Molecular Species Determination of Cyathostomins from Horses in Ireland

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Abstract:

Cyathostomins are globally important equine parasites, responsible for both chronic and acute pathogenic effects. The occurrence of mixed infections with numerous cyathostomin species hinders our understanding of parasite epidemiology, host-parasite dynamics and species pathogenicity. There have been few studies of cyathostomin species occurring in horses in Ireland, where temperate climatic conditions with year-round rainfall provide suitable conditions for infection of grazing animals with bursate nematodes. Here, we amplified and sequenced the ITS-2 region of adult worms harvested at post-mortem from 11 adult horses between August 2018 and June 2020, and recorded species prevalence and abundance of worms recovered from the caecum, right ventral colon and left dorsal colon,

using both BLAST and IDTAXA for taxonomic attribution. Phylogenetic relationships and community composition were also recorded and compared with other relevant studies, including a global meta-analysis. Overall, our results agree with previous studies that there does not seem to be a major difference in cyathostomin species occurrence in equids in different geographical regions. We confirmed the results of other workers in relation to the difficulties in discriminating between *Cylicostephanus calicatus* and *Coronocyclus coronatus* on the basis of ITS-2 sequences.

1. Introduction

Cyathostomins are a group of parasitic helminths that inhabit the gastrointestinal tract. They are obligate parasites which are ubiquitous in equids and of significant pathogenic concern (Boxell *et al*., 2004; Ghafar *et al*., 2022; Hinney *et al*., 2011; Love *et al*., 1999; Morariu *et al*., 2016; Steinbach *et al*., 2006). Currently, there are over 50 recognised species with 40 recorded as infecting horses, and mixed infections are the norm (Lichtenfels *et al*., 2008; Lyons *et al*., 1999). Cyathostomins have a direct life cycle, with infection occurring through ingestion of infective stage (L3) larvae on pasture. Following infection, cyathostomin larvae penetrate into the mucosa of the large intestine, and can remain here in a dormant (hypobiotic) state, emerging to mature and establish patent infection in the lumen after a variable pre-patent period of up to three years (Gibson, 1953; Nielsen and Lyons, 2017). The hypobiotic state is relevant to anthelmintic efficacy and to cyathostomin-associated disease (Boisseau *et al*., 2023; Morariu *et al*., 2016). Typically, of the total worm burden in an individual animal, the majority will comprise hypobiotic larvae in the mucosa (Dowdall *et al*., 2002; Duncan *et al*., 1998; Gibson, 1953; Matthews, 2008). The stimuli responsible for

45 hypobiotic larvae resuming development and completing their life-cycle are unclear, but are
46 thought to involve cross-talk between adult and larval worms, along with interaction with the
47 gut microbiota, the host immune system and environmental disruption such as anthelmintic
48 treatment (Gibson, 1953; Love *et al*., 1999; Matthews, 2008; Walshe *et al*., 2019; Walshe *et*
49 *al*., 2021; Walshe *et al*., 2020). The existence of a large number of species that are typically
50 present in mixed infections highlights the importance of understanding these dynamics, in
51 efforts both to understand the basic biology of cyathostomin infections and the provision of
52 improved control strategies (Bellaw and Nielsen, 2020; Hodgkinson *et al*., 2003).

53 Horses may tolerate light cyathostomin infection without the development of overt clinical
54 signs (Love *et al*., 1999; Morariu *et al*., 2016; Murphy and Love, 1997; Walshe *et al*., 2021).
55 However, high burdens can lead to ill-thrift and poor performance (Murphy and Love, 1997)
56 and in some cases a specific clinical presentation of acute larval cyathostominosis (ALC), which
57 develops in response to synchronous emergence of larvae from the mucosa and involves
58 severe local and systemic inflammation, with high morbidity and potential mortality
59 (Hodgkinson *et al*., 2003; Love *et al*., 1999; Walshe *et al*., 2020). The emergence of encysted
60 cyathostomin larvae is believed to be affected by many factors, including negative inhibition
61 from an established adult worm population, removed following anthelmintic treatment
62 (Steinbach *et al*., 2006; Walshe *et al*., 2019; Walshe *et al*., 2021). ALC has a 50% mortality rate
63 recorded in referral centres (Giles *et al*., 1985; Peregrine *et al*., 2006; Walshe *et al*., 2021).

64 In the past, characterisation of cyathostomin communities relied exclusively on
65 morphological identification of species (Kinsella, 2002; Lichtenfels *et al*., 2008). However, this
66 is laborious, time-consuming and highly specialised (Avramenko *et al*., 2015; Hodgkinson *et*
67 *al*., 2003; Lichtenfels *et al*., 2008), and it has largely been supplanted by molecular species

68 determination, either of individual worms, or using population analysis "Nemabiome"
69 approaches (Avramenko *et al*., 2015).

70 There are just two previously published studies on characterising cyathostomin species in
71 horses in Ireland. The first (Kinsella, 2002), used morphological species determination and the
72 second (Elghryani, 2018) used reverse line blots of the intergenic spacer gene region.
73 Elsewhere, internal transcribed spacer 2 (ITS-2) primers as described by (Gasser et al., 1993)
74 have been widely used as taxonomic markers for cyathostomins. The focus of recent studies
75 have utilised community analysis tools, such as 'Nemabiome' application of ITS-2 sequencing
76 (Avramenko et al., 2015; Courtot et al., 2023; Halvarsson et al., 2023; Nielsen et al., 2022) .

77 We aimed to sequence the ITS-2 region from individual cyathostomins retrieved from
78 naturally infected horses in Ireland in a post-mortem study and to compare the community
79 composition from the colon and caecum to those described in previous studies in Ireland and
80 internationally. We also aimed to explore any difficulties in distinguishing species based on
81 ITS-2 sequencing, to investigate any trends in seasonality in the data, and to compare and
82 contrast two different methods of inferring taxonomy based on ITS-2 sequences, using BLAST
83 and IDTAXA methods.

84

85 ## 2. Materials and Methods

86

87 ### *2.1. Ethical approval*

88 This was a post-mortem study and did not require any specific ethical approval.

89

90 *2.2. Study population*

91 We conducted an abattoir survey between August 2018 and June 2020. A total of eleven
92 equine carcasses were selected at random for examination at two Irish abattoirs. In
93 accordance with food safety legislation, no horse had received anthelmintic treatment within
94 32 days of slaughter. The horses were assessed for clinical abnormalities by a veterinarian
95 prior to inclusion in the study as part of the normal pre-mortem inspection at the abattoir.

96

97 *2.3. Sample processing*

98 Each carcass was processed as per abattoir standard operating procedures. Sampling was
99 undertaken on the same day as euthanasia. To isolate the large intestine, the ileocecal
100 junction was located, tied off with cable ties and dissected. The liver, stomach, spleen,
101 pancreas, and small intestine were removed, and the large intestine was transferred from the
102 processing line to a dissection table.

