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“Old hosts die hard”: the return of the grey wolf (*Canis lupus*) and the re-emerging threat of cardiopulmonary dirofilariosis in northern Italy

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ABSTRACT

Over the past decades, the grey wolf (*Canis lupus*) has expanded its range across Italy, reclaiming historical habitats, including anthropized landscapes. This resurgence raises concerns regarding its potential role in maintaining and spreading pathogens. In this study, we focused on *Dirofilaria immitis*, the causative agent of canine heartworm disease, investigating its prevalence in wolves and exploring the environmental factors influencing infection risk. A total of 488 wolf carcasses collected from northern and central Italy between 2021 and 2024 were analysed. Morphological identification of adult nematodes was conducted using light and scanning electron microscopy, while molecular analyses of mitochondrial COI and 12S rRNA genes were performed to assess genetic variation. Spatial modelling was applied to evaluate infection risk based on environmental factors. *D. immitis* was detected in 3.5% of the sampled wolves, with prevalence varying among regions, reaching 5.2% in Emilia-Romagna. Microfilariae were identified in two cases, confirming the wolves’ potential role as competent hosts. Phylogenetic analysis revealed no significant genetic divergence between *D. immitis* from wolves and those previously recorded in dogs and other hosts. Spatial analysis indicated that infection risk was highest in low-altitude areas of the Po Valley, a historically endemic region for dirofilariosis. These findings suggest that the expanding wolf population may serve as a competent host for *D. immitis*, potentially influencing local epidemiology and complicating control efforts in domestic dogs. Continued surveillance is necessary to assess the impact of wildlife reservoirs on heartworm transmission and public health.

1. Introduction

Vector-borne helminths constitute a significant concern in both veterinary and human medicine, as they can cause diseases in a variety of animal species and some of them can pose zoonotic risks to humans as accidental hosts (Otranto et al., 2013; Perles et al., 2024). Among these, species of the genus *Dirofilaria* have garnered considerable attention due to their impact on animal health and their occasional involvement in human infections. The genus currently comprises 27 described species (Canestri-Trotti et al., 1997), of which *D. immitis* and *D. repens* are the most commonly reported in dog and humans (McCall et al., 2008; Genchi et al., 2011). *Dirofilaria immitis* is the causative agent of heartworm disease (HWD), a severe and potentially fatal cardiopulmonary condition primarily affecting dogs and, to a lesser extent, cats. Clinical manifestations in dogs include respiratory distress, epistaxis, ascites, exercise intolerance, and anorexia (McCall et al., 2008). In contrast, *D. repens* is less pathogenic and typically induces subcutaneous infections in dogs, with rare cases presenting as painless skin nodules (Albanese et al., 2013). Despite its relatively mild impact on canine hosts, *D. repens* is the leading cause of human dirofilariasis, particularly in the Old World (Genchi et al., 2011). Con-

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versely, *D. immitis* is the second most frequent agent of human dirofilariasis globally and the predominant cause in the New World (Dantas-Torres and Otranto, 2013). The geographic distribution of *Dirofilaria* spp. is influenced by the availability of definitive and intermediate hosts, as well as environmental factors, particularly temperature. For instance, the development of *D. immitis* within its mosquito vectors requires temperatures exceeding 14 °C for at least 30 days (Genchi et al., 2009). Climate change and global warming are anticipated to further expand the range of *D. immitis* and *D. repens*, potentially altering their epidemiological patterns (Genchi et al., 2009; Mendoza-Roldan et al., 2020). Recent studies have demonstrated changing distribution patterns of heartworm disease in Europe, with Italy serving as a prominent case study. Over a 10-year period (2009–2019), *D. immitis* established new endemic foci in central and southern Italy, as well as on its islands, while maintaining its presence in traditionally endemic areas such as the Po Valley in the northern region (Mendoza-Roldan et al., 2020), although with lower prevalences in dogs than in the past. Among environmental factors, the epidemiological plasticity of *D. immitis* can also be influenced by the presence of wild competent hosts that may act as reservoirs. In addition to domestic dogs, *D. immitis* is capable of infecting various wild mammals, particularly carnivores. Red foxes (*Vulpes vulpes*) have been identified as hosts in Italy, Spain, and Bulgaria (Morchón et al., 2012), with prevalence rates reaching up to 32% in irrigated regions of Spain (Gortazar et al., 1994). In Italy, a study examining 132 red foxes found that 1.5% harboured *D. immitis* microfilariae (Magi et al., 2008). Other wild hosts documented with *D. immitis* infection in Europe include, for instance, wolves (*Canis lupus*) in Belarus, Italy, and Spain (Genchi et al., 2005; Pascucci et al., 2007; Shimalov and Pen'kevich, 2012); golden jackals (*Canis aureus*) in Bulgaria and Italy (Kirkova et al., 2007; Orioles et al., 2024); and otters (*Lutra lutra*) in Portugal and Spain (Torres et al., 2004). Although most of these infections have been considered epiphenomena associated with dog infection cycles (Otranto et al., 2013), the recent recovery of *D. immitis* adults from Italian wolves in heartworm-endemic areas suggests their role as suitable hosts (Pascucci et al., 2007; Moroni et al., 2020). Embryogram analysis confirmed that wolves can effectively harbour fertile *D. immitis* females. Notably, the examined wolves exhibited a similar level of exposure to heartworm as sympatric unprotected dogs (Moroni et al., 2020). In recent years, the Apennine wolf (*Canis lupus italicus*), a subspecies of the grey wolf, together with the grey wolf, have experienced significant population growth across the Italian peninsula (La Morgia et al., 2022). Over the past few decades, both the size and geographic range of wolf populations in Italy have expanded, with wolves progressively reclaiming their historical habitats, extending from the Apennines to the western and eastern Alps (Marucco et al., 2002). Although wolves generally favour remote locations far from human settlements, sightings near urban areas have become increasingly common, even in densely populated regions (Zanni et al., 2023).

This demographic resurgence has raised concerns about the wolf's potential role in maintaining wild and *peri*-urban cycles of various pathogens that could pose risks to both animal and human health. The focus of the present study has been posed on the possible role of the wolf as a wild reservoir of *D. immitis* in Italy. This is especially pertinent in areas where antifilarial chemoprophylaxis has led to a decline in heartworm prevalence among dogs, suggesting that the wolf's resurgence could influence the local epidemiology of *D. immitis* and potentially undermine control efforts.

2. Materials and methods

2.1. Ethics statement

All samples were gathered in compliance with local regulations. All necropsies were conducted at specialized centres dedicated to wildlife disease control. No specific permission was required to perform the sampling, in accordance with the national Legislative Decree 26/2014.

