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ORIGINAL PAPER

Crenosoma vulpis in wild and domestic carnivores from Italy: a morphological and molecular study

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Abstract *Crenosoma vulpis* is a metastrongyloid nematode primarily associated with respiratory tract infections of red foxes in North America and Europe. Sporadic cases have also been reported in domestic dogs. The present study aimed to provide morphological, molecular, and epidemiological data on the geographical distribution of this nematode throughout Italy. From 2012 to 2014, 12 of the 138 foxes examined, three dogs and one badger scored positive for *C. vulpis*. Forty adults were isolated from foxes and the badger, whereas first-stage larvae were detected in the three dogs. All specimens were morphologically identified as *C. vulpis*, and 28 nematodes were also molecularly characterized by sequencing mitochondrial (12S ribosomal DNA (rDNA)) and nuclear (18S rDNA) ribosomal genes. Four haplotypes were identified based on the 12S rDNA target gene, with the most representative (78.5 %) designated as haplotype I. No genetic variability was detected for the 18S rDNA gene. The molecular identification was consistent with the distinct separation of species-specific clades inferred by the phylogenetic analyses of both mitochondrial and ribosomal genes. Data herein reported indicates that *C. vulpis* has a wide distribution in foxes from southern Italy, and it also occurs in dogs from southern and northern regions of the country. Practitioners should consider the occurrence of this nematode in the differential diagnosis of canine respiratory disease, particularly in dogs living close to rural areas where foxes are present.

Keywords *Crenosoma vulpis* · Red fox · Dog · Morphological identification · 12S rDNA · 18S rDNA · Lungworm

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Introduction

Infections caused by metastrongyloid lungworms are increasingly investigated for their impact on human and animal health (WHO 2012). In veterinary medicine, these nematodes are responsible for respiratory disease of wild and domestic animals (Morgan et al. 2009; Traversa et al. 2010; Brianti et al. 2014a). This is the case for parasitic species infecting felids (e.g., *Aelurostrongylus abstrusus* and *Troglostrongylus brevior*) and canids (*Angiostrongylus vasorum*) in Europe (Brianti et al. 2014b; Spratt 2015). Among this variegate group of nematodes, *Crenosoma vulpis* is endemic in populations of red fox (*Vulpes vulpes*) from temperate regions of North America and Europe (Zeh et al. 1977; Smith 1978; Levine 1980; Sreter et al. 2003; Manfredi et al. 2003; Smith et al. 2003; Jeffery et al. 2004; Nevarez et al. 2005; Saeed et al. 2006), also being reported in dogs (Cobb and Fisher 1992; Bihr and Conboy 1999, Reilly et al. 2000; Unterer et al. 2002; Rinaldi et al. 2007; Barutzki and Schaper 2011) and badgers (*Meles meles*) (Popiołek et al. 2009). Due to their free-roaming behavior, red foxes have been suggested to be the major cause of lungworm dispersal in previously nonendemic areas, and as a potential source of infection to

domestic animals (Otranto et al. 2015). Data about the distribution of *C. vulpis* in dogs from Europe are restricted to single case reports. For instance, in Italy, the parasite has been diagnosed in only two dogs (Rinaldi et al. 2007; Guardone et al. 2013) and in red foxes from northern and central Italy (Manfredi et al. 2003; Magi et al. 2009, 2015). As for most of the metastrongyloid lungworms, the definitive hosts (e.g., red foxes and dogs) become infected following the ingestion of gastropod intermediate hosts (Anderson 2000). Nonetheless, the emergence of infective-stage larvae of metastrongyloids from live or dead snails has been implicated as an alternative transmission pathway for the spread of snail borne diseases (Barçante et al. 2003; Giannelli et al. 2015). Canine crenosomosis may present clinical conditions of differing degrees, from asymptomatic to mild respiratory signs such as bronchitis with mucopurulent discharge and chronic cough (Conboy 2009). The diagnosis of the infection in dogs is based on the retrieval of first-stage larvae (L1) using the Baermann technique, which may present disadvantages due to its laboriousness and good training required for larval identification (Brianti et al. 2012). Scientific information on the epidemiology of *C. vulpis* in Italy is patchy and our study aimed to provide further data on the geographical distribution of this nematode throughout the country. The characterization of nuclear 18S ribosomal DNA (rDNA) and mitochondrial 12S rDNA genes has been performed in order to investigate the phylogenetic relationships between *C. vulpis* and other members of the superfamily Metastrongyloidea.

