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 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

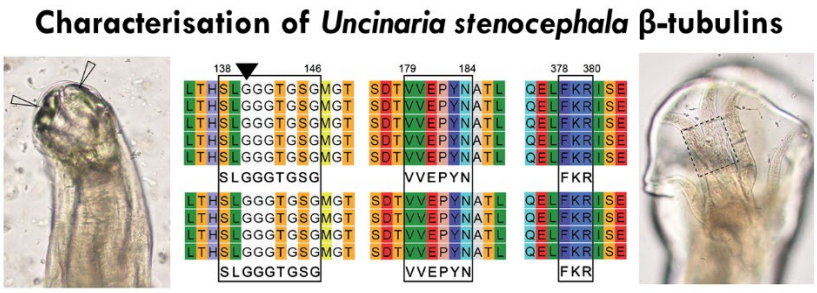
Highlights

Uncinaria stenocephala outnumbers *Ancylostoma caninum* in temperate regions globally

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 so impairing 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).

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. stenocephala tubb-1* (Abdullah et al., 2025). Although clinical treatment failure with benzimidazoles in *U. stenocephala* has not yet been documented, proactive molecular

surveillance is essential. To anticipate emerging resistance, comprehensive characterisation of β-tubulin genes, particularly full-length *tubb-1*, across hookworm species is critical for enabling comparative analyses and early detection of resistance-associated mutations.

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

2. Materials and Methods

2.1. Adult *Uncinaria stenocephala* material

To obtain *Uncinaria stenocephala*, we decided to consider the invasive red fox (*Vulpes vulpes*) that a pest animal in Australia and is regularly culled (Stobo-Wilson et al., 2021). Red foxes are commonly infected by *U. stenocephala* in Australia (Ryan, 1976). Single red fox intestinal tract was donated by a registered shooter in September 2024 (Mardi; 33.2990° S, 151.4084° E); the fox was culled under an ongoing fox control program in Central Coast region of New South Wales, Australia. The gastrointestinal tract was transported on ice to the University of Sydney, stored for 10 days at -80°C prior to dissection. During dissection the gastrointestinal track was thawed, incised longitudinally and content scraped through the researcher's thumb and index finger. The scrapings and small intestinal contents washed with lukewarm tap water into a 150 µm nylon mesh sieve by means of water jet. The washed content was examined in a white metal tray with the aid of stereomicroscope. Nematodes were manually picked against

125 the white tray, washed in phosphate buffered saline (pH=8.0) and stored at -80°C. Hookworms,
126 such as *U. stenocephala*, were identified in accordance with the description of Ransom (1924).

127 **2.2. Genomic DNA extraction and molecular identification**

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

138 **2.3. Whole genome sequencing and assembly**

139 The gDNA obtained from two single *U. stenocephala* was aliquoted (30 µL) into a 1.5 mL
140 Eppendorf tube and submitted to Novogene (HK) Co., Ltd for indexing, library preparation and
141 whole genome sequencing to generate approximately 30 Gb of sequencing data per sample.
142 Sequencing was performed using the Illumina NovaSeq 6000 sequencing platform using 150 bp
143 paired-end sequencing chemistry. Raw FastQ files (JS6871 and JS6872) generated from the
144 same sample were processed in Galaxy Australia (https://usegalaxy.org.au/) (Galaxy
145 Community, 2024). Raw paired-end sequencing reads were examined for quality using *FastQC*
146 (version 0.12.1) (Andrews, 2010) and trimmed using *Trimmomatic* (version 0.36.6) (Bolger et al.,
147 2014) (parameters: "ILLUMINACLIP:TruSeq3-PE.fa:2:30:10 SLIDINGWINDOW:4:20"). Read were
148 mapped using *bwa-mem2* (version 2.2.1) (Li and Durbin, 2009) using simple Illumina mode onto
149 red fox genome (VulVul3) and all mapper reads were removed. The FastQ files were assembled
150 using *SPAdes* (version 4.2.0) (Prjibelski et al., 2020) (parameters: "--cov-cutoff 40.0 -k 77").

151 Contigs were classified using *Kraken* (version 1.1.1) (database: minikraken) (Wood and
152 Salzberg, 2014) and only unclassified contigs were retained. Assembly statistics were
153 generated using *Fasta Statistics* (Seemann and Gladman, 2012).

