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Dirofilaria repens predominates in shelter dogs from South Romania

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ABSTRACT

Canine filarioids are zoonotic vector-borne parasitic nematodes distributed mostly in the tropics and subtropics. The aim of this study was to determine the prevalence, distribution, etiology and genetic variation of canine filarioid infections by three different PCR techniques in four Southern Romanian counties. Blood samples of 300 shelter dogs were screened for infections with canine filarioids by real-time PCR. To determine filarioid species and coinfections, samples positive in the initial screening were further tested by conventional PCR and sequenced. Results indicated that 17% of the tested dogs were positive for at least one filarioid species. The prevalence of *D. repens* infection was 11.7%, significantly higher than that of *D. immitis* (4.7%) and *A. reconditum* (1.3%) ($p \leq 0.003$). The high prevalence of canine filarioid infections represents a challenge to animal and human health in the South of Romania, and they should be constantly monitored.

1. Introduction

Canine vector-borne filarioids are parasitic nematodes belonging to the Filarioidea superfamily and transmitted by arthropods such as mosquitoes or ticks. *Dirofilaria immitis* and *Dirofilaria repens* represent the most important canine vector-borne filarioids due to the cardiovascular disease associated with *D. immitis* and subcutaneous lesions that, in some cases, can be produced by *D. repens* in humans [1]. The infections caused by these nematodes hold an imminent zoonotic potential and importantly, climate change has had a considerable impact on the distribution of *Dirofilaria* species around the world, particularly well documented in Europe [2–4].

Dirofilaria immitis is the most frequently reported canine filarioid infection by veterinary clinicians in Romania since cardiovascular signs are frequently manifested in infected dogs. Several epidemiological studies on dogs show the presence of *D. immitis* in the South, West and East of the country [5–9] and *D. repens* in the South and West of Romania [7,8,10]. While most of these studies have relied on the morphological identification of microfilariae and serological methods, others have used molecular tools such as conventional PCR [8]. The highest prevalence of *D. immitis* and *D. repens* infections in dogs has been reported in the south-eastern county of Tulcea, where *D. immitis* and *D. repens* were detected by conventional PCR in 16% and 19% of the samples, respectively, and serologic assays detected 26–31% positivity in dogs [6,8]. The high abundance of these filarioids in this region can be explained by the high humidity in the Danube Delta, which favours the presence and reproduction of mosquitoes [11].

Filarioid infections hold a zoonotic potential and several cases of human *D. repens* infections have been reported in the southern and western areas of Romania [12–16]. Reports of canine and human

Abbreviations: AL, Alexandria; BLAST, basic local alignment search tool; CI, confidence interval; DJ, Dolj; HRM, high resolution melt analysis; IAL, Ialomița; ITS2, internal transcribed spacer 2; PCR, polymerase chain reaction; RV, Roșiorii de Vede; TS, Dobeta Turnu-Severin; CO, Coinfections with *D. repens* and *D. immitis*.

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infections highlight the considerable proportion of infected dogs in these regions which pose a risk of infection to human populations [12–16].

Taking into consideration that climate change has caused alterations in the geographical distribution of vector-borne diseases, which in turn increases the risk of human infections associated with *D. immitis* and *D. repens*, we consider that studying the epidemiology of these infections in Romania is timely and relevant [2,3,17]. Therefore, the purpose of this study was to evaluate the abundance and distribution of canine filarioid infections in the South of Romania by using highly sensitive molecular tools and to evaluate possible filarioid infections and co-infections in dogs. Both HRM-coupled real-time PCR and conventional PCR methods were used to analyse DNA samples to ensure the high sensitivity and specificity of the reactions.

