Veterinary Parasitology 326 (2024) 110096

Contents lists available at ScienceDirect

Veterinary Parasitology

journal homepage: www.elsevier.com/locate/vetpar

Genetic variability of *Dirofilaria repens* isolates from humans and dogs in Italy

Simona Gabrielli [a,1], Leonardo Brustenga [b,1], Giulia Morganti [b], Lavinia Ciuca [c], Giulia Barlozzari [d], Giulia Rigamonti [b], Margherita Orlandi [e], Monica Sforna [b,*], Fabrizia Veronesi [b]

[a] Department of Public Health and Infectious Diseases, Sapienza University of Rome, Rome, Italy
[b] Department of Veterinary Medicine, University of Perugia, Perugia, Italy
[c] Department of Veterinary Medicine and Animal Production, University of Naples Federico II, Naple, Italy
[d] Istituto Zooprofilattico Sperimentale del Lazio e della Toscana M. Aleandri, Rome, Italy
[e] Freelance Veterinarian for MyLav - La Vallonea, Laboratorio di Analisi Veterinarie s.r.l. Passirana di Rho (MI), Italy

ARTICLE INFO

Keywords:
Dirofilaria repens
MtDNA
Dog
Human
Italy
Genetic variability

ABSTRACT

Dirofilaria repens is a paradigmatic example of an emerging vector-borne pathogen (VBP) in both human and veterinary fields. The spatial expansion and the increasing zoonotic impact of this VBP can be related to several drivers including the genetic structure of parasite populations. Italy is one of the European countries traditionally endemic with the highest incidence of canine and human cases of subcutaneous dirofilariosis. The present study aimed to assess the genetic identity and variability of *D. repens* isolates of human and canine origin from areas of Central Italy, compared with those isolated from different areas of Europe by sequence analysis of mtDNA genes (i.e., 12 S rDNA and cox1). A total of twenty isolates of *D. repens* were obtained from biopsies of subcutaneous and ocular cases of dirofilariosis occurring in 10 dogs and 10 humans. The sequence analysis of 12 S rDNA showed that all the sequences obtained clustered as a monophyletic group with a strong nodal support, indicating that all sequence types represented *D. repens*. The cox1 and the 12 S sequence analysis did not show host-related polymorphisms between human and dog-derived specimens. The sequence analysis of cox1 was performed including 8 additional sequences previously obtained from human and canine isolates in the same areas. Out of the 28 sequences analyzed, 20 were grouped in a haplogroup comprising 15 haplotypes (i.e., DR1, DR2, DR4, DR5, DR7, DR8, DR10-DR18), 2 sequences matched to DR9, reported for the first time in Italy, and 6 showed peculiar polymorphisms that were not previously described. The results obtained have implications for a better understanding of the epidemiology and phylogeography of this emerging vector-borne zoonotic parasite.

1. Introduction

Dirofilaria repens (Spirurida, Onchocercidae) is a mosquito-borne parasite that infects domestic and wild carnivores, localizing in subcutaneous nodules and, less commonly, in other anatomic sites such as the eyes (Simón et al., 2012; Albanese et al., 2013). The most important reservoir of infection is represented by dogs with microfilariaemia, to actively infect the mosquito vectors (Genchi et al., 2011a). Although *D. repens* is traditionally considered a parasite of veterinary concern, it may occasionally be transmitted to humans through anthropozoophilic mosquitoes, such as the Asian tiger mosquito (*Aedes albopictus*) (Capelli et al., 2018). Humans are dead-end hosts in which the parasite causes subcutaneous nodules and "*larva migrans*" syndromes in different tissues (e.g., eyes, lungs, abdominal wall and reproductive apparatus), rarely developing into adult stages (Pupić-Bakrač et al., 2021; Gabrielli et al., 2021). In addition, only 23 microfilaremic cases have been described in humans to date (Pupić-Bakrač et al., 2021; Tasić-Otasevic et al., 2023).

Dirofilaria repens is considered as one of the most evident examples of emerging vector-borne pathogen (VBP) in Europe, with more than 3500 human cases reported in the Old World from 1977 to 2016 (Albanese et al., 2013; Capelli et al., 2018; Genchi and Kramer, 2020; Simón et al., 2012; 2022). In particular, a growing number of cases has been recorded

* Correspondence to: Department of Veterinary Medicine, University of Perugia, San Costanzo Street 4, Perugia 06126, Italy.
E-mail address: monica.sforna@unipg.it (M. Sforna).
[1] These two Authors contributed equally to the work

https://doi.org/10.1016/j.vetpar.2023.110096
Received 9 October 2023; Received in revised form 4 December 2023; Accepted 9 December 2023
Available online 12 December 2023
0304-4017/© 2023 The Author(s). Published by Elsevier B.V. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).

not only in endemic areas of the southern and south-eastern Europe (e.g. Spain, France, Italy, Greece, Ukraine, Romania), but also in previous non endemic ones as central-eastern and northern Europe (e.g. Balkans, Slovakia, Moldova, Russian Federation, Lithuania, Estonia, Latvia, Poland, Hungary, Bulgaria, Norway, Sweden, Finland) (Genchi et al., 2011b; Genchi and Kramer, 2020; Otranto et al., 2013; Sałamatin et al., 2013; Kartashev et al., 2014; Rossi et al., 2015; Capelli et al., 2018; Alsarraf et al., 2021).

The overall expansion of *D. repens* and its growing zoonotic impact, can be attributed to several biotic and abiotic factors, including: i) the improvement in the network of pathological services and the refining of the diagnostic tools, especially for humans (Mendoza-Roldan et al., 2021); ii) the climatic changes favoring the development and survival of larval stages inside the vectors and the development of the mosquitoes themselves (Simón et al., 2017); iii) the introduction of opportunistic and generalist zoophilic feeder vectors (i.e. *Ae. albopictus*); iv) the increased number of pets travelling along with their owners (Cancrini et al., 2007; Capelli et al., 2018). However, also the genetic structure of the parasite populations could affect the epidemiological aspects and clinical presentations of human and animal infections as observed for recently introduced other spirurid species like *Thelazia callipaeda* (Otranto et al., 2005; Zhang et al., 2018). Therefore, the determination of the genetic variability of a broad range of *D. repens* isolates from different hosts and from old and new endemic areas of Europe might be useful to understand both the phylogeography of the parasite and how it affects the epidemiological dynamics of the infection by increasing its zoonotic potential and its ability to spread and adapt to new areas (Laidoudi et al., 2022). This approach could also clarify the clinical impact that different parasitic strains may have. Some Authors already suggested that *D. immitis* might be a complex of cryptic species or that, at least, population-specific differences would exist also having differential zoonotic potential and pathogenic impact, in fact, the existence of a more virulent strain in the North America has been suggested (Dantas-Torres and Otranto, 2013).

