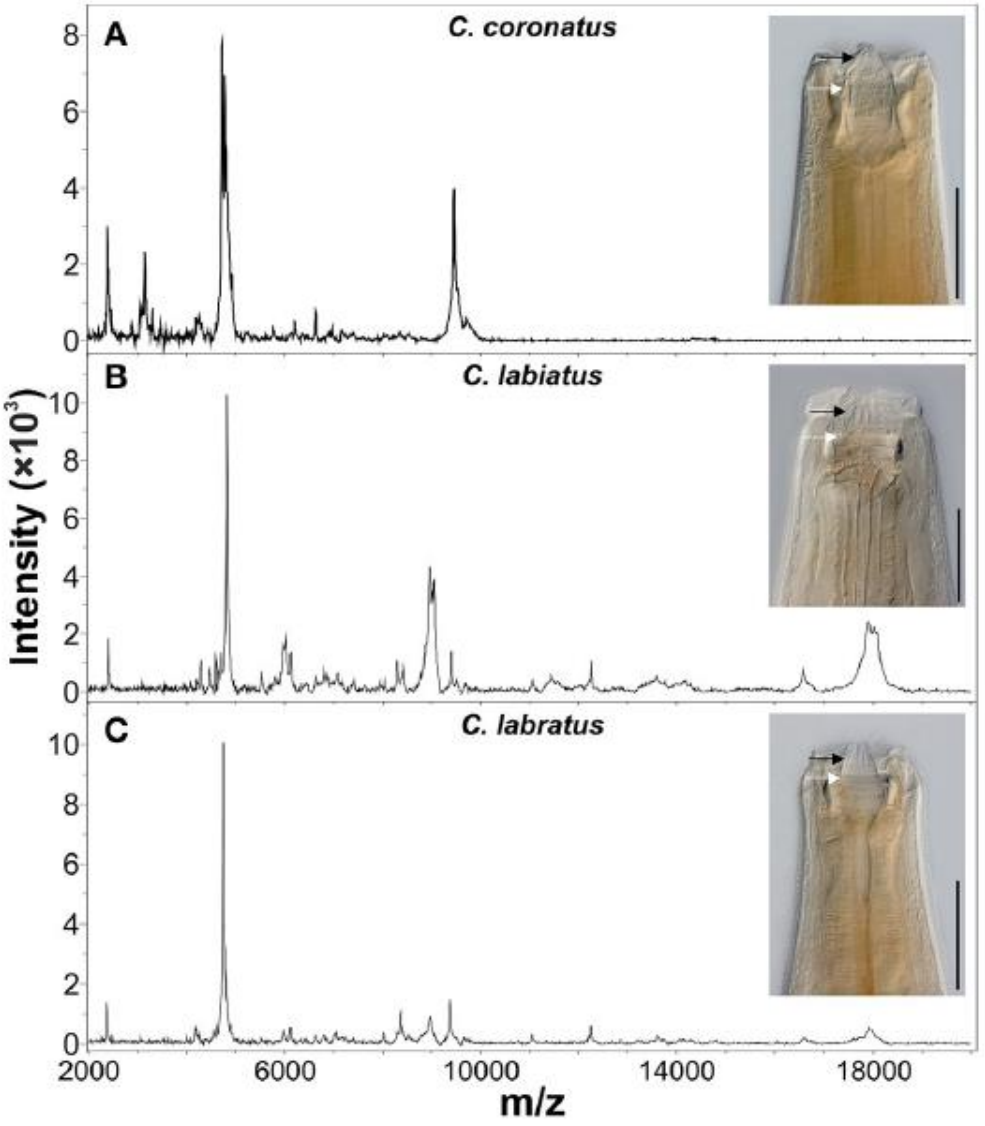

Figure 3-1: Comparison of morphological and proteomic species identification methods. Anterior ends and representative MALDI-TOF MS spectra of three cyathostomin species from the closely related species *Coronocyclus coronatus*, *Coronocyclus labiatus* and *Coronocyclus labratus* are shown. Scale bars represent 100 µm. The x-axes show mass charge ratios while y-axes represent arbitrary intensity units. Spectra were baseline subtracted and smoothed using default parameters in the flexAnalysis software (Bruker Daltonics). Specimen were cleared with lactophenol to improve visibility of structural features of the cuticle. External and internal leaf crown are indicated by black and white arrows, respectively.

MOLECULAR METHODS

To overcome the limitations of morphological identification, research has focused on molecular cyathostomin identification. These methods, once target-sequences are implemented correctly, can be applied independently of the nematode life-stage.

A target locus which proved to be useful in developing genetic markers for diagnostic and phylogenetic purposes is the ribosomal DNA (rDNA) (reviewed by Gasser and Newton, 2000; Chilton, 2004). Eukaryotic nuclear rDNA is organized in clusters of sometimes several hundred repeats. Coding sequences for 18S, 5.8S, and 28S rRNAs are interrupted by the first and second internal-transcribed spacers (ITS-1 and ITS-2) (Long and Dawid, 1980). The similarity of ITS sequences is higher within than among different species (Elder and Turner, 1995). This was also shown for strongyles, where the extent of intraspecific variation was low (0–0.3 %) in comparison to interspecific differences (0.6– 23.7 % for the ITS-1 region, 1.3–56.3 % for the ITS-2 region) (Hung et al., 1999b).

An early approach for molecular species identification based on the ITS-2 locus is the PCR-linked restriction fragment length polymorphism (PCR-RFLP) analysis, which was first used for differentiation of single eggs of the Strongylinae (Campbell et al., 1995) and was then applied to show that the morphologically very similar *Cylicocyclus ashworthi* and *Cylicocyclus nassatus* actually represent separate species (Hung et al., 1997). Another method is the PCR-linked single-strand-conformation-polymorphism technique (SSCP-PCR; Gasser and Monti, 1997), which allows the delineation of 14 strongyle species, including 9 cyathostomins, based on ITS-2 PCR products (Hung et al., 1999a). These methods rely on the DNA from individual worms or eggs and are thus associated with time-consuming procedures if it is desired to screen a representative subset of a strongyle community.

Species identification from mixed parasite DNA from fecal samples and/or copro-cultures was demonstrated after ITS-2 sequences for 28 strongyle species (including 22 cyathostomin species) were determined and species specific primers evaluated for four common species (Hung et al., 1999b). Although, this method theoretically allows species-specific research on pooled samples, it is limited to the identification of only few species.

The variability of the 26S-18S rDNA intergenic-spacer (IGS) was used for species differentiation of 16 cyathostomin species with a range of interspecies variation of 31–56 % (Kaye et al., 1998). The obtained sequences were used to develop a PCRELISA for the identification of six common cyathostomin species (Hodgkinson et al., 2003) and a Reverse-Line-Blot-Assay (RLB) to simultaneously identify 13 strongyle species (Traversa et al., 2007). Both methods have been used to monitor the species composition before and after anthelmintic treatment (Hodgkinson et al., 2005; Cernanská et al., 2009; Ionita et al., 2010; Traversa et al., 2010). Re-evaluation and validation of existing and new oligo-probes increased the number of species that can be identified with RLB to 18 (Cwiklinski et al., 2012). PCR-ELISA and RLB are qualitative methods detecting the presence or absence of the different species. A semi-quantitative approach applying replicates of pooled larvae was positively evaluated to enable screening of many cyathostomin populations in parallel (Kooyman et al., 2016).

Despite being recognized as “a less suitable target than ITS for quick diagnostic tests,” due to its high substitution rates and high possibility of intraspecific polymorphisms, the mitochondrial Cytochrome oxidase c subunit I (COI) is used for species differentiation and could indicate cryptic species (Blouin, 2002). Twentytwo COI sequence haplotypes (overall 10.8 % rate of intraspecific nucleotide difference) were found within *C. nassatus* using specimen from different hosts and geographic origins, while only little variation (0.0–0.6 % differences) was seen in the ITS-2 sequences suggesting cryptic species within *C. nassatus* (Traversa et al., 2008) and maybe other cyathostomin morpho-species as well. Analysis of ITS-1 and ITS-2 sequences of *Cylicostephanus minutus* individuals showed 3.0 and 7.4 % differences also indicating the presence of a cryptic species complex (Hung et al., 1999a). The combination of markers on questionable species appears useful to investigate the occurrence of cryptic species complexes.

Whereas the objective of research on cyathostomin species identification is on the one hand to improve the available diagnostic tools, it aims on the other hand to contribute to the understanding of the phylogenetic relationships between the different taxa. Therefore, three gene loci, the ITS-2, COI and 28S rRNA were compared for their phylogenetic usefulness in

strongyles. It was encountered that the high level of substitution saturation renders COI unsuitable for phylogenetic analysis. The remaining loci, ITS-2 and 28S rRNA, both showed similar groupings of cyathostomins. Combining both loci resulted in a tree with improved bootstrapping support for the internal nodes (McDonnell et al., 2000) pointing towards the importance of the simultaneous application of different molecular markers. This can also be seen in a study analyzing ITS-1 and ITS-2 sequences of 30 strongyle nematode species, including 23 cyathostomin species that questions the widely accepted separation of Strongylinae and Cyathostominae and proposes a framework to systematically analyze future datasets of strongyle nematodes (Hung et al., 2000). Findings consistent with the latter phylogenetic analysis were shown in a study focusing on the genus *Cylicocyclus* which proposed a separation of *Cylicocyclus* in two clades but statistical support for this hypothesis was relatively weak (Bu et al., 2013).

SEROLOGICAL METHODS

Larval cyathostominosis is caused by the simultaneous reactivation and emergence of high numbers of hypobiotic larvae (Love et al., 1999) causing severe pathology. Usually, no eggs are expelled due to absence of adults making coproscopic diagnosis unfeasible (Murphy and Love, 1997). This leads to the aim of prepatent detection of cyathostomin infections using serology to be able to assess the risk of larval cyathostominosis based on the estimation of the mucosal cyathostomin worm burden.

One promising approach identified anti-larval IgG(T) serum antibody responses to two antigen complexes, only elicited by larvae, as potential markers for prepatent cyathostomin infections (Dowdall et al., 2002). Subsequent purification of native antigenic complexes from larvae resulted in higher IgG(T) signals in an ELISA and reduced the number of false positive responses (Dowdall et al., 2003). Further evidence for an immunodiagnostic potential of these markers is given by a study where the mucosal worm burden of naïve and infected horses was assessed and found to significantly correlate with the IgG(T) serum levels. Additionally, sera from horses with clinical suspicion of larval cyathostominosis had significantly increased antigen-specific

IgG(T) levels (Dowdall et al., 2004). One antigenic complex could be identified as cyathostomin gut-associated larval antigen-1 (Cy-GALA-1) and allocated to the species *Cyathostomum pateratum* (McWilliam et al., 2010), followed by the characterization of the orthologous antigens of four additional common cyathostomin species. An ELISA was developed based on recombinant Cy-GALA proteins, which allows the detection of the immune response to cyatostomin larvae. Cross-reactivity to other parasites was not observed and is unlikely, because of the diversity of orthologous GALA sequences of non-cyathostomin species (Mitchell et al., 2016). In the absence of experimental single species infections, crossreactivity between cyathostomin species is hard to evaluate and diagnostic tests should therefore include a panel of different Cy-GALA proteins to detect most larval cyathostomin infections.

If in future available for routine diagnosis this approach could be of clinical relevance and help ruling out or confirming a larval cyathostominosis in horses with unspecific symptoms of wasting or colic. However, due to the lack of species-specificity serological methods will not help gaining detailed knowledge on the role of individual species in larval cyathostominosis.

PROTEOMICS METHOD

The proteome based matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS profiling) species identification of microorganisms has already revolutionized diagnostic microbiology. Species identification is based on the molecular masses of proteins such as ribosomal and other abundant proteins. A small amount of microorganisms or crude extracted intact proteins is transferred to specially designed target plates and allowed to co-crystallize with an inert, UV absorbing matrix such as α-Cyano-4-hydroxycinnamic acid. A pulsed 337 nm laser beam irradiates the samples to form a dense ion plume. The resultant ions are accelerated through a vacuum tube to reach the detector and separated according to their charge/mass (*m/z*) ratio and the time of flight (TOF) for each is measured. The mass range *m/z* 2 000–20,000 is generally applied for species identification through pattern matching of the spectra peaks with that of a reference spectra database. The method is popular due to its cost-effectiveness, reliability and availability of specially designed

linear MALDI machines equipped with software tools and reference databases. This method has been evaluated for a variety of microorganisms such as bacterial, fungal, and viral pathogens (Wieser et al., 2012; Clark et al., 2013). In the past two decades, this technique was established for rapid characterization of eukaryotic cell lines and for species differentiation of protozoan parasites (e.g., *Leishmania*, *Giardia*) and arthropods (e.g., mosquitoes, ticks, tsetse flies) (Hoppenheit et al., 2013; Singhal et al., 2016; Yssouf et al., 2016). Regarding nematodes, first diagnostic use of MALDI-TOF has been described to identify different races of *Ditylensus dipsaci* (Perera et al., 2009) and closely related species of root-knot and seed-gall nematodes (Perera et al., 2005; Ahmad et al., 2012). Despite these developments, extensive studies on the application of MALDI-TOF MS for rapid species identification for helminth have not been reported. Recently, MALDI-TOF MS was applied for rapid species identification of *Trichinella* spp. after adopting a simple formic-acid/acetonitrile extraction from pooled larvae and compilation of a reference database (Mayer-Scholl et al., 2016). This approach could also be extended to cyathostomin species identification. Preliminary data to evaluate the potential for MALDI-TOF MS for cyathostomins revealed distinct patterns for adult individuals of different species **(Figure 3-1)**.

Of course, master-spectra libraries can only be generated with validated, correctly identified material. This requires that proteomic data are obtained from morphologically and molecularly identified individual specimen. For arthropods, this issue can be solved by using always e.g., a wing or leg for proteomic and any other body part for molecular analysis. For nematodes, which are not segmented, this is not trivial since no defined body parts can be reproducibly cut off at exactly the same position without altering the protein spectrum. Therefore, methods need to be developed that reliably allow to conduct both methods using exactly the same starting material, despite the fact that the protein extraction usually involves conditions that damage DNA. Nevertheless, it was possible to extract DNA of sufficient quality from the acetonitrile/formic acid insoluble material to successfully amplify and sequence the ITS-2 region for the three specimen shown in **Figure 3-1**. These were 100, 99, and 97 % identical to Genbank accession numbers JN786951.2, JN786947.2, JN786949 respectively, which confirmed the morphological identification in each case. Possible limitations could be

the different spectra elicited by different development stages, as seen in tick species identification. However, despite changes of the overall MS protein profiles, the ticks could be classified correctly

CONCLUSION

Different approaches have been used over the past decades to improve cyathostomin species delineation. All methods have their advantages and limitations **(Table 3-1)** and none is already fully satisfying for the research questions to be answered and all are far away from applicability in routine laboratory diagnosis. Comprehensive research on different aspects improving the discrimination of individual cyathostomin species, such as inclusion of several molecular markers and additional proteomic profiles could be of great help in the future. This should include the morphological identification together with the description of the genotype (molecular) and phenotype (proteomic) data in association with the currently accepted taxonomic classification. Ideally, morphological, molecular and proteomic data from the same individual should be used to take advantage of all three approaches to identify the complete species spectrum in the Cyathostominae and delineate their phylogenetic relationship.