2. Materials and methods

2.1. Sample collection

Venous blood samples were obtained from 300 stray dogs in municipal shelters from Southern Romania during July 2017. All dogs were apparently healthy and originated from five localities on the outskirts of the cities of Drobeta-Turnu-Severin (44° 37′ 53.9″ N 22° 37′ 55.2″ E), Craiova (44°20′32.4″N 23°43′ 02.6″E), Roșiorii de Vede (44° 07′ 04.8″ N 24° 59′ 58.5″ E), Alexandria (43°57′ 14.5″ N 25° 20′ 43.6″ E) and Slobozia (44° 34′ 18.1″ N 27° 20′ 51.4″ E) (Fig. 1). A map was generated with the sampling locations using the QGis v2.18.0 software (https://qgis.org/en/site/). Sheltered dogs that received no antiparasitic or parasiticidal prophylactic treatments were sampled from the corresponding cities, their outskirts and nearby villages. Blood samples were obtained from the cephalic vein, collected in EDTA tubes, and kept at − 20 °C until DNA extraction was performed. The study was done with the consent of the shelter management and approved by the Ethics committee of the Veterinary Faculty of Iași (reference number: 702). The number of samples collected from each geographical location is described in Table 1.

2.2. DNA Extraction and molecular screening for filarioid DNA

DNA extraction from EDTA blood samples was performed using a commercial kit (Illustra Blood Genomic Prep Mini Spin Kit, GE Healthcare, Buckinghamshire, UK), following the manufacturer's instructions. Extracted DNA was stored at − 20 °C for molecular analysis.

High-resolution melt (HRM)-coupled real-time PCRs were performed using primers that target a 115 bp of the mitochondrial 12 S gene of

