Detection of classic and cryptic *Strongyloides* genotypes by deep amplicon sequencing: A preliminary survey of dog and human specimens collected from remote Australian communities

Meruyert Beknazarova[1]*, Joel L. N. Barratt[2,3]*, Richard S. Bradbury[2], Meredith Lane[2,4], Harriet Whiley[1], Kirstin Ross[1]

* corresponding authors

[1] Environmental Health, Flinders University, South Australia

[2] Division of Parasitic Diseases and Malaria, Centers for Disease Control and Prevention

[3] Oak Ridge Associated Universities, Oak Ridge, Tennessee

[4] Synergy America Inc.

Abstract

Strongyloidiasis is a neglected disease caused by the human infective nematodes *Strongyloides stercoralis, Strongyloides fuelleborni* subsp. *fuelleborni* and *Strongyloides fuelleborni* subsp. *kellyi*. The zoonotic potential of *S. stercoralis* and the potential role of dogs in the maintenance of strongyloidiasis transmission has been a topic of interest and discussion for many years. In Australia, strongyloidiasis is prevalent in remote socioeconomically disadvantaged communities in the north of the continent. Being an isolated continent that has been separated from other regions for a long geological period, knowledge on the diversity of Australian *Strongyloides* genotypes is an important addition to our understanding of the genetic diversity within the genus. Using PCR enrichment combined with Illumina sequencing technology, we sequenced the *Strongyloides SSU* 18S rDNA hyper-variable I and hyper-variable IV regions using *Strongyloides*-specific primers, and a fragment of the mtDNA *cox*1 gene using primers that are broadly specific for *Strongyloides* sp. and hookworms. These loci were amplified from DNA extracted from human and dog faeces, and one human sputum sample. Using this approach, we confirm for the first time that potentially zoonotic *S. stercoralis* genotypes are present in Australia, suggesting that dogs represent a potential reservoir of human strongyloidiasis in remote Australian communities.

Author summary

Strongyloides stercoralis is a soil-transmitted nematode that causes the disease strongyloidiasis. Due to the autoinfective nature of this parasite, it can re-infect a host causing chronic infection. If not diagnosed and treated it can be highly detrimental to human health and has a high mortality rate. Strongyloidiasis is common in remote communities in the north of Australia and has been an issue for decades. Despite various successful intervention programs to treat human strongyloidiasis, the disease remains endemic in those communities. Here for the

first time we looked at the Australian dogs' potential to infect humans and found that they carry two genetically distinct strains of *Strongyloides* spp., one of which also infects humans. This supports the hypothesis that dogs are a potential source for human strongyloidiasis. We also found that dogs in Australia might be carrying unique haplotypes. Whether these new haplotypes are also human infective is to be confirmed by further research.

Keywords: *Strongyloides*, genotypes, dogs, Australia, zoonoses, hookworms, *Ancylostoma*

50 ## Introduction

51 Strongyloidiasis is a neglected disease caused by the human infective nematodes
52 *Strongyloides stercoralis, Strongyloides fuelleborni* subsp. *fuelleborni* and *Strongyloides*
53 *fuelleborni* subsp. *kellyi* (Grove, 1996). Worldwide, it is estimated to infect up to 370 million
54 people and is predominately found in socioeconomically disadvantaged communities (Olsen et
55 al., 2009, Bisoffi et al., 2013, Beknazarova et al., 2016). While *S. stercoralis* is a globally
56 distributed nematode, *S. f. fuelleborni* has thus far only reported in Africa and Southeast Asia
57 and *S. f. kellyi* from Papua New Guinea (PNG) (Thanchomnang et al., 2017, Pampiglione and
58 Ricciardi, 1971, Ashford et al., 1992).

59 In Australia, strongyloidiasis is prevalent in remote communities located across the
60 Northern Territory, Queensland, Western Australia, northern South Australia and northern New
61 South Wales (Page et al., 2016). Current estimates indicate that infection rates in some
62 communities are up to 41% or 60% based on microscopy or serology respectively (Heydon and
63 Green, 1931, Sampson et al., 25-26 June 2003, Miller et al., 2018, Holt et al., 2017). Despite
64 initially successful intervention programs targeting treatment to eliminate human
65 strongyloidiasis in remote Australian communities, the disease remains endemic (Kearns et al.,
66 2017, Page et al., 2006). Reappearance of the infection could possibly be as a result of zoonotic
67 transmission from dog reservoirs given that dogs and humans share a close and intimate cultural
68 bond in rural and remote Indigenous communities of Australia (Constable et al., 2010).

69 The zoonotic potential of *S. stercoralis* infected dogs and their potential role in the
70 maintenance of strongyloidiasis transmission has been a topic of interest and discussion for
71 many years (Beknazarova et al., 2017b, Goncalves et al., 2007, Takano et al., 2009). Molecular
72 data from human and dog derived *S. stercoralis* are useful in understanding the nature of cross
73 infection. There are two regions of the *S. stercoralis* nuclear and mitochondrial genome that are

74 considered to be conserved within the *Strongyloides* genus and can be used as markers for
75 molecular typing of *S. stercoralis*. Hyper-variable regions (HVR) I and IV of the small Subunit
76 (*SSU*) 18s ribosomal DNA and the cytochrome c-oxidase subunit 1 (*cox*1) gene of the
77 mitochondrial DNA (mtDNA) have been widely used to study relationships between *S.*
78 *stercoralis* from different hosts and different geographic locations (Hasegawa et al., 2009,
79 Hasegawa et al., 2010, Jaleta et al., 2017, Nagayasu et al., 2017, Basso et al., 2018). Based on
80 genetic analysis of these genetic loci, it has been recently found that there are two genetically
81 different *S. stercoralis* strains, one is dog and human infective, and the other is dog specific.
82 These data were collected from dogs and humans in Cambodia and Japan (Jaleta et al., 2017,
83 Nagayasu et al., 2017).

84 Being an isolated continent that has been separated from other regions for a long
85 geological period, Australia could represent an interesting addition to our understanding of the
86 genetic diversity within *S. stercoralis* and the *Strongyloides* genus more generally. Indigenous
87 Australians have inhabited the continent for at least 40,000 years and dogs (in the form of
88 dingoes) were likely introduced up to 12,000 years ago (Clutton-Brock, 1995). Given this long
89 period of relative isolation, it might be expected that Australia could harbor unique endemic
90 genotypes or unique sub-species of *S. stercoralis* that have evolved within dog and human
91 populations over this period.

92 Using PCR enrichment combined with Illumina sequencing technology, we sequenced
93 the *Strongyloides SSU* 18S rDNA hyper-variable I and hyper-variable IV regions using
94 *Strongyloides*-specific primers, and a fragment of the mtDNA *cox*1 gene using primers that are
95 broadly specific for *Strongyloides* sp. and hookworms. This approach was applied to DNA
96 extracted from human and dog faeces, and one human sputum sample. The main focus of this
97 study was to genotype Australian human and dog *S. stercoralis* strains to see whether dogs

98 carry human *S. stercoralis* strains and/or vice versa. To our knowledge this is the first time
99 human and dog *S. stercoralis* have been studied in Australia on a molecular level.

100 Methods

101 Study area and faeces collection

102 Dog faecal samples were collected from communities in the Northern Territory,
103 Australia. Dog faeces were collected from the environment (i.e., the ground) in the selected
104 communities and stored in the DESS (dimethyl sulfoxide, disodium EDTA, and saturated
105 NaCL) solution to preserve the DNA (Beknazarova et al., 2017a). For those dog faeces that
106 were collected from the private land, the consent forms were received to collect the samples.
107 The preserved faecal samples were express posted to the Environmental Health laboratory,
108 Flinders University, South Australia, for further analysis.

109 Human faecal and sputum samples were provided by our colleagues at the Royal
110 Darwin Hospital, NT, AusDiagnostics PTY LTD, NSW, and Townsville Hospital, Queensland.
111 While the personal information of the patients remains confidential and unknown, the infection
112 is known to have been locally acquired. Ethics approval from the Social and Behavioural
113 Research Ethics Committee (SBREC) No 6852 dated 1st June 2015 was obtained for collecting
114 dog faeces from the remote communities. Human ethical approval from the Southern Adelaide
115 Clinical Human Research Ethics Committee (SAC HREC) No 309.17 dated 24th January 2018
116 was obtained for comparing *S. stercoralis* DNA extracted from human and dog tissues.

117 DNA extraction

118 Prior to DNA extraction, faecal samples containing DESS were centrifuged for three
119 minutes at 3000 x g rpm using an Orbital 400 Clements (Phoenix, Lidcombe, Australia). The
120 supernatant consisting of the preservative solution was removed. The remaining faecal sample

121 was washed with sterile saline solution. DNA was extracted using the Power Soil DNA
122 isolation kit (QIAGEN, Hilden, Germany) following the manufacturer's instructions with slight
123 modifications that included incubating samples at 56ºC overnight after the cell lysis step,
124 followed by vortexing of samples for three minutes. Approximately 250 milligrams of the pellet
125 was placed into a PowerBead tube containing lysis buffer (included in the Power Soil DNA
126 extraction kit). The remainder of the extraction process was performed according to the
127 manufacturer's instructions. Extracted DNA was stored at -20°C prior to real-time PCR (qPCR)
128 analysis.

129 **Real-time PCR**

130 The samples (273 dog and 4 human DNA samples) were first screened for *Strongyloides*
131 spp. using qPCR in the Environmental Health laboratory at Flinders University, SA, Australia.
132 The real-time PCR assay was adopted from Verweij *et al.* (2009) using *S. stercoralis* - specific
133 primers targeting a 101 base pair region of 18S rRNA. The 20 µL reaction contained 10 µL
134 Supermix (SSoAdvanced, Universal Probes Supermix, Foster City, CA, USA), 1 µL primers
135 and probe mixture (Stro18S-1530F, Stro18S-1630R and Stro18S-1586T FAM) (Bio-Rad
136 Laboratories, CA, USA), 4 µL deionised H_2O, and 5 µL DNA template. All qPCR reactions
137 were performed in triplicate on the Corbett Rotor-Gene 6000 machine (QIAGEN, Hilden,
138 Germany). *S. stercoralis* primers and probes and qPCR conditions used are shown in Table 1.
139 A sample was considered positive when the Ct value was lower than the mean negative Ct
140 minus 2.6 standard deviations of a mean negative control Ct. Positive samples were amplified in
141 every qPCR reaction.

