TITLE: Genetic diversity and population structure of Angiostrongylus vasorum parasites within and between local urban foxes (Vulpes Vulpes)

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1 **Genetic diversity and population structure of *Angiostrongylus***

2 ***vasorum* parasites within and between local urban foxes (*Vulpes***

3 ***vulpes*)**

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19 Declarations of interest: none

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21 **Abstract**

22 *Angiostrongylus vasorum* is a nematode parasite of the pulmonary arteries and heart that infects

23 domestic and wild canids. Dogs (*Canis familiaris*) and red foxes (*Vulpes vulpes*) are the most

24 commonly affected definitive hosts. Recent studies suggest that angiostrongylosis is an emerging

25 disease, and that red foxes may play an important role in the epidemiology of the parasite. Genetic

26 analyses of parasites collected from dogs and foxes throughout Europe have shown that the same

27 parasite haplotypes are commonly shared between different host species. However, the extent of

28 genetic diversity within local *A. vasorum* populations and individual hosts is unknown. The objective of

29 the present study was to assess the occurrence of genetic diversity among *A. vasorum* (a) recovered

30 from different foxes within the Greater London area (a localised population, single worm per fox

31 dataset); and (b) hosted within single foxes (multiple worms per fox dataset). During 2016, *A.*

32 *vasorum* worms were collected from foxes culled for other purposes in London. DNA was extracted

33 from each parasite and a partial fragment of the mitochondrial cytochrome oxidase subunit 1 (mtCOI)

34 gene was amplified and sequenced. Sequences from the single worm dataset were compared with

35 those published elsewhere. Combined, 19 haplotypes were described of which 15 were identified

36 from foxes found in London, indicating that considerable genetic diversity can be detected within a

37 local geographic area. Analysis of the multiple worm dataset identified 22 haplotypes defining worms

38 recovered from just six foxes, emphasising the relevance of wild canines as reservoirs of genetic

39 diversity. This is the first study to explore the genetic complexity of individual fox-hosted *A. vasorum*

40 populations.

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43 **Keywords:** *Angiostrongylus vasorum*; mtCOI gene; dog; genetic variation; red fox; reservoir.

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Highlights

- Foxes may act as wildlife reservoirs of *Angiostrongylus vasorum* for dogs in Europe

- Considerable genetic diversity was found among *A. vasorum* from foxes in Greater London

- First report highlighting genetic diversity of *A. vasorum* within individual foxes

- Foxes may act as reservoirs of genetic diversity of *A. vasorum* in the UK

1. Introduction

Angiostrongylus vasorum is a nematode from the family Metastrongylidae that affects the heart and pulmonary arteries of domestic and wild canids (Jefferies et al., 2010). The dog (*Canis familiaris*) and the red fox (*Vulpes vulpes*) are the main definitive hosts. However, other canid species such as the wolf (*Canis lupus*) and the coyote (*Canis latrans*) have also been described as definitive hosts (Segovia et al., 2001; Bourque et al., 2005), as well as some non-canid species such as the Eurasian badger (*Meles meles* L.) and otter (*Lutra lutra*) (Torres et al., 2001; Santoro et al., 2017). The life cycle of *A. vasorum* is indirect, with various species of gastropod molluscs acting as obligatory intermediate hosts (Morgan et al., 2008). In addition, other animals like frogs and birds can transmit the parasite as paratenic hosts (Bolt et al., 1993; Mozzer and Lima, 2015).

