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Dirofilaria immitis in Bulgaria: the first genetic baseline data and an overview of the current status

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Summary

Dirofilaria immitis, the agent of canine dirofilariosis, is a common parasite of domestic and wild carnivores with zoonotic potential and worldwide distribution, being endemic in many countries. Bulgaria is among European countries recognized as endemic for this heartworm parasite. In the present study, *D. immitis* adults recovered from pulmonary arteries of domestic dog and golden jackal originating from the Pazardzhik region in southern Bulgaria, and from red fox originating from the Plovdiv region in central-southern Bulgaria, were genetically analyzed in nuclear targets. The first PCR amplification of the internal transcribed region 2 (ITS2) of the ribosomal DNA with previously published *D. immitis*-specific primers yielded single fragments in size of 302 bp that is characteristic for these heartworms. PCR products of three isolates, resulted from the second amplification of the 5.8S-ITS2 region (235 bp) with pan-filarioid primers, were subjected to direct DNA sequencing. Identical nucleotide composition was detected across the screened target region for these Bulgarian isolates. When the 5.8S-ITS2 sequences were phylogenetically compared to the GenBank-retrieved *D. immitis* sequences in a worldwide context, the neighbor-joining analysis has shown three discrete clades. The first clade was composed of *D. immitis* isolates from Europe (including the studied Bulgarian samples), Asia and South America, in the second clade samples from Asia and South America were placed, whereas the third clade was formed by two Brazilian dog isolates originated from the north and southeast part of the country. The purpose of the present study was to verify the taxonomic characterization of *D. immitis* nematodes from Bulgaria based on morphology and compare their genetic structure with filariae obtained from the different world regions using molecular assays. It also summarizes previous epidemiological and ecological studies on the parasite distribution and prevalences in different hosts and regions undertaken so far in Bulgaria.

Keywords: *Dirofilaria immitis*; Genetic; Clade; Golden jackal; Red fox; Dog

Introduction

Dirofilariosis is a zoonotic, mosquito-borne disease, caused by nematodes of the superfamily Filarioidea. One of the most pathogenic filarioid nematodes is the canine heartworm *Dirofilaria immitis* (Leidy, 1856) (Nematoda: Onchocercidae), the causative agent of infection that may lead to serious and potentially fatal cardiopulmonary disease, primarily induced by adult heartworms and their antigenic products (Simón *et al*., 2012). Canine dirofilariosis due to *D. immitis* was formerly considered as being a rare disease in humans, but a recent increase in number of infections, particularly after 2000, has resulted in its classification as an emerging

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zoonosis (Traversa *et al*., 2010). The species infects several mammalian species, especially domestic dogs, but in areas where these filariae are endemic, patent infections have also been recorded in wild carnivores as red foxes, jackals, wolves, leopards, coyotes, tigers, lions, ocelots, and less commonly in cats (Otranto & Deplazes, 2019). *D. immitis* can be transmitted by about 60 – 70 mosquito species of the family Culicidae that serve as potential intermediate hosts and vectors (McCall *et al*., 2008). The canine heartworm is widespread in tropical, subtropical and temperate areas, and its endemic occurrence has been reported in many countries of Europe, Asia, Africa, and the Americas (Dantas-Torres & Otranto, 2013; Genchi & Kramer, 2017).

In Europe, the historically endemic region for *D. immitis* infection is geographically restricted to southern countries including Spain, France, Portugal, Greece, Italy and Turkey (Genchi & Kramer, 2009). Nevertheless, the infections caused by this parasite are nowadays emerging in Europe, coinciding with geographic expansion from multiple focal populations in continental south towards the eastern, central and northern European territories (Farkas *et al*., 2020). In the region of Eastern Europe, Bulgaria, Croatia, Romania and Serbia are currently recognized as endemic countries, and sporadic autochthonous cases were also reported from the Czech Republic, Slovakia and Hungary (Genchi *et al*., 2020). Among the main factors that have facilitated the spread of heartworm disease in Europe during the last decades may be the global warming, which caused an increase in abundance of mosquito populations, and lengthening the transmission season (Sassnau *et al*., 2014); the high number of wild hosts, particularly golden jackals, and untreated stray dogs (e.g., Ionică *et al*., 2016; Stoyanova *et al*., 2019); and the launch of the Pet Travel Scheme in 2000, which has allowed easier movement of pet animals throughout the European Union (Trotz-Williams & Trees, 2003).

