Characterisation of β-tubulin isotypes in *Uncinaria stenocephala* and implications for benzimidazole resistance in hookworms

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Abstract

Uncinaria stenocephala is a widespread hookworm of dogs across Europe, Canada, southern Australia, and other temperate regions, where it often outnumbers infections caused by *Ancylostoma caninum*. Although a putative β-tubulin isotype-1 mutation associated with resistance has been detected in *U. stenocephala*, clinical resistance to benzimidazoles has not yet been confirmed. Benzimidazole resistance is primarily linked to single nucleotide polymorphisms (SNPs) in the β-tubulin isotype-1 gene; however, the β-tubulin genes of *U. stenocephala* have not been fully characterised. We aimed to identify β-tubulin genes and confirm the coding sequences for key residues (Q134, F167, E198, and F200) in the β-tubulin isotype-1 gene of the *U. stenocephala* genome. Two *U. stenocephala* specimens were subjected to Illumina sequencing, and species identity was confirmed through morphological and molecular analysis using ITS rDNA and cox-1 markers. Genome assembly revealed the presence of β-tubulin isotype-1 (10 exons) and isotype-2 (9 exons), both homologous to β-tubulins from other hookworms (*A. caninum*, *A. ceylanicum*, *A. duodenale* and *Necator americanus*). The β-tubulin isotype-1 protein sequence of *U. stenocephala* contained two variable residues (S37Q and G441A) compared to other hookworm sequences. While the isotype-2 protein sequence was conserved among *Ancylostoma* species, *U. stenocephala* exhibited six distinct polymorphisms (E39D, T40S, N115S, L130I, A287S, T439G). The benzimidazole-susceptible residues (Q134, F167, E198, F200) were present in the β-tubulin isotype-1 protein sequence. Characterisation of the complete coding regions of β-tubulin isotypes 1 and 2 enables population-level screening for benzimidazole resistance-associated SNPs and provides a foundation for future epidemiological studies in *U. stenocephala*.

Keywords:

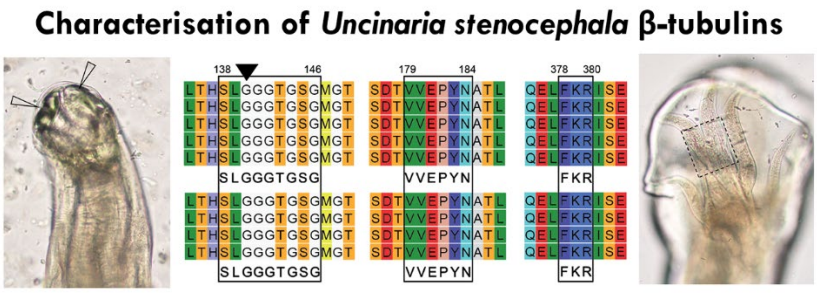
tubb-1, *tubb-2*, whole genome sequence, WGS, mutation, exon, Ancylostomatidate

Highlights

β-tubulin isotypes-1 and -2 were fully characterised in *Uncinaria stenocephala*

Key benzimidazole-susceptible residues were confirmed in β-tubulin isotype-1

β-tubulin isotypes-1 and -2 enables SNP screening and resistance surveillance efforts



1. Introduction

Hookworm nematodes (Ancylostomatidae) cause anaemia in humans, cats, and dogs through gastrointestinal haemorrhages. Until recently, however, anthelmintic resistance had not been a major concern (Geary et al., 2025). Benzimidazoles have been used effectively for over 50 years in companion animals to treat hookworm infections. Their mode of action involves disrupting the assembly of the nematode cytoskeleton and impairing metabolism, phenotypically visible through disrupted development. Clinical resistance to benzimidazoles was first documented in the canine hookworm (*Ancylostoma caninum*) using both *in vivo* (faecal egg count reduction test, FECRT) and *in vitro* (egg hatch assay, EHA) methods (Jimenez Castro et al., 2019; Kitchen et al., 2019). While FECRT and EHA are invaluable, they are inherently low-throughput. High-throughput amplicon sequencing has emerged as a powerful alternative for screening known single nucleotide polymorphisms (SNPs) in tubulin genes, which are critical components of the nematode cytoskeleton. Amplicon metabarcoding and sequencing of the β-tubulin isotype-1 (*tubb-1*) gene have revealed widespread mutations at amino acid positions 134 and 167, which confer benzimidazole resistance in *A. caninum* populations from dogs in North America and Australia (Abdullah et al., 2025; Venkatesan et al., 2023).