Clinical signs associated with *A. vasorum* infection in dogs can be unspecific and highly variable (Di Cesare et al., 2015). However, cardiorespiratory signs are most common , occurring alone or combined with bleeding and neurological disorders. This can eventually lead to death (Morgan et al., 2010; Helm and Morgan, 2017). In foxes, clinical signs of angiostrongylosis have been associated with the respiratory and cardiovascular systems (Jeffery et al., 2004; Morgan et al., 2008). Some studies have reported that infected foxes can present with right ventricular hypertrophy on post-mortem examination (Poli et al., 1984; Morgan et al., 2008), suggesting that the parasite might affect the health and fitness of these animals. In contrast, Jeffery et al. (2004) reported that infected foxes had a lower mean heart mass ratio compared with uninfected foxes. Disseminated cases of angiostrongylosis have recently been identified as cause of death in wild foxes from Italy (Eleni et al., 2014). However, foxes experimentally infected with *A. vasorum* did not show clinical signs during the time observed, other than elevated mean blood eosinophil counts (Webster et al., 2017).

76   *Angiostrongylus vasorum* is widely distributed and, to date, has been found in Europe, Africa, and

77   some areas of North and South America (Jefferies et al., 2009b). The red fox is considered to be the

78   main sylvatic host in Europe (Helm et al., 2010). The parasites' prevalence in foxes and clinical

79   incidence in dogs has been reported to be restricted to endemic foci throughout Europe and North

80   America, only occurring sporadically outside of these foci (Morgan et al., 2005). Despite this, recent

81   studies show that *A. vasorum* is an emerging disease in dogs, since the parasite seems to be

82   spreading within Europe to areas where it has not previously been identified (Helm et al., 2010; Kirk et

83   al., 2014; Maksimov et al., 2017). Prevalence of *A. vasorum* in dogs appears to have increased in

84   recent years, including examples such as Germany (Barutzki et al., 2017).

85

86   Several studies on *A. vasorum* prevalence and distribution have been conducted in dog and fox

87   populations of Great Britain, with endemic foci recognised in the South of England and Wales for

88   more than two decades (Simpson, 1996). Recently, a small number of cases have been reported in

89   the North of England and Scotland (Helm et al., 2015), supporting the hypothesis that *A. vasorum* is

90   an emerging disease (Helm et al., 2010; Kirk et al., 2014). Reasons for these recent increases are

91   unclear, with dog transportation and the expansion of fox ranges suggested (Al-Sabi et al., 2013;

92   Morgan et al., 2009; van Doorn et al., 2009). The distribution of *A. vasorum* is also thought to be

93   influenced by climatic and environmental conditions that may modify parasite population dynamics

94   and activity of its intermediate hosts, snails and slugs (Morgan et al., 2009). Given that the mean

95   winter temperature throughout Great Britain commonly exceeds the limit reported by Jeffery et al.

96   (2004), it can be assumed that transmission is possible in a much greater area than has been

97   described to date (Morgan et al., 2009), suggesting that dog populations will be at greater risk in the

98   future if the disease spreads to its potential (Morgan et al., 2010).

99

100   Wildlife, particularly red foxes, may play an important role in *A. vasorum* epidemiology since they

101   have been identified as a reservoir for canine angiostrongylosis (Bolt et al., 1992; Taylor et al., 2015).

102   Recent genetic analyses have shown that the same *A. vasorum* haplotypes can be found in different

103   species of definitive hosts (Jefferies et al., 2009b, 2010), supporting the importance of wildlife as

104   reservoir hosts. Genetic comparison of parasite populations across broad geographic ranges using

105   markers such as mitochondrial cytochrome oxidase subunit 1 (mtCOI) have identified 24 different

106   haplotypes in parasites recovered from dogs and foxes in Europe (Jefferies et al., 2010), but the

107   occurrence of diversity within local parasite populations, and even within individual hosts, remains

108   unclear. For this reason, this study aimed to define the genetic diversity of *A. vasorum* in foxes from

109   Greater London (1,569 km^2), as well as the diversity within multiple individual wild definitive hosts, in

110   order to contribute to an assessment of the risk that foxes pose to dog health as parasite reservoirs.