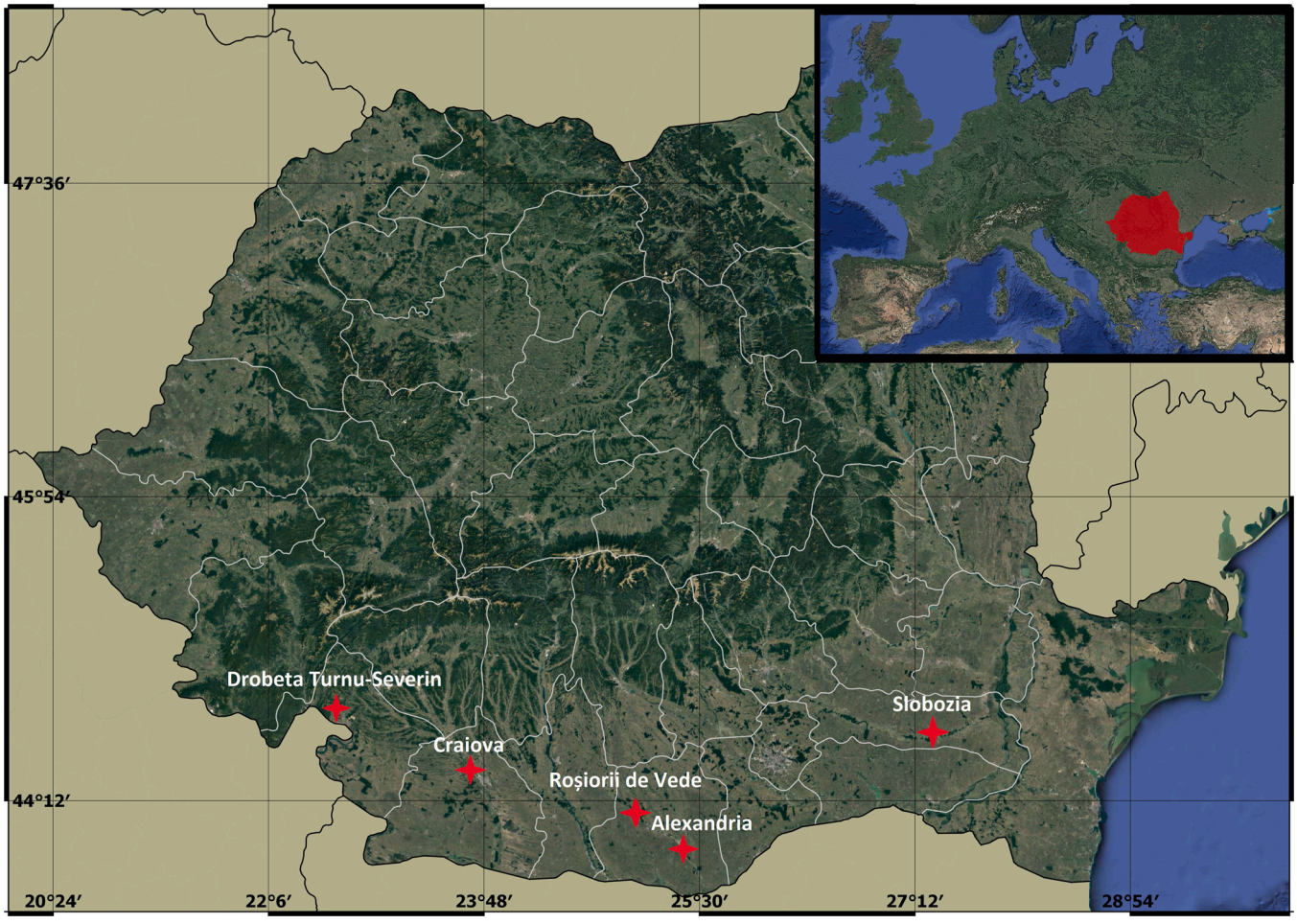

Fig. 1. Sampling locations in Southern Romania indicated as red crosses. Location of Romania in Europe is indicated in the small map.

filarioids (F 5′ -TTTAAACCGAAAAAATATT GACTGAC −3′ and R 5′-AAAAACTAAACAATCATACATGTGCC −3′) [18]. These primers were designed for the detection of *B. malayi, B. pahangi*, and *D. immitis*, and can be used to amplify DNA from other filarioid species using previously described modifications [19]. Reactions were performed in the Applied Biosystems Step One Plus™ Real-Time PCR System (Thermo Fisher Scientific, Massachusetts, USA).

All samples positive by HRM-coupled real-time PCR were tested again with a conventional PCR method to obtain a longer DNA sequence using the pan-filarial primers, DIDR-F1 (5'-AGT GCG AAT TGC AGA CGC ATT GAG-3') and DIDR-R1 (5'-AGC GGG TAA TCA CGA CTG AGT TGA-3') [20]. This reaction has been used to amplify fragments of different length (484–578 bp) of 5.8S-ITS2–28S sequences from *D. immitis* (542 bp), *D. repens* (484 bp), *A. reconditum* (578 bp), and six other filaroid species as described before [20]. Co-infection between canine filarioids was evaluated by running separate PCRs specific to other filarioids [20]. Samples positive for *D. repens* in the HRM-coupled real-time PCR were tested for *D. immitis* and *A. reconditum*. Those dogs with *D. immitis* infection were evaluated for *A. reconditum* and *D. repens*, and dogs positive for *A. reconditum* were tested for *D. immitis* and *D. repens*. The PCR protocols used for this included primers DICOI-F1 (5'-AGT GTA GAG GGT CAG CCT GAG TTA-3' and DICOI-R1 (5'-ACA GGC ACT GAC AAT ACC AAT-3') to detect *D. immitis,* primers DRCOI-F1 (5'-AGT GTT GAT GGT CAA CCT GAA TTA-3') and DRCOI-R1 (5'-GCC AAA ACA GGA ACA GAT AAA ACT-3') to detect *D. repens*, and primers ARCOI-F1 (5'-AGT GTT GAG GGA CAG CCA GAA TTG-3') and ARCOI-R1 (5'-CCA AAA CTG GAA CAG ACA AAA CAA GC-3') to detect *A. reconditum* [20].

2.3. DNA sequencing and phylogenetic analysis

Amplicons obtained from HRM-coupled and conventional PCRs were purified using a commercial kit (EXO-Sap, New England Biolabs Inc., Ipswich, MA, USA) and subsequently sequenced by HyLab Laboratories Ltd. (Rehovot, Israel) using the Big-Dye Terminator v3.1 Cycle Sequencing Kit and an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The 484–578 bp amplicons of the 5.8S-ITS2–28S sequences obtained by conventional PCR using DIDR-F1 and DIDR-R1 primers [20] were used for phylogenetic analysis.