103 Further cable ties were placed at the junction between the caecum and right ventral colon,
104 the junction between the right ventral and left ventral colon, the junction between the left
105 ventral colon and left dorsal colon, and the junction between the left dorsal and right dorsal
106 colon. An incision was made in each of the three intestinal segments (caecum, right ventral
107 colon (RVC) and left dorsal colon (LDC), and the luminal contents were emptied separately
108 into individual 10L graduated buckets. For each section, the mucosa was gently rinsed, and
109 the liquid was added to the bucket containing luminal contents. The contents were then
110 stirred whilst water was added to make a homogeneous suspension to a volume of 10L. Two
111 100ml aliquots were taken from each section, equating to 2% of the total volume. Faeces

112 were collected from the rectum of each horse and placed in a ziplock bag. All samples were
113 placed on ice for transfer to the laboratory.

114

115 *2.4. Faecal Egg Counting*

116 The Mini-FLOTAC technique (Cringoli, 2006) was utilised to count strongyle eggs in faeces.
117 Briefly, 45ml of saturated NaCL was added to 5g of faeces and the flotation chambers were
118 completely filled with the faecal suspension. After 10 minutes, the key was used to turn the
119 reading disc clockwise (about 90°), the sample was examined under the microscope and the
120 eggs per gram calculated. The sensitivity limit for the Mini-FLOTAC is 5 epg.

121

122 *2.5. Luminal worm retrieval*

123 Each aliquot representing 2% of the contents of the caecum, RVC and LDC was examined
124 individually using a modification of the Baermann method by enclosing them in a muslin
125 parcel suspended in water in a conical flask and leaving to sediment for 24 hours. The entire
126 sediment was then examined in detail (using aliquots forming a thin layer in a petri dish) under
127 a dissecting microscope. The remaining sediment was also examined under a dissecting
128 microscope.

129 The total number of worms present in each 200ml sample was enumerated and recorded.
130 Individual worms were removed manually and washed prior to storage. Each worm was
131 placed in a 2ml Eppendorf with >/=50ml PBS and stored at -20˚C. For each section a maximum
132 of 50 worms (range 1-50) was retained for subsequent examination from combined duplicate
133 samples. IBM SPSS (Version 27) was used for descriptive statistics. The Kruskal-Wallis test

134 was utilised for non-parametric data including faecal egg counts (FEC), numbers of worms and
135 species distribution

136

137 *2.6. DNA Extraction and amplification*

138

139 *2.6.1. Genomic DNA extraction from individual nematodes*

140 Individual worms were dissected into a minimum of three parts using a sterile needle. DNA
141 was then extracted from each worm using the Powerfecal Pro extraction kit (Qiagen, Product
142 number 51804), as per the standard operating protocol, with minor adaptations. Specifically,
143 samples were pulse vortexed, as part of the bead beating step, for a minimum of 45 minutes,
144 the dry spin after solution C5 was at 16,600 x G for three minutes, and solution C6 of the kit
145 was put through the filter membrane twice to increase elution yield (Bredtmann *et al*., 2019).
146 After extraction, samples were stored at -20°C until PCR amplification.

147

148 *2.6.2. PCR amplification*

149 PCR reactions were performed in 50µl reaction volumes using ITS-2 primers as described by
150 Gasser *et al*., (1993), (NC1 forward; 5'ACGTCTGGTTCAGGGTTGTT3' and NC2 reverse;
151 5'TTAGTTTCTTTTCCTCCGCT3').

152 The reaction mixture consisted of 10µl of Promega GoTaq Flexi Buffer,(product no. M780A)
153 1.5mM of Promega MgCL2 solution, 0.2mM Promega DNTP PCR nucleotide mix, (product
154 number U151A) 1µM of each forward and reverse primer (Eurofins Genomics) 1.25U of
155 Promega GoTaq G2 Flexi DNA Polymerase, (product no. M780A) 10µl template DNA and

156 15.75µl nuclease free water (Merck Millipore 693520). Amplification steps included initial
157 denaturation at 95°C for 2 minutes, then cycling steps starting at 95°C for 1 minute, annealing
158 at 55°C for 1 minute and extension at 72°C for 1 minute, for 40 cycles, followed by extension
159 at 72°C for 5 minutes.

160 For each run, a negative control containing 10µl nuclease-free water in place of DNA template
161 was included.

162 Amplicons were purified using the Qiagen Qiaquick PCR purification kit (Product.No. 28106)
163 and analysed through Sanger sequencing in both directions using the PCR primers.
164 Sequencing was outsourced to Eurofins Genomics, (Sandyford, Dublin 18, Ireland) and FASTA
165 files were returned to the research group for analysis.

166

167 *2.7. Molecular taxonomy attribution*

168 Aligned sequences were constructed from forward and reverse reads and compared with
169 published nematode sequences in the NCBI database using Clustal Omega.

170 Samples that did not yield a reliable BLAST result, (E value > E^{-10}, low query cover and
171 percentage identity <95%,) were removed from the dataset.

172 As we did not carry out morphological examination of recovered worms, all sequences are
173 uploaded as 'unidentified cyathostomin' on NCBI database and a note accompanying each
174 species outlining its closest hit on BLAST. These nucleotide sequence data is available in the
175 GenBank™ database under the accession numbers: **OR941129 - OR941242.**

176 *2.7.1 Analysis of sequences using Nematode ITS2 database and IDTAXA*

177 Sequences obtained from individual worms were also compared with data in an opensource
178 ITS2 rDNA database for both parasitic and free living nematodes (Wokentine et al., 2020). To
179 infer taxonomy, IDTAXA (Murali et al., 2018) as a component of the DECIPHER (2.22.0)
180 package in R (v4.1.2), and phyloseq package version (1.38.0) through R studio
181 (v2023.12.0.369) was used within the DADA 2 pipeline as described at www.nemabiome.ca .