While mutations in the *tubb-1* gene have been strongly associated with benzimidazole resistance in hookworms, the broader genetic context of β-tubulin genes in nematodes warrants further investigation. Strongyle nematodes, including hookworms, possess four genes encoding β-tubulins (*tubb-1*, *tubb-2*, *tubb-3* and *tubb-4*), in contrast to the model organism *Caenorhabditis elegans*, which also contains the *ben-1* gene and is amenable to genetic studies (Collins et al., 2024; Saunders et al., 2013). Although non-synonymous variants at codons 134, 167, 198 and 200 of the *tubb-1* gene have been associated with benzimidazole resistance, *tubb-1* may not be the sole gene involved (Collins et al., 2024). The causal role of these codon changes was demonstrated through mutations in the *ben-1* gene of *C. elegans*, which confer

resistance to benzimidazoles (Dilks et al., 2020; Dilks et al., 2021; Kitchen et al., 2019; Venkatesan et al., 2023). In *C. elegans*, *ben-1* confers the highest level of resistance, and the loss of a second β-tubulin gene does not alter this resistance level (Collins et al., 2024). In nematodes lacking *ben-1*, resistance is conferred by other β-tubulin genes, with *tubb-1* playing a major, though not exclusive, role. Therefore, it is important to define the full complement of β-tubulin genes, particularly *tubb-1* and *tubb-2*, which are ubiquitously expressed in nematode cells, and to screen for mutations that may either confer or contribute to benzimidazole resistance (Collins et al., 2024; Gandasegui et al., 2024; Saunders et al., 2013; Venkatesan et al., 2023). Currently, no additional β-tubulin isotypes (*tubb-3* and *tubb-4*) have been identified in hookworms.

Hookworm nematodes are among the common intestinal parasites of companion animals, particularly dogs and cats, alongside roundworms and whipworms (Lightner et al., 1978). In these hosts, the primary pathogenic hookworms are *A. caninum* in dogs and *Ancylostoma tubaeforme* in cats (Miller, 1968). However, in cooler temperate regions, *Uncinaria stenocephala*, traditionally considered less pathogenic, predominates. Unlike the blood-feeding *Ancylostoma* species that cause anaemia, *U. stenocephala* infections are associated with protein-losing enteropathy (Walker and Jacobs, 1985). Recognised as the 'northern hookworm', *U. stenocephala* is widely distributed across Europe, Canada, southern Australia, and New Zealand, where it often outnumbers *Ancylostoma* spp. infections (Beveridge, 2002; Gibbs, 1961; Xhaxhiu et al., 2011).

Given the recent evidence of benzimidazole resistant-associated mutation in *U. stenocephala*, a deeper molecular characterisation of its β-tubulin genes is needed. Building on molecular tools developed for *A. caninum*, Stocker et al. (2023) demonstrated that a *tubb-1*-targeting assay could also amplify *U. stenocephala tubb-1*, making it suitable for screening potential genetic markers of benzimidazole resistance in this species. A study from Australia reported the presence of the canonical F167Y benzimidazole resistance mutation in *U.*

98 *stenocephala tubb-1* (Abdullah et al., 2025). Although clinical treatment failure with
99 benzimidazoles in *U. stenocephala* has not yet been documented, proactive molecular
100 surveillance is essential. To anticipate emerging resistance, comprehensive characterisation of
101 β-tubulin genes, particularly full-length *tubb-1*, across hookworm species is critical for enabling
102 comparative analyses and early detection of resistance-associated mutations.

103 The aim of this study was to characterise the *tubb-1* and *tubb-2* genes of *U.*
104 *stenocephala* and to identify key amino acid residues potentially associated with benzimidazole
105 resistance. To achieve this, we isolated adult *U. stenocephala* specimens and performed
106 whole-genome sequencing. Using a comparative approach with available hookworm genomic
107 data from *A. caninum*, *Ancylostoma ceylanicum*, *Ancylostoma duodenale*, and *Necator*
108 *americanus*, we identified and characterised the coding sequences of *tubb-1* and *tubb-2*. To
109 facilitate high-throughput screening of benzimidazole-susceptible residues in TUBB-1 (Q134,
110 F167, E198, and F200), we reviewed existing primer sequences and in silico assessed their
111 suitability for *U. stenocephala* and other hookworm species.

112

113 **2. Materials and Methods**

114 **2.1. Adult *Uncinaria stenocephala* material**

115 To obtain *Uncinaria stenocephala*, we decided to consider the invasive red fox (*Vulpes*
116 *vulpes*) a non-native species with significant ecological impact in Australia and is regularly
117 culled (Stobo-Wilson et al., 2021). Red foxes are commonly infected by *U. stenocephala* in
118 Australia (Ryan, 1976). Single red fox intestinal tract was donated by a registered shooter in
119 September 2024 (Mardi; 33.2990° S, 151.4084° E); the fox was culled under an ongoing fox
120 control program in Central Coast region of New South Wales, Australia. The gastrointestinal
121 tract was transported on ice to the University of Sydney, stored for 10 days at -80°C prior to
122 dissection. During dissection the gastrointestinal track was thawed, incised longitudinally and
123 content scraped through the researcher's thumb and index finger. The scrapings and small

124 intestinal contents washed with lukewarm tap water into a 150 µm nylon mesh sieve by means
125 of water jet. The washed content was examined in a white metal tray with the aid of
126 stereomicroscope. Nematodes were manually picked against the white tray, washed in
127 phosphate buffered saline (pH=8.0) and stored at -80°C. Hookworms, such as *U. stenocephala*,
128 were identified in accordance with the description of Ransom (1924).