DNA sequences were evaluated with the MEGA X software [21] and compared for similarity with sequences available in GenBank, using BLAST (https://pubmed.ncbi.nlm.nih.gov/2231712/). Identity was assigned to the closest GenBank accession with at least 97% of similarity to the identified organism. The number of strains for each filaroid species was estimated using the DNAsp V6 software [22] and representative sequences from each location were chosen for phylogenetic analysis. Phylogenetic analyses were performed using the MEGA X software [21] with the maximum likelihood method and the Tamura 3-parameter nucleotide substitution model using 1000 bootstraps [23,24]. A. reconditum, Onchocerca volvulus, and Mansonella ozzardii were used as outgroups.

2.4. Statistical analysis

Prevalence and 95% confidence intervals were calculated using the WinPepi v11.65 software (available at: http://www.brixtonhealth.com/pepi4windows.html) [25]. Differences of prevalence between each identified filarioid species and the difference of prevalence of each species between different counties were analysed using the Pearson's Chi-square test, and the WinPepi v11.65 software. The Bonferroni correction for *p* value was applied for multiple comparisons.

3. Results

3.1. Molecular diagnosis and statistical analysis

Seventeen percent of the tested dogs (52/300) were positive for *D. repens*, *D. immitis* or *A. reconditum* as obtained by the HRM-coupled real-time and conventional PCRs and confirmed by DNA sequencing. The total prevalence of infection for *D. repens* was 11.66% (n = 36, 95% CI: 8.55%−16.22%), 4.66% (n = 14, 95% CI: 2.57%−7.71%) for *D. immitis* and 1.33% (n = 4, 95% CI: 0.36%−3.38%) for *A. reconditum*. The prevalence of *D. repens* infections was significantly higher than that of *D. immitis* or *A. reconditum* ($p \leq 0.007$, $\chi^2 = 27.6$). Moreover, no significant differences were found in the prevalence of *D. repens*, *D. immitis* or *A. reconditum* between the collection sites (Table 1). Only three dogs from the regions of Mehedinti and Dolj were coinfected with *D. repens* and *D. immitis*.

3.2. Phylogenetic analysis

Dirofilaria immitis sequences obtained in this study were between 99.5% and 100% identical among themselves. They were 100% identical to ITS2 sequences deposited in GenBank obtained from red foxes (*Vulpes vulpes*) from Slovakia (MN596213) and dogs (*Canis familiaris*) from Turkey (KF273906). *D. repens* sequences were between 97% and 100% identical to each other and 100% identical to other ITS2 deposited in GenBank from dogs studied in Lithuania (MH469230) and Tunisia (KR676387). Only one *A. reconditum* sequence longer than 100 bp was obtained and was 100% identical to a sequence from a Brazilian dog (KX932122).

The maximum likelihood phylogenetic tree constructed with the 5.8-ITS2–28S sequences obtained in this study showed a close similarity to sequences from other geographical locations (Fig. 2). *D. repens* sequences from dogs in this analysis clustered together with other sequences of *D. repens* from other European countries such as the Czech Republic and Lithuania, and also with sequences from more distant countries like India, Tunisia and the United States (dog imported from the Netherlands). Comparably, *D. immitis* sequences from our investigation clustered together with *D. immitis* sequences from relatively close countries such as Turkey and Lithuania, and also with sequences from Chile or India.

4. Discussion

In this study, we present a comprehensive analysis of filarioid infections in stray dogs from different regions of Southern Romania using conventional and real-time PCR techniques. An overall 17% prevalence of canine infections with *D. repens*, *D. immitis* or *A. reconditum* was obtained, which was higher for some species than previous reports from Romania.

Dirofilaria repens was found in 11.7% of the dogs, higher than 4.25% previously reported in West Romania by the Knott's test and conventional PCR [10], 6.2% in the South of Romania by the Knott's test [7] and 6.92% in the Center of the country as obtained by conventional PCR [8]. The higher prevalence found in the current study can be explained by different sampling locations and the use of different diagnostic methods. While Ionica et al. used conventional PCR, other studies have mostly relied on the morphological identification of microfilaria in blood, which is less sensitive than molecular assays [8]. In contrast, we used an HRM-coupled real-time PCR which has been demonstrated to have a higher sensitivity compared to other tools [19].