Table 3-1: Comparison of methods for cyathostomin species identification.

Identification method		Life stage	Usefulness and limitations	References
MORPHOLOGICAL IDENTIFICATION				
		Eggs	No species differentiation possible	Lichtenfels, 1975, 2008; Dvojnos and Kharchenko, 1994; Tolliver, 2000; Lichtenfels et al., 2008; Kharchenko et al., 2009; Kornaś et al., 2009; Santos et al., 2016
		Larvae	L3 can be allocated to different larvae types, but not to individual species	
		Adults	Identification keys published but species identification is difficult for inexperienced workers	
MOLECULAR METHODS				
Marker	**Method**			
ITS-1 and ITS-2	PCR and sequencing	All	Species identification and phylogenetic analysis, identification of cryptic species Sometimes only small differences between closely related species Not applicable for mixed samples, isolation of DNA from individual specimen necessary	Campbell et al., 1995; Chilton et al., 1997; Hung et al., 1999a, 2000; Bu et al., 2013
	SSCP-PCR	All	Delineation of 14 Strongylida species (9 Cyathostomin species), Isolation of DNA from individual specimen necessary	Gasser and Monti, 1997
	PCR-RFLP	All	Distinction of Strongylinae eggs, Distinction of two Cyathstomin species (*C.ashworthi, C.nassatus*). Isolation of DNA from individual specimen necessary, established for larvae	Campbell et al., 1995; Hung et al., 1997
IGS	PCR-ELISA	All	Screening for 6 cyathostomin species in mixed samples possible. Established for eggs and larvae	Hodgkinson et al., 2003, 2005
	RLB	All	Differentiation of up to 18 common species, less time consuming and costly than other molecular methods, screening of strongyle population before and after anthelmintic treatment possible, mixed samples possible, but more viable for individual worms; semi-quantitative approach possible	Traversa et al., 2007; Ionita et al., 2010; Cwiklinski et al., 2012; Kooyman et al., 2016
COI	PCR and sequencing	All	Investigation of intraspecies genetic variability, identification of cryptic species, not applicable for mixed samples, isolation of DNA from individual specimen necessary	Hung et al., 1999a; Traversa et al., 2008
SEROLOGICAL METHODS				
Protein-based ELISA		Larvae	Pre-patent detection of four common cyathostomin species possible from serum, no cyathostomin species differentiation possible	Mitchell et al., 2016
PROTEMICS METHOD				
		Potentially all	Method is established for bacteria, fungi, several species of arthropods; only one study on nematodes (*Trichinella* spp.), protocols and reference spectra data base have to be established	Karger et al., 2012; Yssouf et al., 2013, 2016; Mayer-Scholl et al., 2016; Singhal et al., 2016;

AUTHOR CONTRIBUTIONS

CB performed MALDI-TOF experiments and literature surveys. CB and JK drafted and edited the manuscript. TK contributed microphotographs. TK, JM, and GS contributed to writing of the manuscript. CB, JK, JM, and GS designed the general outline.

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4 Concurrent Proteomic Fingerprinting and Molecular Analysis of Cyathostomins

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4.2 Supporting Information

4.2.1 Supporting information, Text 1

The ITS-2 region was amplified by PCR with primers NC1 (5'-ACGTCTGGTTCAGGGTTGTT-3') and NC2 (5'-TTAGTTTCTTTTCCTCCGCT-3'). [1] Amplification of the COI region was performed using the primers COI_Nema_Fw (5'-GAAAGTTCTAATCATAARGATATTGG-3') and COI_Nema_Rv (reverse; 5'-ACCTCAGGATGACCAAAAAAYCAA-3').[2] Each reaction contained 200 µM dNTPs, 0.5 µM of each primer pair, 0.4 U Phusion Hot Start II High-fidelity DNA polymerase (Thermo Scientific) and 2 µl template DNA in 20 µl 1 × HF buffer. The PCR runs included initial denaturation at 98 °C for 30 s, 40 cycles of denaturation at 98 °C for 10 s, annealing at 55°C for 30 s and elongation at 72 °C for 30 s, followed by a final extension at 72°C for 5 min. PCR products were directly purified with DNA Clean & Concentrator™-5 (Zymo Research).

Molecular cloning of PCR products into the pSC-B-amp/kan vector was performed by applying the StrataClone Blunt PCR Cloning Kit (Agilent Technologies). Plasmid DNA was isolated from liquid bacterial cultures with either ZYPPY™-96 Plasmid Miniprep Kit (Zymo Research) or EasyPrep® Pro Plasmid Miniprep Kit (Biozym). Sequencing of plasmid DNA was performed by LGC Genomics (Berlin). Before further analyses, primer sequences were removed from the data.

DNA sequences were aligned either codon-wise using MUSCLE as implemented in Mega7 (COI sequences) or using MAFFT with the Q-INS-i option (ITS-2), which uses RNA secondary structure predictions to optimize the alignment. [3-5] The COI sequences were split into two different alignments representing either for codon positions 1 and 2 or position 3 using DAMBE software. [6] The latter was also applied to identify potential substitution saturation in the alignments by applying the test according to Xi et al..[7] Then, alignments were concatenated with FASconCAT-G creating a phylip formatted supermatrix and a partition file. After manually editing the partition file to apply the DNA+G model to each partition, phylogenetic analysis was performed using RAxML 8.2.10 on the CIPRES portal in the rapid bootstrapping mode to identify the phylogram with the highest likelihood and bootstrap support values for nodes in a single step (-f a options). [8, 9] The resulting tree topology was used in a second run of RAxML to constrain the topology of the phylogram and calculate

independent node support values using the Shimodaira-Hasegawa likelihood ratio test (SH LRT). Separate trees were calculated for the ITS-2 alignment (one partition), the COI alignment (two partitions) and the combination of both datasets (three partitions). An additional phylogenetic tree was calculated using only ITS-2 sequences but including additional sequences obtained from GenBank™ with the same approach.

To calculate pairwise identities of between individual orthologues sequences, p-distance matrices were calculated from alignments of COI and ITS-2 sequences using the dist.dna function in the "ape" package.[10] Pairwise deletion was set to FALSE and in order to obtain uncorrected distances not based on modeling e.g. reversions, the method was set to "raw". Identities were calculated from distances using the formula 100-100×pdistance. Identities were categorized depending on whether they represented comparisons within the same species or OTU. Differences between such categories were analyzed for significance using a Kruskal-Wallis test followed by Dunn's post hoc test comparing all pairs of categories.

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4.2.2 Supporting figures

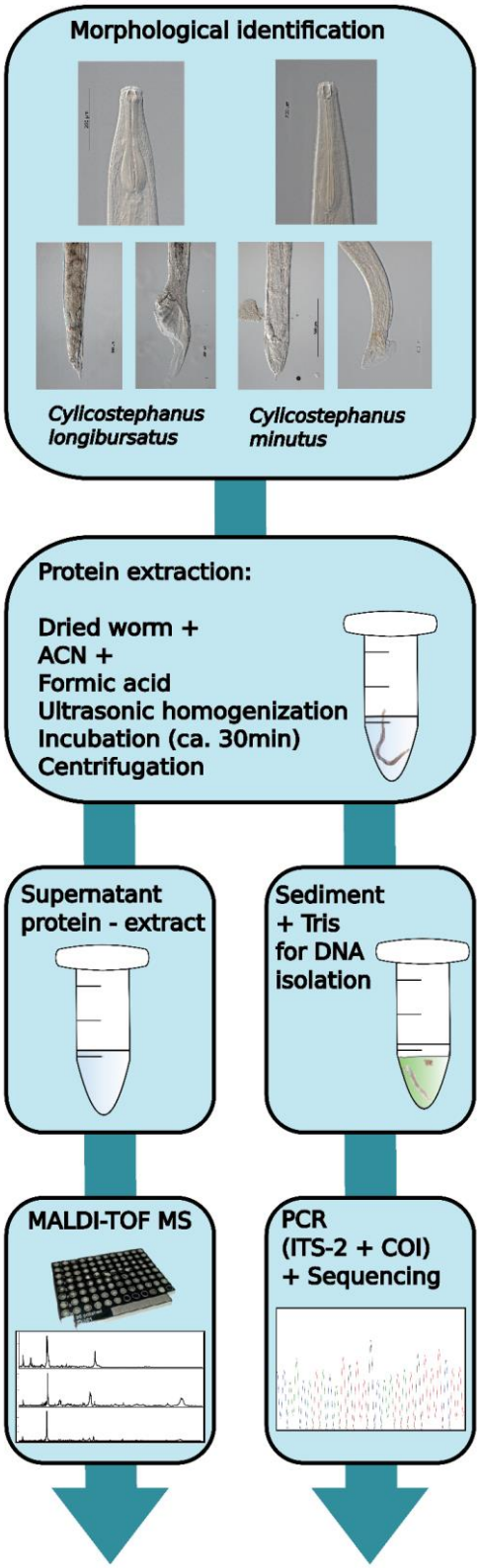

Figure 4-5: Schematic workflow of sample preparation procedure and experiments.

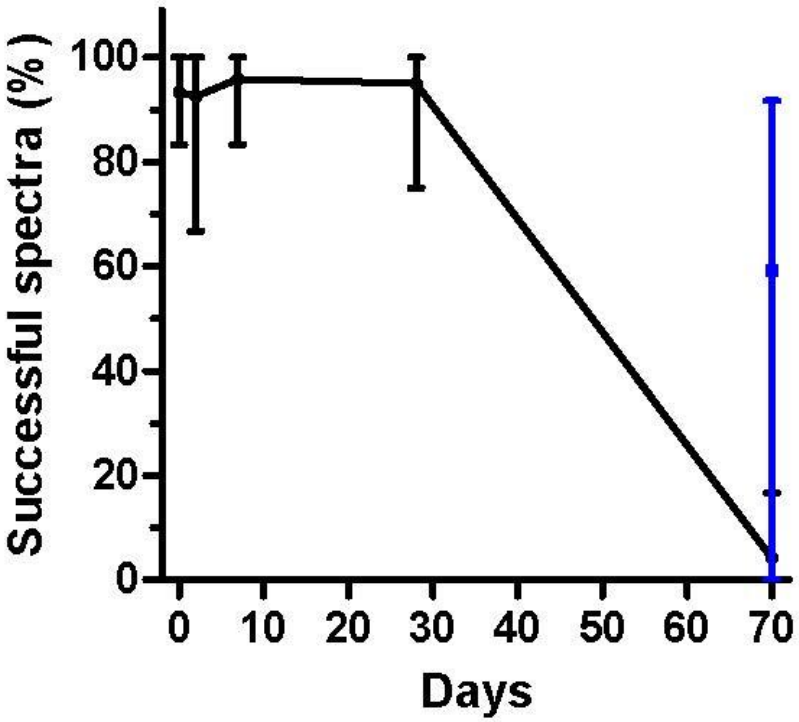

Figure 4-6: Percentage of spectra above the intensity threshold of 4 000 arbitrary units out of 12 technical replicates of 10 biological replicates. Data were obtained using 55 % (black) or 65 % of the available laser power. All values represent mean ± range for 10 individual worms.

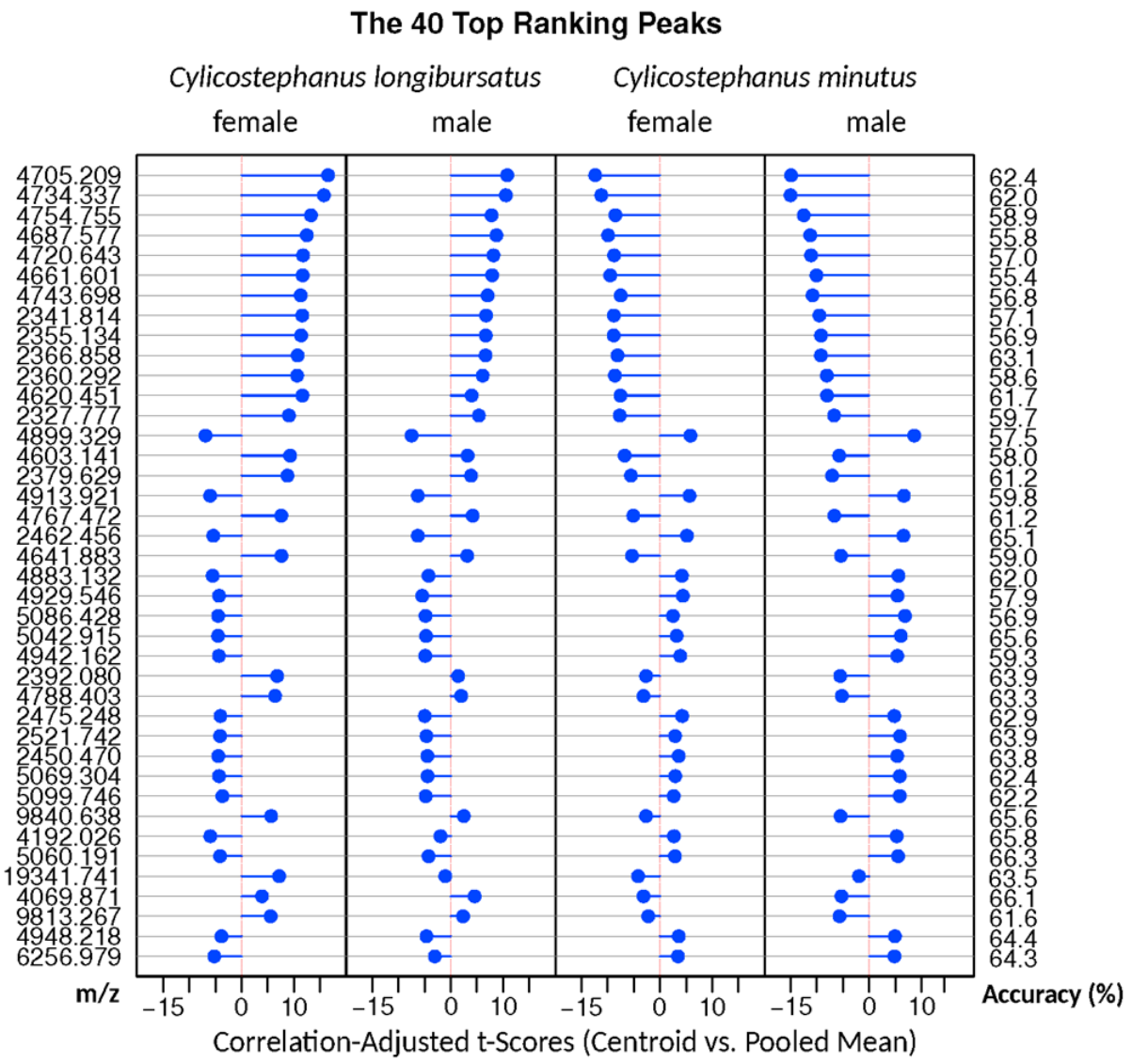

Figure 4-7: Ranking of the top 40 peaks (*m/z*) after linear discriminant analysis to identify *Cylicostephanus longibursatus* and *Cylicostephanus minutus* and their sex. The peak with the highest variation between the groups species is ranked first, the length and direction of the horizontal lines visualize the correlation-adjusted *t*-scores of the centroid versus the pooled mean of each listed peak. The different sex of the same species can be identified with the same peaks, although with varying discriminatory power.

