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Dogs are reservoir hosts of the zoonotic *Dirofilaria* sp. 'hongkongensis' and potentially of *Brugia* sp. Sri Lanka genotype in Sri Lanka

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ABSTRACT

In 2016, the World Health Organization declared Sri Lanka as having successfully eliminated lymphatic filariasis as a public health concern. However, in recent decades, several infections with subperiodic filarial species suggestive of zoonotic infections have been recorded across the country. The arthropod-borne filarioids *Dirofilaria repens, Brugia malayi, Brugia ceylonensis*, and *Acanthocheilonema reconditum* are historically known to be endemic in dogs in Sri Lanka. Despite this, limited information on the prevalence, diversity, and predictors of filarial infections in dogs in the country has resulted in suboptimal control and prevention of these parasites, some of which are known to be zoonotic. To address this, whole blood and metadata were collected and analysed from 423 pet dogs across three geo-climatic zones within Sri Lanka. Blood samples were screened using the Modified Knott's Test (MKT) and PCR followed by Sanger sequencing. Multivariable logistic regression models were used to assess predictors for canine filarial infections. *Dirofilaria* sp. 'hongkongensis' (*Dirofilaria* sp. HK) and *Brugia* sp. Sri Lanka (SL) genotype were identified infecting dogs. The overall prevalence of filarial infection in pet dogs by PCR was 36.9% (95% CI 32.3–41.7%, $n = 156$), compared to 18.8% (95% CI 15.2–22.9%, $n = 79$) detected using the MKT. >80% of filarial-positive dogs were infected by *Dirofilaria* sp. HK, while the remaining dogs were infected by *Brugia* sp. SL genotype. Increasing age ($p < 0.001$) and residing in the low-country wet zone ($p < 0.001$), which includes regions that were endemic for human filariasis in Sri Lanka, were associated with filarial infections in dogs. No clear pathognomonic signs for filarial infection were identified, indicating that dogs act as reservoirs for these potentially zoonotic pathogens. Given the morphological similarity of *Dirofilaria* HK and *Brugia* sp. SL microfilariae with those of *D. repens* and *B. malayi*, respectively, it is likely that these species have been misidentified in the past. Prevention and control measures of these potentially zoonotic canine filarial infections are highly advocated to safeguard both canine and human health.

1. Introduction

Several filarial species in the Family Onchocercidae are known to infect the domestic dog (*Canis lupus familiaris*) worldwide. In the tropics, *Acanthocheilonema reconditum*, *Dirofilaria* sp. 'hongkongensis' (*syn* '*Candidatus* Dirofilaria 'hongkongensis'), *Dirofilaria immitis,* and *Dirofilaria repens* are the main reported species [1–5]. Infections with *Brugia* species, such as *Brugia malayi*, *Brugia pahangi*, and *Brugia ceylonensis* have been described to a lesser extent in dogs and are primarily prevalent in southern and southeastern Asia [6,7]. Mosquitoes belonging to the subfamily Culicinae act as vectors for most canine filariae [8,9] while some filarioid species, such as *A. reconditum,* are known to have flea or louse vectors [10].

Infection with *D. immitis* in dogs is well described as it causes severe pathology due to the adults' predilection sites in the cardiopulmonary system [11]. In contrast, *D. repens*, *Dirofilaria* sp. 'hongkongensis', and *A. reconditum* have subcutaneous or intra-peritoneal predilection sites [12,13] and are rarely found to cause significant pathology in dogs. The clinicopathological manifestations of *Brugia* infection in dogs are scarcely reported; however, *B. pahangi* is known to infect the lymphatic system causing associated pathology [14].

Most of these canine filariae can also infect humans, for example, *Dirofilaria repens* and *Dirofilaria* sp. 'hongkongensis' can cause human subcutaneous dirofilariasis, whilst *D. immitis* infrequently generates

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vascular-pulmonary lesions [15]. *Brugia malayi* is one of the causative agents of human lymphatic filariasis (LF), and is considered a neglected tropical disease by the World Health Organization (WHO), accounting for nearly 10% of global LF infections [16].

The earliest reported case of canine filariasis in Sri Lanka dates back to 1934 [17]. From then onwards, several case reports and epidemiological surveys have identified *D. repens* [18–22], *B. malayi* [20–22] *B. ceylonensis* and *A. reconditum* [23] in dogs from Sri Lanka. Due to the restricted geographical range investigated in prior studies alongside the suboptimal molecular identification techniques employed, the reliable identification of canine filariae within this country has to date, been prevented [19,21–25]. No previous studies have used the mitochondrial cytochrome oxidase I (*cox*-1) gene for filarial molecular characterisation in Sri Lanka, a significant shortcoming given that this is the preferred barcoding gene for reliable species identification in this group [26].

Records of human filarial infections in Sri Lanka date back to the twelfth century CE [27] when LF was endemic along the country's western and southern coasts. At this time, the original predominance of *B. malayi* was displaced by *Wuchereria bancrofti* following World War II [27] LF caused by *B. malayi* was reported to have been eliminated from Sri Lanka during the latter part of 1960 [28] and in 2016, the World Health Organization declared Sri Lanka to have successfully eliminated LF as a public health concern. However, *Brugia* infections re-emerged in the early 2000s [29] after nearly four decades of apparent quiescence [30]. The (re)-emerging *Brugia* species were nocturnally sub-periodic in contrast to the nocturnally periodic former species [31], suggesting a

