Angiostrongylus vasorum from South America and Europe represent distinct lineages

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SUMMARY

Angiostrongylus vasorum is a nematode parasite of sylvan and domestic species of the family Canidae. It has a broad but patchy distribution worldwide, and there is evidence for geographical spread and increasing incidence of infection in recent years. While historically *Angiostrongylus*-like nematodes identified in dogs and foxes have been described as *A. vasorum* in Europe and *Angiocaulus raillieti* in South America, more recent taxonomic revision has amalgamated these into a single species, *A. vasorum*. Here we report, for the first time, the molecular characterization of isolates of *A. vasorum* from Germany, Portugal, Denmark and the United Kingdom on the basis of the mitochondrial COI gene and the second ribosomal internal transcribed spacer. When compared with isolates from Brazil, sequence analysis revealed 2 distinct genotypes. Estimated rates of evolution based on COI sequences for both nematode and host are consistent with the hypothesis that the presence of *A. vasorum* in South America is a result of an ancient evolutionary event. *Angiostrongylus vasorum* in South America potentially represents a separate species to that observed in Europe.

Key words: *Angiostrongylus vasorum*, *Angiocaulus raillieti*, Brazil, co-evolution, COI, dog, Europe, fox, ITS-2, nematode, phylogenetics.

INTRODUCTION

The mollusc-transmitted parasitic nematode *Angiostrongylus vasorum* (Nematoda: Metastrongyloidea) has been reported to infect various species of canid definitive host (Bolt *et al.* 1994) including dogs (*Canis lupis familiaris*), red foxes (*Vulpes vulpes*), pampas foxes (*Pseudalopex gymnocerus*) (Fiorello *et al.* 2006), hoary zorro (*Pseudalopex vetulus*) (Lima *et al.* 1994), crab-eating foxes (*Dusicyon thous*), wolves (*Canis lupis*) (Segovia *et al.* 2001) and coyotes (*Canis latrans*) (Bourque *et al.* 2005). Its distribution extends through Europe (Bolt *et al.* 1992; Gortazar *et al.* 1998; Sreter *et al.* 2003; Staebler *et al.* 2005) and South America (Lima *et al.* 1994; Fiorello *et al.* 2006) and has also been reported in isolated foci in Canada (Jeffery *et al.* 2004) and Uganda (Bwangamoi, 1972). Recent reports suggest an increasing incidence and broader distribution of this nematode in Europe (Morgan *et al.* 2005, 2008). There have also been reports of imported cases into non-endemic countries such as Australia and the USA (Williams *et al.* 1985; Tebb *et al.* 2007). Pathogenesis of the infection is highly variable with respiratory disease most common but bleeding and neurological disorders can also occur which can be fatal (Koch and Willesen, 2009).

Historically, much confusion has surrounded the taxonomic status of *Angiostrongylus vasorum* and *Angiocaulus raillieti* particularly in relation to South America. *Angiostrongylus raillieti* was first described by Travassos (1927) and was identified in *D. thous* in southern Brazil. Dougherty (1946) also reported this species in *D. thous* and domestic dogs, while decades later, Goncalves (1961) identified *Angiostrongylus vasorum* in *D. thous* in Columbia and in domestic dogs in Brazil. To add to this confusion, Grisi (1971) re-described *Angiostrongylus raillieti* as *Angiocaulus raillieti*. The validity of the species status of *Angiocaulus raillieti* has been questioned throughout the last century, with Dougherty (1946) and Rosen *et al.* (1970) reporting that *Angiocaulus raillieti* was likely to be synonymous with *Angiostrongylus vasorum*. Most recently Costa *et al.* (2003) published a re-description of *Angiostrongylus vasorum* with particular reference to South America and included *Angiocaulus raillieti* as a synonym of this species on the basis of morphological similarity. In summary, the most recent taxonomic revision recognizes only one species infecting hosts throughout the world, *Angiostrongylus vasorum*.

The purpose of this study was therefore to characterize various isolates of *A. vasorum* from Europe and South America to determine the level of molecular variation within the second ribosomal internal

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transcribed spacer (ITS-2) and the mitochondrial cytochrome c oxidase subunit I (COI) locus and to assess whether *A. vasorum* in South America may represent separate species. Both genetic loci have previously been considered suitable candidate markers for species differentiation in nematodes (Romstad *et al.* 1998; Blouin, 2002). Two hypotheses for the presence of *A. vasorum* in South America are also considered. The appearance of *A. vasorum* in South America has been either (i) a recent event, potentially as a result of the importation of dogs or exotic gastropod species, or (ii) an ancient event, potentially as a result of the evolutionary radiation of the definitive host species or host transfer.

