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ORIGINAL PAPER

Mitochondrial haplotypes of *Aelurostrongylus abstrusus* and *Troglostrongylus brevior* (Nematoda, Metastrongyloidea) from domestic and wild felids

Donato Traversa[1] · **Fabrizia Veronesi**[2] · **Anastasia Diakou**[3] · **Raffaella Iorio**[1] · **Giulia Simonato**[4] · **Federica Marcer**[4] · **Angela Di Cesare**[1]

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Abstract The lungworm *Aelurostrongylus abstrusus* is the most important respiratory parasite of domestic cats. Pulmonary aelurostrongylosis has been reported in wild felids, though unequivocally evidence of wildlife infection by *A. abstrusus* is scant. Recently, *Troglostrongylus brevior*, a lungworm usually infecting wild felids, has been described in domestic cats from Mediterranean areas. The present work evaluates the sequence variation of an informative region within the gene encoding the mitochondrial cytochrome *c* oxidase subunit 1 of *A. abstrusus* and *T. brevior*, in order to provide novel information on the genetic make-up of these lungworms. Parasitic stages of *A. abstrusus* and *T. brevior* were collected from domestic and wild hosts (i.e., domestic cat, European wildcat, caracal, serval, and lion) from Italy, Greece, and South Africa. Five (HI-HV) and four (HI-HIV) haplotypes were recorded for *A. abstrusus* and *T. brevior*, respectively, mostly shared between domestic and wild felids in different geographical areas. The phylogenetic analysis showed that all haplotypes of *A. abstrusus* and *T. brevior* clustered as monophyletic groups with a strong nodal support, indicating that all haplotypes identified were distinct from each other. All sequence types represent two distinct species, *A. abstrusus* and *T. brevior*, and these genetic convergences are also detected within and among populations of these nematodes, irrespective of their hosts and geographical origin. The occurrence of *A. abstrusus* and *T. brevior* haplotypes in different hosts from the same regions and between different countries indicates that the same lungworm populations circulate in domestic and wild hosts under the same routes of transmission.

Keywords *Aelurostrongylus abstrusus* · *Troglostrongylus brevior* · Cytochrome *c* oxidase subunit 1 · Epidemiology · Felids

Introduction

The cat lungworm *Aelurostrongylus abstrusus* (Nematoda, Metastrongyloidea) has been considered for a long time the only metastrongyloid nematode affecting the respiratory tract of the domestic cat (*Felis silvestris catus*) (Di Cesare et al. 2015a). This parasite is transmitted by intermediate hosts represented by terrestrial mollusks and causes subclinical to severe respiratory signs in infected cats (Traversa and Di Cesare 2016). Although the clinical scenario may be relevant, life-threatening cases of cat aelurostrongylosis are very few (Traversa and Di Cesare 2013). The international literature reports several cases of *A. abstrusus* or *Aelurostrongylus* spp. in felid species other than the domestic cat (Traversa 2014; Di Cesare et al. 2016; Giannelli et al. 2016). Nonetheless, there is few morphological and/or molecular evidence unequivocally showing that *A. abstrusus* infects a broader range of hosts (Traversa 2014). For instance, the ability of *A. abstrusus* to cause pulmonary damages in the European wildcat (*Felis silvestris silvestris*) has been ultimately shown only recently (Veronesi et al. 2016). Also, a

✉ Donato Traversa
dtraversa@unite.it

[1] Faculty of Veterinary Medicine, University of Teramo, Località Piano D'Accio, snc, Teramo, Italy

[2] Department of Veterinary Medicine of Perugia, Via San Costanzo 4, 06126 Perugia, Italy

[3] School of Veterinary Medicine, Faculty of Health Sciences, Aristotle University of Thessaloniki, 54124 Thessaloniki, Greece

[4] Department of Animal Medicine, Production and Health, University of Padua, Viale dell'Università 16, Legnaro, Padua, Italy

definitive evidence that *A. abstrusus* may infect lions (*Panthera leo*) has been published only in 2016, along with the first report of aelurostrongylosis in servals (*Leptailurus serval*) and caracals (*Caracal caracal*) (Di Cesare et al. 2016).