129 **2.2. Genomic DNA extraction and molecular identification**

130 Genomic DNA (gDNA) was isolated using the Monarch® Spin gDNA Extraction Kit (New
131 England Biolabs, Australia). The gDNA was stored at -20°C. The isolated DNA was subject to two
132 separate PCR assays. The PCR amplifying ITS rDNA used NC16 [S0863] (5'- AGT TCA ATC GCA
133 ATG GCT T -3') and NC2 [S0864] (5'- TTA GTT TCT TTT CCT CCG CT -3') (Chilton et al., 2003) The
134 partial *cox*1 was amplified with JB3 [S0361] (5'- TTT TTT GGG CAT CCT GAG GTT TA -3') and JB4.5
135 [S0362] (5'- TAA AGA AAG AAC ATA ATG AAA ATG -3') (Bowles et al., 1992). Amplicons of
136 expected size (~800 bp, ITS rDNA; ~450 bp, *cox*1) were submitted for Sanger sequencing
137 (Macrogen, Korea). Sequences were assembled and compared to available sequences in CLC
138 Main Workbench 25.0.1 (CLC bio, Qiagen, Australia). As a reference of *U. stenocephala* ITS
139 rDNA sequence we used (Nadler et al., 2000) and *cox*1 we used AJ407939 (Hu et al., 2002).

140 **2.3. Whole genome sequencing and assembly**

141 The gDNA obtained from two single *U. stenocephala* was aliquoted (30 µL) into a 1.5 mL
142 Eppendorf tube and submitted to Novogene (HK) Co., Ltd for indexing, library preparation and
143 whole genome sequencing to generate approximately 30 Gb of sequencing data per sample.
144 Sequencing was performed using the Illumina NovaSeq 6000 sequencing platform using 150 bp
145 paired-end sequencing chemistry. Raw FastQ files (JS6871 and JS6872) generated from the
146 same sample were processed in Galaxy Australia (https://usegalaxy.org.au/) (Galaxy
147 Community, 2024). Raw paired-end sequencing reads were examined for quality using *FastQC*
148 (version 0.12.1) (Andrews, 2010) and trimmed using *Trimmomatic* (version 0.36.6) (Bolger et al.,
149 2014) (parameters: "ILLUMINACLIP:TruSeq3-PE.fa:2:30:10 SLIDINGWINDOW:4:20"). Read were

150 mapped using *bwa-mem2* (version 2.2.1) (Li and Durbin, 2009) using simple Illumina mode onto
151 red fox genome (VulVul3) and all mapper reads were removed. The FastQ files were assembled
152 using *SPAdes* (version 4.2.0) (Prjibelski et al., 2020) (parameters: "--cov-cutoff 40.0 -k 77").
153 Contigs were classified using *Kraken* (version 1.1.1) (database: minikraken) (Wood and
154 Salzberg, 2014) and only unclassified contigs were retained. Assembly statistics were
155 generated using *Fasta Statistics* (Seemann and Gladman, 2012).

156 **2.4. Identification of β-tubulin genes in the genome assemblies**

157 The *tubb-1* and *tubb-2* genes and their corresponding proteins were retrieved from
158 hookworm genomes at 'WormBase ParaSite' (Howe et al., 2016; Howe et al., 2017). The genes
159 were identified in *A. caninum, A. ceylanicum, A. duodenale* and *Ne. americanus* with and
160 addition of an outgroup (*Ni. braziliensis*) (International Helminth Genomes, 2019; Schwarz et
161 al., 2015). The reference sequences for *tubb-1* (ANCCAN_08153, Acey_s0035.v2.g13228,
162 ANCDUO_05511, Necator_chrII.pre1.g8352 and NBR_0000799801) and *tubb-2*
163 (ANCCAN_23405, Acey_s0432.v2.g1106, Necator_chrII.pre1.g8352 and Nippo_chrII.g5566)
164 were aligned and genes structure identified. There was no *tubb-2* for *A. duodenale* localised due
165 to partial coverage of the genome.