142 **Conventional PCR for amplification of *SSU* HVR-I and HVR-IV, and *cox*1 sequences**

143 Extracted DNA from samples that were qPCR positive for *Strongyloides* spp. (47 dog
144 and four human DNA samples) were shipped on dry ice to the Centers of Disease Control and
145 Prevention (CDC), Georgia, USA for conventional PCR, sequencing and bioinformatics
146 analysis. Hyper-variable regions (HVR) I and IV in the small Subunit (*SSU*) 18S ribosomal
147 DNA and a fragment of the mitochondrial cytochrome c-oxidase subunit 1 (*cox*1) gene were
148 amplified using conventional PCR and then sequenced using Illumina technology. All PCR
149 reactions were performed on a GeneAmp PCR System 9700 Thermo Cycler, version 3.12
150 (Applied Biosystems, USA). *S. stercoralis* primers and PCR conditions used for qPCR and
151 conventional PCR are shown in Table 1. For the *cox*1 gene, PCR reactions were performed in a
152 total volume of 50 µL containing 10 µL NEB 5X Q5® Buffer (New England BioLabs, USA),
153 10 µL NEB 5X Q5® High GC Enhancer (New England BioLabs, USA), 4 µL NEB
154 Deoxynucleotide Solution Mix (10 mM each nt) (New England BioLabs, USA), 1 µL Q5®
155 High-Fidelity DNA Polymerase (New England BioLabs, USA), 2.5 µL forward primer
156 (SSP_COX1_F), 2.5 µL reverse primer (SSP_COX1_R), 18 µL deionised H_2O, and 2 µL DNA
157 template. For the HVR-I and HVR-IV regions, PCR reactions were performed in a 25 µL
158 reaction containing 12.5 µL of NEBNext® Q5® Hot Start HiFI PCR Mastermix, MO543L
159 (New England BioLabs, USA), 1.5 µL forward primer (NEW_HVR_I_F or NEW_HVR_IV_F),
160 1.5 µL reverse primer (NEW_HVR_I_R or NEW_HVR_IV_R), 7.5 µL deionised H_2O, and 2
161 µL DNA template.
162
163
164 *Insert Table 1 here*
165
166

167 The amplified PCR products were separated by 1.5% agarose gel electrophoresis and stained
168 with ethidium bromide. The stained DNA bands were visualised by UV illumination using a
169 Ugenious 3 (SYNGENE, Japan). For quality control, each PCR run was accompanied by a
170 positive control containing *Strongyloides* genomic DNA as template, a non-template control
171 containing autoclaved sterile water instead of template, and a negative control containing DNA
172 extracted from a parasite-free specimen. Amplicons for each of the three markers were also
173 generated for *Strongyloides ratti* as an additional control for the sequencing and *in silico*
174 analysis steps.

175 **Next Generation Sequencing (NGS)**

176 Ten microliters of PCR amplicon was purified and normalized for concentration prior to
177 library preparation using SequalPrep Normalization Plate Kit (Thermo Fisher Scientific, USA).
178 DNA libraries were prepared using the NEBNext Ultra DNA Library Prep Kit for Illumina
179 (New England BioLabs, USA), and NEBNext Multiplex Oligos for Illumina Index kit (New
180 England BioLabs, USA). The sequencing reactions were prepared using the MiSeq reagent
181 Nano Kit v2 (PE250bp), and performed on the Illumina MiSeq platform (Illumina).

182 ***In silico* analysis**

183 The Illumina reads were analyzed using Geneious (www.geneious.com) by means of a
184 workflow that performed read quality control, assembly of contigs and genotype assignment. As
185 part of this workflow, quality trimming to a minimum phred score of 20 and removal of adapter
186 sequence was performed using BBDuk (v 37.64). Reads less than 50 bases in length were
187 discarded. Paired reads were then merged using BBMerge (v 37.64) and all reads (merged and
188 unmerged) were mapped to a reference sequence. For *cox*1 amplicons, reads were mapped to a
189 *S. stercoralis* sequence with the GenBank (GB) accession number LC050212.1. For *SSU* HVR-

190 I and *SSU* HVR-IV, reads were mapped to a *S. stercoralis* with the accession AF279916.2.
191 Prior to mapping, each reference sequence was trimmed to the length of the amplicon,
192 excluding the primer sequences. Mapping was performed using the Geneious mapper under the
193 Medium sensitivity / Fast default settings. Reads that successfully mapped (and their mates)
194 were retained for *de novo* assembly. These reads were assembled using the Geneious *de novo*
195 assembler with the following customized parameters; minimum overlap: 50 bases, minimum
196 overlap identity: 100%, maximum number of mismatches per read: 0%, and the maximum
197 number of ambiguities: 1. Contigs were split if coverage fell below 50 and sub-variants with
198 coverage less than 50 were not considered. Contigs were trimmed to the primer sequence using
199 the 'Trim Ends' function in Geneious. Contigs were validated by taking the trimmed and
200 merged reads and mapping them back to the contigs generated using the Geneious mapper, and
201 the following custom parameters: minimum mapping quality: 30, minimum overlap: 150 bases,
202 minimum overlap identity: 100%, maximum number of mismatches: 0%, and the maximum
203 number of ambiguities: 1. Contigs were split or discarded if the coverage fell below 300 to 500
204 depending on the depth obtained for a particular specimen (500 for specimens that obtained
205 >10k reads, 300 for specimens with <10k reads). Finally, genotypes were assigned by
206 performing a local BLASTN search (within Geneious) against a database constructed from all
207 unique *Strongyloides* sp. 18S and *cox*1 sequences available in GenBank and the DNA Databank
208 of Japan. A set of homologous sequences from several other roundworms (parasitic and free-
209 living) were also included in this database. Sequences were only added to the BLASTN
210 database if they overlapped our 217 base pair *cox*1 amplicon by more than 95%. This included
211 several previously published *cox*1 haplotypes that had matching *SSU* HVR-I and HVR-IV
212 genotypes assigned (Jaleta et al., 2017).

213 Any specimens for which *Strongyloides ratti* sequences were detected as part of this
214 workflow were considered to be at risk of cross contamination from our positive control and
215 potentially from other specimens included in the study. Any contaminated specimens were
216 excluded from further analysis.

217 **Construction of a *cox*1 cluster dendrogram**

218 Sequences of *cox*1 were exported from Geneious as a fasta file and were aligned using
219 the 'msa' package in R. The 'dist.alignment' function from the 'seqinr' package was used to
220 compute a pairwise identity matrix, considering gaps in the identity measure. The resulting
221 matrix was clustered using the agglomerative nested clustering approach performed with the
222 'agnes' R package, using "euclidean" distances and the "average" clustering method. A cluster
223 dendrogram was generated using the 'ggtree' R package. Vector images (i.e. graphics) used for
224 annotation of the dendrogram were either generated in house at CDC or obtained from PhyloPic
225 (http://phylopic.org).

226 ## Results

227 **Real-time PCR, conventional PCR and sequencing**

228 We screened 273 dog and four human samples using real-time and conventional PCR.
229 47 dog and four human samples that were positive by qPCR were selected for sequencing of
230 their *cox*1, *SSU* HVR-I and HVR-IV regions. In some cases, useable sequences were not
231 generated due to poor amplification of the PCR product. Sequence data was obtained for 24
232 specimens including four human specimens and twenty dog specimens. The complete genotype
233 of all specimens analyzed in this study is shown in Figure 1 and summarized in Table 2.

234 *Insert Figure 1 here*

235

236 *Insert Table 2 here*

237

238 **_SSU_ HVR-I haplotypes detected among _S. stercoralis_ from Australian dogs and humans**

239 Jaleta *et al.* (2017) previously sequenced the *SSU* HVR-I region of *S. stercoralis* worms
240 from Cambodian dog and human specimens and identified five different haplotypes (HVR-I
241 haplotypes I-V) (Jaleta et al., 2017). A recent study of European dogs identified a new
242 haplotype from the HVR-I region (haplotype VI) (Basso et al., 2018). In our Australian samples
243 we found haplotype II in both human and dog samples and haplotype IV in dog samples only,
244 which is consistent with the findings from Jaleta *et al.* (2017). We also identified haplotype VI
245 in a single Australian dog. Following the genotype nomenclature developed by Jaleta *et al.*
246 (2017) and Basso *et al.* (2018), in addition to these six haplotypes previously described (Jaleta
247 et al., 2017, Basso et al., 2018), we discovered two new HVR-I haplotypes; haplotypes VIII and
248 X (Figure 2, Table 1). Due to the existence of noteworthy similarities (> 99% in all cases)
249 between sequences of *S. stercoralis*, *Strongyloides procyonis*, a sequence assigned to
250 *Strongyloides* sp. Okayama (GB: LC038066.1), and our novel dog sequences, we expanded the
251 Jaletta *et al*. typing scheme to include these sequences. This involved inclusion of haplotypes
252 that could not be confidently assigned to *S. stercoralis* given the information on hand, yet are
253 highly similar to known *S. stercoralis* 18S haplotypes. This adjustment was also required
254 because a sequence attributed to *S. procyonis* (GB: AB272234.1) possesses HVR-I haplotype
255 IV, which is identical to an *S. stercoralis* genotype assigned to a Cambodian dog (GB:
256 KU724124.1). For details, refer to Figure 2 and Table 3.