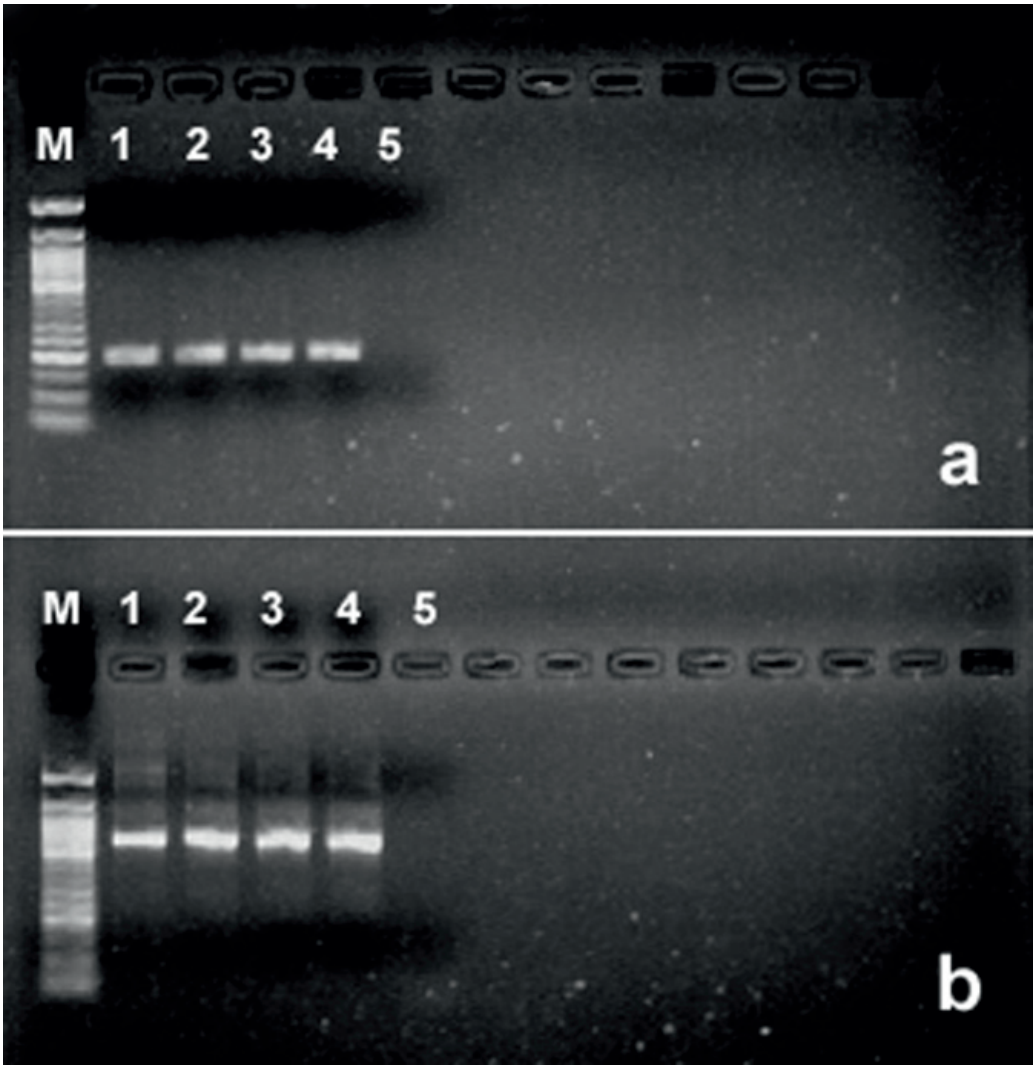

Fig. 1. Gel electrophoresis of PCR products in a 1.5% agarose gel using *Dirofilaria immitis*-specific primers for ITS2 region (**1a**) and pan-filarioid primers for ITS2 region (**1b**). Lane M: MW marker; lane 1, sample 1: *D. immitis* DNA (dog); lane 2, sample 2: *D. immitis* DNA (jackal); lane 3, sample 3: *D. immitis* DNA (fox), lane 4, sample 4: positive control (*D. immitis* DNA); lane 5, sample 5: negative control.