Materials and methods

Sample source and processing

From January 2012 to December 2014, carcasses of 138 red foxes (82 males and 56 females) shot during the hunting seasons and a road-killed badger were collected in different regions of southern Italy (Table 1) and inspected for lungworms. Data on the gender, age, and origin were recorded, and carcasses were delivered to the Istituto Zooprofilattico Sperimentale del Mezzogiorno–Avellino Unit, Italy, to the Department of Veterinary Medicine and Animal Productions (University of Napoli, Italy) and to the Department of Veterinary and Public Health (University of Messina, Italy), stored in plastic bags at 4 °C, until necropsy. In addition, three dogs from the Basilicata, Emilia Romagna, and Veneto regions were presented to local practitioners due to a productive cough and dyspnea and were subjected to bronchoscopic examination.

Adult nematodes (n=40) were detected upon necropsy in the bronchi of the foxes and badger, whilst L1 by the Baermann technique, in the bronchoalveolar lavage (BAL) and in fecal samples of dogs (MAFF 1986). Nematodes were stored in 70 % ethanol and sent to the Parasitology Unit of the Department of Veterinary Medicine (University of Bari, Italy) to be morphologically and molecularly identified. In particular, one male and one female lungworm were clarified in lactophenol, measured, photographed, and drawn. The other specimens were examined by mounting the anterior and posterior parts, with glycerol-ethanol, on a slide. Adult nematodes and L1 were identified at species level according to morphological keys (Craig and Anderson 1972; Soulsby 1982; Jančev and Genov 1988). All microscopic images and measures were taken using a digital image processing system (AxioVision rel. 4.8, Carl Zeiss, Germany). The central part of adult specimens and L1 larvae were kept in 70 % ethanol and phosphate-buffered saline (PBS), respectively, until molecular processing.

Molecular procedures and analyses

Genomic DNA from adult worms and L1 (Table 1) was extracted using a commercial kit (DNeasy Blood & Tissue Kit, Qiagen, GmbH, Hilden, Germany), in accordance with the manufacturer's instructions. Partial fragment of mitochondrial 12S rDNA (~330 bp) and nuclear 18S rDNA (~1700 bp) genes were amplified using two sets of primers (Fila_12SF: 5′-CGGGAGTAAAGTTTTGTTTAAACCG-3′ and Fila_12SR: 5′-CATTGACGGATGGTTTGTACCAC-3′; NC18SF1: 5′-AAAGATTAAGCCATGCA-3′ and NC5BR: 5′-GCAGGTTCACCTACAGAT-3′, respectively). Each reaction consisted of 4 μl genomic DNA (~100 ng) and 46 μl of PCR mix containing 2.5 mM $MgCl_2$, 10 mM Tris–HCl (pH 8.3) and 50 mM KCl, 250 μM of each dNTP, 50 pmol of each primer and 1.25 U of AmpliTaq Gold (Applied Biosystems). Samples without DNA were included as negative controls. The 18S and 12S rDNA genes were amplified using the following conditions: 95 °C for 10 min (first polymerase activation and denaturation), followed by 35–40 cycles of 95 °C for 30–60 s (denaturation); 57°–58 °C for 30–60 s (annealing), 72 °C for 60 s (extension); and a final extension at 72 °C for 7 min, respectively. The amplicons were purified and sequenced, in both directions using the same primers as for PCR, employing the Taq Dye Deoxy Terminator Cycle Sequencing Kit (v.2, Applied Biosystems) in an automated sequencer (ABI-PRISM 377). Sequences were compared, using Basic Local Alignment Search Tool (BLAST – http://blast.ncbi.nlm.nih.gov/Blast.cgi), with those available in the GenBank database. The percentage of nucleotide variation among sequences was calculated by pairwise comparison (Kimura 2-parameter model) (Kimura 1980) by using MEGA6software (Tamura et al. 2013). In order to investigate the phylogenetic relationships with other metastrongyloids, the sequences of mitochondrial and nuclear genes herein generated were aligned, using ClustalW, with

Table 1 *Crenosoma vulpis* found in different hosts and regions of Italy and their molecular identification