257 *Insert Figure 2 here*

258

259 *Insert Table 3 here*

260

261 ***SSU* HVR-IV haplotypes detected among *S. stercoralis* from Australian dogs and humans**

262 In the Australian samples we identified HVR-IV haplotype A in both humans and dogs,
263 and haplotype B only in dogs, as previously observed (Jaleta et al., 2017). Supporting the
264 findings of Jaleta *et al*. (2017), our results also showed that haplotype II of HVR-I is found in
265 combination with haplotype A of HVR-IV, and haplotype IV of the HVR-I region is only found
266 in combination with haplotype B of the HVR-IV region (Jaleta et al., 2017). We observed that a
267 unique sequence attributed to *S. stercoralis* had been submitted to GenBank in 1993, and this
268 was assigned to haplotype C due to its historic value (GB: M84229.1). Given the strong
269 similarity between HVR-IV sequences of *S. procyonis* and *S. stercoralis* and the fact that HRV-
270 I haplotye IV (Jaleta *et al*. 2017) is also found in *S. procyonis* SSU sequences, we assigned the
271 HVR-IV sequence from *S. procyonis SSU* DNA to haplotype D (GB: AB272234.1 and
272 AB205054.1). A HVR-IV genotype 99% similar to the *Strongyloides* sp. Okayama (GB:
273 LC038066.1) was detected in Dog 45. Therefore, the HVR-IV sequence of *Strongyloides* sp.
274 Okayama was assigned to haplotype H, and the sequence from Dog 45 was assigned to
275 Haplotype I. Consequently, four new haplotypes detected in four Australian dog samples were
276 assigned to HVR-IV haplotypes E, F, G and I (Figure 1, Table 4).

277

278 *Insert Table 4 here*

279 **Clustering of *Strongyloides stercoralis* based on *cox*1 sequences**

280 A 217 base pair fragment of *cox*1 was sequenced from 20 Australian specimens
281 including those from 18 dogs and two humans, plus the *S. ratti* control (21 *cox*1 sequences in
282 total). Multiple *cox*1 types were obtained from a single specimen in many cases, revealing

283 infections caused by multiple helminth species and multiple *S. stercoralis* genotypes in a single
284 host (Figure 1). Dendrogram construction by agglomerative nested clustering revealed three
285 distinct *S. stercoralis* clades, including one occupied predominantly by worms possessing the
286 II/A *SSU* genotype, which constituted sequences obtained from dogs and humans. Four *cox*1
287 sequences obtained in this study (one from each of human 1, human 3, dog 6, and dog 18), were
288 assigned to the dog and human-infecting *S. stercoralis* clade. A *S. stercoralis* clade occupied
289 mostly by specimens possessing the I/B and V/B *SSU* genotypes was also apparent (one
290 specimen possessed the IV/B genotype), representing dog infections only (dog clade 1). None
291 of the Australian specimens were assigned to this clade (Figure 1). A final *S. stercoralis* clade
292 containing *cox*1 sequences obtained from only dogs (dog clade 2) was also dominated by
293 specimens possessing the I/B and V/B genotypes, though two specimens were also assigned the
294 IV/B. A single *cox*1 sequence from each of dogs 6 and 7 was assigned to this clade (Figure 1).

295 **Cryptic *cox*1 sequences potentially from *Strongyloides* sp. helminths that could not be**
296 **assigned to a genus or species**

297 Sequences were obtained from two dog fecal specimens (dogs 22 and 43) that potentially
298 belong to a *Strongyloides* sp. helminth but could not be confidently assigned to a species given
299 the information available. Two *cox*1 sequences were obtained for dog 22. One of these clustered
300 with a *cox*1 sequences from *Strongyloides mirzai* (GB: AB526307.1), a helminth that infects a
301 Japanese pit viper. The second sequence from dog 22 clustered between the *S. mirzai* clade and
302 the *S. fuelleborni* clade yet also clustered in a position immediately basal to all hookworms
303 (Figure 1). This sequence also obtained a nearest BLASTN hit to a *Necator* sp. sequence (GB:
304 AB793563.1), though its next best hit based on an online BLASTN search was to a sequence
305 from *S. stercoralis* (GB: LC179452.1). Three unique *cox*1 sequences were obtained from dog
306 43 (GB: MK434220, MK434221; MK434222), one clustering with *S. mirzai* and a second

307 clustering with two sequences of the *Strongyloides* sp. 'loris' clade (GB: LC197958.1,
308 LC197946.1). When submitted to an online BLASTN search, the sequence clustering alongside
309 *S. mirzai* also obtained top hits to free living nematodes (*Ektaphelenchus* sp. and
310 *Bursaphelenchus populi*, GB: JX979197.1 and HQ699854.1 respectively), yet several of its top
311 hits were also to *S. fuelleborni cox*1 sequences. The third sequence from dog 43 clustered
312 between the *Strongyloides* sp. 'loris' clade and a clade containing all *S. stercoralis* sequences
313 (Figure 1).

314 **Mixed genotype infections with *Strongyloides stercoralis***

315 Two samples had mixed *S. stercoralis* infections where a complete genotype was
316 obtained (a sequence for *cox*1, HVR-I and HVR-IV); dog 6 and dog 18. When examining the
317 number of reads that mapped to each haplotype for these specimens (not shown), for dog 6
318 approximately 20% of reads were assigned to haplotype II and 80% to haplotype IV for the
319 HVR-I region. For the HVR-IV region, approximately 20% of reads were assigned to haplotype
320 A and 80% to haplotype B. With this information it was deduced that this dog was infected with
321 two strains of *S. stercoralis*, one from the human / dog clade (genotype II/A) and another from a
322 dog-specific clade (genotype IV/B). Interestingly, two *cox*1 sequences were obtained from this
323 dog, one assigned to the human / dog clade and another assigned to dog clade 2, supporting our
324 deduction. Dog 18 was also infected with two types of *S. stercoralis*, with a genotype of II + VI
325 / A + E assigned to this specimen. Given that the number of reads assigned to each of these
326 types fell between 40% and 50%, it is difficult to link the HVR-I types identified here to their
327 corresponding HVR IV type. It should also be noted that while the specimen from dog 18
328 possessed two 18S genotypes, evidence was only found for a single *cox*1 sequence. There were
329 two *S. stercoralis* strains found in the HVR-IV region of the dog 13. Approximately 50% of
330 reads were assigned to the haplotype A and 50% to a new haplotype G.

331 **Detection of non-*Strongyloides* sp. *cox*1 sequences and mixed helminth infections**

332 Twenty-three *cox*1 sequences were attributed to *Ancylostoma* spp. and one dog (dog 6)
333 was infected with an *Ancylostoma* sp. clustering closely with *Anyclostoma ceylanicum* (Figure
334 1, cyan clade), and two distinct genotypes of *S. stercoralis*. Sequences were obtained from dogs
335 14, 15 and 16 that belong to a *Metastrongylus*-like helminth, possibly *Metastrongylus salmi*.
336 Two sequences attributed to *Ancylostoma caninum* were also obtained from dog 15, and a
337 fourth sequence belonging to an *Ancylostoma* sp. was also detected in this dog. A sequence was
338 obtained from dog 37 that obtained BLASTN hits to *S. fuelleborni* sequences (e.g., GB:
339 AB526303.1, 86.2% identity). Agglomerative nested clustering placed this sequence in a
340 position basal to all *Strongyloides* and hookworm sequences included in this analysis. This *cox*1
341 sequence also obtained close BLASTN hits (87% identity) to *Aphelenchoides* sp. (GB:
342 KX356839.1) and *Bursaphelenchus luxuriosae* (AB097863.1) which are free living
343 mycophagous and/or potentially plant parasitic nematodes. The *cox*1 from dog 28 does not
344 appear to be helminth in origin and most closely resembles a *cox*1 sequence from a rotifer;
345 *Macrotrachela quadricornifera* (GB: JX184003.1), which served as a convenient outgroup for
346 the clustering analysis (Figure 1).

347 **The presence of *Strongyloides* and other helminths in remote Australian communities**

348 *Strongyloides stercoralis* was detected in 10 specimens from seven remote communities in
349 Australia by genotypic analysis. While all specimens included in this study tested positive for *S.*
350 *stercoralis* using a published real-time PCR assay (Verweij *et al.* 2009), deep sequencing found
351 no evidence of *S. stercoralis* infection in several cases where instead, an infection with another
352 helminth (usually *Ancylostoma* spp.) was confirmed. Some of these sequences clustered closely
353 to *A. ceylanicum* yet appeared to be distinct. However, it should be noted that these sequences
354 were identical to a published sequence assigned to *A. caninum* (GB: AJ407962.1) which did not

355 overlap completely with our amplicon (50 bases short), so we cannot be sure they possess the
356 same sequence type. This necessitates the conservative assignment of these sequences to
357 *Ancylostoma* sp. Alternatively, most non-*Strongyloides* spp. sequences obtained from dogs were
358 more confidently assigned to *Ancylostoma caninum* (Figure 2). Two dogs from community 2
359 were also infected with *S. stercoralis* while in one dog from community 5, a cryptic
360 *Strongyloides* sp. possessing a unique *SSU* genotype for both HVR-I and HVR-IV was detected
361 (dog 22, genotype VIII/F). Interestingly, a *Metastrongylus*-like *cox*1 sequence was detected in
362 all dogs from community 3 that were tested, and a single dog (dog 15) from this same
363 community was infected with *A. caninum* and at least one other *Ancylostoma* sp. (Figure 2, cyan
364 clade). We propose that sequences obtained from community 6 are from environmental
365 organisms, possibly representing extraneous contaminants given that one represents a rotifer-
366 like sequence (dog 28) (GB: MK434225) and the other obtained BLASTN hits to free-living
367 nematodes (dog 37) (GB: MK434224). Community 4 and community 7 are represented by a
368 single typed specimen each (dog 18 and dog 45 respectively), that include unique sequences
369 from a helminth that we can confidently assign to the genus *Strongyloides* (Figure 1).

