



## Research paper

# 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 Cyathostominae (Nematoda, Strongyloidea) parasitising equines represent a diverse group currently including 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 haplotype groups (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 *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 non-interbreeding genospecies within *Cys. calicatus*, which needs further confirmation using additional samples from diverse geographical origins.

## 1. Introduction

Cyathostomins (Nematoda, Strongyloidea) are considered to be the most important equine parasites, due to their global occurrence, their

widespread and emerging resistance against all available classes of anthelmintics, and due to the clinical disease named larval cyathostomiasis, which affects horses of all age groups (Matthews, 2008). Acute clinical cases might be fatal despite comprehensive treatment; chronic

**Abbreviations:** ca, of *Cys. calicatus*; co, of *Cor. coronatus*; COI, cytochrome oxidase I; Cor., *Coronocyclus*; Cys., *Cylicostephanus*; HG, haplotype group; IGS, intergenic spacer; 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*; OTU, operational taxonomic unit; SH-aLRT, Shimodaira–Hasegawa approximate likelihood ratio test

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cases are characterised by intermittent diarrhoea and wasting (Corning, 2009; Love et al., 1999). These parasites have a direct life cycle with adults residing in the cecum and colon of the host, where they produce eggs, which are shed with faeces. On pasture, larvae hatch and moult twice to third stage larvae which infect horses after ingestion by the host. After oral uptake, larvae penetrate the intestinal wall and reside as histotropic stages where they develop to fourth stage larvae during the up to 14 weeks. However, this development can be inhibited for example if the larvae have previously been exposed to cold temperatures. This phase also named hypobiosis can last for at least 2.5 years (Gibson, 1953). Upon an unknown stimulus, simultaneous emergence of inhibited larvae from the mucosa into the intestinal lumen is triggered, which causes inflammation and the clinical presentation of larval cyathostomiasis (Love et al., 1999).

Currently, 50 species of the subfamily Cyathostominae infecting all equid species, including donkeys and zebras are recognised (Lichtenfels et al., 2008). Globally, only few experts can reliably identify these, only up to 22 mm sized parasites based on morphological characteristics, mainly of the buccal capsule (e.g. the internal and external leaf crown) and the bursa (Dvojnós and Kharchenko, 1994; Lichtenfels, 1975; Tolliver, 2000; Lichtenfels et al., 2008). Other options for diagnosis on a species level are currently very limited (Bredtmann et al., 2017).

The systematics of this extraordinary diverse group of nematode species has been revised multiple times over the years including re-description of species (Lichtenfels, 1975; Lichtenfels et al., 2002; Lichtenfels et al., 2008; Lichtenfels et al., 1998) and evidence for cryptic species in some morphospecies (Bredtmann et al., 2019; Hung et al., 1999b). Molecular and morphology-based phylogenetic approaches apparently came to distinct topologies but the morphology-based analyses were only presented at workshops and have never been published (Lichtenfels et al., 2002; Lichtenfels et al., 2008). Marker sequences that have been used in the past to diagnose cyathostomins include internal transcribed spacer 1 and 2 (ITS-1, ITS-2), intergenic spacer, cytochrome oxidase I (COI) and the 16S mitochondrial rRNA gene (Hung et al., 1999a; Hung et al., 1999b; Gasser et al., 2004; Cwiklinski et al., 2012; Hodgkinson et al., 2001; McDonnell et al., 2000; Traversa et al., 2008). In the present project, it was decided to focus on ITS-2 and COI sequences since (i) a combination of nuclear and mitochondrial markers had been shown to be advantageous in comparison to the use of only one of the genomes (Ramünke et al., 2018), (ii) the ITS-2 had been used successfully to delineate the phylogeny of cyathostomins (Hung et al., 1999a) and (iii) both markers were previously used to identify potential cryptic species and determine intra-species variability (Hung et al., 1999b; Traversa et al., 2008). In the ITS-2 based molecular phylogenetic analysis, resolution was low and members of the genera *Cylicostephanus* Ihle, 1922 and *Coronocylcus* Hartwich, 1986 were not reliably placed into separate, statistically supported clusters (Hung et al., 1999a). However, combination with more variable COI sequences was assumed to improve the resolution of phylogenetic analyses.

