Molecular Speciation of Cyathostomins From Horses In Ireland

Orla Byrne, Disha Gangotia, Annetta Zintl, Liam Kiser, Olivia Boxall, Daniel McSweeney, Fiona O'Neill, Stacey Dunne, Breanna Rose Lamb, Nicola Walshe, Grace Mulcahy.

UCD School of Veterinary Medicine and Conway Institute, University College Dublin, Dublin, Ireland.

Corresponding author: orla.byrne1@ucdconnect.ie;

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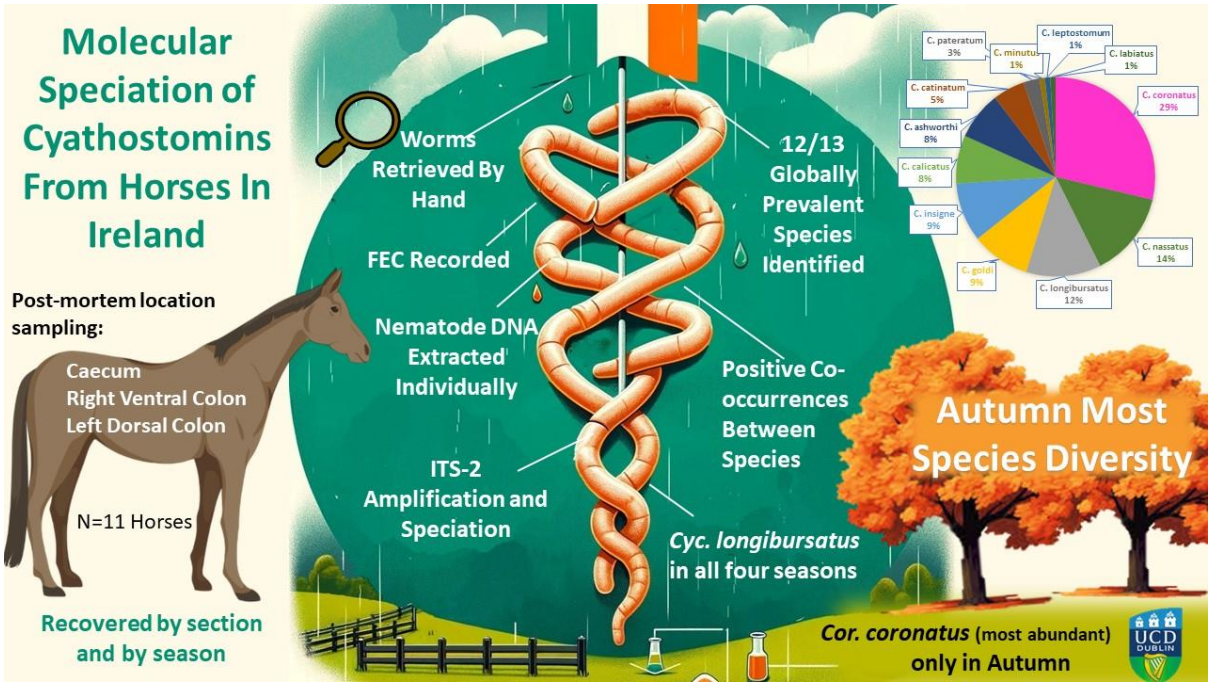
Graphical Abstract:



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 provides 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. Seasonal distribution, 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 research, in 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 studies in relation to the difficulties in discriminating between *Cylicostephanus calicatus* and *Cor. coronatus* on the basis of ITS-2 sequences. We found a positive correlation between the occurrence of *Cyathostomum pateratum* and *Cyc. calicatus* ($p < 0.01$). *Cor. coronatus*, the most abundant species, was only recovered from horses sampled in autumn. Although this study is limited in terms of sample size, it provides a platform for furthering understanding of the epidemiology and pathogenesis of equine cyathostomin infections in temperate climates.

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 hypobiotic larvae resuming development and completing their life-cycle are unclear, but have been hypothesised to involve cross-talk between adult and larval worms, along with interaction with the gut microbiota, the host immune system and environmental disruption such as anthelmintic treatment (Gibson, 1953; Love *et al*., 1999; Matthews, 2008; Walshe *et al*., 2019; Walshe *et al*., 2021; Walshe *et al*., 2020). The nature of communication between adult and larval cyathostomins, and the extent to which this can occur between species, is not well-understood. The existence of a large number of species that are typically present in mixed infections highlights the importance of understanding these dynamics, in efforts both to understand the basic biology of cyathostomin infections and the provision of improved control strategies (Bellaw and Nielsen, 2020; Hodgkinson *et al*., 2003).

Horses may tolerate light cyathostomin infection without the development of overt clinical signs (Love *et al*., 1999; Morariu *et al*., 2016; Murphy and Love, 1997; Walshe *et al*., 2021). However, high burdens can lead to ill-thrift and poor performance (Murphy and Love, 1997) and in some cases a specific clinical presentation of acute larval cyathostominosis (ALC), which develops in response to synchronous emergence of larvae from the mucosa and involves severe local and systemic inflammation, with high morbidity and potential mortality (Hodgkinson *et al*., 2003; Love *et al*., 1999; Walshe *et al*., 2020). The emergence of encysted cyathostomin larvae is believed to be affected by many factors, including negative inhibition from an established adult worm population, removed following anthelmintic treatment (Steinbach *et al*., 2006; Walshe *et al*., 2019; Walshe *et al*., 2021). Administration of anthelmintics has also been shown to have an adverse impact on the gut microbiota, either transiently, in normal animals (Walshe *et al*., 2019) or more severely in ALC, with severe typhlocolitis developing (Walshe *et al*., 2021). ALC has a 50% mortality rate recorded in referral centres (Giles *et al*., 1985; Peregrine *et al*., 2006; Walshe *et al*., 2021).

In the past, characterisation of cyathostomin communities relied exclusively on morphological identification of species (Kinsella, 2002; Lichtenfels *et al*., 2008). However, this is laborious, time-consuming and highly specialised (Avramenko *et al*., 2015; Hodgkinson *et al*., 2003; Lichtenfels *et al*., 2008), and it has largely been supplanted by molecular speciation, either of individual worms, or using population analysis “Nemabiome” approaches (Avramenko *et al*., 2015).

82 There are just two published studies on characterising cyathostomin species in horses in
83 Ireland. The first (Kinsella, 2002), used morphological speciation and the second (Elghryani,
84 2018) used reverse line blots of the intergenic spacer gene region. Elsewhere, internal
85 transcribed spacer 2 (ITS-2) primers as described by (Gasser *et al*., 1993), have been used for
86 molecular diagnostics of equine nematode infections, reviewed by (Ghafar *et al*., 2022).

87 We aimed to sequence the ITS-2 region from individual cyathostomins retrieved from
88 naturally infected horses in Ireland in a post-mortem study and to compare the community
89 composition to those described in previous studies in Ireland and internationally.