MATERIALS AND METHODS

Nematode isolates

Adult *A. vasorum* ($n=7$ nematodes) were collected post-mortem from the pulmonary artery or right cardiac ventricle of foxes and dogs from Denmark, Germany, Portugal and the United Kingdom and stored at −20 °C until DNA was extracted. Individual adult worms were morphologically identified to species level using light microscopy according to Costa *et al.* (2003). Extracted genomic DNA from *Angiostrongylus cantonensis* ($n=2$ nematodes) collected from the Philippines was kindly provided by Chris Wade and Ian Fontanilla, University of Nottingham, UK.

DNA extraction and PCR amplification

Individual worms were macerated with a sterile pipette tip before DNA was extracted using a QIAamp® tissue kit (QIAGEN, Germany) following the manufacturer's instructions. PCR amplification was conducted using the previously described primers COIF 5′ TAAAGAAAGAACATAATGAAAATG 3′ and COIr 5′ TTTTTTGGGCATCCTGAGGTTTAT 3′ for a partial region of the COI gene (Bowles *et al.* 1993; Hu *et al.* 2002), and NC2 5′ TTAGTTTCTTTTCCTCCGCT 3′ and NC1 5′ ACGTCTGGTTCAGGGTTGTT 3′ (Gasser *et al.* 1993) for the entire ITS-2. PCR assays were performed in a final reaction volume of 25 μl that consisted of 2·5 μl of 10× polymerase buffer (TrisCl, KCl, $(NH_4)_2SO_4$, 15 mM $MgCl_2$, pH 8·7) (QIAGEN, Germany), 0·5 μl of dNTPs (10 mM each), 0·5 μl of each primer (12·5 ng/ml), 0·1 μl of EasyTaq DNA polymerase (5 U/ml) (QIAGEN, Germany), 19·4 ml of dH_2O and 2 μl of DNA. Reactions were thermo-cycled at 94 °C for 5 min, followed by 40 cycles of (94 °C for 30 sec, 55 °C for 30 sec and 72 °C for 1 min) followed by a final extension step of 72 °C for 5 min. PCR products were electrophoresed on a 1% (w/v) agarose gel and visualized using ethidium bromide and UV illumination. Amplified products were purified using a QIAquick PCR Purification Kit (QIAGEN, Germany). Sequencing reactions were performed using an ABI Prism DyeTerminator Cycle Sequencing Core kit (Applied Biosystems, USA) and sequence data were analysed using BioEdit v7.0.5 (http://www.mbio.ncsu.edu/BioEdit). Independent amplification and sequencing was conducted at least twice for each sample to minimize the risk of nucleotide errors as a consequence of PCR and sequencing artifact.

Sequence alignment and phylogenetic analysis

The ITS-2 nucleotide sequences for isolates from Denmark, UK, Portugal and Germany were aligned with sequences of *A. vasorum* from Brazil (Caldeira, R. L., Carvalho, O. S., Graeff-Teixeira, C., Lima, W. S., Monteiro, E., Simpson, A. J. G. and Lenzi, H. L., unpublished data) available from the GenBank database (Accession numbers DQ028994, DQ028995, DQ028996) together with sequences of *A. costaricensis* (DQ028987, DQ028988, DQ028989, DQ028990, DQ028991, DQ028992, DQ028993) and *A. cantonensis*. *Aelurostrongylus abstrusus* (DQ372965) was initially used as an outgroup species (also a member of the family Angiostrongylidae). Homologous COI nucleotide and translated amino acid sequences for isolates from Europe were also aligned with those from Brazilian isolates (Caldeira *et al.* unpublished data), along with other members of the order Rhabditida (*Caenorhabditis elegans* (AY171203), *Caenorhabditis briggsae* (EU407797), *Ancylostoma tubaeforme* (AJ407940), *Ancylostoma duodenale* (NC003415), *Heterorhabditis bacteriophora* (NC008534)) and *Aphelenchoides xylocopae* (AJ537512) as an outgroup species. Sequence alignments were conducted using CLUSTALw and further edited manually using BioEdit v7.0.5. Sequences for *A. vasorum* were deposited in the GenBank database under Accession numbers EU493161-67 for COI and EU627592-98 for the ITS-2 sequences. ITS-2 sequences for *A. cantonensis* isolates from the Philippines were deposited under Accession numbers EU636007 and EU636008.

Phylogenetic relationships based on a 275 bp region of the ITS-2 and a 360 bp region of the COI gene were determined using MEGA 4 (maximum parsimony and neighbor-joining) (Tamura *et al.* 2007) and Phylip (Phylogeny Inference Package) 3.67 (maximum likelihood) (Felsenstein, 2005). The Close-Neighbor-Interchange and maximum composite likelihood algorithms were used for maximum parsimony and neighbor-joining analyses respectively and default settings were used for each program. At least 1000 bootstrap replicates were used to infer statistical support at branch nodes. Estimates of pair-wise percentage differences between nucleotide sequences were calculated using the maximum composite likelihood method (MEGA 4).