166 Local BLAST databases from the assembled contigs of *U. stenocephala* were created and
167 queried with the reference *tubb-1* and *tubb-2* genes sequences. Candidate contigs were aligned
168 to the reference alignments and exon-intron boundaries identified. Alignments and local BLAST
169 searches were performed in CLC Main Workbench v 25.0.1 (CLC bio, Qiagen, Australia).

170 Sequence reads were mapped to the putative *tubb-1* and *tubb-2* using *bwa-*
171 *mem2* (version 2.2.1) (Li and Durbin, 2009) using simple Illumina mode. *Samtools mpileup*
172 (Version 1.15.1) (Danecek et al., 2021) was used to input variants into *VarScan* (Version 2.4.2)
173 (Koboldt et al., 2012) (parameters: --min-coverage 20 --min-reads2 2 --min-avg-qual 15 --min-
174 var-freq 0.2 --min-freq-for-hom 0.75 --p-value 0.05). The resulting BAM and VCF files were
175 visualised using *IGV* (version 2.18.1). VCF file was processed to select only SNP within the

exons of *tubb-1* and *tubb-2*. The variant detection was performed in Galaxy Australia (https://usegalaxy.org.au/) (Galaxy Community, 2024).

To evaluate the binding of the primers targeting *A. caninum tubb-1* (ACB1_167_F: 5'-GGY GCA GGA AAC AAC TG -3', ACB1_167_R: 5'- CTT TGG TGA GGG GAC AAC A -3', ACB1_200_F: 5'-GTR GTG GAG CCA TAC AAT GC -3' and ACB1_200_R: 5'- GGC ATG AAG AAG TGA AGA CGT -3') we first mapped them onto the reference *A. caninum tubb-1* and then in an multiple sequence alignment that included the *U. stenocephala tubb-1* we evaluated the number of mismatches across the primer binding region. The primers were originally development for amplicon metabarcoding to consider the frequency of the SNPs that code for the nonsynomynous mutations at 134, 167, 198 and 200 amino acid residue of *A. caninum tubb-1* responsible for benzimidazole resistance (Jimenez Castro et al., 2019; Venkatesan et al., 2023).

2.5. Data availability

The *U. stenocephala* sequences were submitted to GenBank under the following accession numbers: PV848420 and PV848421 (ITS rDNA), PV848422 and PV848423 (*cox*1), PV133906 (*tubb-1*) and PV862047 (*tubb-2*). Raw FastQ sequence data were deposited at SRA NCBI BioProject: PRJNA1281746 (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1281746). The additional data are available at LabArchives (https://dx.doi.org/10.25833/ffcf-2175).

3. Results

3.1. Morphological and molecular confirmation of *Uncinaria stenocephala*

Four hookworms (5-7 mm) were extracted from the small intestinal contents of a red fox (*V. vulpes*, TSF_1). The hookworms (2 males, 2 females) were consistent with the morphology of *U. stenocephala* (**Figure 1**; Ransom (1924))

Two *U. stenocephala* (TSF_1.1, TSF_1.2) were molecularly characterised at ITS rDNA and *cox*1 gene. Sequenced ITS rDNA (818 nt) were identical to each other and to the reference

202 sequence of *U. stenocephala* (AF194145). Comparison of the new *U. stenocephala* ITS rDNA
203 with *U. stenocephala* ITS2 rDNA data from amplicon metabarcoding data from dog faeces
204 (Abdullah et al., 2025) revealed 95.8% to 100% match across 260 nt. The sequenced partial
205 *cox*1 (393 nt) sequence from the two *U. stenocephala* differed by a single synonymous mutation
206 (99.8% identity), and they were 99.2-99.5% identical to the reference *U. stenocephala* sequence
207 (AJ407939).

208 **3.2 Sequencing and assembly of *Uncinaria stenocephala* genome**

209 Sequencing of TSF_1.1 (JS6871) and TSF_1.2 (JS6872) generated 117,718,741 and
210 102,660,825 paired-end reads (150 bp), respectively, with average GC contents of 46% and
211 45%. This yielded a total of 35.2 Gb for TSF_1.1 and 30.6 Gb for TSF_1.2. After quality trimming,
212 117,358,176 (99.7%) and 102,353,896 (99.7%) high-quality paired-end reads were retained.
213 There were 4% and 23% sequence reads that mapped to the red fox (VulVul3) genome that were
214 removed from TSF_1.1 and TSF_1.2, respectively. The final datasets comprised 31.0 Gb for
215 TSF_1.1 and 21.6 Gb for TSF_1.2, both with a GC content of 45-46%. Each sample was
216 assembled independently. The TSF_1.1 assembly had N50 of 2,203 bp and L50 of 32,460
217 contigs. We then removed potential bacterial or viral contaminant contigs (TSF_1.1: 0.2% of the
218 contigs) to produce final assembly contigs (TSF_1.1: N50 of 2,090 bp and L50 of 32,766 contigs)
219 (**Table 1**). The TSF_1.2 assembly had N50 of 8,683 bp and L50 of 2,153 contigs, following
220 decontamination it had N50 of 8,431 bp and L50 of 2,065 contigs (**Table 1**).