370 Discussion and conclusions

371 In a recent study on *S. stercoralis* from humans and dogs in Cambodia, two genetically
372 distinguishable *S. stercoralis* populations were identified based on the HVR-IV region. The
373 HVR-IV haplotype A strain was found to be dog and human infective, while HVR-IV
374 haplotype B strain was shown to be dog specific (Jaleta et al., 2017). In our study we observed
375 the same phenomenon, that HVR-IV haplotype A is associated with strains infective for both
376 humans and dogs, while HVR-IV haplotype B is restricted to strains that are only infectious to
377 dogs. Supporting earlier findings, our results also showed that haplotype II of HVR-I is found in
378 combination with haplotype A of the HVR-IV region, and haplotype IV of the HVR I region is

379 only found in combination with haplotype B, which is specific to dogs. One dog possessing the
380 HVR-I haplotype IV (dog 6) had a mixed *S. stercoralis* infection, presumably with worms of
381 the genotype II/A (a type infectious to dogs and humans) and others with the genotype IV/B (a
382 dog specific genotype). The detection of two *cox*1 sequences that cluster in the dog / human and
383 dog specific clades respectively supports this assessment. In an Australian dog, we also
384 identified HVR-I haplotype VI which has only been previously reported in European dogs.
385 Interestingly, this dog (dog 18) also had a mixed genotype infection that included a novel *S.*
386 *stercoralis* HVR-IV genotype (haplotype E), that was linked to a *cox*1 sequence clustering in
387 the dog / human *S. stercoralis* clade.

388 In agreement with previous reports, the current study demonstrated that the *SSU* HVR
389 IV region in the *SSU* rDNA can be used to detect within species differences that correspond
390 with the genetic clades that appear when the same specimens are analyzed at the *cox*1 locus
391 (Hasegawa et al., 2016, Jaleta et al., 2017) (Figure 1). To support analysis of the *cox*1 locus by
392 deep sequencing, the *cox*1 PCR assay described here was designed so that merged paired-end
393 Illumina reads span the entire length of the amplicon. This greatly reduces the complexity of *in*
394 *silico* analysis when mixed *cox*1 genotypes are encountered. A trade-off of using a short
395 amplicon for this analysis is that it may capture less diversity. Additionally, short sequences are
396 of limited use in phylogenetic analysis. However, it should be noted that phylogenies are only
397 truly relevant for constructing the evolutionary history of taxa and because evolutionary
398 analysis was not the objective of this study, agglomerative nested clustering was used to group
399 *cox*1 sequences based on their pairwise sequence identity. Despite its limitations, our *cox*1
400 assay clearly resolved the dog and human infective *S. stercoralis* genotypes into a clade that is
401 distinct from the dog-specific types, which was our primary objective, allowing us to compare
402 our results to those obtained in previous studies. Furthermore, we show that the *cox*1 fragment

403 amplified here clearly distinguishes *S. stercoralis*-derived *cox*1 amplicons from other helminth
404 species including *S. fuelleborni* and multiple species of hookworm.

405 As part of this study, we modified the typing scheme initially reported by Jaleta *et al*.
406 (2017) to include certain *SSU* sequences previously published in GenBank in order to
407 accommodate our novel genotypes. The rationale for these additions was several fold (Table 2
408 and Table 3). Firstly, we observe that a sequence published in 1993 by Putland *et al.* (1993)
409 (GB: M84229.1) was distinct from other *S. stercoralis* sequences that have been mentioned in
410 the literature since, differing from HVR-IV haplotype A by one SNP at position 125 (Figure 2)
411 (Putland et al., 1993). Consequently, the HVR-IV region of this sequence was added to the
412 typing scheme as haplotype C due to its historic value (Figure 2). The HVR-I sequence of
413 M84229.1 is so drastically different to that of other *S. stercoralis* haplotypes (and to that of any
414 other *Strongyloides* spp. in general), and given that it has not been reported in the literature
415 since its initial publication, it was not added to the typing scheme. Next, we observed that *S.*
416 *stercoralis SSU* HVR-I haplotype IV (reportedly a dog-specific type) is also found in sequences
417 assigned to *S. procyonis* from a Japanese badger (GB: AB272234.1). To reconcile this
418 observation, we incorporated the HVR-IV region of sequences assigned to *S. procyonis* into the
419 typing scheme, referring to them as haplotype D (Table 3, GB: AB272234.1 and AB205054.1).
420 This also meant that the HVR-I sequence of the *S. procyonis SSU* (GB: AB205054.1) became
421 HVR-I haplotype VII. Hence, the new *SSU* HVR-I genotype from dog 22 (GB: MK468661)
422 became type haplotype VIII. A novel SSU HVR-I genotype from dog 45 was also discovered as
423 part of this study (GB: MK468662), and its sequence was most similar to the *SSU* HVR-I region
424 from *Strongyloides* sp. Okayama, isolated from a Japanese striped snake (GB: LC038066.1). As
425 this sequence was already in GenBank prior to the commencement of this study, the HVR-I
426 region of LC038066.1 was assigned to haplotype IX, while the novel sequence obtained from

427 dog 45 was assigned to haplotype X (GB: MK468662). As haplotypes A to D for HVR-IV had
428 been assigned to other sequences, the novel genotypes discovered in dogs 18, 22 and 13 were
429 assigned to HVR-IV haplotype E, F and G respectively (GB: MK468674, MK468675, and
430 MK468676). Finally, the HVR-IV sequence from *Strongyloides* sp. Okayama
431 (GB:LC038066.1) was assigned to haplotype H because it obtained a nearest match to the
432 HVR-IV regions sequenced from dog 45, which was consequently assigned to haplotype I (GB:
433 MK468677). Also note that all HVR-I and HVR-IV types discussed above (both novel and
434 previously published) are more similar to each other than they are to the corresponding *SSU*
435 regions from *S. ratti*. Consequently, they do not provide enough information on their own to
436 make confident species assignments (Figure 2).

437 Ultimately, while the typing scheme developed by Jaleta *et al.* (2017) was originally
438 designed to consider *S. stercoralis* genotypes alone, the detection of several novel cryptic
439 genotypes that: (1) cannot be confidently assigned to a species, (2) are nonetheless greater than
440 99% similar to each other and to known *S. stercoralis* genotypes and (3), are genotypes detected
441 in the same host (dogs), means that the adjustments made here represent the most
442 straightforward solution to the issue at hand. Moreover, being an isolated continent, it is
443 possible that Australian dogs might be infected with genetically distinct *S. stercoralis* strains
444 (Cawood and Korsch, 2008). Consequently, it is not unreasonable to suggest that some of the
445 cryptic dog *Strongyloides* genotypes described herein (i.e., from dogs 18, 22, 13 and 45) might
446 be attributable to truly novel *S. stercoralis* genotypes restricted to Australia. However, a larger
447 sample number is needed along with additional sequences and morphological analysis of
448 multiple specimens before these sequences can be assigned to a species of helminth. Similarly,
449 it is plausible that the *cox*1 sequences obtained from dog 43 are attributable to a helminth

450 belonging to the genus *Strongyloides*, yet the lack of any *SSU* sequences associated with this
451 specimen makes it difficult to draw any solid conclusions in that regard.

452 This study employed an alteration of the Jaleta *et al.* (2017) genotyping assay developed
453 at the Centers for Disease Control and Prevention (J. Barratt) for adaptation to NGS
454 technologies. The assay was designed to genotype *Strongyloides* sp. and potentially detect
455 mixed helminth infections (e.g., hookworm and *Strongyloides*) when applied directly to DNA
456 extracted from faeces and other biological specimens. This method has great advantages over
457 previous genotyping techniques in that it can be undertaken directly from faecal DNA extracts
458 and does not require culture of larvae for extraction of DNA from individual worms.
459 Furthermore, the depth of sequencing provided by NGS allows the detection of all genotypes in
460 a single sample (Zahedi et al., 2017).

461 This study had a number of limitations including the collection of dog stool from the
462 environment where it could have potentially become contaminated with extraneous
463 environmental organisms or their DNA. As noted in Table 1, the *cox*1 PCR employed in this
464 study also detects multiple hookworm species, and was even found to amplify the *cox*1
465 sequence of a *Metastrongylus*-like helminth. This may represent an advantage of the method if
466 simultaneous detection and genotyping of multiple pathogenic intestinal nematodes from dogs
467 and humans is required, although results such as this should be viewed with caution. Given the
468 sensitivity of deep sequencing, we suspect that detection of *cox*1 sequences resembling those of
469 *Metastrongylus salmi* could be attributable to the consumption of pig offal by dogs in
470 community 3. While *Metastrongylus* sp. are known to occasionally infect other species
471 including humans (Calvopina et al., 2016), the genus is generally thought to be specific to pigs.
472 We also note that the genotype from dog 45 closely resembles that of a reptile-infecting
473 *Strongyloides* sp. and its presence in a dog could be due to consumption of reptiles or reptile

474 feces by the dog. Consequently, these cases may represent an incidental finding rather than true
475 infections with a *Metastrongylus*-like helminth or a *Strongyloides* sp. resembling those found in
476 reptiles. Similarly, the *cox*1 assay described here detected DNA from potentially free – living
477 nematodes. A sequence obtained from dog 37 received a BLASTN hit to *S. fuelleborni* (GB:
478 AB526306.1), though with only 86.2% identity. However, agglomerative nested clustering
479 placed this sequence in a position basal to all *Strongyloides* and hookworm sequences included
480 in this analysis. This sequence also obtained close BLASTN hits (87% identity) to
481 *Aphelenchoides* sp. (GB: KX356839.1) and *Bursaphelenchus luxuriosae* (AB097863.1) which
482 are mycophagous and/or potentially plant parasitic nematodes. This sequence could therefore
483 represent a free-living nematode that came into contact with the fecal specimens in the
484 environment between when the stool was passed and collected. Surprisingly, a sequence similar
485 to one obtained from a rotifer; a free-living extremely distant relative of nematodes, was
486 detected in the specimen from dog 28 using this assay. This also likely represents contamination
487 of the stool specimen from the local environment prior to its collection.