111   The objective of the present study was to assess the genetic diversity of *A. vasorum* (a) hosted in

112   foxes within Greater London area; and (b) hosted within individual foxes. Our hypothesis was that

113   foxes are likely to be reservoirs of genetic diversity for *A. vasorum* since their diet is likely to lead to (i)

114   significant and repeated parasite exposure and, in the absence of routine de-worming (ii)

115   accumulation of multiple adult worms. Thus, the opportunity for sex between genetically distinct

116   worms and successful reproduction would be high in foxes.

117

2. Methods

119   *2.1. Parasite isolation*

120   Red foxes were culled as part of a routine pest control programme in the Greater London urban area

121   throughout 2016 (Supplementary Fig. 1). All foxes were shot by a skilled marksman and sampling did

122   not rely on trapping or targeting weaker or older animals. In total 175 adult foxes were admitted to the

123   study, removing host age as a variable and maximising the opportunity to detect infected individuals.

124   These animals were subsequently examined as part of an opportunistic surveillance scheme at the

125   Royal Veterinary College (RVC). Each individual was sent in a sealed bag, which included the date

126   and postcode of the area where it was killed. Post-mortem examinations were carried out within 48h

127   of arrival at the RVC and a unique ID number was assigned to each carcass. No animals were culled

128   specifically for this project and ethical review was not required.

129

130   Worms were recovered from lungs, heart and pulmonary arteries following the protocol detailed in

131   Morgan et al. (2008) and identified microscopically as *A. vasorum* based on morphological description

132   (Costa et al., 2003). Worms were counted and measured before being preserved in RNAlater as

133   described by the manufacturer (ThermoFisher Scientific™; UK) and stored at -20°C for genetic

134   analysis.

135

136 *2.2. DNA extraction*

137 DNA was extracted in two rounds. Initially, total genomic DNA was extracted from 83 worms, each

138 representing a separate fox host (Dataset 1). Subsequently, DNA was extracted from a further 49

139 worms including between 7 and 10 worms from each of six foxes (Dataset 2, plus one sequence per

140 fox from Dataset 1). Genomic DNA was extracted using a DNeasy Blood & Tissue Kit (QIAGEN,

141 Germany) following the manufacturer's protocol for extraction from animal tissue (Spin-Column

142 Protocol).

143

144 *2.3. PCR amplification and sequencing*

145 PCR was carried out targeting a partial region of the mtCOI locus (~710 bp) using the primers LCO

146 (5'-GGTCAACAAATCATAAAGATATTGG-3') and HCO (5'-TAAACTTCAGGGTGACCAAAAAATCA-

147 3') as described previously by Jefferies et al. (2010). The final reaction volume was 25 μL and

148 contained 2 μL extracted DNA, 0.1 μL of each primer (100 μM stock; Sigma, Crawley, UK), 12.5 μL of

149 MyTaq™ Mix (2x) (Bioline Reagents Ltd; UK) and 10.3 μL molecular grade water (Sigma). Genomic

150 DNA extracted previously from *A. vasorum* and molecular grade water were used as positive and

151 negative controls, respectively. The thermal cycling conditions were adapted from Jefferies et al.

152 (2010), including an initial denaturation of 5 min at 95 °C, followed by 35 cycles of 45 sec at 95 °C, 1

153 min at 50 °C and 1 min at 72 °C, and a final extension step of 10 min at 72 °C.

154

155 PCR products were resolved by agarose gel electrophoresis using 1% (w/v) UltraPure™ Agarose

156 (Invitrogen™; Paisley, UK) and TBE (Tris/Borate/EDTA) buffer (0.5x) with 0.005% (v/v) SafeView

157 Nucleic Acid Stain (NBS Biologicals Ltd; Cambridgeshire, UK). PCR amplicons of the anticipated size

158 were purified using the QIAquick PCR Purification kit (QIAGEN, Germany) following the

159 manufacturer's protocol. DNA concentration was measured using a Nanodrop® ND-1000

160 (ThermoScientific™) and diluted using molecular grade water to obtain a final sample of 20 μL volume

161 and 25 ng/μL concentration. Samples were subjected to Sanger chain-terminating dideoxynucleotide

162 sequencing (GATC Biotech, Constance, Germany) using the primer LCO. Worms presenting unique

163 polymorphisms were re-analysed (repeating both PCR and sequencing steps) to ensure the absence

164 of false diversity as a consequence of PCR mutation. In recognition of the diploid, and thus potentially

165 heterozygous *A. vasorum* genomes sampled, sequence traces were manually annotated to identify

166 the dominant haplotype from each worm.