154 **2.4. Identification of β-tubulin genes in the genome assemblies**

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

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

168 Sequence reads were mapped to the putative *tubb-1* and *tubb-2* using *bwa-*
169 *mem2* (version 2.2.1) (Li and Durbin, 2009) using simple Illumina mode. *Samtools mpileup*
170 (Version 1.15.1) (Danecek et al., 2021) was used to input variants into *VarScan* (Version 2.4.2)
171 (Koboldt et al., 2012) (parameters: --min-coverage 20 --min-reads2 2 --min-avg-qual 15 --min-
172 var-freq 0.2 --min-freq-for-hom 0.75 --p-value 0.05). The resulting BAM and VCF files were
173 visualised using *IGV* (version 2.18.1). VCF file was processed to select only SNP within the
174 exons of *tubb-1* and *tubb-2*. The variant detection was performed in Galaxy Australia
175 (https://usegalaxy.org.au/) (Galaxy Community, 2024).

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

185 **2.5. Data availability**

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

191

192 **3. Results**

193 **3.1. Morphological and molecular confirmation of *Uncinaria stenocephala***

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

197 Two *U. stenocephala* (TSF_1.1, TSF_1.2) were molecular characterised at ITS rDNA and
198 *cox*1 gene. Sequenced ITS rDNA (818 nt) were identical to each other and to the reference
199 sequence of *U. stenocephala* (AF194145). Comparison of the new *U. stenocephala* ITS rDNA
200 with *U. stenocephala* ITS2 rDNA data from amplicon metabarcoding data from dog faeces
201 (Abdullah et al., 2025) revealed 95.8% to 100% match across 260 nt. The sequenced partial

202 *cox*1 (393 nt) sequence from the two *U. stenocephala* differed by a single synonymous mutation
203 (99.8% identity), and they were 99.2-99.5% identical to the reference *U. stenocephala* sequence
204 (AJ407939).

205 **3.2 Sequencing and assembly of *Uncinaria stenocephala* genome**

206 Sequencing of TSF_1.1 (JS6871) and TSF_1.2 (JS6872) generated 117,718,741 and
207 102,660,825 paired-end reads (150 bp), respectively, with average GC contents of 46% and
208 45%. This yielded a total of 35.2 Gb for TSF_1.1 and 30.6 Gb for TSF_1.2. After quality trimming,
209 117,358,176 (99.7%) and 102,353,896 (99.7%) high-quality paired-end reads were retained.
210 There were 4% and 23% sequence reads that mapped to the red fox (VulVul3) genome that were
211 removed from TSF_1.1 and TSF_1.2, respectively. The final datasets comprised 31.0 Gb for
212 TSF_1.1 and 21.6 Gb for TSF_1.2, both with a GC content of 45-46%. Each sample was
213 assembled independently. The TSF_1.1 assembly had N50 of 2,203 bp and L50 of 32,460
214 contigs. We then removed potential bacterial or viral contaminant contigs (TSF_1.1: 0.2% of the
215 contigs) to produce final assembly contigs (TSF_1.1: N50 of 2,090 bp and L50 of 32,766 contigs)
216 (**Table 1**). The TSF_1.2 assembly had N50 of 8,683 bp and L50 of 2,153 contigs, following
217 decontamination it had N50 of 8,431 bp and L50 of 2,065 contigs (**Table 1**).

218 The TSF_1.1 assembly yielded a contig (NODE_5499; length 7,645 nt, coverage 19.8) that
219 contained complete *tubb-1* gene using blast searches with other *tubb-1* from hookworms as a
220 query. The *tubb-2* was represented by three contigs, one contig (NODE_15465; length 4,291 nt,
221 coverage 30.3) included all but first exon and was truncated at the first intron. The first exon with
222 partial first intron was found on two contigs, where the first exon was 100% identical between
223 the contigs (NODE_82476; length 558 nt, coverage 29.6; NODE_38650; length 1,780 nt,
224 coverage 24.1). The *tubb-2* complete coding sequence was fused from the contigs, the
225 nucleotide sequence is truncated in the first exon. There were no contigs that would contain
226 putative *tubb-1* and *tubb-2* in the TSF_1.1 assembly.