In the context of a larger study aiming to obtain molecular and proteomic data for a large set of cyathostomin species, very high similarity between ITS-2 sequences of *Coronocylcus coronatus* [Looss, 1900] Hartwich, 1986 and *Cylicostephanus calicatus* [Looss, 1900] Cram, 1924 in combination with intra-species variants was observed. These species were also placed in close phylogenetic proximity by two previous molecular phylogenetic analyses (Hung et al., 1999a; McDonnell et al., 2000). Therefore, the present study aimed to characterise the molecular diversity and relationship of *Cor. coronatus* and *Cys. calicatus* using a large set of morphologically identified specimens from two different geographic regions and different host species.

## 2. Materials and methods

### 2.1. Collection of worms

Adult worms were collected from eight German horses (*Equus ferus caballus*) during necropsy and from faeces of five species of equines kept at the Askania 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 the macrocyclic lactone product "Univerm" (0.2% aversectin C, PharmBioMed, Moscow, Russia) as described recently (Bredtmann et al., 2019). Adult worms were identified morphologically (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 specimens are provided in Table S1.

### 2.2. DNA isolation, PCR and sequencing

After DNA extraction with the NucleoSpin® Tissue XS Kit (MACH-EREY-NAGEL, Düren, Germany), PCRs targeting the 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 Table S2 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 PCR fragment of the individual worm was sequenced by LGC Genomics (Berlin).

### 2.3. Phylogenetic analyses

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 IQ-TREE (Schmidt et al., 2014) on the IQ-TREE server (<http://iqtree.cibiv.univie.ac.at>). The ModelFinder option of IQ-TREE (Kalyaanamoorthy et al., 2017) was set to auto-determination of the best model and models with FreeRate heterogeneity were included. Ultrafast bootstrapping (1000 bootstrapped alignments) (Hoang et al., 2017) and the Shimodaira-Hasegawa approximate likelihood ratio test (SH-aLRT) (1000 replicates) (Guindon et al., 2010) were chosen to obtain node support statistics. The corresponding command in IQ-TREE for ITS-2 sequences was: `iqtree -s infile.fas -st DNA -m TESTNEW -bb 1000 -alrt 1000`. For the protein coding COI sequences, separate models were fitted for codon positions 1 and 2 vs. codon position 3 and the command line was: `iqtree -s COI_FcC.infile.fas -spp partition_file.txt -pre infile.fas -m TESTNEW -bb 1000 -alrt 1000`. A combined ITS-2/COI tree was calculated using three partitions (ITS-2, COI codon position 1&2, COI codon position 3) and the command `iqtree -s infile.fas -st DNA -spp partition_file.txt -pre infile.fas -m TESTNEW -bb 1000 -alrt 1000`.

In order to calculate identities between sequences the `dna.dist` function with the method "raw" identities from the R package `ape` version 5.0 was used in R 3.4.3.

## 3. Results and discussion

### 3.1. Analysis of internal transcribed spacer 2 sequences

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 and 41 of these *Cor. coronatus* specimens showed an additional PCR product of 369–370 bp (A). The sequences of these two 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

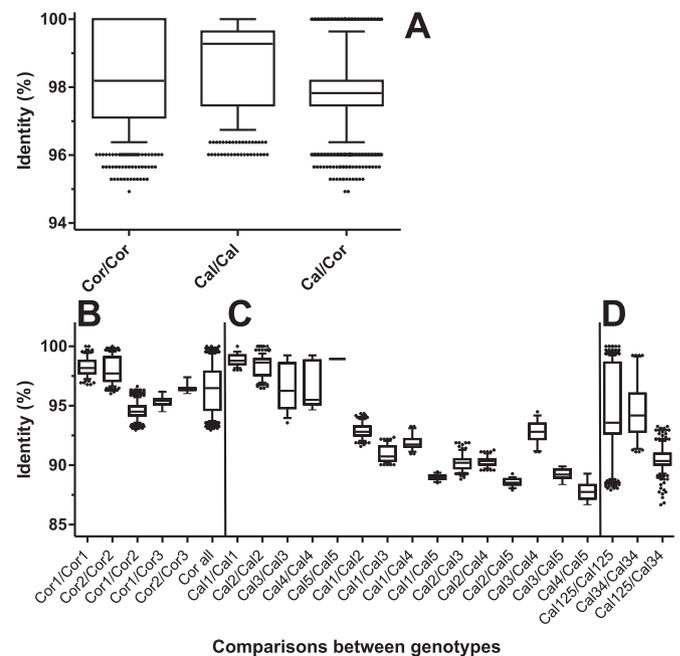
370 bp (A) fragment while 17 specimens showed a 281 bp (B) fragment. In this case, the deletion encompassed the positions 110–198 (89 bp). The number of *Cor. coronatus* A fragment haplotypes was 25, while 10 B fragment haplotypes were counted. For *Cys. calicatus*, 20 A fragment and 9 B fragment haplotypes 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* (Fig. 1A). 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 smaller B fragments and the homologous regions of the larger A fragments (excluding the indel region) was 95.0–100% even between the morphospecies *Cor. coronatus* and *Cys. calicatus*. When focusing only on the insertions of 92 or 89 bp that were present only in the larger A fragment, the between-species comparison revealed only 84.3–94.6% identity. 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 (Fig. 2 and Fig. S1). 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 specimens with double bands (A plus B) separated from the sequences from specimens showing only the B amplicon (Fig. 2 and Fig. S1). 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 (nHGAc0). The majority of *Cys. calicatus* sequences was clustered in two groups containing 36 (nHGAc1) and 8 (nHGAc2) 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 (Fig. 2). 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 (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).