In the past few years, another lungworm, i.e., *Troglostrongylus brevior* (Nematoda, Metastrongyloidea), has been unexpectedly recorded from domestic cats living in Mediterranean countries, i.e., Spain, Italy, and Greece (rev. in Di Cesare et al. 2015a). These records of *T. brevior* in domestic cats were surprising because nematodes of the genus *Troglostrongylus* usually infect wild felids (Anderson 2000; Brianti et al. 2014). This nematode, also transmitted by slugs and snails, has been firstly described from two species of wild felids (Gerichter 1949). After a local report of few years later in an European wildcat (*F. s. silvestris*) and in a cat defined as "feral" and of unknown origin from central Italy (Paggi 1959), *T. brevior* was not described again until few years ago, when single records in domestic cats were published from Ibiza (Jefferies et al. 2010a) and Sicily (Brianti et al. 2012). Since then, *T. brevior* has been described in *F. s. catus* from Apennine areas of Italy, Greece, and European islands (Di Cesare et al. 2015b, c, d; Tamponi et al. 2014; Traversa et al. 2014, 2015; Diakou et al. 2014, 2015). Indeed, clinical importance of troglostrongylosis in domestic cats is high, because *T. brevior* is potentially deadly, especially in kittens and young animals (Brianti et al. 2012; Diakou et al. 2014; Di Cesare et al. 2014a, 2015b; Traversa et al. 2014; Crisi et al. 2015). At the moment, there is uncertainty on the factual origin of this "novel" lungworm in domestic cats, because it is unknown whether *T. brevior* usually infect cats but it has been most often mistaken for *A. abstrusus*, or if *T. brevior* is currently changing its host affiliation from wild felids to domestic hosts (Brianti et al. 2012; Di Cesare et al. 2015a). Though there are pathological, epidemiological, and clinical evidences suggesting that *T. brevior* is presently spreading in populations of domestic cats as a result of a spill-over from wild felids (Traversa and Di Cesare 2013; Di Cesare et al. 2015a, d), no molecular data have been thus far generated to support this hypothesis. At the same time, further information is necessary to investigate the origin of *A. abstrusus* and routes of transmission in felids other than the domestic cat. Given the merit in improving knowledge on the genetic features of lungworm populations infecting felid species, the present work evaluated the sequence variation of an informative region within the gene encoding the mitochondrial cytochrome *c* oxidase subunit 1 (*cox*1) of *A. abstrusus* and *T. brevior*, isolated from domestic and wild felids from different areas.

Materials and methods

First stage larvae (L1) and single adult stages of *A. abstrusus* (n. 29 samples) and *T. brevior* (n. 24 samples) were obtained from naturally infected domestic cats and wild felids during previous studies carried out in different regions of Italy, Greece, and South Africa (Table 1).

DNA extracted from adult parasites using the QIAamp Tissue Kit (QIAGEN GmbH, Germany) after disruption with liquid nitrogen, and from larval samples using the QIAampDNA stool Mini Kit (QIAGEN GmbH, Germany) after three freeze (−80 °C)/thaw (100 °C) cycles, was subjected to a PCR specific for an internal region of the *cox*1 gene of metastrongylids as previously described (Brianti et al. 2012). A negative-control sample containing all of the reaction reagents and sterile distilled water to substitute for the template was added to each PCR run. All amplicons obtained were purified using a QIAquick gel extraction kit (QIAGEN, GmbH, Hilden, Germany) and sequenced directly using BigDye Terminator v.3.1 chemistry (Applied Biosystems, USA). The sequences were compared with those of the DNA of other nematodes available in the GenBank™ using the nucleotide-nucleotide "Basic Local Alignment Search Tool" (BLAST), and analyzed using Data Analysis in Molecular Biology and Evolution version 4.5.55 (DAMBE) and Mega Evolutionary Genetic Analysis version 7.0.20 (MEGA7) software.

The evolutionary relationships of generated sequences were analyzed using the neighbor-joining (NJ) method (Saitou and Nei 1987) using the Tajima-Nei model (Tajima and Nei 1984). The evolutionary distances were computed by MEGA7 (Kumar et al. 2016). The bootstrap consensus trees inferred from over 8000 replicates were taken to represent the evolutionary history of the taxa analyzed (Felsenstein 1985). *Toxocara cati* (GenBank Accession number NC_010773.1) was chosen as out-group.