2.2. Study area

We collected wild wolf carcasses in northern and central Italy, in Piedmont, Lombardy, Emilia-Romagna, Tuscany, Marche, and Umbria regions (Fig. 1). The study area is characterized by a huge environmental heterogeneity, with elevations ranging from 0 to above 1,500 m a. s. l. in the Alpine and Apennine mountainous chains. Accordingly, a wide variety of habitats are included. Mediterranean maquis in the western coastal areas, mixed forests, sparse agricultural patches and pastures in the hilly and mountainous ranges, and large, intensely cultivated and urbanized landscapes in the large plain of the Po Valley. Human density is heterogeneously distributed, mostly concentrated in the lower elevations. The annual average land surface temperature ranges from the 10.5 °C of Alpine and Apennine peaks to the 16.4 °C of lowlands. The wolf population experienced a rapid expansion in both range and numbers during the last few decades, with the highest densities being reported in the Apennine regions and the lowest in the Po Valley (Fardone et al., 2025; La Morgia et al., 2022).

2.3. Data collection

In total, 488 wolves carcasses were collected, of these 322 had GPS coordinates. The carcasses were reported to the police authorities responsible for the recovery of wild fauna, who delivered them to authorised institutes to carry out necropsy investigations and health sampling for epidemiological surveillance, as previously described (Dini et al., 2024). At the arrival of each carcass to institutes, a first form containing the following information was filled: subject's identification data with the attribution of a unique ID code, the discovery location (reported as GPS coordinates or municipality), the sex, the weight (in kg). For subjects in an advanced state of decomposition, the designation "ND" (Not Detectable) was assigned. The age of the animal was estimated based on dental development, body size and weight (Brasington et al., 2023). Here, all individuals were aged using 3 categories as follows: class 1: <12 months; class 2: 1–2 years; class 3: >2 years.

The biometric information and phenotypic characteristics were also recorded. After finalizing the external examination, the carcass was placed in decubitus to proceed with its flaying. A complete necropsy was performed and all organs were individually inspected and assessed, before being sampled for further laboratory analysis. Specifically fort this study, the aortic arch and pulmonary arteries were systematically opened and inspected, along with the heart and all cardiac chambers. The presence of *D. immitis* adults was assessed during the necroscopic procedure. Furthermore, in two positive wolves carcasses which were well conserved, and fluid unclothed blood was available for collection, the modified Knott's test (Knott, 1939) was used starting from 1 ml of fluid blood to detect circulating microfilariae.

2.4. Morphological characterization

All specimens of adult filariods recovered from the positive wolves were thoroughly washed in saline solution and subsequently fixed in ethanol for further examination, except for four (2 males and 2 females) well-preserved specimens; these were meticulously rinsed and cleaned before being fixed in 10% buffered formalin to prepare them for scanning electron microscopy. For scanning electron microscopy (SEM), anterior and posterior portions of the selected nematodes were dehydrated through a graded ethanol series, subjected to critical point drying, sputter-coated

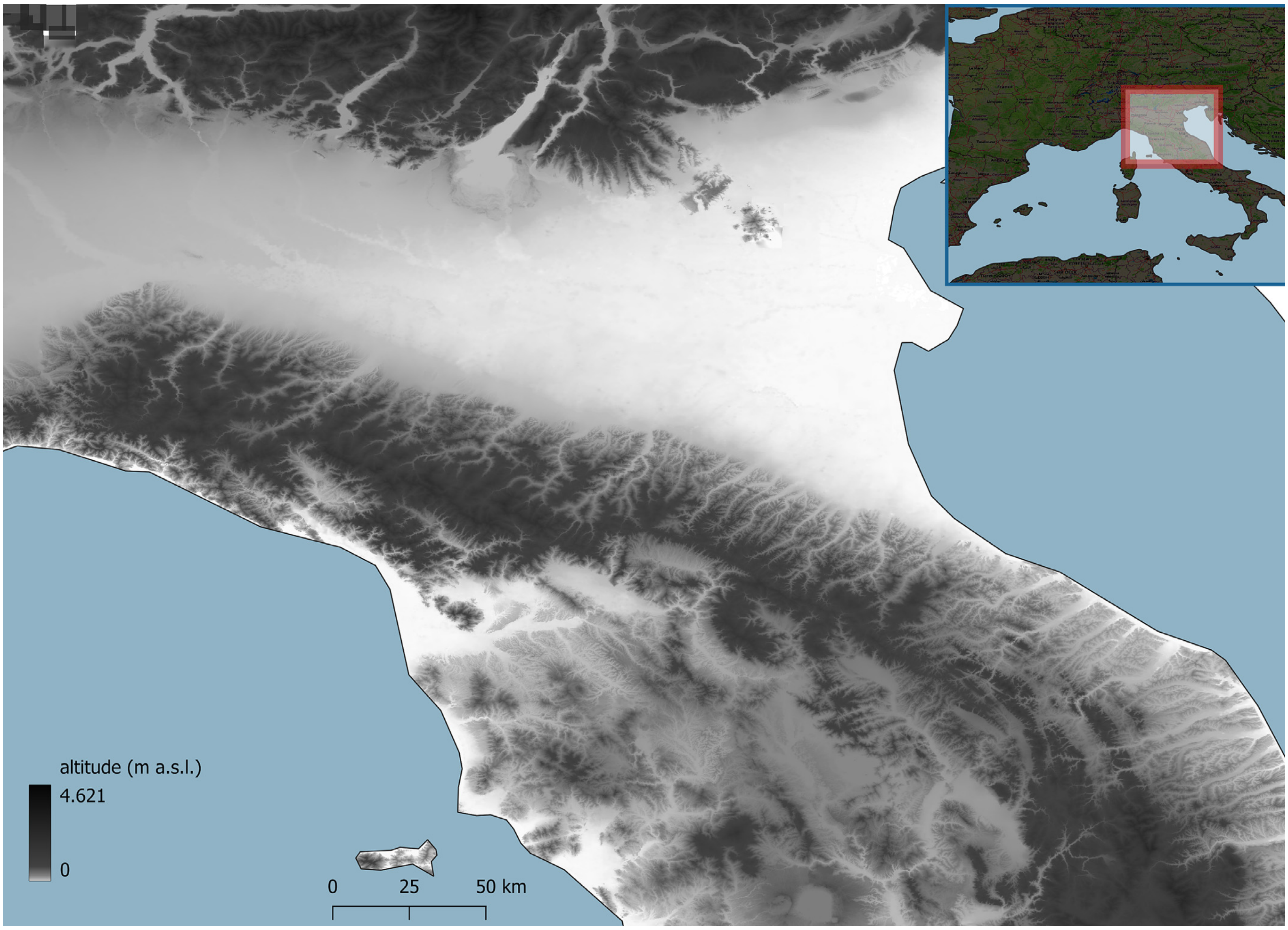

Fig. 1. Study area altitudinal gradient (main map) and its position in southern Europe (top-right rectangle).

with gold palladium, and observed using a Phenom XL G2 Desktop SEM (Thermo Fisher Scientific, Eindhoven, 120 The Netherlands) operating at 5 kV (Davidovich et al., 2022).

After ethanol fixation, the total length of each specimen was measured. Two cuts were then made: one at one-third of the body length from the cephalic end, and another two centimetres from the caudal end to ensure the entire tail was included. The two sections of the specimens were then clarified in Amman's lactophenol to facilitate detailed morphological examination. Morphological features such as oesophagus, vulvar opening, the presence and the number of pre-cloacal and post cloacal papillae, and the spicules has been considered to assess the morphological identification of the specimens collected. At the cephalic cut site, before mounting, an additional thin slice of approximately 1 mm of worm tissue was excised to collect material for DNA extraction. This tissue was stored at −20 °C until further analysis.