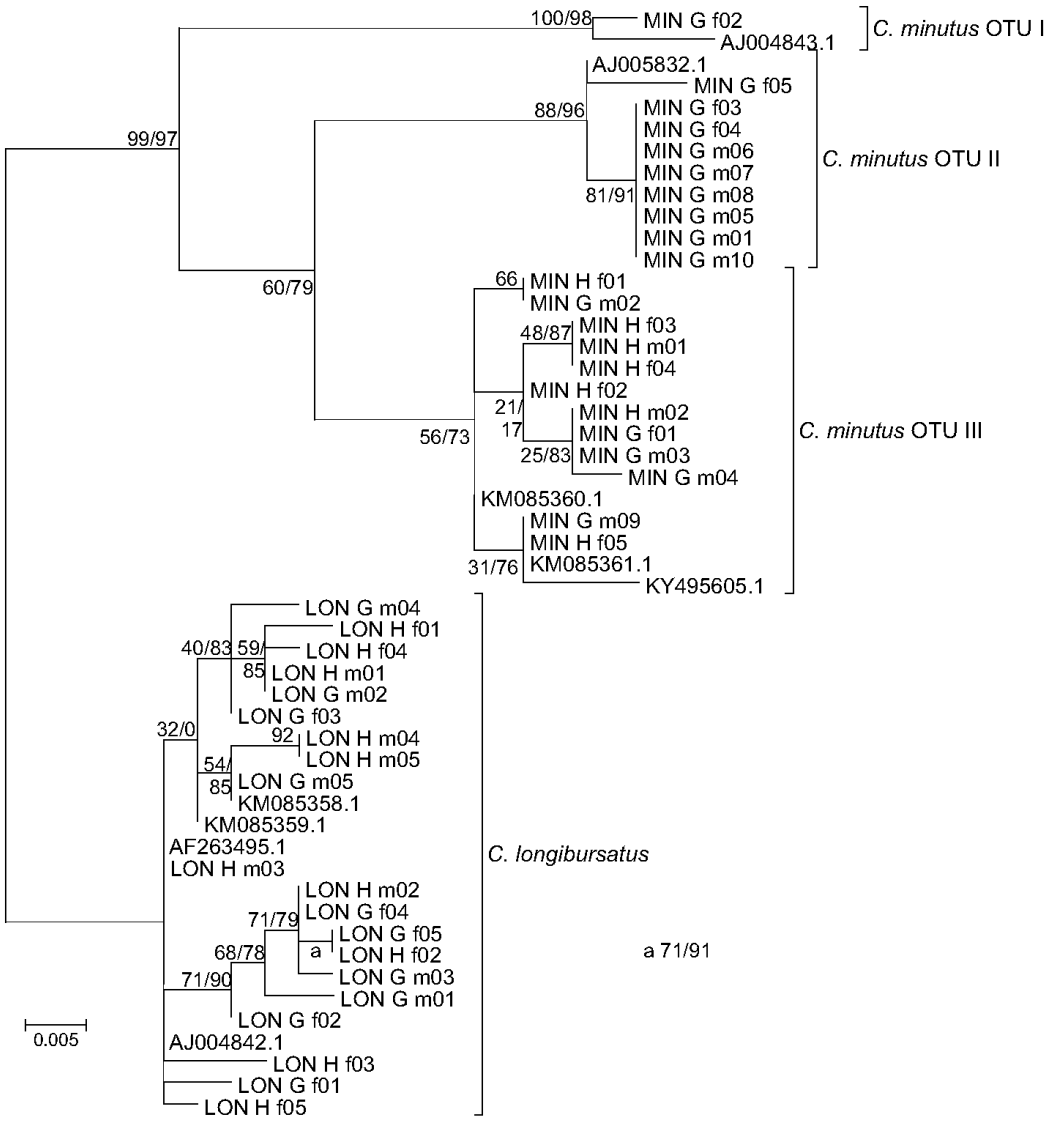

Figure 4-8: Maximum likelihood phylogenetic tree for *Cylicostephanus longibursatus* and *Cylicostephanus minutus* using ITS-2 sequences only. The different operational taxonomic units (OTUs) within *C. minutus* are indicated. Node support is given as bootstrap values before and results of the Shimodaira-Hasegawa likelihood ratio test behind the slash. If both values were identical, only a single number is provided. The scale bar represents 0.005 substitutions per site. Specimen collected within the present study are labeled using morphospecies identification (MIN or LON), the origin (G, Germany; H, Ukraine), the sex (m, male; f, female) and an individual number. Sequences obtained from GenBank™ are labeled with their accession numbers.

5 Nuclear and mitochondrial marker sequences reveal close relationship between *Coronocyclus coronatus* and a potential *Cylicostephanus calicatus* cryptic species complex

Full details of the scientific findings of this chapter have been subjected to peer review in December 2018 and have been accepted for publication in *Infection Genetics and Evolution* in July 2019. In compliance with the Elsevier Sharing policy, an author created version (preprint) without any editorial changes is provided below.

The final publication can be accessed via:

https://doi.org/10.1016/j.meegid.2019.103956

5.1 Publication

Nuclear and mitochondrial marker sequences reveal close relationship between *Coronocyclus coronatus* and a potential *Cylicostephanus calicatus* cryptic species complex

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Abstract

The Cyathostomins (Nematoda, Strongyloidea) parasitizing equines represent a diverse group currently including more than 50 species. However, their taxonomy has been repeatedly revised and occasionally the presence of cryptic genospecies was suggested. Moreover, molecular- and morphology-based phylogenetic analyses give divergent results. For instance, molecular data have suggested close relationship between *Coronocyclus coronatus* and *Cylicostephanus calicatus*, although morphology-based taxonomy places them in different genera. Here, nuclear (internal transcribed spacer 2, ITS-2) and mitochondrial (cytochrome oxidase I, COI) sequences were obtained from the same individual, morphologically identified worms. In both morphospecies, two ITS-2 sequences types were observed: In *Cor. coronatus*, a small PCR product of 278 bp (nuclear haplotype group nHGBco) was always present but often in combination with a larger 369-370 bp fragment (nHGAco). In *Cys. calicatus*, either a large 370 bp product (nHGAca) or a short 281 bp amplicon (nHGBca) were found, but never both. Sequence identity between morphospecies was up to 100 %. The smaller differed from the larger fragments by deletion of the region 110-198 in Cor. coronatus and 112-203 in *Cys. calicatus*. In COI, three and five mitochondrial HGs, mtHG1co-mtHG3co and mtHG1ca-mtHG5ca were identified for *Cor. coronatus* and *Cys. calicatus*, respectively. In *Cor. coronatus*, there was no particular association of mtHG with nuclear genotypes (only nHGBco vs. both nHGBco plus nHGAco). In contrast, in *Cys. calicatus* the nHGAca was always associated with the mtHG1ca, mtHG2ca or mtHG5ca whereas nHGBca was exclusively associated with mtHG3ca or mtHG4ca. Despite up to 100 % identity in the nHGs, no mixing of mtHGs was observed between both species. Clear separation of certain nHGs with particular mtHGs in *Cys. calicatus*, despite the fact that the same host individuals were infected with both groups simultaneously, suggests presence of two noninterbreeding genospecies within *Cys. calicatus*, which needs further confirmation using additional samples from diverse geographical origins.

Abbreviations: ca, of *Cys. calicatus*; co, of *Cor. coronatus*; COI, cytochrome oxidase I; *Cor.*, *Coronocyclus*; *Cys., Cylicostephanus*; HG, haplotype group; ITS-2, internal transcribed spacer 2; mtHG, mitochondrial haplotype group; nHG, nuclear haplotype group; nHGAca, nuclear haplotype group A of *Cys. calicatus*, nHGBca, nuclear haplotype group B of *Cys. calicatus*; nHGAco, nuclear haplotype group A of *Cor. coronatus*; nHGBco, nuclear haplotype group B of *Cor. coronatus*.

Introduction

The subfamily Cyathostominae (Nematoda, Strongyloidea) contains important gastrointestinal parasites of equines and currently more than 50 species infecting equines are recognised (Lichtenfels et al., 1998, 2002, 2008). The systematic of this group has been revised multiple times over the years including redescription of species and evidence for cryptic species in some morphospecies (Bredtmann et al., 2019; Hung et al., 1999b) and options for diagnosis on a species level are currently very limited (Bredtmann et al., 2017). Molecular and morphology-based phylogenetic approaches apparently came to quite distinct topologies; but the morphology-based analyses were only presented on workshops and have never been published (Lichtenfels et al., 2002, 2008). In the ITS-2 based molecular phylogenetic analysis, resolution was quite poor and e.g. members of the genera *Cylicostephanus* Ihle, 1922 and *Coronocyclus* Hartwich, 1986 were not reliably placed into separate, statistically supported clusters (Hung et al., 1999a). The present study aimed to characterise the molecular diversity and relationship of two closely related species *Coronocyclus coronatus* (Looss, 1900) Hartwich, 1986 and *Cylicostephanus calicatus* (Looss, 1900) Cram, 1924 that were placed in close phylogenetic proximity by two molecular phylogenetic analyses (Hung et al., 1999a; McDonnell et al., 2000).

Materials and methods

Adult worms were collected from eight German horses (*Equus ferus caballus*) during necropsy and from faeces of five species of equines kept at the Askana Nova Biosphere reserve, Ukraine, i.e. a domestic horse (*Equus caballus*), a wild Przewalski's horse (*Equus ferus przewalskii*), a donkey (*Equus asinus*), a Turkmenian kulan (*Equus hemionus kulan*) and a Burchell's zebra (*Equus quagga burchelli*) after anthelmintic treatment with a macrocyclic lactone drug (aversectin C) as described recently (Bredtmann et al., 2019). Adult worms were identified morphologically according to Lichtenfels et al. (2008); specimens identified as *Cor. coronatus* and *Cys. calicatus* were included into this study. Details regarding numbers, host origin and sex of specimen are provided in **Supplementary Table 5-1**.

After DNA extraction, PCRs targeting the internal transcribed spacer 2 (ITS-2) (Gasser et al., 1993) and a partial cytochrome oxidase I (COI) (Duscher et al., 2015) fragments were

conducted using a high-fidelity DNA polymerase (Phusion II, Thermofisher Scientific) as detailed in **Supplementary Table 5-2** and in Bredtmann et al. (2019). PCR products were cloned into the pSC-B-amp/kan vector (Strataclone Blunt Cloning Kit, Agilent) and one clone with insert per individual worm was sequenced by LGC Genomics (Berlin).

Sequences were aligned using MAFFT with consideration of the predicted RNA secondary structure (Q-INS-I option) for ITS-2 (Katoh et al., 2017) and MUSCLE for COI (Edgar, 2004) followed by maximum likelihood phylogenetic analyses conducted using RAxML (Stamatakis, 2014) as described recently (Bredtmann et al., 2019). For COI sequences, separate models were fitted for codon positions 1 and 2 vs. codon position 3.

Results and discussion

In total, 60 *Cor. coronatus* and 63 *Cys. calicatus* specimens were included into this study. ITS-2 amplification was successful for all specimens. For all *Cor. coronatus*, a 278 bp PCR (B) fragment was amplified but 41 of these *Cor. coronatus* specimens showed an additional (A) PCR product of 369-370 bp. Both PCR products differed by deletion of the bases in positions 112-203 (92 bp) of the A fragment. For *Cys. calicatus*, 46 specimens showed amplification of a 370 bp (A) fragment while 17 specimens showed a 281 bp (B) fragment. In this case, the deletion encompassed the positions 110-198 (89 bp). The number of *Cor. coronatus* A fragment haplotypes was 25, while 10 B fragment haplotypes were counted. For *Cys. calicatus*, 20 A fragment and 9 B fragment haplotypes were identified. The overall identity between fragments of the same morphospecies was in the range of 94.9-100 % for *Cor. coronatus* and 96.0–100 % for *Cys. calicatus* (**Figure 5-1 A**). However, identity of ITS-2 sequences between both morphospecies was also 94.9–100 % indicating that ITS-2 sequences are not suitable to discriminate between *Cor. coronatus* and *Cys. calicatus*. Identity of the B fragments and the corresponding regions of the larger A fragments was 95.0-100 % even between the morphospecies. In contrast, the between-species comparison for the region between positions 110 and 203, present only in the A fragments, was only 84.3-94.6 %.

In a maximum likelihood phylogenetic analysis based on ITS-2 sequences, the A and the B fragments formed separate clusters and these were used to root one cluster with the other (**Figure 5-2** and **Supplementary Figure 5-4**). In the cluster containing the smaller B fragments, there was virtually no substructuring observable and the *Cor. coronatus* and *Cys. calicatus*

sequences were not separated from each other nor were the *Cor. coronatus* sequences from specimen with double bands (A plus B) separated from the sequences from specimen showing only the B amplicon (**Supplementary Figure 5-4**). Thus, for each species only one B type nuclear haplotype group (nHGBco and nHGBca) was identified. In contrast, for the A fragments distinct subclusters with moderate statistical support were defined as major nHGs. One cluster contained 33 *Cor. coronatus* nHG A sequences (nHGAco). The majority of *Cys. calicatus* sequences was clustered in two groups containing 36 (nHGAca1) and 8 (nHGAca2) sequences, respectively. A few *Cor. coronatus* and *Cys. calicatus* sequences were not included in these nHGs but positioned with low statistical support in the A fragment subtree (**Figure 5-2**). The complex PCR fragment pattern in *Cor. coronatus* (1 vs. 2 products) cannot be explained by hybridisation with *Cys. calicatus* since no nHGAca was found in any *Cor. coronatus*, as identified by morphology and COI sequence. There was no obvious differentiation between geographic regions (Ukraine vs. Germany) or equine host species. The fact that the ITS-2 sequences were not able to discriminate two species of cyathostomins that were not even placed in the same genus (see Lichtenfels et al., 2008) confirms previous findings that the ITS-2 is not a reliable diagnostic marker on the species level as revealed by data on the ruminant parasitic nematode genus *Cooperia* (Ramünke et al., 2018).