Results

All DNA extracts produced amplicons of ~300 bp, with no intraspecific or interspecific variations for either species. Amplicons of all *A. abstrusus* and *T. brevior* isolates from different hosts and geographical areas were successfully sequenced and no nucleotide variations were found in the reverse and forward sequences for each isolate. The alignment of all sequences resulted in a total of 272 analyzed characters including 212 conserved and 60 variable sites, of which 54 were parsimony-informative and 6 were singletons, with an overall pairwise distance of 0.091. The nucleotide frequencies were 22.39% (A), 43.99% (T), 11.92% (C), and 21.71% (G), with an overall transitions/transversion bias of 0.043. Phylogenetic analyses revealed well-defined clades for both *A. abstrusus* and *T. brevior*, including all haplotypes identified, that were distinct from each other. The phylograms showed that the five haplotypes of *A. abstrusus* clustered with a strong nodal support in a monophyletic group, as well as the

Table 1 Specimens (adult worm or first stage larvae), animal, host, and geographical origin of *Aelurostrongylus abstrusus* and *Troglostrongylus brevior* subjected to a molecular study to investigate the haplotypic variations from different hosts in various countries

N	Sample	Location	N. animal	Identity	Reference
Domestic cat—*Felis silvestris catus*					
1	Adult worm	Umbria, Central Italy	DC1	Aa	Traversa et al. 2015
2	Adult worm			Tb	
3	L1	Umbria, Central Italy	DC2	Aa	Di Cesare et al. 2015d
4	L1		DC3	Tb	
5	L1		DC4	Tb	
6	L1		DC5	Tb	
7	L1		DC6	Aa	
8	L1		DC7	Aa	
9	L1	Latium, Central Italy	DC8	Aa	
10	L1		DC9	Aa	
11	L1		DC10	Aa	
12	L1		DC11	Tb	
13	L1		DC12	Tb	
14	L1	Abruzzo, Central Italy	DC13	Aa	
15	L1		DC14	Aa	
16	L1		DC15	Tb	
17	L1		DC16	Tb	
18	L1	Friuli, Northern Italy	DC17	Aa	
19	L1		DC18	Tb	
20	L1		DC19	Tb	
21	L1	Crete, Greece	DC20	Tb	Diakou et al. 2014
22	L1	Athens, Greece	DC21	Aa	Diakou et al. 2015
23	L1		DC22	Aa	
24	L1		DC23	Tb	
25	L1	Mykonos, Greece	DC24	Aa	
26	L1		DC25	Tb	
27	L1	Skopelos, Greece	DC26	Aa	
European Wildcat—*Felis silvestris silvestris*					
1	Adult worm	Marche, Central Italy	WC1	Tb	Veronesi et al. 2016
2	Adult worm			Tb	
3	Adult worm			Tb	
4	L1			Tb	
5	L1	Marche, Central Italy	WC2	Tb	
6	Adult worm	Umbria, Central Italy	WC3	Tb	
7	Adult worm	Umbria, Central Italy	WC4	Tb	
8	Adult worm			Aa	
9	L1			Aa	
10	Adult worm	Umbria, Central Italy	WC5	Tb	
11	Adult worm			Aa	
12	L1			Aa	
13	Adult worm	Umbria, Central Italy	WC6	Tb	
14	Adult worm			Tb	
15	Adult worm			Aa	
16	L1	Umbria, Central Italy	WC7	Aa	
17	L1	Umbria, Central Italy	WC8	Aa	
18	Adult worm	Latium, Central Italy	WC9	Aa	
19	L1	Latium, Central Italy	WC10	Aa	
18	Adult worm	Friuli-VG, Northern Italy	WC11	Tb	Unpublished

Table 1 (continued)

N	Sample	Location	N. animal	Identity	Reference
		African wild felids—*Caracal caracal*			
1	L1	Teniwka, South Africa	CC1	Aa	Di Cesare et al. 2016
2	L1	Gondwana, South Africa	CC2	Aa	
3	L1	Jukani, South Africa	CC3	Aa	
		African wild felids—*Leptailurus serval*			
1	L1	Tenikwa, South Africa	LS1	Aa	Di Cesare et al. 2016
		African wild felids—*Panthera leo*			
1	L1	Gondwana, South Africa	PL1	Aa	Di Cesare et al. 2016
2	L1	Gondwana, South Africa	PL2	Aa	

DC domestic cat, *WC* European wildcat, *CC Caracal caracal*, *LS Leptailurus serval*, *PL Panthera leo*, *Aa A. abstrusus*, *Tb T. brevior*

four haplotypes of *T. brevior* (Fig. 1). These features indicate that all sequence types represented two distinct species, i.e., *A. abstrusus* and *T. brevior*, and these genetic convergences were also detected within and among populations of these nematodes (i.e., haplotypes), irrespective of their hosts and geographical origin (Fig. 1).