488 Several unique sequences assigned to *Strongyloides* spp., *Ancylostoma* spp.,
489 *Metastrongylus* sp. and some unknown helminth species were detected for the first time using
490 the technology described here. We propose that some of the cryptic *Strongyloides* sp.
491 genotypes we encountered are potentially unique to the Australian continent and may have
492 diverged from southeast Asian *Strongyloides* populations as a result of vicariance. Discovery of
493 a *cox*1 sequence that clusters most closely to a *Strongyloides* sp. identified from a slow loris
494 might support this (dog 43, GB: MK434221), given that lorises are endemic to southeast Asia.
495 Aside from these cryptic types, several examples of the classic *S. stercoralis* genotypes
496 described by Jaleta *et al.* (2017) were detected. Consequently, our study was able to
497 independently support previous reports of at least two genetically distinct groups of *S.*

498 *stercoralis*; one infecting both dogs and humans and another group that is specific to dogs.
499 While this does not prove the potential of dogs as a reservoir of *S. stercoralis*, it supports the
500 hypothesis of zoonotic transmission in remote Australian communities. As discussed previously
501 and with respect to the One Health approach, we suggest that humans and dogs should be
502 treated concomitantly in these communities to control strongyloidiasis (Beknazarova et al.,
503 2017b, Jaleta et al., 2017). Ultimately, we confirm for the first time that potentially zoonotic *S.*
504 *stercoralis* genotypes are present in Australia and suggest that dogs represent a potential
505 reservoir of human strongyloidiasis in remote Australian communities.

506 **Author Contributions**

507 MB: Designed study, obtained ethics approvals, collected specimens, extracted DNA, ran real-
508 time PCR, optimized and ran PCR assays for *SSU* HVR-I, HVR-IV and *cox*1, performed
509 Illumina library preparation, performed *in-silico* analysis, data analysis and interpretation, wrote
510 and reviewed draft manuscripts; JB: Designed PCR assay for *SSU* HVR-I, HVR-IV and *cox*1,
511 performed *in silico* analysis and data interpretation, constructed cluster dendrogram, wrote and
512 reviewed manuscript drafts; RB: Designed study, reviewed manuscript drafts, obtained funding,
513 reviewed manuscript drafts; ML: Carried out PCR product normalization, prepared Illumina
514 libraries for deep sequencing, and sequenced amplicons; HW: Provided PhD supervision of
515 MB, designed study, obtained ethics approvals, obtained funding, reviewed manuscript drafts;
516 KR: Provided PhD supervision of MB, designed study, obtained ethics approvals, obtained
517 funding, reviewed manuscript drafts.

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619 ## Supporting Information Legends

620 **S1 Table. GenBank accession numbers of the *SSU* HVR I and IV and *cox*1 haplotypes**.

621 The GenBank accession numbers are provided for the *SSU* HVR I and IV haplotypes generated

622 in this study (MK468654-MK468677). The GenBank accession numbers are provided for the

623 *cox*1 haplotypes generated in this study (MK434217- MK434258).

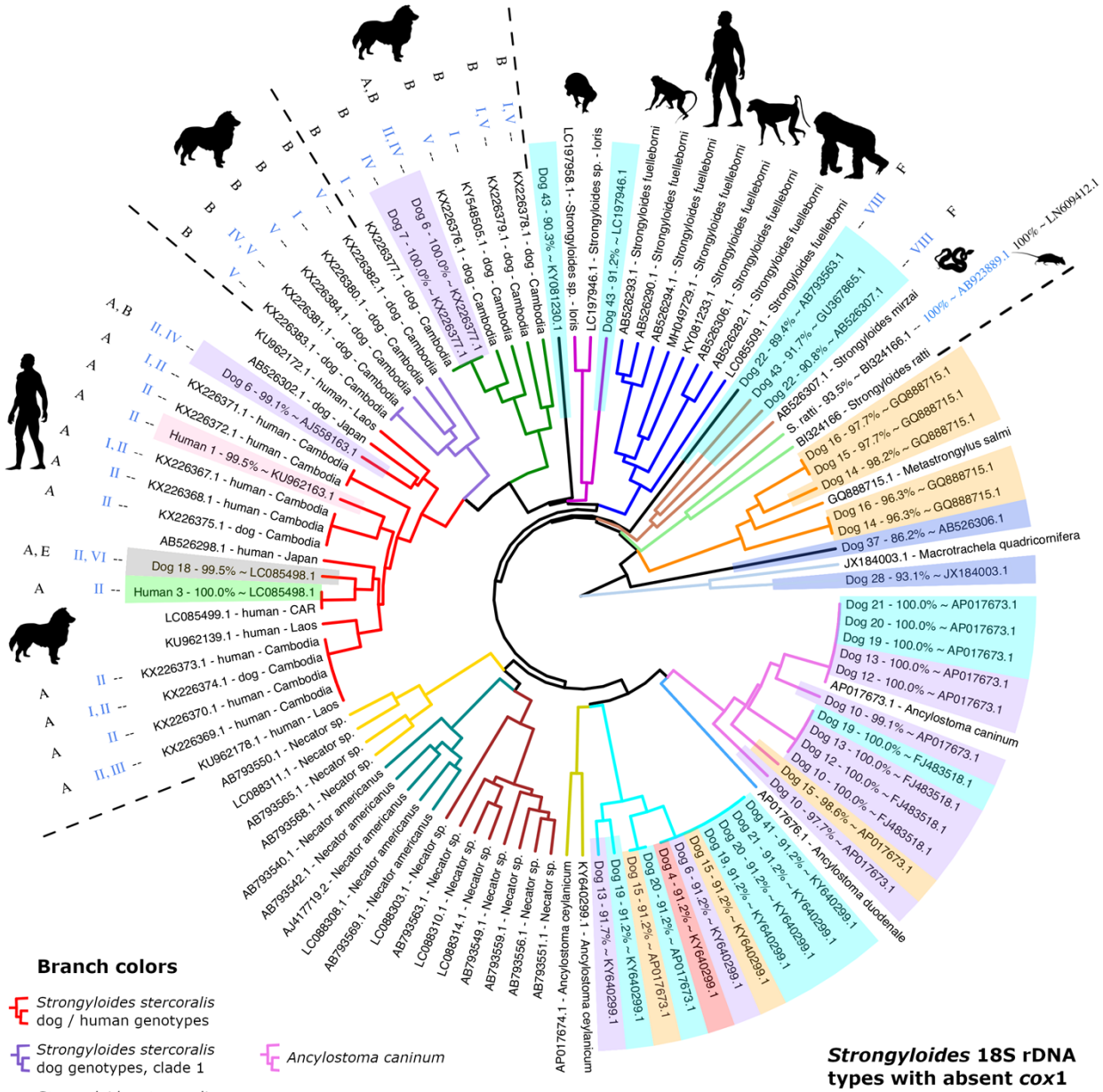
Dog 43 - 90.3% ~ KY081230.1
LC197958.1 - Strongyloides sp. - loris
LC197946.1 - Strongyloides sp. - loris
Dog 43 - 91.2% ~ LC197946.1
AB526293.1 - Strongyloides fuelleborni
AB526290.1 - Strongyloides fuelleborni
AB526294.1 - Strongyloides fuelleborni
MH049729.1 - Strongyloides fuelleborni
KY081233.1 -Strongyloides fuelleborni
AB526306.1 - Strongyloides fuelleborni
AB526282.1 - Strongyloides fuelleborni
LC085509.1 - Strongyloides fuelleborni
Dog 22 - 89.4% ~ AB793563.1
Dog 43 - 91.7% ~ GU367865.1
Dog 22 - 90.8% ~ AB526307.1
AB526307.1 - Strongyloides mirzai
BI324166 - Strongyloides ratti
S. ratti - 93.5% ~ BI324166.1
Dog 16 - 97.7% ~ GQ888715.1
Dog 15 - 97.7% ~ GQ888715.1
Dog 14 - 98.2% ~ GQ888715.1
GQ888715.1 - Metastrongylus salmi
Dog 16 - 96.3% ~ GQ888715.1
Dog 14 - 96.3% ~ GQ888715.1
Dog 37 - 86.2% ~ AB526306.1
JX184003.1 - Macrotrachela quadricornifera
Dog 28 - 93.1% ~ JX184003.1
100% ~ AB923889.1
100% ~ LN609412.1
Dog 21 - 100.0% ~ AP017673.1
Dog 20 - 100.0% ~ AP017673.1
Dog 19 - 100.0% ~ AP017673.1
Dog 13 - 100.0% ~ AP017673.1
Dog 12 - 100.0% ~ AP017673.1
AP017673.1 - Ancylostoma caninum
Dog 10 - 99.1% ~ AP017673.1
Dog 19 - 100.0% ~ FJ483518.1
Dog 13 - 100.0% ~ FJ483518.1
Dog 12 - 100.0% ~ FJ483518.1
Dog 10 - 100.0% ~ FJ483518.1
Dog 15 - 98.6% ~ AP017673.1
Dog 10 - 97.7% ~ AP017673.1
AP017676.1 - Ancylostoma duodenale
Dog 41 - 91.2% ~ KY640299.1
Dog 21 - 91.2% ~ KY640299.1
Dog 20 - 91.2% ~ KY640299.1
Dog 19 - 91.2% ~ KY640299.1
Dog 15 - 91.2% ~ KY640299.1
Dog 6 - 91.2% ~ KY640299.1
Dog 4 - 91.2% ~ AP017673.1
Dog 20 - 91.2% ~ AP017673.1
Dog 15 - 91.2% ~ KY640299.1
Dog 19 - 91.2% ~ KY640299.1
Dog 13 - 91.7% ~ KY640299.1
KY640299.1 - Ancylostoma ceylanicum
AP017674.1 - Ancylostoma ceylanicum
AB793551.1 - Necator sp.
AB793556.1 - Necator sp.
AB793559.1 - Necator sp.
AB793549.1 - Necator sp.
LC088314.1 - Necator sp.
LC088310.1 - Necator sp.
AB793563.1 - Necator sp.
LC088303.1 - Necator sp.
AB793569.1 - Necator sp.
LC088308.1 - Necator americanus
AJ417719.2 - Necator americanus
AB793542.1 - Necator americanus
AB793540.1 - Necator americanus
AB793568.1 - Necator sp.
AB793565.1 - Necator sp.
LC088311.1 - Necator sp.
AB793550.1 - Necator sp.
KU962178.1 - human - Laos
KX226369.1 - human - Cambodia
KX226370.1 - human - Cambodia
KX226374.1 - dog - Cambodia
KX226373.1 - human - Cambodia
KU962139.1 - human - Laos
LC085499.1 - human - CAR
Human 3 - 100.0% ~ LC085498.1
Dog 18 - 99.5% ~ LC085498.1
AB526298.1 - human - Japan
KX226375.1 - dog - Cambodia
KX226368.1 - human - Cambodia
KX226367.1 - human - Cambodia
Human 1 - 99.5% ~ KU962163.1
KX226372.1 - human - Cambodia
KX226371.1 - human - Cambodia
Dog 6 - 99.1% ~ AJ558163.1
AB526302.1 - dog - Japan
KU962172.1 - human - Laos
KX226383.1 - dog - Cambodia
KX226381.1 - dog - Cambodia
KX226384.1 - dog - Cambodia
KX226380.1 - dog - Cambodia
KX226382.1 - dog - Cambodia
KX226377.1 - dog - Cambodia
Dog 7 - 100.0% ~ KX226377.1
Dog 6 - 100.0% ~ KX226377.1
KX226376.1 - dog - Cambodia
KY548505.1 - dog - Cambodia
KX226379.1 - dog - Cambodia
KX226378.1 - dog - Cambodia
Branch colors
Strongyloides stercoralis dog / human genotypes
Strongyloides stercoralis dog genotypes, clade 1
Strongyloides stercoralis dog genotypes, clade 2
Strongyloides sp. 'loris' and similar types
Strongyloides fuelleborni
Strongyloides mirzai and similar types
Strongyloides ratti
Matastrongylus salmi and similar types
Macrotrachela quadricornifera (a rotifer) and similar types
Ancylostoma caninum
Ancylostoma duodenale
Ancylostoma sp.
Ancylostoma ceylanicum
Necator sp. clade 1
Necator americanus
Necator sp. clade 2
Other taxa
Specimen shading
Community 1 - Northern Territory, Australia
Community 2 - Northern Territory, Australia
Community 3 - Northern Territory, Australia
Community 4 - Northern Territory, Australia
Community 5 - Northern Territory, Australia
Community 6 - Northern Territory, Australia
Community 7 - Northern Territory, Australia
Human - New South Wales, Australia
Human - Northern Territory, Australia
Human - Queensland, Australia
Strongyloides 18S rDNA types with absent cox1 sequence
HVR I
HVR IV
Dog 13 - A, G
Dog 32 - A
Dog 45 X I
Human 2 - A
Sputum II A