2.5. Molecular characterization and phylogenetic analysis

One specimen for each positive wolf underwent molecular analysis. Genomic DNA extraction was performed using Pure Link® Genomic DNA Mini kit (Invitrogen, Life Technologies, Carlsbad, CA, USA), according to the manufacturer's protocol. PCR amplification of each DNA sample was performed using primer sets targeting the 12S mitochondrial rRNA (Diro12S-F/ Diro12S-R), amplifying a 480 bp amplicon, and Cytochrome *c* Oxidase I gene (COI) (Diro-cox1-F/Diro-cox1-R) amplifying a 800 bp amplicon (Suzuki et al., 2015), generic for *Dirofilaria* spp. For both genes, a reaction volume of 25 µl, containing 12.5 µl 2× Dream Taq Hot Start Green PCR Master Mix (Thermo Scientific), 9.6 µl ddH2O, 0.20 µl (0.4 µM final concentration) of each primer, and 2.5 µl template DNA were used. For amplification, an initial denaturation step at 94 °C for 5 min was followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 54 °C for 30 s and extension at 72 °C for one min. Final extension was performed at 72 °C for 7 min. In all the PCRs, sterile water was included as negative control, while as positive control DNA of *D. repens* from child testicular infection (Ugolini et al., 2022) was employed.

Amplifications were performed in a T-personal thermal cycler (Bio metra, Gottingen, Germany). The PCR products were electrophoresed on 1.5% agarose gel stained with SYBR Safe DNA Gel Stain (Thermo Fisher Scientific, Carlsbad, CA, USA) in 0.5 × TBE. For sequencing, the amplicons were excised and purified by Nucleo-Spin Gel and PCR Cleanup (Mackerey-Nagel, Düren, Germany), and sequenced with an ABI 3730 DNA analyzer (StarSEQ, Mainz, Germany).

The resulting sequences were assembled with Contig Express (VectorNTI Advance 11 software, Invitrogen, Carlsbad, CA, USA), and the consensus sequences were compared with published data by BLAST tools (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Sequence alignments were carried out by BioEdit 7.2.5 (Hall, 1999); p-distance and the maximum-likelihood (ML) tree were calculated by MEGA 7 (Kumar et al., 2016). For 12S mtDNA phylogenetic tree was based on Hasegawa-Kishino-Yano model (HKY) (Hasegawa et al., 1985), and discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.2303), while for COI mtDNA Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei, 1993) with a Gamma distribution (TN + G) was employed. For both ML trees a bootstrap of 1000 replicate was applied.

2.6. Spatial covariates annotation

To investigate the environmental factors influencing the probability of wolves being infected by *D. immitis*, we conducted spatial analyses on the 322 collected wolf carcasses with ascertained geolocation. Among environmental factors, we considered altitude, mean land surface temperature, and availability of surface water bodies for their know role as ecological drivers of the density of mosquitos of genera *Aedes, Culex, Culiseta, Coquillettidia,* and *Anopheles* (Khalin et al., 2021; Zittra and Waringer, 2014), all potential vectors of *D. immitis* (Morchón et al., 2012; Simón et al., 2012). Altitude was derived from a surface model online raster database (EU-DEM v1.0, expressed in m a.s.l.). For land surface temperature, we used the MODIS/Terra Land Surface Temperature/Emissivity Monthly L3 Global 0.05Deg CMG V006, averaging the monthly rasters of 2021 to get the average annual temperature estimates. Finally, we derived the spatial distribution of surface water bodies (lakes and lagoons, expressed as occupied surface) and that of water body lines (rivers, expressed as length) from the European Environmental Agency (https://www.eea.europa.eu/data-and-maps/data/wise-wfd-spatial/surface-water-body), using the most recent available product (2016). We also considered the spatial variation of human development as a proxy of domestic dog presence (main *D. immitis* reservoir host), using the Human Footprint index of 2020 provided by Mu et al. (2022).

Each carcass was assigned covariate values based on the above-mentioned environmental data. To capture the environmental conditions experienced by each individual wolf, we created circular buffers centred on each carcass's location with a radius of 5,129 m, representing the average home range size of wolves in Italy (Mattioli et al., 2018). The mean values of each environmental covariate within the buffer were then computed and assigned to each carcass.

2.7. Spatial analysis and wolf dirofilariosis prevalence predictions

To assess the relationship between dirofilariosis prevalence and the selected environmental predictors, we first evaluated potential multicollinearity among the spatial covariates. A strong negative correlation was detected between altitude and both human presence ($r = -0.8$) and mean land surface temperature ($r = -0.9$). Since, among these three predictors, altitude was the primary independent variable, we retained altitude and excluded human footprint and temperature from further analysis.

We used a Generalized Additive Mixed Model (GAMM) with a binomial distribution and logit link function to model the probability of dirofilariosis, thus including as response variable the carcass dirofilariosis status (positive or negative). Besides the environmental covariates (altitude, water body density, and river density), we also included the Julian date of the carcass discovery (a continuous count of days from 1st to 365th day of the year), to capture possible seasonal variations in the infection, and the individual sex and age class as control predictors. We included a smooth function of latitude and longitude as control predictor to control for spatial autocorrelation. Finally, the year was included as random intercept to account for potential yearly variability in dirofilariosis prevalence. These predictive variables were used to build a GAMM with a full-model structure. We used the dredge function ('MuMIn' package in R) to fit a set of models with all possible combinations of the variables of the full model and selected the best model by using the Akaike's information criterion (Zuur et al., 2009). We retained the smooth function of latitude and longitude in all combinations to account for spatial autocorrelation in any best model being possibly selected. Models with ΔAIC < 2 were assumed to be as good as the minimum AIC model. Among the models with ΔAIC < 2, the one with the least variables was chosen as the best model (Symonds and Moussalli, 2011).

To predict the prevalence of dirofilariosis across the study area, we applied the best model to a spatial grid with a resolution of 1 km, covering central and northern Italy. For each grid cell, we extracted environmental covariates and assigned default values for non-spatial variables being possibly included in the best model. We then used the best GAMM to predict dirofilariosis risk at each grid point. This process allowed us to create a continuous surface representing the predicted dirofilariosis risk.

2.8. Data accessibility

All data supporting this study are available as an open-access dataset at https://doi.org/10.17632/bcc9dzxdnw.1.

The sequences obtained in this study have been deposited in GenBank under the accession numbers PV523598- PV523608 (11 sequences of12S rDNA) and PV524378- PV524388 (11 sequences of COI).

3. Results

3.1. Positive cases in northern Italy, different burden of infection and evidence of microfilaremia

Our final sample included 488 wolves collected between 2021 and 2024. Age classification was determined for 478 out of 488 analysed carcasses (98.0%), with 34.5% assigned to age class 1, 33.1% to age class 2, and 32.4% to age class 3. Sex identification was successful for 477 individuals (97.7%), of which 43.2% were female and 56.8% were male. Regarding geographic distribution, 39.3% of the sampled wolves originated from Emilia-Romagna, 7.2% from Lombardy, 32.0% from Piedmont, 4.5% from Marche, 7.2% from Tuscany, and 9.8% from Umbria.