In Bulgaria, there is a clear trend of raising *D. immitis* prevalences in dogs and wild carnivores over the last decade (e.g., Panayotova-Pencheva *et al*., 2016; Iliev *et al*., 2017; Stoyanova *et al*., 2019). Nonetheless, no data are available so far about the genetic structure and polymorphism of *Dirofilaria* heartworms circulating in the country. Molecular methods were before successfully employed for identifying different filarioid parasites, especially by amplifying sequences of ribosomal DNA spacers by polymerase chain reactions using species-specific and universal primers for members of the superfamily Filarioidea (e.g., Gasser *et al*., 1996; Mar *et al*., 2002; Nuchprayoon *et al*., 2005; Rishniw *et al*., 2006). Hence, the purpose of this study was to verify the taxonomic characterization of selected canine heartworms from Bulgaria based on morphology, and to compare their genetic structure with filariae obtained from the different world regions using molecular assays. Additionally, previous epidemiological and ecological studies on *D. immitis* distribution and prevalences in different hosts and regions undertaken so far in Bulgaria are summarized.

Material and Methods

The study was carried out on adult heartworms collected from pulmonary arteries from three animals: a domestic dog (*Canis familiaris* L.) and a golden jackal (*Canis aureus* L.) originating from the Pazardzhik region, and from a red fox (*Vulpes vulpes* L.) from the Plovdiv region. Both regions are located in southern Bulgaria. The dog was provided dead for helminthological necropsy by the owners. The wild animals were hunted during the season 2014. Morphometric data and morphological characterization of adult helminths recovered from three hosts were provided in a previous study of Panayotova-Pencheva *et al*. (2016), and resulted in *D. immitis* taxonomic classification.

For molecular assays, genomic DNA was extracted using the DNeasy Blood & Tissue Kit 250 (QIAGEN, Germany) according to the manufacturer's instructions. The second internal transcribed spacer (ITS2) of the ribosomal DNA was first amplified by conventional PCR using species-specific primers D.imm-F1 (CATCAGGTGATGATGTGATGAT) and D.imm-R1 (TTGATT GGATTTTAACGTATCATTT), designed by Rishniw *et al*. (2006), that amplify the expected products only for *D. immitis* (size of 302 bp). For DNA sequencing, ITS2 and adjacent nuclear regions (5.8S, 28S) were then amplified with pan-filarioid primers DIDR-F1 (AGTGCGAATTGCAGACGCATTGAG) and DIDR-R1 (AGCGGGTAATCACGACTGAGTTGA), described also by Rishniw *et al*. (2006), and spanning a region of 542 bp for *D. immitis*.

The PCR products of the three isolates determined for sequencing were visualized on a 1.5 % agarose gel and then purified by NucleoSpin gel and PCR Clean-up (Macherey-Nagel, Germany). Amplicons were sequenced in both directions using a dye terminator cycle sequencing kit by Sanger sequencing in the commercial company at the Scientific Park of the Comenius University in Bratislava (Slovakia).

The obtained nucleotide sequences were manually edited and aligned using Clustal Omega multiple sequence alignment program (Sievers *et al*., 2011), and compared to the GenBank sequences by nucleotide BLAST program. The evolutionary branching patterns were generated by MEGA 7 software (Kumar *et al*., 2016) using the neighbor-joining method. The evolutionary distances were computed using the Tamura-3-parameter method (Tamura, 1992). All nucleotide positions with less than 95 % site coverage were eliminated from phylogenetic evaluation. Nucleotide sequences derived from the 5.8S-ITS2 region for three studied carnivore isolates were deposited in GenBank under the accession numbers MN596211, MN596213, and MN596214.

Ethical Approval and/or Informed Consent

No animals were killed for the purpose of this study.