Figure Legends

Figure 1. Dendrogram of clustered *cox*1 amplicons from Australian dog and human specimens

This dendrogram includes *cox*1 sequences generated in this study and a selection of previously published *cox*1 sequences that overlap with our 217 base pair *cox*1 amplicon by 100%. Specimens analyzed as part of this study are shaded according to their site of collection. Branches are color coded according to their identity; either a species assignment, a proposed genus assignment, or their *S. stercoralis* genotype. When available, *Strongyloides* sp. *cox*1 sequences are annotated with their associated *SSU* genotypes, with their HVR-I type shown in blue and their HVR-IV type shown in black. Specimens for which a *cox*1 sequence was not obtained are shown in a table embedded in the figure (bottom right), which includes two specimens possessing unique 18S genotypes; dog 13 (HVR-IV, type G) and dog 45 (HVR-I, type X). A dash (-) shown in this table indicates failed amplification and/or sequencing of that marker. 'Sputum' refers to the sole sputum sample from a human patient (human 4) included in this study. Sequences published in previous studies that are not from *S. stercoralis* are labelled with their GenBank and/or DNA Data Bank of Japan accession numbers followed by their species name. *Strongyloides stercoralis* sequences from previous studies are labelled with their accession number, host species, and country of origin. Note that 'CAR' means Central African Republic. Names of specimens collected as part of this study begin with a host name and a unique number assigned in this study, followed by a percentage similarity to (~) a near BLASTN hit identifiable by its accession number.

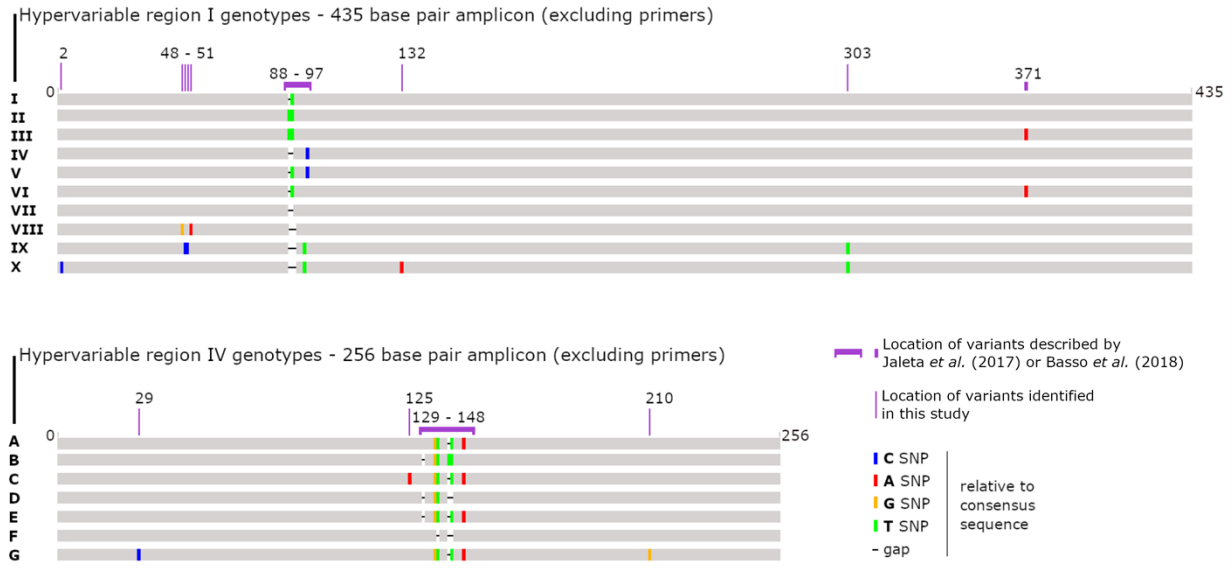

Figure 2. Schematic detailing the proposed modifications to the previously described *S. stercoralis* genotyping scheme

A graphical representation of the novel *Strongyloides* genotypes discovered in this study compared to haplotypes identified in previous reports. The location of sites that were genotypically informative based on the original genotyping method described by Jaletta *et al.* (2017) and Basso *et al.* (2018) are indicated, as well as new SNP/indel sites that have been incorporated into the typing scheme based on the results of this study (Jaleta et al., 2017, Basso et al., 2018). For hypervariable region I, we introduce two novel types (VIII and X), and assign new haplotype names to published sequences that had not been previously considered in this typing scheme (VII and IX). For hypervariable region IV, we introduce three novel types (E, F, G and I), and assign new haplotype names to published sequences that had not been previously considered in this typing scheme (C, D and H) (see Tables 3 and 4 for details).

Tables

Table 1: Primers and probes and PCR conditions

Primer	Amplicon	Sequence	Reaction conditions
Stro18S-1530F Stro18S-1630R Stro18S-1586T FAM	rDNA 101 bp	5'-GAATTCCAAGTAAACGTAAGTCATTAGC-3' 5'-TGCCTCTGGATATTGCTCAGTTC-3' 5'-ACACACCGGCCGTCGCTGC-3'-BHQ1	**Step 1:** 95°C for 15 min, **Step 2:** 95°C for 15 s, **Step 3:** 60°C for 30 s. Repeat steps two and three 40 times.
SSP_COX1_F * SSP_COX1_R *	mtDNA 270 bp	5'-TTTGATCCTAGTTCTGGTGGTAATCC-3' 5'-GTAGCAGCAGTAAAATAAGCACGAGA-3'	**Step 1:** 98°C for 30 s, **Step 2:** 98°C for 10 s, **Step 3:** 60°C for 10 s, **Step 4:** 72°C for 10 s, **Step 5:** 72 °C for 2 min. Repeat steps two to four 45 times.
NEW_HVR_I_F NEW_HVR_I_R	rDNA 500 bp	5'-GCTCATTATAACAGCTATAGACTACACGGTA-3' 5'-CCACAACAATCATTTTATGCACTTGG-3'	**Step 1:** 98°C for 30 s, **Step 2:** 98°C for 10 s, **Step 3:** 60°C for 10 s, **Step 4:** 72°C for 10 s, **Step 5:** 72 °C for 2 min. Repeat steps two to four 45 times.
NEW_HVR_IV_F NEW_HVR_IV_R	rDNA 320 bp	5'-CGGGCCGGACACTATAAGG-3' 5'-ATCTCTAAACAGGAACATAATGATCACTAC-3'	**Step 1:** 98°C for 30 s, **Step 2:** 98°C for 10 s, **Step 3:** 63°C for 10 s, **Step 4:** 72°C for 10 s, **Step 5:** 72 °C for 2 min. Repeat steps two to four 45 times.