227 **3.3 Characterisation of *tubb-1* and *tubb-2* of *Uncinaria stenocephala***

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

234 The *U. stenocephala tubb-1* gene was 4,011 nt long including 10 exons homologous to
235 other *btub-1* form hookworms (**Figure 1A**). The largest intron between the exon 4 and 5 was
236 1,688 nt, shorter compared to the intron from *A. caninum tubb-1* (46 nt: ANCCAN_08153 and
237 1217 nt: DQ459314). The *U. stenocephala btub-2* gene has 9 exons homologous to those of
238 other hookworms (**Figure 1B**). VarScan has detected 9 independent heterozygous nucleotide
239 position in TSF_1.1 and TSF_1.2 within *tubb-1* of *U. stenocephala*. Within *tubb-2* of *U.*
240 *stenocephala* there were 48 and 32 heterozygous positions in TSF_1.1 and TSF_1.2,
241 respectively.

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

249 We then compared the primers designed by (Jimenez Castro et al., 2019) for the
250 amplification of the canonical SNPs in *tubb-1* of *A. caninum* with that of *U. stenocephala* (**Figure**
251 **2A**). The ACB1_167_F primer sequence was a perfect match, while ACB1_167_F had single,
252 G>A, mismatch in the middle of the primer sequence compared to the *U. stenocephala tubb-1*.
253 Both ACB1_200_F and ACB1_200_R had single mismatch each compared to the *U.*

254 *stenocephala tubb-1*. The mismatch in the ACB1_200_R is at the 3' terminal nucleotide position,
255 T>C.
256
257 **4. Discussion**
258 We successfully characterised the coding sequences of two representatives of the β-
259 tubulin gene family in *U. stenocephala*. Both the *tubb-1* and *tubb-2* genes are highly conserved
260 among hookworms and retain an identical exon–intron structure. Access to the complete *U.*
261 *stenocephala tubb-1* sequence enabled us to confirm the presence of key residues (Q134,
262 F167, E198, and F200) that are associated with benzimidazole susceptibility in hookworms
263 (Venkatesan et al., 2023). The corresponding TUBB-1 protein sequence was found to be
264 homologous to the partial *U. stenocephala* sequences previously identified in amplicon
265 metabarcoding studies (Abdullah et al., 2025; Stocker et al., 2023; Stocker et al., 2024).
266 Anthelmintic resistance in companion animals is an emerging concern, and in the canine
267 hookworm *A. caninum*, it has become an established reality (Geary et al., 2025; von Samson-
268 Himmelstjerna et al., 2021). This study represents the first successful sequencing of the full
269 *tubb-1* gene in the northern hookworm *U. stenocephala*. Characterisation of both *tubb-1* and
270 *tubb-2* now enables population-level screening for resistance-associated SNPs in *U.*
271 *stenocephala*, similar to the foundational work for *A. caninum* by (Schwenkenbecher et al.,
272 2007).
273 The European red fox is a widespread invasive species in Australia (Stobo-Wilson et al.,
274 2021). The prevalence of *U. stenocephala* in red foxes was reported to range from 18.2% to
275 30.6% (Coman, 1973; Dybing et al., 2013; Ryan, 1976). Using ITS rDNA and
276 partial *cox*1 sequences, we demonstrated that *U. stenocephala* isolates from red foxes are
277 genetically indistinguishable from those found in domestic dogs (Abdullah et al., 2025; Górski
278 et al., 2023). Given that wild red foxes are not exposed to anthelmintic treatments, their high
279 infection rates could represent a substantial refugium for benzimidazole-susceptible

280 hookworms. However, the detection of the *tubb-1* F167Y SNP, associated with benzimidazole
281 resistance, in *U. stenocephala* from an urban dog in Australia by Abdullah et al. (2025) suggests
282 that the epidemiology of this parasite in dogs and foxes may be more complex and distinct than
283 currently assumed.