Five (HI-HV) and four (HI-HIV) haplotypes were recorded for *A. abstrusus* and *T. brevior*, respectively (Table 2). The percent identity matrix among *A. abstrusus* and *T. brevior* haplotypes ranged from 92.78% (HII vs HV) to 99.64% (HI vs HIII) and from 97.11% (HIV vs HIII and HI) to 98.92% (HII vs HIII and HI), respectively. The most prevalent *A. abstrusus* and *T. brevior* haplotypes were HIII (*n* = 15/29; 51.7%) and HI (*n* = 7/29; 24.1%), and HII (*n* = 10/24; 41.7%) and HI (*n* = 8/24; 33.3%), respectively.

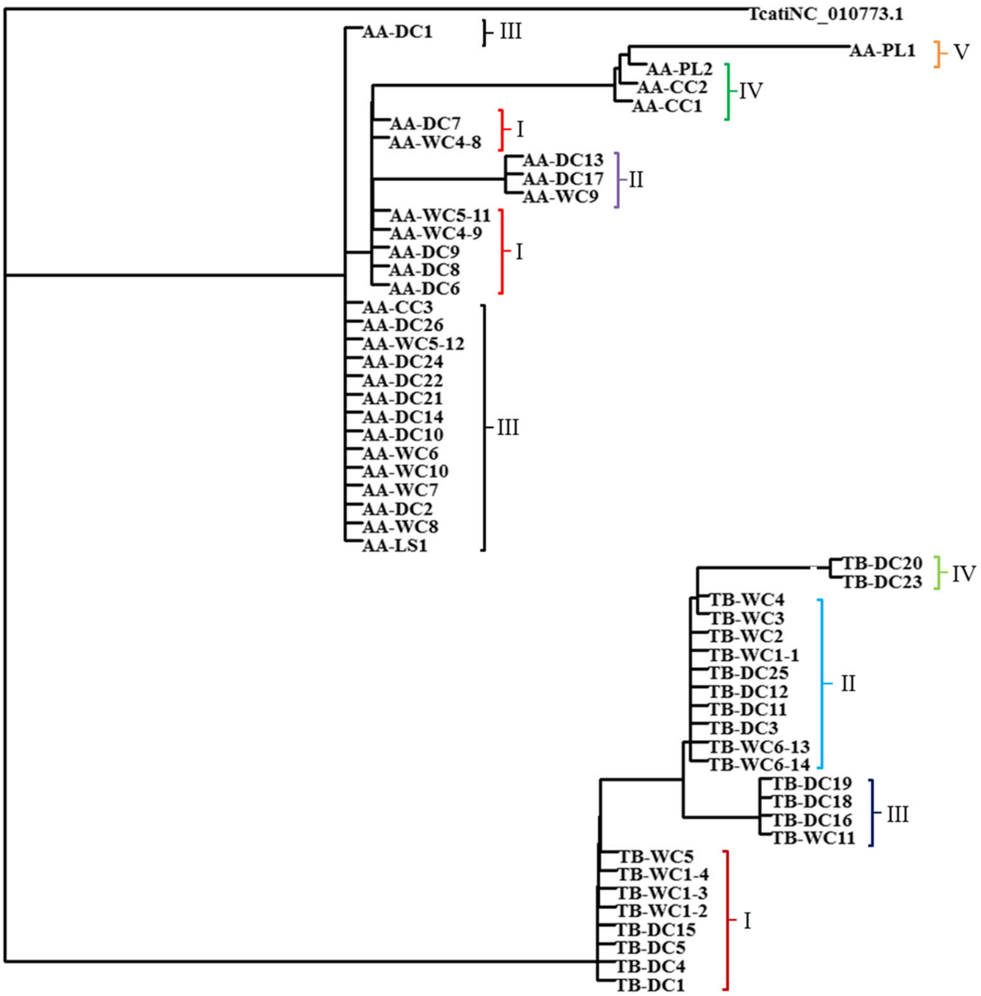
The most diffused haplotypes HI and HIII of *A. abstrusus* were common in both domestic and wild cats from the same regions of Italy and, moreover, HIII was recorded in domestic cats of Greece and in two wild felids of South Africa (Table 3). HIV and HV were found only in wild felids from South Africa. The most common haplotypes of *T. brevior* were HI, HII, and HIII. The HI of *T. brevior* was found in domestic and wild cats from Central Italy, while HII and HIII were present in domestic and wild cats from Central Italy, in domestic cats from Mykonos (Greece) (HII) and in domestic and wild cats from the North of Italy (HIII). HIV was found only in domestic cats from Greece (Table 3).