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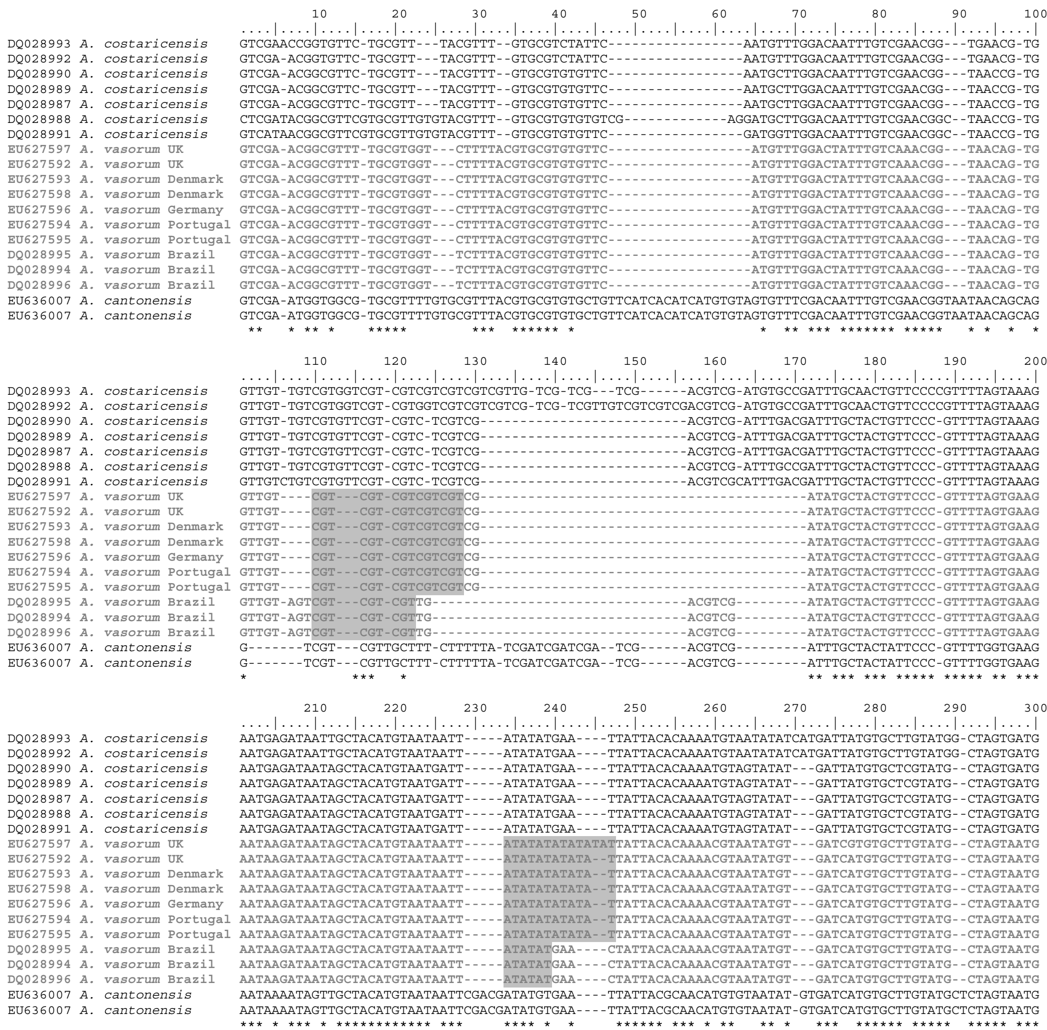

Fig. 1. Alignment of a 300 bp region of the ITS-2 sequences of *Angiostrongylus vasorum* isolates from Brazil and Europe (shown in grey), along with *A. costaricensis* and *A. cantonensis*. Microsatellites that discriminate between *A. vasorum* genotypes are highlighted. Asterisks indicate conserved nucleotides across all aligned sequences.