Therefore, a more variable mitochondrial marker with superior barcoding properties was included in the analysis (Blouin, 2002). Amplification and sequencing of a 653 bp COI fragment was achieved for 59 specimens of *Cor. coronatus* (56 different haplotypes) and 53 of *Cys. calicatus* (49 haplotypes). In contrast to the ITS-2 data, the COI-based phylogram was able to clearly separate *Cor. coronatus* and *Cys. calicatus*. The analysis further identified two major mitochondrial HGs, mtHG1co and mtHG2co, with 23 and 35 sequences, respectively (**Figure 5-3** and **Supplementary Figure 5-5**). A single *Cor. coronatus* COI sequence was not assigned to a cluster and was considered to belong to an additional mtHG3co. In *Cys. calicatus*, 5 mtHGs were identified with 14 mtHG1ca, 23 mtHG2ca, 9 mtHG3ca, 5 mtHG4ca and 2 mtHG5ca sequences (**Figure 5-3** and **Supplementary Figure 5-5**).

These mtHGs were then mapped back to the ITS-2 tree (**Figure 5-2** and **Supplementary Figure** 5-4). The two major *Cor. coronatus* mtHGs were both found in specimens showing only the nHGBco or both, nHGBco plus nHGAco. This shows obviously free interbreeding between the

two nuclear genotypes and the two major mtHGs. Apparently, ITS-2 variants of different length are present in the *Cor. coronatus* populations. Since rRNA genes are usually present in more than one cluster in the genome, such variation in ITS length can occur in a single genome as previously described e.g. in *Ancylostoma duodenale* (Demeler et al., 2013).

In contrast to *Cor. coronatus*, the five *Cys. calicatus* mtHGs were distributed very unevenly among the two different nHGs. While all specimen with the nuclear nHGA1ca or nHGA2ca belonged to the mtHG1ca, mtHG2ca or mtHG5ca, all specimens with the nHGBca were associated with the mtHG3ca and mtHG4ca. This strict association of certain nuclear and mitochondrial HGs suggests that there is no or little gene flow between these groups. Since the parasites came from the same host individuals, i.e. both *Cys. calicatus* genospecies were found to co-infect the German and Ukrainian horses, as well as the kulan, while in specimens from the Przewalski's horse, donkey and zebra only the nHGAca was found, it is reasonable to assume that the different *Cys. calicatus* genospecies do not interbreed and might represent different cryptic parasite genospecies.

Although the mitochondrial COI sequences provide a much better resolution in comparison to ITS-2 sequences, they alone are obviously not sufficient to correctly delineate species boundaries. In terms of raw sequence identity (**Figure 5-1 B-D**) as well as phylogenetic position (**Figure 5-3** and **Supplementary Figure 5-5**), the different mtHGs within *Cys. calicatus* show a degree of dissimilarity that would be comparable with a status as discrete species. In particular, the distance between mtHG5ca on one and mtHG1ca/mtHG2ca on the other hand is larger than distance between mtHG1ca/mtHG2ca and mtHG3ca/mtHG4ca. However, combined analysis of nuclear and mitochondrial marker sequences strongly suggests that mtHG1ca/mtHG2ca together with mtHG5ca belong to one and mtHG3ca/mtHG4ca to a second species. However, investigation of additional specimens from different geographical origins is required to confirm this hypothesis.

In conclusion, the data set presented here shows high genetic similarity of *Cor. coronatus* and *Cys. calicatus* despite the fact that morphology-based taxonomy places them in different genera. Neither nuclear ITS-2 nor mitochondrial COI sequences alone were able to identify genospecies correctly while combined analysis provided a better resolution indicating the existence of discrete subspecies in what is currently assigned as *Cys. calicatus*.

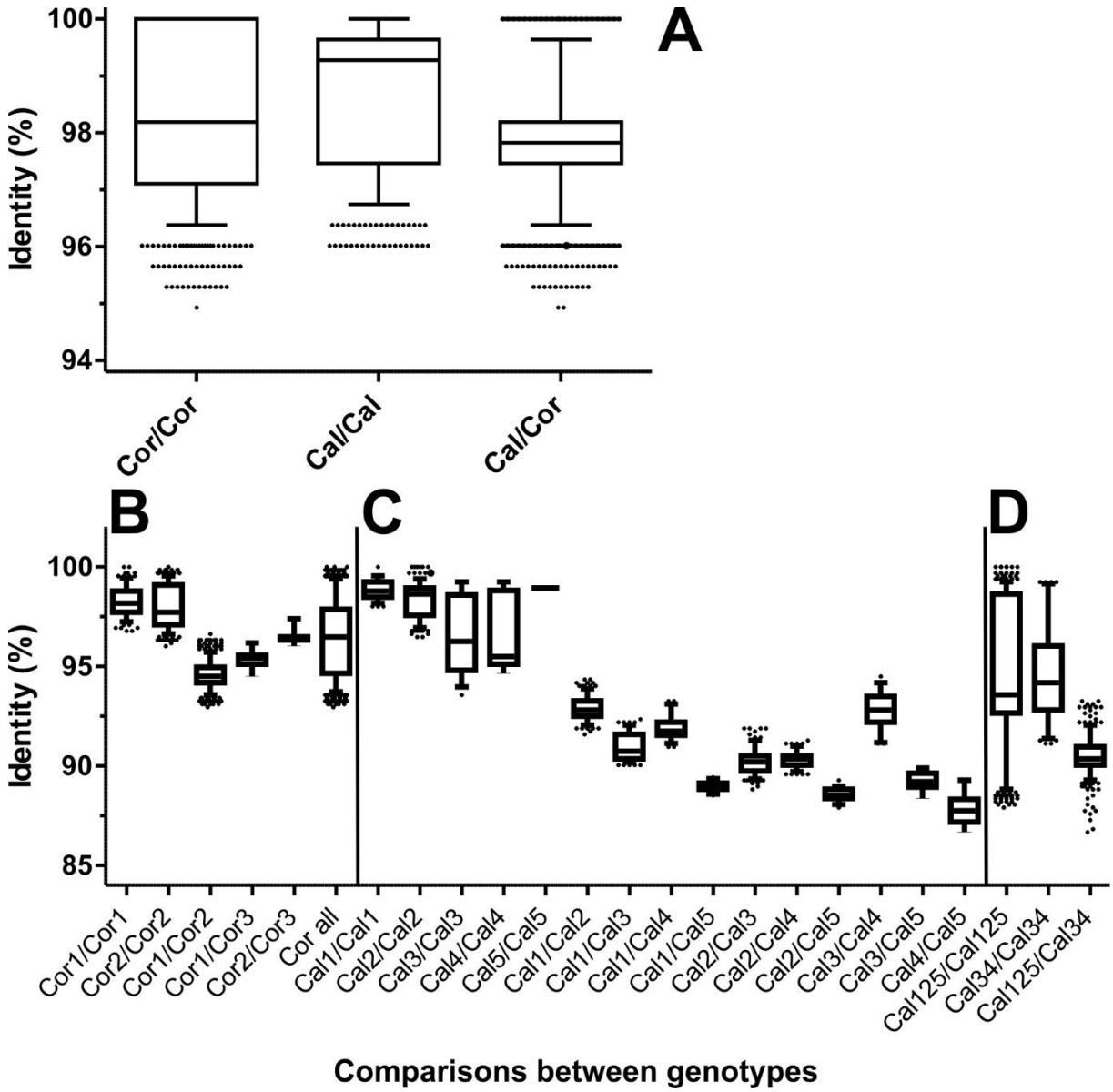

Figure 5-1: Comparison of sequence identity between different species on the ITS-2 sequence (A) or between different cytochrome oxidase I (COI) mitochondrial genotypes of the same species (B-D). Identities were calculated using dist.dna function and plotted as boxplots (medians and 25 %/75 % percentiles) with whiskers showing the 95 % percentiles and outliers shown by dots. Abbreviations on the x-axis indicate the species *Coronocyclus coronatus* (Cor) and *Cylicostephanus calicatus* (Cal) in (A) or the mitochondrial haplotype groups Cor1-Cor3 (mtHG1co-mtHG3co) for *Cor. coronatus* and Cal1-Cal5 (mtHG1ca-mtHG5ca) for *Cys. calicatus* in (B, C) In (B), "Cor all" represents all possible comparisons between any *Cor. coronatus* sequence obtained in this study. In (D), *Cys. calicatus* mitochondrial genotypes were grouped into potential genospecies with the mtHG1ca, mtHG2ca and mtHG5ca (Cor125) being associated with the nuclear haplotype groups nHGAca, while mtHG3 and mtHG4(Cor34) were associated with nHGBca.

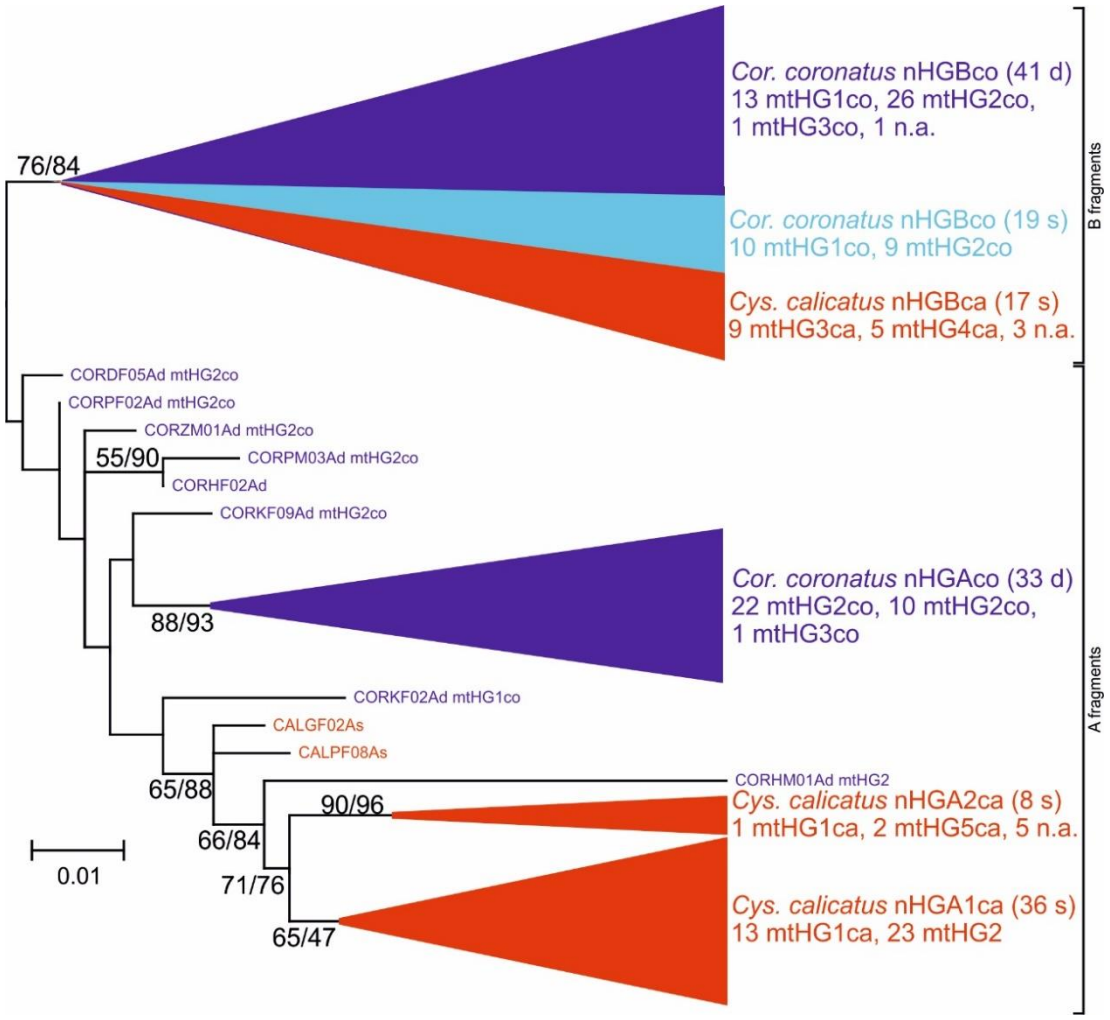

Figure 5-2: A maximum likelihood phylogenetic tree was calculated using aligned ITS-2 sequences. The scale bar represents 0.01 substitutions per site and node support was obtained by rapid bootstrapping (1000 replicates) before and the Shimodaira-Hasegawa likelihood ratio test after the slash. Numbers in brackets behind the species names indicate the number of specimen and if a single (s) or a double (d) ITS-2 fragment were amplified. In addition to the nuclear haplotype groups (nHG), the number of cytochrome oxidase I (COI) mitochondrial haplotype groups for each nHG is provided (mtHG1co-mtHG3co for *Coronocyclus coronatus* and mtHG1ca–mtHG5ca for *Cylicostephanus calicatus*). The "n.a." indicates no successful amplification of the COI fragment from some samples. Individual specimen that were not assigned to one of the major HGs are designated according to the following code: COR/CAL for the species *Cor. coronatus* and *Cys. calicatus*; G, H, P, K, Z for the hosts German horse, Ukrainian horse, Przewalski's horse, kulan and zebra, respectively; F/M for female or male; a number indicating the individual specimen; A/B for the large and small PCR fragments and s/d indicating whether one or two PCR products in the ITS-2 PCR were obtained. If no mtHG is provided, this information was not available. The large ITS-2 A fragment (369-370 bp) and the small B fragments (278 for *Cor. coronatus* and 381 bp for *Cys. calicatus*), but not the sequences derived from each species, form distinct subtrees. In *Cys. calicatus* showing only the ITS-2 version B, only the mtHG3ca and mtHG4ca were found while *Cys. calicatus* for which only the ITS-2 A variant was amplified, only mtHG1ca, mtHG2ca and mtHG5ca were detected.