Discussion

The present results show that the same haplotypes of *A. abstrusus* (HI-HIII) infecting domestic cats in Italy occur in wildcats living in the same regions (Veronesi et al. 2016). Interestingly, the most common haplotype of *A. abstrusus* (i.e., HIII) was also here found in domestic cats from Greece and in two wild felids from South Africa, a Country where two further haplotypes (HIV and HV) were described in lions and in a caracal (Table 3). The results here presented support the recent hypothesis that high prevalence of cat aelurostrongylosis in some areas may broaden the host range of *A. abstrusus* (Di Cesare et al. 2015a; Veronesi et al. 2016). In fact, it should be taken into account that the presence of *A. abstrusus* in wildcats is uncommon, as shown by most recent studies carried out in other regions of Italy (Beraldo et al. 2014; Falsone et al. 2014) and Germany (Krone et al. 2008). Therefore, as *A. abstrusus* haplotypes here found in *F. s. silvestris* are widely spread in domestic cats living in the same regions (Tables 2 and 3), a spill-over may occur from domestic hosts to wildlife in territories with high prevalence of cat aelurostrongylosis (Di Cesare et al. 2015d).

Also, it is here shown that the same populations of *T. brevior* co-infect *F. s. catus* and *F. s. silvestris* in the same study areas of Europe. No haplotypes of this nematode resulted confined in either hosts with a single exception represented by one haplotype (HIV) recorded in two regions of Greece, i.e., Crete island and Attica (Athens) (Table 3). It is not known why *T. brevior* has been diagnosed in domestic cats only in the past few years. It is undoubted that the increase in the number of troglostrongylosis cases in domestic cats could be due to an increased awareness of this parasitosis (Brianti et al. 2014). However, several evidence have indicated that biological and epizootiological drivers may potentially change the host affiliation of lungworms and have indicated that *T. brevior* occurs in domestic cats living in areas that offer suitable routes of development and transmission from the wild reservoirs (Di Cesare et al. 2015a). The most important epizootiological indication is that in the vast majority of the cases *T. brevior* has been up to now isolated from domestic cats living in regions where populations of *F. s. silvestris* live in sympatry and are infected by this lungworm with high percentage (Di Cesare

Fig. 1 Phylogenetic tree based on the neighbor-joining analysis of partial *cox*1 sequence data for *Aelurostrongylus abstrusus* (haplotypes HI-HV) and *Troglostrongylus brevior* (haplotypes HI-HIV). Number of aligned sequences: 54. Number of sites for each sequences: 277. The tree is rooted by outgroup *Toxocara cati* GenBank accession number NC_010773.1



et al. 2015a). The occurrence of the same haplotypes of *T. brevior* in domestic and wild cats indicates that this lungworm finds its way to parasitize *F. s. catus* under certain circumstances and that a spill-over of *T. brevior* from wild to domestic hosts occurs in some regions of Mediterranean areas. The possible reasons for such epizootiological modifications have been thoroughly described in a recent article (Di Cesare et al. 2015a) and will not be discussed further.