### 3.2. Cytochrome oxidase I sequence analysis

Due to the missing resolution obtained by analysis of the ITS-2 sequences, a more variable mitochondrial marker with superior barcoding properties was included in the analysis (Blouin, 2002; McDonnell et al., 2000). 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*. For *Cor. coronatus*, the analysis further identified two major mitochondrial HGs, mtHG1co and mtHG2co, with 23 and 35 sequences, respectively (Fig. 3 and Fig. S2). 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 (Fig. 3 and Fig. S2).

These mtHGs were then mapped back to the ITS-2 tree (Fig. 2). The two major *Cor. coronatus* mtHG1co and mtHG2co were both found in specimens showing only the nHGBco or both, nHGBco plus nHGAc0. This shows obviously free interbreeding between the two nHGs 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



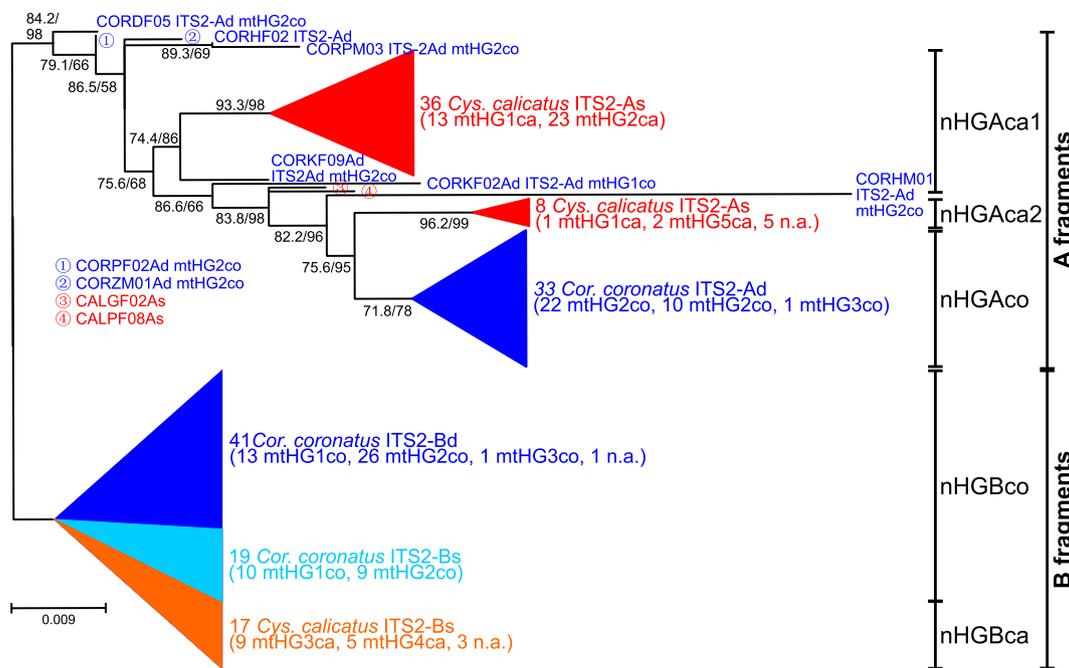
**Fig. 1.** Comparison of sequence identity between different species on the internal transcribed spacer 2 (ITS-2) sequence (A) or between different cytochrome oxidase I (COI) mitochondrial genotypes of the same species (B–D). Identities were calculated using dist.dna function and plotted as boxplots (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 nHGAc1, while mtHG3 and mtHG4(Cor34) were associated with nHGBca.

distributed very unevenly among the two different nHGs. While all specimens 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 nHGAc1 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 (Fig. 1B–D) as well as phylogenetic position (Fig. 3 and Fig. S2), the different mtHGs within *Cys. calicatus* show a degree of dissimilarity that would be comparable with a status as discrete species. In particular, distance between mtHG5ca on one and mtHG1ca/mtHG2ca on the other hand is larger than the distance between mtHG1ca/mtHG2ca and mtHG3ca/mtHG4ca.