Molecular evolutionary rates

To test the hypotheses that the introduction of *A. vasorum* into South America has either been a recent or an ancient event, further analysis of the COI gene was conducted to measure the evolutionary rate and estimated divergence times of the two *A. vasorum* genotypes and their canid hosts. COI sequences for *A. vasorum* from Brazil and Europe were analysed along with those for *C. elegans* and *C. briggsae* as previously described, using *H. bacteriophora* and *A. xylocopae* as outgroup species. Partial fox COI nucleotide and amino acid sequences (homologous region to the *A. vasorum* sequences) were obtained from the GenBank database for the known Brazilian host species *Dusicyon thous* (AF028193) and *Pseudalopex vetulus* (AF028196) and the European species *Vulpes vulpes* (AF028206). *Urocyon cinereoargenteus* (AF028204), *Canis lupus* (AF028189), *Canis latrans* (AF028188) and *Canis familiaris* (AY656741) were also included for analyses using *Procyon lotor* (AM711899), a member of the suborder Caniformia, as an outgroup species.

Estimates of divergence times were calculated using MEGA 4. The equality of evolutionary rate based on nucleotide sequences for nematode and canid species was calculated separately using the Tajima's relative rate test (Tamura *et al.* 2007). A *p*-value less then 0·05 was used to reject the null hypothesis of equal rates between lineages. If there was no significant violation of a 'molecular clock', then a calibration was applied to each group. A separate calibration was performed for each of the nematodes and canids to infer times of divergence.

RESULTS

ITS-2

Alignment of the *A. vasorum* ITS-2 sequences with *A. costaricensis* and *A. cantonensis* (alignment length = 300 bp, 84 of which were variable and 73 parsimony-informative) revealed the presence of 2

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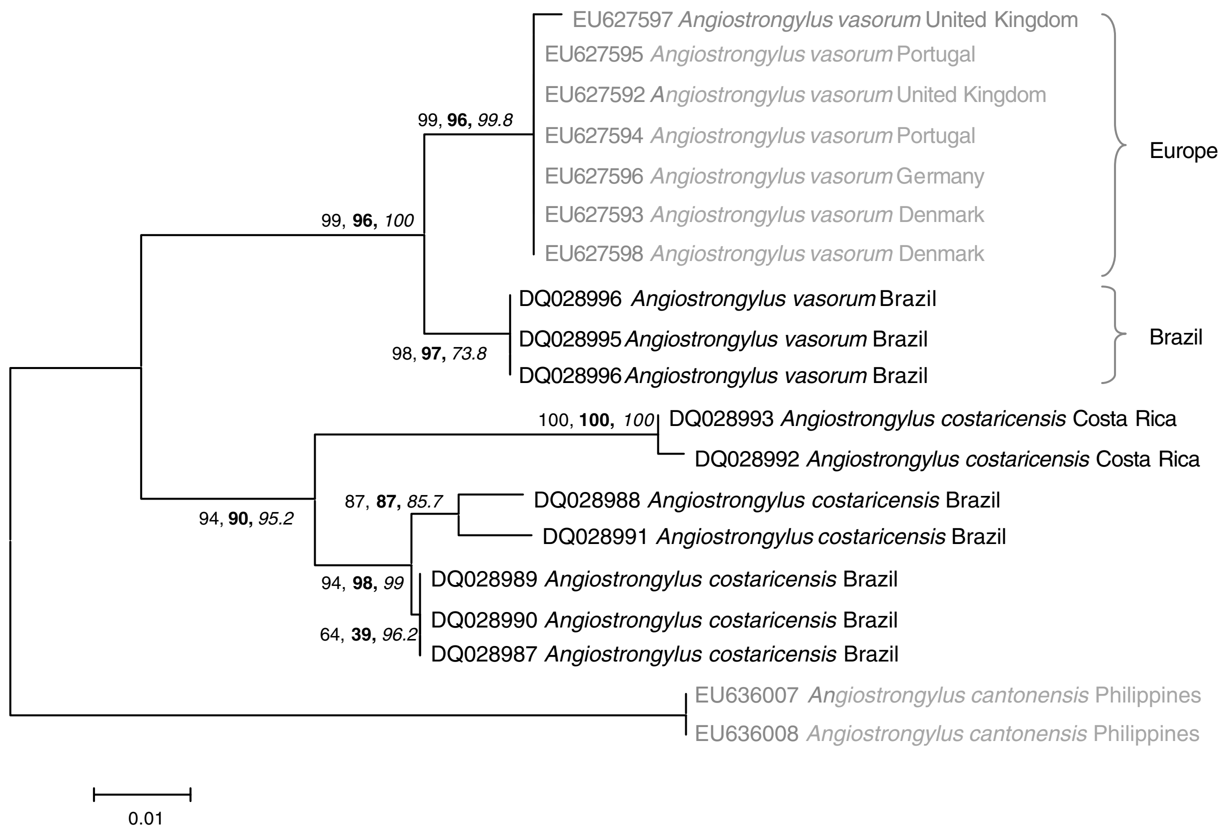