167

168 *2.4. Sequence alignment, phylogenetic and population analysis*

169 Partial mtCOI sequences were curated using CLC Main Workbench (v6.0.2) and aligned with

170 published sequences derived from dog, fox and coyote hosts (Jefferies et al., 2010; GenBank

171 accession numbers GQ982734-GQ982876) using ClustalW with default parameters. The

172 *Angiostrongylus costaricense* mtCOI sequence (GenBank accession number KX378965.1) was used

173 as an outgroup.

174

175 MEGA version 6.0.6 was used to infer phylogeny in this study (Tamura et al., 2013). Using default

176 parameters in MEGA, the TN93+G model was identified as optimal based on the Bayesian

177 information criterion (BIC). Subsequently, a Maximum Likelihood (ML) tree was generated with 1,000

178 bootstrap iterations. The tree was left unrooted. Neighbor-joining (NJ) and Maximum Parsimony (MP)

179 phylogenies were created for comparison, also using 1,000 bootstrap iterations. Sequence alignments

180 were imported into the program NETWORK, version 5.0.0.1 (Bandelt *et al.* 1999) and haplotype

181 networks were calculated using default parameters with parsimonious single nucleotide

182 polymorphisms (SNPs) identified in the mtCOI sequences. Parameters defining genetic diversity were

183 calculated using DnaSP version 5.10.

184

185 All sequences generated in this work have been made publically available under the accession

186 numbers LT990053-LT990148.

187

188 **3. Results**

189 *3.1. DNA extraction*

190 A total of 175 foxes were sampled during 2016 in Greater London, including between one and seven

191 foxes from each of 30 postcode districts. Of these foxes, 107 (61.1%) were found to contain *A.*

192 *vasorum* worms (Martineau et al., manuscript in preparation). A panel of 83 foxes were used for

193 parasite DNA extraction, including one worm per fox and excluding those worms which exhibited

194 obvious signs of degradation ('single worm per fox', Dataset 1). Comparison of the foxes used in the

195   study revealed that 58% were female and 42% were male, and 45% were considered to be adult

196   (skeletally mature) compared with 55% juveniles. Six individual foxes within this group that were

197   found to contain at least seven worms were chosen for more detailed analysis, including a mix of

198   sexes, locations and ages (Table 1). Up to ten worms were selected from each fox (as available) for

199   use in the second 'multiple worms per fox' dataset (Dataset 2), providing a total of 55 worms in

200   addition to one other worm per fox from Dataset 1.

201

202   *3.2. PCR and sequence analysis*

203   In total, 138 worms from 83 different foxes were analysed. Of 83 worms processed in Dataset 1, 59

204   (71.1%) produced full length amplicon sequences which passed quality control (CLC Main

205   Workbench, default parameters). For Dataset 2, 37 quality sequences were derived from 55 worms

206   (67.3%), supplemented by one additional sequence per fox extracted from Dataset 1 (n=43).