182

183 2.8 Phylogenetic analiysis

184 MEGA11, and iTOL (https://itol.embl.de/) were used for phylogenetic tree creation. MEGA11
185 was used for MUSCLE alignment and trimming of sequences. A tree was created using the
186 Maximum likelihood algorithm with 1,000 bootstrap replications and pairwise deletion for
187 gaps. (Tamura et al., 2021). The evolutionary history was inferred by using the Maximum
188 Likelihood method and Tamura-Nei model(Tamura and Nei, 1993). The bootstrap consensus
189 tree inferred from 1000 replicates (Felsenstein, 1985) is taken to represent the evolutionary
190 history of the taxa analyzed. Branches corresponding to partitions reproduced in less than
191 50% bootstrap replicates are collapsed. Initial tree(s) for the heuristic search were obtained
192 automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise
193 distances estimated using the Tamura-Nei model, and then selecting the topology with
194 superior log likelihood value. Seven sequences were removed at the alignment section of the
195 phylogenetic analysis as they were too short when aligned with other species. There were a
196 total of 203 positions in the final dataset. Visualisation of the phylogenetic tree was
197 conducted using iTOL V6.8.1(Letunic and Bork, 2021)

198

199 ## 3. Results

200

201 *3.1. Study cohort and worm recovery*

202 Table 1 provides the details of horses included in the study, together with their strongyle FEC.
203 number of worms, and the areas from which worms for molecular identification were
204 retained. The horses included in the study ranged from yearlings to twenty-three years old
205 with a mean of 7.7 years of age, and a median of 6 years. There were four females and seven
206 males. Five were Thoroughbred, and six Irish Sport Horse. FEC ranged from 0-1,670 eggs per
207 gram (epg). Nematodes were retrieved from two horses with a FEC of 0. Conversely, high
208 FEC did not always correlate with high luminal burden of nematodes. Numbers of worms
209 recovered from the caecum were lower than those recovered from LDC (P=0.009). No other
210 significant differences in worm recovery between compartments was noted. Up to 14
211 individual worms from each intestinal compartment, per horse, had taxonomy inferred to
212 species level by BLAST and IDTAXA. For all but one horse, worms from two or three
213 compartments were included in the taxonomic analysis.

214

215 *3.2. BLAST analysis of ITS-2 amplified sequences*

216 A total of 116 samples were extracted, amplified and compared using the BLAST algorithm
217 with sequences in the NCBI database. Another 43 samples did not provide adequate
218 sequence information for analysis Of the 116 sequences analysed, 43 samples had a 100%
219 identity score with their presumed species. The remaining samples had taxonomy inferred
220 based on similarity scores of > 95% similarity, and E,= 10^{-10} . The amount of samples that fell

221 within the percentage similarity values outlined previously (Mitchell et al., 2019) are outlined
222 as follows: >99% n= 46, >98% n= 16, >97% n= 5, >96% n= 3.. (Supplementary File).

223

224 3.3 IDTAXA analysis of amplified sequences and comparison with BLAST results.

225 A total of 103 of the original 116 consensus Sanger sequences gave valid outputs from the
226 nemabiome.ca workflow using the ITS2 database using IDTAXA to infer taxonomy. Thirteen
227 sequences did not pass quality control cut-offs for the IDTAXA algorithm because they fell
228 below the 60% taxonomic classification confidence threshold. Of the 103 sequences meeting
229 the threshold, 83 (80.5%) were assigned to species level by IDTAXA and gave the same result
230 as BLAST using >95% similarity, (Table 2). Of the remainder, 13 were identified only to genus
231 level by IDTAXA, but as the same genus as BLAST. A further six were presumptively identified
232 as *Cylicostephanus calicatus* by BLAST and *Coronocylus coronatus* by IDTAXA. One sequence
233 identified on BLAST as *Cor. coronatus* was assigned as unclassified *Cyathostomum* spp. using
234 IDTAXA.

235

236 *3.3. Species recovered, abundance and prevalence*

237 The species abundance in each of the three intestinal compartments is shown in Figure 1.
238 *Cor. coronatus* was the most abundant species recovered (29%). Between them, *Cor.*
239 *coronatus*, *Cylicocyclus nassatus* and *Cylicostephanus longibursatus* accounted for more than
240 half (54%) of the entire dataset. Although there was no significant difference in species
241 distribution across the three intestinal compartments, *Cylicocyclus insigne* was equally

242 abundant to *Cor. coronatus* in the LDC. This compartment also had the most diverse range
243 of species (n=11), with the caecum having the least diversity (n=8).

244 Figure 2 outlines the composition per horse of presumed species. It outlines the species found
245 in each of the 11 horses, represented as a percentage of the total samples amplified from
246 each individual. The actual number of worms that each segment represents is also included
247 in the figure, overlaying each segment. This figure provides an insight into the species
248 composition differences between individuals from the samples that were obtained in this
249 study. For the purposes of this analysis, *Strongylus vulgaris* (horse 5) was included in this
250 analysis.

251 The prevalence of individual species and the richness of cyathostomin communities in this
252 study population is illustrated in Figure 3. *Cor. coronatus* and *Cyc. longibursatus* were the
253 most prevalent, found in seven of the eleven horses (63.63% prevalence), *Cyc. nassatus* was
254 present in six horses (54.54%) and *Cylicostephanus goldi* in five horses (45.45%). *Cyc. insigne,*
255 *Cylicostephanus calicatus, Cylicocyclus ashworthi* and *Cyathostomum catinatum* were each
256 recorded in four horses, at a prevalence of 36.36%. *Cyathostomum pateratum* was found in
257 three horses (27.27% prevalence) with *Cylicostephanus minutus, Coronocyclus. labiatus* and
258 *Cylicocyclus leptostomum* in one animal each, comprising 9.09% prevalence.

259 Minimal richness from the samples taken ranged from two to seven species per individual,
260 with a mean of 4.27 +/- 0.604 (SEM). The modal species richness was two and five species
261 equally, with a median species richness of five within this dataset. Over 50% (54.5%) of the
262 horses sampled had >5 cyathostomin species present. One large strongyle, *Strongylus*
263 *vulgaris,* was identified (Horse 5) but not included in the dataset.

264

265 *3.4 Analysis in relation to previous local and global data*

266 Next, (Figure 4) we compared the composition of cyathostomin species found in our study
267 with data from a previous study in Ireland (Kinsella, 2002) and a global meta-analysis (Bellaw
268 and Nielsen, 2020). Twelve cyathostomin species were common to our study, Kinsella, 2002,
269 and the global meta-analysis. Notably, *Cor. labratus* was not identified in either of the Irish
270 datasets, but features in the global meta-analysis. Our study presumptively identified 12 of
271 the 13 most prevalent global species, (Bellaw and Nielsen, 2020), using two different
272 methods of inferring taxonomy from ITS-2 sequences.

273

274 *3.6. Phylogenetic analysis of species*

275 Sequences were used for construction of a Maximum Likelihood phylogenetic tree (Figure 5)
276 which was rooted at the midpoint. Figure 5 unveiled some interspecies occurrences within
277 branches, notably between *Cyc. calicatus* and *Cor. coronatus*.

278

279 **4. Discussion**

280 There was no direct relationship between strongyle faecal egg count and worm burden in the
281 carcases examined, which was not unexpected (Chapman *et al*., 2003a; Chapman *et al*.,
282 2003b; Nielsen *et al*., 2010). We recovered worms from horses with a 0 EPG count, which
283 has been well-documented previously (Gibson, 1953; Love *et al*., 1999; Matthews, 2008;
284 Walshe *et al*., 2019; Walshe *et al*., 2020).