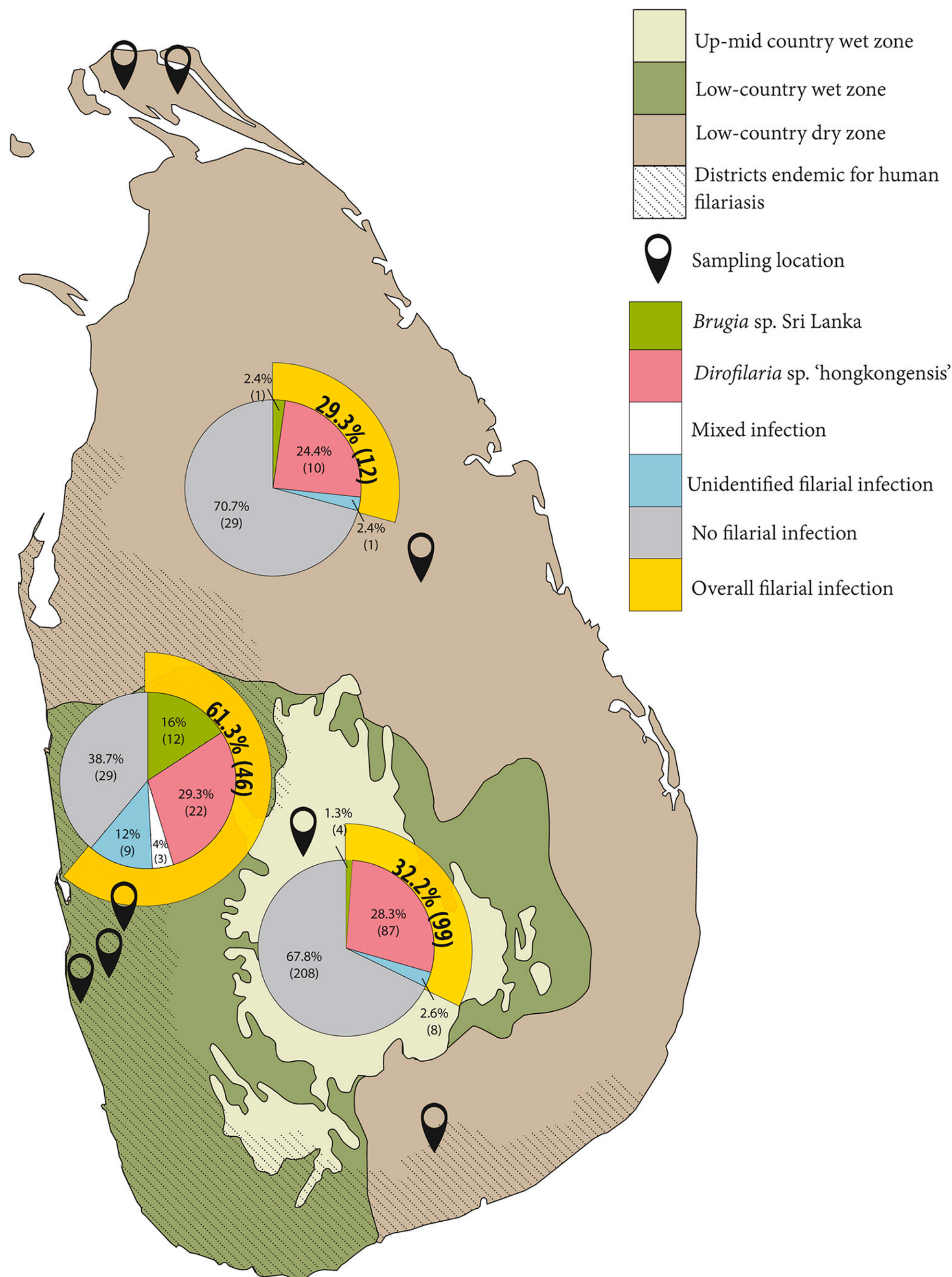

Fig. 1. Apparent prevalence of filarial species in dogs in different geo-climatic zones in Sri Lanka. The black drop-pins indicate the sampling locations, and the hatched area shows the districts endemic for human filariasis in Sri Lanka. Pie charts represent apparent prevalence of filarial infections per zone, the number of dogs is indicated in parenthesis.

zoonotic origin [32]. The detection of *Brugia* microfilaraemic dogs in Sri Lanka, coupled with their close association with cases of human infection by *Brugia* [21,33], suggests that Sri Lankan canines may act as reservoir hosts for current human *Brugia* infections. This study aims to fill knowledge gaps on the prevalence, predictors, and genetic diversity of canine filarial infections in Sri Lanka, which is crucial for implementing effective prevention and control measures to safeguard the health of both dogs and humans.

2. Materials and methods

2.1. Study sites and sampling

Sri Lanka (latitudes 5°55′ - 9°51′ N and longitudes 79°41′ - 81°53′ E) is an island with a tropical climate that can be categorised into three distinct geoclimatic zones; low-country dry zone, low-country wet zone, and mid-up-country wet zone based on the geographical relief and mean annual precipitation [34]. Eight veterinary clinics/hospitals (Fig. 1) across these three geo-climatic zones were selected based on local veterinary networks. Within each clinic, pet dogs were sampled excluding emergency admissions. For multi-pet households, only one dog per household was included. From each dog, 2–3 ml of whole blood was collected in ethylenediaminetetraacetate (EDTA) tubes through cephalic or lateral saphenous venepuncture and stored at −20 °C at the University of Peradeniya, Sri Lanka. Sample size calculations were based either on the assumed prevalence of each filarial species based on published data [21] assuming 95% confidence and 5% precision or on demonstrating freedom from the reported filarial species in the region, assuming a diagnostic test sensitivity of 75% and specificity of 95% using the ‘epiR’ package [35] and ‘Epitools’ web platform (https://epitools.ausvet.com.au/), respectively (Supplementary File B). The higher number of samples required was used moving forward. This study was approved by the Committee for Ethical Clearance on Animal Research of the Faculty of Veterinary Medicine and Animal Science, University of Peradeniya, Sri Lanka (VERC/20/07).

2.2. Collection of data

Data were collected from April 2020 to March 2021. Following consent, individual animal data including, age, sex, neutering status, breed, ectoparasitic and deworming treatment formulation and frequency were collected from the owner of each participating dog using a paper-based structured questionnaire. The complete list of demographic and clinical data collected is available in Supplementary File A. Clinical manifestations such as body condition, skin pathology (e.g., erythema, nodules, alopecia), liver and spleen abnormalities (e.g., splenomegaly and hepatomegaly), mucous membrane colour, body temperature, and lymph node abnormalities were obtained through a physical examination by a veterinarian. Tick, flea, and/or louse infestations were identified by examining the whole-body surface (including interdigital spaces) for approximately 5 mins. If at least one tick, flea, or louse was found, the pet was considered to have an active infestation by the respective arthropod species. The dog's body condition was assessed according to the WSAVA five-scale body condition score (BCS) chart [36].

2.3. Modified Knott's test and identification of microfilariae

The collected blood samples were transported on ice to the University of Melbourne, Australia where a modified Knott's test (MKT) was performed to diagnose and concentrate microfilariae. In brief, 1 ml of blood in EDTA was mixed with 9 ml of 2% formalin to lyse RBCs and centrifuged at 500*g* for 5 min in an Avanti J-15R centrifuge, (Beckman Coulter Life Sciences, USA), concentrating microfilariae in the sediment. The supernatant was discarded, and a drop of the sediment was mounted on a glass slide with a drop of 0.1% methylene blue solution and covered with a cover slip, prior to screening under a CX43 Biological Microscope, Olympus Life Sciences (Japan) at x40, x100 and x400 magnifications to detect microfilariae. Microfilariae were identified using Lan-Chou 1933, Schacher 1962, Lindsey 1965, Laurence & Simpson 1971, Tongu 1974, Seo 1976, Purnomo et al., 1977, Orihel et al., 1997, and Magnis et al., 2013 [37–45].

2.4. DNA extraction and molecular screening

Extraction of DNA from canine whole blood was performed with the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol at the University of Peradeniya, Sri Lanka. The extracted DNA was stored at −20 °C until shipped to the University of Melbourne, Australia on ice. The DNA extracts were screened with minor modifications using a PCR [46] targeting a portion of the mitochondrial *cox*-1 gene of filariae. The PCRs were performed in 20 μl reactions containing 0.2 mM dNTP, 0.5 units of HotStarTaq™ DNA Polymerase (Qiagen, Hilden, Germany), x1 PCR buffer (Qiagen), 0.5 μM of each forward (COIfilF) and reverse (COIfilR) primers. The 12S ribosomal RNA (12S rRNA) gene was also amplified for ~20% of each filarial species identified using the *cox*-1 barcoding gene, using previously published primers 12SF and 12SnemR2 [47,48] in 20 μl reactions containing dNTPs, primers and PCR buffer in concentrations similar to the *cox*-1 gene PCR reaction, but with 1.5 units of HotStarTaq DNA Polymerase (Qiagen, Hilden, Germany) per reaction. All PCRs were performed in a SimpliAmp™, Thermo Fisher Scientific (USA) thermocycler according to the respective thermal profiles in Table 1. A known *D. immitis* DNA sample was used as a positive control along with a no template reaction control in all PCRs conducted. PCR products were visualised on a 1.5% agarose gel with Gel Red® nucleic acid stain (Biotium, USA) in a ChemiDoc XRS+ visualisation system (Bio-Rad Laboratories, Inc., USA) using Image Lab, version 6.1.0 (Bio-Rad Laboratories, Inc., USA) software. PCR products of all *cox*-1 gene positive samples and 12S rRNA gene were purified using ExoSAP-IT (Applied Biosystems™, USA) according to the manufacturer's protocol and Sanger sequenced by Macrogen, (Seoul, Republic of Korea). DNA sequences were visualised and edited using Geneious Prime® 2022.2.1 and identified by comparison against sequences from the GenBank database (NCBI) using the nucleotide basic local alignment search tool (nBLAST) [49].

2.5. Data analysis

The data was transferred from the paper-based questionnaire to a Microsoft Excel® for Microsoft 365 MSO (Version 2212 Build 16.0.15928.20196) for storage, checking and cleaning. Statistical analyses were performed using R statistical software version 4.2.0 in R studio version 2023.03.1 [50]. The packages' “**dplyr**” [51], “**janitor**” [52], were used for data cleaning and exploration.

For the description of the study population, dogs were categorised according to four age groups, as <6 months, 6 months - 2 years, >2–6 years, and > 6 years. Two breed categories were defined including dogs of local breeds and their crosses in one group and exotic breeds, such as German Shepherds, Rottweilers, etc., and their crosses in the other. Based on the body condition, the dogs were categorised as low if BCS < 3 and normal/high if BCS ≥ 3.