To date, studies on the genetic structure of *D. repens* received scant attention from the scientific community (Aher et al., 2016; Laidoudi et al., 2022; Alsarraf et al., 2023a) compared to *D. immitis* (Alsarraf et al., 2023a,b). Few genotyping studies have been performed by PCR amplification and sequencing of genetic markers used also in other spirurid species (Otranto et al., 2005; Lefoulon et al., 2015; Rojas et al., 2018), particularly barcoding mitochondrial (mtDNA) markers (i.e. cytochrome C oxidase subunit I (*cox*1), dehydrogenase subunit I (NADH), 12 S rDNA) and /or ribosomal genes (rDNA), (i.e., internal spacer transcripts, ITS1, and ITS2) (Ferri et al., 2009; Laidoudi et al., 2022; Alsarraf et al., 2023a).

The single nucleotide polymorphisms (SNPs) detected in the sequences of the 18S-ITS1–5.8 S region and the complete mtDNA allowed to suggest the existence of two further species of *Dirofilaria*, Candidatus *Dirofilaria hongkongensis*, responsible for both human and canine subcutaneous dirofilariosis in China and India and *Dirofilaria* sp. "Thailand II" responsible in Thailand of feline infections (To et al., 2012; Dantas-Torres and Otranto, 2020). Furthermore, a recent survey conducted on European and Middle-east isolates allowed to identify 18 haplotypes of *D. repens* named DR1 through DR18, differentiated on the basis of SNPs of mtDNA markers, using a *cox*1 fragment of 936 bp obtained overlapping two smaller fragment of 411 bp and 525 bp and a NADH fragment of 516 bp. DR1 was identified as the dominant haplotype and a close relationship between haplotypes from new endemic region of north-eastern Europe and the old ones of the south-eastern Europe, excluding Italy was detected (Laidoudi et al., 2022; Alsarraf et al., 2023a).

To date the genotyping of few isolates of Italian origin allowed to speculate about the occurrence of a segregation of some haplotypes (DR14, DR15, DR17), found exclusively in Italy and in none of the other European countries, however the small number of analyzed samples (n. 3) did not allow a reliable and definitive evaluation of the circulating mt haplotypes (Alsarraf et al., 2023a). Recently 8 sequences of the mtDNA *cox*1 gene were obtained in central Italy from 5 humans (Gabrielli et al., 2021) and 3 from dogs (Barlozzari et al., 2021) respectively, but no haplotype analysis was assessed.

Italy is one of the "sentinel" countries for monitoring the incidence of subcutaneous dirofilariosis in Europe; in fact, the numerous human cases (over 320 in 10 years) and the high *D. repens* infestation rates in the canine population provides evidence that *D. repens* is emerging (Otranto et al., 2011a; Otranto et al., 2011b; Simón et al., 2012; Albanese et al., 2013; Genchi and Kramer, 2020).

The aim of the present study was to explore the genetic variability within human and canine *D. repens* isolates collected from different geographical locations of Central Italy, considered historically endemic for *D. repens* with a medium prevalence (range 1%−12.1%) (Cringoli et al., 2001; Pampiglione et al., 2001; Scaramozzino et al., 2005; Traversa et al., 2010; Magi et al., 2012; Sauda et al., 2018; Macchioni et al., 2020), using 2 mtDNA genes (cox1 and 12 S rDNA). These molecular markers have been selected because they are suitable for inferring population differences and for conducting phylogenetic analysis at different taxonomic levels (Suzuki et al., 2015; Alsarraf et al. 2023a,b). In addition, the use of *cox*1 allowed us to include in the phylogenetic analysis the clinical *D. repens* isolates of human and canine origin collected in Central Italy in the previous publications of Gabrielli et al. (2021) and Barlozzari et al. (2021).

2. Materials and methods

2.1. Dirofilaria repens isolates

In the present study, a total of 20 *D. repens* specimens, of which 10 from dogs and 10 from humans from endemic areas of central Italy (Umbria, Latium, Campania and Tuscany regions) were obtained.

The specimens were searched, on the database repositories of the Pathology Services of the Veterinary Teaching Hospitals (OVUDs) of the Department of Veterinary Medicine of Perugia (Umbria region) and Naples (Campania region), Istituto Zooprofilattico Sperimentale of Latium and Tuscany M. Aleandri (Latium region), the Human Hospital Santa Maria della Misericordia of Perugia (Umbria region) and the Department of Public Health and Infectious Diseases of the Sapienza University of Rome (Lazio region) (personal collection of S. Gabrielli), records of biopsies of subcutaneous nodular or ocular lesions, and DNA extracted from worms or whole blood samples collected for clinical diagnosis or research purposes. The stored formalin-fixed and paraffin-embedded (FFPE) blocks of tissues of dogs and humans were included in the study if previous histopathological examinations identified subcutaneous infections by *D. repens* based on morphological criteria (e.g. 13–15 μm thick cuticular layer and longitudinal striations in the form of ridges on the external surface of the cuticle of the worms observed). The FFPE tissue blocks were cut to obtain four-to-six 10 μm-thick sections for each one to be submitted to DNA extraction. DNA extracted from worm and whole blood samples of this study had previously tested positive for *D. repens* using Filarioid cox1 PCR amplification and sequencing.

2.2. Isolation of genomic DNA and amplification of the 12 S rDNA and cox1 loci

The sections of each FFPE block were deparaffinized at room temperature by immersion washing twice for 30 min each in 1 mL of xylene and rinsed twice with 1 mL of 100% ethanol for 5 min. The samples were centrifuged at 10,000 × *g* for 5 min and the fluid was decanted between each change. Total genomic DNA was extracted with the ExgeneTM FFPE Tissue DNA Kit (GeneAll, Seoul, Korea), according to the manufacturer's protocol and submitted to PCR amplification targeting two fragments of mtDNA genes: the cox1, to assess the haplotypes present in the Italian population; and the 12 S rDNA used as secondary target to double-check the taxonomical identification.

Table 1
Identificative number (Genbank accession number), region and host of origin, year of collection and location of *Dirofilaria repens* included in the study.