With respect to *D. immitis* prevalence in the studied population, adult parasites were detected in 17 out of 488 wolves, corresponding to an overall prevalence of 3.5% across the investigated area. These included 10 males and 7 females, all belonging to age classes 2 (5 individuals) and 3 (12 individuals). The prevalence varied across sampling regions, with no positive cases detected in Tuscany, Umbria, or Marche (Central Italy). In contrast, Emilia-Romagna exhibited the highest prevalence (5.2%), followed by Piedmont (3.8%) and Lombardy, where only one case was recorded out of 35 individuals (2.8%) (Table 1).

Adult parasites were recovered and processed for species identification in 15 cases (Fig. 2), revealing a mean intensity of three worms per host (range: 1–22) (Fig. 2B–D). Parasite's sex was established in 13 cases of infection, and female to male sex ratio (F:M) was calculated for cases of mixed-sex infection (9 cases). F:M sex ratio ranged from 0.5 to 2 with a mean value of 1.04. In the remaining four cases only males or females were recovered.

Finally, the Knott test performed in two cases, allowed the assessment of microfilariae presence. In both cases, *D. immitis* microfilariae were detected based on their morphological characteristics (Fig. 2E).

3.2. Morphological features of D. immitis in wolf

Worms elongated in shape, filiform, with cephalic extremity tapering anteriorly and rounded (Fig. 3A). Terminal oral opening,

Table 1
Total number of wolves examined, with their respective positivity and Confidence Interval (CI), divided by age class, sex and region of origin.

		Examined wolves	Positive wolves (%)	CI (95%)
Age class	1: <12 months	165	0 (0%)	–
	2: 1–2 years	158	6 (3.8%)	[0.87–6.73]
	3: >2 years	155	11 (7.1%)	[3.02–10.98]
	ND	10	0 (0%)	–
Sex	M	271	10 (3.7%)	[1.51–5.89]
	F	206	7 (3.4%)	[0.98–5.82]
	ND	11	0 (0%)	–
Region	Emilia-Romagna	192	10 (5.2%)	[2.12–8.28]
	Piedmont	156	6 (3.8%)	[0.85–6.75]
	Lombardy	35	1 (2.8%)	[0–8.25]
	Marche	22	0 (0%)	–
	Tuscany	35	0 (0%)	–
	Umbria	8	0 (0%)	–

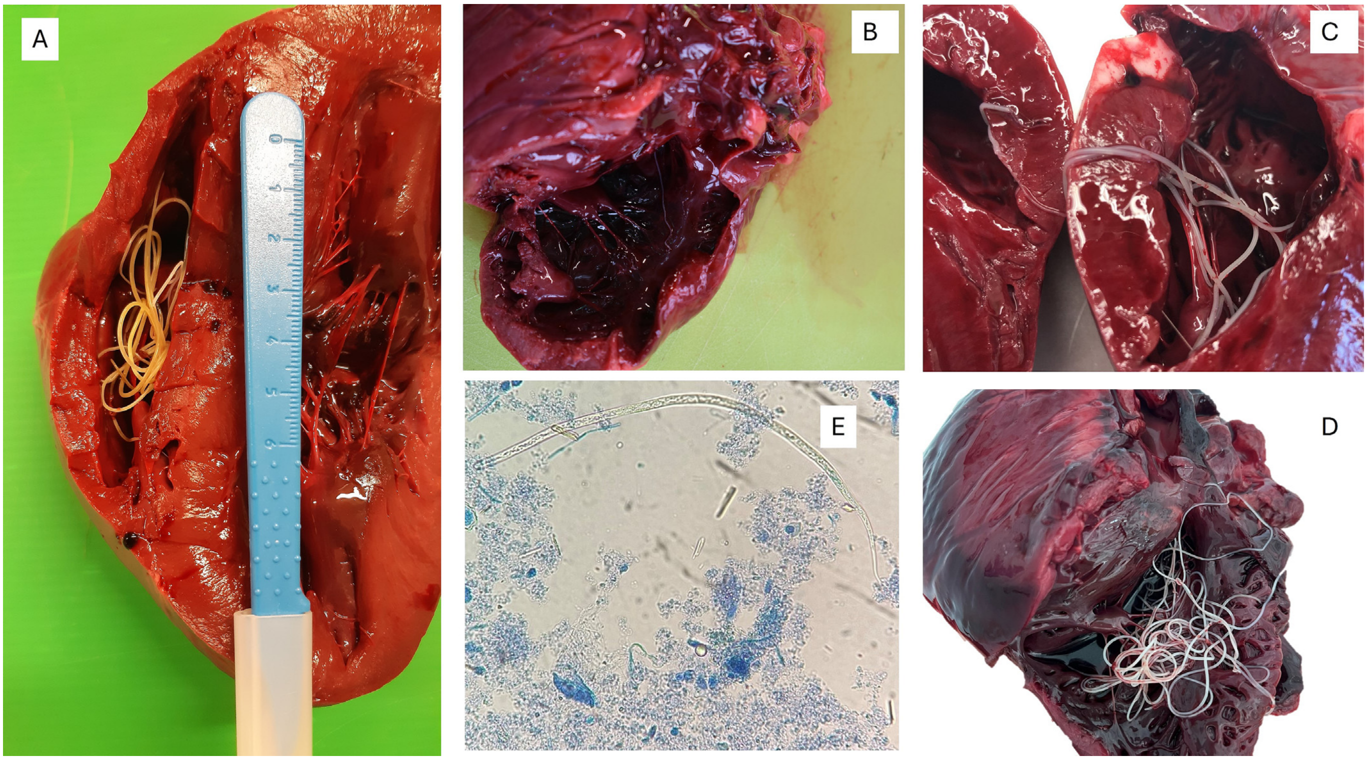

Fig. 2. Pathological and parasitological findings of positive wolves, with different stages of infestation: (A) longitudinal section of a wolf's heart showing *Dirofilaria immitis* infestation in the right ventricle; (B) right ventricle of a wolf showing low intensity of infestation (the yellow arrow indicates a male worm); (C) right ventricle of a wolf showing medium intensity of infestation (8 worms); (D) right ventricle of a wolf showing high intensity of infestation (22 worms); (E) *Dirofilaria immitis* microfilaria at Knott Test. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

circular, without lips, surrounded by 4 pairs of small cephalic papillae and 2 lateral amphids (Fig. 3B). Oesophagus divided in muscular and glandular regions, but without clear demarcation between these regions.

Males 13.5 cm (11.0–16.0) long and 0.04 cm (0.03–0.05) wide. Cuticle with fine transversal cuticular striation and spiralled cuticular ridges (Fig. 3C). Spicules unequal, with large spicule lying in channel formed by small spicule (Fig. 3C, D). Paired pre-cloacal papillae, a pair of small ad-cloacal papillae, a pair of post-cloacal ventral papillae, large post-cloacal ventro-lateral papillae, and ventral phasmids (Fig. 3E).