285 We did not attempt morphological identification of the worms recovered in our study,
286 because of the inherent difficulties in doing so and our lack of expertise in this area. Our

287 taxonomic classifications, therefore, are inferred solely on the basis of sequence information.
288 Clearly, reliance on molecular data only for this purpose raises the possibility of errors due to
289 errors in sequence databases, judgement calls when there is similarity to more than one
290 species, and the inability to distinguish between some species on the basis of one locus (in
291 this case ITS-2) alone. In order to validate and provide for greater reliability from our results,
292 we used two distinct methods for inferring taxonomy from sequence information, BLAST, and
293 IDTAXA.

294 IDTAXA is a method of taxonomic classification based on sequences of marker genes from
295 organisms found in microbiome samples or other sources. It involves assigning sequences to
296 a reference taxonomy containing sequences from known members of a reference dataset,
297 and avoids overclassification of sequences from organisms which may not be present in the
298 reference set (Murali et al., 2018). A database of ITS2 rDNA sequences from both parasitic
299 and free-living nematodes is publicly available, (Workentine et al., 2020) and is now widely
300 used in "nemabiome" studies in order to characterise a mixed population of parasitic
301 nematodes within a single host, or host population, including in horses (Ghafar et al., 2022;
302 Halvarsson et al., 2023; Poissant et al., 2021; Sargison et al., 2022) following high-throughput
303 parallel sequencing and metabarcoding. However, the same bioinformatics pipeline using
304 DADA 2 can be used to assign taxonomy to data obtained by conventional Sanger sequencing.
305 The results obtained in our study from BLAST of individual sequences and from the DADA 2
306 IDTAXA output gave identical results to species level for 82 (80.6%) of worms and results
307 consistent to genus level for a further 12 (12.6%). For the remaining seven sequences, (6.8%)
308 there was inconsistency in distinguishing between *Cor. coronatus* and *Cyl. calicatus*, reflecting
309 difficulties in using ITS-2 sequences to distinguish between these potentially cryptic species
310 in other studies (Bredtmann et al., 2019,(Louro et al., 2021).

311

312 Overall, given the consistency between taxonomic assignment by BLAST, and by IDTAXA, in
313 this study, together with the difficulty and potential for error in morphological identification
314 of strongyle worms of horses, we suggest that comparison of two sequence-based methods
315 of taxonomic classification is useful in further determining the taxonomic relationships
316 between these parasites. The characterisation of additional loci may also be useful in some
317 cases (Courtot et al., 2023)

318

319 This study detected sequences similar to twelve of the thirteen most common species globally
320 (Figure 4), as compared to Bellaw and Nielsen's meta-analysis (Bellaw and Nielsen, 2020). It
321 should be noted that prevalence as outlined in this study is based on a 2% sample of contents
322 of each intestinal compartment sampled and thus is likely to underestimate prevalence.
323 Among the nineteen species identified in the morphological study by Kinsella (2002), we
324 detected twelve (Figure 4). Notably, the prevalence of *Cya. catinatum* was 36% in our study,
325 in contrast to 100% prevalence in Kinsella's work and 94% in the global meta-analysis.
326 Elghryani (2018), found *Cyc. nassatus* as the most prevalent among the twelve species
327 identified. The high abundance and prevalence of *Cyc. nassatus,* occurring in 64% (n=7) of the
328 horses sampled, is consistent among all three Irish studies and the global meta-analysis. The
329 prevalence findings between the three studies from Ireland broadly reflect one another. A
330 notable observation was the absence of the twelfth most prevalent species as identified by
331 (Bellaw and Nielsen, 2020), *Cor. labratus,* which was absent from the Irish studies, and has
332 not been detected either in some studies in similar climates-Wales and Scotland(Mitchell et

333 al., 2019; Sargison et al., 2022). This species has however, been recorded in England and
334 France (Cwiklinski *et al*., 2012; Peachey *et al*., 2018).

335 *Cyc. calicatus* and *Cor. coronatus* are interspersed within the ML tree. This reflects their close
336 relationship, as previously identified, and the limitations of using the ITS-2 locus to
337 differentiate the subsets of these species (Courtot *et al*., 2023; Hung *et al*., 2000; Louro *et al*.,
338 2021).

339 Across the three intestinal compartments examined in this study, the number of worms
340 retrieved from the caecum was significantly lower than that recovered from LDC (P =0.009).
341 This was also observed in a previous Irish study(Kinsella, 2002). (Ogbourne, 1976), assessed
342 abundance of cyathostomins postmortem and found 50% in the ventral colon and 45% in the
343 dorsal colon. The worm burden in the caecum represented only 5% of the overall
344 cyathostomin burden. Similarly, (Stancampiano *et al*., 2010), observed that the majority of
345 small strongyles were concentrated in the ventral colon (67.34%) and less frequently in the
346 caecum (27.52%) and dorsal colon (5.14%). While this distribution was also reflected by
347 Kinsella (2002), the converse was found for encysted stages of cyathostomins, as recorded by
348 (Nielsen *et al*., 2021) who documented 40% of EL3/LL3/L4 in the caecum, and 38%/22% in the
349 ventral and dorsal colon respectively. This recurrent recording of biological preference of
350 luminal cyathostomins for the colon over the caecum, and the inverse for encysted larvae
351 may reflect the slower transit time of the colon and the natural peristalsis of food through
352 the digestive tract, enabling passive transport and a longer exposure to the intestinal wall
353 necessary to facilitate larval encystment (Geor *et al*., 2013; Miyaji *et al*., 2008).

354

355 Over half of the horses in this study had more than five cyathostomin species recovered from
356 them (Figure 2).(Abbas et al., 2023; Reid et al., 1995)

357

358 ## 5. Limitations

359 The most important limitation of this study is sample size. This is a descriptive study, which
360 affects the potential for statistical power. Conclusions from observations within any study
361 with a limited sample size must take this into account. Results are considered in the context
362 that only two nematodes were retrieved for sampling from horse eight. Notably, it is clear
363 from this research and other studies that the ITS-2 locus cannot resolve all isolates of *Cor.*
364 *coronatus* and *Cyc. calicatus,* pointing to the need for a different locus for further molecular
365 work, such as COX1, or a multi-locus approach (Courtot *et al*., 2023; Ghafar *et al*., 2022). We
366 acknowledge also the value of examining seasonality in relation to cyathostomin species
367 prevalence and abundance, as well as potential interactions, positive or negative between
368 species. Variability of seasonal patterns among species, in particular, is an understudied facet
369 of cyathostomin biology which may be crucial for comprehensive characterisation of
370 pathogenesis and epidemiology of cyathostomin-related disease, as this tends to have a
371 seasonal distribution of presentation(Abbas et al., 2023; Reid et al., 1995). Other studies,
372 (Salle et al., 2018) have begun to explore interactions among cyathostomin communities, and
373 this also has a potential bearing on the pathogenesis and epidemiology of cyothostomin-
374 related disease. Our study was too small to make reliable observations on these points.