221 The TSF_1.1 assembly yielded a contig (NODE_5499; length 7,645 nt, coverage 19.8) that
222 contained the complete *tubb-1* gene using blast searches with other *tubb-1* from hookworms as
223 a query. The *tubb-2* was represented by three contigs, one contig (NODE_15465; length 4,291
224 nt, coverage 30.3) included all but the first exon and was truncated at the first intron. The first
225 exon with partial first intron was found on two contigs, where the first exon was 100% identical
226 between the contigs (NODE_82476; length 558 nt, coverage 29.6; NODE_38650; length 1,780 nt,
227 coverage 24.1). The *tubb-2* complete coding sequence was fused from the contigs, the

228 nucleotide sequence is truncated in the first exon. There were no contigs that would contain the
229 complete putative *tubb-1* and *tubb-2* in the TSF_1.1 assembly.

230 **3.3 Characterisation of *tubb-1* and *tubb-2* of *Uncinaria stenocephala***

231 The putative *tubb-1* and *tubb-2* of *Uncinaria stenocephala* genes were used to reference
232 map the available sequence reads. A total of 1,774 and 1,256 reads were successfully mapped
233 to the *tubb-1*, resulting in an average coverage of 59.7 and 41.6 bases per position across the
234 covered regions from TSF_1.1 and TSF_1.2, respectively. Similarly, a total of 2,335 and 1,610
235 reads were successfully mapped to the *tubb-2*, resulting in an average coverage of 68.7 and
236 47.2 bases per position across the covered regions from TSF_1.1 and TSF_1.2, respectively.

237 The *U. stenocephala tubb-1* gene was 4,011 nt long including 10 exons homologous to
238 other *btub-1* form hookworms (**Figure 1A**). The largest intron between the exon 4 and 5 was
239 1,688 nt, shorter compared to the intron from *A. caninum tubb-1* (46 nt: ANCCAN_08153 and
240 1217 nt: DQ459314). The *U. stenocephala btub-2* gene was 4649 nt long and had 9 exons
241 homologous to those of other hookworms (**Figure 1B**). VarScan has detected 9 independent
242 heterozygous nucleotide position in TSF_1.1 and TSF_1.2 within *tubb-1* of *U. stenocephala*.
243 Within *tubb-2* of *U. stenocephala* there were 48 and 32 heterozygous positions in TSF_1.1 and
244 TSF_1.2, respectively.

245 The coding protein sequence of *U. stenocephala* TUBB-1 had two amino acid changes
246 when compared to other hookworms (S37Q and G441A), the remaining animo acid sequence
247 was identical with other hookworms. The TUBB-2 protein of *U. stenocephala* had five amino
248 acid changes compared to the TUBB-2 from *Ancylostoma* species: E39D, T40S, N115S, L130I
249 and A287S. In addition, there is a single change shared with *N. americanus* TUBB-2 (T439G).
250 Both TUBB-1 and TUBB-2 proteins included tubulin and β-tubulins protein motifs:
251 VPRAVLVDLEP, SLGGGTGSG, VVEPYN and the β-tubulin specific motif FKR (**Figure 1C**).

252 We then compared the primers designed by (Jimenez Castro et al., 2019) for the
253 amplification of the canonical SNPs in *tubb-1* of *A. caninum* with that of *U. stenocephala* (**Figure**

254 **2A**). The ACB1_167_F primer sequence was a perfect match, while ACB1_167_R had single,
255 G>A, mismatch in the middle of the primer sequence compared to the *U. stenocephala tubb-1*.
256 Both ACB1_200_F and ACB1_200_R had single mismatch each compared to the *U.*
257 *stenocephala tubb-1*. The mismatch in the ACB1_200_R is at the 3' terminal nucleotide position,
258 T>C.
259

260 **4. Discussion**

261 We successfully characterised the coding sequences of two representatives of the β-
262 tubulin gene family in *U. stenocephala*. We show, that both the *tubb-1* and *tubb-2* genes are
263 highly conserved among hookworms and retain an identical exon–intron structure. Access to
264 the complete *U. stenocephala tubb-1* sequence enabled us to confirm the presence of key
265 residues (Q134, F167, E198, and F200) that are associated with benzimidazole susceptibility in
266 hookworms (Venkatesan et al., 2023). The corresponding TUBB-1 protein sequence was found
267 to be homologous to the partial *U. stenocephala* sequences previously identified in amplicon
268 metabarcoding studies (Abdullah et al., 2025; Stocker et al., 2023; Stocker et al., 2024).
269 Anthelmintic resistance in companion animals is an emerging concern, and in the canine
270 hookworm *A. caninum*, it has become an established reality (Geary et al., 2025; von Samson-
271 Himmelstjerna et al., 2021). This study represents the first successful sequencing of the full
272 *tubb-1* gene in the northern hookworm *U. stenocephala*. Characterisation of both *tubb-1* and
273 *tubb-2* now enables population-level screening for resistance-associated SNPs in *U.*
274 *stenocephala*, similar to the foundational work for *A. caninum* by (Schwenkenbecher et al.,
275 2007).