Identificative number	Region of origin	Host of origin	Year of collection	Nodule location
OR413192	Umbria	Human	2021	Left abdomen
OR413193	Latium	Human	2016	Subcutaneous
OR413194	Latium	Human	2002	Ocular
OR413195	Latium	Human	2017	Subcutaneous
OR413196	Latium	Human	2015	Subcutaneous
OR413197	Latium	Human	2008	Subcutaneous
OR413198	Latium	Human	2005	Ocular
OR413199	Latium	Human	2002	Subcutaneous
OR413200	Latium	Human	2010	Ocular
OR413220	Latium	Human	2006	Ocular
MW525256 *	Latium	Human	2018–2020	Cheek
MW525257 *	Latium	Human	2018–2020	Lung
MW525258 *	Latium	Human	2018–2020	Lung
MW525259 *	Latium	Human	2018–2020	Calf muscle
MW525260 *	Latium	Human	2018–2020	Ocular
MT345574 * *	Latium	Dog	2019	Testicle
MT345575 * *				Blood
MT345576 * *	Latium	Dog	2019	Blood
OR413181	Umbria	Dog	2020	Temporal region
OR413182	Umbria	Dog	2015	Trunk
OR413183	Tuscany	Dog	2013	Chest
OR413184	Tuscany	Dog	2013	Axilla
OR413185	Umbria	Dog	2019	Anterior right leg
OR413186	Umbria	Dog	2020	Dorsal region
OR413187	Umbria	Dog	2018	Left trunk
OR413188	Campania	Dog	2015	N.a.
OR413189	Campania	Dog	2018	N.a.
OR413190	Campania	Dog	2019	N.a.

* sequences from Gabrielli et al., 2021
* * sequences from Barlozzari et al., 2021

The fragment (330 bp) coding for the 12 S rDNA gene was amplified using the primers Fila12SF 5'- CGGGAGTAAAGTTTTGTTTAAACCG – 3' and Fila12SR 5'- CATTGACGGATGGTTTGTACCAC – 3' (Latrofa et al., 2015), whereas the fragment (209 bp) coding for the cox1 was amplified using the primers DRCOI-F1 5'- AGTGTTGATGGTCAACCTGAATTA – 3' and DRCOI-R1 5'- GCCAAAACAGGAACAGATAAAACT – 3' (Rishniw et al., 2006). The amplifications for both the genetic targets were carried out on a total volume of 25 μL as follows: 12.5 μL of BlasTaq 2x PCR MasterMix (Applied Biological Materials, Richmond, Canada), 1 μL of each of the forward and reverse 10 μM primers, 5 μL of template DNA and Nuclease-Free water (Promega, Madison, USA) to final volume. Thermal cycling for each of the targets were as follows: for the 12 S fragment an initial denaturation at 95 °C for 2 s followed by 40 cycles of denaturation at 95 °C for 1 min, annealing at 58 °C for 1 min and elongation at 72 ° for 1 min, with a final elongation step at 72 °C for 7 min; for the cox1 fragment an initial denaturation at 94 °C for 2 min followed by 40 cycles of denaturation at 94 °C for 45 s, annealing at 47 °C for 45 s and elongation at 72° for 1 min and 30 s, with a final elongation step at 72 °C for 5 min. Negative controls using nuclease-free water were added in each round of amplification. Amplicon quality and product size were checked in SafeView (Applied Biological Materials, Richmond, Canada) stained 2% agarose gel.

2.3. Sequencing and typing of Dirofilaria repens isolates

The DNA amplicons obtained from the two genetic markers amplifications were outsourced to BioFab Research laboratories (www.biofabresearch.com/it/) for purification and Sanger sequencing in both directions. All sequencing data were handled with MEGA v.11 software (Tamura et al., 2021). Electropherograms were visually inspected to check the quality of the sequencing run and to exclude the various forms of heteroplasmy (Pizzirani et al., 2020; Lucentini et al., 2020).

Table 2
Sequences of Dirofilaria repens analysed (20 derived from the present work, 5 from the work of Gabrielli et al., 2021 and 3 from Barlozzari et al., 2021). Geographical origin, host, number of specimens examined and haplotype identified are reported. Hg identifies the samples that belong in the broad haplogroup, whereas N.D. (not determinable) identifies the samples that have polymorphisms not shared by any of the described haplotypes.

Region of origin	Host/*N°* of specimens	*N°* specimens - haplotype
Campania	Dog/3	2 - Hg1 - N.D.
Latium	Human /14	9 - Hg2 - DR93 - N.D.
	Dog/3	3 - Hg
Tuscany	Dog / 2	2 - Hg
Umbria	Human /1	1 - Hg
	Dog /5	3 - Hg2 - N.D.

All the 20 new cox1 sequences from the current study were deposited in GenBank (http://www.ncbi.nlm.nih.gov/genbank) under the accession numbers reported in Table 1. An alignment of the sequences produced, along with 5 from parasites isolated between 2018 and 2019 from human patients (MW525256.1; MW525257.1; MW525258.1; MW525259.1; MW525260.1) by Gabrielli et al. (2021), and 3 sequences from parasites isolated in 2018 from two dogs (MT345574.1; MT345575.1; MT345576.1) by Barlozzari et al. (2021), was produced (Supplementary File 1) to perform phylogenetic analyses. All the 28 Italian isolates were compared with isolates from different areas of Europe to identify the presence of the haplotypes described by Alsarraf et al. (2023a).

Phylogenetic analyses were carried out as reported by Alsarraf et al. (2023a) using the Maximum Likelihood method and Tamura 3-parameter model (Tamura, 1992); initial trees for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Tamura 3 parameter model, and then selecting the topology with superior log likelihood value, over a bootstrap replication of 4000. A cox1 sequence from *Dirofilaria immitis* (KF918393.1) was chosen as out-group. A Median Joining Network of the cox1 sequences was performed using PopART 1.7 (Bandelt et al., 1999).

3. Results

All amplified products produced single amplicon of the expected size, whereas no visible bands were visible in negative controls. All the amplicons were successfully sequenced and no discrepancy (i.e. nucleotide variation) was found in the reverse and forward sequence for each isolate.

All the sequences from both the targets were attributed to *D. repens* by using the Nucleotide Basic Local Alignment Search Tool (nBLAST) (https://blast.ncbi.nlm.nih.gov/Blast.cgi, accessed on 01 September 2023) with percentages of homology ranging from 98% to 100%. No host-related polymorphisms were shown in the two targets.

Phylogenetic analyses of 12 S rDNA showed that all the sequences clustered with a relatively strong nodal support in a monophyletic group of *D. repens* (data not showed).

The electropherogram cleaning of the cox1 *locus* sequences gave back shorter fragments of 152 bp to be used for the phylogenetic analysis, due to the damage and degradation of the DNA obtained by FFPE tissues used in the present study. The 152 bp fragments obtained completely encompassed in the 411 bp fragment used from Alsarraf et al. (2023a) to define haplotypes and allowed for the reliable identification of DR3, DR6 and DR9 haplotypes. The short fragment did not allow for the discrimination of the remaining 15 haplotypes (i.e. DR1, DR2, DR4, DR5, DR7, DR8, DR10-DR18), which have therefore been treated as a broad haplogroup (Hg).