Fig. 2. Midpoint-rooted phylogenetic tree constructed with ITS-2 sequences and inferred using the neighbor-joining method. Numbers above branches represent bootstrap percentages of 1000 replicates (neighbor-joining, **maximum parsimony**, *maximum likelihood*). Scale bar represents number of nucleotide substitutions per site for NJ analysis and new sequences from this study are shown in grey.

microsatellites $(CGT)_n$ and $(AT)_n$, (Fig. 1) and could be used to discriminate isolates from Brazil (AT_3 and CGT_3) and isolates from Europe (AT_{6-7} and CGT_5). The same microsatellites were also observed in *A. costaricensis* (AT_3 and CGT_{2-6}) and *A. cantonensis* (AT_2 and CGT_2). Intraspecific variation based on pair-wise distance, ranged from 0 to 3·5%, and distinct genotypes were observed for *A. vasorum* (Brazil and Europe) and *A. costaricensis* (2 from Brazil and 1 from Costa Rica). Pairwise differences of 2–2·3% existed between *A. vasorum* genotypes, with 5 transitions, 3 transversions and 7 insertion/deletions observed. The average genetic difference on the basis of the partial ITS-2 between each of the 3 *Angiostrongylus* species ranged from 12·1 to 29·3%.

Initial alignment of the *Angiostrongylus* species sequences with *Aelurostrongylus abstrusus* for phylogenetic analysis proved problematic due to a high level of sequence divergence between taxa (data not shown). Phylogenetic trees were therefore constructed without the use of an outgroup species. The grouping of geographically separate isolates of *A. vasorum* was supported by high bootstrap values (>73%) for neighbor-joining, maximum parsimony and maximum likelihood analyses and all isolates of *A. vasorum* formed a distinct clade separate from the other *Angiostrongylus* species (Fig. 2). The phylogenetic relationship of *A. vasorum* with *A. costaricensis* or with *A. cantonensis* could not be accurately resolved due to the absence of an outgroup species.

COI

Alignment of the partial COI sequences for the *A. vasorum* isolates (alignment length = 360 bp, 34 of which were variable and 28 parsimony-informative) and amino acid sequences (2 of 120 variable) also revealed 2 distinct groups, namely isolates from Brazil and Europe (data not shown). Variable nucleotide positions are shown in Table 1. This was again supported by phylogenetic analysis using other nematode species (Fig. 3). Pair-wise differences for the COI between these groups ranged from 8·7–10·3% and within groups was 0·5% for isolates from Europe and 1·6% for those from Brazil. Three individual haplotypes (each differing by 1 to 6 nucleotide substitutions) were observed for each of the isolates from Europe (A, B, C) and from Brazil (X, Y, Z) (Fig. 3). As a means of comparison, genetic variation between *Ancylostoma duodenale* and *Ancyclostoma tubaeforme* was 14·7% (27/360 bp variable) and between *Caenorhabditis elegans* and *Caenorhabditis briggsae* was 17·3% (52/360 bp variable).

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Table 1. Variable nucleotide positions within the COI gene of *Angiostrongylus vasorum* from Brazil and Europe

A. vasorum isolate	Nucleotide position 010	025	040	055	067	073	100	103	106	115	127	130	160	163	172	184	190	211	217	253	259	265	280	292	295	297	301	306	307	310	316	325	340	342
Brazil 5642	A	A	A	G	T	G	T	G	C	G	T	G	A	T	T	C	G	A	G	A	A	G	G	C	G	A	G	A	G	G	A	G	G	A
Brazil 5421	A	A	A	G	T	G	T	G	T	G	T	G	A	T	T	C	G	A	G	A	A	G	G	C	G	A	G	A	G	G	G	G	G	T
Brazil 5641	A	G	A	A	G	G	T	G	T	A	T	G	A	T	T	C	G	A	G	A	A	G	G	T	G	A	G	A	G	G	G	G	G	C
EU493161 Portugal	G	T	G	G	G	A	G	A	T	G	T	A	G	T	G	G	A	G	T	G	G	G	A	G	T	G	T	G	A	A	A	A	A	A
EU493162 Portugal	G	T	G	G	G	A	G	A	T	G	T	A	G	T	G	G	A	G	T	G	G	G	A	G	T	G	T	G	A	A	A	A	A	A
EU493165 Denmark	G	T	G	G	G	A	G	A	T	G	T	A	G	T	G	G	A	G	T	G	G	A	A	G	T	G	T	G	A	A	A	A	A	A
EU493167 Germany	G	T	G	G	G	A	G	A	T	G	T	A	G	T	G	G	A	G	T	G	G	A	A	G	T	G	T	G	A	A	A	A	A	A
EU493166 Denmark	G	T	G	G	G	A	G	A	T	G	T	A	G	T	G	G	A	G	T	G	G	A	A	G	T	G	T	G	A	A	A	A	A	A
EU493165 Denmark	G	T	G	G	G	A	G	A	T	G	T	A	G	T	G	G	A	G	T	G	G	A	A	G	T	G	T	G	A	A	A	A	A	A
EU493163 UK	G	T	G	G	G	A	G	A	T	G	T	A	G	T	G	G	A	G	T	G	G	A	A	G	T	G	T	G	A	A	A	A	A	A
EU493164 UK	G	T	G	G	G	A	G	A	T	G	C	G	G	C	G	G	A	G	T	G	G	A	A	G	T	G	T	G	A	A	A	A	A	A

