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Genetic variability of *Eucoleus aerophilus* from domestic and wild hosts

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ABSTRACT

Eucoleus aerophilus (syn. *Capillaria aerophila*) is a trichuroid nematode affecting domestic and wild carnivores and, sometimes, humans. This parasite has a worldwide distribution and may cause significant clinical disease in pet animals. The present paper investigates the sequence variation in partial mitochondrial cytochrome *c* oxidase subunit 1 (*cox*1) gene of *E. aerophilus* isolates from pets and wild animals from different countries. Forty-four egg pools of *E. aerophilus* were collected from dogs, cats and foxes from Italy, while seventeen adult stages of *E. aerophilus* were obtained from red foxes and beech martens from Portugal, Romania, Serbia and UK. Fifteen different haplotypes were characterized and five were shared between pets in Italy and wildlife from Europe. The remaining haplotypes were either confined only in hosts or countries, or in a given host from a country. The phylogenetic analysis showed that all haplotypes clustered as a monophyletic group with a strong nodal support, indicating that all sequence types represented *E. aerophilus*. The results here presented have implications for a better understanding of the epidemiology, phylo-geography and clinical impact of *E. aerophilus*. In particular, the geographic distribution of *E. aerophilus* haplotypes in different host species and geographic regions, and their variation in terms of pathogenic impact and zoonotic role, warrant further investigations.

1. Short communication

Adults of the trichuroid nematode *Eucoleus aerophilus* (syn. *Capillaria aerophila*) live embedded underneath the epithelium of bronchi and trachea of pets (e.g. cats and dogs) and wildlife (e.g. mustelids and wild canids) (Gherman et al., 2002; Morgan et al., 2008; Conboy, 2009; Traversa et al., 2010; Di Cesare et al., 2012a, 2012b). Females of *E. aerophilus* lay non-embryonated eggs, which are coughed, swallowed and shed into the environment *via* the faeces. It is thought that the eggs reach the infective stage in the environment but there is some uncertainty as to whether the life cycle of *E. aerophilus* is direct or indirect. It has been hypothesized that earthworms could act as a facultative intermediate hosts or paratenic hosts, but studies ultimately demonstrating the role of these invertebrates in the biology of *E. aerophilus* are lacking (Anderson, 2000; Conboy, 2009; Traversa et al., 2010).

E. aerophilus has been recently described in pets from countries of Europe and America, in which it may cause bronchovesicular sounds, sneezing, wheezing, and chronic dry productive cough (Conboy, 2009; Campbell and Little, 1991; Burgess et al., 2008; Traversa et al., 2009, 2010; Mircean et al., 2010; Di Cesare et al., 2011, 2012b). Additionally, this nematode may mimic lung cancer in humans (Lalosević et al., 2008).

Although there have been several recent studies that have examined various aspects of the biology, morphology, diagnosis and therapy of lung eucoleosis (Traversa et al., 2011, 2012; Di Cesare et al., 2012a), the molecular characteristics of *E. aerophilus* recovered from companion and wild animals remain to be investigated.

This work evaluated the sequence variation in partial mitochondrial cytochrome *c* oxidase subunit 1 (*cox*1) gene of *E. aerophilus*, isolated from pets and wild animals in different countries, in order to provide a foundation for further studies aiming at improving knowledge of this parasitosis.

Individual egg pools of *E. aerophilus* were collected from faeces of naturally infected dogs (*n.* 33), cats (*n.* 10) and foxes (*n.* 1) from different regions of Italy. Seventeen single adult stages of *E. aerophilus*

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Table 1
Specimens of *Eucoleus aerophilus* examined. Geographical origin, host, number of specimens examined and haplotype identified are reported.

Geographical origin	Host/N° of specimen	N° specimens/haplotypes
Italy	Dog/33	27/I
		2/III
		1/IV
		1/V
		1/VI
		1/VIII
	Cat/10	5/I
		3/II
		2/VII
	Fox/1	1/IX
Serbia	Fox/3	2/I
		1/II
Romania	Fox/4	2/I
		1/IV
		1/X
Portugal	Fox/3	1/III
		1/IV
		1/VII
	Beech marten/3	1/III
		1/X
		1/XV
	Fox/3	1/XIII
		1/XI
		1/XIV
Canada	Fox/1	1/XII

were obtained from 14 red foxes (*Vulpes vulpes*) (4 from Romania, 3 from Portugal, 3 from Serbia, 3 from UK and 1 from Canada) and 3 beech martens (*Martes foina*) from Portugal (Table 1).

Egg batches (from 5 to 20 eggs per batch) and adult stages were individually subjected to the extraction of genomic DNA. PCRs specific for a ~344 bp fragment internal to the *cox*1 gene of *E. aerophilus* were performed as previously described (Di Cesare et al., 2012a).

A semi-nested PCR protocol was applied to increase the sensitivity of the assay on those DNA samples from adults (i.e. 5 samples) or eggs (i.e. 39 samples) which were negative at the first PCR, in order to achieve a specific amplification of a 299-bp-long fragment within the *E. aerophilus cox*1 gene (Di Cesare et al., 2012a). PCR products were purified using Ultrafree-DNA columns (Jetquick Genomed GmbH, Löhne, Germany) and then sequenced directly. Sequences were determined in both directions with the same primers individually as for the PCR, and then aligned using the ClustalX and analysed with sequences available in GenBank™ using Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1997).