Out of the 28 sequences analyzed in the present study 20 (MT345574.1, MT345575.1, MT345576.1, MW525256.1, MW525257.1, MW525260.1, OR413181, OR413183, OR413184,

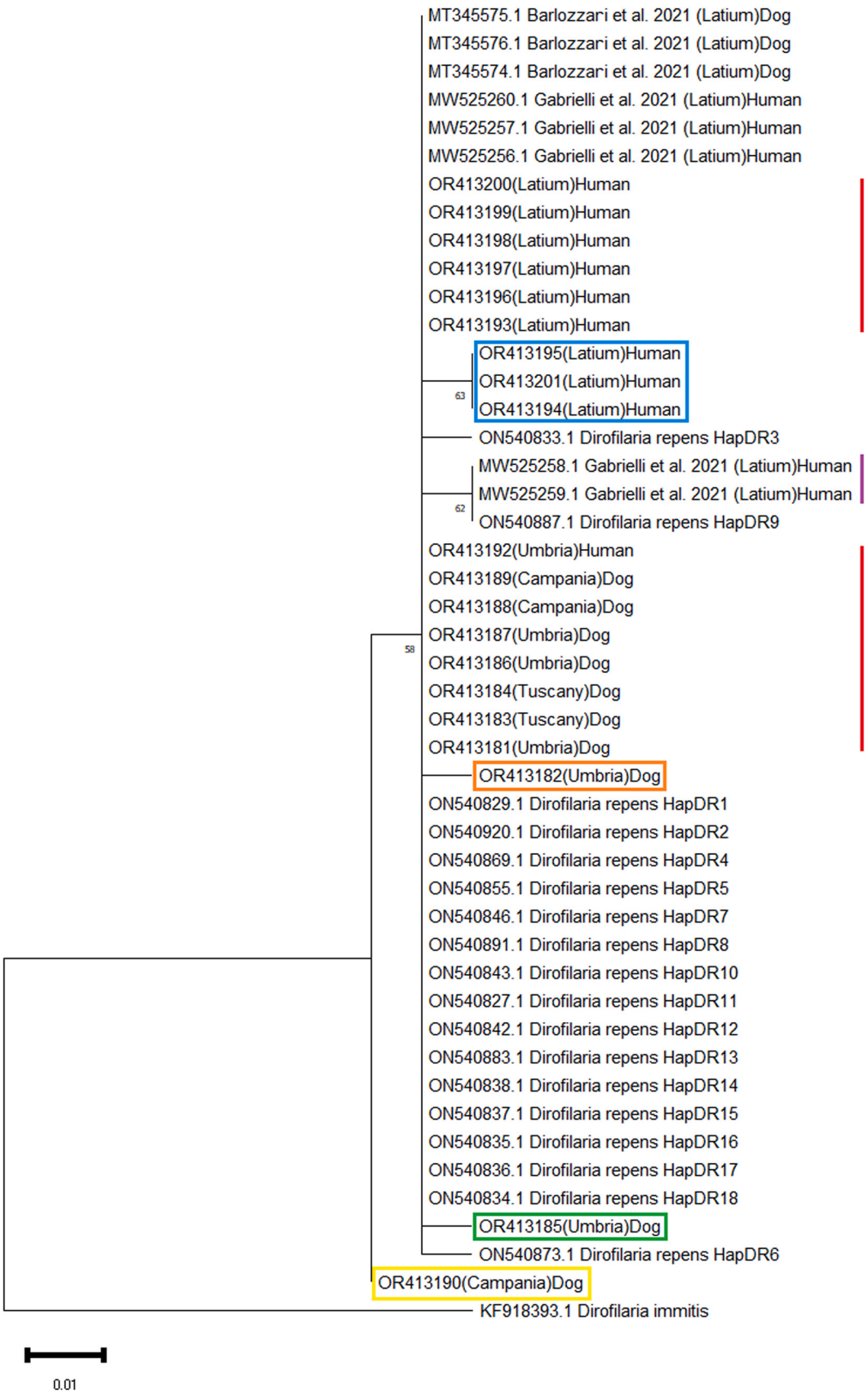

Fig. 1. Phylogenetic tree with the highest log likelihood (−322.49). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 44 nucleotide sequences. Codon positions included were 1st+ 2nd+ 3rd+Noncoding. There were a total of 152 positions in the final dataset.