90

91 ## 2. Materials and Methods

92

93 *2.1. Ethical approval*

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

95

96 *2.2. Study population*

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

103 Samples were taken in September, October, February, March and June, with samples taken
104 in February designated as winter samples, March as spring, June as summer, and
105 September/October as autumn.

106

107 *2.3. Sample processing*

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

Further cable ties were placed at the junction between the caecum and right ventral colon, the junction between the right ventral and left ventral colon, the junction between the left ventral colon and left dorsal colon, and the junction between the left dorsal and right dorsal colon. An incision was made in each of the three intestinal segments (caecum, right ventral colon (RVC) and left dorsal colon (LDC)), and the luminal contents were emptied separately into individual 10L graduated buckets. For each section, the mucosa was gently rinsed, and the liquid was added to the bucket containing luminal contents. The contents were then stirred whilst water was added to make a homogeneous suspension to a volume of 10L. Two 100ml aliquots were taken from each section, each equating to 1% of the volume within each container. Faeces were collected from the rectum of each horse and placed in a ziplock bag. All samples were placed on ice for transfer to the laboratory.

2.4. Faecal Egg Counting

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

2.5. Luminal worm retrieval

Each duplicate 1% aliquot of the caecum, RVC and LDC was examined individually using a modification of the Baermann method. The contents of each aliquot were enclosed in a muslin parcel suspended in water in a conical flask and left to sediment for 24 hours. The entire sediment was then examined in detail (using aliquots forming a thin layer in a petri

137 dish) under a dissecting microscope. The remaining sediment was also examined under a
138 dissecting microscope.

139 The total number of worms present in each 100ml sample was enumerated and recorded.
140 Individual worms were removed manually and washed prior to storage. Each worm was
141 placed in a 2ml Eppendorf with >/=50ml PBS and stored at -20˚C. For each section a maximum
142 of 50 worms (range 1-50) was collected for subsequent examination from combined duplicate
143 samples

144

145 *2.6. DNA Extraction and amplification*

146

147 *2.6.1. Genomic DNA extraction from individual nematodes*

148 Individual worms were dissected into a minimum of three parts using a sterile needle. DNA
149 was then extracted from each worm using the Qiagen Powerfecal Pro extraction kit as per the
150 standard operating protocol, with minor adaptations. Specifically, samples were pulse
151 vortexed for a minimum of 45 minutes, the dry spin after solution C5 was at 16,600 x G for
152 three minutes, and solution C6 of the kit was put through the filter membrane twice to
153 increase elution yield (Bredtmann *et al*., 2019). After extraction, samples were stored at -20°C
154 until PCR amplification.

155

156 *2.6.2. PCR amplification*

157 PCR reactions were performed in 50µl reaction volumes using ITS-2 primers as described by
158 Gasser *et al*., (1993), (NC1 forward; 5'ACGTCTGGTTCAGGGTTGTT3' and NC2 reverse;
159 5'TTAGTTTCTTTTCCTCCGCT3').

160 The reaction mixture 10µl of Promega GoTaq Flexi Buffer, 1.5mM of Promega MgCL2 solution,
161 0.2mM Promega DNTP PCR nucleotide mix, 1µM of each forward and reverse primer (Eurofins
162 Genomics) 1.25U of Promega GoTaq G2 Flexi DNA Polymerase, 10µl template DNA and
163 15.75µl nuclease free water. Amplification steps included initial denaturation at 95°C for 2

164 minutes, then cycling steps starting at 95°C for 1 minute, annealing at 55°C for 1 minute and
165 extension at 72°C for 1 minute, for 40 cycles, followed by extension at 72°C for 5 minutes.

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

168 Amplicons were purified using the Qiagen Qiaquick PCR purification kit and sequenced in both
169 directions using the PCR primers (Eurofins Genomics).

170

171 *2.7. Molecular speciation*

172 Consensus sequences were constructed from forward and reverse reads and compared with
173 published nematode sequences in the NCBI database using Clustal Omega (Clustal Omega <
174 Multiple Sequence Alignment < EMBL-EBI).

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

177

178 *2.8. Data analysis*

179 Data was analysed using SPSS (Version 27) for descriptive statistics and to evaluate statistical
180 significance. Significance was determined using T-tests and ANOVA. The Kruskal-Wallis test
181 was utilised for non-parametric data including faecal egg counts (FEC), numbers of worms and
182 species distribution. Spearman rank correlation coefficient was calculated and created using
183 R functions, Excel and Inkscape (v.1.3, https://inkscape.org/release/inkscape-1.3/). The
184 ggplot2 package in R (version 4.3.1 - https://cran.r-project.org/bin/windows/base/old/),
185 Excel and Inkscape were used for data visualisation. MEGA11, IQtree web server
186 (http://iqtree.cibiv.univie.ac.at/) and iTOL (https://itol.embl.de/) were used for phylogenetic
187 tree creation. SPSS (V27) was used for Chi Squared, T-tests and ANOVA analyses. MEGA11
188 was used for MUSCLE alignment and trimming of sequences. A Maximum Likelihood tree was
189 created using MEGA, IQtree and iTOL to create a tree with 1,000 ultrafast bootstrap
190 replications and pairwise deletion for gaps/missing data. In order to construct a simplified
191 tree to compare multiple species, representative sequences were selected for each species.

192

193 ## 3. Results

194

195 The genetic data from this study has been uploaded to GenBank under the accession
196 numbers OR941129 - OR941242.

197

198 *3.1. Study cohort and parasitism levels*

199 Table 1 provides the details of horses included in the study, together with their strongyle FEC
200 and number of worms recovered in a 1% sample of the total luminal contents. The horses
201 included in the study ranged from yearlings to twenty-three years old with a mean of 7.7 years
202 of age, a median of 6 years. There were four females and seven males. Five were
203 thoroughbred, and six Irish Sport Horse. FEC ranged from 0-1,670 eggs per gram (epg).
204 Nematodes were retrieved from two horses with a FEC of 0. Conversely, high FEC did not
205 always correlate with high luminal burden of nematodes. Measurement of worm burdens
206 revealed a negative binomial distribution. Five horses were sampled in autumn and two
207 horses were sampled in spring, summer and winter, respectively.

208

209 *3.2. Species recovered, abundance and prevalence*

210 Table 2 shows the number of worms recovered in each intestinal compartment in this study,
211 and compared to those from a previous study on horses in Ireland (Kinsella, 2002). In both
212 studies, numbers recovered from the caecum were significantly lower than from the colonic
213 compartments (our study: P=0.009, Kinsella P=0.05). The LDC yielded the highest number of
214 worms in our study. There was no significant difference between the numbers recovered from
215 the RVC and LDC.

216 Table 3 shows the numbers of worms speciated from each intestinal compartment for each
217 horse. Up to 14 individual worms from each intestinal compartment, per horse, were
218 successfully speciated. For all but one horse, worms from two or three compartments were
219 speciated.