207

208   Sequences in Dataset 1 were aligned with the published *A. vasorum* mtCOI sequences GQ982734-

209   GQ982876 in an alignment which comprised 588 bp. Topology of ML, NJ and MP trees derived using

210   these data was comparable, although branch structure was unstable as a consequence of limited

211   sequence diversity (see Supplementary Fig. 2 for an example). A summary of the genetic parameters

212   calculated for each of the datasets is presented in Table 2. There were 15 parsimony informative

213   nucleotide polymorphisms in total. Nineteen SNP haplotypes were identified (Table 2), of which 14

214   were detected in UK foxes (Fig. 1A). Eight SNP haplotypes were described in the UK for the first time,

215   of which six had been described previously in canines from other countries. All haplotypes described

216   previously from UK dogs with the exception of GQ982772 were detected here in foxes from the

217   Greater London area with no evidence of spatial haplotype clustering detected. The four most

218   common haplotypes described previously from Europe were all detected in London foxes, as was the

219   haplotype identified from Canadian canines. Dataset 2 included 43 sequences after quality control,

220   providing an alignment that included 574 nucleotides. There were 16 polymorphisms in total, only 10

221   of which were informative, and 22 different haplotypes were identified (Table 2). Despite the large

222   number of haplotypes in dataset 2, overall nucleotide diversity was lower (Table 2). Non-parsimony

223   informative SNPs were confirmed by repeat PCR and sequencing. A haplotype network constructed

224   using the "multiple worms" dataset identified the occurrence of considerable genetic diversity within

7

225 individual foxes in London, with sequences obtained from separate worms collected from six different

226 foxes presenting between two and nine haplotypes per fox (Table 1; Fig. 1B). Comparison of

227 haplotype occurrence revealed two common examples, both of which were detected in half of the

228 foxes analysed (F22, F46 and F94), despite these foxes coming from different postcode areas.

229 However, these postcodes were relatively close to each other, all located in northern Greater London

230 region (Supplementary Fig. 1). In contrast, fox F017, which had been culled in postcode W6 (west

231 Greater London) and was the most isolated and western of the foxes sampled, presented a distinct

232 series of haplotypes.

233

234 Alignment of the reference sequences and Datasets 1 and 2 revealed a total of 26 haplotypes, seven

235 of which came from Dataset 2 and were new. A panel of 21 parsimonious SNPs were identified

236 across the sequenced amplicon range, 19 featuring two variants and two featuring three variants

237 (Table 3).

238

239 4. Discussion

240 This study provides detailed analysis of genetic diversity within *A. vasorum* collected from a restricted

241 geographic area for the first time. It is also the first report to describe multiple haplotypes infecting

242 individual foxes at a specific time, emphasising their relevance as a reservoir of genetic diversity with

243 the potential for genetic exchange between *A. vasorum*.

244

245 The occurrence of *A. vasorum* among the foxes sampled in this study was notably high. One or more

246 worms were detected in 61% of foxes sampled, higher than reported previously from British foxes

247 (7.3%, varying from 0% to 23% by region; Morgan et al., 2008). A comparable sample set from

248 domestic dogs was not available, but recent publications suggest a lower occurrence in this or

249 equivalent populations across Europe (Helm et al., 2010; Kirk et al., 2014; Maksimov et al., 2017),

250 likely a consequence of better controlled diets, administration of anthelmintic products and reduced

251 access to intermediate/paratenic hosts of *A. vasorum*.

252

253 Several genomic loci have previously been investigated as genetic markers for *A. vasorum* including

254 the second internal transcribed spacer (ITS-2) region and fragments of the mtCOI (Jefferies et al.,

255 2009a, 2010; Gasser et al., 2012), with the latter proving to be more informative (Blouin, 2002). The

256 same region was used among others by Jefferies et al., (2010), who reported the presence of multiple

257 haplotypes commonly shared between different host species in Europe and Canada. Here, a greater

258 density of sampling was undertaken from a more spatially restricted local area, Greater London, to

259 explore the occurrence of rarer haplotypes. Comparison of multiple mtCOI sequences permitted the

260 detection of all but five mtCOI haplotypes described previously from Europe and North America. An

261 explanation for this could be that London is a highly populated urban area with a large number of

262 domestic dogs (PFMA, 2016). Following the introduction of the pet passport (European Union