Geographical origin	Host (*n*)	Localization	Number and sex	Developmental stage	N°/12S haplotype
Basilicata	*Vulpes vulpes* (1)	Bronchi	1 (F)	Adult	1/I
	Meles meles (1)	Bronchi	1 (F)	Adult	1/I
	Canis familiaris (1)	Bronchi	1	Larva	na
Calabria	*Vulpes vulpes* (1)	Bronchi	8 (F), 8 (M)	Adult	3/I; 1/II; 1/IV
Campania	*Vulpes vulpes* (9)	Bronchi	17 (F), 2 (M)	Adult	15/I; 3/II; 1/III
Reggio Emilia	*Canis familiaris* (1)	Bronchi	1	Larva	1/I
Sicily	*Vulpes vulpes* (1)	Bronchi	2 (F), 1 (M)	Adult	1/I
Veneto	*Canis familiaris* (1)	Feces	3	Larva	na

na not available

those available in the GenBank database (Larkin et al. 2007). Maximum Likelihood (ML) and neighbor-joining analyses were conducted using the gamma distribution (+*G*) and *p*-distance model, respectively, for both 12SrDNA and 18SrDNA genes by MEGA6 software (Tamura et al. 2013). For each analysis, the bootstrapped confidence interval was based on 5000 replicates. Sequences of *Nematodirus oiratianus* (NC024639) and *Nippostrongylus brasiliensis* (AJ920356) were used as outgroups for the 12S rDNA and 18S rDNA genes, respectively.

Results

Of the 138 red foxes examined, 12 (8.7 %) scored positive for lungworms. All adult specimens from wild animals, and L1 collected from dogs, were identified as *C. vulpis*. The studied specimens from a red fox in Sicily were characterized by the following morphology: male, body 4.6 mm long; anterior end bearing six small lips; cuticle with 15-fold distinct in anterior 1.0 mm of body; interrupted longitudinal cuticular ridges extending over entire body surface (Figs. 1a and 2a); deirids minute, situated at 70 and 80 μm from anterior end; excretory pore at 90 μm from anterior end; esophagus muscular 227 μm long, with maximum width at posterior third 42 μm (Figs. 1a and 2a); nerve ring at 100 μm from anterior end; tail 109 μm long; right and left spicules, brownish, equal in shape and size, 369 μm long, with pointed asymmetrically expanded distal end (Figs. 1b and 2b); gubernaculum 136 μm long. Female (without absent body part) 12.4 mm long; maximum body width 437 μm; vulva situated at 7 mm from posterior end; tail conical 128 μm long, with rounded tip (Fig. 1c); phasmids subterminal; uteri containing numerous first-stage larvae 233–251 μm ($n=5$) long and 15–16 μm ($n=5$) wide (Fig. 2c). L1 of *C. vulpis* collected by the Baerman technique from dog measured in mean 352.4±2.4 μm. They were featured by a bluntly conical anterior end and a straight-pointed tail, lacking any kinks or spines. The esophagus was filiform (Fig. 3).

PCR amplification of each target region from individual DNA samples resulted in amplicons of the expected size. Overall, 28 sequences were obtained for 12S rDNA gene, and four distinct haplotypes (i.e., named as haplotypes I–IV) were identified. Haplotype I was identified in specimens

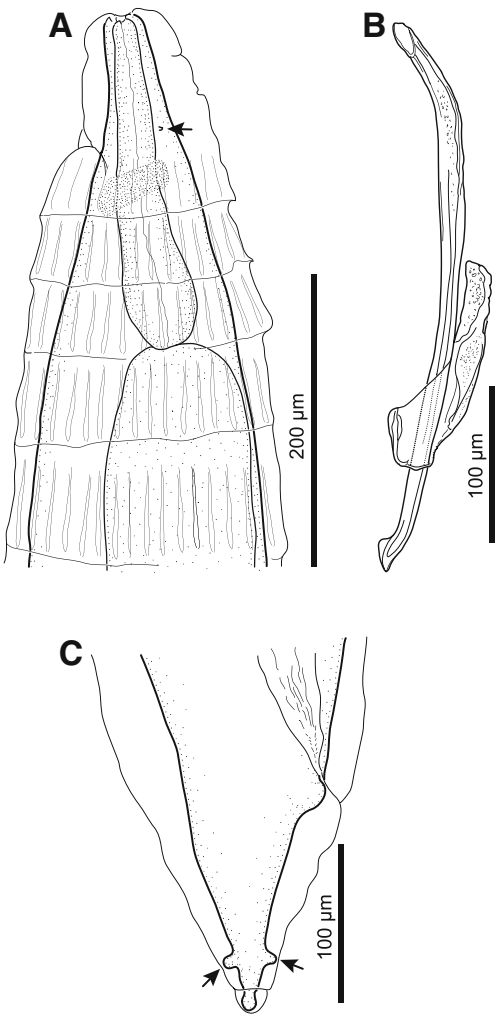

Fig. 1 *Crenosoma vulpis*. **a** Anterior end, male, lateral view, note deirid (*arrow*). **b** Spicule and gubernaculum, lateral view. **c** Tail, female, lateral view, note phasmids (*arrows*)