Females 21.5 cm (16.8–25.0) long, 0.05 cm (0.40–0.60) wide. Vulva in anterior end, with an elliptical and transverse vulvar opening, delimited by fine, but not prominent, lips (Fig. 3F). In mature specimens uterus filled with microfilariae at different developmental stages (Fig. 3G). Rounded terminal end; anus subterminal with incurved opening, toward posterior extremity (Fig. 3H). Pair of latero-ventral phasmids with circular openings.

3.3. Mitochondrial genetic variability between wolf and dog *Dirofilaria immitis*

From the 15 specimens for which DNA extraction was performed, PCR amplification of both genes (12S mtDNA and COI) yielded positive results. However, complete and readable sequences were obtained from 11 specimens (for both genes), and these were further analysed by phylogeny. Regarding the BLAST results, the nucleotide sequences obtained from both genes showed 99.4–100% similarity with *D. immitis* sequences deposited in GenBank.

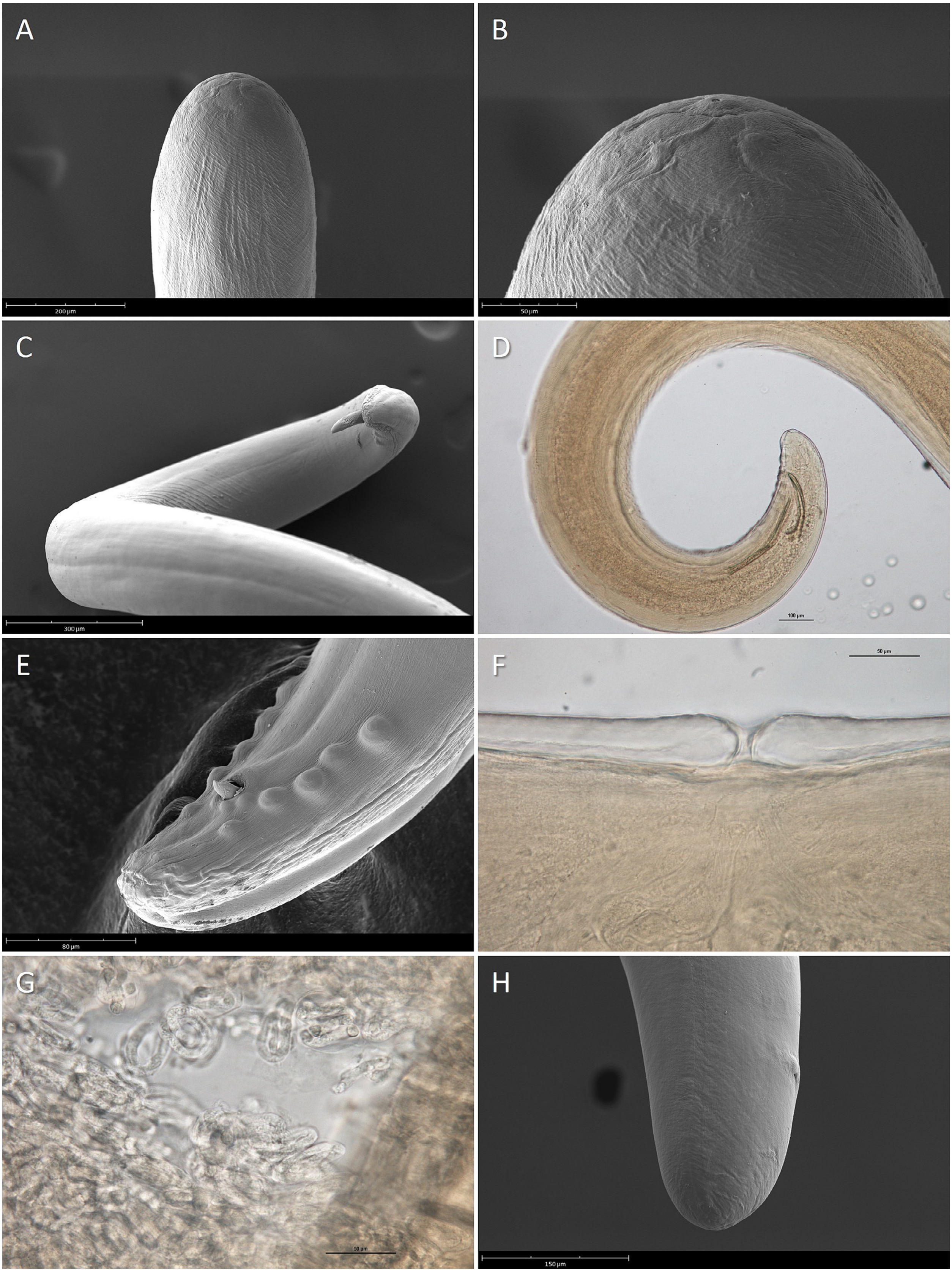

Fig. 3. Adult stages of *D. immitis* from the right ventricle and pulmonary artery of *C. lupus*: (A) anterior end with fine cuticular striations; (B) detail of anterior end showing circular oral aperture and cephalic papillae; (C) male posterior end showing spiralled cuticular striations and everted spicule; (D) male posterior end showing unequal spicules; (E) detail of caudal papillae of male specimen, with partially everted spicule; (F) detail of vulvar opening in female specimen; (G) detail of microfilariae in uterus; H) female posterior end with rounded extremity and subterminal anus.

Sequence alignment revealed small point mutations in one or more sequences, depending on the gene. Specifically, all COI sequences exhibited a nucleotide substitution at position 343 when compared to *D. immitis* sequences from dogs in northern Italy (AM749228-9), southern Italy (FN391553), and other parts of the world. Unfortunately, the only available *D. immitis* sequence from a wolf in Italy is partial and lacks this region, preventing direct comparison.

Sample 243/23 2 M (Accession number PV523601) showed an insertion of a thymine (T) at position 370 in the mitochondrial 12S rRNA gene. This was identified through alignment with the complete mitochondrial genome reference sequence NC_005305. This insertion was absent in all other wolf and Italian dog sequences. However, the same mutation was present in *D. immitis* sequences obtained from a human in Brazil (HQ504423) and a red panda in China (EU182327).

For the 12S rRNA ML tree, sequences from six different species across four genera within the subfamily Onchocercinae were used. For the COI marker, sequences from seven species spanning five genera within Onchocercinae were analysed (Fig. 4). In both ML trees, *Ascaris lumbricoides* (JN801161) was chosen as the outgroup. Phylogenetic analyses of both genes placed our sequence within the *Dirofilaria immitis* clade. In both phylogenetic analyses, the tree topology was strongly supported by high bootstrap values. Accession numbers are showed in the phylogenetic tree.