Univariable associations of filarial infection, host and environmental factors, and clinical signs (e.g., age, breed, sex, neutering status, presence of ticks, fleas, or lice, ectoparasiticide treatment status, deworming status, geo-climatic zone, presence of pale mucosae, and fever) were assessed using binomial logistic regression in the ‘stats’ package [50].

Directed acyclic graph (DAG) constructed with DAGitty v3.0 [53] were used to inform the multivariable analysis. (Supplementary File B). Environment and host variables with a $p < 0.2$ in univariable analysis were selected for the multivariable analysis. Variables related to clinical signs were excluded due to co-infections with other vector-borne

Table 1
Primers and cycling conditions used to amplify *cox-1* and *12S rRNA* genes of filaria species.

Target gene, expected amplicon length, and [Reference (s)]	Primers	Stage	Temperature (°C)	Duration	Number of cycles
cox-1, ~670 bp [46]	COIfilF – 5′ TGA TTG GTG GTT TTG GTA A 3′	Initial denaturation	95	5 min	1
		Denaturation	94	45 s	40
	COIfilR – 5′ ATA AGT ACG AGT ATC AAT ATC 3'	Annealing	52	45 s	
		Extension	72	60s	
		Final extension	72	5–7 min	1
12S rRNA gene, ~520 bp [47,48]	12SF – 5′ GTT CCA GAA TAA TCG GCT A 3′	Initial denaturation	95	5 min	1
		Denaturation	94	45 s	40
	12SnemR2–5' CTA CCA TAC TAC AAC TTA CGC 3'	Annealing	52	45 s	
		Extension	72	90s	
		Final extension	72	7 min	1

pathogens. Correlation between variables were assessed in a pairwise matrix with the Cramers V test in "**creditmodel**" [54] package in R version 4.2.0 and visualised with "**corrplot**" package [55] before including them in the multivariable model. If variables showed >75% correlation, only one of the pair was included in the analysis. The selected variables were used as the fixed effects and sampling veterinary clinics as random effects in a generalized linear mixed-effect model (GLMM) using "**lme4**" package [56] in R to determine predictors for filarial infection in Sri Lankan dogs. The "**sjPlot**" [57] package in R was used to obtain odds ratios for the models. Model selection was performed by iterative backward stepwise elimination considering $p < 0.1$ to be significant. The model was validated with the "**DHARMa**" package in R [58] using simulated residual plots.

2.6. Phylogenetics and sequence type network construction

Available mitochondrial *cox*-1 and 12S rRNA gene sequences of ≥400 bp in the GenBank database as of 3rd October 2022 [59] for *Dirofilaria* and *Brugia* species were downloaded and imported into Geneious Prime® 2022.2.1. The sequence types (ST) identified within this study and the relevant GenBank database sequences were aligned using MAFFT algorithm to generate separate alignment for each species-gene combination. The alignments were exported as FASTA files which subsequently converted to the NEXUS format with MEGA 11 (version 11.0.13) [60]. Sequence types of each alignment were identified using DnaSPv6 software (version 6.12.03) [61] by excluding sites with gap/missing or ambiguous data. The output of identified STs was saved in a NEXUS file. One or two sequences belonging to each ST were selected for phylogenetic inference.

For phylogenetic inference, the selected sequences were aligned with the MAFFT algorithm in Geneious Prime® and exported to a FASTA file. The best nucleotide substitution model for each alignment was determined with maximum likelihood analysis, including 1st, 2nd, and 3rd codon positions in MEGA 11. The model with the lowest Bayesian Information Criterion (BIC) value was selected for phylogenetic inference. For all alignments, number of nucleotide substitutions were 2 with gamma distributed rates. For Bayesian phylogenetic inference (BI) selected FASTA alignments were converted to NEXUS format for MrBayes, that includes a code block with instructions for Bayesian inference with Mesquite: a modular system for evolutionary analysis (version 3.70) software [62]. Bayesian phylogenetic inference was performed using MrBayes 3.2.7 separately on each alignment. Each BI was performed with two million Markov Chain Monte Carlo (MCMC) generations, sampling every 100th generation with four chains by allowing for transitions and transversions with gamma-distributed rates. Phylogenetic inference by the Neighbour-Joining (NJ) distance method was performed in MEGA 11 (Version 11.0.13) for the selected alignments. The NJ analysis was performed with 2000 bootstrap replications using the Tamura-Nei model, based on best nucleotide substitution models determined above, including both transition and transversions with gamma-distributed rates.

For *cox*-1 sequence type network construction, each ST from each geographical location was selected and aligned with the MAFFT algorithm and converted to the NEXUS file as mentioned above. The geographical locations of each sequence were added in a 'trait block' to the NEXUS file manually. Separate minimum spanning networks (at epsilon =0) were constructed for *Dirofilaria* and *Brugia cox*-1 alignments in PopART version 1.7 [63] and the resulting networks were edited in Adobe Illustrator Version 27.3.1 (Adobe, United States).

3. Results

A total of 423 blood samples were collected from the up-mid-country wet zone (n = 307, 72.6%), low-country wet zone (n = 41, 9.7%), and low-country dry zone (n = 75, 17.7%). From our study population, over 60% (n = 265) of sampled dogs were local breed dogs, whilst the dogs of exotic breeds (n = 147, 34.8%) consisted of German Shepherd (n = 53), Rottweiler (n = 19), Labrador Retriever (n = 19), Dobermann (n = 9), Pomeranian (n = 8), Dachshund (n = 6), Shih Tzu (n = 5), Golden Retriever (n = 5), Terrier breed (n = 5), Rhodesian Ridgeback (n = 5), Boxer (n = 4), Dalmatian (n = 3), Bull Mastiff (n = 2), Siberian Husky (n = 1), Cocker Spaniel (n = 1), and exotic-exotic breed crosses (n = 2). The study dogs' geo-climatic zone, age, sex, neuter status, breed, tick, flea and louse infestation status, and use of ectoparasiticides and deworming treatments are summarised in Table 2. Over 60% (n = 265) of the dogs were dewormed and nearly 45% (n = 189) had received ectoparasite treatment. Details of deworming and ectoparasiticide treatments used on the dogs within this study are summarised in Supplementary File B. Overall, 37.1% (95% CI 32.5–41.9%, n = 157) of study dogs tested positive for filarial infection by cPCR and/or MKT. At the MKT, 18.8% (95% CI 15.2–22.9%; 79/420) dogs scored positive for microfilariae (Table 3). Unsheathed microfilariae, with a hooked tail ('umbrella handle'-like) and two cephalic space nuclei were detected in 18.6% (95% CI 15–22.6% n = 78) of samples (Fig. 2A). Only 0.5% (95% CI 0.1–1.7%, n = 2) of samples had sheathed microfilariae with two terminal nuclei resembling those of *Brugia* species (Fig. 2B). No microfilariae of *D. immitis,* or *A. reconditum,* were identified. Screening with PCR amplifying the *cox*-1 gene revealed 36.9% (95% CI 32.3–41.7%, n = 156) of samples to be positive for filarial DNA. Filarial infection results according to age group, sex, neutering status, breed group, tick, flea, and louse infestation, geoclimatic zone, ectoparasiticide treatment and deworming status through PCR and MKT are summarised in Table 4.

All 156 samples positive by the *cox*-1 gene PCR produced amplicons that were sequenced by Sanger sequencing, of which 137 sequences were of good quality. The majority (86.1%, n = 118) of sequences obtained in the present study were identified as *Dirofilaria* (accession nos. For our sequences were **OR019675, OR019676, OR019677**), whilst only 19 sequences herein obtained were identified as *Brugia* (accession nos. For these sequences were **OR019673** and **OR019674**) when compared to GenBank sequences using nBLAST. All our *Dirofilaria*

Table 2
Dogs divided by geo-climatic zone, age-group, sex, neuter-status, breed group, ectoparasiticide usage, deworming treatment, and tick, flea, and louse infestation.