220 The species abundance in each of the three intestinal compartments is shown in Figure 1.
221 *Coronocyclus coronatus* was the most abundant species recovered (29%). Between them,
222 *Cor. coronatus*, *Cylicocyclus nassatus* and *Cylicostephanus longibursatus* accounted for more
223 than half (54%) of the entire dataset. Although there was no significant difference in species
224 distribution across the three intestinal compartments, *Cylicocyclus insigne* was equally
225 abundant to *Cor. coronatus* in the LDC. This compartment also had the most diverse range
226 of species (n=11), with the caecum having the least diversity (n=8).

227 The prevalence of individual species and the richness of cyathostomin communities in this
228 study population is illustrated in Figure 2. *Cor. coronatus* and *Cyc. longibursatus* were the
229 most prevalent, being found in seven of the eleven horses (63.63% prevalence), *Cyc. nassatus*
230 was present in six horses (54.54%) and *Cylicostephanus goldi* in five horses (45.45%). *Cyc.*
231 *insigne, Cylicostephanus calicatus, Cylicocyclus ashworthi* and *Cyathostomum catinatum*
232 were each recorded in four horses, at a prevalence of 36.36%. *Cyathostomum pateratum* was
233 found in three horses (27.27% prevalence) with *Cylicostephanus minutus, Coronocyclus.*
234 *labiatus* and *Cylicocyclus leptostomum* in one animal each, comprising 9.09% prevalence.

235 Species richness ranged from two to seven species per individual, with a mean of 4.27 +/-
236 0.604 (SEM). The modal species richness was two and five species equally, with a median
237 species richness of five within this dataset. Over 50% (54.5%) of the horses sampled had >5
238 cyathostomin species present. One large strongyle, *Strongylus vulgaris* was identified (Horse
239 5) but not included in the dataset.

240

241 *3.3. Community composition*

242 Figure 3 is a heatmap (Spearman correlation) of species co-occurrence. The analysis identified
243 significant positive correlation (0.86) between *Cya. pateratum* and *Cyc. calicatus* ($p < 0.01$). A
244 negative correlation was observed between *Cyc. insigne* and *Cyc. calicatus,* although this did
245 not reach significance ($p = 0.08$). *Cor. coronatus* showed trends towards positive co-
246 occurrences with *Cyc. nassatus* (0.46), *Cyc. goldi* (0.51), *Cyc. calicatus* (0.55), *Cyc. ashworthi*
247 (0.46), *Cya. pateratum* (0.33) and *Cyc. minutus* (0.41). *Cor. labiatus* had the most negative
248 correlations with seven negative interactions recorded to *Cyc. minutus* (-0.1), *Cya. pateratum*

(-0.19), *Cya. catinatum* (-0.23), *Cyc. calicatus* (-0.23), *Cyc. goldi* (-0.27), *Cyc.nassatus* (-0.28), and *Cyc. nassatus* (-0.36).

Next, (Figure 4) we compared the prevalence of cyathostomin species found in our study with that found in a previous study in Ireland (Kinsella, 2002) and a global meta-analysis (Bellaw and Nielsen, 2020). Twelve cyathostomin species were common to our study, Kinsella, 2002, and the global meta-analysis. There were no species identified in Ireland that were not identified in the latter. Broadly, the most prevalent species in the meta-analysis and in Kinsella, 2002, were also highly represented in our study. Prevalence as reported by Bellaw and Nielsen, 2020, was highest for *Cya. catinatum* (94%), *Cyc. nassatus* (91%), and *Cyc. longibursatus* (89%). However, we found *Cor. coronatus* and *Cyc. nassatus* to have the joint highest prevalence, occurring in 64% (n=7) of the horses sampled. Kinsella, 2002, recorded four species at 100% prevalence, namely, *Cya. catinatum, Cyc. nassatus, Cyc. calicatus* and *Cyc. longibursatus*. Of the 35 species recorded in the meta-analysis, our study identified 34% of species, while Kinsella, 2002, identified 54%. The ten most prevalent species in both Irish studies matched those outlined in the global meta-analysis, albeit with some differences in the ranking of the top ten species. The other Irish study (Elghryani, 2018) did not have sufficient prevalence data for comparative purposes. The most notable absence in our study from the top species in the meta-analysis is *Cor. labratus*, which was the 12th most prevalent species recorded globally but has not been identified in any of the three Irish studies mentioned here.

3.4. Seasonal variation

Figure 5 shows the seasonal variation in abundance recorded in our study. Notably, the most highly abundant species, *Cor. coronatus*, was recorded only during horses sampled during autumn. The greatest number of species (nine) were also identified in autumn samples. *Cyc. longibursatus* was the only species recorded in all four seasons. Seven of the species were observed in only one season.

278 *3.5. Phylogenetic analysis of species*

279 Sequences were used for construction of a Maximum Likelihood phylogenetic tree (Figure 6A)
280 which was rooted at the midpoint. This unveiled some interspecies occurrences within
281 branches, notably between *Cyc. calicatus* and *Cor. coronatus*.

282 Figure 6B shows a simplified phylogenetic tree, where consensus sequences for individual
283 cyathostomins sequenced at the ITS-2 region were used for phylogenetic analysis. *Cya.*
284 *pateratum* and *Cyc. goldi* branched together, as did *Cor. labiatus* and *Cyc. Insigne,* and *Cyc.*
285 *leptostomum* with *Cyc. Nassatus. Cyc. calicatus* and *Cor. coronatus* branch off together from
286 the root of the tree.

287

288 ## 4. Discussion

289 *4.1. Strongyle egg counts*

290 There was no direct relationship between strongyle faecal egg count and worm burden, which
291 was not unexpected (Chapman *et al*., 2003a; Chapman *et al*., 2003b; Nielsen *et al*., 2010) but
292 indicates the importance of understanding the potential differences between individual
293 cyathostomin species in terms of differences in biotic potential. We recovered worms from
294 horses with a 0 EPG count, which has been well-documented previously (Gibson, 1953; Love
295 *et al*., 1999; Matthews, 2008; Walshe *et al*., 2019; Walshe *et al*., 2020). As expected, we
296 observed a negative binomial distribution in the analysis of the egg shedding in our
297 population.

298

299 *4.2. Species abundance*

300 This study detected twelve of the thirteen most common species globally (Figure 4), as
301 compared to Bellaw and Nielsen's meta- analysis (Bellaw and Nielsen, 2020). Among the
302 nineteen species identified in the morphological study by Kinsella (2002), we detected twelve.
303 Notably, *Cya. Catinatum's* prevalence was 36% in our study, contrasting to 100% prevalence
304 in Kinsella's work and 94% in the global meta-analysis. Elghryani (2018), found *Cyc. nassatus*
305 as the most prevalent among the twelve species identified. The high abundance and
306 prevalence of *Cyc. nassatus* is consistent among all three Irish studies and the global meta-

307 analysis. Overall, the prevalence findings between the three studies from Ireland broadly
308 reflect one another (Figure 3). A notable observation was the absence of the twelfth most
309 prevalent species as identified by (Bellaw and Nielsen, 2020), *Cor. labratus,* which was absent
310 from the Irish studies. This species has been recorded in the UK and France (Cwiklinski *et al*.,
311 2012; Peachey *et al*., 2018).