Molecular evolutionary rates

Alignment of the COI sequences of the South American fox species with *Vulpes vulpes* and the *Canis* species revealed 87/360 bp and 58/360 bp variable respectively. Lower levels of amino acid variation were observed, with only 1/120 variable between *Dusicyon thous* and *Vulpes vulpes*. Using *Procyon lotor* as an outgroup, the equality evolutionary rate between *Dusicyon thous* and *Vulpes vulpes* was 0·78 ($p=0{\cdot}37634$ with 1 degree of freedom) and *Pseudalopex vetulus* and *Vulpes vulpes* was 1·14 ($p=0{\cdot}28575$), suggesting similar rates of evolution between these lineages. Based on previous studies of fossil evidence and genetic analysis, divergence between the *Vulpes* species and the South American fox species occurred over 10 million years ago (Wang *et al*. 2004). A linearized evolutionary history was inferred using the neighbor-joining method (Fig. 4A).

The equality of evolutionary rate (x^2) for *A. vasorum* partial COI sequences from Europe and South America was determined as 0·80 ($p=0{\cdot}37109$ with 1 degree of freedom) using Tajima's relative rate test and *Heterorhabditis bacteriophora* as an outgroup species. The rate between *C. elegans* and *A. vasorum* was 1·85 ($p=0{\cdot}17357$), between *C. briggsae* and *A. vasorum* was 0·18 ($p=0{\cdot}66824$) and between *C. briggsae* and *C. elegans* was 1·14 ($p=0{\cdot}28575$), thus suggesting a similar rate of evolution across each of these lineages. A linearized neighbor-joining tree was produced assuming equal evolutionary rates (Fig. 4B) and molecular clocks were set based on the upper and lower limits of the estimated divergence time of *C. elegans* and *C. briggsae*, 20–120 million years ago (Gupta and Sternberg, 2003; Stein *et al*. 2003). The divergence between the South American and European *A. vasorum* was therefore estimated to have occurred between 11 and 67 million years ago (Fig. 4B).

The estimated time of the divergence of the European and Brazilian *A. vasorum* is therefore similar to the postulated divergence time for the South American canids from *V. vulpes* and predates the separation of the *Canis* and South American canid groups. This observation is consistent with the ancient introduction of *A. vasorum* into South America as it co-evolved with its canid host, although slower molecular evolutionary rates were observed for the parasite when compared to the host.

DISCUSSION

For the first reported time, European isolates of *A. vasorum* have been genotyped at a mitochondrial and nuclear genomic region and compared with South American-derived specimens. This analysis defined 2 separate phylogenetic clades, representing putatively distinct genetic populations from Brazil

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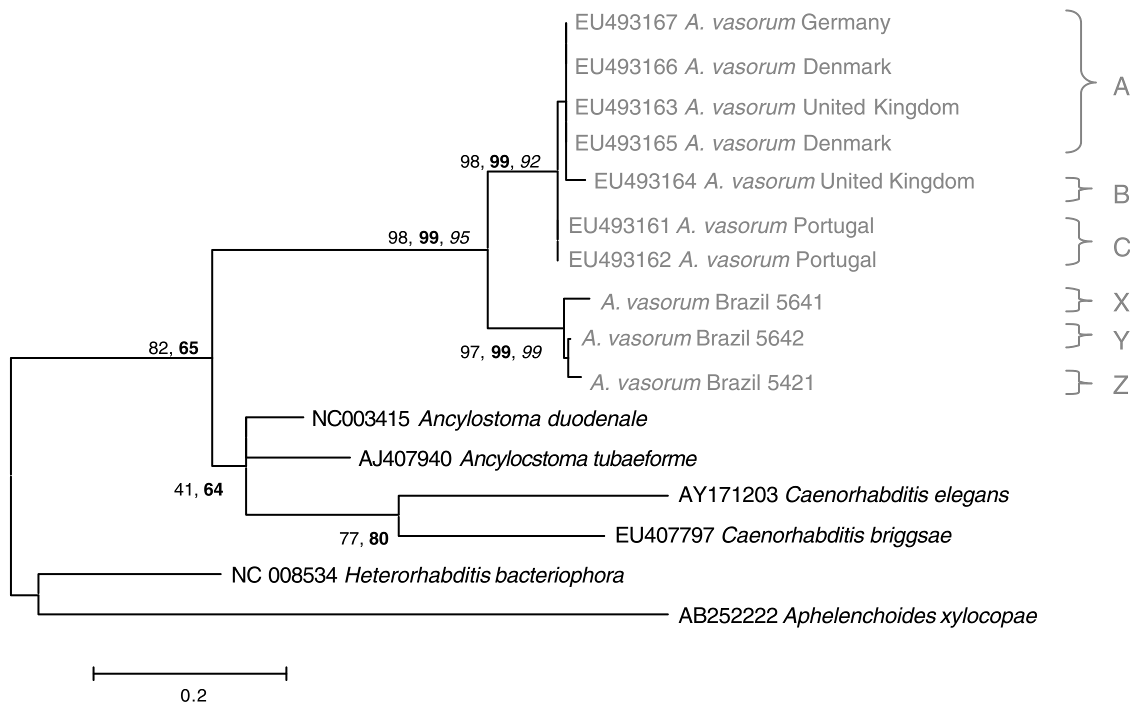