Variable	Category	n (%)
Geo-climatic zone	Up-mid-country wet zone	307 (72.6)
	Low-country wet zone	75 (17.7)
	Low-country dry zone	41 (9.7)
Age group	<6 months	28 (6.6)
	6–24 months	146 (34.5)
	> 2–6 years	115 (27.2)
	> 6 years	113 (26.7)
	Unknown	21 (5)
Sex	Female	189 (44.7)
	Male	216 (51.1)
	Unknown	18 (4.3)
Neuter status	Intact	313 (74)
	Neutered	81 (19.1)
	Unknown	29 (6.9)
Breed	Exotic	147 (34.8)
	Local	265 (62.6)
	Unknown	11 (2.6)
Tick infestation	Absent	273 (64.5)
	Present	121 (28.6)
	Unknown	29 (6.9)
Flea infestation	Absent	175 (41.4)
	Present	219 (51.8)
	Unknown	29 (6.89)
Louse infestation	Absent	391 (92.4)
	Present	5 (1.2)
	Unknown	27 (6.4)
Treatment against ectoparasites	Given	189 (44.7)
	Not given	145 (34.3)
	Unknown	89 (21)
Deworming treatment	Given	265 (62.6)
	Not given	71 (16.8)
	Unknown	87 (20.6)

sequences showed 99.8% nucleotide identity to reference sequences of *Dirofilaria* sp. 'hongkongensis' in GenBank (reference accession nos. **OP185210**, **OL314721**, and **KX265050**) with 100% query coverage. Our *Brugia* sequences showed 97.3–99.7% nucleotide identity to just one *B. malayi* reference sequence isolated from dogs in Tamil Nadu, India (GenBank accession **MN564741**), whilst all other *B. malayi* reference sequences in GenBank showed a nucleotide identity <96.5% with the *Brugia* sequences detected in this study.

A total of 377 sequences were downloaded from the GenBank database for *Dirofilaria* spp. and 31 STs were identified (Supplementary File B). For *Brugia* spp. 45 sequences were downloaded from GenBank database and 23 STs were identified (Supplementary File B). The closest genetic relation of *Dirofilaria* sp. 'hongkongensis' was to *D. repens* with 12–21 single nucleotide polymorphisms (SNP) observed throughout a stretch of 333 bases on the *cox*-1 gene. The sequence type network for *Dirofilaria* spp. demonstrated *D. repens* STs to be distributed in Europe, Africa, Central and Eastern Asia, while *Dirofilaria* sp. 'hongkongensis' were primarily detected from the Indian subcontinent (Fig. 3).

The closest relative of the *Brugia* sp. Sri Lanka genotype identified in the current study was *B. malayi* which had 18–26 SNP differences across 425 bases. Sequence type networks for *Brugia* demonstrated that *B. malayi* sequences were restricted to Southeast Asia while *Brugia* sp. Sri Lanka was limited to the Indian subcontinent (Fig. 4).

Figs. 5 and Supplementary File B show the phylogenetic relationships attained using Bayesian inference and NJ methods for the genus *Dirofilaria* utilising the *cox*-1 and 12S rRNA genes, respectively. Phylogeny using Bayesian inference of *cox*-1 gene sequences of *Dirofilaria* identified six major clades with a high degree of support (posterior probability 0.96–1). The sequences in this study clustered with the *Dirofilaria* sp. 'hongkongensis' sequences with a posterior probability of 0.99. Comparable results were obtained through NJ analysis with 98% bootstrap support for the *Dirofilaria* sp.' hongkongensis'. With Bayesian inference for the 12S rRNA gene, the *Dirofilaria* sp. 'hongkongensis'

Table 3
Filarial infections detected with Modified Knott's Test (MKT) and conventional PCR (cPCR) followed by Sanger sequencing.

Filarial species identified	cPCR followed by Sanger sequencing No. of positives (%)	MKT No. of positives (%)
Dirofilaria sp. 'hongkongensis'	118 (27.9)	77 (18.3)
Brugia sp. Sri Lanka	19 (4.5)	1 (0.2)
Dirofilaria sp. & *Brugia* sp.	–	1 (0.2)
Unidentified filariae	19 (4.5)	–
Overall	156 (36.9)	79 (18.8)

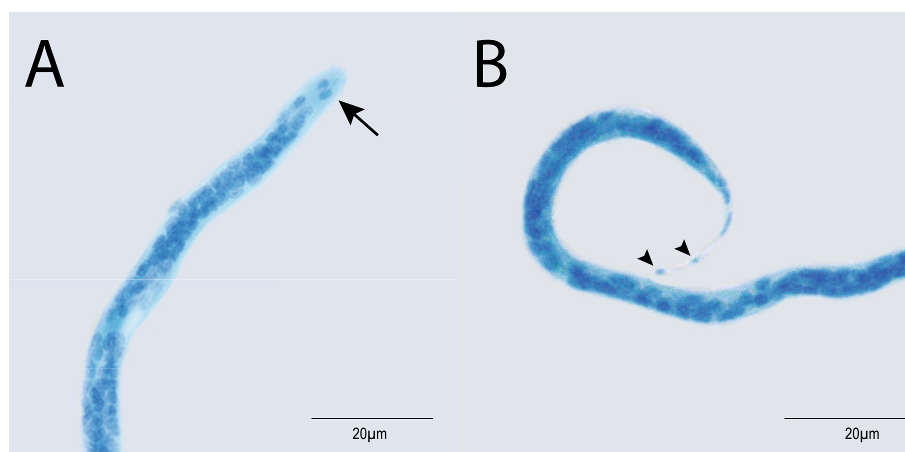

Fig. 2. A) Cephalic nuclei (black arrow) of *Dirofilaria* and B) terminal nuclei (black arrowheads) of *Brugia* microfilariae identified in the Knott's test sediment stained with 0.1% methylene blue. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 4
Dogs diagnosed with filarial infections by conventional PCR (cPCR) followed by Sanger sequencing and Modified Knott's Test (MKT) divided by age group, sex, neutering status, breed group, tick, flea, and louse infestation, geoclimatic zone, and treatment against helminths and ectoparasites.

Variable	cPCR			MKT		
	Total	Positives	% (95% CI)	Total	Positives	% (95% CI)
Age group						
< 6 months	28	1	3.6 (0.1 – 18.3)	28	0	0 (0 -12.3)
6 – 24 months	146	45	30.8 (23.5 – 39)	143	17	11.9 (7.1 – 18.4)
> 2 – 6 years	115	47	40.9 (31.8 – 50.4)	115	24	20.9 (13.9 – 29.4)
> 6 years	113	54	47.8 (38.3 – 57.4)	113	35	31 (22.6 – 40.4)
Unknown	21	9	42.9 (21.8 – 66)	21	3	14.3 (3 – 36.3)
Sex						
Female	189	59	31.2 (24.7 - 38.4)	188	31	16.5 (11.5 - 22.6)
Male	216	90	41.7 (35 - 48.6)	214	46	21.5 (16.2 - 27.6)
Unknown	18	7	38.9 (17.3 - 64.3)	18	2	11.1 (1.4 - 34.7)
Neutering status						
Intact	313	114	36.4 (31.1 - 42)	310	56	18.1 (13.9 - 22.8)
Neutered	81	29	35.8 (25.5 - 47.2)	81	18	22.2 (13.7 - 32.8)
Unknown	29	13	44.8 (26.5 - 64.3)	29	5	17.2 (5.9 - 35.8)
Breed						
Exotic	147	55	37.4 (29.6 - 45.8)	146	24	16.4 (10.8 - 23.5)
Local	265	96	36.2 (30.4 - 42.3)	263	54	20.5 (15.8 - 25.9)
Unknown	11	5	45.5 (16.8 - 76.6)	11	1	9.1 (0.2 - 41.3)
Tick infestation						
Absent	273	101	37 (31.3 - 43)	271	54	19.9 (15.3 - 25.2)
Present	121	46	38 (29.4 - 47.3)	120	22	18.3 (11.9 - 26.4)
Unknown	29	9	31 (15.3 - 50.8)	29	3	10.3 (2.2 - 27.4)
Flea infestation						
Absent	175	69	39.4 (32.1 - 47.1)	172	32	18.6 (13.1 - 25.2)
Present	219	78	35.6 (29.3 - 42.4)	219	44	20.1 (15 - 26)
Unknown	29	9	31 (15.3 - 50.8)	29	3	10.3 (2.2 - 27.4)
Louse infestation						
Absent	391	144	36.8 (32 - 41.8)	388	74	19.1 (15.3 - 23.3)
Present	5	4	80 (28.4 - 99.5)	5	3	60 (14.7 - 94.7)
Unknown	27	8	29.6 (13.8 - 50.2)	27	2	7.4 (0. 9 - 24.3)
Geoclimatic zone						
Up-mid country wet zone	307	98	31.9 (26.7 - 37.5)	305	58	19 (14.7 - 23.8)
Low country wet zone	75	46	61.3 (49.4 - 72.4)	74	16	21.6 (12.9 - 32.7)
Low country dry zone	41	12	29.3 (16.1 - 45.5)	41	5	12.2 (4.1 - 26.2)
Treatment against ectoparasites						
Given	189	72	38.1 (31.1 - 45.4)	188	36	19.2 (13.8 - 25.5)
Not given	145	51	35.2 (27.4 - 43.5)	144	32	22.2 (15.7 - 29.9)
Unknown	89	33	37.1 (27.1 - 48)	88	11	12.5 (6.4 - 21.3)
Deworming treatment						
Not given	71	28	39.4 (28 - 51.8)	71	19	26.8 (16.9 - 38.6)
Given	265	97	36.6 (30.8 - 42.7)	262	47	17.9 (13.5 - 23.1)
Unknown	87	31	35.6 (25.7 - 46.6)	87	13	14.9 (8.2 - 24.2)