312

313 *4.3. Phylogenetic analysis*

314 *Cyc. calicatus* and *Cor. coronatus* branch off together at the root of the tree 6B and are also
315 interdispersed in the ML tree (Figure 6A). This reflects their close relationship, as previously
316 identified, and the limitations of using the ITS-2 locus to differentiate the subsets of these
317 species (Courtot *et al*., 2023; Hung *et al*., 2000; Louro *et al*., 2021).

318

319 *4.4. Characterisation of cyathostomin communities, and correlation with other properties in*
320 *our study population.*

321

322 *4.4.1. Distribution across intestinal compartments*

323 Across the three intestinal compartments examined in this study, the number of worms
324 retrieved from the caecum was significantly lower than that recovered from LDC (P =0.009).
325 (Ogbourne, 1976), assessed abundance of cyathostomins postmortem and found 50% in the
326 ventral colon and 45% in the dorsal colon. The worm burden in the caecum represented only
327 5% of the overall cyathostomin burden. Similarly, (Stancampiano *et al*., 2010), observed that
328 the majority of small strongyles were concentrated in the ventral colon (67.34%) and less
329 frequently in the caecum (27.52%) and dorsal colon (5.14%). While this distribution was also
330 reflected by Kinsella (2002), the converse was found for encysted stages of cyathostomins, as
331 recorded by (Nielsen *et al*., 2021) who documented 40% of EL3/LL3/L4 in the caecum, and
332 38%/22% in the ventral and dorsal colon respectively. This recurrent recording of biological
333 preference of luminal cyathostomins for the colon over the caecum, and the inverse for
334 encysted larvae may reflect the slower transit time of the colon and the natural peristalsis of

335 food through the digestive tract, enabling passive transport and a longer exposure to the
336 intestinal wall necessary to facilitate larval encystment (Geor *et al*., 2013; Miyaji *et al*., 2008).

337

338 *4.4.2. Community composition*

339 Over half of the horses in this study had more than five cyathostomin species recovered from
340 them (Figure 2). We found a significant positive correlation between the occurrence of *Cyc.*
341 *nassatus* and *Cyc. insigne* (Figure 3), which supports similar findings by (Sallé *et al*., 2018).

342

343 *4.4.3. Seasonality*

344 Samples were taken from each quarter of the year to account for seasonal factors of
345 cyathostomin distribution. *Cyc. longibursatus* was consistently recovered in all four seasons.
346 The most abundant species, *Cor. coronatus*, was identified only in autumn, as was the case
347 for *Cyc. calicatus*, which share a close relationship at the ITS-2 region (Hung *et al*., 2000; Louro
348 *et al*., 2021). Increased abundance of *Cor. coronatus* in autumn was also noted in a recent
349 study in Saskatchewan which found a significantly higher proportional abundance of *Cor.*
350 *coronatus* in the autumn compared to the spring (Saworski, 2023). Our findings also reflect
351 those of Kinsella (2002), who found significantly more cyathostomin species recorded in
352 spring and autumn than in other seasons. A Louisiana based study also reflected these
353 seasonal findings, with autumn recording the greatest species diversity (Chapman *et al*.,
354 2003b). Variability of seasonal patterns among species is an understudied facet of
355 cyathostomin biology which may be crucial for comprehensive characterisation of
356 pathogenesis and epidemiology of cyathostomin-related disease, as this tends to have a
357 seasonal distribution of presentation (Reid *et al*., 1995).

358

359 ## 5. Limitations

360

361 The most important limitation of this study is sample size. This is a descriptive study, which
362 affects the potential for statistical power. Conclusions from observations within any study

363 with a limited sample size must take this into account. Results from Table 3 are considered in
364 the context that only two nematodes were retrieved for sampling from horse eight. Notably,
365 it is clear from this research and other studies that the ITS-2 locus cannot resolve all isolates
366 of *Cor. coronatus* and *Cyc. calicatus,* pointing to the need for a different locus for further
367 molecular work, such as COX1, or a multi-locus approach (Courtot *et al*., 2023; Ghafar *et al*.,
368 2022).

369

370 ## 6. Conclusions

371 This study has produced new insights into cyathostomin species occurrence, interactions,
372 community composition and seasonality in horses in Ireland, with comparisons to previous
373 studies in this locality a global meta-analysis.

374 It is likely that communication occurs not only between cyathostomin species and stages, but
375 also between the helminths, gut microbiota and the host, as observed within other systems
376 within the gastrointestinal tract (Gieryńska *et al*., 2022). Post-mortem analysis and
377 identification in naturally-infected hosts, as conducted here, will continue to have a role to
378 play in advancing understanding of cyathostomin biology and pathogenesis, in parallel with
379 population-based approaches. More extensive studies involving both population and post-
380 mortem studies are warranted in order to enhance risk assessment, biomarker-based
381 diagnostic tools and treatment protocols (Walshe *et al*., 2020). It is also useful in investigating
382 potentially cryptic species as seen in this study with *Cor. coronatus* and *Cyc. calicatus.*

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

386

387 ## Author contributions

388 Conceptualization, G.M., N.W., O.B.; writing- original draft preparation, O.B. – review and
389 editing, G.M., N.W., O.B. and A.Z.; laboratory work and bioinformatics- O.B., D.G.; student
390 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. 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		
Horse	**Age**	**Breed**	**Sex**	**Sampling Date**	**FEC (epg)**	**RVC**	**LDC**	**Caecum**
1	16	ISH	F	Sep-18	1550	79	188	10
2	8	ISH	M (gelding)	Sep-18	0	11	3	1
3	1	TB	M (colt)	Sep-18	1670	205	155	12
4	10	ISH	F	Oct-18	25	201	245	23
5	1	TB	M (colt)	Oct-18	905	590	33	16
6	6	ISH	M (colt)	Feb-20	225	2	26	0
7	2	TB	M (colt)	Feb-20	800	1	249	2
8	12	ISH	M	Mar-20	50	0	2	0
9	23	TB	F	Mar-20	85	9	24	1
10	3	TB	F	Jun-20	715	1410	3328	33
11	3	ISH	M	Jun-20	0	40	56	53
Total	-	-	-	-	-	2548	4309	151
Mean	7.7+/- 2.124	-	-	-	547.73 +/- 188.83	231.63 +/- 129.21	391 +/- 295*	13.73 +/- 5.1*
Range	1-23	-	-	-	0-1670	0-1410	2-3328	0-53

Table 2: Comparison of numbers of cyathostomins recovered from the caecum, RVC and LDC in our study and with a previous study in horses in Ireland from Kinsella (2002). In both studies, the number of worms recovered from the caecum was significantly lower, (our study: P=0.009, Kinsella P=0.05). The greatest number of worms was recovered from RVC in the 2002 study, whereas LDC yielded the greatest number overall in our study. Total worms in our study refers to 1% sample aliquot which was taken from each section of the gastrointestinal tract sampled. Kinsella retrieved 250ml aliquots from 15 sections of the Caecum, ventral colon and dorsal colon.