3.4. High risk for dirofilariosis detected in Po Valley flat areas by GAMM

The best GAMM on the likelihood of wolves being positive for dirofilariosis included only the altitude and the individual age class. Overall, the model explained 39.5% of the deviance in dirofilariosis, with an adjusted R-squared of 0.246, indicating a moderate fit. The predictors being excluded from the best model such as river density, water body presence, and Julian date had a negligible effect on the dirofilariosis prevalence variability in wild wolves. The age class, although included in the best model, did not have a statistically significant effect on dirofilariosis prevalence ($p = 1$ for wolves of both age class 2 and 3 compared to those of class 1, and $p = 0.16$ for wolves of class 2 compared to those of class 3). The effect of the spatial smooth term, included to account for potential spatial autocorrelation, was not statistically significant ($p = 0.569$), suggesting no strong spatial pattern in dirofilariosis prevalence after accounting for altitude and age. Conversely, altitude was significantly associated with the likelihood of *Dirofilaria* infection ($p = 0.018$). The estimated degrees of freedom for altitude were 3.354, indicating that the relationship was moderately complex but well-captured by the smooth term. The highest dirofilariosis prevalence occurred at the sea level itself, then steeply decreased at growing altitudes, reaching minimum levels from 90 m a.s.l. onwards (Fig. 5). The model detected a second slight increase in the response around 270 m a.s.l., but with confidence intervals comprising zero.

Using the best model, we predicted the spatial distribution of dirofilariosis prevalence across the study area (Fig. 6). The highest risk was predicted for the eastern parts of the Po Valley, the lowest portion of this large flat area, suggesting that this region could be a hotspot for dirofilariosis in wild wolves, with decreasing risk at higher altitudes outside of the valley.

4. Discussion

In Europe, wolves recolonized marginal areas between the early 1990s and mid-2010s, driven by forest regeneration and rural abandonment (Cimatti et al., 2021; Miller et al., 2014). In Italy, legal protection since 1971, along with habitat recovery and prey availability, facilitated their expansion beyond undisturbed areas into anthropized landscapes (Zanni et al., 2023). Today, wolves are found in *peri*-urban areas and even the densely populated Po Valley, recovering much of their historical range. Moreover, the growing presence of insect vectors, including the introduction of non-native species such as *Aedes albopictus*, favoured by climate change, further complicates the situation, as they facilitate the spread of vector-borne nematodes among domestic and wild carnivores wherever ecological conditions are suitable. (Cancrini et al., 2003; Otranto and Deplazes, 2019).

In the present study we analysed a large sample of approximately 500 wolves, from various regions of the central-northern Apennines and the western Alps. To the best of our knowledge, this is the first large scale study to assess the prevalence of cardiac dirofilariosis in wolves in Italy, and likely in Europe. The first confirmed case of *D. immitis* infection in the Italian wolf population was documented in 2007, when Pascucci et al. reported its occurrence in an Apennine wolf (*Canis lupus italicus*) and suggested a possible fatal outcome due to high parasitic burden. At the time, the detection of *D. immitis* in this species was regarded as an epiphenomenon in the epidemiology of dirofilariosis, given the relatively low population size of the Apennine wolf. However, the authors postulated that this canid, as an ancestor of domestic dogs, could act as a competent reservoir host, sustaining prolonged microfilaremia and thereby contributing to the transmission dynamics of the parasite. It was not until 2020 that Moroni et al., through an investigation of a large wolf population in the Piedmont region, provided first likely evidence of wolf's ability to act as a competent host, with embryogram analysis confirming the successful reproductive capacity of *D. immitis*in wolves.

The detection of microfilaremic wolves in the present study, despite being tested only in a limited subsample of animals, further supports this hypothesis, confirming their ability to sustain microfilaremia and potentially contribute to the transmission of *D. immitis* to vector populations.

The overall prevalence of *D. immitis* infection based on necropsy evidence in this study was 3.5% across the investigated regions. This value is higher than previously reported infection rates in European wolves, though available data are limited and based on small sample sizes. Specifically, prevalence was recorded at 1.42% in Serbia ($n = 70$) (Penezić et al., 2014), 2.1% in Spain ($n = 47$) (Segovia et al., 2001), and a single positive case out of 18 examined wolves in Bulgaria (Georgieva et al., 2001).

Regarding the Italian peninsula, the findings of this study indicate an increasing prevalence of *D. immitis* infection in wolves from the Piedmont region, with a recorded prevalence of 3.8%, compared to the 1.42% reported by Moroni et al. (2020). Additionally, positive cases were identified in Lombardy (2.8%) and Emilia-Romagna (5.2%), confirming the presence of the parasite in wolves inhabiting the Po Valley. This region is historically considered hyperendemic for heartworm (HW) infection, with historical prevalence rates in prophylactically untreated dogs reaching 50–80% (Genchi et al., 1998). In 1998, Genchi et al. reported a prevalence of 7–12% in dogs presented for routine veterinary care along Italy's northern border, suggesting the progressive spread of the parasite into previously unaffected areas. More recently, Mendoza-Roldan et al. (2020) provided the evidence of a shift in prevalence patterns towards southern Italy. Their study, based on serological data collected over a decade (2009–2019), suggested an overall "apparent" decline in HW prevalence in northern Italy (2.3%) compared to historical records, likely inferable to increased clinical awareness and the widespread adoption of chemoprophylactic programs. However, the true infection rate among untreated (and, typically, untested) dogs remains unclear, suggesting that hyperendemic foci may still persist in the Po Valley. This area, located between 45°N