Fig. 3. Rooted phylogenetic tree constructed using partial COI gene sequences and inferred using the neighbor-joining method. Numbers above branches represent bootstrap percentages of 1000 replicates (neighbor-joining, **maximum parsimony**, *maximum likelihood*). Individual haplotypes are represented by A-C for Europe isolates and X-Z for Brazil isolates. Scale bar represents number of nucleotide substitutions per site and new sequences from this study are shown in grey.

and Europe. Whilst comparable morphology has been described for both *A. vasorum* in Europe (Guilhon and Cens, 1973) and Brazil (Grisi, 1971; Costa *et al.* 2003), the level of genetic variation between these populations suggests potential cryptic speciation. The synonomy of *A. vasorum* and *A. raillieti* as a single species, as proposed by Costa *et al.* (2003) may therefore be premature, as 2 separate species may actually exist pending further biological evidence.

Detection of cryptic species, that is, morphologically similar, yet genetically distinct species has been previously reported for multiple nematode taxa (Romstad *et al.* 1998; Blouin, 2002) and taxonomic changes within genera have also been postulated based on genetic differences (e.g., Mattiucci and Nascetti, 2006). Similarly, sequence data could be used to delimit species within the genus *Angiostrongylus*; however, this would require analysis using a larger sample size and the inclusion of additional species before definitive conclusions can be made on the taxonomic status of this group. The multiple genotypes observed for *A. costaricensis* may also represent cryptic species and require further taxonomic delineation.

The multiple microsatellites observed between and within the species of *Angiostrongylus* studied, make the ITS-2 a potentially useful diagnostic marker, supporting previous research by Caldeira *et al.* (2003). Similar discrimination of the South American and European isolates of *A. vasorum* was also observed within the COI gene with a range of 8·7 to 10·3% nucleotide variation between isolates within the South American and European groups, respectively. Such a level of variation supports classification as separate species and is in agreement with mitochondrial sequence differences reported between other co-generic nematode species (Blouin *et al.* 1998). The high number of haplotypes observed using the COI gene makes this a promising candidate for population genetics-based studies.

The genetic variation observed between South American and European populations of *A. vasorum* is consistent with the hypothesis that the appearance of *A. vasorum* in South America is an ancient event. Divergence of the South American foxes from the *Vulpes* and *Canis* groups of canids based on fossil and molecular evidence is estimated to have occurred over 10 million years ago (Wang *et al.* 2004). Based on evolutionary divergence times for *C. elegans* and *C. briggsae*, the divergence between the South American and European *A. vasorum* populations is predicted to have occurred between 11 and 67 million years ago, a time frame that is closer to the canid divergence then the comparatively recent introduction of the first dogs into South America less than 10 thousand years ago. Interestingly, the most ancestral fox species, namely those belonging to the genus