*Broadly specific primers for amplification of *Strongyloides* sp. *cox*1 sequences as well as those of several hookworms

Table 2: Human and dog samples analyzed in this study and their complete genotype

Sample	HVR-I haplotype	HVR-IV haplotype	*Cox*1 Accessions
Human 1	Haplotype II	Haplotype A	MK434219†
Human 2	NA (excluded due to *S. ratti* contamination)	Haplotype A	NA
Human 3	Haplotype II	Haplotype A	MK434218
Sputum (Human 4)	Haplotype II	Haplotype A	NA
Dog 4	NA	NA	MK434258†
Dog 6	Haplotype II and Haplotype IV	Haplotype A and Haplotype B	MK434255 MK434256† MK434257†
Dog 7	Haplotype IV	Haplotype B	MK434254
Dog 10	NA	NA	MK434251 MK434252† MK434253†
Dog 12	NA	NA	MK434249 MK434250
Dog 13	NA	Haplotype A and Haplotype G*	MK434246 MK434247 MK434248†
Dog 14	NA (excluded due to *S. ratti* contamination)	NA	MK434244† MK434245†
Dog 15	NA	NA	MK434240† MK434241† MK434242† MK434243†
Dog 16	NA	NA	MK434238† MK434239†
Dog 18	Haplotype II and Haplotype VI	Haplotype A and Haplotype E*	MK434237†
Dog 19	NA	NA	MK434233 MK434234 MK434235† MK434236†
Dog 20	NA	NA	MK434230

			MK434231† MK434232†
Dog 21	NA	NA	MK434228 MK434229†
Dog 22	Haplotype VIII*	Haplotype F*	MK434226† MK434227†
Dog 28	NA	NA	MK434225†
Dog 32	NA	Haplotype A	NA
Dog 37	NA	NA	MK434224†
Dog 41	NA	NA	MK434223†
Dog 43	NA	NA	MK434220† MK434221† MK434222†
Dog 45	Haplotype X*	Haplotype I*	NA

* Novel *SSU* haplotypes, †Novel *cox*1 sequences

Note: All GenBank Accession numbers associated with the sequences generated in this study are provided in Supplementary Table S1

Table 3. HVR I genotypes assigned to *Strongyloides* sp. based on current data

Haplotypes	GenBank Accession/s	Notes
I	AB923888.1, AF279916.2, AJ417023.1, MK468654	Found in dogs and humans.
II	KF926659.1, MK468655, MK468656, MK468657, MK468658	Found in dogs and humans.
III	AB453315.1	Fund in dogs and humans.
IV	AB272234.1, KU724124.1, MK468663	This haplotype has been detected in dogs though not in humans. It has also been described in a badger where it was assigned to *S. procyonis.*
V	Jaleta *et al*. (2017)	Described by Jaleta *et al*. (2017) but the sequence of this type was not available in GenBank prior to this study. This is potentially a dog-specific haplotype.
VI	AB453316.1, AB453314.1, MH932098.1, MH932099.1, MH932100.1, MK468660	Assigned only to *S. stercoralis*. Identified in dog 18 from this study. Described predominantly in dogs but also in a chimpanzee (AB453314.1).
VII	AB205054.1	A *S. procyonis* sequence greater than 99% similar to *S. stercoralis* Haplotype I and Haplotype IV.
VIII	MK468661	Novel *Strongyloides* sp. sequence from Dog 22 most similar to Haplotype VII.
IX	LC038066.1	*Strongyloides* sp. Okayama isolated from a Japanese striped snake. This sequence is greater than 99% identical to sequences of *S. stercoralis* and *S. procyonis*. Similar to Haplotype X (see below) identified in an Australian dog.
X	MK468662	Sequence identified in dog 45 in present study. Most similar to *Strongyloides* sp. Okayama (Haplotype IX).

Table 4. HVR IV genotypes assigned to *Strongyloides* sp. based on current data

Haplotypes	GenBank Accession/s	Notes
A	KY081221.1, KU724128.1, KU724125.1, LC085483.1, LC085482.1, LC085481.1, KU962182.1, KU962181.1, KU962180.1, KU962179.1, AB923888.1, KF926662.1, KF926661.1, AF279916.2, MH932097.1, MH932097.1, MH932095.1, KY081223.1, AB526826.1, AB453316.1, AB453315.1, AB453314.1, MK468664 - MK468671	Identified in dogs, humans and chimpanzees. A *S. stercoralis*- specific haplotype
B	KU724129.1	Identified only in dogs. Consistently assigned to *S. stercoralis*
C	M84229.1	This sequence was published in GenBank in 1993 and assigned to *S. stercoralis*. It has not appeared in the literature since based on our knowledge, though we assigned to genotype C for its historic value. It shares one SNP difference to type A (Figure 2).
D	AB272234.1, AB205054.1	Includes two sequences assigned to *S. procyonis*. This type is 99% similar to Haplotype B.
E	MK468674	Novel genotype identified in dog 18. Most similar to Haplotype A.
F	MK468675	Novel genotype identified in dog 22. Most similar to Haplotype D.
G	MK468676	Novel genotype identified in dog 13. Most similar to Haplotype A.
H	LC038066.1	*Strongyloides* sp. Okayama isolated from a Japanese striped snake.
I	MK468677	Novel genotype identified in dog 45. Most similar to Haplotype H.