An interesting exception is represented by *T. brevior* HIV that has been here found only in domestic cats from two Greek regions (Table 3). The European wildcat is present in Crete (Diakou et al. 2014) but, unfortunately, the absence of any information on the occurrence of *T. brevior* in wildlife of this island prevents from any further analysis or considerations. A future availability and analysis of *T. brevior* isolates from wildcats of Crete would allow to perform detailed comparisons on the genetic features of these populations. Importantly, *F. s. silvestris* is not recorded in Mykonos island, where *T. brevior* HIV was found in domestic cats (Diakou et al. 2015). These results need to be interpreted with caution because few specimens were examined from these areas and it is plausible that also the other haplotypes of this lungworm occur in domestic cats from the same regions. For instance, domestic cats from Mykonos island might harbor *T. brevior* HII, that has also been recorded in wildcats and *F. s. catus* from continental Europe (Tables 2 and 3). As wildcats are not present in Mykonos (Diakou et al. 2015), these isolates may originate from movements of pets and/or introduction of paratenic hosts, as previously discussed (Diakou et al. 2015). Although it is possible that *T. brevior* HIV occurs where the other sub-populations are spread, at the same time its apparent confinement due to geographical barriers, i.e., a major cause for parasite segregation and confined evolution of isolated genetic populations (Traversa et al. 2008), should be taken into account. Other than the obvious natural barriers of the Island of Crete, one should consider that the region of Attica, where *T. brevior* was recorded, is separated from the North by four mountains, ranging from 468 to 1.413 m altitude. Because the presence of the sea at West, East, and South of Attica, this area is somehow isolated from the rest of continental Greece. Furthermore, there is a large area extending to the North of Attica where no wildcats have ever been recorded

Table 2 Number (N°) of specimens and haplotypes (H) of *Aelurostrongylus abstrusus* and *Troglostrongylus brevior* from different hosts and geographical origins

Species	Host/ N°specimen	N° specimens/H	Specimens
			Italy
Aa	DC/10	4/I	AA-DC6, AA-DC7, AA-DC8, AA-DC9
		2/II	AA-DC13, AA-DC17
		4/III	AA-DC1, AA-DC2, AA-DC10, AA-DC14
	WC/9	3/I	AA-WC4(8), AA-WC4(9), AA-WC5(11)
		1/II	AA-WC9
		5/III	AA-WC5(12), AA-WC6, AA-WC7, AA-WC8, AA-WC10
Tb	DC/10	4/I	TB-DC1, TB-DC4, TB-DC5, TB-DC15
		3/II	TB-DC3, TB-DC11, TB-DC12
		3/III	TB-DC16, TB-DC18, TB-DC19
	WC/11	4/I	TB-WC1(2), TB-WC1(3), TB-WC1(4), TB-WC5
		6/II	TB-WC1(1), TB-WC2, TB-WC3, TB-WC4, TB-WC6(13), TB-WC6(14)
		1/III	TB-WC11
			Greece
Aa	DC/4	4/III	AA-DC21, AA-DC22, AA-DC24, AA-DC26
Tb	DC/3	1/II	TB-DC25
		2/IV	TB-DC20, TB-DC23
			South Africa
Aa	CC/2	1/III	AA-CC3
		2/IV	AA-CC1, AA-CC2
	LS/1	1/III	AA-LS1
	PL/2	1/IV	AA-PL2
		1/V	AA-PL1

DC domestic cat, *WC* European wildcat, *CC Caracal caracal*, *LS Leptailurus serval*, *PL Panthera leo*, *Aa A. abstrusus*, *Tb T. brevior*

(Yamaguchi et al. 2015), and this could represent a barrier for the flow of wildcat haplotypes to domestic hosts. At the same time, this could be a possible explanation for the segregation of *T. brevior* HIV only in domestic hosts since a long time.

The occurrence of *A. abstrusus* HIII in wildlife from South Africa clearly shows that this haplotype is able to circulate among several species of felids, even from countries far from each other. Conversely, the absence of *A. abstrusus* HIV and HV in Europe could be due to a geographical confinement of these populations in the southern hemisphere and/or only in large wild felids. Unfortunately, the absence of information on the genetic make-up of lungworms in domestic cats from the same African territories prevents any further comment on the potential circulation of the same parasite populations in wild and domestic hosts, as here found for two study countries of Europe.