demonstrated a posterior probability of 0.97 and with NJ analysis a bootstrap support of 96%.

Figs. 6 and Supplementary File B show the phylogenetic relationships attained using Bayesian inference and NJ methods for the genus *Brugia* utilising the *cox*-1 and 12S rRNA genes, respectively. For the genus *Brugia*, the Bayesian phylogenetic relationships distinguished five separate clades with four clades with a high degree of support (posterior probability 0.98–1). The remaining clade contained sequences from this study and Tamil Nadu (India) *B. malayi* (GenBank accession **MN564741**) which had marginal support (posterior probability = 0.94). However, this clade was well distinguished from other clades of the tree. A similar result was obtained using NJ analysis, with 99% bootstrap support for *Brugia* sp. Sri Lanka.

Univariable analyses identified that residing in the in the low-country wet zone, older age, male sex, and louse infestation were associated with filarial infections ($p \leq 0.2$) (Table 5). Association of filarial infections with clinical signs in dogs are summarised in Supplementary File B. None of the variables were highly correlated (Supplementary File B). The final multivariable model is shown in Table 6. On average across all clinics, increasing age (OR 1.1 95% CI 1.1–1.2, $p < 0.001$), male dogs (OR 1.5 95% CI 1–2.3, $p = 0.077$), and dogs residing in the low-country wet zone (OR 4.2 95% CI 2.4–7.3, $p < 0.001$) had higher odds of filarial infection.

4. Discussion

In this study we report data detailing the first comprehensive epidemiological investigation on filarioids of pet dogs in Sri Lanka

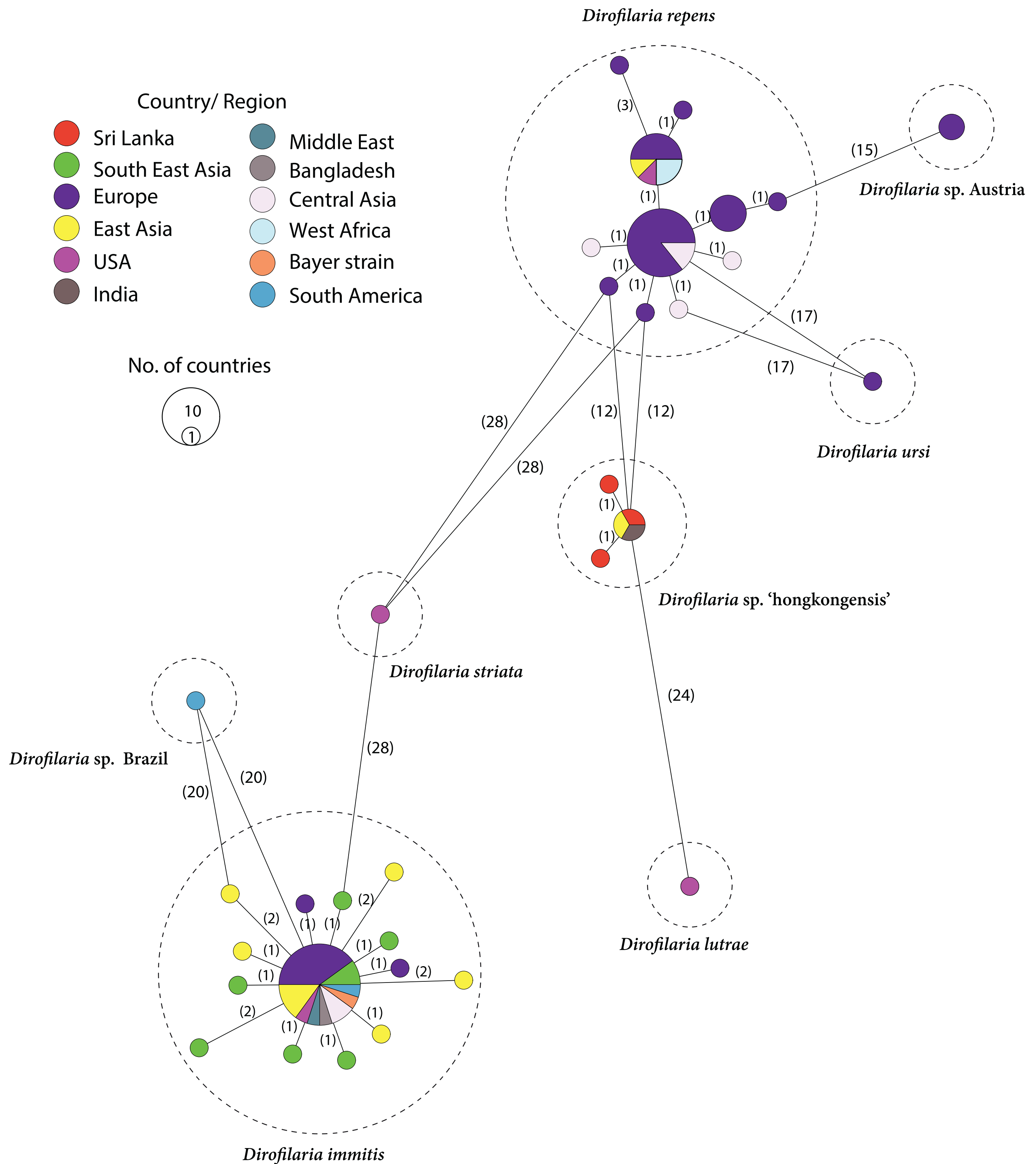

Fig. 3. Available *Dirofilaria* sequence types (STs) in the GenBank database and their geographical distribution indicating intra and inter-specific mutations/genetic diversity across a 333 bp region of the mitochondrial *cox*-1 gene inferred according to the minimum spanning networks (at epsilon = 0). The number of nucleotide differences between each ST is indicated in parentheses.