284 Although there is currently no conclusive clinical evidence of benzimidazole treatment
285 failure in *U. stenocephala*, the detection of the F167Y SNP in the *tubb-1* gene by Abdullah et al.
286 (2025) provides early warning of potential resistance. This finding suggests that the conditions
287 for resistance are present, and it may only be a matter of time before the frequency of this SNP
288 increases to levels associated with clinical drug failure. In the absence of phenotypic validation
289 of the F167Y SNP as a resistance marker in *U. stenocephala*, we must remain vigilant and
290 anticipate the spread of benzimidazole resistance, as has occurred in *A. caninum* populations
291 in Australia and North America (Abdullah et al., 2025; Geary et al., 2025; Leutenegger et al.,
292 2024; Leutenegger et al., 2023; Venkatesan et al., 2023).

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

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

319 **5. Conclusion**

320 Benzimidazole- and multidrug-resistant *A. caninum* are present in domestic dog
321 populations across North America and Australia (Abdullah et al., 2025; Kitchen et al., 2019;
322 Venkatesan et al., 2023)**.** In contrast, although *U. stenocephala* is the predominant hookworm
323 species in canines from cooler regions such as continental Europe, southern Australia, and
324 likely other under-sampled areas, data on its distribution and resistance profile remain scarce.
325 By characterising the complete coding regions of *tubb-1* and *tubb-2*, we enable population-level
326 screening for benzimidazole resistance-associated SNPs and lay the groundwork for
327 epidemiological studies in the 'northern hookworm' *U. stenocephala*. Genetic screening
328 revealed widespread SNPs in *A. caninum tubb-1* linked to benzimidazole resistance, indicating
329 that these alleles circulated within populations long before clinical drug failures were
330 recognised by veterinary parasitologists (Geary et al., 2025). Geary et al. (2025)recently
331 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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517

518

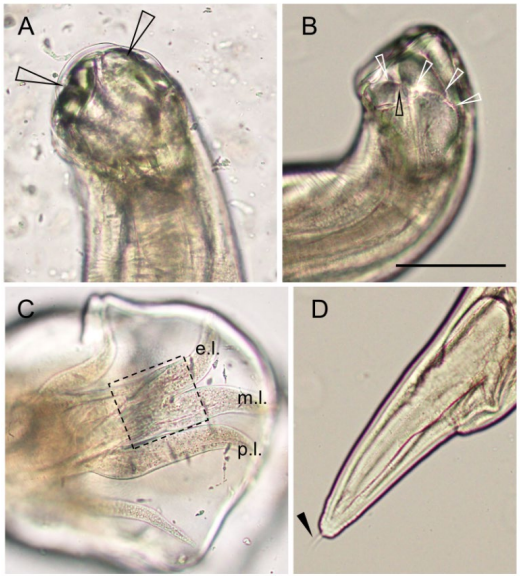
519 **Table 1. Assembly of the whole genome sequencing of *Uncinaria stenocephala***