276 The European red fox is a widespread invasive species in Australia (Stobo-Wilson et al.,
277 2021). The prevalence of *U. stenocephala* in red foxes was reported to range from 18.2% to
278 30.6% (Coman, 1973; Dybing et al., 2013; Ryan, 1976). Using ITS rDNA and
279 partial *cox*1 sequences, we demonstrated that *U. stenocephala* isolates from red foxes are

280 genetically indistinguishable from those found in domestic dogs (Abdullah et al., 2025; Górski
281 et al., 2023). Given that wild red foxes are not exposed to anthelmintic treatments, their high
282 infection rates could represent a substantial refugium for benzimidazole-susceptible
283 hookworms. However, the detection of the *tubb-1* F167Y SNP, associated with benzimidazole
284 resistance, in *U. stenocephala* from an urban dog in Australia by Abdullah et al. (2025) suggests
285 that the epidemiology of this parasite in dogs and foxes may be more complex and distinct than
286 currently assumed.

287 Although there is currently no conclusive clinical evidence of benzimidazole treatment
288 failure in *U. stenocephala*, the detection of the F167Y SNP in the *tubb-1* gene by Abdullah et al.
289 (2025) provides early warning of potential resistance. This finding suggests that the conditions
290 for resistance are present, and it may only be a matter of time before the frequency of this SNP
291 increases to levels associated with clinical drug failure. In the absence of phenotypic validation
292 of the F167Y SNP as a resistance marker in *U. stenocephala*, we must remain vigilant and
293 anticipate the spread of benzimidazole resistance, as has occurred in *A. caninum* populations
294 in Australia and North America (Abdullah et al., 2025; Geary et al., 2025; Leutenegger et al.,
295 2024; Leutenegger et al., 2023; Venkatesan et al., 2023).

296 DNA polymerase fidelity is highly sensitive to mismatches at the 3' end of PCR primers,
297 particularly when the 3' terminal nucleotide is incorrectly base-paired, as this can hinder the
298 initiation of extension by the enzyme (Kwok et al., 1990). In the case of the ACB1_200_R primer,
299 a T>C mismatch at the 3' end poses a potential risk for PCR failure due to impaired primer
300 extension. However, previous studies have shown that this mismatch did not interfere with
301 successful amplification of the *U. stenocephala tubb-1* amplicon using the
302 ACB1_200_F/ACB1_200_R primer pair (Abdullah et al., 2025; Stocker et al., 2023; Stocker et al.,
303 2024; Venkatesan et al., 2023). With our characterisation of the *U. stenocephala tubb-1* gene
304 and comparative analyses with other hookworm species, there is now an opportunity to refine
305 these primers, either through degeneracy or redesign, to better accommodate sequence

306 variability. The availability of genome sequencing data and expanded hookworm reference
307 datasets further enhances the potential for improved diagnostic accuracy and data reliability in
308 high- to medium-throughput molecular applications (Ilík et al., 2024).

309 We concluded that the identified protein sequnces belong to *U. stenocephala* β-tubulin,
310 specifically TUBB-1 and TUBB-2. Burns (1991) established three protein motifs common to all
311 tubulins, the VPRA(V/I)(F/L)(V/L)DLEP, S(F/L/A)AGGTGSG and VV(E/N)PYN motifs. These motifs
312 were detected in all TUBB-1 and TUBB-2 from hookworms, with a sinlge exception. There was a
313 deviation at the third residue of the second motif (A140G) aparently shared across all
314 hookworms. All β-tubulin posses the motive F(K/R)R motif that was conserved across all
315 hookworms. Whereas α- and γ-tubulin have KFD or RKR motif instead, respectively (Burns,
316 1991). Burns (1991) considered the hypervariable regions of the tubulin genes to infer functional
317 residues. The divergent TUBB-1 residues and four of the six divergent TUBB-2 residues (E39D,
318 T40S, A287S, T349G) of *U. stenocephala* were located in hypervariable regions and so unlikely
319 to impact the function of the β-tubulins. The benzimidazole-susceptible residues Q134, F167,
320 E198, and F200 were present in *U. stenocephala* TUBB-1 and were homologues to those in other
321 hookworm sequences.