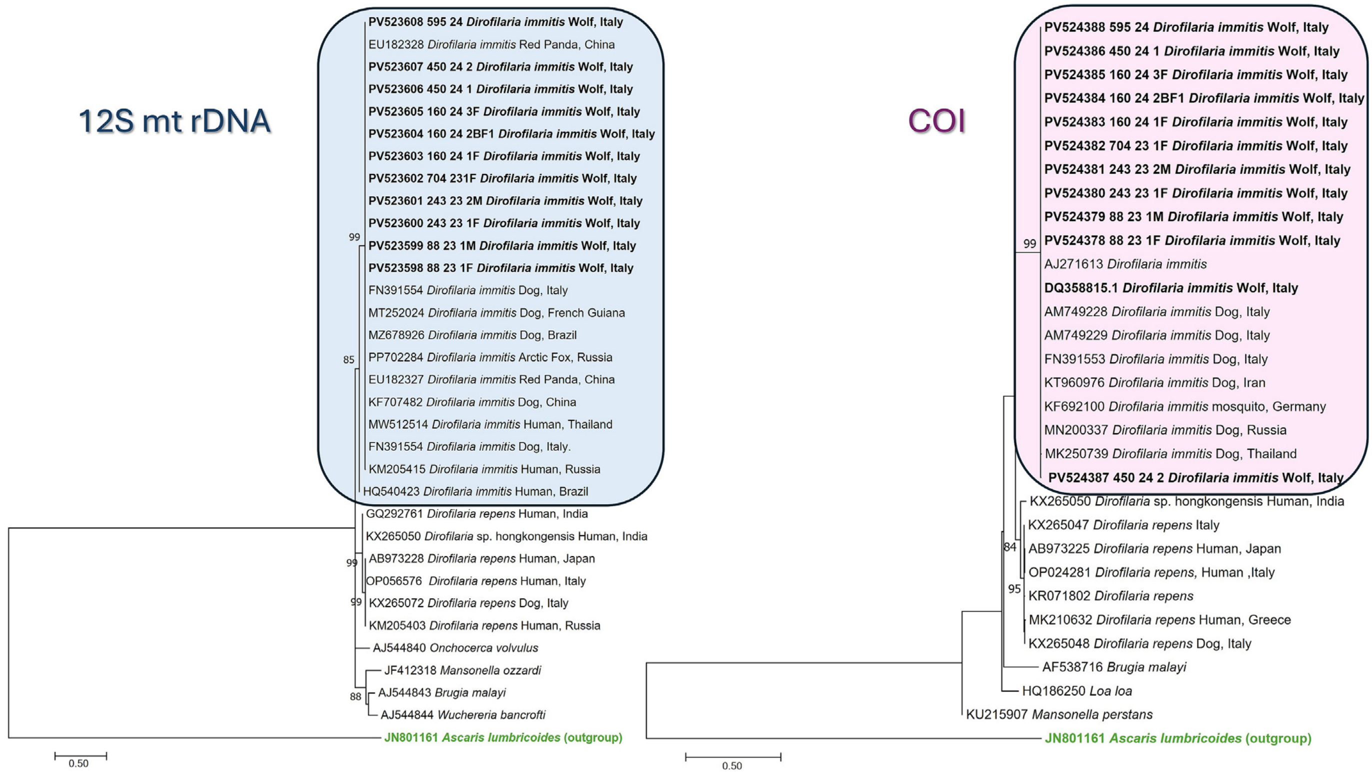

Fig. 4. Molecular Phylogenetic analysis of the two mitochondrial markers: the evolutionary history was inferred by using the Maximum Likelihood method based on the Hasegawa-Kishino-Yano model for 12S mtDNA (left), while for COI gene (right) was based on the Tamura-Nei model, only bootstrap values >75 are displayed in the figure.

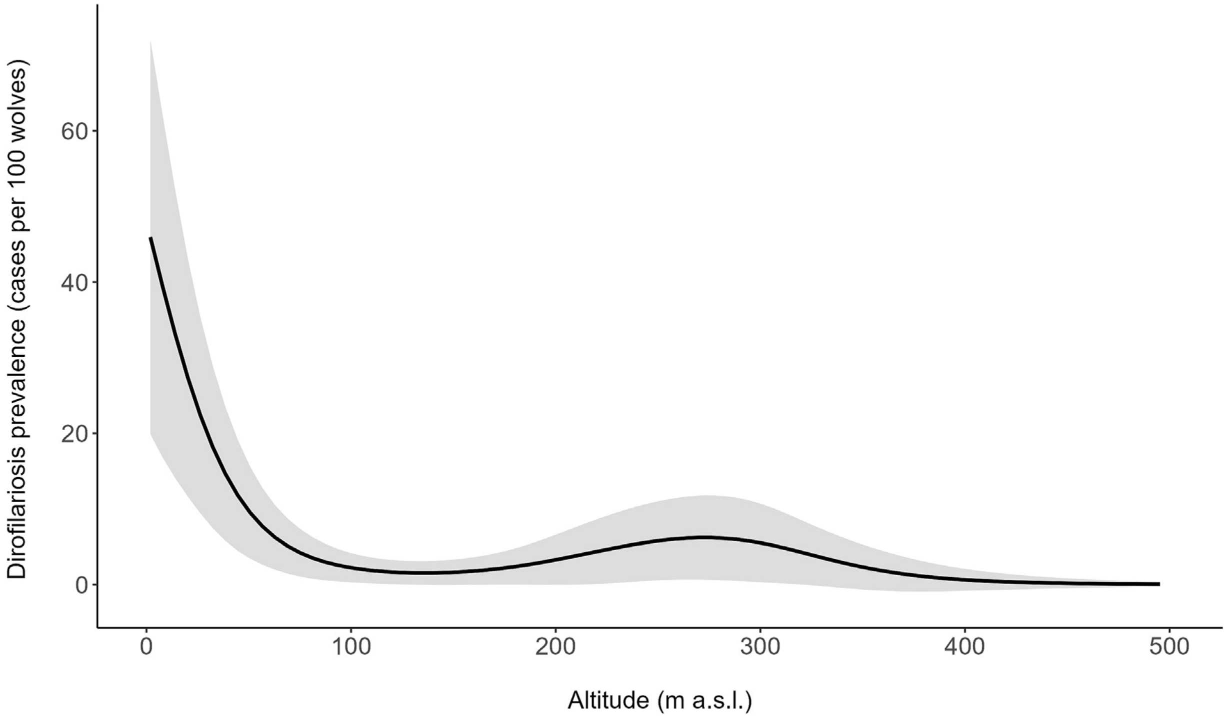

Fig. 5. Variation of dirofilariosis prevalence in wolf carcasses analysed in northern and central Italy along the altitudinal gradient, as predicted by the best Generalized Additive Mixed Models (see the text for more details). Gray-shaded areas represent 95% confidence intervals of the prediction.

and 46°N in Northern Italy, is an extensive and ecologically homogeneous lowland characterized by high rainfall, elevated relative humidity, and intensive agricultural activity. In the present study, altitude showed a significantly negative effect on the likelihood of *Dirofilaria immitis* infection ($p = 0.018$), with all positive cases, except for one in a low-altitude hilly area, detected along the lowland border of the Apennine ridge. The strong collinearity among altitude, average temperature, and human footprint prevents us