375

376

377 ## 6. Conclusions

378 This study has produced new insights into cyathostomin species occurrence, interactions and,
379 community composition in horses in Ireland, with comparisons to previous studies in this
380 locality and a global meta-analysis. It also displays a high level of agreement in assigning
381 taxonomy from ITS-2 sequences, based on BLAST and IDTAXA, which we believe is useful in
382 increasing the reliability of studies using molecular information to study cyathostomin
383 communities.

384 Post-mortem analysis and identification in naturally-infected hosts, as conducted here, will
385 continue to have a role to play in advancing understanding of cyathostomin biology and
386 pathogenesis, in parallel with population-based approaches. More extensive studies involving
387 both population and post-mortem studies are warranted in order to enhance risk assessment,
388 biomarker-based diagnostic tools and treatment protocols (Walshe *et al*., 2020). It is also
389 useful in investigating potentially cryptic species as seen in this study with *Cor. coronatus* and
390 *Cyc. calicatus.*

391 Studies on cyathostomin population dynamics are a crucial step in understanding the complex
392 picture of cyathostomin infections in the face of pervasive anthelmintic resistance and their
393 potential to advance mechanistic understanding of gut health in horses.

394

395 ## Author contributions

396 Conceptualization, G.M., N.W., O.B.; writing- original draft preparation, O.B. – review and
397 editing, G.M., N.W., O.B. and A.Z.; laboratory work and bioinformatics- O.B., D.G., J.C.;
398 laboratory contributions, L.K., O.BX., D.McS., F.ON., S.D., BR.L. All authors have read and
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Conflict of interest:

The authors declare no conflict of interest.

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Tables

Table 1: Horse details, FEC and number of worms recovered and speciated from each intestinal compartment. RVC= Right Ventral Colon and LDC = Left Dorsal Colon. * = P < 0.05. Mean calculations are +/- the SEM (standard error of the mean). The number of worms recovered accounts for a 1% subsection of the total luminal contents of each animal sampled. Numbers of worms recovered from the caecum were lower than those recovered from LDC (P=0.009). No significant difference was recorded between RVC or from composite RVC and LDC colon to the caecum (P=0.291). FEC represents eggs per gram (epg) of faecal material).

Horse Details						Number of worms recovered			Speciated Worms		
Horse	**Age**	**Breed**	**Sex**	**Sampling Date**	**FEC (epg)**	**RVC**	**LDC**	**Caecum**	**RVC**	**LDC**	**Caecum**
1	16	ISH	F	Sep-18	1550	79	188	10	2	9	-
2	8	ISH	M (gelding)	Sep-18	0	11	3	1	5	-	-
3	1	TB	M (colt)	Sep-18	1670	205	155	12	8	2	4
4	10	ISH	F	Oct-18	25	201	245	23	3	13	-
5	1	TB	M (colt)	Oct-18	905	590	33	16	3	-	4
6	6	ISH	M (colt)	Feb-20	225	2	26	0	1	4	-
7	2	TB	M (colt)	Feb-20	800	1	249	2	-	4	1
8	12	ISH	M	Mar-20	50	0	2	0	-	2	-
9	23	TB	F	Mar-20	85	9	24	1	3	3	-
10	3	TB	F	Jun-20	715	1410	3328	33	8	5	14
11	3	ISH	M	Jun-20	0	40	56	53	4	5	9
Total	-	-	-	-	-	2548	4309	151	37	47	32
Mean	7.7+/-2.124	-	-	-	547.73 +/- 188.83	231.63 +/- 129.21	391 +/- 295*	13.73 +/- 5.1*	3.36 +/- 0.83	4.27 +/- 1.16	2.91 +/- 1.4
Range	1-23	-	-	-	0-1670	0-1410	2-3328	0-53	0-8	0-13	0-14

Table 2. Comparison of BLAST and IDTAXA identification of ITS sequences. We compared the degree to which BLAST, with >95% sequence identity, and the IDTAXA algorithm gave comparable results using our ITS-2 sequences.

Sequences for which taxonomy inferred by BLAST	Agreement on Species using IDTAXA	Agreement on Genus level, IDTAXA	Inability to distinguish between *Cor. coronatus* and *Cyl. calicatus*	Species BLAST, different genus IDTAXA	Did not pass IDTAXA Quality Threshold
Cor. coronatus	26	2	7	1 (unclassified Cyathostomum).	5
Cyl. nassatus	15				
Cyl. goldi	11				
Cyl. insigne	11				
Cyl. longibursatus	9	1		3	3
Cyl. ashworthi	3	6			
Cya. pateratum	2	1			
Cya. catinatum	2	3		2	2
Cor. labiatus	1				
Cyl. leptostomus	1				
Cyl. minutus	1				
S. vulgaris	1				
Cyl. calicatus					2

592

Figure Legends

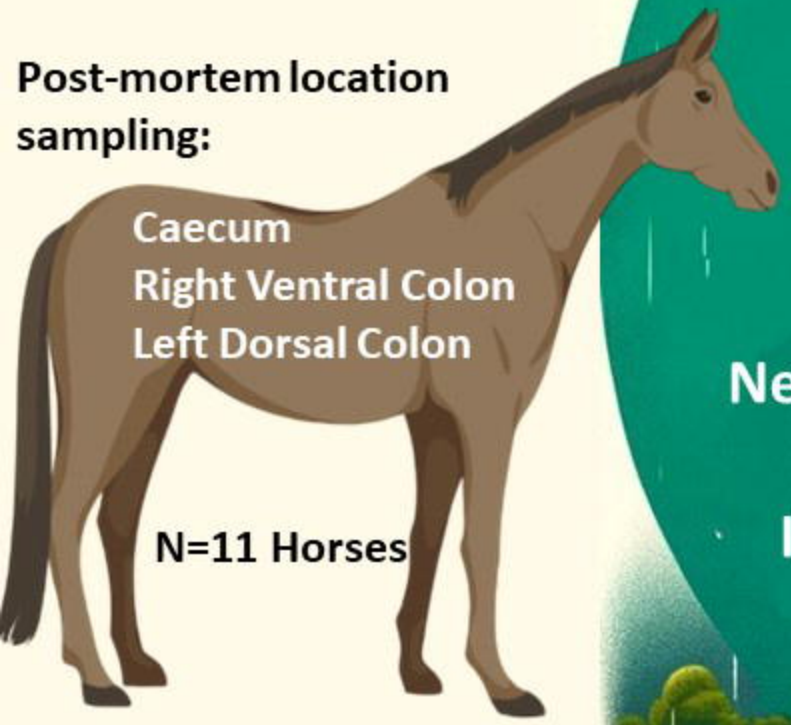
Figure 1: Species abundance data from samples obtained from Right Ventral Colon (a) Left Dorsal Colon (b), Caecum (c) and across all three compartments (d). *Cor. coronatus* was the most abundant species recovered, at 29% of total nematodes speciated. Between them, *Cor. coronatus, Cyc. nassatus* and *Cyc. longibursatus* accounted for more than half (54%) of the entire dataset. The LDC had the most diverse range of species (n=11), with the caecum having the least diversity (n=8).