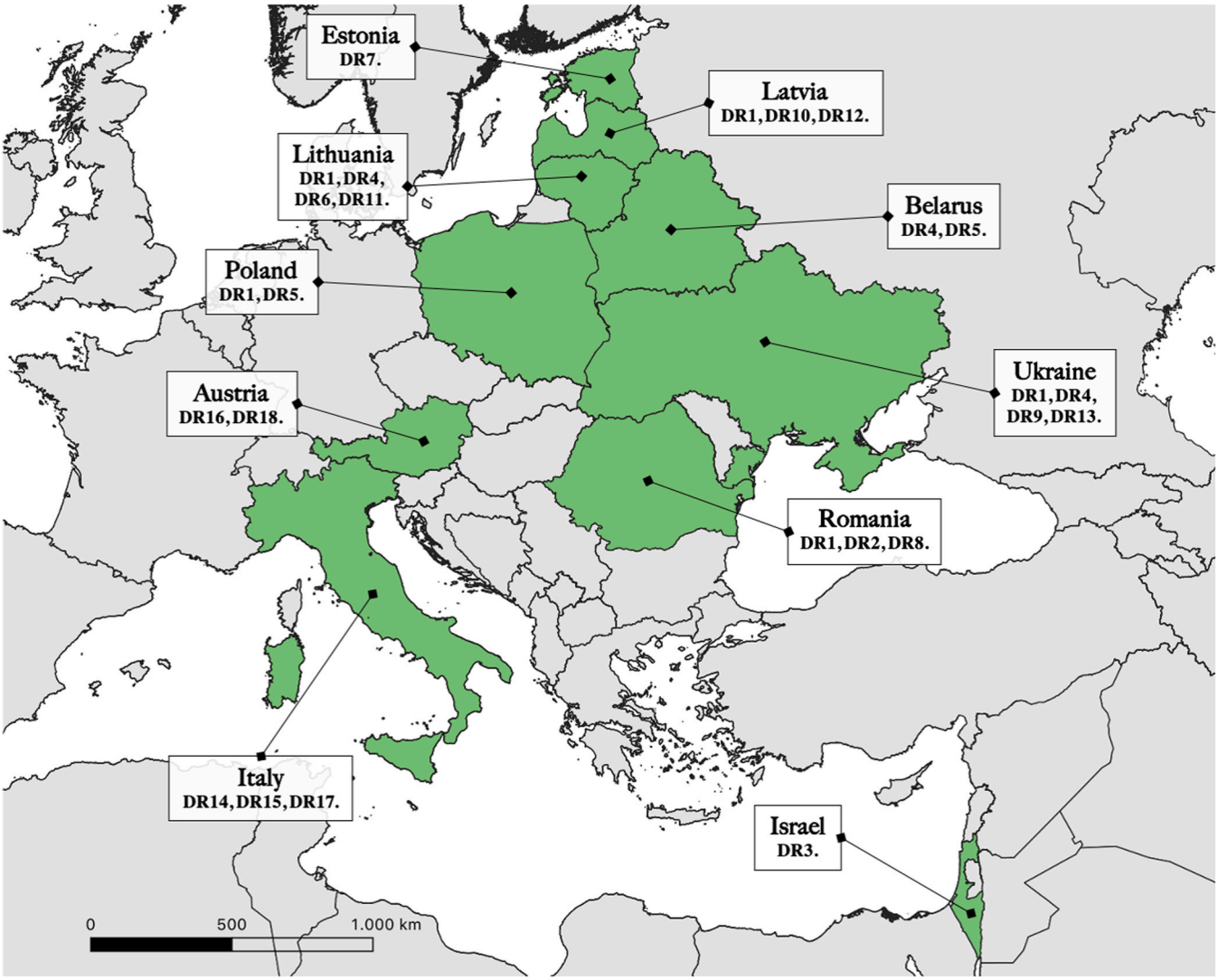

Fig. 2. Distribution of the 18 haplotypes of *Dirofilaria repens* identified by Alsarraf et al. (2023a) across European and Middle-east countries.

OR413186, OR413187, OR413188, OR413189, OR413192, OR413193, OR413196, OR413197, OR413198, OR413199, OR413200) were grouped in the Hg, whereas 2 sequences (MW525258.1, MW525259.1) were matched to DR9, reporting it for the first time in Italy (Table 2). It is notable, however, that 6 sequences showed peculiar single nucleotide polymorphisms (SNPs) that were not present in any of the previously described haplotypes. Among these, three sequences (OR413182, OR413185, OR413190), all from dog hosts, have peculiar mutations that differentiate them whereas three, all from human hosts, shared the same mutations (OR413194, OR413195, OR4132201), allowing to speculate the existence of 4 additional haplotypes from Italy. The phylogenetic relations between the cox1 sequences analyzed in this work and the haplotypes described by Alsarraf et al. (2023a) are summarized in Fig. 1, whereas the Median Joining Network output can be found in Supplementary File 2.

4. Discussion

The present work explored the genetic variability of *D. repens* population by sequence analysis of the mtDNA genes cox1 and 12 S rDNA. Both those loci have proven to be useful for refining information on the biology and epidemiology of other Spirurida-like *T. callipaeda*, *Onchocerca lupi*, *D. repens* and *D. immitis*, as well as of other nematodes (e.g. strongylids and ascarids), because of their relatively rapid evolutionary rates (Otranto and Eberhard, 2011c; Yilmaz et al., 2016; Alsarraf et al., 2021, 2023a,b); phylogenetic analysis of this study was conducted at the cox1 *locus*, in order to compare our sequences with representative Italian *D.repens* isolates from animals and humans.

A total of 28 *D. repens* sequences were analyzed in the present work, representing the largest study conducted in Italy to date and also the largest referred to human isolates (n. 15) from traditional endemic areas of southern Europe.

The overall results of the phylogenetic analysis conducted at the cox1 *locus* on the 28 sequences considered in the present work, and on the 3 Italian isolates reported by Alsarraf et al. (2023a), are consistent with a structured population of *D. repens* among the country.

To date, 18 haplotypes have been delineated, with a variable distribution across European and Middle-eastern countries, as summarized in Fig. 2. A wider haplotype diversity in *D. repens* collected from Italy was observed if compared with other European countries previously investigated, in which few haplotypes (from 1 to 4) were recovered. As mentioned above, the low number of haplotypes and genetic diversity described in south-eastern and central-eastern European countries may be the result of the relatively recent introduction of the parasite, however these records need to be interpreted with caution because few specimens (average 10) were examined for each country and only one sequence type detected for given haplotypes (Alsarraf et al., 2023a).

The genetic analysis conducted on the Italian isolates revealed the

presence of at least 4 out of the 18 haplotypes delineated by Alsarraf et al. (2023a): the DR9, that was previously described only in Ukraine, was also found in the samples analysed in this study; whereas is possible that the 20 analyzed sequences clustered in the haplogroup belonged to either DR14, DR15 or DR17 haplotypes, found exclusively in Italy by Alsarraf et al. (2023a). Unfortunately, given the reduced fragment length, most these 20 sequences could only be included in a broad haplogroup (Hg) that grouped 15 haplotypes. Among them, the DR1 is the most frequently detected and geographically distributed haplotype in the natural reservoirs and needs to be ascertained whether it is also linked to human cases (Alsarraf et al., 2023a); the haplotypes DR2, DR4, DR5, DR7, DR8, DR10-DR18, were isolated in other countries of south-eastern and central-eastern Europe (e.g. Austria, Romania, Lithuania, Latvia, Poland), that represent areas of active geographical expansion of *D. repens* (Capelli et al., 2018; Alsarraf et al., 2023a). Based on the presented results it can be speculated that the genetic diversity of *D. repens* population in Italy might be furtherly complex. Indeed, four new haplotypes of *D. repens* were herein delineated by the cox1 peculiar SNPs detected and additional genetic diversity might be present but not highlighted because of the shortness of the sequences used for the analysis.

These results are rather unexpected for the studied areas, in fact it could be speculated that the intra-population genetic variability of *D. repens* was lower in the regions investigated (e.g. Umbria, Tuscany, Campania and Latium), since they represent foci of transmission with apparently little opportunity for genetic diversification and great opportunity for inbreeding of the parasite. Moreover, an additional higher genetic variability not recorded could be observed in the future, due to technical issues which occurred in this work. Indeed, the processed FFPE tissues, allowed to recover a consistent number of isolates, but on the other side the formalin fixation and the paraffin embedding process degraded significantly the quality of the DNA, allowing for the amplification of shorter fragments that limited the quality of the genetic analysis. Therefore, is possible that some polymorphic sites from different specimens could not be mapped due to the short length of the sequences generated.

Genetic homogeneity within *D. repens* supports the hypothesis that there are no genetic differences between human-infecting parasites and dog-infecting parasites, at least in the investigated loci, also supporting the existence of common patterns of transmission, but according to the considerations above, further multi-locus phylogenetic analyses, including both mitochondrial and nuclear loci, are needed to highlight the possible presence of a host-related genetic variability within *D. repens* isolates from human and animal hosts (Yilmaz et al., 2019). On the other hand, the low-level of intra-specific genetic variability observed could be related to the occasional transmission of the parasite to human as well as to the low pathogenicity shown in this host. Some Authors (Nazar et al., 2017; Parsa et al., 2020) suggested the hypothesis that the parasite has recently extended its range to humans, however no specific studies have been conducted on the evolutionary history of *Dirofilaria* spp. and further evidences should be need.