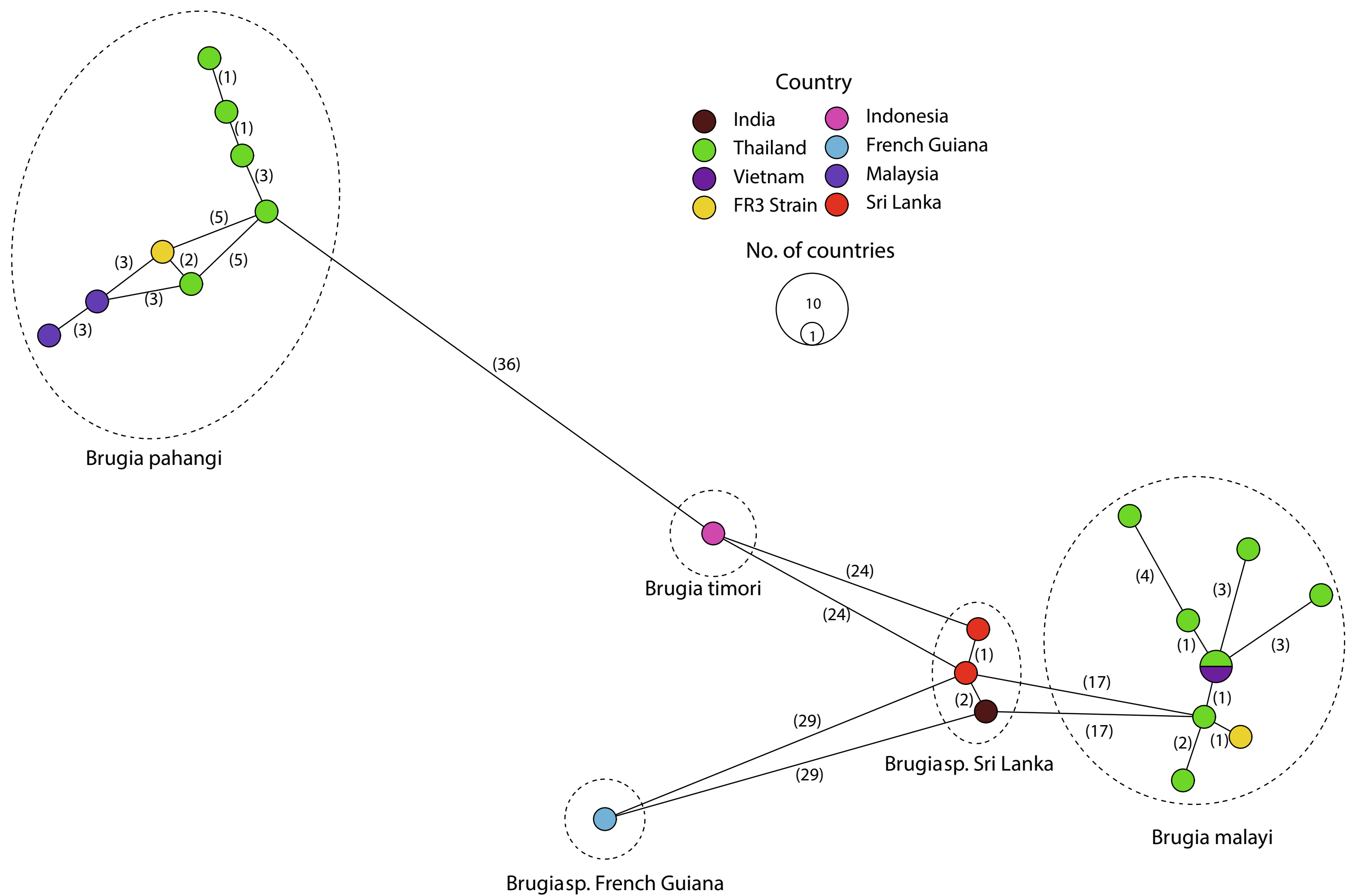

Fig. 4. Available *Brugia* sequence types (STs) in the GenBank database and their geographical distribution indicating intra and inter-specific mutations/genetic diversity across a 425 bp region of the mitochondrial *cox*-1 gene inferred according to the minimum spanning networks (at epsilon = 0). The number of nucleotide differences between each ST is indicated in parentheses.

through parasitological, molecular, phylogenetic, and statistical techniques. Molecular methods were fundamental in demonstrating the high endemicity of the zoonotic *Dirofilaria* sp.' hongkongensis' and *Brugia* sp. Sri Lanka genotype. Over one-third of pet dogs in this study were infected with *Dirofilaria* sp. 'hongkongensis' accounting for >80% of the total infections, placing this parasite as the main filarioid species infecting dogs in the country.

The current understanding of the epidemiology of filarial infections in Sri Lanka is hindered by a lack of information on parasite species identification and definitive host association with mammals. Previous investigations have reported the occurrence of *D. immitis* [64], *A. reconditum*, *B. ceylonensis* [23], *B. malayi*, and *D. repens* [19,21,22] from dogs in Sri Lanka. While the single case of *D. immitis* was found in an imported dog [64], all the other filarioids are considered as endemic to the country, despite *A. reconditum* having not been reported since 1962, when it was first described in Sri Lanka [23].

Notably, our study provides molecular characterisation of two filarial species that affect pet dogs in Sri Lanka. We identify *D. repens* in Sri Lanka as belonging to *Dirofilaria* sp. 'hongkongensis' and uncover a distinct *Brugia* genotype closely related to *B. malayi*, separate from all those reported elsewhere, with the exception of a single case in South India [65]. Human subcutaneous dirofilariasis has been widespread throughout the country since its first report in humans in 1962 [66] with Sri Lanka recording the second highest number of cases in the world [67]. The total caseload recorded in Sri Lanka is likely an underestimation of the actual infection rate, as all cases reported are done so through passive surveillance. While subcutaneous dirofilariasis in humans is primarily caused by *D. repens* in Europe and Central Asia, including Russia [68–76], recent evidence suggests that *Dirofilaria* sp. 'hongkongensis' may actually be responsible for the majority of human subcutaneous *Dirofilaria* infections in some parts of Asia [3,77–79]. The high prevalence of this zoonotic pathogen herein reported in the domestic canine population, together with the abundance of mosquito vectors, increases the risk of human infections in the country.

Based on our findings, it is highly probable that the *Brugia* species herein reported is *B. ceylonensis*, which has previously been isolated from dogs in Sri Lanka [23]. Nevertheless, the absence of genetic data from the original morphological description of this parasite and the lack of adult parasites in our study prevents definitive conclusions. These findings are particularly noteworthy given that recent studies have revealed an increased number of human infections with *B. malayi* in Sri Lanka [22,23,31].