Region	Range This Study	Total Worms This Study	% Of Total	Total Worms Kinsella 2002	% Of Total Kinsella 2002
Caecum	0-53	151	2.1	511	6.9
RVC	0-1410	2548	36.3	4064	55.5
LDC	2-3328	4309	61.4	2744	37.4
Total Across Compartments	0-3328	7008	-	7319	-

Table 3: Numbers of worms speciated from each intestinal compartment for each horse. Mean numbers are +/- SEM). Up to 14 individual worms were speciated from each area, and in total, 116 individual worms were sequenced with sufficient data quality for speciation.

Horse	RVC	LDC	Caecum
1	2	9	-
2	5	-	-
3	8	2	4
4	3	13	-
5	3	-	4
6	1	4	-
7	-	4	1
8	-	2	-
9	3	3	-
10	8	5	14
11	4	5	9
Total	37	47	32
Mean	3.36 +/- 0.83	4.27 +/- 1.16	2.91 +/- 1.4
Range	0-8	0-13	0-14

Figures

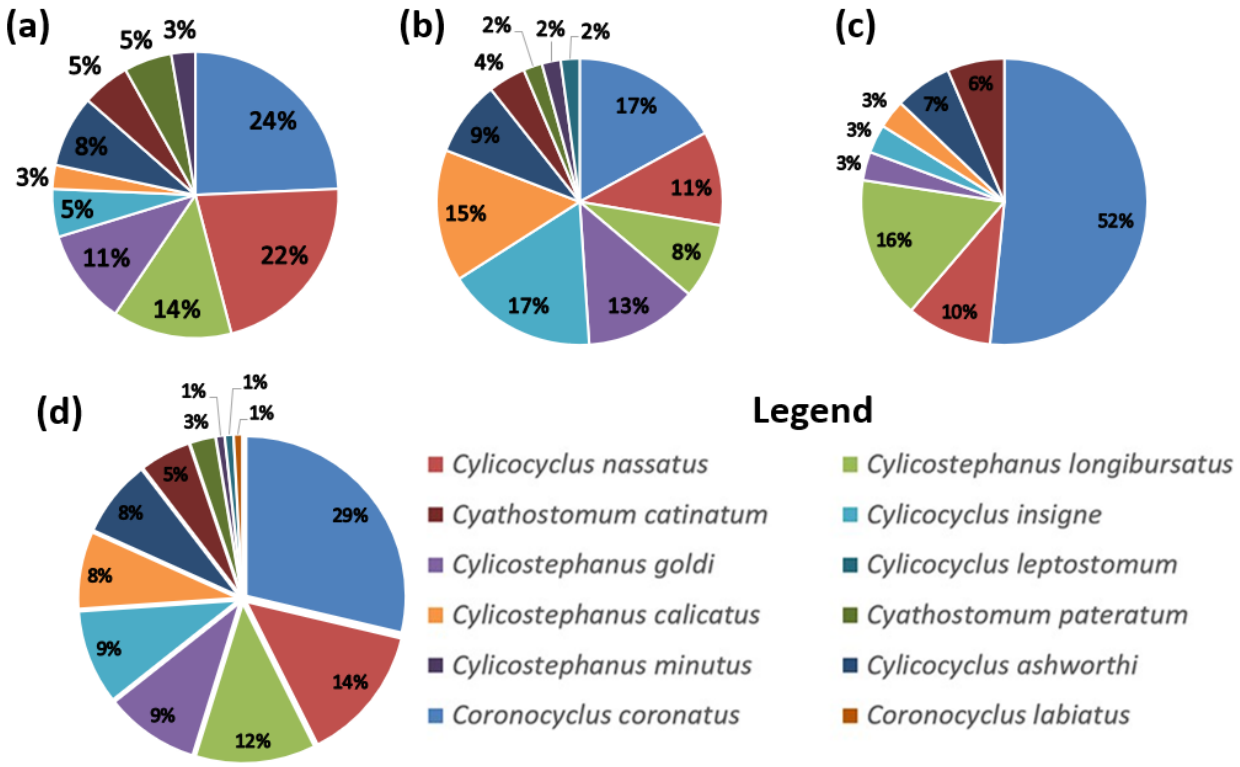


Figure 1: Species abundance data from samples obtained from Right Ventral Colon (a) Left Dorsal Colon (b), Caecum (c) and across all three compartments (d) . *Cor. coronatus* was the most abundant species recovered, at 29% of total nematodes speciated. Between them, *Cor. coronatus*, *Cyc. nassatus* 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).

Horse	*Coronocyclus coronatus*	*Cylicocyclus nassatus*	*Cylicostephanus longibursatus*	*Cylicostephanus goldi*	*Cylicocyclus insigne*	*Cylicostephanus calicatus*	*Cylicocyclus ashworthi*	*Cyathostomum catinatum*	*Cyathostomum pateratum*	*Cylicostephanus minutus*	*Strongylus vulgaris*	*Coronocyclus labiatus*	*Cylicocyclus leptostomum*
1	■		■	■		■			■				
2	■			■									
3	■	■				■	■			■			
4	■	■	■	■		■	■		■				
5	■		■			■		■	■		■		
6		■	■		■								
7			■		■		■					■	
8		■			■								
9			■					■					
10	■	■		■	■		■	■					
11	■	■	■	■				■					■

Figure 2: Prevalence of individual cyathostomin species and richness of cyathostomin communities in this study population. Filled sections depict species found in each horse. The mean species richness across samples was 4.27 +/- 0.604 (S.E.M). Richness ranged from 2-7 species per horse. There was a median of five species within this dataset. Over 50% (54.5%) of the horses sampled had >5 cyathostomin species. *Coronatus coronatus* and *Cylicostephanus longibursatus* were the most prevalent in 7 of 11 subjects (63.63% prevalence), *Cylicocyclus nassatus* was present in 6 horses (54.54%) and *Cylicostephanus goldi* was present in 5 horses (45.45%). The least prevalent were *Cylicostephanus minutus, Coronocyclus labiatus* and *Cylicocyclus leptostomum* were in one animal each, comprising 9.09% prevalence. One large strongyle, *Strongylus vulgaris* was identified but not included in the cyathostomin dataset.