5. Conclusions

The results obtained in the present study expand the knowledge on the genetic diversity of *D. repens* across Europe. In conclusion, although few studies have previously investigated the genetic diversity of *Dirofilaria* spp., the present results showed a higher diversity in *D. repens* mt haplotypes than in *D. immitis* in Italy (Alsarraf et al., 2023b). Such diversity could be associated with a more rapid expansion of *D. repens* infection rather than *D. immitis* infection in humans and animals.

Funding

This research was funded by the University of Perugia under the program “Ricerca di Base 2019”.

CRediT authorship contribution statement

Morganti Giulia: Supervision, Methodology, Formal analysis. **Brustenga Leonardo:** Visualization, Methodology, Formal analysis. **Gabrielli Simona:** Writing – original draft, Supervision, Conceptualization. **Veronesi Fabrizia:** Writing – original draft, Validation, Supervision, Conceptualization. **Sforna Monica:** Validation, Conceptualization. **Orlandi Margherita:** Methodology, Investigation. **Rigamonti Giulia:** Methodology, Formal analysis. **Barlozzari Giulia:** Methodology, Investigation. **Ciuca Lavinia:** Methodology, Investigation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

Our gratitude goes to the clinician Francesco Albanese who provided us canine cases included in the study, Dr. Jairo Alfonso Mendoza Roldan for the technical support and the Master Degree student Martina Proietti for support in laboratory procedures.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.vetpar.2023.110096.

References

Aher, A.M., Caudill, D., Caudill, G., Butryn, R.S., Wolf, D., Fox, M., Blake, D.P., Cunningham, M.W., 2016. Prevalence, genetic analyses, and risk factors associated with heartworm (*Dirofilaria immitis*) in wild coyotes (*Canis latrans*) from Florida, USA. J. Wildl. Dis. 52, 785–792.

Albanese, F., Abramo, F., Braglia, C., Caporali, C., Venco, L., Vercelli, A., Ghibaudo, G., Leone, F., Carrani, F., Giannelli, A., Otranto, D., 2013. Nodular lesions due to infestation by *Dirofilaria repens* in dogs from Italy. Vet. Dermatol. 24, 255–256.

Alsarraf, M., Levytska, V., Mierzejewska, E.J., Poliukhovych, V., Rodo, A., Alsarraf, M., Kavalevich, D., Dwuznik-Szarek, D., Behnke, J.M., Bajer, A., 2021. Emerging risk of *Dirofilaria* spp. infection in Northeastern Europe: high prevalence of *Dirofilaria repens* in sled dog kennels from the Baltic countries. Sci. Rep. 11, 1068 https://doi.org/10.1038/s41598-020-80208-1.

Alsarraf, M., Baneth, G., Bogucka-Kocka, A., Ciuca, L., Dwużnik-Szarek, D., Fuehrer, H. P., Kloch, A., Kołodziej, P., Levytska, V., Mierzejewska, E.J., Mihalca, A.D., Ionică, A. M., Mushynskyi, A., Nachum-Biala, Y., Alsarraf, M., Bajer, A., 2023a. Haplotypes of *Dirofilaria repens* from Poland and selected countries of Central, North-Eastern Europe and the Middle East: an evaluation on the relation between the genetic diversity and the geographic distribution of the fast-spreading parasite. Vet. Parasitol. 315, 109882 https://doi.org/10.1016/j.vetpar.2023.109882.

Alsarraf, M., Carretón, E., Ciuca, L., Diakou, A., Dwużnik-Szarek, D., Fuehrer, H.P., Genchi, M., Ionică, A.M., Kloch, A., Kramer, L.H., Mihalca, A.D., Miterpáková, M., Morchón, R., Papadopoulos, E., Pękacz, M., Rinaldi, L., Alsarraf, M., Topolnytska, M., Vismarra, A., Zawistowska-Deniziak, A., Bajer, A., 2023b. Diversity and geographic distribution of haplotypes of *Dirofilaria immitis* across European endemic countries. Parasites Vectors 16, 325.

Bandelt, H.J., Forster, P., Röhl, A., 1999. Median-joining networks for inferring intraspecific phylogenies. Mol. Biol. Evol. 16 (Issue 1), 37–48.

Barlozzari, G., Felice, T., Salvato, L., Conti, R., De Liberato, C., Furzi, F., Gabrielli, S., Scarpulla, M., 2021. Usual or unusual presentations of *Dirofilaria repens* in two sibling dogs: a case report. Parasitol. Res. 120, 109–115.

Cancrini, G., Scaramozzino, P., Gabrielli, S., Di Paolo, M., Toma, L., Romi, R., 2007. *Aedes albopictus* and *Culex pipiens* implicated as natural vectors of *Dirofilaria repens* in Central Italy. J. Med. Entomol. 44, 1064–1066.

Capelli, G., Genchi, C., Baneth, G., Bourdeau, P., Brianti, E., Cardoso, L., Danesi, P., Fuehrer, H.P., Giannelli, A., Ionică, A.M., Maia, C., Modrý, D., Montarsi, F., Krücken, J., Papadopoulos, E., Petrić, D., Pfeffer, M., Savić, S., Otranto, D., Poppert, S., Silaghi, C., 2018. Recent advances on *Dirofilaria repens* in dogs and humans in Europe. Parasit. Vectors 11, 663. https://doi.org/10.1186/s13071-018-3205-x.

Cringoli, G., Rinaldi, L., Veneziano, V., Capelli, G., 2001. A prevalence survey and risk analysis of filariosis in dogs from the Mt. Vesuvius area of southern Italy. Vet. Parasitol. 102, 243–252.

Dantas-Torres, F., Otranto, D., 2013. Dirofilariosis in the Americas: a more virulent *Dirofilaria immitis*? Parasit. Vectors 6, 288. https://doi.org/10.1186/1756-3305-6-288.

Dantas-Torres, F., Otranto, D., 2020. On the validity of "*Candidatus Dirofilaria hongkongensis*" and on the use of the provisional status Candidatus in zoological nomenclature. Parasit. Vectors 3, 287. https://doi.org/10.1186/s13071-020-04158-3.

Ferri, E., Barbuto, M., Bain, O., Galimberti, A., Uni, S., Guerrero, R., Ferté, H., Bandi, C., Martin, C., Casiraghi, M., 2009. Integrated taxonomy: traditional approach and DNA barcoding for the identification of filarioid worms and related parasites (Nematoda). Front. Zool. 7 (1) https://doi.org/10.1186/1742-9994-6-1.