Pairwise comparison (Pwc%) and the transition (Ts)/transversion (Tv) ratio (*R*) among sequences were calculated by Kimura 2-parameter model (Kimura, 1980) using MEGA5 (Tamura et al., 2011). The open reading frames were confirmed by conceptual translation using the invertebrate mitochondrial code by MEGA5 (Tamura et al., 2011). The evolutionary relationships of taxa belonging to Capillariinae available in GenBank™, representative haplotypes previously sequenced for *E. aerophilus* (JQ905052 to JQ905059) (Di Cesare et al., 2012a) and sequences herein generated were included in the analysis. The evolutionary history was carried out using the neighbour-joining (NJ) method (Saitou and Nei, 1987) using the Tajima–Nei model (Tajima and Nei, 1984) and maximum likelihood method based on the Kimura 2-parameter model (Kimura, 1980). The evolutionary distances were computed by MEGA5 (Tamura et al., 2011). The bootstrap consensus trees inferred from over 8000 replicates were taken to represent the evolutionary history of the taxa analysed (Felsenstein, 1985). *Thelazia callipaeda* (AM042549) was chosen as out-group. The nucleotide sequences of *E. aerophilus* are available in the GenBank databases (JQ905052-JQ905059, KF479371-KF479377).

DNA extracts produced amplicons of the expected size according to the PCR protocol used, i.e. 344 bp and 299 bp for 17 and 44 samples respectively, with no intraspecific variations. Sixty-one amplicons of all *E. aerophilus* coming from different geographical areas were successfully sequenced and no discrepancy (i.e. nucleotide variation) was found in the reverse and forward sequence for each isolate. Both *cox*1 haplotypes previously determined (i.e., I–VIII, Di Cesare et al., 2012a) and seven new haplotypes (herein designed as IX–XV) were characterized (Table 1). The most prevalent haplotypes were I ($n = 36$; 59%), II and III ($n = 8$; 13.1%), followed by all the other eleven haplotypes ($n = 17$; 27.8%). The three most diffused haplotypes and haplotypes IV and VII were common in dogs and cats from Italy and wildlife from Romania, Portugal and Serbia. The other haplotypes were detected with varying frequency in animal species or countries (Table 1).

The alignment of the sequences resulted in a total of 299 characters including 274 conserved and 25 variable sites, of which 21 were singletons and four parsimony-informative (data not shown). The majority of the variable sites ($n = 18$; 72%) was at the third codon position, whereas the remaining ($n = 7$; 28%) was at the first and second codon positions. The sequences had an AT content of 67.2% and a bias at the third codon position to A/T (i.e., 86%). Nucleotide variations were represented by transitions (Ts) rather than transvertions (Tv) with a Ts/Tv ratio of 1.8 in the nucleotidic sequences.

Amino-acid sequences had an open reading frame in first position without stop codon, showing synonymous intraspecific nucleotide variations with the exception of four nucleotide substitutions that occurred at second and two at the third positions. The mean intraspecific nucleotide difference was 1.5%, ranging from 0.4 to 5.1% in haplotype VIII (e.g., dog, Italy) *vs* haplotype XIV (e.g., fox, UK), respectively. Phylogenetic analyses with the two independent algorithms revealed a well-defined clade, including all haplotypes identified. Although there is a relatively high number of distinct haplotypes, the phylograms showed that the 15 haplotypes clustered with a relatively strong nodal support in a monophyletic group to the exclusion of those representing other Capillariinae. These results indicate that all sequence types represented one *Eucoleus* species (Fig. 1). The dendrogram obtained with the NJ was consistent with that derived from the maximum likelihood analysis (data not shown). Additionally, the genetic convergence was also detected within and among populations of these nematodes, irrespective of their hosts and geographical origin.

All here generated haplotypes but VIII showed a 98–100% identity to the *cox*1 sequences of *E. aerophilus* KC341988-KC341991 obtained from Italian and Swiss red foxes (Guardone et al., 2013). Haplotype VIII displayed homology of ~96% with these aforementioned sequences, although the phylogenetic analysis (Fig. 1) demonstrated that this isolate belonged to the same *E. aerophilus* clade.

Overall, this information illustrates that some sub-populations of this parasite co-infect pets and wildlife, as demonstrated by the fact that all haplotypes but two detected in wild carnivores from Romania, Serbia and Portugal were found to infect companion animals in Italy as well (Table 1, Fig. 1). Interestingly, recent works have suggested a spreading of lung eucoleosis in companion dogs and cats in Italy and other countries (Traversa et al., 2009, 2010, 2012; Di Cesare et al., 2011). Reduction of natural habitats and new food sources in urban settings induce wildlife (e.g. feral cats, foxes, mustelids) to move into new hospitable environments, such as the suburbs and cities, favouring the spreading of parasites, and increasing the risks of zoonotic pathogens, e.g. fleas, extra-intestinal and intestinal helminthes (Dryden et al., 1998; Deplazes et al., 2004; Blagburn and Dryden, 2009; Traversa et al., 2010). Also, conurbations increasingly provide large numbers of suitable habitats and anthropogenic food sources for wild animals. Hence, the