**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 dirofilariasis, 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-filaroid 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 northeast 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-filaroid 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*) from the Plovdiv region. Both regions are located in southern Bulgaria. The dog was provided dead for helminthological necropsy by the Plovdiv Regional Public Health Laboratory. The golden jackal was provided as part of the zoo’s necropsy and the red fox was provided as part of a research project. Both regions are considered part of the superfamily Filarioidea (e.g., Gasser et al., 1996; Mar et al., 2002; Nuchprayoon et al., 2005; Rishniw et al., 2006). 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.

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-filaroid primers produced a band at position around 550 bp confirmed to *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; Yildirim et al., 2007) thus exactly matched the nucleotide composition of the Bulgarian isolates. Within this cluster, only two isolates (GB
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
. (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.
| 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) |
|              | Red foxes (78)  | Stara Zagora | HW antigen test | 7.4 |          |
|              | Golden jackals (45) | Stara Zagora | Necropsy | 12.0 |          |
|              | Wolves (18)     | Stara Zagora | Necropsy | 5.2 |          |
|              |                 |           | Necropsy | 4.4 |          |
|              |                 |           | Necropsy | 5.5 |          |
| 2001 – 2006  | Dogs (487)      | various regions | Knott’s test | 8.6 | Kirkova et al. (2007) |
|              | Red foxes (113) |                 | HW antigen test | 9.2 |          |
|              | Golden jackals (56) |                 | Necropsy | 3.0 |          |
|              |                 |                 | Necropsy | 8.9 |          |
| 2011         | Dogs (240)      | Ruse | Knott’s test | 8.7 | Kostadinov (2012) |
|              |                 |                 | HW antigen test | 8.7 |          |
|              |                 |                 | 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) |
|              | Golden jackals (56) | Stara Zagora | Necropsy | 29.1 | Panayotova-Pencheva et al. (2016) |
|              |                 | Haskovo | (8/2) |          |
|              |                 | Burgas | (5/3) |          |
|              |                 | Pazardzhik | (1/1) |          |
|              |                 | Sofia | (2/1) |          |
|              |                 | Silistra | 26.7 |          |
|              | Red foxes (113) | Pazardzhik | 44.4 |          |
|              |                 | Plovdiv | 40.9 |          |
|              |                 | Silistra | (5/2) |          |
|              |                 | Sliven | (9/3) |          |
| 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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