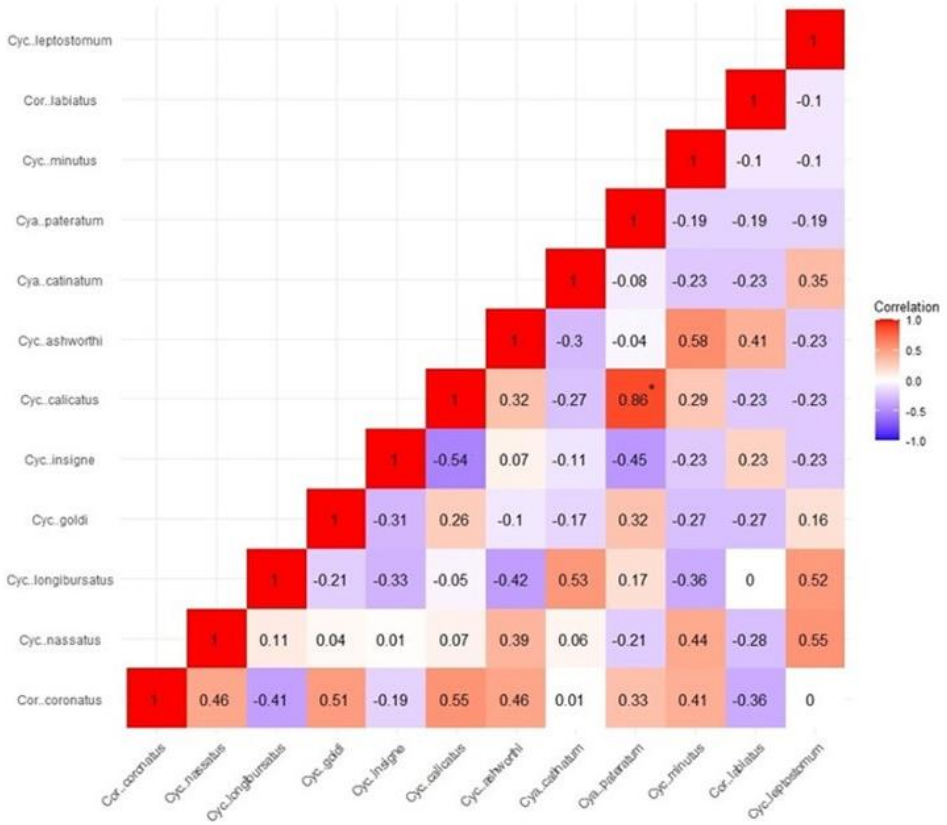
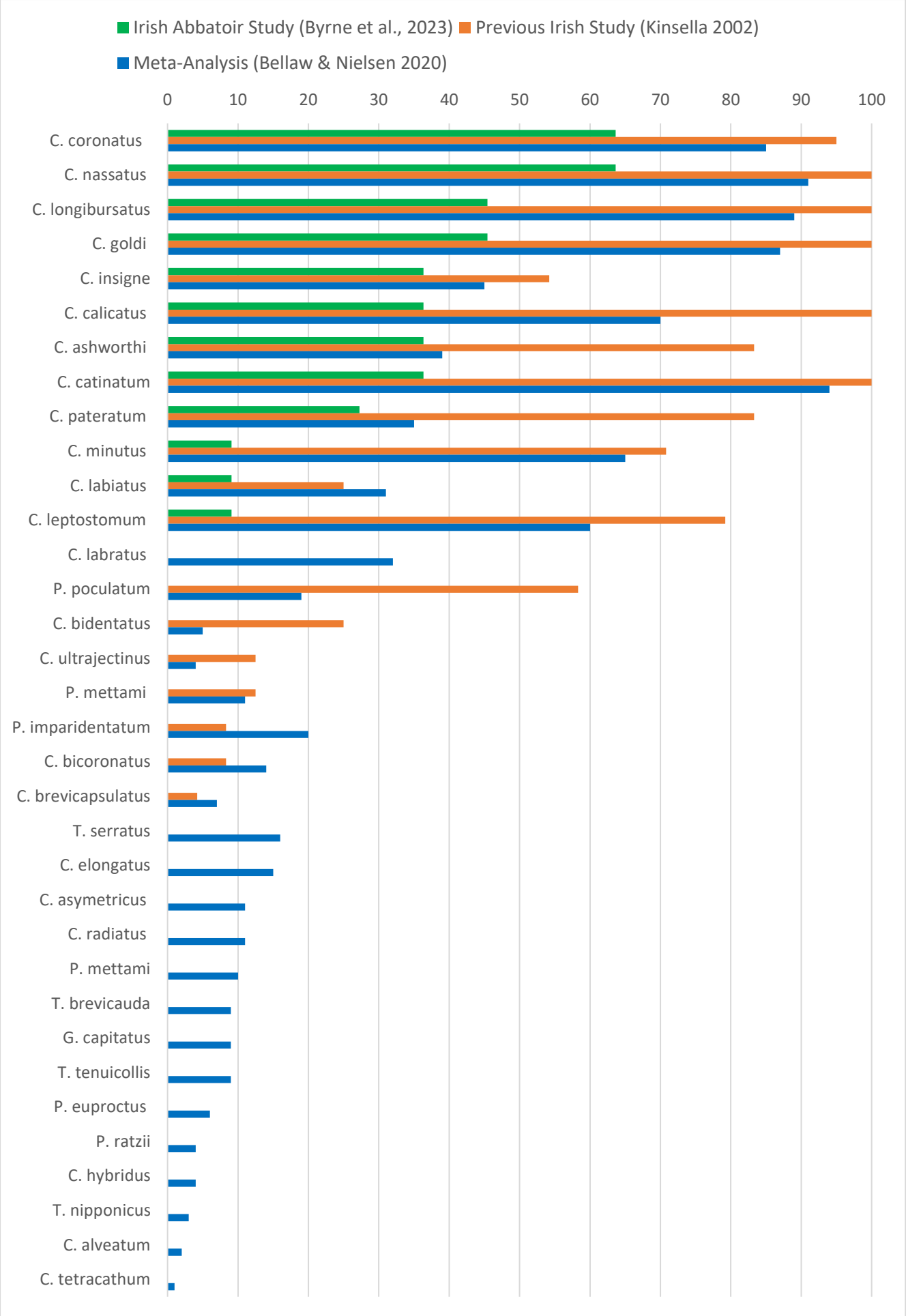


Figure 3: Heatmap of species co-occurrence using Spearmann correlations. This heatmap indicated a significant positive correlation (0.86) between *Cyathostomum pateratum* and *Cylicostephanus calicatus* ($p < 0.01$). A negative correlation was observed between *Cylicocylus insigne* and *Cylicostephanus calicatus,* although this did not reach significance ($p = 0.08$).