Gabrielli, S., Mangano, V., Furzi, F., Oliva, A., Vita, S., Poscia, R., Fazii, P., Di Paolo, J., Marocco, R., Mastroianni, C.M., Bruschi, F., Mattiucci, S., 2021. Molecular identification of new cases of human Dirofilariosis (*Dirofilaria repens*) in Italy. Pathogens 10, 251. https://doi.org/10.3390/pathogens10020251.

Genchi, C., Mortarino, M., Rinaldi, L., Cringoli, G., Traldi, G., Genchi, M., 2011a. Changing climate and changing vector-borne disease distribution: the example of *Dirofilaria* in Europe. Vet. Parasitol. 176, 295–299.

Genchi, C., Kramer, L.H., Rivasi, F., 2011b. Dirofilarial infection in Europe. Vector Borne Zoonotic Dis. 11, 1307–1317.

Genchi, C., Kramer, L.H., 2020. The prevalence of *Dirofilaria immitis* and *D. repens* in the old world. Vet. Parasitol. 280, 108995 https://doi.org/10.1016/j.vetpar.2019.108995.

Kartashev, V., Afonin, A., González-Miguel, J., Sepúlveda, R., Simón, L., Morchón, R., Simón, F., 2014. Regional warming and emerging vector-borne zoonotic dirofilariosis in the Russian Federation, Ukraine, and other post-Soviet states from 1981 to 2011 and projection by 2030. Biomed. Res. Int. 2014, 858936 https://doi.org/10.1155/2014/858936.

Laidoudi, Y., Bedjaoui, S., Latrofa, M.S., Fanelli, A., Dantas-Torres, F., Otranto, D., 2022. Genetic and geographical delineation of zoonotic vector-borne helminths of canids. Sci. Rep. 12 https://doi.org/10.1038/s41598-022-10553-w.

Latrofa, M.S., Lia, R.P., Giannelli, A., Colella, V., Santoro, M., D'Alessio, N., Campbell, B. E., Parisi, A., Dantas-Torres, F., Mutafchiev, Y., Veneziano, V., Otranto, D., 2015. *Crenosoma vulpis* in wild and domestic carnivores from Italy: a morphological and molecular study. Parasitol. Res. 114, 3611–3617.

Lefoulon, E., Bain, O., Bourret, J., Junker, K., Guerrero, R., Cañizales, I., Kuzmin, Y., Satoto, T.B., Cardenas-Callirgos, J.M., de Souza Lima, S., Raccurt, C., Mutafchiev, Y., Gavotte, L., Martin, C., 2015. Shaking the Tree: Multi-locus Sequence Typing Usurps Current Onchocercid (Filarial Nematode) Phylogeny. PLoS Negl. Trop. Dis. 9, e0004233 https://doi.org/10.1371/journal.pntd.0004233.

Lucentini, L., Plazzi, F., Sfriso, A.A., Pizzirani, C., Sfriso, A., Chiesa, S., 2020. Additional taxonomic coverage of the doubly uniparental inheritance in bivalves: Evidence of sex-linked heteroplasmy in the razor clam *Solen marginatus* (Pulteney, 1799), but not in the lagoon cockle *Cerastoderma glaucum* (Bruguière, 1789). J.Z.S.E.R, 58, 561–570.

Macchioni, F., Sed, G., Cecchi, F., 2020. Canine filarial infections in an area of Central Italy (Tuscany-Latium border) historically free from the disease. Vet. Parasitol. Reg. Stud. Rep. 20, 100404 https://doi.org/10.1016/j.vprsr.2020.100404.

Magi, M., Guardone, L., Prati, M.C., Tozzini, G., Torracca, B., Monni, G., Macchioni, F., 2012. Canine filarial infections in Tuscany, central Italy. J. Helminthol. 86, 113–116.

Mendoza-Roldan, J.A., Gabrielli, S., Cascio, A., Manoj, R.R.S., Bezerra-Santos, M.A., Benelli, G., Brianti, E., Latrofa, M.S., Otranto, D., 2021. Zoonotic Dirofilaria immitis and Dirofilaria repens infection in humans and an integrative approach to the diagnosis. Acta Trop. 223, 106083 https://doi.org/10.1016/j.actatropica.2021.106083.

Nazar, N., Lakshmanan, B., Jayavardhanan, K.K., 2017. Molecular characterization of human *Dirofilaria* isolates from Kerala. Indian. J. Med. Res. 146, 528–533.

Otranto, D., Testini, G., De Luca, F., Hu, M., Shamsi, S., Gasser, R.B., 2005. Analysis of genetic variability within *Thelazia callipaeda* (Nematoda: Thelazioidea) from Europe and Asia by sequencing and mutation scanning of the mitochondrial cytochrome c oxidase subunit 1 gene. Mol. Cell. Probes 19, 306–313.

Otranto, D., Brianti, E., Gaglio, G., Dantas-Torres, F., Azzaro, S., Giannetto, S., 2011a. Human ocular infection with *Dirofilaria repens* (Railliet and Henry, 1911) in an area endemic for canine dirofilariasis. Am. J. Trop. Med. Hyg. 84 (6), 1002–1004. https://doi.org/10.4269/ajtmh.2011.10-0719.

Otranto, D., Diniz, D.G., Dantas-Torres, F., Casiraghi, M., Almeida, I.N.F., Almeida, L.N., dos Santos, J.N., Furtado, A.P., de Almeida Sobrinho, E.F., Bain, O., 2011b. Human intraocular filariasis caused by *Dirofilaria* sp. nematode. Brazil Emerg. Infect. Dis. 17, 863–866.

Otranto, D., Eberhard, M.L., 2011c. Zoonotic helminths affecting the human eye. Parasit. Vectors 4, 41. https://doi.org/10.1186/1756-3305-4-41.

Otranto, D., Dantas-Torres, F., Brianti, E., Traversa, D., Petrić, D., Genchi, C., Capelli, G., 2013. Vector-borne helminths of dogs and humans in Europe. Parasit. Vectors 6, 16. https://doi.org/10.1186/1756-3305-6-16.

Pampiglione, S., Rivasi, F., Angeli, G., Boldorini, R., Incensati, R.M., Pastormerlo, M., Pavesi, M., Ramponi, A., 2001. Dirofilariasis due to *Dirofilaria repens* in Italy, an emergent zoonosis: report of 60 new cases. Histopathology 38, 344–354.

Parsa, R., Sedighi, A., Sharifi, I., Bamorovat, M., Nasibi, S., 2020. Molecular characterization of ocular dirofilariasis: a case report of *Dirofilaria immitis* in south-eastern Iran. BMC Infect. Dis. 20, 520 https://doi.org/10.1186/s12879-020-05182-5.