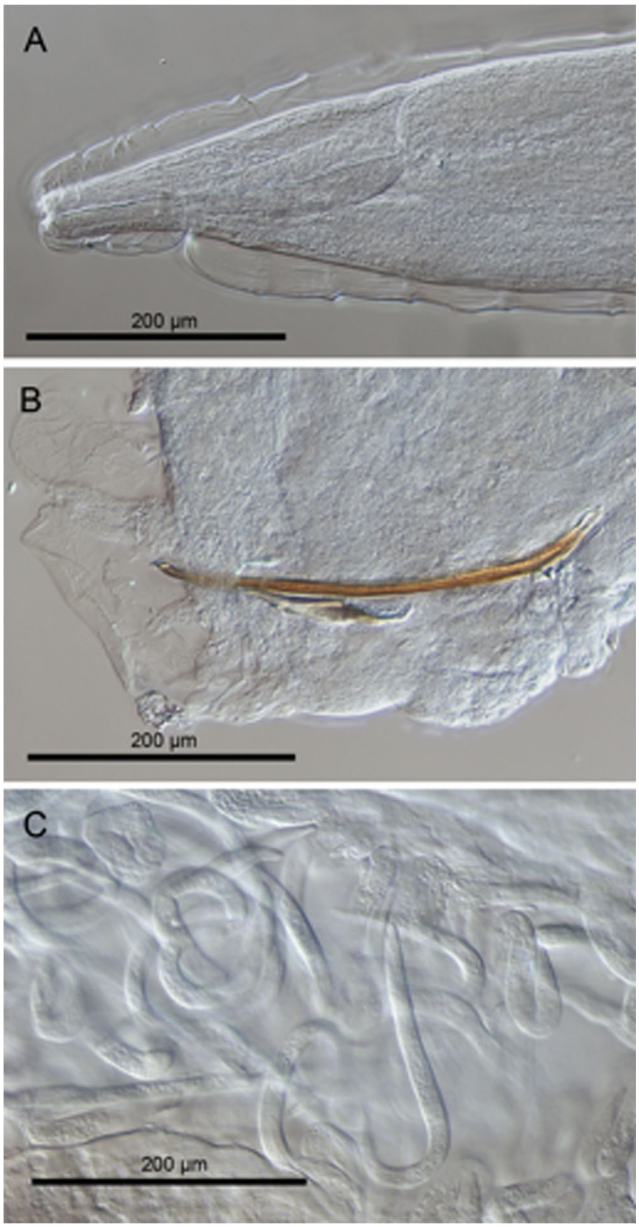

Fig. 2 *Crenosoma vulpis*. **a** Anterior end, lateral view, male. **b** Poster end, male, lateral view. **c** First-stage larvae *in uteri*

collected from each vertebrate host and was the most prevalent (n=22; 78.5 %), followed by haplotype II (n=4; 14.3 %) (Table 1). The nucleotide sequence variation, upon pairwise comparison, ranged from 0.3 to 0.9 % (mean value 0.5 %) and the highest nucleotide difference was recorded between haplotypes II and III, from the Basilicata and Campania regions, respectively. Analysis of the 18S rDNA gene revealed no nucleotide variability among the 29 sequences obtained from the specimens examined, were unrelated to their host and geographic origin and displaying a 100 % nucleotide identity with 18S rDNA sequences of *C. vulpis* in GenBank (AJ920367). No 12S rDNA sequences of *C. vulpis* were available in the GenBank database, and the BLAST analysis of this gene

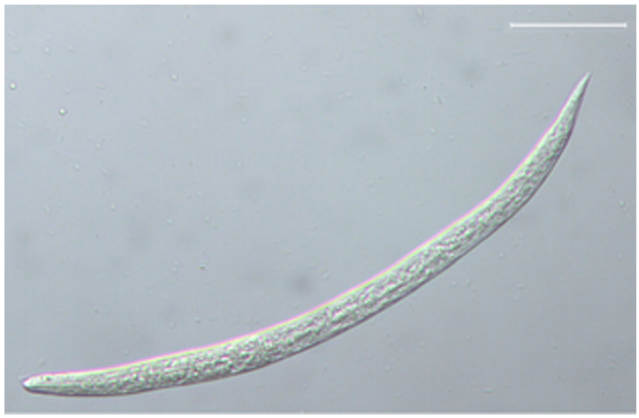

Fig. 3 First-stage larva of *Crenosoma vulpis*, detected at the Baermann test (*scale bar*=50 μm)

revealed the highest nucleotide identity with that of *A. abstrusus* (i.e., 85 %, JX519458).