	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

520 * putative bacterial and viral contaminants removed via Kraken

521

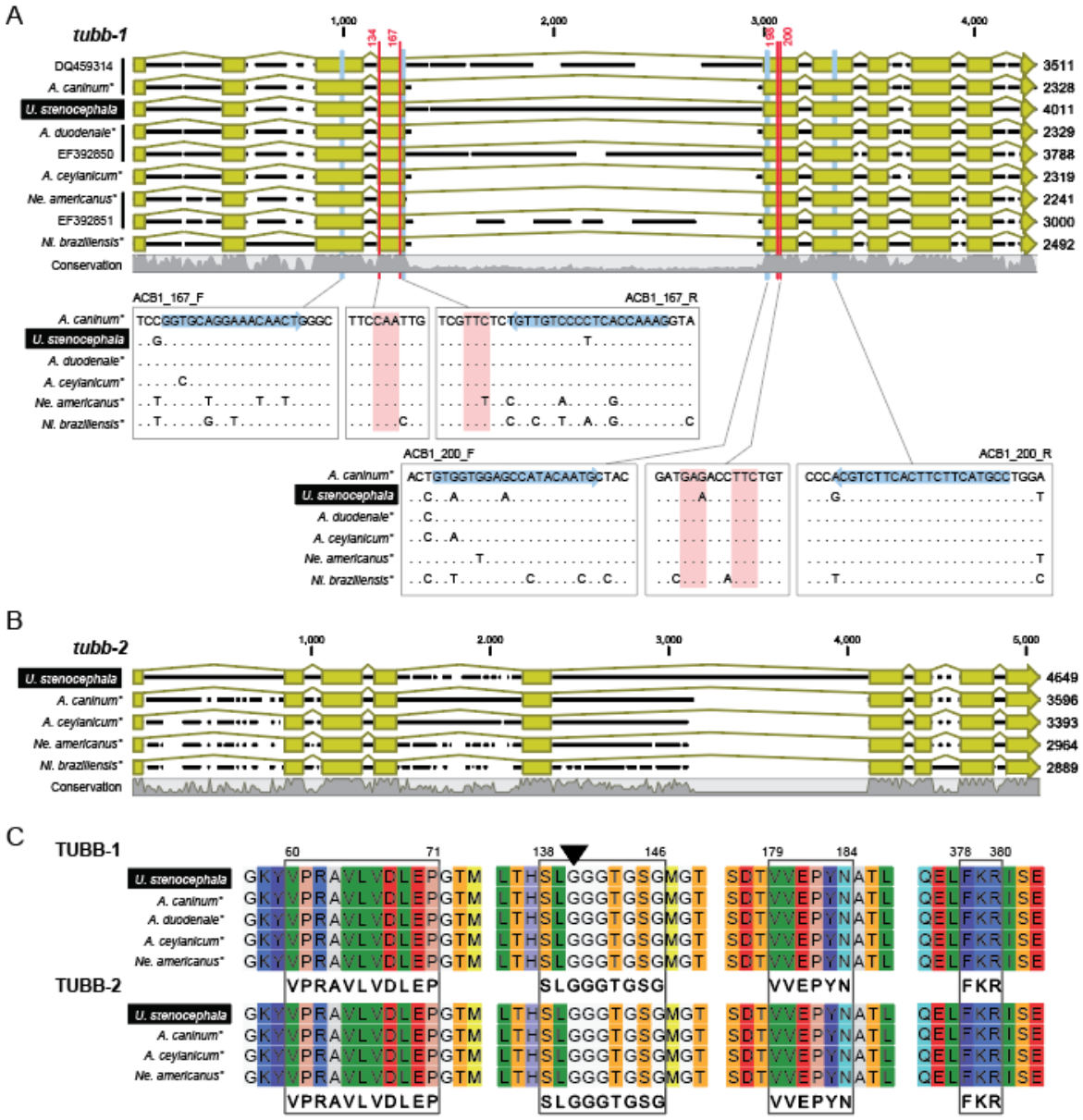
522 **Figures:**



523

524 **Figure 1: Morphology of sampled *Uncinaria stenocephala*.** (A) Dorso-ventral view of the
525 anterior end. Bicuspid cutting plates are visualised with an arrow for each cutting plate. (B) The
526 lateral view of the anterior end. The internal cephalic papilla is labelled with a black arrow. The
527 white arrows signify the boundary line between the thick ventral portion and the thinner dorsal
528 portion of the lateral wall of the oral capsule. This boundary crosses through the internal
529 cephalic papilla which is consistent with *U. stenocephala*. Scale bar is representative of 100
530 µm.

531



532

533 **Figure 2: Comparison of *Uncinaria stenocephala* structure of *tubb-1* and *tubb-2* genes with**

534 **other hookworms.** (A) Gene sequence showing exon-intron boundaries of *tubb-1* of

535 hookworms. Regions where primers targeting *A. caninum tubb-1* (ACB1_167_F: 5'-GG**Y** GCA

536 GGA AAC AAC TG -3', ACB1_167_R: 5'- CTT TGG TGA GGG GAC AAC A -3', ACB1_200_F: 5'- GT**R**

537 GTG GAG CCA TAC AAT GC -3' and ACB1_200_R: 5'- GGC ATG AAG AAG TGA AGA CGT -3') and

538 benzimidazole-susceptible residues in *tubb-1* (Q134, F167, E198, and F200) are enlarged to

539 show nucleotide sequences (identical residues to the top sequence as dots). (B) Gene

540 sequence showing exon-intron boundaries of *tubb-2* of hookworms. Names with (*) indicate

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