### 3.3. Combined analysis of cytochrome oxidase and internal transcribed spacer 2 sequences

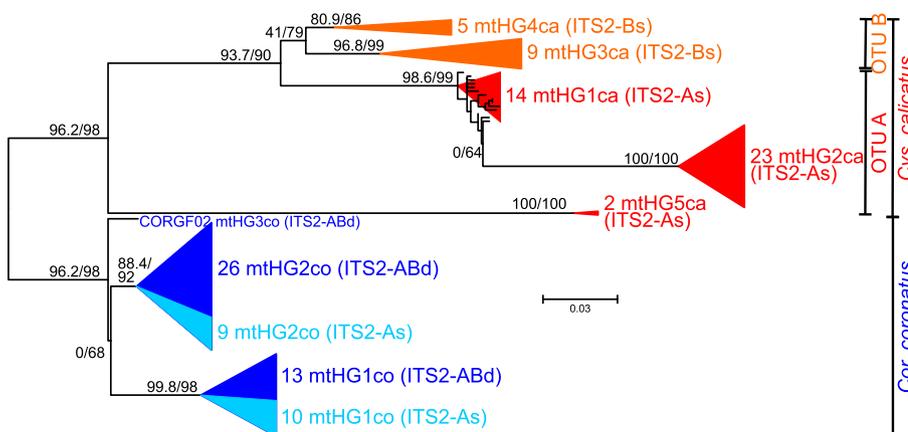
Finally, a combined analysis of nuclear and mitochondrial genotypes was performed. The complex PCR fragment pattern in *Cor. coronatus* (one vs. two products) cannot be explained by hybridisation with



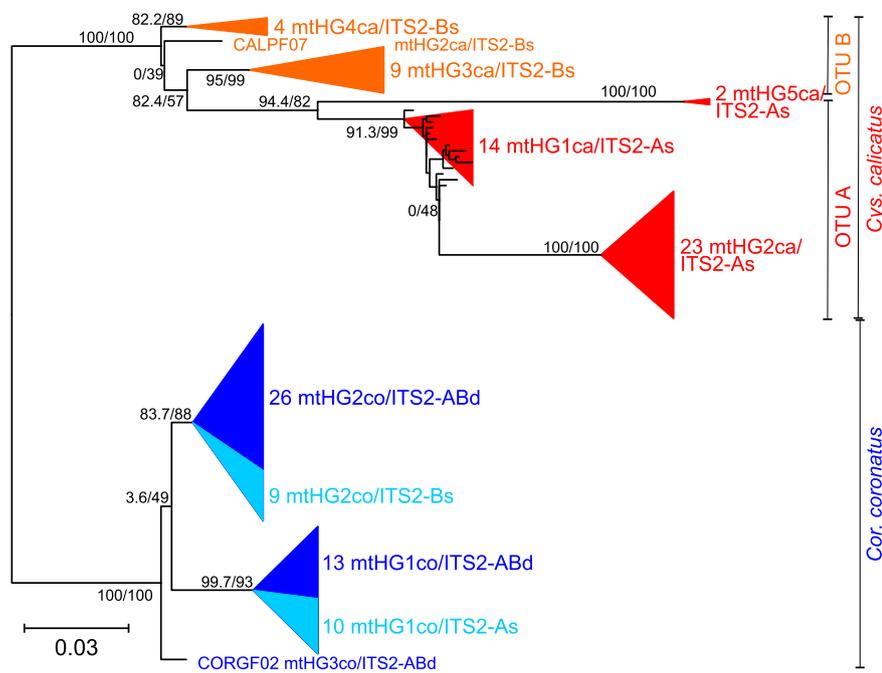
**Fig. 2.** A maximum likelihood phylogenetic tree was calculated using aligned internal transcribed spacer 2 (ITS-2) sequences. The scale bar represents 0.009 substitutions per site and node support was obtained by the Shimodaira-Hasegawa likelihood ratio test before and ultrafast bootstrapping (1000 replicates) after the slash. Numbers before the species names indicate the number of specimens in the group. The ITS-2 types ITS2A and ITS2B are indicated behind the species name or the specimen designation and are followed by a letter indicating whether single (s) or double (d) ITS-2 fragments 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 specimens 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. If no mtHG is provided for individual specimens, 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.