Pizzirani, C., Viola, P., Gabbianelli, F., Fagotti, A., Simoncelli, F., Di Rosa, I., Salvi, P., Amici, A., Lucentini, L., 2020. First evidence of heteroplasmy in Grey Partridge (*Perdix perdix*). Avian Res 11, 27.

Pupić-Bakrač, A., Pupić-Bakrač, J., Beck, A., Jurković, D., Polkinghorne, A., Beck, R., 2021. *Dirofilaria repens* microfilaremia in humans: case description and literature review. One Health 13, 100306. https://doi.org/10.1016/j.onehlt.2021.100306.

Rishniw, S.C., Barr, K.W., Simpson, M.F., Frongillo, M., Franz, J.L., Alpizar, D., 2006. Discrimination between six species of canine microfilariae by a single polymerase chain reaction. Vet. Parasitol. 135, 303–314.

Rojas, A., Sanchis-Monsonis, G., Alic, A., Hodzic, A., Otranto, D., Yasur-Landau, D., Martínez-Carrasco, C., Baneth, G., 2018. *Spirocerca vulpis* sp. nov. (Spiruridae: Spirocercidae): description of a new nematode species of the red fox, *Vulpes vulpes* (Carnivora: Canidae). Parasitology 21, 1-12.

Rossi, A., Peix, A., Pavlikovskaya, T., Sagach, O., Nikolaenko, S., Chizh, N., Kartashev, V., Simón, F., Siles-Lucas, M., 2015. Genetic diversity of *Dirofilaria* spp. isolated from subcutaneous and ocular lesions of human patients in Ukraine. Acta Trop. 142, 1–4.

Sałamatin, R., Pavlikovska, T., Sagach, O., Nikolayenko, S., Kornyushin, V., Kharchenko, V., Masny, A., Cielecka, D., Konieczna-Sałamatin, J., Conn, D., Golab, E., 2013. Human dirofilariasis due to *Dirofilaria repens* in Ukraine, an emergent zoonosis: epidemiological report of 1465 cases. Acta Parasitol. 58, 592–598.

Sauda, F., Malandrucco, L., Macrì, G., Scarpulla, M., De Liberato, C., Terracciano, G., Fichi, G., Berrilli, F., Perrucci, S., 2018. *Leishmania infantum, Dirofilaria* spp. and other endoparasite infections in kennel dogs in central Italy. Parasite 25, 2. https://doi.org/10.1051/parasite/2018001.

Scaramozzino, P., Gabrielli, S., Di Paolo, M., Sala, M., Scholl, F., Cancrini, G., 2005. Dog filariosis in the Lazio region (Central Italy): first report on the presence of *Dirofilaria repens*. BMC Infect. Dis. 5, 75–81.

Simón, F., Siles-Lucas, M., Morchón, R., González-Miguel, J., Mellado, I., Carretón, E., Montoya-Alonso, J.A., 2012. Human and Animal Dirofilariasis: the Emergence of a Zoonotic Mosaic. Clin. Microbiol. Rev. 25 (3), 507–544.

Simón, F., Gonzalez-Miguel, J., Diosdado, A., Gomez, P.J., Morchon, R., Kartashev, V., 2017. The complexity of zoonotic filariasis episystem and its consequences: a multidisciplinary view. Biomed. Res. Int. 2017, 1–10.

Simón, F., Diosdado, A., Siles-Lucas, M., Kartashev, V., Gonzalez-Miguel, J., 2022. Human dirofilariosis in the 21st century: a scoping review of clinical cases reported in the literature. Transbound. Emerg. Dis. 69, 2424–2439.

Suzuki, J., Kobayashi, S., Okata, U., Matsuzaki, H., Mori, M., Chen, K.-R., Iwata, S., 2015. Molecular analysis of *Dirofilaria repens* removed from a subcutaneous nodule in a Japanese woman after a tour to Europe. Parasite 22, 2. https://doi.org/10.1051/parasite/2015002.

Tamura, K., 1992. Estimation of the number of nucleotide substitutions when there are strong transition-transversion and G + C-content biases. Mol. Biol. Evol. 9, 678–687.

Tamura, K., Stecher, G., Kumar, K., 2021. MEGA11: molecular evolutionary genetics analysis version 11. Mol. Biol. Evol. 38, 3022–3027.

Tasić-Otasevic, S., Golubović, M., Trichei, S., Zdravkovic, D., Jordan, R., Gabrielli, S., 2023. Microfilaremic *Dirofilaria repens* Infection in Patient from Serbia. Emerg. Infect. Dis. 29 (12), 2548–2550. https://doi.org/10.3201/eid2912.230796.

To, K.K., Wong, S.S., Poon, R.W., Trendell-Smith, N.J., Ngan, A.H., Lam, J.W., Tang, T. H., Chong, A.K., Kan, J.C., Chan, K.H., Yuen, K.Y., 2012. A novel Dirofilaria species causing human and canine infections in Hong Kong. J. Clin. Microbiol. 50, 3534–3541.

Traversa, D., Aste, G., Milillo, P., Capelli, G., Pampurini, F., Tunesi, C., Santori, D., Paoletti, B., Boari, A., 2010. Autochthonous foci of canine and feline infestations by *Dirofilaria immitis* and *Dirofilaria repens* in central Italy. Vet. Parasitol. 169, 128–132.

Yilmaz, E., Fritzenwanker, M., Pantchev, N., Lendner, M., Wongkamchai, S., Otranto, D., Kroidl, I., Dennebaum, M., Le, T.H., Anh Le, T., Ramünke, S., Schaper, R., von Samson-Himmelstjerna, G., Poppert, S., Krücken, J., 2016. The mitochondrial genomes of the zoonotic canine filarial parasites *Dirofilaria (Nochtiella) repens* and *Candidatus Dirofilaria (Nochtiella) Honkongensis* provide evidence for presence of cryptic species. PLoS. Negl. Trop. Dis. 10, e0005028 https://doi.org/10.1371/journal.pntd.0008347.

Yilmaz, E., Wongkamchai, S., Ramünke, S., Koutsovoulos, G.D., Blaxter, M.L., Poppert, S., Schaper, R., von Samson-Himmelstjerna, G., Krücken, J., 2019. High genetic diversity in the *Dirofilaria repens* species complex revealed by mitochondrial genomes of feline microfilaria samples from Narathiwat, Thailand. Transbound. Emerg. Dis. 66, 389–399.

Zhang, X., Shi, Y.L., Han, L.L., Xiong, C., Yi, S.Q., Jiang, P., Wang, Z.X., Shen, J.L., Cui, J., Wang, Z.Q., 2018. Population structure analysis of the neglected parasite *Thelazia callipaeda* revealed high genetic diversity in Eastern Asia isolates. PLoS Negl. Trop. Dis. 12, e0006165 https://doi.org/10.1371/journal.pntd.0006165.