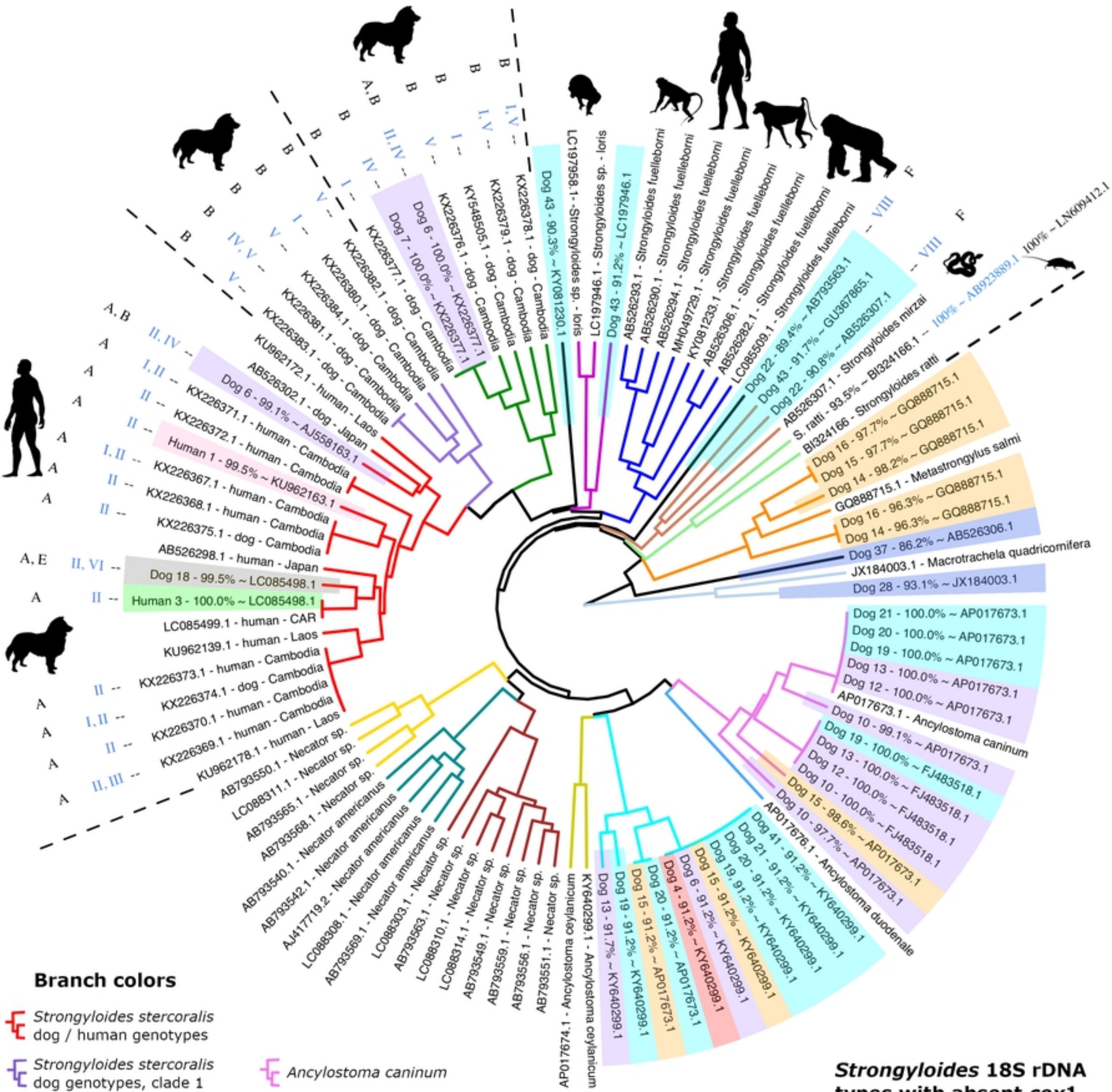
Dog 43 - 90.3% ~ KY081230.1
LC197958.1 - Strongyloides sp. - loris
LC197946.1 - Strongyloides sp. - loris
Dog 43 - 91.2% ~ LC197946.1
AB526293.1 - Strongyloides fuelleborni
AB526290.1 - Strongyloides fuelleborni
AB526294.1 - Strongyloides fuelleborni
MH049729.1 - Strongyloides fuelleborni
KY081233.1 - Strongyloides fuelleborni
AB526306.1 - Strongyloides fuelleborni
AB526282.1 - Strongyloides fuelleborni
LC085509.1 - Strongyloides fuelleborni
Dog 22 - 89.4% ~ AB793563.1
Dog 43 - 91.7% ~ GU367865.1
Dog 22 - 90.8% ~ AB526307.1
AB526307.1 - Strongyloides mirzai
S. ratti - 93.5% ~ BI324166.1
BI324166 - Strongyloides ratti
Dog 16 - 97.7% ~ GQ888715.1
Dog 15 - 97.7% ~ GQ888715.1
Dog 14 - 98.2% ~ GQ888715.1
GQ888715.1 - Metastrongylus salmi
Dog 16 - 96.3% ~ GQ888715.1
Dog 14 - 96.3% ~ GQ888715.1
Dog 37 - 86.2% ~ AB526306.1
JX184003.1 - Macrotrachela quadricornifera
Dog 28 - 93.1% ~ JX184003.1
Dog 21 - 100.0% ~ AP017673.1
Dog 20 - 100.0% ~ AP017673.1
Dog 19 - 100.0% ~ AP017673.1
Dog 13 - 100.0% ~ AP017673.1
Dog 12 - 100.0% ~ AP017673.1
AP017673.1 - Ancylostoma caninum
Dog 10 - 99.1% ~ AP017673.1
Dog 19 - 100.0% ~ FJ483518.1
Dog 13 - 100.0% ~ FJ483518.1
Dog 12 - 100.0% ~ FJ483518.1
Dog 10 - 100.0% ~ FJ483518.1
Dog 15 - 98.6% ~ AP017673.1
Dog 10 - 97.7% ~ AP017673.1
AP017676.1 - Ancylostoma duodenale
Dog 41 - 91.2% ~ KY640299.1
Dog 21 - 91.2% ~ KY640299.1
Dog 20 - 91.2% ~ KY640299.1
Dog 19, 91.2% ~ KY640299.1
Dog 15 - 91.2% ~ KY640299.1
Dog 6 - 91.2% ~ KY640299.1
Dog 4 - 91.2% ~ KY640299.1
Dog 20 - 91.2% ~ AP017673.1
Dog 15 - 91.2% ~ AP017673.1
Dog 19 - 91.2% ~ KY640299.1
Dog 13 - 91.7% ~ KY640299.1
KY640299.1 - Ancylostoma ceylanicum
AP017674.1 - Ancylostoma ceylanicum
AB793551.1 - Necator sp.
AB793556.1 - Necator sp.
AB793559.1 - Necator sp.
AB793549.1 - Necator sp.
LC088314.1 - Necator sp.
LC088310.1 - Necator sp.
AB793563.1 - Necator sp.
LC088303.1 - Necator sp.
AB793569.1 - Necator americanus
LC088308.1 - Necator americanus
AJ417719.2 - Necator americanus
AB793542.1 - Necator americanus
AB793540.1 - Necator americanus
AB793568.1 - Necator sp.
AB793565.1 - Necator sp.
LC088311.1 - Necator sp.
AB793550.1 - Necator sp.
KU962178.1 - human - Laos
KX226369.1 - human - Cambodia
KX226370.1 - human - Cambodia
KX226374.1 - dog - Cambodia
KX226373.1 - human - Cambodia
KU962139.1 - human - Laos
LC085499.1 - human - CAR
Human 3 - 100.0% ~ LC085498.1
Dog 18 - 99.5% ~ LC085498.1
AB526298.1 - human - Japan
KX226375.1 - dog - Cambodia
KX226368.1 - human - Cambodia
KX226367.1 - human - Cambodia
Human 1 - 99.5% ~ KU962163.1
KX226372.1 - human - Cambodia
KX226371.1 - human - Cambodia
Dog 6 - 99.1% ~ AJ558163.1
AB526302.1 - dog - Japan
KU962172.1 - human - Laos
KX226383.1 - dog - Cambodia
KX226381.1 - dog - Cambodia
KX226384.1 - dog - Cambodia
KX226380.1 - dog - Cambodia
KX226382.1 - dog - Cambodia
KX226377.1 - dog - Cambodia
Dog 7 - 100.0% ~ KX226377.1
Dog 6 - 100.0% ~ KX226377.1
KX226376.1 - dog - Cambodia
KY548505.1 - dog - Cambodia
KX226379.1 - dog - Cambodia
KX226378.1 - dog - Cambodia
100% ~ LN609412.1
100% ~ AB923889.1
Branch colors
Strongyloides stercoralis dog / human genotypes
Strongyloides stercoralis dog genotypes, clade 1
Strongyloides stercoralis dog genotypes, clade 2
Strongyloides sp. 'loris' and similar types
Strongyloides fuelleborni
Strongyloides mirzai and similar types
Strongyloides ratti
Matastrongylus salmi and similar types
Macrotrachela quadricornifera (a rotifer) and similar types
Ancylostoma caninum
Ancylostoma duodenale
Ancylostoma sp.
Ancylostoma ceylanicum
Necator sp. clade 1
Necator americanus
Necator sp. clade 2
Other taxa
Specimen shading
Community 1 - Northern Territory, Australia
Community 2 - Northern Territory, Australia
Community 3 - Northern Territory, Australia
Community 4 - Northern Territory, Australia
Community 5 - Northern Territory, Australia
Community 6 - Northern Territory, Australia
Community 7 - Northern Territory, Australia
Human - New South Wales, Australia
Human - Northern Territory, Australia
Human - Queensland, Australia
Strongyloides 18S rDNA types with absent cox1 sequence
HVR I
HVR IV
Dog 13 - A, G
Dog 32 - A
Dog 45 - X I
Human 2 - A
Sputum II A

Figure 1

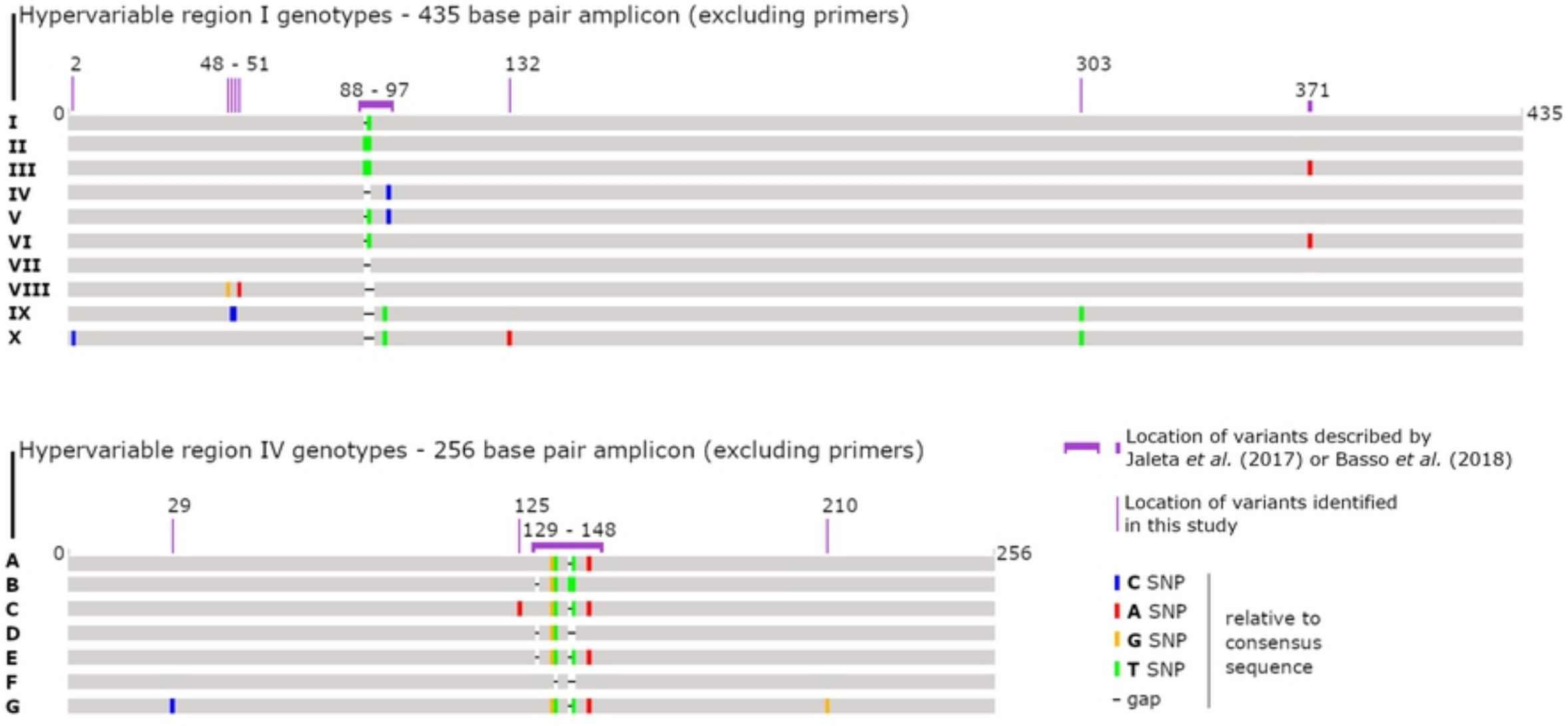
Hypervariable region I genotypes - 435 base pair amplicon (excluding primers)
2
48 - 51
88 - 97
132
303
371
0
435
I
II
III
IV
V
VI
VII
VIII
IX
X
Hypervariable region IV genotypes - 256 base pair amplicon (excluding primers)
29
125
129 - 148
210
0
256
A
B
C
D
E
F
G
Location of variants described by Jaleta et al. (2017) or Basso et al. (2018)
Location of variants identified in this study
C SNP
A SNP
G SNP
T SNP
- gap
relative to consensus sequence

Figure 2