Figure 2: 100% stacked bar graph of the species distribution per horse. Species richness ranged from 2-7 per horse. Each horse represents a stacked column, identified on the X- axis. The species are represented by coloured sections illustrated as a percentage of the nematodes obtained from each horse. The actual number of samples from each species is also illustrated within each segment.

Figure 3: Prevalence of cyathostomin species as compared with a previous study in Ireland (Kinsella 2002) and a global meta-analysis (Bellaw and Nielsen, 2020). We identified sequences similar to twelve cyathostomin species recorded in both the previous Irish study and the global meta-analysis. There were no species identified in either study in Ireland that were not identified within the global meta-analysis. Generally, the most prevalent species in the two previous studies were also highly prevalent in our study.

Figure 4: Full phylogenetic tree of sequences from recovered worms. IDtaxa similarity sequences for individual cyathostomins were used for phylogenetic analysis. MEGA11 was used for MUSCLE alignment and trimming of sequences. A tree was created using the Maximum likelihood algorithm with 1,000 bootstrap replications and pairwise deletion for gaps. This tree unveiled species that located within groupings of other species, notably *Cylicostephanus calicatus* and *Coronocyclus coronatus.*

Molecular Species Determination of Cyathostomins from Horses In Ireland



Recovered individually by section

Worms Retrieved By Hand

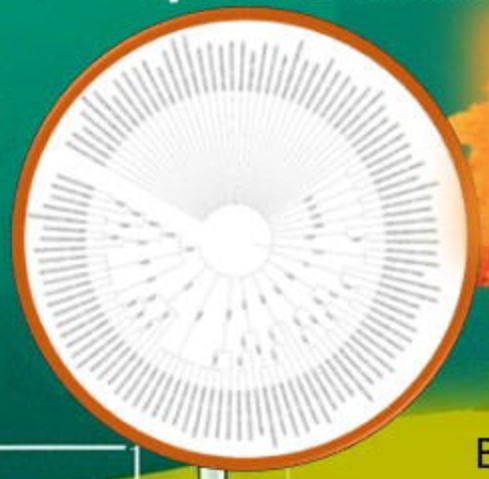
FEC Recorded

Nematode DNA Extracted Individually N=116

ITS-2 Amplification and Sanger Sequencing

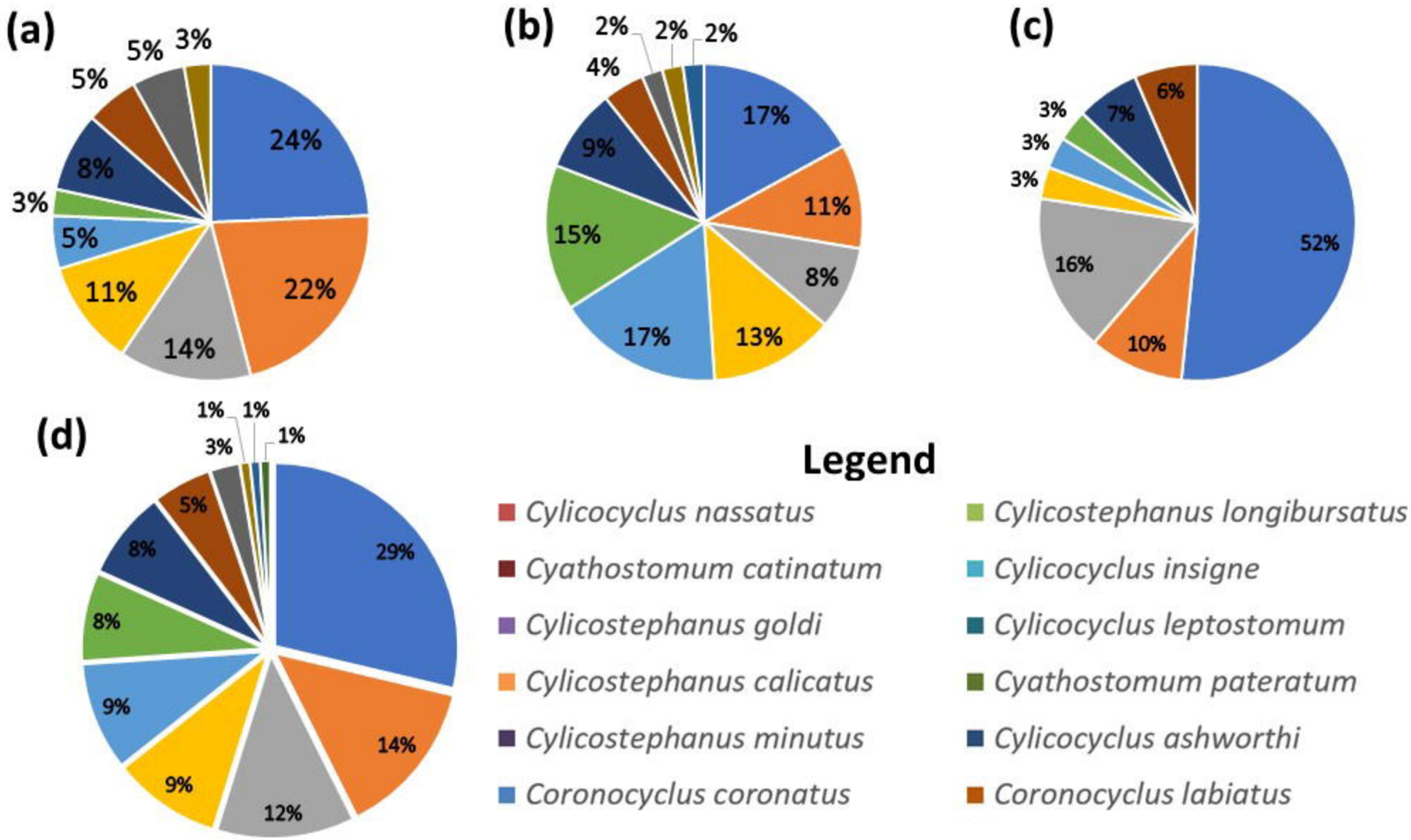
IDTAXA and BLAST Species Comparison

12/13 Globally Prevalent Species Identified



Phylogenetic Tree Analysis

Byrne *et al.*, 2024


(a)
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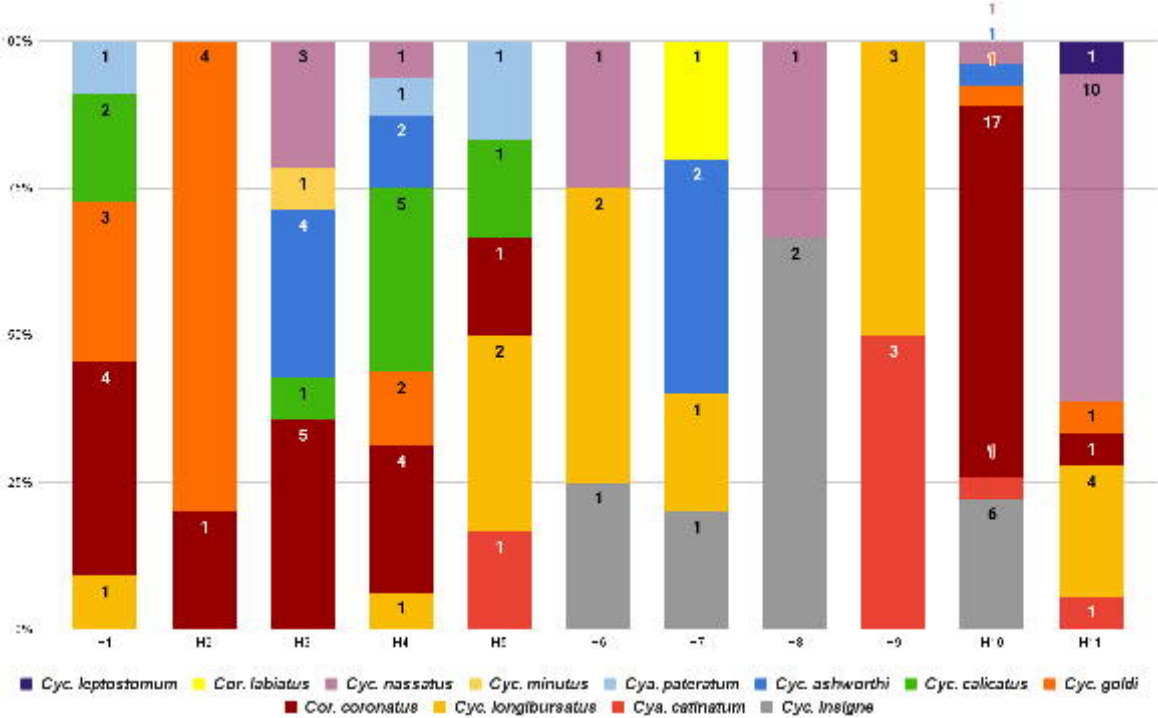
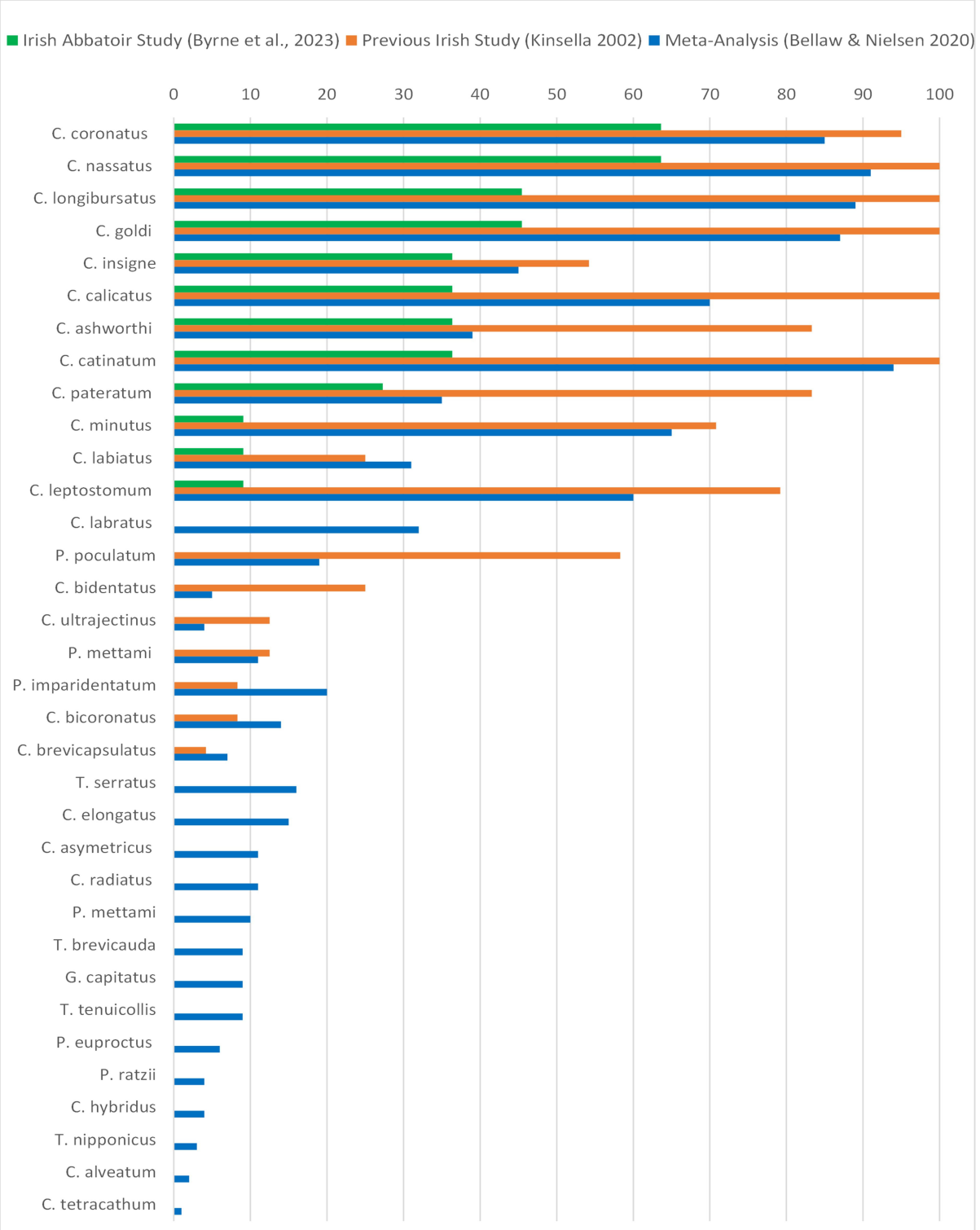
Legend
Cylicocyclus nassatus
Cyathostomum catinatum
Cylicostephanus goldi
Cylicostephanus calicatus
Cylicostephanus minutus
Coronocyclus coronatus
Cylicostephanus longibursatus
Cylicocyclus insigne
Cylicocyclus leptostomum
Cyathostomum pateratum
Cylicocyclus ashworthi
Coronocyclus labiatus


Cyc. leptostomum
Cor. labiatus
Cyc. nassatus
Cyc. minutus
Cya. paterratum
Cyc. ashworthi
Cyc. calicatus
Cyc. goldi
Cor. coronatus
Cyc. longibursatus
Cya. cattnatum
Cyc. insigne