The percentage of *D. repens*-positive dogs from the counties of

Table 1
Detection of infections with *Dirofilaria repens*, *Dirofilaria immitis* and *Acanthoheilonema reconditum* in dogs from the south of Romania. Infections are separated by city (Dobeta Turnu-Severin, Craiova, Roșiorii de Vede, Alexandria and Slobozia) and county (Mehedinți, Dolj, Teleorman and Ialomița).

County	City	Total			D. repens							D. immitis
	pos/tot	%	95% CI		pos/tot	%	95% CI	Statistical significance*				pos/tot
								CR	RV	AL	SL	
Mehedinți	TS	15/ 64	23.44%	13.75–35.69	12/64	18.75%	10.08–30.46	p = 1.000	p = 0.236	p = 1.000	p = 0.370	1/64
Dolj	CR	23/ 100	23%	15.17–32.49	14/100	14%	7.87–22.37		p = 0.657	p = 1.000	p = 0.961	10/100
Teleorman	RV	2/ 50	4%	0.49–13.71	2/50	4%	0.49–13.71			p = 0.984	p = 1.000	0/50
	AL	9/ 41	21.95%	10.56–37.61	6/41	14.63%	5.57–29.17				p = 1.000	3/41
Ialomița	SL	2/ 45	4.44%	0.54–15.15	2/45	4,44%	0.54–15.15					0/45
Total		51/ 300	17%	12.93–21.74	36/300	12%	8.55–16.22					14/300

TS – Dobeta Turnu-Severin, CR - Craiova, RV - Roșiorii de Vede, AL - Alexandria, SL – Slobozia
CO – Coinfections with *D. repens* and *D. immitis*
*Corrected for multiple comparisons (Bonferroni)

Mehedinți (South-West), Dolj (South-West) and Teleorman (South) was 18.8%, 14% and 8.8%, respectively. These results were similar to the prevalence of 13.7% in Dolj and 9.8% in Teleorman reported in 2014 [8]. A study published in 2021 reports a much lower prevalence of 2.66% in Dolj and 9.5% in Mehedinți. However, this difference could be explained by the fact that it was carried on both stray and owned dogs (mostly owned) and the detection of microfilaremia was by the Knott's test and only confirmed by conventional PCR [26]. Epidemiological studies on dogs, from countries to the south of Romania reported a higher prevalence of *D. repens*. One study from neighbouring Serbia from 2008 reported that 49.2% of dogs were infected with *D. repens* as shown by the Knotťs test [27] while another study from 2012, in a neighbouring region of Serbia, reported that 17.2% of the dogs were microfilaremic with *D. repens* and 42.6% were seropositive dogs by ELISA [28]. A study from Bulgaria in 2016 reported an 18% prevalence of *D. repens* using the Knotťs test [29]. The lower prevalence of this nematode species in our study might be explained by a difference of climate and also by the fact that the studies were done in different years.

The overall prevalence of *D. immitis* infection in the current study was 4.7%, similar to the 6.2% prevalence in owned dogs from the South and Center of Romania as detected in 2010 and 2011 [8]. A previous study conducted on randomly selected dogs (guard, pet, shelter, stray, and hunting dogs) in all regions of Romania showed a seroprevalence of 3.3% of *D. immitis* by ELISA [6]. However, two other studies on stray and owned dogs using the Knott's method, from the South of the country reported a much higher percentage of infection, between 12.6% and 23% [5,7]. Although both these studies were carried out in the south of Romania, the higher prevalence observed in those studies may be explained that they were carried out in different counties than our work. Moreover, in those studies, sampling was done in different years and using different sampling methods. In the present study, 10% of the dogs from the county of Dolj had *D. immitis* infections, close to the 7.8% reported in this location using conventional PCR [8]. In addition, a 14% seroprevalence was found in the county of Dolj in 2012 in randomly selected dogs [6]. The higher positivity in the previous study compared to our data, may be explained again by occult and amicrofilaremic infections produced by filarioid females, which lead to amicrofilaremia [30]. A recent study from 2021 reports a prevalence of *D. immitis* of 1.3% in Dolj and 7.1% in Mehedinți, in owned and stray dogs (mostly owned). This is different than the 10% in Dolj and 1.5% in Mehedinți reported in this study. This could be explained by sampling both owned and stray dogs (mostly owned) and by the use of the Knott's test for the initial determination of infection before PCR [26].