263 Regulation 998/2003) many of these dogs travel to/from Europe, creating opportunities to import

264 different parasite strains and facilitating the spread of novel haplotypes throughout the UK. Further,

265 new haplotypes have been described for the first time. The inclusion of additional markers is likely to

266 have resulted in detection of even greater haplotype diversity. Published analyses of genetic diversity

267 within closely related parasites such as *A. cantonensis* are not directly comparable, but work with loci

268 such as mitochondrial cytochrome b and partial coding sequences of a 66 kDa protein have revealed

269 considerable haplotype diversity, with detectable geographic structure (Eamsobhana et al., 2013;

270 Peng et al., 2017). The work described here reinforces the geographic split between Europe and

271 North America, but reveals no notable geographic structure within European *A. vasorum* populations

272 (Jefferies et al., 2009b).

273

274 In agreement with previous reports (Jefferies et al., 2010), this study has confirmed that dogs and

275 foxes can share common *A. vasorum* haplotypes, supporting the suggestion that foxes act as wild

276 reservoirs of *A. vasorum* for domestic dog populations (Bolt et al., 1992). Comparison of diversity

277 between worms from different local foxes identified notable levels of polymorphism, while sequencing

278 multiple worms from individual hosts has demonstrated that foxes also act as reservoirs of genetic

279 diversity for *A. vasorum*. A minimum of two *A. vasorum* haplotypes were found to infect an individual

280 fox, with one fox hosting nine different haplotypes at the time of sampling. In the absence of

281 comparable sampling from domestic dogs it is not possible to determine whether wild canines harbour

282 more diverse parasite populations. *Angiostrongylus vasorum* infection of definitive hosts appears to

283 be chronic and animals remain infected and shedding larvae for long periods (Al-Sabi et al., 2013;

284 Webster et al., 2017). A study with experimentally infected dogs has reported that larval excretion

285 may occur for over three weeks, despite anthelmintic treatment, and shedding of larvae in untreated

286 animals could last for at least 300 days (Oliveira-Júnior et al., 2006; Schnyder et al., 2010). This long

287 shedding period offers the possibility of cross-fertilisation between different haplotypes if present.

288 Thus, the suggestion that foxes harbour a greater number of more genetically diverse worms

289 increases the chances of genetic segregation/recombination and emergence of new haplotypes,

290 conferring *A. vasorum* the ability to evolve more rapidly.

291

292 Two of the foxes sampled in dataset 2 were culled in the same postcode area. The worms hosted

293 within these foxes did not share the same haplotypes, suggesting a high haplotype diversity within a

294 small urban area. Alternatively, some foxes culled from separated postcodes in datasets 1 and 2 were

295 shown to host some shared haplotypes. The lack of information on the actual foraging range of these

296 foxes does not allow us to infer whether they were infected by a similar source due to overlapping

297 territories, or whether similar haplotypes were found in different locations. Sampling a higher number

298 of foxes from single postcode areas would have allowed the detection of such geographic

299 associations. Only six animals were selected to study genetic diversity within individual foxes. Despite

300 this sample size being small, it was sufficient to confirm the genetic variation hosted within individual

301 foxes, which had not previously been investigated.

302

5. Conclusions

304 In conclusion, this study emphasizes the importance of sequencing multiple worms within individual

305 definitive hosts. Results showed that individual foxes were infected by genetically diverse *A. vasorum*

306 parasites. As adult *A. vasorum* can persist within definitive hosts for extended periods when left

307 untreated, it is reasonable to assume that genetically diverse worms harboured within wild foxes may

308 be facilitating the emergence of new haplotypes through cross-fertilisation. Therefore, foxes are

309 shown to be reservoirs not only of *A. vasorum* for domestic dogs, but also of parasite genetic

310 diversity. More studies are needed to understand *A. vasorum* genetic diversity within individual foxes

311 with appropriate comparisons from domestic dogs, and how this may influence the emergence of new

312 haplotypes. Moreover, further studies sequencing multiple worms per animal from other definitive host

313 species would be desirable to understand the role of other species as genetic reservoirs of the

314 parasite.