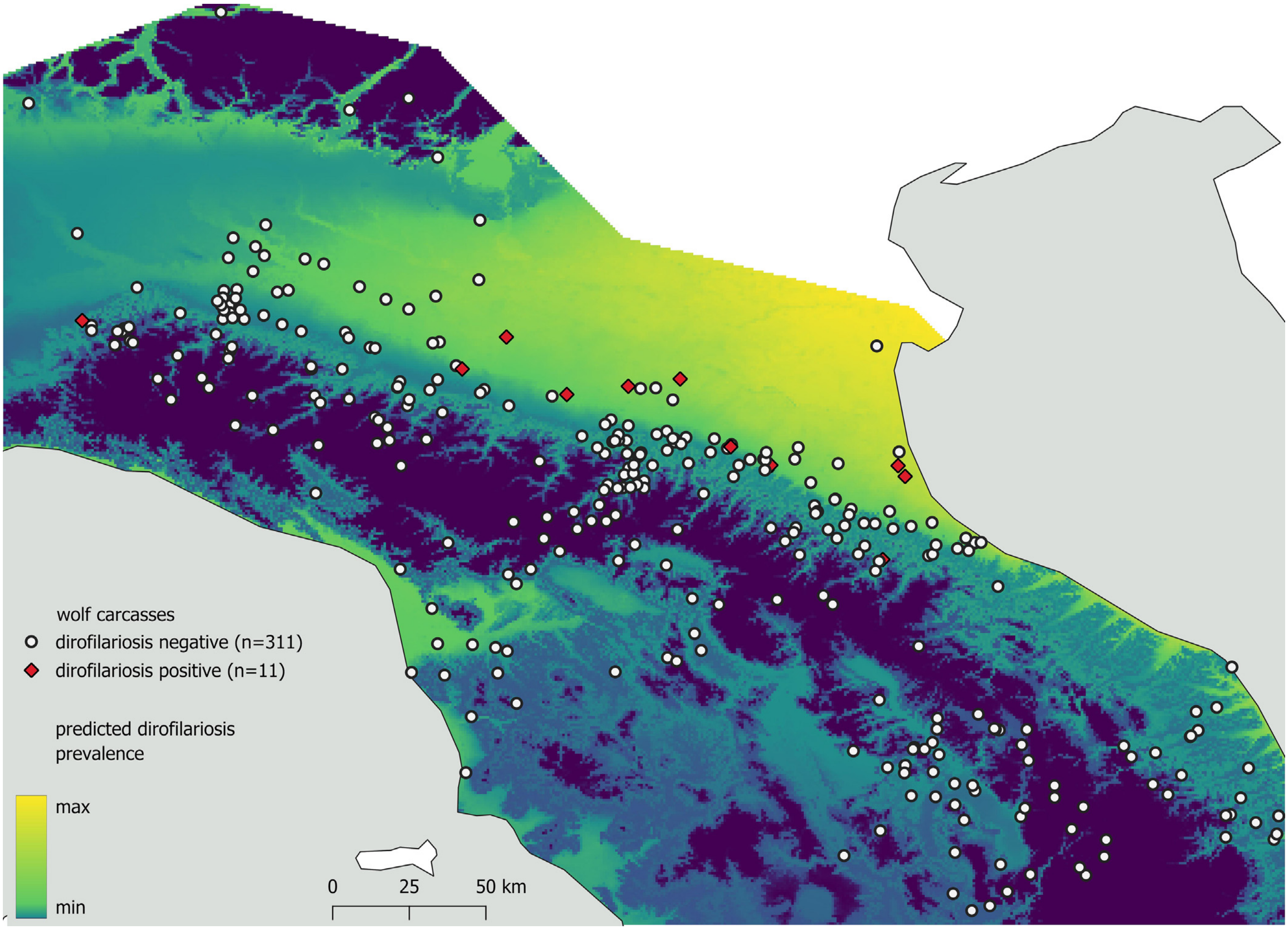

Fig. 6. Dirofilariosis prevalence risk in wild wolves in northern and central Italy as predicted by the best Generalized Additive Mixed Models (see the text for more details). White dots and red squares represent the locations where filariasis-negative and positive wolf carcasses, respectively, were discovered. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

from disentangling the direct ecological drivers of this pattern. Higher infection rates in lowlands may result from the greater presence of domestic dogs, linked to human activity, from warmer temperatures favouring vector survival, or from other environmental factors inherently associated with low-altitude areas; most likely, a combination of these. Despite this uncertainty, our spatial model provided a valuable risk map, clearly delineating areas of higher prevalence and identifying the eastern Po Valley, the lowest section of this vast plain, as the region of highest predicted risk (Fig. 6). A limitation of the present study is the lack of GPS coordinates for wolves sampled from the Piedmont region, which prevented their inclusion in the GAMM analysis. However, in this region, only wolves from lowland provinces (specifically, Alessandria and Asti) tested positive for *Dirofilaria,* likely associated with an increased risk of vector exposure (Moroni et al., 2020).

This pattern reflects local exposure risks, reinforcing the notion that this area, characterized by dense vector populations, serves as a hotspot for dirofilariosis in wild wolves, according to historical data documenting a high prevalence of the disease in dogs over previous decades (Canestri Trotti et al., 1986; Genchi et al., 1988; Poglayen et al., 1988; Poglayen et al., 1996; Pietrobelli et al., 1998).

Interestingly, the parasitic burden observed in this study ranged from 1 to 22 adult nematodes, with a mean intensity of three nematodes. This range of infection levels is consistent with Moroni et al. (2020), which have reported similar or higher parasitic load among wolf populations. Interestingly, in the present study, the majority of dirofilariosis cases had mixed-sex infections, and the mean F:M sex ratio equaled 1, suggesting that wolves are competent hosts, capable of maintaining microfilaremia, highlighting their suitability as reservoir for *Dirofilaria* infection, as already suggested (Szentiványi and Garamszegi, 2024). On the contrary, red foxes and badgers usually show low parasitic intensity, low prevalence of microfilaremia, and limited presence of adult worms of both sexes (Ionică et al., 2017).

Most of the samples obtained in this study have been morphologically identified, providing further microscopic (light microscopy and SEM) information of *D. immitis* specimens in host different from dogs, and confirming the morphological features of previous descriptions (Furtado et al., 2010). Moreover, the identification of the nematodes was confirmed through molecular analysis, and potential genetic variability between the wolf-derived specimens in this study and *D. immitis* sequences from other hosts was evaluated through phylogenetic analyses of two mitochondrial markers. The minor differences observed in sequence alignment (of both 12S mitochondrial rRNA and COI) did not result in any phylogenetic separation at ML trees between our sequences and those from dogs, humans, mosquitoes, and other mammals. Several studies have analysed the haplotype diversity of *D. immitis* and *D. repens* using mitochondrial markers such as COI, 12S rRNA, and NADH dehydrogenase subunit 1 in different animal hosts and locations (Laidoudi et al., 2022; Alsarraf et al., 2023). Notably, *D. repens* exhibits greater haplotype diversity than *D. immitis*, with the latter characterized by only five haplotypes, primarily differentiated by geographic origin rather than host specificity (Alsarraf et al., 2023). Whole genome sequencing has further confirmed this pattern by revealing distinct differences in heartworm populations across continents. These findings suggest that geographic factors are the primary drivers of genetic variation of *D. immitis* worldwide. At the same time, the absence of clear host specificity, supports the hypothesis of ongoing transmission between domestic animals and wildlife (Power et al., 2024). Notably, wolves and coyotes (*Canis latrans*) carry heartworm burdens similar to those found in domestic dogs. This lends support to the hypothesis that wild canids (especially ancestral wolves and coyotes), were the original hosts of *D. immitis* in earlier evolutionary periods (Power et al., 2024).

The high mitochondrial genetic similarity between the *D. immitis* specimens sequenced in this study and those previously identified in dogs suggests a shared transmission pattern between these two species. Based on the observed prevalence, it can be inferred that wolves, previously restricted to mountainous regions, likely acquired the infection while recolonizing high-risk lowland areas such as the Po Valley. These wolves may now act as a reservoir for *D. immitis*, supporting the persistence of the parasite in the environment and facilitating its spread to untreated dogs. This scenario may complicate the epidemiological landscape of canine heartworm disease, pose challenges for the implementation of prophylactic measures in domestic dogs, and ultimately increase the risk of infection in untreated dog populations that do not receive regular macrocyclic lactone-based chemoprophylaxis. Additionally, the potential zoonotic implications of *D. immitis* circulation in wildlife further underscore the need for integrated surveillance strategies.

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