Irish Abbatoir Study (Byrne et al., 2023)
Previous Irish Study (Kinsella 2002)
Meta-Analysis (Bellaw & Nielsen 2020)
0
10
20
30
40
50
60
70
80
90
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

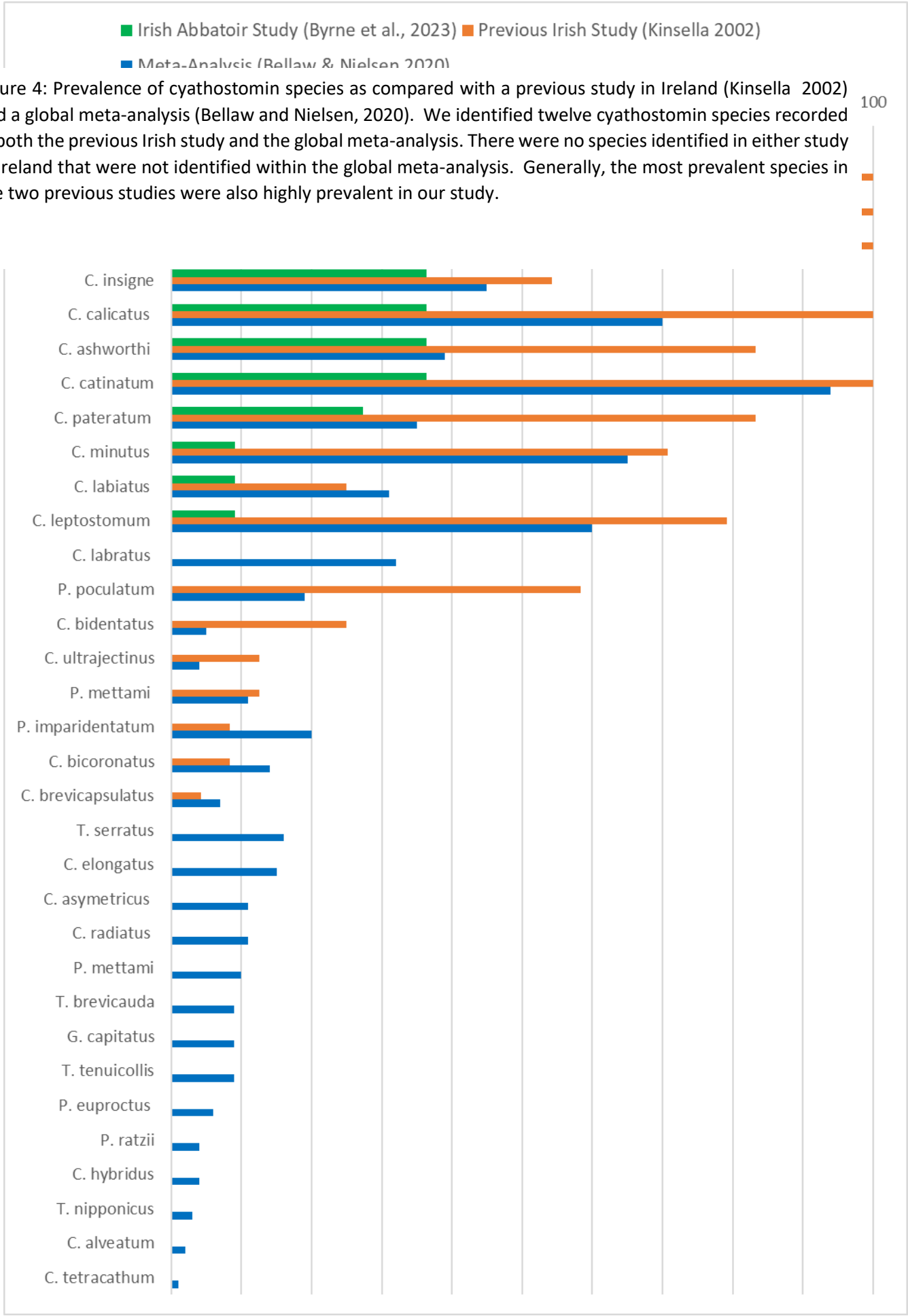


Figure 4: Prevalence of cyathostomin species as compared with a previous study in Ireland (Kinsella 2002) and a global meta-analysis (Bellaw and Nielsen, 2020). We identified twelve cyathostomin species recorded in both the previous Irish study and the global meta-analysis. There were no species identified 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.

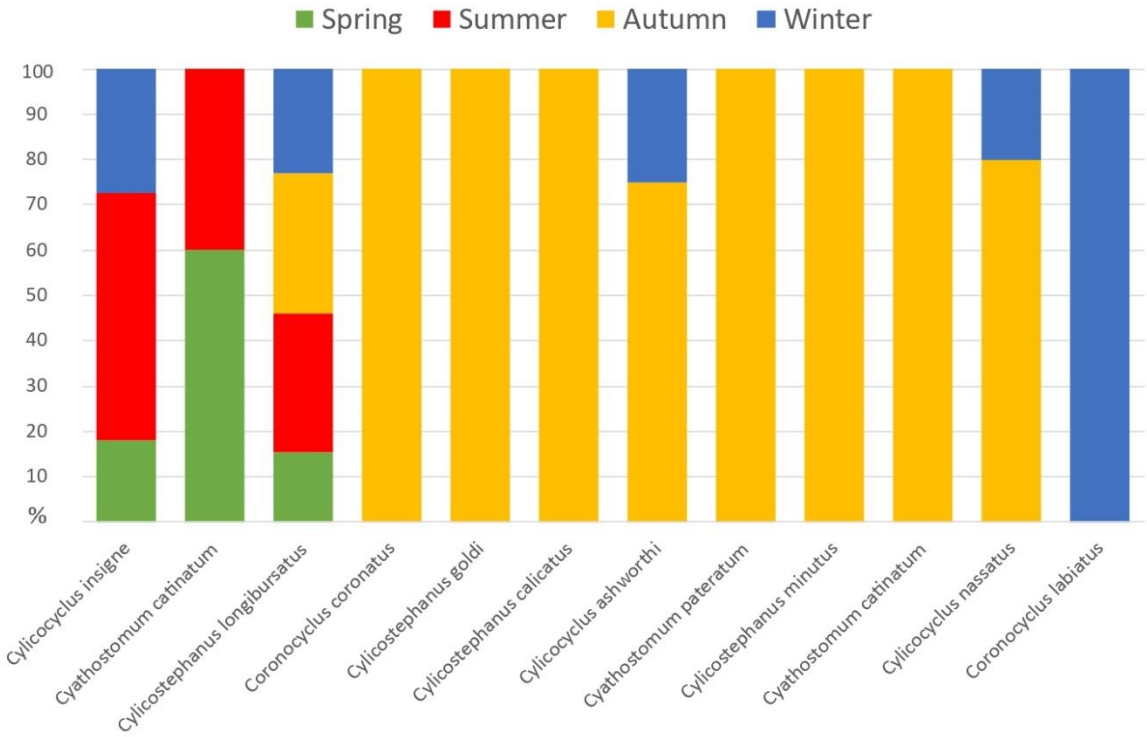


Figure 5: Seasonal variation observed in the recovery of cyathostomin species from the large intestine (n=116). Winter= Dec-Feb, Spring= Mar-May, Summer= June-Aug, Autumn= Sept-Nov. Sampling months include February, March, June, September and October. The greatest diversity of species (nine) was recovered in the Autumn. *Cylicostephanus. longibursatus* was the only species recovered in all four seasons. Seven species were observed in one season only.