The prevalence of canine *D. immitis* infections reported in countries to the South of Romania is considerably higher than the prevalence we report here. Studies on stray and owned dogs from Serbia reported infections from 7.2% to 12.3% with *D. immitis* [27,28], whereas studies from Bulgaria also on stray and owned dogs, reported infection rates ranging from 7.4% to 15% [29,31,32]. These variations may arise from sampling locations with different climate conditions with potential differences in vectors, and periods of vector activities [4].

The prevalence of *A. reconditum* in our study was 1.3% which is in line with previous findings of 2% in owned dogs from the south of Romania [8] and 0.91% in owned dogs imported to Germany from Romania [33]. In addition, a low prevalence of *A. reconditum* (0.33%) has also been reported in red foxes from Romania [34]. Similarly, a low prevalence of *A. reconditum* infection in owned dogs was reported in Serbia (2.1%) in 2008 and in Greece (1.3%) in 2016 [27,35].

Differences in the prevalence of canine filarioid infections between countries reflect environmental and demographic variations. While climate and the way the dogs are kept are two important factors explaining the changes in the prevalence between countries, other factors, such as the number of stray dogs not treated with anti-filarial drugs, the abundance of wetlands or rivers, the presence of compatible mosquito vectors and their numbers, their activity periods, as well as governmental efforts to control mosquito vectors, and the research methodology used in the studies may also influence the results [36,37].

Dirofilaria immitis, *D. repens* and *A. reconditum* infections have been reported in several wild animals in Romania. *D. repens* has been reported in jackals (*Canis aureus*), red foxes, wolves (*Canis lupus*), and weasels (*Mustela nivalis)*; while *D. immitis* was reported in jackals, red foxes, and wildcats (*Felis silvestris*). Lastly, *A. reconditum* has only been reported in red foxes [34,38]. Wildlife animals represent important reservoirs for filarioid infections. Therefore, monitoring these parasites in wild hosts is highly relevant to understanding how these parasites spread [34,38].

Canine filarioid infections also represent a public health concern. *D. repens* and *D. immitis* can be transmitted to humans with serious consequences on human health [39,40]. Several cases of human *D. repens* infections have been reported in Romania, between 2012 and 2018 [12–16]. A seroepidemiological study reported an 18.2% human seroprevalence for *D. immitis* in Bucharest located in the south of Romania [41]. It is clear that a significant part of the human population in the south of Romania is exposed to filarioid infections. Because dogs are considered the main reservoir for these parasites, human *D. immitis* and *D. repens* infections are likely a consequence of the high proportion of the filarioids in dogs from South Romania. Comprehensive and

D. immitis						A. reconditum							CO
%	95% CI	Statistical significance*				pos/tot	%	95% CI	Statistical significance*				
		CR	RV	AL	SL				CR	RV	AL	SL	
1.56%	0.04–8.40	p = 0.205	p = 1.000	p = 1.000	p = 1.000	3/64	4.69%	0.98–13.09	p = 1.000	p = 0.604	p = 0.819	p = 0.712	1
10%	4.9–17.62		p = 0.036	p = 1.000	p = 0.053	1/100	1%	0.03–5.45		p = 1.000	p = 1.000	p = 1.000	2
0%	0–7.11			p = 0.267	p = 1.000	0/50	0%	0–7.11			p = 1.000	p = 1.000	0
7.32%	1.54–19.92				p = 0.327	0/41	0%	0–8.6				p = 1.000	0
0%	7.87					0/45	0%	0–7.87					0
4.66%	2.57–7.71					4/300	1.33%	0.36–3.38					3