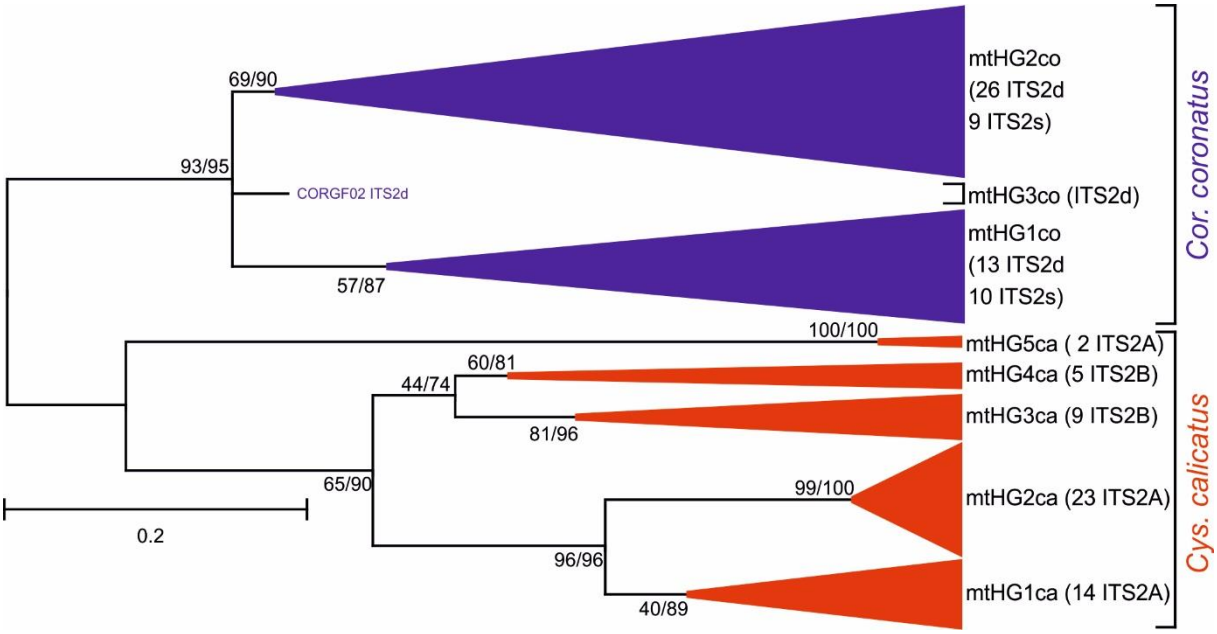

Figure 5-3: Representation of a maximum likelihood phylogenetic tree calculated from the cytochrome oxidase I (COI) sequences. The scale bar indicates a distance of 0.2 substitutions per site and node support values before and behind the slash were obtained by rapid bootstrapping and the Shimodaira-Hasegawa likelihood ratio test. In addition to the major mitochondrial haplotype groups mtHG1co-mtHG3co for *Coronocyclus coronatus* and mtHG1ca-mtHG5ca for *Cylicostephanus calicatus*, the number of different nuclear haplotypes/genotypes in the individual clusters is provided in brackets. For *Cor. coronatus*, ITS2s and ITS2d stand for specimen from which one (ITS2B) or two (ITS2A plus ITS2B) ITS-2 variants were amplified. In the case of *Cys. calicatus*, the HGs ITS2A and ITS2B are indicated.

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5.2 Supporting Information

5.2.1 Supporting figures

Figure 5-4: Maximum likelihood phylogenetic tree for ITS-2 sequences obtained from *Coronocyclus coronatus* and *Cylicostephanus calicatus*. Individual specimen that were not assigned to one of the major HGs are designated according to the following code: COR/CAL for the species *Cor. coronatus* and *Cys. calicatus*; G, H, P, K, Z for the hosts German horse, Ukrainian horse, Przewalski's horse, kulan and zebra, respectively; F/M for female or male; a number indicating the individual specimen; A/B for the large and small PCR fragments and s/d indicating whether one or two PCR products in the ITS-2 PCR were obtained. If available, the mitochondrial haplotype group for each specimen is provided (mtHG1co-mtHG3co for *Cor. coronatus* and mtHG1ca-mtHG5ca for *Cys. calicatus*). In brackets, the number of individuals within a cluster with either a single (s) nHG or two nHGs (d) is shown. Support values at notes represent rapid bootstrapping values (1000 replicates). This supplemental figure shows the complete tree that is provided in a condensed format in **Figure 5-2**.

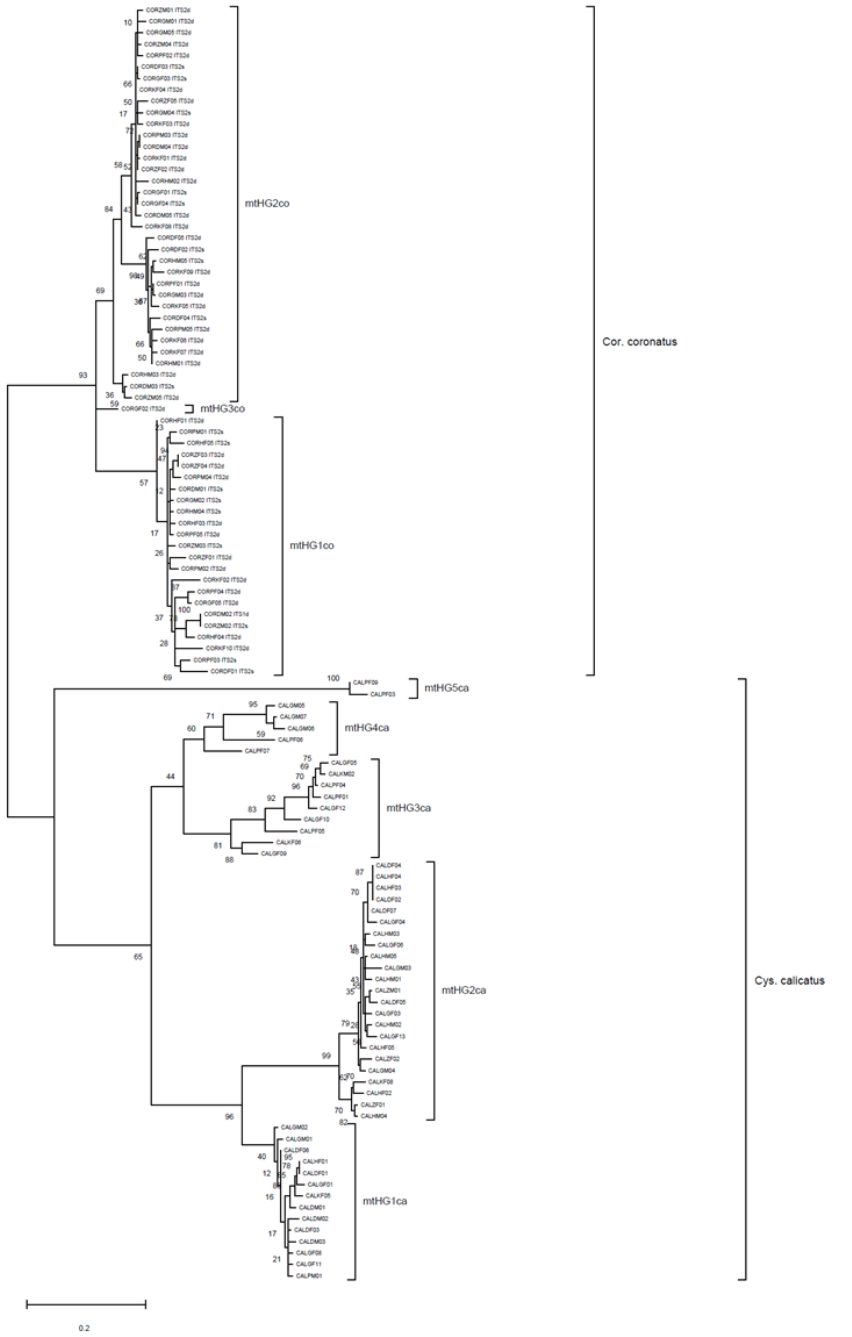

Figure 5-5: Maximum likelihood phylogenetic tree based on cytochrome oxidase I (COI) sequences for *Coronocyclus coronatus* and *Cylicostephanus calicatus*. Individual specimen that were not assigned to one of the major HGs are designated according to the following code: COR/CAL for the species *Cor. coronatus* and *Cys. calicatus*; G, H, P, K, Z for the hosts German horse, Ukrainian horse, Przewalski's horse, kulan and zebra, respectively; F/M for female or male; a number indicating the individual specimen; A/B for the large and small PCR fragments and s/d indicating whether one or two PCR products in the ITS-2 PCR were obtained. For *Cor. coronatus*, the nuclear genotype is indicated (ITS2s with one amplicon vs. ITS2d with two amplicons). For *Cys. calicatus*, all specimens in the cluster mtHG1ca, mtHG2ca and mtHG5ca had the nuclear haplotype group A (nHGAca) while all specimens in the clusters mtHG3ca and mtHG4ca had the nHGBco. Node support was calculated by rapid bootstrapping (1000 replicates). This supplemental figure shows the complete tree that is provided in a condensed format in **Figure 5-3**.

5.2.2 Supporting tables

Table 5-1: Description of parasite specimen

Species	Host	Origin	Sex[a]	n[b]	Code	COI[c]	ITS-2[d]
Coronocyclus coronotus	Donkey	Ukraine	f	01	COR_D_f01	Y	Bs
Coronocyclus coronotus	Donkey	Ukraine	f	02	COR_D_f02	Y	Bs
Coronocyclus coronotus	Donkey	Ukraine	f	03	COR_D_f03	Y	Bs
Coronocyclus coronotus	Donkey	Ukraine	f	04	COR_D_f04	Y	Bs
Coronocyclus coronotus	Donkey	Ukraine	f	05	COR_D_f05	Y	Ad+Bd
Coronocyclus coronotus	Donkey	Ukraine	m	01	COR_D_m01	Y	Bs
Coronocyclus coronotus	Donkey	Ukraine	m	02	COR_D_m02	Y	Bs
Coronocyclus coronotus	Donkey	Ukraine	m	03	COR_D_m03	Y	Bs
Coronocyclus coronotus	Donkey	Ukraine	m	04	COR_D_m04	Y	Ad+Bd
Coronocyclus coronotus	Donkey	Ukraine	m	05	COR_D_m05	Y	Ad+Bd
Coronocyclus coronotus	Horse	Germany	f	01	COR_G_f01	Y	Bs
Coronocyclus coronotus	Horse	Germany	f	02	COR_G_f02	Y	Ad+Bd
Coronocyclus coronotus	Horse	Germany	f	03	COR_G_f03	Y	Bs
Coronocyclus coronotus	Horse	Germany	f	04	COR_G_f04	Y	Bs
Coronocyclus coronotus	Horse	Germany	f	05	COR_G_f05	Y	Ad+Bd
Coronocyclus coronotus	Horse	Germany	m	01	COR_G_m01	Y	Ad+Bd
Coronocyclus coronotus	Horse	Germany	m	02	COR_G_m02	Y	Bs
Coronocyclus coronotus	Horse	Germany	m	03	COR_G_m03	Y	Ad+Bd
Coronocyclus coronotus	Horse	Germany	m	04	COR_G_m04	Y	Bs
Coronocyclus coronotus	Horse	Germany	m	05	COR_G_m05	Y	Ad+Bd
Coronocyclus coronotus	Horse	Ukraine	f	01	COR_H_f01	Y	Ad+Bd
Coronocyclus coronotus	Horse	Ukraine	f	02	COR_H_f02	N	Ad+Bd
Coronocyclus coronotus	Horse	Ukraine	f	03	COR_H_f03	Y	Ad+Bd
Coronocyclus coronotus	Horse	Ukraine	f	04	COR_H_f04	Y	Ad+Bd
Coronocyclus coronotus	Horse	Ukraine	f	05	COR_H_f05	Y	Bs
Coronocyclus coronotus	Horse	Ukraine	m	01	COR_H_m01	Y	Ad+Bd
Coronocyclus coronotus	Horse	Ukraine	m	02	COR_H_m02	Y	Ad+Bd
Coronocyclus coronotus	Horse	Ukraine	m	03	COR_H_m03	Y	Ad+Bd
Coronocyclus coronotus	Horse	Ukraine	m	04	COR_H_m04	Y	Bs
Coronocyclus coronotus	Horse	Ukraine	m	05	COR_H_m05	Y	Bs
Coronocyclus coronotus	Kulan	Ukraine	f	01	COR_K_f01	Y	Ad+Bd
Coronocyclus coronotus	Kulan	Ukraine	f	02	COR_K_f02	Y	Ad+Bd
Coronocyclus coronotus	Kulan	Ukraine	f	03	COR_K_f03	Y	Ad+Bd
Coronocyclus coronotus	Kulan	Ukraine	f	04	COR_K_f04	Y	Ad+Bd
Coronocyclus coronotus	Kulan	Ukraine	f	05	COR_K_f05	Y	Ad+Bd
Coronocyclus coronotus	Kulan	Ukraine	f	06	COR_K_f06	Y	Ad+Bd
Coronocyclus coronotus	Kulan	Ukraine	f	07	COR_K_f07	Y	Ad+Bd
Coronocyclus coronotus	Kulan	Ukraine	f	08	COR_K_f08	Y	Ad+Bd
Coronocyclus coronotus	Kulan	Ukraine	f	09	COR_K_f09	Y	Ad+Bd
Coronocyclus coronotus	Kulan	Ukraine	f	10	COR_K_f10	Y	Ad+Bd
Coronocyclus coronotus	Przewalski's	Ukraine	f	01	COR_P_f01	Y	Ad+Bd
Coronocyclus coronotus	Przewalski's	Ukraine	f	02	COR_P_f02	Y	Ad+Bd
Coronocyclus coronotus	Przewalski's	Ukraine	f	03	COR_P_f03	Y	Bs
Coronocyclus coronotus	Przewalski's	Ukraine	f	04	COR_P_f04	Y	Ad+Bd
Coronocyclus coronotus	Przewalski's	Ukraine	f	05	COR_P_f05	Y	Ad+Bd
Coronocyclus coronotus	Przewalski's	Ukraine	m	01	COR_P_m01	Y	Bs
Coronocyclus coronotus	Przewalski's	Ukraine	m	02	COR_P_m02	Y	Ad+Bd
Coronocyclus coronotus	Przewalski's	Ukraine	m	03	COR_P_m03	Y	Ad+Bd
Coronocyclus coronotus	Przewalski's	Ukraine	m	04	COR_P_m04	Y	Ad+Bd