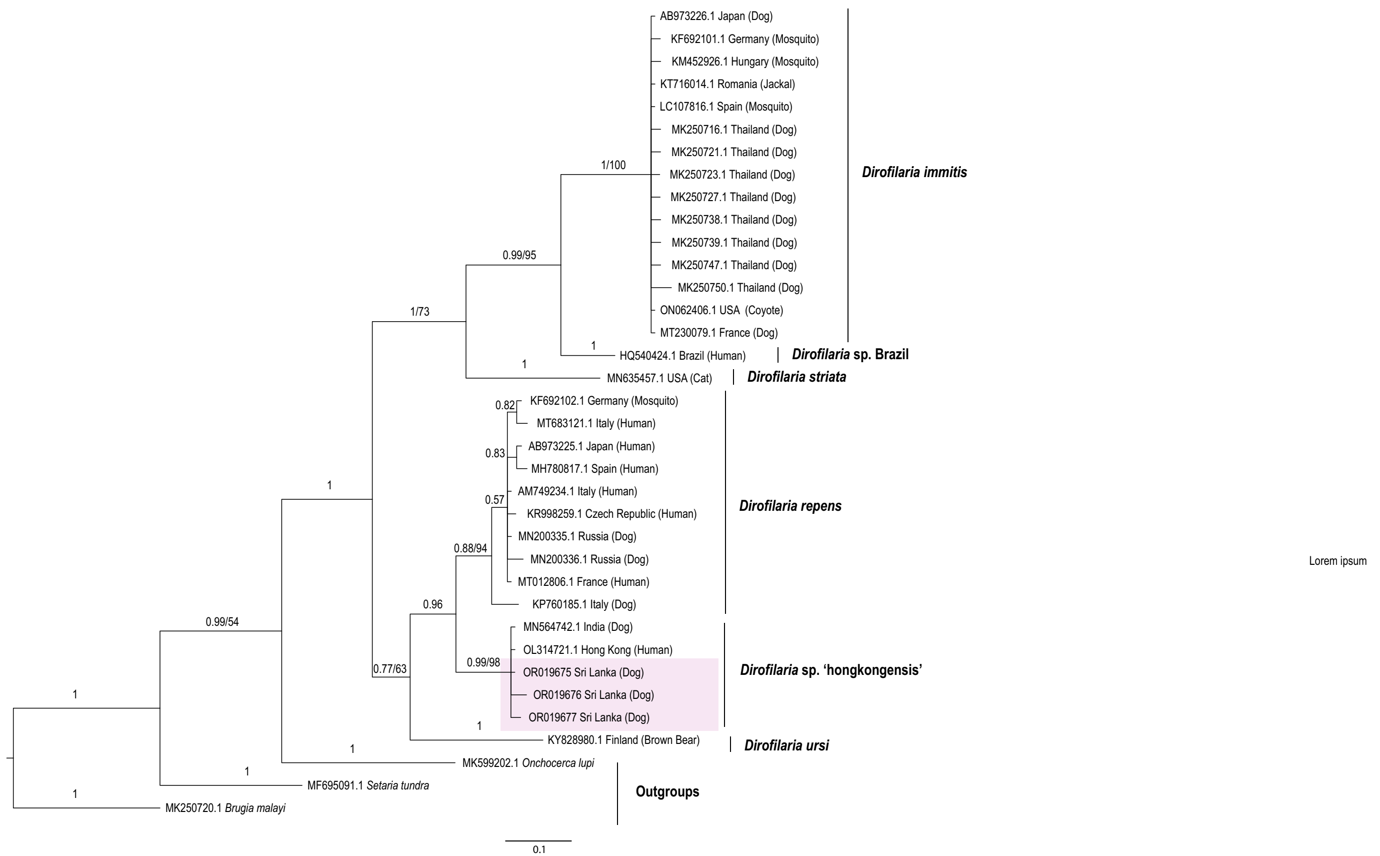

Fig. 5. Bayesian and neighbour-joining phylogenetic inference for a 333 bp segment of the mitochondrial *cox*-1 gene of various *Dirofilaria* species. The posterior probability values and bootstrap support (where available) for tree branches are indicated, and the highlighted region indicates the sequences generated in this study.

In Sri Lanka, *B. malayi* was reported as having been eliminated around 1967 [80] as a result of reducing breeding locations alongside concurrent control of malaria vectors with the use of the insecticide dichlorodiphenyltrichloroethane [27,80]. The surveillance studies in the late 1980s [28] further confirmed the absence of *B. malayi,* as no *Brugia* microfilariae were found after examining over 50,000 blood smears from humans. Nevertheless, since the year 2000, sporadic cases of *Brugia* infection in humans have been reported in Sri Lanka [81]. Even though the infections were attributed to *B. malayi*, a clear taxonomic description of this re-emergent *Brugia* was not possible [24,29,31,82]. The nucleotide identity of the 5S ribosomal RNA intergenic spacer and internal-transcribed spacer −2 (ITS-2) genetic regions of *Brugia* microfilariae isolated from seven human individuals from Sri Lanka had only 96% and 97% identity to *B. malayi* [82]. The *Brugia* sp. Sri Lanka genotype identified in this study demonstrated significant phylogenetic differences to the *B. malayi* clade at both the *cox*-1 and 12S rRNA genes. Interestingly, the *B. malayi* identified from Tamil Nadu, India [65] has a higher nucleotide identity with the *Brugia* sp. Sri Lanka of this study, than the Southeast Asian *B. malayi*, suggesting this genotype might be limited to the Indian subcontinent. Additionally, the re-emergent *Brugia* sp. in Sri Lanka, is nocturnally sub-periodic [31] in contrast to the nocturnally periodic *B. malayi* infections identified in the past. Previous cases of sub-periodic *B. malayi* displayed a broad host range spanning domestic animals (especially dogs and cats) as well as wild species, such as the dusky leaf-monkey (*Trachypithecus obscurus*), pangolin (*Manis javanica*) and Asian palm civet (*Paradoxurus hermaphroditus*) [32]. These factors are highly suggestive of a zoonotic origin for this re-emergent *Brugia* species.

It is noteworthy that >90% of the documented *Brugia* cases in humans were reported from the low country wet zone, where we identified a higher prevalence of canine *Brugia* infection. Consequently, these data strongly suggest that the resurgence of this *Brugia* species is of animal origin and could potentially be attributed to *B. ceylonensis*, as hypothesized in earlier investigations [21,29]. However, the lack of *cox*-1 and 12S rRNA genes sequences from *Brugia* in Sri Lanka in public repositories has prevented the comparison of our sequences with previous records of human *Brugia* infection in the country.

Multivariable analysis identified dogs living in the low-country wet zone to be at a higher likelihood of filarial infection compared to other regions of the country. Nevertheless, this association with the geo-climatic zone might not be due to geographical and climatic factors alone, as this region is home to nearly half of the Sri Lankan human population [83]. A high human population density in this area has contributed to dense housing, accumulation of garbage and suboptimal drainage systems, increasing the availability of micro-breeding habitats (e.g., roof gutters, coconut shells, discarded tyres etc.) for vector mosquitoes compared to less densely populated regions [84]. High human population density and availability of food waste can also

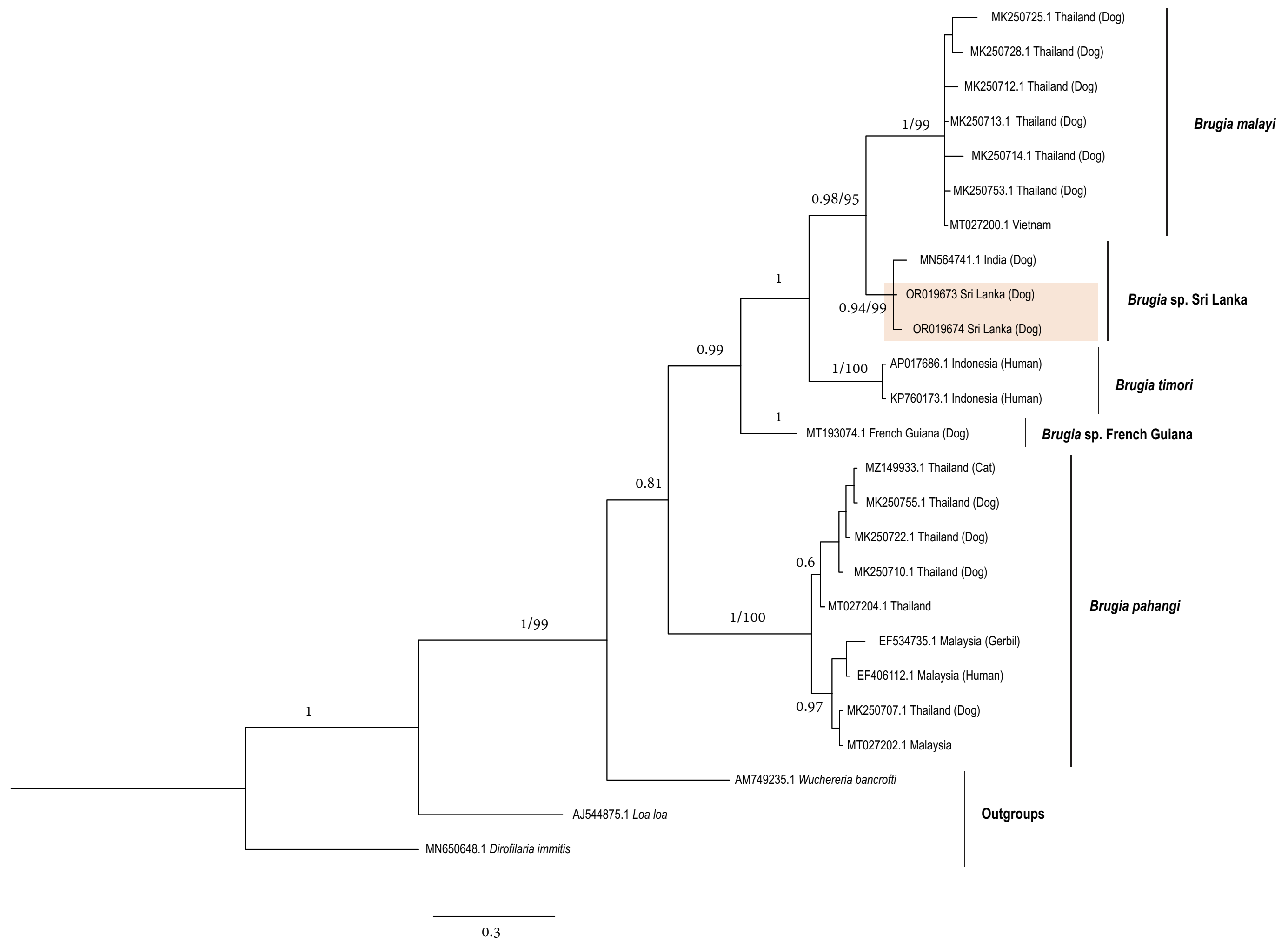

Fig. 6. Bayesian and neighbour-joining phylogenetic inference for a 425 bp segment of the *cox*-1 gene of various *Brugia* species. Posterior probability values and bootstrap support (where available) for tree branches are indicated, and the highlighted region indicates the sequences generated in this study.