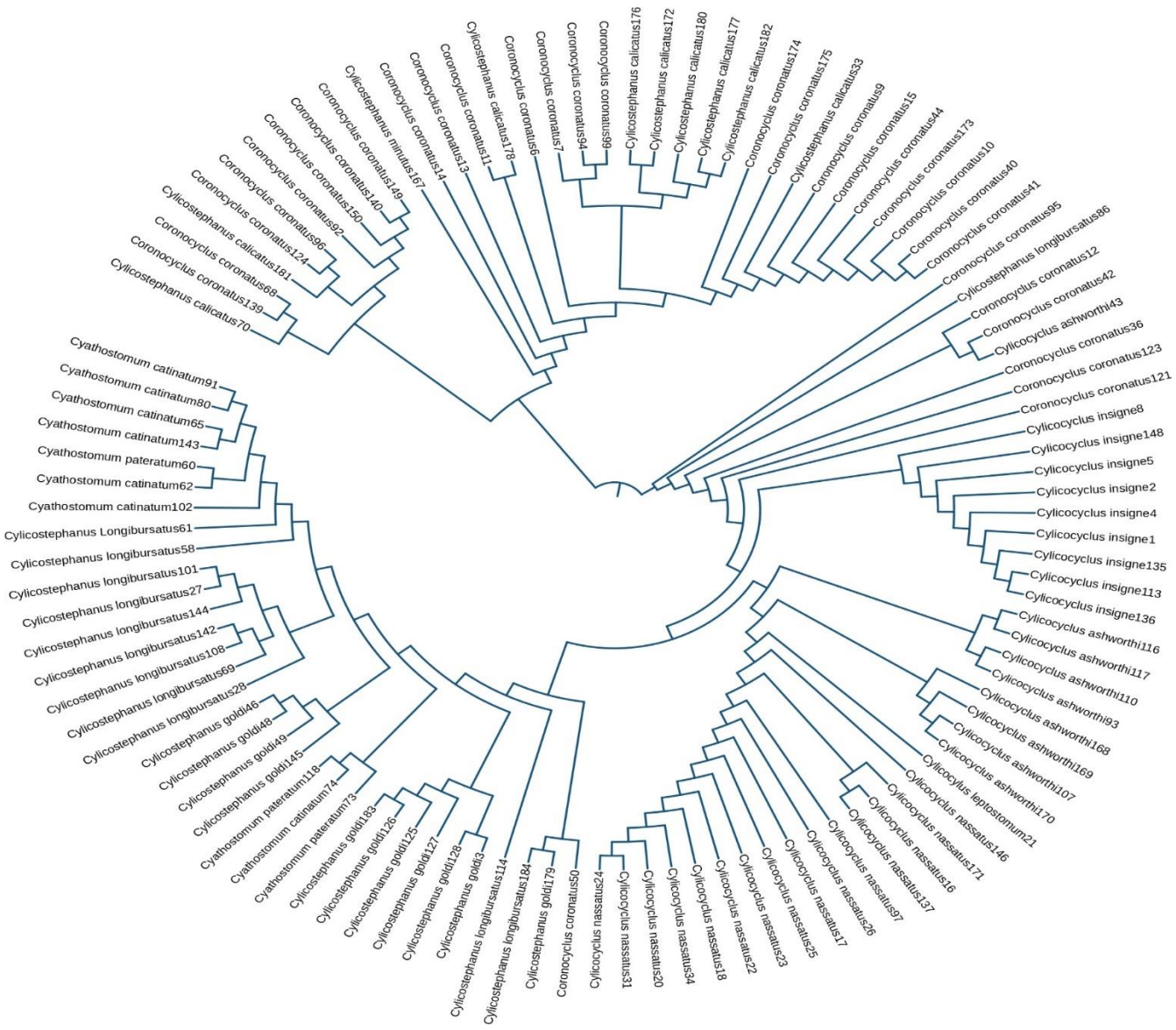


Figure 6A: Full phylogenetic tree of sequences from recovered worms. Consensus sequences for individual cyathostomins were used for phylogenetic analysis. MEGA11 was used for MUSCLE alignment and trimming of sequences. A tree was created using the Maximum likelihood algorithm with 1,000 bootstrap replications and pairwise deletion for gaps. This tree unveiled species that located within groupings of other species, notably *Cylicostephanus calicatus* and *Coronocyclus coronatus.*

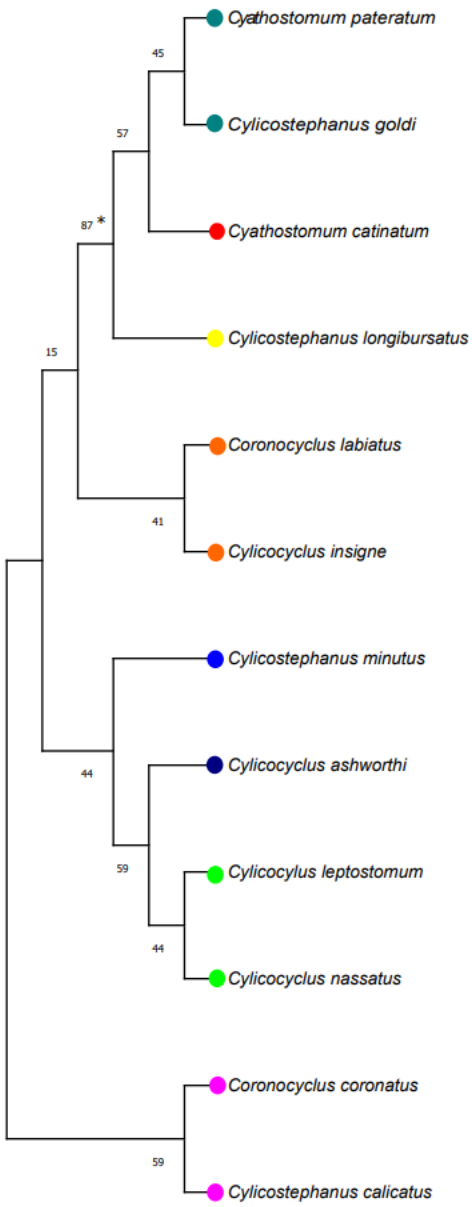


Figure 6B: A species-specific tree was created (5B) and a representative sequence was selected by creating ML trees of each species and selecting the midpoint sequence. *Cya. pateratum* and *Cyc. goldi* branched together, as did *Cor. labiatus* with *Cyc. insigne* and *Cyc. leptostomum* with *Cyc.nassatus. Cyc. calicatus* and *Cor. coronatus* diverge together at the root of the tree, reflecting their close relationship as identified by Louro (2021) and the discrepancy speciating the two using the ITS-2 locus. The asterix (*) denotes a bootstrapping value of more than 80%, indicating confidence that the species within this branch are truly more closely related to each other than to the species on the rest of the tree.