Species	Host	Origin	Sex[a]	n[b]	Code	COI[c]	ITS-2[d]
Coronocyclus coronatus	Przewalski's	Ukraine	m	05	COR_P_m05	Y	Ad+Bd
Coronocyclus coronatus	Zebra	Ukraine	f	01	COR_Z_f01	Y	Ad+Bd
Coronocyclus coronatus	Zebra	Ukraine	f	02	COR_Z_f02	Y	Ad+Bd
Coronocyclus coronatus	Zebra	Ukraine	f	03	COR_Z_f03	Y	Ad+Bd
Coronocyclus coronatus	Zebra	Ukraine	f	04	COR_Z_f04	Y	Ad+Bd
Coronocyclus coronatus	Zebra	Ukraine	f	05	COR_Z_f05	Y	Ad+Bd
Coronocyclus coronatus	Zebra	Ukraine	m	01	COR_Z_m01	Y	Ad+Bd
Coronocyclus coronatus	Zebra	Ukraine	m	02	COR_Z_m02	Y	Bs
Coronocyclus coronatus	Zebra	Ukraine	m	03	COR_Z_m03	Y	Bs
Coronocyclus coronatus	Zebra	Ukraine	m	04	COR_Z_m04	Y	Ad+Bd
Coronocyclus coronatus	Zebra	Ukraine	m	05	COR_Z_m05	Y	Ad+Bd
Cylicostephanus calicatus	Donkey	Ukraine	f	01	CAL_D_f01	Y	As
Cylicostephanus calicatus	Donkey	Ukraine	f	02	CAL_D_f02	Y	As
Cylicostephanus calicatus	Donkey	Ukraine	f	03	CAL_D_f03	Y	As
Cylicostephanus calicatus	Donkey	Ukraine	f	04	CAL_D_f04	Y	As
Cylicostephanus calicatus	Donkey	Ukraine	f	05	CAL_D_f05	Y	As
Cylicostephanus calicatus	Donkey	Ukraine	f	06	CAL_D_f06	Y	As
Cylicostephanus calicatus	Donkey	Ukraine	f	07	CAL_D_f07	Y	As
Cylicostephanus calicatus	Donkey	Ukraine	m	01	CAL_D_m01	Y	As
Cylicostephanus calicatus	Donkey	Ukraine	m	02	CAL_D_m02	Y	As
Cylicostephanus calicatus	Donkey	Ukraine	m	03	CAL_D_m03	Y	As
Cylicostephanus calicatus	Horse	Germany	f	01	CAL_G_f01	Y	As
Cylicostephanus calicatus	Horse	Germany	f	02	CAL_G_f02	N	As
Cylicostephanus calicatus	Horse	Germany	f	03	CAL_G_f03	Y	As
Cylicostephanus calicatus	Horse	Germany	f	04	CAL_G_f04	Y	As
Cylicostephanus calicatus	Horse	Germany	f	05	CAL_G_f05	Y	Bs
Cylicostephanus calicatus	Horse	Germany	f	06	CAL_G_f06	Y	As
Cylicostephanus calicatus	Horse	Germany	f	07	CAL_G_f07	N	Bs
Cylicostephanus calicatus	Horse	Germany	f	08	CAL_G_f08	Y	As
Cylicostephanus calicatus	Horse	Germany	f	09	CAL_G_f09	Y	Bs
Cylicostephanus calicatus	Horse	Germany	f	10	CAL_G_f10	Y	Bs
Cylicostephanus calicatus	Horse	Germany	f	11	CAL_G_f11	Y	As
Cylicostephanus calicatus	Horse	Germany	f	12	CAL_G_f12	Y	Bs
Cylicostephanus calicatus	Horse	Germany	f	13	CAL_G_f13	Y	As
Cylicostephanus calicatus	Horse	Germany	m	01	CAL_G_m01	Y	As
Cylicostephanus calicatus	Horse	Germany	m	02	CAL_G_m02	Y	As
Cylicostephanus calicatus	Horse	Germany	m	03	CAL_G_m03	Y	As
Cylicostephanus calicatus	Horse	Germany	m	04	CAL_G_m04	Y	As
Cylicostephanus calicatus	Horse	Germany	m	05	CAL_G_m05	Y	Bs
Cylicostephanus calicatus	Horse	Germany	m	06	CAL_G_m06	Y	Bs
Cylicostephanus calicatus	Horse	Germany	m	07	CAL_G_m07	Y	Bs
Cylicostephanus calicatus	Horse	Ukraine	f	01	CAL_H_f01	Y	As
Cylicostephanus calicatus	Horse	Ukraine	f	02	CAL_H_f02	Y	As
Cylicostephanus calicatus	Horse	Ukraine	f	03	CAL_H_f03	Y	As
Cylicostephanus calicatus	Horse	Ukraine	f	04	CAL_H_f04	Y	As
Cylicostephanus calicatus	Horse	Ukraine	f	05	CAL_H_f05	Y	As
Cylicostephanus calicatus	Horse	Ukraine	m	01	CAL_H_m01	Y	As
Cylicostephanus calicatus	Horse	Ukraine	m	02	CAL_H_m02	Y	As
Cylicostephanus calicatus	Horse	Ukraine	m	03	CAL_H_m03	Y	As
Cylicostephanus calicatus	Horse	Ukraine	m	04	CAL_H_m04	Y	As
Cylicostephanus calicatus	Horse	Ukraine	m	05	CAL_H_m05	Y	As
Cylicostephanus calicatus	Kulan	Ukraine	f	01	CAL_K_f01	N	As

Species	Host	Origin	Sex[a]	n[b]	Code	COI[c]	ITS-2[d]
Cylicostephanus calicatus	Kulan	Ukraine	f	02	CAL_K_f02	N	As
Cylicostephanus calicatus	Kulan	Ukraine	f	03	CAL_K_f03	N	Bs
Cylicostephanus calicatus	Kulan	Ukraine	f	04	CAL_K_f04	N	As
Cylicostephanus calicatus	Kulan	Ukraine	f	05	CAL_K_f05	Y	As
Cylicostephanus calicatus	Kulan	Ukraine	f	06	CAL_K_f06	Y	Bs
Cylicostephanus calicatus	Kulan	Ukraine	f	07	CAL_K_f07	N	Bs
Cylicostephanus calicatus	Kulan	Ukraine	f	08	CAL_K_f08	Y	As
Cylicostephanus calicatus	Kulan	Ukraine	m	01	CAL_K_m01	N	As
Cylicostephanus calicatus	Kulan	Ukraine	m	02	CAL_K_m02	Y	Bs
Cylicostephanus calicatus	Przewalski's	Ukraine	f	01	CAL_P_f01	Y	Bs
Cylicostephanus calicatus	Przewalski's	Ukraine	f	02	CAL_P_f02	N	As
Cylicostephanus calicatus	Przewalski's	Ukraine	f	03	CAL_P_f03	Y	As
Cylicostephanus calicatus	Przewalski's	Ukraine	f	04	CAL_P_f04	Y	Bs
Cylicostephanus calicatus	Przewalski's	Ukraine	f	05	CAL_P_f05	Y	Bs
Cylicostephanus calicatus	Przewalski's	Ukraine	f	06	CAL_P_f06	Y	Bs
Cylicostephanus calicatus	Przewalski's	Ukraine	f	07	CAL_P_f07	Y	Bs
Cylicostephanus calicatus	Przewalski's	Ukraine	f	08	CAL_P_f08	N	As
Cylicostephanus calicatus	Przewalski's	Ukraine	f	09	CAL_P_f09	Y	As
Cylicostephanus calicatus	Przewalski's	Ukraine	m	01	CAL_P_m01	Y	As
Cylicostephanus calicatus	Zebra	Ukraine	f	01	CAL_Z_f01	Y	As
Cylicostephanus calicatus	Zebra	Ukraine	f	02	CAL_Z_f02	Y	As
Cylicostephanus calicatus	Zebra	Ukraine	m	01	CAL_Z_m01	Y	As

[a]f, female;m, male

[b]Consecutive number starting with one for every combination of host and sex.

[c]Y, COI sequence available; N, COI sequence not available

[d]A, large PCR fragment; B, short PCR fragment; s, single fragment; d, double fragments.

Table 5-2: Primer and PCR conditions

					Initial denaturation	40 PCR cycles			Final extension	
Target	Primer (5'->3'), 0.5 µM	Volume	Enzyme	Buffer	98 °C	98 °C	55 °C	72 °C	72 °C	Reference
IST-2[a]	NC1 ACGTCTGGTTCAGGGTTGTT NC2 TTAGTTTCTTTTCCTCCGCT	20 µl	0.4 U Phusion[c]	HF with 200 µM dNTPs	30 s	10 s	30 s	30 s	5 min	Gasser et al., 1993
COI[b]	COI_Nema_Fw GAAAGTTCTAATCATAARGATATTGG COI_Nema_Rv ACCTCAGGATGACCAAAAAAYCAA	20 µl	0.4 U Phusion[c]	HF with 200 µM dNTPs	30 s	10 s	30 s	30 s	5 min	Duscher et al., 2015

[a]ITS-2, internal transcribed spacer

[b]COI, cytochrome oxidase I

[c]Phusion Hot Start II High-fidelity DNA polymerase (ThermoFischer)

6 Discussion

6.1 The publications and how they interlink

In the publication entitled “Nematode Species Identification – Current Status, Challenges and Future Perspectives for Cyathostomins” (Bredtmann et al., 2017) the advantages and limitations of different methods for species identification in cyathostomins are summarised and discussed. Similar to other authors, it was concluded, that the currently existing methods to allow species-specific research are not sufficient and novel methods to overcome the current knowledge gaps are highly needed (Lichtenfels et al., 2008; Love et al., 1999; Nielsen et al., 2014a). In this publication, a first outlook on the potentially useful method of the proteomic profiling with MALDI-TOF MS was given. Although routinely applied for bacteria, fungi and even insects, only few publications on the use in nematodes are available (Ahmad et al., 2012; Mayer-Scholl et al., 2016; Perera et al., 2009, 2005) and the existing protocols were not entirely transferable to the cyathostomins.

As a result, in the second publication entitled “Concurrent Proteomic Fingerprinting and Molecular Analysis of Cyathostomins” (Bredtmann et al., 2019b), a protocol and subsequent bioinformatics workflow for cyathostomin species identification with MALDI-TOF MS and concurrent molecular analysis of the same individual worms were described and the resolution of the molecular and proteomic methods compared. Furthermore, the cryptic species complex of *Cys. minutus* was examined and results indicate that the systematic of the cyathostomin species is more complicated than anticipated.

The complexity of species is also discussed in the last publication entitled “Nuclear and mitochondrial marker sequences reveal close relationship between *Coronocyclus coronatus* and a potential *Cylicostephanus calicatus* cryptic species complex” (Bredtmann et al., 2019a). In this publication two cyathostomin species of different genera have been shown to have up to 100 % sequence identity in the nuclear marker ITS-2.

6.2 Research purposes

The *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (Terrestrial Manual)* aims to set laboratory standards for OIE listed diseases and other diseases of global concern (OIE, 2018). Although cyathostomins/cyathostominosis are not listed, the principles for the development of diagnostic methods should be applicable. The first principle is that, in order to obtain relevant data, the sampling and test methods must suit defined purposes (OIE, 2013). According to the current species-specific knowledge gaps, several intended purposes can be defined and will be discussed with regards to the methods available.

6.2.1 Validity of species and genera and implications on phylogeny

Before any conclusions on species and genera can be meaningful, the validity of species and genera must be determined. During the work for this thesis, presumable cryptic species were encountered, which raise the question of the species delimitation in the diverse group of cyathostomins.

The concept of species delimitation describes the separation of one species from another. The definition of the species and species delimitation affect all areas of biology and are extensively discussed without consensus (Agapow et al., 2004; Bock, 2004; Camargo and Sites, 2018; Zhang et al., 2013). Historically, the cyathostomin species were described and assigned to the genera based on morphological features (Dvojnos and Kharchenko, 1994; Lichtenfels et al., 2008; Tolliver, 2000). Molecular methods allowed to extend the existing knowledge on a genetic basis up to the identification of 'Operative Taxonomic Units' (OTUs), which were also found during the research for this thesis.

Whereas the sequences of both molecular markers (COI and ITS-2) obtained for the specimens from *Cys. longibursatus* were very homogenous and had high genetical similarities, the specimens from *Cys. minutus* were designated to three OTUs) (Bredtmann et al., 2019b). Two OTUs have been described before, namely OTU I and II (Hung et al., 1999b). The third OTU matches sequences in GenBank™ that have been deposited without an associated publication. Since the present study focussed on the establishment of a protocol for proteomic analysis of cyathostomins, further investigation of these OTUs was out of the scope of this publication. However, to explore the phylogenetic significance of the three OTUs, they should be placed in a molecular context with more species. This has been done previously for OTU I and II, which always cluster together in different phylogenetic trees of one publication (Hung

et al., 2000), but separate in a dendrogram after manual alignment in another publication (Hung et al., 1999a). This exemplary demonstrates the need for a methodological consensus for phylogenetic analyses.

Similarly, a potential cryptic species complex was described for *Cys. calicatus* (Bredtmann et al., 2019a). For this species two morphologically different forms of the buccal capsule have been described before: “Forma major” and “Forma minor” or “mouth open” and “mouth shut” (Kharchenko, 1997; Tolliver, 2000). Unfortunately, it is not possible to trace back our specimen to these varieties. If it would have been possible to assign these two genospecies to the morphological varieties, the term “cryptic species” would not be correct, since a morphological differentiation of both species would be possible for the trained person. Morphological reassessment of molecularly described cryptic species can lead to new morphological features for identification, as reported for two previously described cryptic darkling beetle species (Zúñiga-Reinoso and Benítez, 2015).

Furthermore, *Cys. calicatus* and *Cys. minutus* are easily confused morphologically (Tolliver, 2000). A phylogenetic analysis based on the molecular data of both species (including the different genospecies) should be performed to analyse if the morphological similarities can be recapitulated using molecular data. One could speculate that the cryptic species of both publications might actually represent the confused species, but these sequence data are not identical (unpublished data).

An additional species complex has been proposed for *Cyc. ashworthi* based on phylogenetic trees using combined ITS-1 and ITS-2 sequences (Bu et al., 2013). In the published phylogenetic trees, two clusters of *Cyc. ashworthi* are shown, separated with relatively low support values (55 and 59 %) by *Cyc. leptostomum*, which is unlikely to be morphologically confused with *Cyc. ashworthi*. Faint differences in the male genital cone were observed during this study, but not described in detail and require morphological re-examination (Bu et al., 2013).

No cryptic species, but a high genetic variability (overall 10.8 % differences) for the mitochondrial COI marker have been described for the species *Cyc. nassatus*. Specimen were collected from different host species in different global regions and the occurrence of specific

haplotypes in specific host species only led to the hypothesis of the existence of different populations of this species (Traversa et al., 2008). This hypothesis still needs to be verified but could further complicate the systematics of cyathostomins.

Similarly to our results, these data question the current species delimitation of the cyathostomin species. No single easy way can be proposed to overcome these discrepancies and questions. Several methods have been described to bioinformatically delimit species and in most investigations only a few methods are applied (Carstens et al., 2013). To find a trustful method, it is suggested to apply a wide range of methods and stick to the hypothesis with the most congruent results across the methods applied (Carstens et al., 2013). However, it has been advised against drawing conclusions from falsely delimited species that do not actual represent evolutionary lineages (Carstens et al., 2013). To overcome this pitfall, the easiest way proposed in literature, is to focus research on the higher taxa or the supraspecific groups, such as genus in this case (Agapow et al., 2004).

Likewise, research on genera and subsequently on phylogeny can only be meaningful, when the genera are clearly defined, which is also a topic of discussion for the present thesis: The two species *Cys. calicatus* and *Cor. coronatus* were shown to have very high identities despite being morphologically assigned to different genera (Bredtmann et al., 2019a). This is in line with previous research, where both species cluster closely together in phylogenetic trees based on different methods (Hung et al., 2000; McDonnell et al., 2000). In addition, during a collaboration project, these species still cluster together when analysed in context with four other species from the genera *Cylicostephanus* and *Coronocyclus* (Louro, Kuzmina, Bredtmann, Madeira de Carvalho, von Samson-Himmelstjerna, Krücken, unpublished data).

Uncertainties regarding the different genera were also found in other studies: The different species of the genus *Cylicocyclus* were studied in detail regarding their ITS-1 and ITS-2 regions, revealing two different clades within this genus: The species *Cyc. elongatus* and *Cyc. ultrajectinus* were clustering more closely with *Poteriostomum imparidentatum* and *Petrovinema poculatum*, which led to the suggestion that these two species may belong to another genus (Bu et al., 2013).