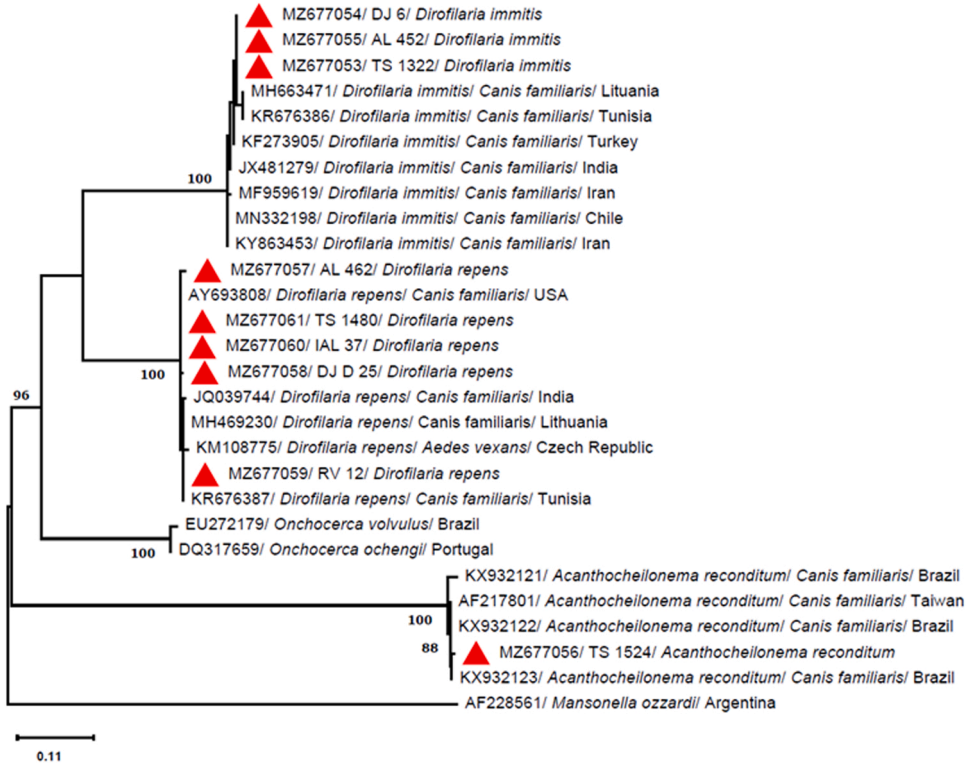

Fig. 2. Maximum likelihood phylogenetic tree of canine filarioid 5.8S-ITS2–28S sequences obtained in the present study and compared to other sequences deposited in GenBank. Sequences obtained in the present study are marked with red triangles and include GenBank accession numbers with the sampling location marked as DJ (Dolj), AL (Alexandria), IAL (Ialomița), RV (Roșiorii de Vede), and TS (Dobeta Turnu-Severin). Only bootstrap values above 60% are shown.

updated epidemiological studies focused on canine filarioid infections, like the study presented here, describe an indirect yet accurate indication of the risk for human exposure to these infections.

5. Conclusions

A high percentage of canine filarioid infection was found in stray dogs from Southern Romania, with *D. repens* having the highest abundance, followed by *D. immitis* and *A. reconditum*. Canine filarioid infections caused by *D. repens* and *D. immitis* represent a challenge for animal and human health in Romania due to insufficient awareness of these infections and a changing climate that modifies the prevalence and distribution of vector-borne diseases. There is a growing need to further monitor these zoonotic infections and to understand the factors that influence their distribution and prevalence.

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CRediT authorship contribution statement

Andrei Alexandru Cimpan: Conceptualization, Investigation, Methodology, Formal analysis, Writing – original draft. **Gad Baneth:** Conceptualization, Project Administration, Resources, Writing – review & editing. **Yaarit Nachum-Biala:** Project administration, Writing – review & editing. **Liviu Miron:** Writing – review & editing. **Alicia Rojas:** Conceptualization, Investigation, Methodology, Supervision, Writing – review & editing.

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Conflict of interest

The authors declare they have no competing interest.

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