Results and Discussion

The first DNA amplification of three examined Bulgarian adult isolates from domestic dog, golden jackal and red fox (herein assigned as DD-B, GJ-B, RF-B) using *D. immitis*-specific primers for the ITS-2 region generated single fragments in size around 300 bp, characteristic for these filariae (302 bp according to Rishniw *et al*., 2006) (Fig. 1a). The second DNA amplification of the 5.5S-ITS-2-28S region using pan-filarioid primers produced a band at position around 550 bp conformed to Rishniw *et al*. (2006) report where a single fragment of 542 bp was determined for *D. immitis* (Fig. 1b). The PCR products were then subjected to direct sequencing in both directions and the consensus sequences screened against the GenBank database using the BLAST algorithm has verified the *D. immitis* categorization for the three isolates under study.

Given that double peaks were detected throughout a second half of the obtained sequence patterns (spanning partially ITS2 and 28S region), only segments in length of 182 bp for ITS2 and 53 bp for 5.8S were taken for phylogenetic evaluation. Identical nucleotides were detected across the resolved nuclear region for the three Bulgarian isolates. When the sequences were compared to the worldwide GenBank-retrieved sequences available for the 5.8S-ITS2 region in *D. immitis*, three main clades were identified in the neighbor-joining phylogram (though not supported by high bootstrap values due to subtle intraspecific differences – 64 %, 54 %, and 65 %, respectively). As seen in Fig. 2, the Bulgarian samples clustered with the major *D. immitis* group consisting of isolates from geographically distinct areas in European, Asian and South American continents, specifically from Portugal, Turkey, Brazil, Iran and China. The only retrieved *D. immitis* sequences from Europe (GB accession numbers LN626266, LN626267) from continental Portugal (Ferreira *et al*., 2015) and from the geographically closer Mediterranean region (Kayseri province, Turkey; Yildrim *et al*., 2007) thus exactly matched the nucleotide composition of the Bulgarian isolates. Within this cluster, only two isolates (GB