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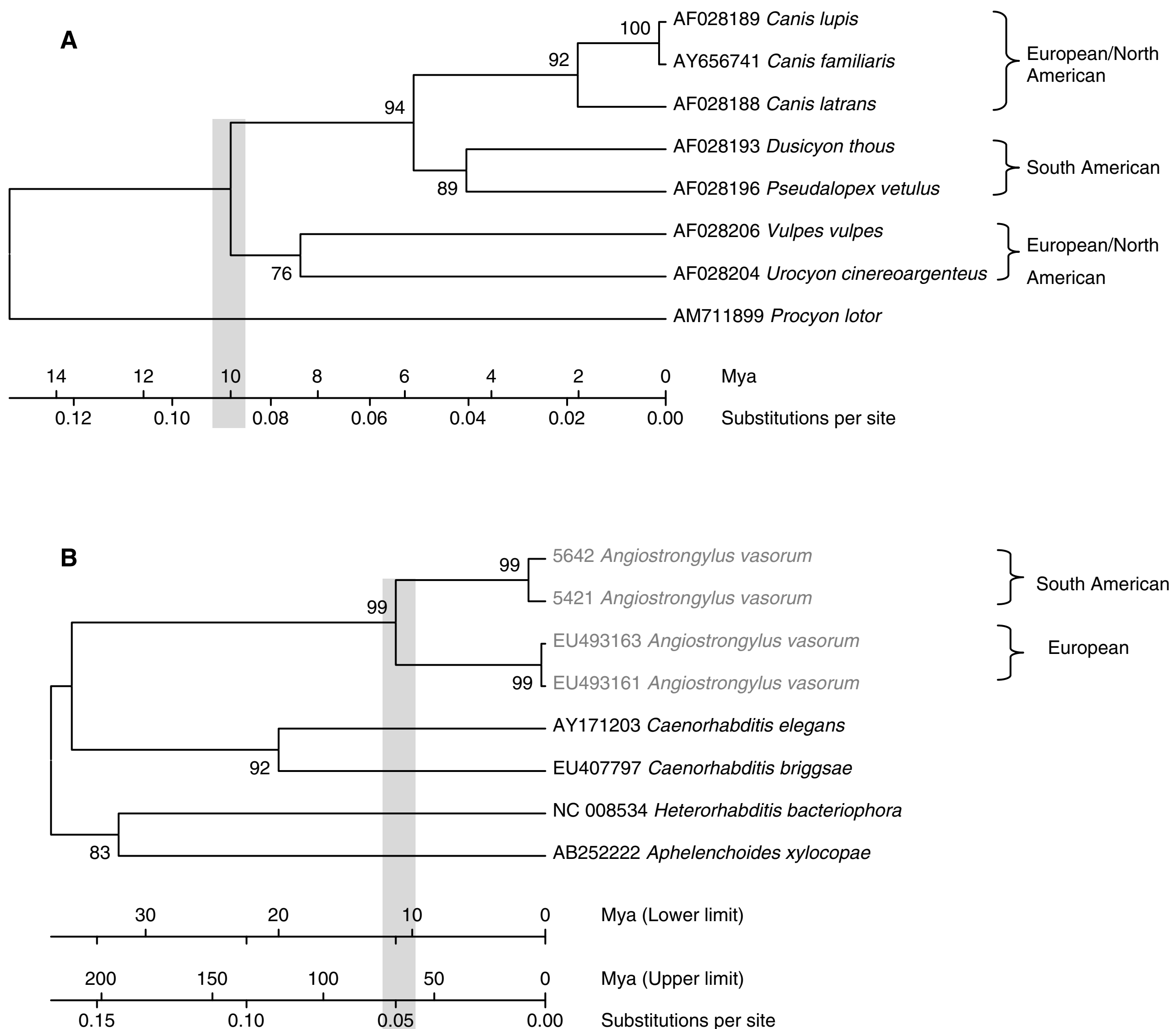

Fig. 4. Evolutionary rates between *Angiostrongylus vasorum* and its fox host inferred using the neighbor-joining method. Phylogenetic trees were constructed using partial COI gene sequences (360 bp) for the canid species (A) and *A. vasorum* and other nematode species (B). Each tree was linearized assuming equal evolutionary rates in all lineages. Shading represents the estimated divergence times for *Vulpes vulpes* from other canid species (A) and South American and European *A. vasorum* (B). Evolutionary distances were computed using Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. The clock calibration (time/node height) to convert distance to time was 108·36 for A and 224·21 (lower limit) and 1345·35 (upper limit) for B.

Urocyon (Wang *et al.* 2004), have been reported to be infected with an *Angiocaulus gubernaculatus*-like nematode (Faulkner *et al.* 2001), which may share a common ancestor with *A. vasorum*. Genetic characterization of this nematode species is imperative to further confirm the evolutionary history of *A. vasorum*.

Infections in domestic dogs may complicate such a hypothesis of host/parasite co-evolution, as dogs may become infected with either genotype depending on the geographically distinct sylvatic life cycle to which they are exposed. This may be reflected in potential host switching between *Vulpes vulpes* and the *Canis* related species in the same geographical locations and similarly, *A. vasorum* from the South American canids may have recently infected *C. lupis familiaris*. Ultimately, multiple host switching events have likely occurred throughout the evolutionary history of *A. vasorum* considering the diverse range of species capable of becoming infected with this parasite.

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