315

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431

Tables

Table 1. Foxes included in the multiple worms per fox dataset (dataset 2). S = number of worm sequences included in the analysis (including one additional sequence from Dataset 1 per fox); H = number of haplotypes identified within each fox.

Fox code	Postcode culled	Age	Sex	N° worms analysed	**S**	**H**
F17	W6	ADULT	Male	10	6	5
F20	NW3	ADULT	Male	7	6	4
F22	N16	JUVENILE	Male	10	10	5
F45	IG1	ADULT	Male	10	7	2
F46	IG1	ADULT	Female	10	11	9
F94	E7	JUVENILE	Female	8	7	3

Table 2. Summary of genetic parameters calculated for the single and multiple worm datasets

Alignment	Size (bp)	N	S	k	π Jukes Cantor	H	Hd
Single worm (Dataset 1)	588	202	15 (15)	1.989	0.156	19	0.826
Multiple worm (Dataset 2)	574	43	16 (10)	2.916	0.005	22	0.882

N = number of sequences tested; S () = number of variant sites detected, with the number of parsimony-informative variant sites shown in parentheses; k = average number of pairwise differences; π = nucleotide diversity calculated with the Jukes Cantor correction; H = number of haplotypes detected; Hd = haplotype diversity.

444

445 **Table 3. Summary of parsimonious single nucleotide polymorphisms (SNPs) detected in sequenced mtCOI PCR amplicons.**

	Alignment position (bp)																				
	4	47	131	179	293	308	326	332	337	368	**371**	395	413	419	**422**	428	478	501	520	528	531
Major	A	T	T	A	A	G	T	G	G	T	T	T	T	T	G	G	T	G	T	A	G
Minor	G	C	C	G	G	A	C	A	A	C	C	A	C	A	A	A	A	T	C	G	A
Minor (2)	-	-	-	-	-	-	-	-	-	-	G	-	-	-	T	-	-	-	-	-	-

446

447

448 Figure legends

449 Fig. 1. Haplotype NETWORKs based on partial mtCOI sequences from *A. vasorum* recovered from

450   London foxes. (A) mtCOI sequences from Dataset 1 (single worm per fox) compared with

451   published sequences derived from parasites hosted by dogs, foxes and a coyote (accession

452   numbers GQ982734-GQ982876). The diameter of the circle is proportional to the number of

453   individuals presenting each haplotype. The colour of each node indicates geographic origin and

454   worm host. (B) mtCOI sequences from Dataset 2 (multiple worms per fox), including between 7

455   and 11 worms recovered from each of six London foxes. The diameter of the circle is

456   proportional to the number of individuals presenting each haplotype. The colour of each node

457   indicates host identity. Nodes circled in red were not previously detected in Dataset 1.

458

459

460 Supplementary Fig. 1. The location of foxes sampled in this study at the time of culling used in

461   datasets 1 (single worm per fox, black squares; the number indicates the sample size per

462   postcode) and 2 (multiple worms per fox, red circles) from the Greater London area.

463

464 Supplementary Fig. 2. An example of a Maximum Likelihood phylogenetic tree representing Dataset

465   1, illustrating relationships between the single worm dataset from London (highlighted) and

466   published sequences (accession numbers GQ982734-GQ982876). GenBank sequence suffixes

467   include the initial of the country of parasite origin and the initial of the host it was isolated from.

468   Specifically, c = Canada, d = Denmark, f = France, g = Germany, I = Ireland, n = Netherlands,

469   p= Portugal, and u = UK; while F = fox, C = coyote, D = dog. NJ and MP phylogenies presented

470   similar topologies, with a lack of structure within each branch.

471