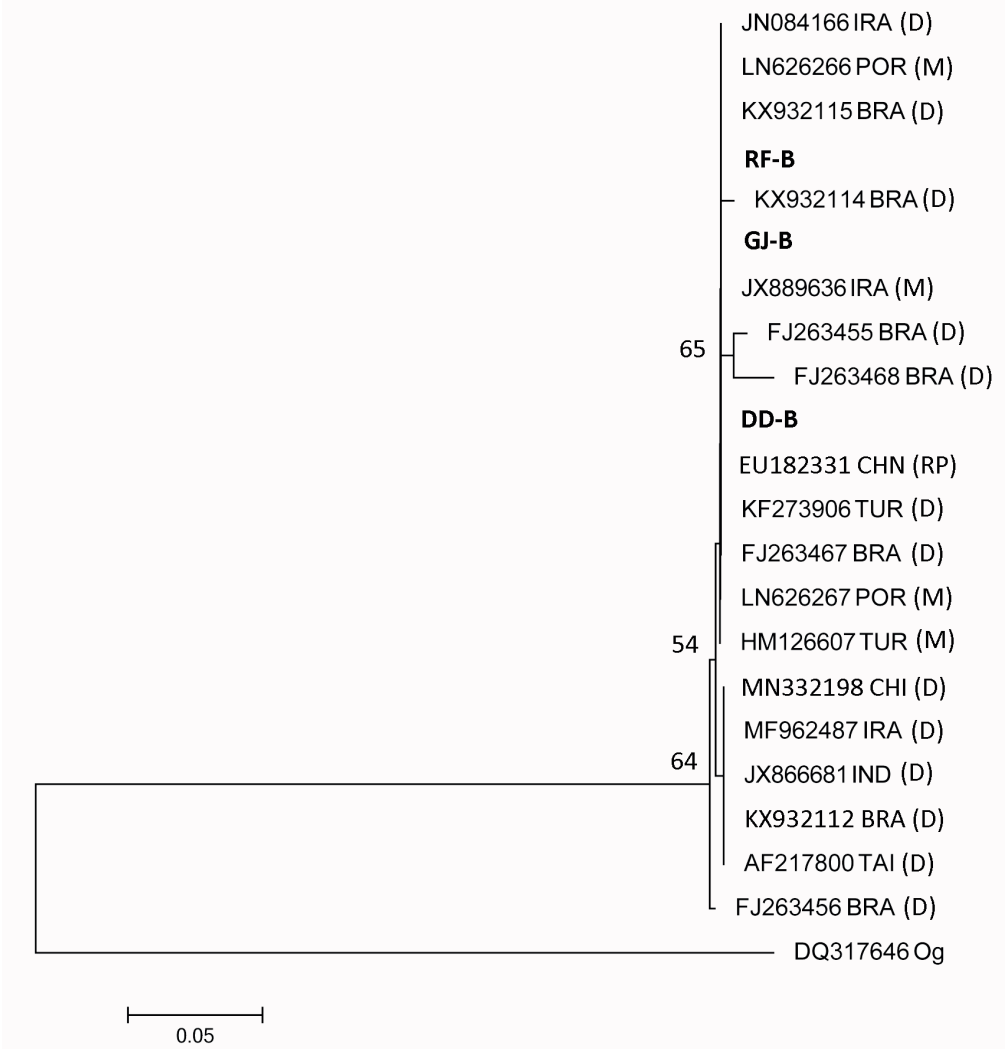

Fig. 2. Neighbor-joining (NJ) phylogram generated from ITS2 and 5.8S (231 bp) sequences showing the relationships among the *Dirofilaria immitis* from Bulgaria and GenBank reference conspecific sequences. *Onchocerca gibsonii* (Og) was used as the outgroup. Numbers next to the branches indicate the bootstrap value calculated from 1,000 replicates. The evolutionary distances were computed using the Tamura 3-parameter method and are in the units of the number of base substitutions per site. Bulgarian isolates involved in the phylogram: *D. immitis* from domestic dog (DD-B), golden jackal (GJ-B), and red fox (RF-B). Geographical origins of the referenced isolates: POR - Portugal, TUR - Turkey, IRA - Iran, CHN - China, IND - India, TAI - Taiwan, BRA - Brazil, CHI - Chile. Hosts and vectors of the referenced isolates (their labels are in brackets following the country codes next to the phylogram): D - dog, RP - red panda, M - mosquito.

accession numbers FJ263456, KX932114) from north of Brazil (Marajo Island) (Furtado *et al*., 2009) exhibited one nucleotide substitution in different sites. A single nucleotide polymorphism (G/A at position 207) was responsible for separating this cluster from the second clade that contained isolates from Asia and South America, specifically from India, Taiwan, Iran, Turkey, Brazil, and Chile (revealing 99.6 % similarity with the ´Bulgarian´ cluster). The third, most differentiated clade, was formed by the two dog isolates from the Marajo Island and the state of Rio de Janeiro, southeast of Brazil (GB accession numbers FJ263468, FJ263455; Furtado *et al*., 2009) that showed 97.7 % and 98.6 % sequence similarity, respectively, to the 'Bulgarian' cluster.

The obtained data pointed out for the homogeneous genetic structure of *D. immitis* in Bulgaria despite the involvement of two wildlife and one domestic host species that extended to the European and Mediterranean scale (continental Portugal, Turkey) in the nuclear region examined. Previous studies of the ITS2 regions conducted on a variety of helminths revealed that while intraspecific variation may occur, it is much smaller than interspecific variation and is often restricted to single nucleotide polymorphism (e.g., Conole *et al*., 1999; Huby-Chilton *et al*., 2001; Jenkins *et al*., 2005). Such aspect was also confirmed in this study where this type of polymorphism was common in the Genbank-retrieved *D. immitis* sequences derived from three continents.

The first cases of heartworm in dogs from various regions of Bulgaria were reported by Kanev *et al*. in 1996. In 2001, Georgieva *et*