322 **5. Conclusion**

323 Benzimidazole- and multidrug-resistant *A. caninum* are present in domestic dog
324 populations across North America and Australia (Abdullah et al., 2025; Kitchen et al., 2019;
325 Venkatesan et al., 2023)**.** In contrast, although *U. stenocephala* is the predominant hookworm
326 species in canines from cooler regions such as continental Europe, southern Australia, and
327 likely other under-sampled areas, data on its distribution and resistance profile remain scarce.
328 By characterising the complete coding regions of *tubb-1* and *tubb-2*, we enable population-level
329 screening for benzimidazole resistance-associated SNPs and lay the groundwork for
330 epidemiological studies in the 'northern hookworm' *U. stenocephala*. Genetic screening
331 revealed widespread SNPs in *A. caninum tubb-1* linked to benzimidazole resistance, indicating

that these alleles circulated within populations long before clinical drug failures were recognised by veterinary parasitologists (Geary et al., 2025). Geary et al. (2025)recently proposed a set of action points to address resistance in *A. caninum*; we argue that these recommendations are equally relevant and urgently needed for both *A. caninum* and *U. stenocephala*.

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Author contributions: CRediT

Thomas Stocker: Conceptualization, Formal analysis, Investigation, Writing – original draft. **Jan Šlapeta**: Conceptualization, Funding acquisition, Project administration, Validation, Writing – review and editing.

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522 **Table 1. Assembly of the whole genome sequencing of *Uncinaria stenocephala***

	TSF_1.1	TSF_1.1*	TSF_1.2	TSF_1.2*
L50	32,460	32,766	2,152	2,065
N50	2,203	2,090	8,683	8,431
Number of bp	341,439,219	333,472,904	62,113,770	57,967,112
Number of sequences	1,039,638	1,037,453	50,108	49,593
GC content (%)	45.5	45.6	44.6	44.7

523 * putative bacterial and viral contaminants removed via Kraken

524

525

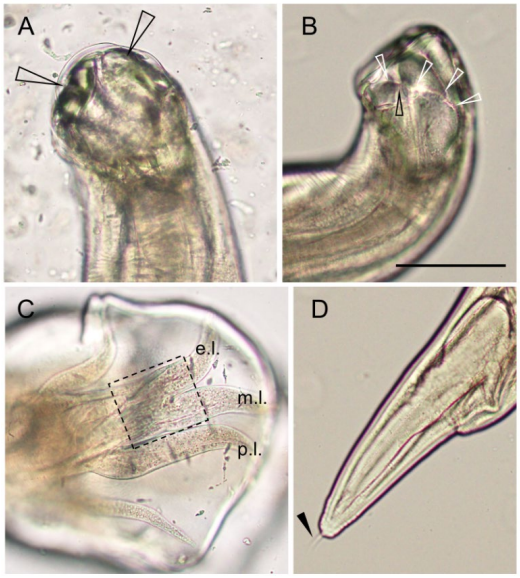

Figure 1: Morphology of sampled *Uncinaria stenocephala*. (A) Dorso-ventral view of the anterior end. Bicuspid cutting plates are visualised with an arrow for each cutting plate. (B) The lateral view of the anterior end. The internal cephalic papilla is labelled with a black arrow. The white arrows signify the boundary line between the thick ventral portion and the thinner dorsal portion of the lateral wall of the oral capsule. This boundary crosses through the internal cephalic papilla which is consistent with *U. stenocephala*. (C) Male copulatory bursa with the medio-lateral (m.l.) ray is roughly equal in width to the externo-lateral ray (e.l.) and the postero-lateral ray (p.l.). (D) Female posterior end with a caudal bristle (arrowhead). Scale bar for A-D , 100 µm.