For several studies the nuclear ribosomal ITS-2 marker was analysed, which has been proven useful for phylogenetic studies (Chilton et al., 1997; Hung et al., 2000). In our study, it was shown for *Cor. coronatus* that there are eventually two varying copies of this locus within one

specimen (Bredtmann et al., 2019a). This might question the phylogenetic trees that are already based on this data and should be considered for future research: The occurrence of different haplotypes of this locus should be evaluated, since neglecting the occurrence of a second fragment leads to conflicting results and will give an inaccurate estimation of phylogenetic relationships. Furthermore, the occurrence of cryptic species, such as in the case of *Cys. calicatus*, requires re-evaluation of existing preliminary trees and analysis of data in a wider context.

To overcome the limitations of the use of single markers, research is directed towards analyses containing several markers: Different phylogenetic tree-building methods were previously used to calculate phylogenetic trees based on the molecular markers of the nuclear ribosomal ITS-1 and ITS-2 sequences and eventually complemented by a model of the secondary structure of the ITS-2 of 30 strongyloid nematode species (including 23 cyathostomin species). No concordant results were obtained for the placement of different species within these trees and no consistent clusters for the different genera were formed (Hung et al., 1999; Hung et al., 2000). A combination of the nuclear ITS-2 marker and the mitochondrial markers of the large ribosomal RNA (l-rRNA) sequence and COI have furthermore been applied to investigate the phylogenetic relationship between different cyathostomin and strongylin species (McDonnell et al., 2000). Also, this research failed in separating the individual cyathostomin species into distinct genera, and similarly to our results, it was shown, that the phylogenetic information derived from COI sequences is limited due to its high substitution rate at the third codon position. However, the combined use of ITS-2 and l-rRNA appeared to be useful in the study above, whereas in an investigation, focussing on the D3 domain of the l-rRNA of 21 equine strongyle species (including 15 cyathostomin species), no complete resolution of the subfamilies was achieved (Zhang et al., 2007).

The next higher level of molecular species delimitation is the investigation of larger datasets, such as the mitochondrial genomes with approximately 13,000 bp. In the past years, the costs for next generation sequencing decreased and the computational powers increased tremendiously. As a result, the mitochondrial genomes of several cyathostomin and strongylin species were sequenced and analysed. Based on this research, it was suggested that

Triodontophorus spp., currently classified as a member of the Strongylinae, are more closely related to the Cyathostominae (Gao et al., 2017a, 2017b).

So far, conclusions from both morphological and molecular studies on the phylogeny of cyathostomins are not consistent and it has been proposed to revise the classification of subfamilies (Lichtenfels et al., 2008). It should be noted that the methods applied throughout all these studies differed and comparison of the studies based on different analytical methods is questionable. A methodological consensus for phylogenetic analyses of cyathostomins is necessary and ultimately dependent on the application of a combination of meaningful set of markers.

6.2.2 Species identification

The efficacy of an anthelmintic compound against specific target organisms has to be demonstrated in many countries when applying for marketing authorisations (Duncan et al., 2002). Currently, this is mostly done by experts via morphological identification of adult nematodes in critical and controlled tests. Globally, only few trained experts for the morphological identification are available, particularly for equine parasites. Although identification keys have been published (Dvojnos and Kharchenko, 1994; Lichtenfels et al., 2008; Tolliver, 2000), this method will always rely on these experts, is highly dependent on the individual researcher's experience and is therefore not universally applicable. For respective work concerning other taxa, such as bumblebees, even the accuracy of the expert´s identification is questionable (Austen et al., 2016). Consequently, a more objective method to identify the L4, immature/preadult and adult parasite stages is needed. One possible method that was evaluated in this thesis is species-identification using MALDI-TOF MS. Under the assumption that cyathostomin species are sufficiently defined, a master-spectra library could be set up and further individuals could be identified with matching of protein-patterns.

It should be noted that only differentiation of two species (including a cryptic species complex) was evaluated using MALDI-TOF MS in this study. For future use, it must be evaluated if a reliable species identification is still possible after inclusion of additional cyathostomin species in the master spectra library. A possible pitfall could be that the intra-species variability of spectra is as high as the inter-species variability and no specific pattern can be identified for each species. This would lead to incorrect identification. When the spectra that were used for evaluation were compared with the master-spectra library (extended by preliminary spectra

of other cyathostomin species), one mismatch with another cyathostomin species was identified pointing towards potential limitations of current methods that need to be addressed in the future.

With regards to the high variability of spectra, the identification of species-specific peaks appears to be useful. However, no peaks that occur in each individual of the species have been identified in the context of only two morphospecies. Identification and differentiation were moreover due to a combination of occurrence and absence of peaks and only in adults. The applicability of the same patterns for different life-stages, such as eggs and larvae (especially immature L4) needs to be evaluated. The use of single eggs or larvae for protein extraction and spotting appears to be highly ambitious. However, successful MALDI MS spectra were also acquired for legs of insects (Yssouf et al., 2013).

Although MALDI-TOF MS is routinely used for species identification of bacteria and has also been applied to other organisms, only a few publications on the application to parasitic nematodes are available. Therefore, the first important step was to establish a suitable protocol that allows to verify the morphological identification of cyathostomins with objective molecular identification. This was necessary due to the absence of well-defined single-species infections and the minute size of biological material of individual worms. As it has already been discussed in the associated publication (Bredtmann et al., 2019b), halving the worm was not possible to avoid bias of potentially different protein patterns, when not separated at the exactly the same position. Noteworthy, just recently the use of the anterior ends ("heads") of specimens from *Dirofilaria immitis*, *D. repens*, *Ascaris lumbricoides* and *A. suum* for MALDI-TOF MS was described and applied for comparing the protein peak patterns of the closely related species from the same genus (Nagorny et al., 2019).

In principle, MALDI MS based species identification involves only three steps: (1) Spotting of the sample on a target plate and overlaying with matrix, (2) MALDI MS measurements and (3) identification through pattern matching with the data base. Expenditures of time for this are accounted with about 2-5 minutes per sample in the case of microbial species identification (Murugaiyan and Roesler, 2017). Although the expenditures of time for these three steps are comparable between bacteria and nematodes, the differences in sample preparation should be considered: Whereas bacteria can be directly spotted from culture,

protein extraction from nematodes requires several steps that have to be performed manually and are time-consuming.

To allow high throughput identification of cyathostomins with MALDI-TOF MS, sample preparation needs to be optimised. One possibility would direct spotting of crushed/manually grinded worms. Direct spotting has for example been performed with haemolymph from different tick species to identify other vector transmitted diseases (Yssouf et al., 2015). If acquisition of high-resolution mass spectra is possible with this method, it should be considered that the resulting spectra likely differ from the ones acquired using other sample-preparation techniques. Regarding future data acquiring and evaluation, not only the possibility of acquiring spectra needs to be evaluated but also the potential to discriminate between species needs to be re-evaluated. The bioinformatics workflow proposed here could be used as a template for this purpose.

Particularly the step of homogenisation with an ultrasonic device is time consuming and prone to flaws and cross-contamination should be optimised in several aspects: Due to the minute material in a volume of 30 to 50 µl in a 1.5 ml tube, the tube needs to be hold manually and care has to be taken that the tip of the device is not touching the tube itself and that the extraction solution including the worm are not catapulted out of the tube. The tip of the device has to be cleaned after handling of each sample, but no alcohol must get into contact with the sample. In addition, ultrasonic homogenisation is exhausting and dangerous for the executing person and operational safety for ear protection and to protect from vibrations has to be respected (Bundesministerium für Justiz und Verbraucherschutz, 2007).

When matching the obtained spectra with the whole MALDI Biotyper Realtime Classification database, extended by in-house spectra and spectra from other cyathostomin species, some spectra matched with low score values with bacterial species. It was assumed that this might be due to the intestinal origin of the specimens and due to contamination e.g. with bacteria during sampling. The samples used were fixed in 70 % ethanol prior freezing and subsequent protein extraction. This should usually prevent colonisation by bacteria but bacteria attached to the specimens might be processed throughout the protocol and contribute to the signal. Another option could be incubation with broad-spectrum bactericidal antibiotics, as it was for example done in another publication with phenoxymethylpenicillin (penicillin V) (Nagorny et al., 2019).

To critically conclude, MALDI-TOF MS offers a potentially useful tool for species identification of adult cyathostomins in critical or controlled trials as required for marketing authorisations for anthelmintic drugs. Once a master spectra library is set up, this method allows bioinformatics-based species identification without expert training. However, as stated above, the method needs to be further evaluated with additional species in order to validate the method. Additionally, although the main procedure of MALDI is not time-consuming, the prior sample preparation needs to be optimised or automated in order to allow high-throughput identification of a representative number of specimens.

6.2.3 Clinical impact

Of clinical interest is the diagnosis of infection with encysted larvae, before clinical symptoms appear and serological detection methods, based on cyathostomin gut-associated larval antigens are promising to indirectly detect presence of larvae. It is, however unlikely that all species are equally pathogenic (Nielsen et al., 2014b) and the predominant pathogenic ones should be identified. For this, the characterisation of species in clinical and/or fatal disease is necessary. An attempt in this direction has been made based with the development of a PCR-ELISA for six cyathostomin species that has been evaluated in 17 clinical cases. Larvae were individually submitted to the assay and between 10 and 111 larvae were examined per case (Hodgkinson et al., 2003). Given, that clinical disease is characterised by mass excystation of larvae, these numbers appear very low and conclusions on the contribution of individual species to clinical disease should be carefully evaluated. Since only up to six species can be identified with this assay, the number of species involved in these clinical cases could have been higher. The importance of examination of a representative subset of the population was determined in other studies, where the number of detected species markedly differed, depending on the number of counted worms (Chapman et al., 2003): Whereas an average of only ten species where identified from a total number 200 examined worms, an average of 26 species was detected when entire 5 % aliquots of the large intestinal content with about 600 to 4800 (average of 2200) worms were examined. Therefore, the extension of the PCR-ELISA method to more species-specific probes (including rare species as well) and the application on pooled larvae would be beneficial. This also accounts for the RLB method, which can at least identify up to 18 common species and is applicable on pooled samples as well (Traversa et al.,

2007). If at one point, differences in the pathogenicity of different species can be shown and these species can be diagnosed separately, this could maybe lead to a more selective strategy in anthelmintic treatment.

6.2.4 Anthelmintic treatment and anthelmintic resistance

Anthelmintic treatment is supposed to target all susceptible parasites although there might be some differences in susceptibility of histotropic stages between parasite species. If some species can be considered less pathogenic, treatment might not be necessary. This might decrease the frequency of anthelmintic treatments and consequently the selection pressure for AR.

Regarding AR, research needs to be directed towards the diagnostics of the species-specific occurrence and the underlying mechanisms for the different drug classes. The gold-standard to diagnose AR is the Faecal Egg Count Reduction Test (FECRT). Here, the number of strongyle eggs per gram faeces before and after anthelmintic treatment is counted and the relative reduction of strongyle eggs in the faeces is calculated. Since eggs of the strongyle type are indistinguishable, the result of this test can only generally indicate towards AR for all strongyle species.

However, in some publications it is stated that the relative prevalence of the most common species did not change during the past decades, despite the use of anthelmintic drugs and emerging resistance (Chapman et al., 2002; Kaplan, 2002). The studies the author refers to, are mainly morphological studies where only subsets of the whole parasite species composition in the large intestine of the equine host was examined or they were based on RLB, where only up to 18 common species, and mostly only the 13 species most common species are detected. Consequently, the relevance of these studies with regards to rare species has to be considered as limited.

6.3 Outlook

Another approach that is used to study microbial communities, is the deep amplicon sequencing of the 16S rDNA. This concept has been transferred to explore the gastro-intestinal “nemabiome” of cattle based on the ITS-2 rDNA locus and allows to determine the species composition from L3 that were cultured from faeces collected in the field (Avramenko et al., 2015). A similar approach would be useful for cyathostomins as well; however, as the research

of this thesis has shown, the ITS-2 locus might not be sufficient to evaluate the true species composition, since identities of ITS-2 sequences of different species can be up to 100 %. Consequently, the COI locus would be preferable. The aim of such studies will be to evaluate the feasibility of this method for equine parasites and establish a protocol that has been tested on different equine populations. Once established, this method could help gaining insight into the dynamics and development of cyathostomin species compositions of horse populations under influence of different factors such as anthelmintic treatment. Furthermore, an improved insight into the intra-species variability might be obtained and haplotypic groups within the species could be detected. Since molecular methods are highly sensitive, the chance for detection of rare species from cultured L3 is much higher than with other methods. Furthermore, this approach would facilitate research on the species composition of different equine host species.

7 Summary

Molecular and proteomic species characterisation of cyathostomins

Cyathostomins are currently considered to be the most important equine parasites, due to their ubiquitous prevalence, their ability to cause potentially fatal larval cyathostominosis and their widespread and progressing anthelmintic resistance. Multi-species infections, comprising usually more than 15 species simultaneously, impede research on the contributions of individual species to the pathogenesis as well as development of anthelmintic resistance in individual species. Research in cyathostomins e.g. on multi-species composition and species-specific pathogenicity is currently hampered by insufficient diagnostic tools. Particularly universally applicable methods, that are non-invasive and life-stage independent are needed. Molecular methods appear useful but can be resource and time-consuming, and some are limited to specific species, only. Proteomics methods, such as matrix-assisted laser desorption/ionisation-time-of-flight mass spectrometry (MALDI-TOF MS) allow species identification on the basis of matching protein patterns of organisms.

In this thesis a detailed evaluation of the application of MALDI-TOF MS on cyathostomins is given. A protocol based on protein extraction with equal volumes of acetonitrile and formic acid, followed by ultrasonic homogenisation and DNA isolation after buffering with Tris-buffer was established and successfully applied. It was demonstrated that this protocol was applicable to repeatably obtain high-quality mass spectra from cyathostomins that were freshly collected during necropsy and immediately fixed in 70 % ethanol. The mass spectra of two closely related species, namely *Cylicostephanus longibursatus* and *Cylicostephanus calicatus,* were examined with a bioinformatics workflow using freely available software. Despite their close relationship, all examined specimen of both species were reliably differentiated from each other. Mass peaks were sorted according their influence on discrimination. Although a rate of identification of close to 100 % was reached, it should be noted that no single peak alone occurred only in one of the two species as it would be required to be used as biomarker for species identification. Of importance for this work is the fact that this protocol allows to retrieve protein extracts of whole worms and to perform subsequent DNA extraction for PCR. As a result, the worms that were identified morphologically, could be used for protein profiling and molecular characterisation. Two different markers, the internal transcribed spacer (ITS) 2 and cytochrome oxidase I (COI) were

applied and revealed three clusters within *Cylicostephanus minutus*. Since two of the clusters were already described as potential cryptic species of *Cys. minutus* and designated as operational taxonomic units (OTU) I and II, the third was designated OTU III. A total of 22 individual worms of this species were examined and one, nine and twelve specimens could be assigned to OTU I, II, and III, respectively. For each marker and species/OTU, the sequences were compaired pairwise. The identities within the same species/OTUs were higher than between the OTUs. Identities between species and OTUs were lower for COI locus than for ITS-2 locus, indicating higher inter-species variability for COI. No identitification of the different OTUs in the proteomic analysis was possible, which could be linked to the low number of specimen available of each OTU. However, MALDI-TOF MS represents a step towards a universally applicable method for species identification of cyathostomins, provided the technical infrastructure as well as a comprehensive master spectra library is available. To set up a proteomic master spectra library, the individual species should be examined in parallel molecularly to detect cryptic species.