The here reported evidence for bridging infections by lungworms from and to domestic and wild cats due recent changes in the biology and phenology of intermediate and definitive hosts (Di Cesare et al. 2015a) is not surprising if one considers that other recent studies have provided similar evidence for other cardio-respiratory nematodes. In fact, the same haplotypic sub-populations of the respiratory trichuroid *Capillaria aerophila* (syn. *Eucoleus aerophilus*) have recently been found to be shared between wild animals (e.g., foxes, beech martens) and companion cats and dogs, thus indicating the existence of common patterns of transmission for extra-intestinal nematodes (Di Cesare et al. 2014b). Analogously, molecular analyses using mitochondrial and nuclear DNA markers confirmed that *Angiostrongylus vasorum* genotypes are well mixed between dogs and foxes in Europe supporting the hypothesis that transmission occurs between wild and domestic canids (Jefferies et al. 2010b). Along with the increasing records of *A. vasorum* in dogs and foxes, genetically identical populations of this nematode have been found in wolves from Italy (Eleni et al. 2013).

Another similar overflow between domestic and wild felids has been recently proposed to explain the presence of *Ancylostoma tubaeforme* in the Iberian lynx from Spain, as the transmission of monoxenous parasites is related to host density and remaining lynx populations in the study region are very small (Millán and Blasco-Costa 2012).

Table 3 Detailed distribution of *Aelurostrongylus abstrusus* and *Troglostrongylus brevior* haplotypes from different hosts and geographical origins

Species	Haplotype	Hosts	Locations
Aelurostrongylus abstrusus	I	Domestic cat	Umbria, Central Italy
			Latium, Central Italy
		Wild cat	Umbria, Central Italy
	II	Domestic cat	Abruzzo, Central Italy
			Friuli, Northern Italy
		Wild cat	Latium, Central Italy
	III	Domestic cat	Umbria, Central Italy
			Latium, Central Italy
			Abruzzo, Central Italy
			Athens, Greece
			Mykonos, Greece
			Skopelos, Greece
		Wild cat	Umbria, Central Italy
			Latium, Central Italy
		Caracal	Jukani, South Africa
		Serval	Tenikwa, South Africa
	IV	Caracal	Tenikwa, South Africa
			Gondwana, South Africa
		Lion	Gondwana, South Africa
	V	Lion	Gondwana, South Africa
Troglostrongylus brevior	I	Domestic cat	Umbria, Central Italy
			Abruzzo, Central Italy
		Wild cat	Marche, Central Italy
			Umbria, Central Italy
	II	Domestic cat	Umbria, Central Italy
			Latium, Central Italy
			Mykonos, Greece
		Wild cat	Marche, Central Italy
			Umbria, Central Italy
	III	Domestic cat	Abruzzo, Central Italy
			Friuli, Northern Italy
		Wild cat	Friuli, Northern Italy
	IV	Domestic cat	Crete, Greece
			Athens, Greece

Analogously, sympatric domestic cat populations have been indicated as a potential reservoir for *A. tubaeforme* infection for the lynx based on the existence of shared hookworm haplotypes (Millán and Blasco-Costa 2012).

In conclusion, as previously seen for other extra-intestinal and intestinal parasites of companion animals, the present information illustrates that some sub-populations of the felid lungworms *A. abstrusus* and *T. brevior* are shared between common hosts and other closely-related animals. Recent works have suggested a spreading of felid lungworms and a change in host affiliation of *T. brevior*, at least in some Mediterranean areas (Di Cesare et al. 2014a; Diakou et al. 2015; Traversa et al. 2014, 2015). The present study support the existence of common patterns of transmission for haplotypes shared among different felid hosts. Further studies would be warranted to evaluate these epizootiological affiliations and modifications in broader geographic areas, for instance implementing knowledge on the current occurrence of *A. abstrusus* and *T. brevior* in large felids. Moreover, appropriate diagnostic approach and specific control measures in the domestic cat populations living into or near the areas were *T. brevior* is spreading are encouraged in order to prevent further transmission of this harmful parasite in domestic felid populations. Simultaneously, the control of the infection by *A. abstrusus* in domestic cats is warranted to avoid the spreading of the infection also in wild hosts, where it can cause pulmonary lesions and damage (Veronesi et al. 2016).

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical statement Procedures applied to collect parasitic stages analyzed in the present study are not within the context of EU legislation for animal experimentations. Samples were collected by veterinarians and caused no suffering to the animals. All applicable international, national, and/or institutional guidelines for the care of animals were followed. This article does not contain any studies with human participants performed by any of the authors.

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