The phylogenetic analyses of sequences herein examined and those of other metastrongyloids were concordant in clustering *C. vulpis*12S rDNA haplotypes in a paraphyletic group, to the exclusion of other metastrongyloid nematodes. The 18S rDNA sequence type of *C. vulpis* herein identified, and that of the same species available from GenBank, clustered with other *Crenosoma* species in a monophyletic clade (Fig. 4a and b). All representative haplotypes/sequence types of *C. vulpis* obtained have been deposited in the GenBank database (18S rDNA: KR920038; 12S rDNA: KR920039-KR920042).

Discussion

Crenosoma vulpis has been herein identified in wild (i.e., red foxes and badger) and domestic carnivores (i.e., dogs) from different regions of Italy and provide a phylogenetic account of this taxon within the superfamily Metastrongyloidea (Table 1).

C. vulpis is the first cause of pulmonary parasitic infections in foxes (Magi et al. 2009), with an overall prevalence up to 15.8 % in central Italy (Magi et al. 2015), which is higher than that herein recorded (i.e., 8.7 %). This difference may indicate that *C. vulpis* is less prevalent in southern Italy or it is expanding southward, as is the case for *A. vasorum* (Simin et al. 2014). Accordingly, the detection of *C. vulpis* in three dogs from geographically distinct regions (e.g., Basilicata, Emilia Romagna, and Veneto) suggests that the infection is most likely endemic in different dog populations of Italy, also considering only two reports in dogs from Campania and Liguria region (Rinaldi et al. 2007; Guardone et al. 2013). Conversely, the parasite is endemic in canine populations from the UK (Cobb and Fisher 1992; Reilly et al. 2000), Switzerland (Unterer et al. 2002), and Germany, where *C. vulpis* L1 were detected in 6.0 % of the 810 dogs examined