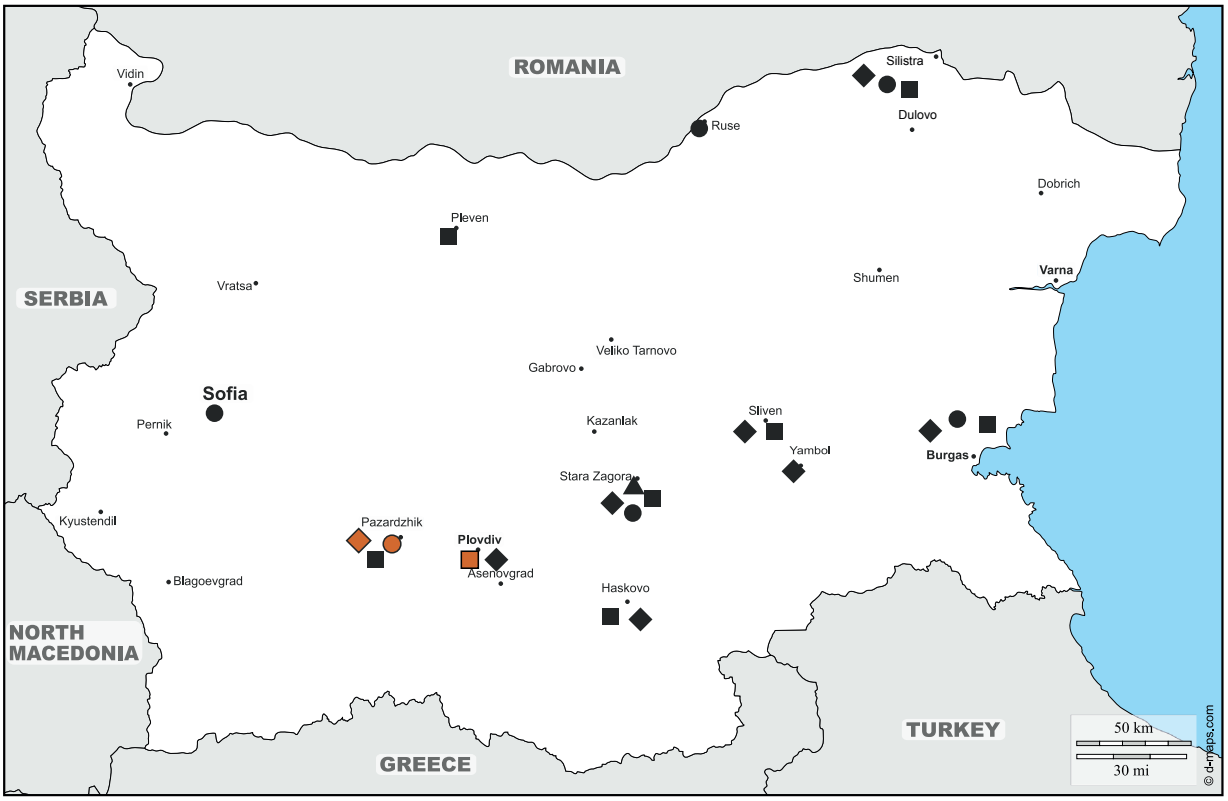

Fig. 3. Geographical distribution of *Dirofilaria immitis* in dogs (circles), red foxes (squares), golden jackals (rhombs) and wolf (triangle) recorded in the territory of Bulgaria. Orange marks represent *D. immitis* isolates analysed in the present study.

al. (2001) confirmed a presence of *D. immitis* in hearts and lungs of necropsied red foxes, golden jackals and one wolf (Table 1). Since then, the parasite has been detected in carnivores from several regions of Bulgaria with the highest occurrence in southern and lowland parts of Bulgaria, especially in areas of the Upper Thracian Plain and Danubian Plain. These areas were often situated near wet or marshy grounds, enlarged by the restoration of landscapes for rice cultivation over the last decade in the southern parts of the country, and provided excellent conditions for the reproduction and proliferation of the mosquito vectors. Further, climate changes and the enhanced movement of dogs and people across Europe may also have contributed to the increased geographical dispersal of dirofilariosis in Bulgaria according to Panayotova-Pencheva *et al*. (2016). Review on the recorded prevalences of *D. immitis* in domestic and wild-living carnivores in the country is presented in Table 1 and its spatial distribution in Figure 3.