A close examination of two additional species, *Coronocyclus coronatus* and *Cylicostephanus calicatus* has been performed in this study**.** Although both species are placed in different genera based on morphological descriptions, sequence identities on the ITS-2 locus were up to 100 %. This stresses the need for the employment of the COI marker that allowed, together with the ITS-2 marker, delineation of both species. Furthermore, different nuclear and mitochondrial haplotypes were identified for each species. For *Cyc. calicatus*, two different genospecies were identified based on two ITS-2 variants, which are associated with distinct mitochondrial haplotypes and point towards a cryptic species complex of at least two non-interbreeding species.

In short, it should be summarised, that the current methods for species identification of cyathostomins are not sufficient and that the species complexity of cyathostomins is even more complicated than anticipated. To establish MALDI-TOF MS as a new method for species identification, molecular research should be performed to correctly delineate cyathostomin species that can be subsequently used for establishing a master spectra library for protein profiling.

8 Zusammenfassung

Molekulare and proteomische Spezieschарakterisierung von Cyathostominen

Aufgrund ihres ubiquitären Vorkommens, ihrer Fähigkeit die potenziell tödliche larvale Cyathostominose zu verursachen und wegen der weit verbreiteten und fortschreitenden Anthelmintikaresistenz, werden Cyathostomine derzeit als die wichtigsten Pferdeparasiten angesehen. Multispeziesinfektionen, die üblicherweise mehr als 15 Spezies umfassen, erschweren die Erforschung der Beteiligung der einzelnen Spezies an der Krankheitsentwicklung, so wie auch an der Entwicklung von Anthelmintikaresistenz in einzelnen Spezies. Die Erforschung von Cyathostominen, beispielsweise hinsichtlich der Zusammensetzung der vorherrschenden Multispezieskomplexe aber auch der speziesspezifischen Pathogenität ist derzeit durch unzureichende diagnostische Methoden eingeschränkt. Insbesondere werden universell anwendbare Methoden, die nicht-invasiv und unabhänging vom Lebenszyklus des Parsiten sind, benötigt. Molekulare Methoden erscheinen hilfreich, können jedoch material- und zeitaufwendig sein und sind bisher auf bestimmte Spezies beschränkt. Proteomische Methoden, wie die Matrix-assistierte Laser Desorptions/Ionisations – Flugzeit Massenspektrometrie (MALDI-TOF MS) ermöglichen die Identifikation einzelner Spezies anhand des Abgleiches von Proteinmustern von Organismen.

Im Rahmen dieser Dissertation wurde MALDI-TOF MS zur Anwendung bei Cyathostominen evaluiert. Es wurde ein Protokoll, basierend auf Proteinextraktion mittels gleicher Volumina von Acetonitril und Ameisensäure, gefolgt von Ultraschallhomogenisierung und DNA Isolation nach Pufferung mit Trispuffer etabliert und erfolgreich angewandt. Es konnte gezeigt werden, dass dieses Protokoll anwendbar ist, um wiederholbar qualitativ hochwertige Massenspektren von Cyathostominen, welche während Sektionen frisch gesammelt und in 70 %igen Ethanol fixiert wurden, zu erhalten. Die Massenspektren zweier nah verwandter Spezies, *Cylicostephanus longibursatus* und *Cylicostephanus calicatus*, wurden mittels frei erhältlicher Software untersucht. Trotz ihrer nahen Verwandtschaftbeziehung konnten alle untersuchten Exemplare verlässlich voneinander unterschieden werden. Die ermittelten Massenhöchstwerte wurden anhand ihres Einflusses zur Unterscheidung sortiert. Obwohl eine Identifikationsrate von annhähernd 100 % erreicht wurde, sollte beachtet werden, dass kein Massenhöchstwert ausschließlich in einer Spezies vorkommt und deshalb kein einzelner Marker zu Speziesidentifizierung herangezogen werden kann.

Von besonderer Bedeutung für diese Arbeit ist, dass dieses Protokoll sowohl die Gewinnung von Proteinextrakten vollständiger Würmer, als auch von DNA für nachfolgende PCR-basierte Analysen ermöglicht. Dadurch konnten morphologisch identifizierte Würmer sowohl zur Erstellung von Proteinprofilen, als auch zur molekularen Charakterisierung herangezogen werden. Zwei unterschiedliche Marker, der interne transkribierte Platzhalter 2 (ITS-2) und die erste Cytochromoxidase-Untereinheit (COI) wurden verwendet, und damit drei taxonomische Gruppierungen innerhalb der Art *Cylicostephanus minutus* identifiziert. Da zwei Gruppierungen bereits als Operationale taxonomische Einheiten (OTU) I und II beschrieben wurden, wurde die Dritte als OTU III bezeichnet. Insgesamt wurden von dieser Spezies 22 Individuen untersucht, von denen ein Exemplar OTU I, neun Exemplare zu OTU II und zwölf Ememplare zu OTU III zugeordnet werden konnten. Jeder dieser Marker wurde paarweise miteinander verglichen. Die Ähnlichkeit innerhalb der gleichen Spezies/OTU waren größer als zwischen den verschiedenen OTUs. Die Ähnlichkeit zwischen Spezies und OTUs waren für den COI Lokus geringer als für den ITS-2 Lokus, was auf eine höhere Variabilität der Spezies am COI Lokus hinweist. Die Identifikation der OTUs bzw. die entsprechende Differenzierung der untersuchten Würmer mittels proteomischer Analyse war nicht möglich, was auf die geringe Anzahl an Exemplaren für jedes OTU zurück zu führen sein könnte. Nichtsdestoweniger stellt das hier etablierte MALDI-TOF MS-basierte Protokoll einen ersten wesentlichen Schritt in die Richtung einer universell anwendbaren Methode zur Speziesdifferenzierung von Cyathostominen dar. Ein wichtiger nächster Schritt ist die Etablierung einer umfassenden Referenzspektrensammlung. Um eine proteomische Referenspektrendatenbank zu erstellen, sollten die individuellen Spezies parallel molekular untersucht werden um eventuelle kryptische Spezies zu festzustellen.

Zwei weitere Spezies, *Coronocyclus coronatus* and *Cylicostephanus calicatus* wurden im Rahmen dieser Dissertation eingehend untersucht: Obwohl beide Spezies anhand von morphologischen Beschreibungen in verschiedene Gattungen eingeordnet werden, stimmten die Nukleotidsequenzen am ITS-2 Lokus bis 100 % überein. Dies verdeutlicht die Notwendigkeit der Anwendung des COI Markers, der zusammen mit dem ITS-2 Marker eine Unterscheidung beider Spezies ermöglichte. Des Weiteren wurden für beide Spezies unterschiedliche nukleare und ribosomale Haplotypen identifiziert. Bei *Cyc. calicatus* konnten

zwei ITS-2 Varianten, die mit unterscheidlichen mitochondrialen Haplotypen einhergehen, bestimmt werden und deuten auf einen nicht hybridisierenden Komplex kryptischer Spezies hin.

In Kürze ist zusammenzufassen, dass die derzeitigen Methoden zur Speziesbestimmung von Cyathostominen nicht ausreichen und, dass die Komplexität der Cyathostominspezies wesentlich ausgeprägter ist, als bisher angenommen wurde. Um MALDI-TOF MS als neue Identifierungsmethode zu etablieren, ist es notwendig die Spezies molekular abzugrenzen und nachfolgend zur Erstellung von Referenzspektren heranzuziehen.

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10 Publications

10.1 Publications in peer-reviewed journals

Project related:

Bredtmann, C. M., Krücken, J., Murugaiyan, J., Balard, A., Hofer, H., Kuzmina, T. A., von Samson-Himmelstjerna, G., **Concurrent Proteomic Fingerprinting and Molecular Analysis of Cyathostomins**. *Proteomics* 2019, 19, 1800290. https://doi.org/10.1002/pmic.201800290

Bredtmann CM, Krücken J, Murugaiyan J, Kuzmina T and von Samson-Himmelstjerna G (2017) **Nematode Species Identification—Current Status, Challenges and Future Perspectives for Cyathostomins**. *Front. Cell. Infect. Microbiol.* 7:283. https://doi.org/10.3389/fcimb.2017.00283

Christina M. Bredtmann; Jürgen Krücken; Tetiana A. Kuzmina; Mariana Louro; Luís M. Madeira de Carvalho; G. von Samson-Himmelstjerna, **Nuclear and mitochondrial marker sequences reveal close relationship between *Coronocyclus coronatus* and a potential *Cylicostephanus calicatus* cryptic species complex**, *Infection, Genetics and Evolution* https://doi.org/10.1016/j.meegid.2019.103956

Further publications:

M. Schediwy, S. Balmer, **C.M. Bredtmann**, D. Hadorn, P.J. Bless, G. Rosato, T. Sydler, M. Harisberger, R. Graage, H. Saura-Martinez, H. Posthaus, C. Gurtner, **Reviving post-mortem diagnostics as a tool to increase porcine herd health and strengthen early detection of pig diseases – the PathoPig project 2014-2016,** *Schweizer Archiv für Tierheilkunde* 2018, 60(6):375-384. https://doi.org/10.17236/sat00164

Rajnish Sharma, Lisa L. Loseto, Sonja K. OstertagMatilde Tomaselli, **Christina M. Bredtmann**, Colleen Crill, Cristina Rodríguez-Pinacho, Dayna Schultz, Dongyun Jung, Kshitiz Shrestha, Prateek Jindal, Emily J. Jenkins, **Qualitative risk assessment of impact of *Toxoplasma gondii* on health of beluga whales, Delphinapterus leucas, from the Eastern Beaufort Sea, Northwest Territories,** *Arctic Science*, 2018, 4(3): 321-337, https://doi.org/10.1139/as-2017-0037

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10.2 Abstracts of oral or poster presentations in conference proceedings

Christina Bredtmann, Jayaseelan Murugaiyan, Tetiana Kuzmina, Jürgen Krücken, Georg von Samson-Himmelstjerna (2018) **Establishing a protocol for simultaneous comparative molecular and proteomic species identificatiom of individual cyathostomin worms.** 28th Annual Meeting of the German Society for Parasitology, Berlin, Germany, March 21st -24th 2018, Programme & Abstract Book, ISBN 978-3-9816508-7-7, page 95

Christina Bredtmann, Jürgen Krücken, Jayaseelan Murugaiyan, Tetiana Kuzmina, Georg von Samson-Himmelstjerna (2017) **Concurrent proteomic profiling and molecular characterization of cyathostomins.** 26th International Conference of the World Association for the Advancement of Veterinary Parasitology, Kuala Lumpur, Malaysia, September 4th- 8th 2017, Abstract Book, page 248

Christina Bredtmann, Jürgen Krücken, Tetiana Kuzmina, Jayaseelan Murugaiyan, Georg von Samson-Himmelstjerna (2017) **Cryptic species in *Cylicostephanus calicatus*: Application of molecular and proteomic methods for study of cyathostomins.** 26th International Conference of the World Association for the Advancement of Veterinary Parasitology, Kuala Lumpur, Malaysia, September 4th- 8th 2017, Abstract Book, page 431

C. Bredtmann, J. Krücken, J. Murugaiyan, G. von Samson-Himmelstjerna (2017) **Etablierung molekularer und proteomischer Methoden zur Differenzierung von Cyathostominae beim Pferd.** Annual meeting of the German Society for Veterinary Medicine (DVG; Working group Parasitology), Hannover, Germany, June 12th – June 14th 2017, Programme & Abstract book ISBN 978-3-86345-372-5, pages 96-97

C. Bredtmann, J. Murugaiyan, J. Krücken, Tetiana A. Kuzmina, U. Rösler, G. von Samson-Himmelstjerna (2016) **Evaluierung molekularer und proteomischer Methoden zur Differenzierung von kleinen Strongyliden beim Pferd.** Annual meeting of the German Society for Veterinary Medicine (DVG; Working group Parasitology), Berlin, Germany, May 2nd – 4th 2016, Programme & Abstract book ISBN 978-3-86345-311-4, pages 115-116

C. Bredtmann, J. Murugaiyan, T.A. Kuzmina, J. Krücken, G. von Samson-Himmelstjerna (2016) Evaluation of molecular and proteomic methods for comprehensive species identification of Cyathostomines. 27th Annual Meeting of the German Society for Parasitology, Göttingen, Germany, March 9th –12th 2016, Programme & Abstract book, page 153

11 Disclosure of the own share in the body of work

The share of the authors who were involved in the publications of this work is listed under the following criteria:

1. Idea and concept of the study
2. Design of experiments
3. Test execution
4. Analysis of test data
5. Compilation of the manuscript

Nematode Species Identification—Current Status, Challenges and Future Perspectives for Cyathostomins

1. Bredtmann, Krücken, von Samson-Himmelstjerna
2. Murugaiyan, Bredtmann
3. Bredtmann, Murugaiyan, Kuzmina
4. Bredtmann, Krücken, Murugaiyan
5. Bredtmann, Krücken, Murugaiyan, Kuzmina, von Samson-Himmelstjerna

Concurrent Proteomic Fingerprinting and Molecular Analysis of Cyathostomins

1. von Samson-Himmelstjerna, Krücken
2. Bredtmann, Murugaiyan, Krücken, Kuzmina
3. Bredtmann, Murugaiyan
4. Bredtmann, Balard, Krücken, Hofer
5. Bredtmann, Krücken

Nuclear and mitochondrial marker sequences reveal close relationship between *Coronocyclus coronatus* and a potential *Cylicostephanus calicatus* cryptic species complex

1. Kuzmina, Bredtmann, Krücken, von Samson-Himmelstjerna
2. Bredtmann, Kuzmina
3. Bredtmann, Kuzmina, Louro
4. Krücken, Louro, Bredtmann
5. Krücken & Bredtmann (shared 1st authorship), Kuzmina, Madeira de Carvalho, von Samson-Himmelstjerna

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14 Statement of Authorship

Except where reference is made in the text, this dissertation contains no material published elsewhere or extracted in whole or in part from a dissertation presented by me for another degree or diploma. No other person´s work has been used without due acknowledgement in the main text of the dissertation. This dissertation has not been submitted for the award of any other degree or diploma in any other tertiary institution.

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