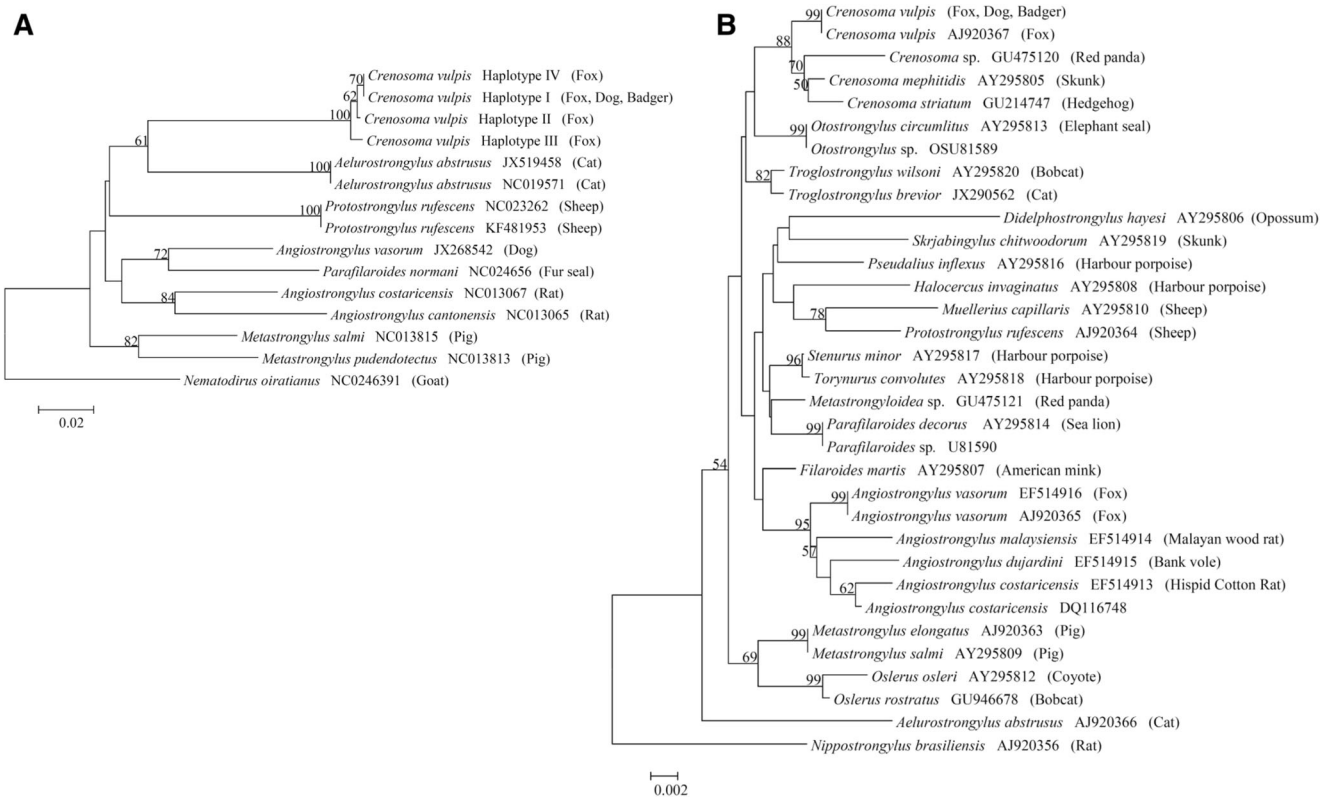

Fig. 4 Phylogenetic trees based on ribosomal 12S rDNA (**c**) and 18S rDNA (**b**) DNA sequence data for *Crenosoma vulpis* along with those of other metastrongyloids available in the GenBank database. The trees were constructed using the neighbor-joining (NJ) method, and bootstrap values are based on 5000 replicates, only bootstraps >50 % are indicated

(Barutzki and Schaper 2003). The expansion of *C. vulpis* infection to southern Europe could be a consequence of the existence of a sylvatic life cycle in red foxes, which is ultimately responsible for the transfer to dogs. In addition, red foxes may be effective spreaders of the parasite across European countries due to their ability to cross geographical borders (Manfredi et al. 2003; Magi et al. 2009, 2015; Taubert et al. 2009; Barutzki and Schaper 2011; Guardone et al. 2013). Changes in temperature and humidity may generate suitable environments for the reproduction of different species of gastropod intermediate hosts, as suggested for the increasing reports of *A. vasorum* (Morgan et al. 2005). Similarly, badgers were previously found positive for *C. vulpis* infection, also suggesting that this animal species may act as a bridging host between the sylvatic and urban cycle (Rudolph 1968; Popiołek et al. 2009).

The genetic data herein reported were consistent with the morphological identification of *C. vulpis* and provide further information for future molecular epidemiological studies of this nematode. For example, the retrieval of three out of four 12S rDNA haplotypes in a single red fox individual from Calabria indicates a significant level of genetic variability of this nematode within host populations. The existence of genetic variants of *C. vulpis* may be due to the inbreeding of specimens from different hosts or geographical areas of the same host or to a higher mutation rate of the mitochondrial gene region (Avise 1994). These hypotheses are supported by the higher nucleotide variability detected between haplotypes II and III (i.e., 0.9 %) for *C. vulpis* collected from red foxes from close geographical regions (i.e., Calabria and Campania). Furthermore, the high prevalence of 12S rDNA haplotype I (78.5 %) from red foxes may indicate a recent spreading of the parasite in the animal population as also inferred by the detection of this haplotype also in dogs and in the badger. This is also supported by the presence of a single 18S rDNA sequence type among all isolates, which was identical to that previously detected in foxes (Chilton et al. 2006). On the whole, the finding of the unique 18S rDNA sequences type and of the 12S rDNA haplotype I in foxes, dogs, and a badger throughout the country suggests that foxes have most likely played a role in spreading *C. vulpis* in previously nonendemic areas (Tolnai et al. 2015). Since no molecular data is available for *C. vulpis* specimens in any of the previous reports, it is not possible to identify which haplotypes/sequence types of this lungworm are circulating in Europe and in the USA.

Based on data herein generated, the epidemiology of *C. vulpis* is most likely underestimated, especially in dog

populations. This may be due to the infrequent use of Baermann examination in veterinary practice and/or because fecal flotation often fails to detect *C. vulpis* infections (Conboy 2009). Finally, the results of this study suggest that the distribution of this nematode is likely to increase due to closer roaming of foxes to the urbanized area (Sreter et al. 2003).

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

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