Two herein examined wildlife isolates were recovered from golden jackal and red foxes, which are regarded as potential important reservoirs that could naturally widen the distribution area of filariae in Europe (Genchi & Kramer, 2020). The rapid spread of the golden jackals over the past decades throughout Europe addresses the issue of its involvement in the sylvatic cycle for a variety of pathogens in the newly colonized territories, including *Dirofilaria* spp. (Otranto *et al*., 2015). Since 1980´s, the jackal numbers have steadily increased in Europe and its current distribution range covers most of southeastern Europe and parts of eastern and central Europe, with animals occasionally being documented also in the north (Estonia, Lithuania) and in the west (Switzerland), far from the established Balkan populations (Arnold *et al*., 2012; Trouwborst *et al*., 2015). In light of recent positive findings of *D. immitis* in golden jackals and red foxes from Hungary (Tolnai *et al*., 2014), it might be possible that infected wild canids arrived from Romania (Ionică *et al*., 2017) or Serbia (Penezić *et al*., 2014) where *Dirofilaria* infections were recorded, which accentuates their role as reservoir hosts in the dissemination of these nematodes.

The present study provided the first evidence about the genetic structure of zoonotic *D. immitis* nematode in Bulgaria through the analysis of the partial 5.8-ITS2 nuclear region, with no polymorphism found among three animal isolates. Further studies will be aimed at screening of the additional gene segments in causative agents of canine dirofilariosis in Bulgaria and adjacent countries of southeastern Europe to better elucidate dissemination patterns linked to their recent expansion across the region.

Conflict of Interest

The authors declare that they have no conflict of interest.

Table 1. Review on the occurrence of *Dirofilaria immitis* in dogs and wild-living carnivores in Bulgaria.

Study year(s)	Host species (n)	Locality	Detection method	Prevalence % (ex/pos)	Reference
1991 – 1996	Dogs (341)	various regions	Knott´s test	5.3	Kanev *et al*. (1996)
1998	Dogs (20)	Stara Zagora	Necropsy	10.0	Gerogieva *et al*. (1999)
1997 – 1999	Dogs (258)	Stara Zagora	Knott´s test	7.4	Georgieva *et al*. (2001)
			HW antigen test	7.4	
	Dogs (40)		Necropsy	12.0	
	Red foxes (78)		Necropsy	5.2	
	Golden jackals (45)		Necropsy	4.4	
	Wolves (18)		Necropsy	5.5	
2001 – 2006	Dogs (487)	various regions	Knott´s test	8.6	Kirkova *et al*. (2007)
			HW antigen test	9.2	
	Red foxes (113)		Necropsy	3.0	
	Golden jackals (56)		Necropsy	8.9	
2011	Dogs (240)	Sofia	Knott´s test	8.7	Kostadinov (2012)
			HW antigen test	8.7	
		Ruse	Knott´s test	15.7	
			HW antigen test	15.7	
2012 – 2013	Red foxes (87)	various regions	Necropsy	27.6	Mirchev *et al*. (2013)
2015	Dogs (167)	Stara Zagora	HW antigen test	16.2	Pantchev *et al*. (2015)
2012 – 2013	Dogs (27)	Burgas	Necropsy	(8/4)	Panayotova-Pencheva *et al*. (2016)
		Pazardzhik		(5/3)	
		Silistra		(1/1)	
		Sofia		(2/1)	
	Red foxes (113)	Burgas		26.7	
		Pazardzhik		44.4	
		Plovdiv		40.9	
		Silistra		(5/2)	
		Sliven		(9/3)	
		Stara Zagora		(8/2)	
		Haskovo		(1/1)	
	Golden jackals (56)	Burgas		29.1	
		Pazardzhik		58.7	
		Pleven		27.8	
		Plovdiv		48.1	
		Silistra		44.4	
		Sliven		18.2	
		Stara Zagora		(5/3)	
		Haskovo		(4/4)	
		Yambol		14.3	
2013 – 2014	Dogs (33)	Sofia	Knott´s test	15.2	Radev *et al*. (2016)
			HW antigen test	15.2	
2012 – 2017	Dogs (367)	Stara Zagora	HW antigen test	34.3	Iliev *et al*. (2017)
2017 – 2018	Dogs (80)	Sofia	HW antigen test	31.3	Stoyanova *et al*. (2019)

n=number of examined; HW = heartworm; un = unknown; (ex/pos) = *D. immitis* examined/positive if less than 10 samples were collected

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