contribute to an increase in the number of stray dogs that can act as reservoirs for these pathogens. However, in a previous large-scale study in Asia, human and animal population density and human/pet ratio were not associated with an increased exposure of dogs and cats to zoonotic parasites, including filarial nematodes [85]. The impact of water on the spread of filarial worm infections in dogs has been well documented [27]. For instance, the breeding of *Mansonia* spp. mosquitoes that vector *Brugia* (including *B. ceylonensis*) in Sri Lanka are known to prefer stagnant water bodies with aquatic vegetation [27]. As a result, the prevalence of *Brugia* infections is generally lower in up-mid country regions with hilly terrain and in low-country dry zones with arid climates where such stagnant water bodies are less common. Nevertheless, data on the abundance of such micro-habitats for mosquito breeding across the island is unavailable; thus, it remains unclear as to what extent such factors contribute to filarial prevalence in Sri Lanka.

Increasing age was a significant predictor of infection which can be attributed to a prolonged exposure to vectors in comparison with younger animals, causing a larger proportion of older dogs to be infected. In addition, male dogs were found to have higher odds of filarial infection potentially due to their territorial nature, with a concurrent increase in their exposure time outdoors and thereby to mosquitoes. In addition, higher blood concentrations of androgenic hormones in males can contribute to poorer immunity [86,87], which may increase their susceptibility to parasitic infections [88] compared to females. Interestingly, similar multivariable associations with age and sex with filarial infections were shown in Cringoli et al. (2001) [89] for dogs in Italy.

Except for pruritis, pale mucous membrane, and hepato- or splenomegaly, our study did not identify associations between clinical signs and filarial infections. Similarly, clinical manifestations of subcutaneous dirofilariasis by *D. repens* in dogs are known to be unremarkable or to manifest as cutaneous lesions [13], and clinicopathological manifestations of *Dirofilaria* sp. 'hongkongensis' in dogs have not been documented to date. The clinical presentation of *Brugia* spp. in dogs is also poorly defined. Nevertheless, Snowden and Hammerberg (1989) [14] observed clinicopathological manifestations with *B. pahangi* infection to be associated with the peripheral lymphatic system, such as transient lymphadenomegaly, limb oedema, and lymphatic fibrosis. The lack of a direct association between clinical signs and filarial worm infections

Table 5
Univariable analysis of associations between filarial infections, geo-climatic zone, host factors, deworming and ectoparasiticide treatment in dogs in Sri Lanka. (CI = confidence interval, SE = standard error).

Variable	Category	Estimate ± SE	Odds ratio (95% CI)	*P*-value
Geo-climatic zone	Up-mid-country wet zone		Reference	
	Low-country wet zone	1.2 ± 0.27	3.3 (2–5.7)	<0.001
	Low-country dry zone	−0.14 ± 0.36	0.9 (0.4–1.7)	0.701
Age (years)		0.11 ± 0.03	1.1 (1.1–1.2)	<0.001
Breed	Exotic		Reference	
	Local	−0.04 ± 0.21	1 (0.6–1.5)	0.87
Sex	Female		Reference	
	Male	0.47 ± 0.21	1.6 (1.1–2.4)	0.024
Neuter status	Intact		Reference	
	Neutered	−0.04 ± 0.26	1 (0.6–1.6)	0.876
Tick infestation	Absent		Reference	
	Present	0 03 ± 0.23	1 (0.7–1.6)	0.902
Flea infestation	Absent		Reference	
	Present	−0.19 ± 0.21	0.8 (0.6–1.3)	0.372
Louse infestation	Absent		Reference	
	Present	1.91 ± 1.12	6.8 (1–133.4)	0.088
Use of Ectoparasite treatment	Not given		Reference	
	Given	0.15 ± 0.23	1.2 (0.7–1.8)	0.518
Use of deworming treatment	Not given		Reference	
	Given	−0.1 ± 0.27	0.9 (0.5–1.6)	0.704

Table 6
Odds ratios and coefficient estimates of the generalized linear mixed model on filarial infections in Sri Lankan pet dogs (CI = confidence interval, SE = standard error).

Variable	Estimate ± SE	Odds Ratio (95% CI)	P-value
(Intercept)	−1.62 (± 0.24)		
Age (years)	0.13 (± 0.03)	1.1 (1.1–1.2)	**<0.001**
Sex			**0.077**
Female		Reference	
Male	0.4 (± 0.22)	1.5 (1–2.3)	
Geo-climatic zone			**<0.001**
Up-mid-country wet zone		Reference	
Low-country wet zone	1.42 (± 0.29)	4.2 (2.4–7.4)	
Low-country dry zone	−0.02 (± 0.4)	1 (0.4–2.1)	

highlighted in this study, suggests that dogs act as suitable reservoir hosts for these pathogens.

Due to COVID-19 travel restrictions experienced during the sampling period we were able to obtain more samples from the up-mid country wet zone, and this might have introduced a sampling bias. However, the target sample size across all three geoclimatic zones was exceeded by 24 samples and sampling was performed from provinces where nearly 65% of the population of Sri Lanka lives. In addition, we were not able to detect pre-patent and mono-sex infections using our detection methods, as in these contexts microfilaremia is not present. Therefore, it is likely that the overall prevalence of filarial infection in Sri Lankan pet dogs is higher than that herein reported.

Future work could build upon the epidemiological data accrued in the present study by employing novel next-generation sequencing diagnostic approaches that demonstrate an improved ability to characterise vector-borne pathogen coinfections in humans, animals, and arthropod vectors [90–92].

5. Conclusions

In this study, we provide the first comprehensive epidemiological data on filarioids infecting pet dogs in Sri Lanka, utilising a combination of parasitological, molecular, phylogenetic, and statistical techniques. With this approach we were able to report a high prevalence of zoonotic filarial infections in dogs and identify the causative agents as *Dirofilaria* sp. 'hongkongensis' and *Brugia* sp. Sri Lanka genotype. Considering the high prevalence of these parasites in dogs and the (re)-emergence of filarial infections in humans, it is crucial to promptly identify the species of filarial parasites causing infections in animals and humans, to thereby elucidate reservoirs of infection and develop appropriate prevention strategies. A synergistic effort from both veterinary and public health authorities is therefore recommended to enact effective control of zoonotic filarial infections in dogs and humans in Sri Lanka.

Author agreement

All co-authors participated in this study and agreed with the submission and subsequent revisions submitted by the corresponding author.

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CRediT authorship contribution statement

Ushani Atapattu: Conceptualization, Methodology, Formal analysis, Investigation, Resources, Data curation, Writing – original draft. **Anson V. Koehler:** Formal analysis, Writing – review & editing. **Lucas G. Huggins:** Supervision, Writing – review & editing. **Anke Wiethoelter:** Methodology, Formal analysis, Supervision, Writing – review & editing. **Rebecca J. Traub:** Conceptualization, Supervision, Writing – review & editing. **Vito Colella:** Conceptualization, Methodology, Formal analysis, Supervision, Writing – review & editing.

Declaration of Competing Interest

None.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.onehlt.2023.100625.

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