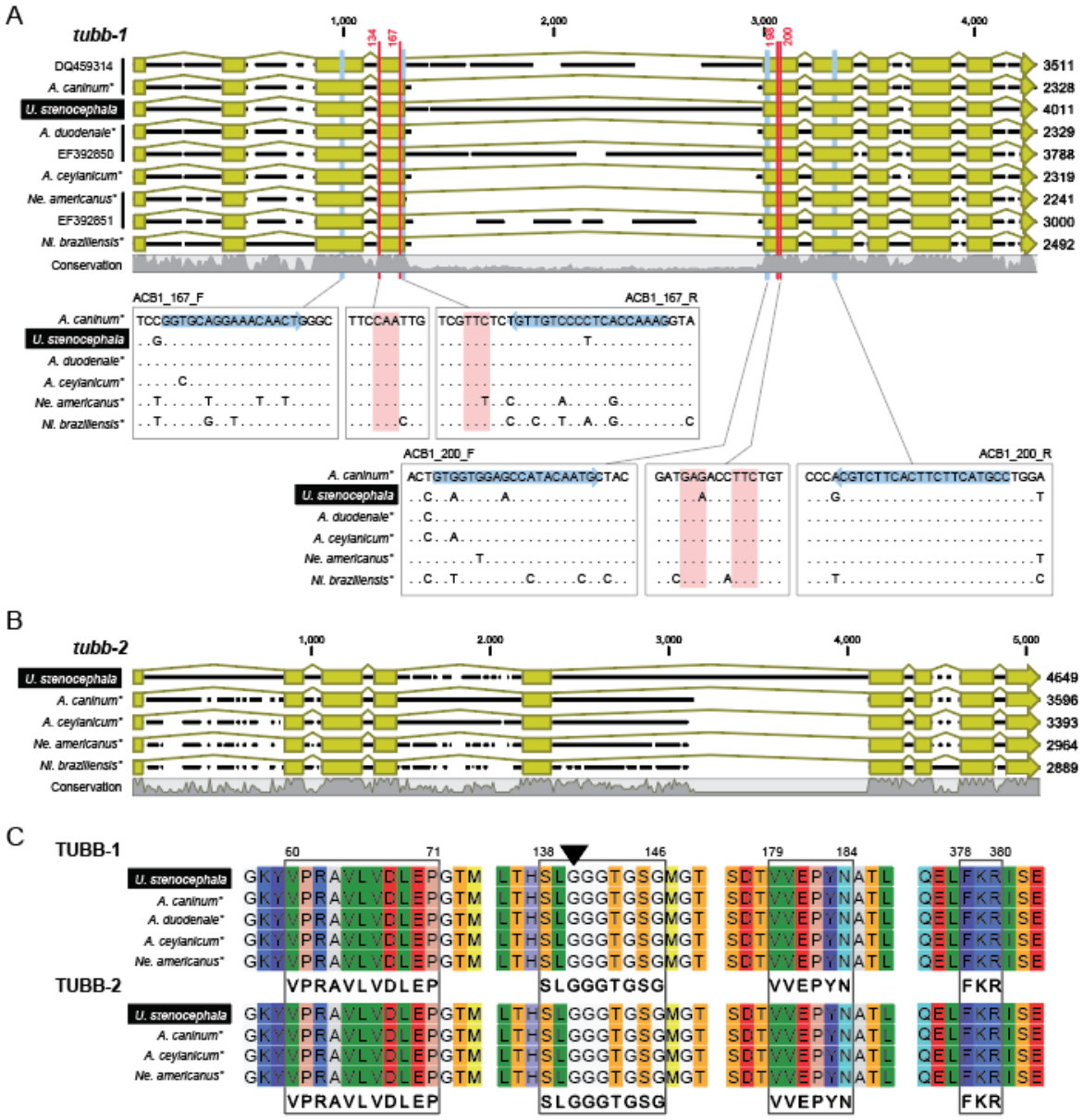

Figure 2: Comparison of *Uncinaria stenocephala* structure of *tubb-1* and *tubb-2* genes with other hookworms. (A) Gene sequence showing exon-intron boundaries of *tubb-1* of hookworms. Regions where primers targeting *A. caninum tubb-1* (ACB1_167_F: 5'-GG**Y** GCA GGA AAC AAC TG -3', ACB1_167_R: 5'- CTT TGG TGA GGG GAC AAC A -3', ACB1_200_F: 5'- GT**R** GTG GAG CCA TAC AAT GC -3' and ACB1_200_R: 5'- GGC ATG AAG AAG TGA AGA CGT -3') and benzimidazole-susceptible residues in *tubb-1* (Q134, F167, E198, and F200) are enlarged to show nucleotide sequences (identical residues to the top sequence as dots). (B) Gene sequence showing exon-intron boundaries of *tubb-2* of hookworms. Names with (*) indicate

549 that sequence data were obtained from WormBase Parasite, *tubb-1* (ANCCAN_08153,
550 Acey_s0035.v2.g13228, ANCDUO_05511, Necator_chrII.pre1.g8352 and NBR_0000799801) and
551 *tubb-2* (ANCCAN_23405, Acey_s0432.v2.g1106, Necator_chrII.pre1.g8352 and
552 Nippo_chrII.g5566), otherwise sequences are labelled by GenBank accession number. (C)
553 Amino acid alignment regions of TUBB-1 and TUBB-2 showing the tubulin motifs
554 VPRA(V/I)(F/L)(V/L)DLEP, S(F/L/A)AGGTGSG and VV(E/N)PYN motifs. TUBB-1 second motive
555 devaiates from the standard motive (A140G, arrowhed). The β-tubulin conserved protein motive
556 F(K/R)R motif in preset across all sequences.
557