Irish Abbatoir Study (Byrne et al., 2023)
Previous Irish Study (Kinsella 2002)
Meta-Analysis (Bellaw & Nielsen 2020)
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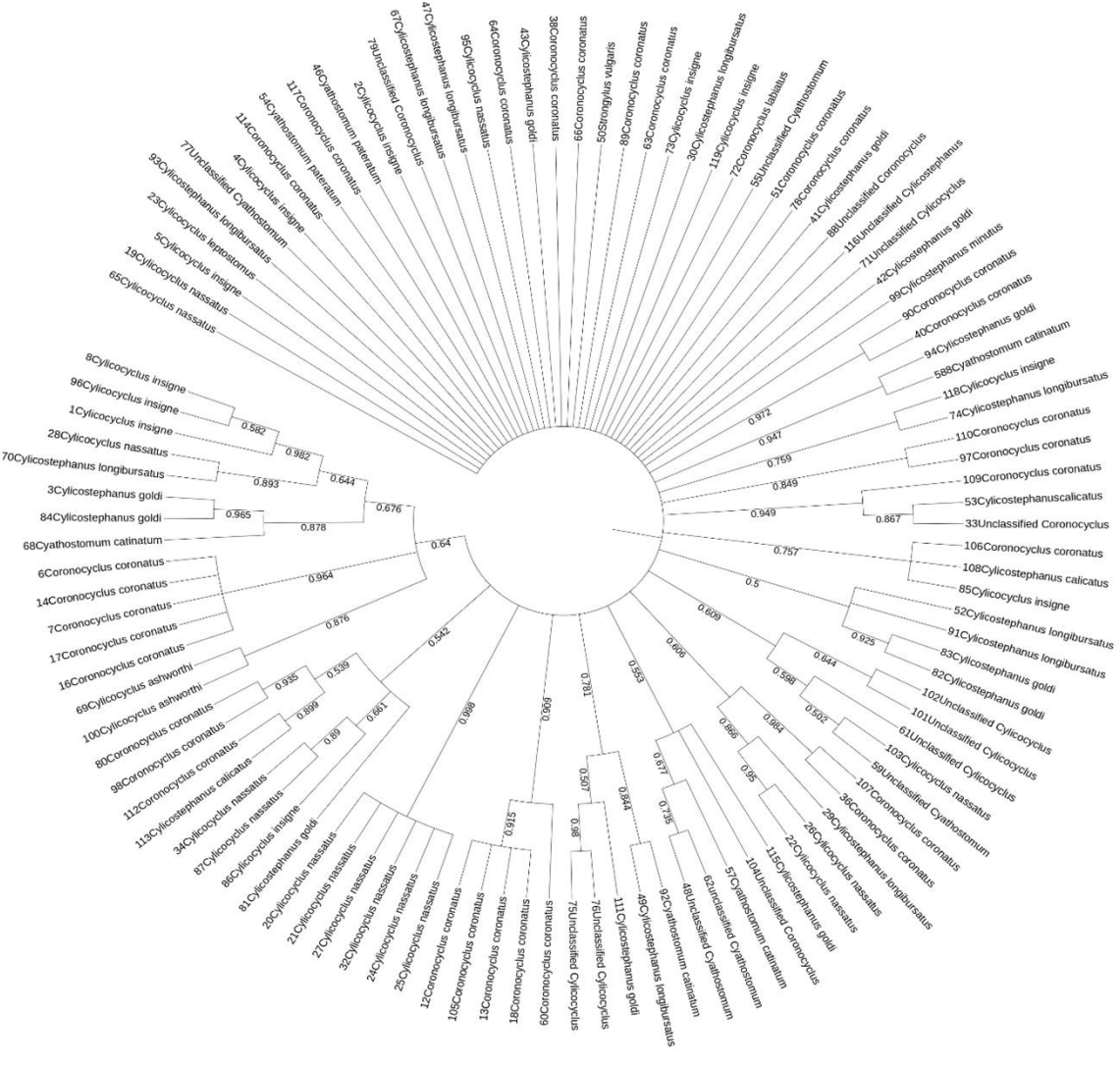
100
C. coronatus
C. nassatus
C. longibursatus
C. goldi
C. insigne
C. calicatus
C. ashworthi
C. catinatum
C. pateratum
C. minutus
C. labiatus
C. leptostomum
C. labratus
P. poculatum
C. bidentatus
C. ultrajectinus
P. mettami
P. imparidentatum
C. bicoronatus
C. brevicapsulatus
T. serratus
C. elongatus
C. asymetricus
C. radiatus
P. mettami
T. brevicauda
G. capitatus
T. tenuicollis
P. euproctus
P. ratzii
C. hybridus
T. nipponicus
C. alveatum
C. tetracathum



38Coronocyclus coronatus
66Coronocyclus coronatus
50Strongylus vulgaris
89Coronocyclus coronatus
63Coronocyclus coronatus
73Cylicocyclus insigne
30Cylicostephanus longibursatus
119Cylicocyclus insigne
72Coronocyclus labiatus
55Unclassified Cyathostomum
51Coronocyclus coronatus
78Coronocyclus coronatus
41Cylicostephanus goldi
88Unclassified Coronocyclus
116Unclassified Cylicostephanus
71Unclassified Cylicocyclus
42Cylicostephanus goldi
99Cylicostephanus minutus
90Coronocyclus coronatus
40Coronocyclus coronatus
94Cylicostephanus goldi
588Cyathostomum catinatum
118Cylicocyclus insigne
74Cylicostephanus longibursatus
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97Coronocyclus coronatus
109Coronocyclus coronatus
53Cylicostephanuscalicatus
33Unclassified Coronocyclus
106Coronocyclus coronatus
108Cylicostephanus calicatus
85Cylicocyclus insigne
52Cylicostephanus longibursatus
91Cylicostephanus longibursatus
83Cylicostephanus goldi
82Cylicostephanus goldi
102Unclassified Cylicocyclus
101Unclassified Cylicocyclus
61Unclassified Cylicocyclus
103Cylicocyclus nassatus
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107Coronocyclus coronatus
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29Cylicostephanus longibursatus
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22Cylicocyclus nassatus
115Cylicostephanus goldi
104Unclassified Coronocyclus
57Cyathostomum catinatum
62unclassified Cyathostomum
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92Cyathostomum catinatum
49Cylicostephanus longibursatus
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113Cylicostephanus calicatus
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80Coronocyclus coronatus
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7Coronocyclus coronatus
14Coronocyclus coronatus
6Coronocyclus coronatus
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3Cylicostephanus goldi
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79Unclassified Coronocyclus
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47Cylicostephanus longibursatus
95Cylicocyclus nassatus
64Coronocyclus coronatus
43Cylicostephanus goldi
0.972
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