*Cys. calicatus* since no nHGAc was found in any *Cor. coronatus*, as identified by morphology and COI sequence. Therefore, a combined analysis of ITS-2 and COI data was conducted for all specimens for which both sequences were available. For *Cor. coronatus* ITS-2 sequences, only the B fragment was included since this was present in all specimens. The combined tree in Fig. 4 (and Fig. S3) obtained in this analysis was able to clearly separate *Cor. coronatus* from *Cys. calicatus* and in addition also clearly separated the two nHGs of *Cys. calicatus* into two groups, which was not the case using the COI sequences alone in Fig. 3. The specimens with the larger ITS-2 A bands were now assigned to an operational taxonomic unit (OTU) A and those with the smaller band to OTU B. Although these data do suggest that both OTUs

represent closely related but independent species, additional marker sequences need to be included since OTU B is paraphyletic regarding OTU A. Additional markers might include the previously used IGS and 16S rRNA sequences that both have been proven to contain informative sequence variations (Cwiklinski et al., 2012; McDonnell et al., 2000). However, both markers are closely physically linked to the ITS-2 and COI sequences used here. While physical linkage cannot be avoided regarding the analysis of mitochondrial markers, combination of unlinked nuclear markers would be presumably more informative than combination of linked nuclear markers. Unlinked nuclear markers could provide evidence for isolation between genotypes, which is difficult to prove using markers with close physical proximity. Using the latter, it is



**Fig. 3.** Representation of a maximum likelihood phylogenetic tree calculated from the cytochrome oxidase I (COI) sequences. The scale bar indicates a distance of 0.03 substitutions per site and node support values before and behind the slash were obtained by the Shimodaira-Hasegawa likelihood ratio test and ultrafast bootstrapping. 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 specimens 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.



**Fig. 4.** Combined maximum likelihood phylogenetic tree based on cytochrome oxidase I (COI) and internal transcribed spacer 2 (ITS-2) sequences. Genotypes are designated according to mitochondrial haplotype groups (mtHGs) and presence of large (ITS2As), small (ITS2Bs) or both (ITS2ABd) are indicated. The scale bar represents 0.03 substitutions per site. Node support in terms of the results of the Shimodaira-Hasegawa likelihood ratio test and ultrafast bootstrapping are provided before and after the slash, respectively. The number of sequences in each genotype is given before the designation. Individual sequences are labelled with the specimen designation as described for Fig. 2.

very difficult to demonstrate recombination between markers.

Instead of using several independent genetic regions, the present study focused on investigating more specimens from different geographical origins. Both approaches will be required to confirm the presence of cryptic species. This approach was chosen since the initial aim was the combined analysis of molecular and proteomic markers and sample processing was optimised for this approach as described recently (Bredtmann et al., 2019). Obtaining enough protein and DNA for amplification of multiple markers was not possible so far. Unfortunately, proteomic analysis then turned out to be impossible using most of the samples included in the present study since it depends on specimens collected freshly during necropsies (Bredtmann et al., 2019) while the majority of samples included here were obtained from faeces post treatment.

### 3.4. Conclusions

Regarding the overall aim to obtain a reliable list of valid species of Cyathostominae and even Strongylidae infecting equines as well as a phylogenetic tree representing the most likely evolutionary history, the present study is of course only a small piece in a large puzzle. To obtain a final picture, more genetic loci, sequences of specimen from more geographic regions and more samples from equines other than domestic horses will be required. The ongoing project on molecular and proteomic characterisation of specimens identified morphologically by a recognised expert aims to contribute to this longterm goal. Additional approaches such as meta-barcoding as recently developed for ruminant gastrointestinal nematodes will presumably contribute an additional aspect to further characterise the epidemiology of this group of highly variable parasites.

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. The data of both markers together clearly separated *Cor. coronatus* from *Cys. calicatus* but also indicated the existence of discrete genospecies in what is currently assigned to the morphospecies *Cys. calicatus*.

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.meegid.2019.103956>.

### Declaration of Competing Interest

None.

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