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A cross-sectional study on phlebotomine sand flies in relation to disease transmission in the Republic of Kosovo

Betim Xhekaj[1] | **Ina Hoxha**[2] | **Katharina Platzgummer**[2] | **Jovana Stefanovska**[3] | **Vít Dvořák**[4] | **Markus Milchram**[5] | **Adelheid G. Obwaller**[6] | **Wolfgang Poeppl**[7] | **Nesade Muja-Bajraktari**[8] | **Julia Walochnik**[2] | **Attila J. Trájer**[9] | **Kurtesh Sherifi**[1] | **Aleksandar Cvetkovikj**[3] | **Edwin Kniha**[2]

[1]Faculty of Agriculture and Veterinary, University of Prishtina "Hasan Prishtina", Prishtina, Kosovo

[2]Institute of Specific Prophylaxis and Tropical Medicine, Center for Pathophysiology, Infectiology and Immunology, Medical University Vienna, Vienna, Austria

[3]Department of Parasitology and Parasitic Diseases, Faculty of Veterinary Medicine-Skopje, Ss. Cyril and Methodius University in Skopje, Skopje, North Macedonia

[4]Department of Parasitology, Faculty of Science, Charles University Prague, Prague, Czech Republic

[5]Institute of Zoology, Department of Integrative Biology and Biodiversity Research, University of Natural Resources and Life Sciences, Vienna, Austria

[6]Division of Science, Research and Development, Federal Ministry of Defence, Vienna, Austria

[7]Department of Dermatology and Tropical Medicine, Military Medical Cluster East, Austrian Armed Forces, Vienna, Austria

[8]Faculty of Mathematics and Natural Sciences, University of Prishtina "Hasan Prishtina", Prishtina, Kosovo

[9]University of Pannonia, Sustainability Solutions Research Lab, Veszprém, Hungary

Correspondence
Edwin Kniha, Institute of Specific Prophylaxis and Tropical Medicine, Center for Pathophysiology, Infectiology and Immunology, Medical University Vienna, Vienna, Austria.
Email: edwin.kniha@meduniwien.ac.at

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Abstract

Sand flies (Diptera: Psychodidae: Phlebotominae) are blood-feeding insects that transmit the protozoan parasites *Leishmania* spp. and various arboviruses. The Balkan region, including the Republic of Kosovo, harbours a diverse sand fly fauna. Vector species of *Leishmania infantum* as well as phleboviruses are endemic; however, recent data are scarce. We performed a cross-sectional study to update the current sand fly distribution in Kosovo and assess biological as well as environmental factors associated with sand fly presence. CDC light trapping was conducted at 46 locations in 2022 and 2023, specifically targeting understudied regions in Kosovo. Individual morphological species identification was supported by molecular barcoding. The occurrence data of sand flies was used to create distribution maps and perform environmental analyses, taking elevation, wind speed and climate-related factors into account. In addition, PCR-based blood meal analysis and pathogen screening were conducted. Overall, 303 specimens of six sand fly species were trapped, predominated by *Phlebotomus neglectus* (97%). Barcodes from eight of nine known endemic sand fly species were obtained. Combining our data with previous surveys, we mapped the currently known sand fly distribution based on more than 4000 specimens at 177 data points, identifying *Ph. neglectus* and *Ph. perfiliewi* as the predominant species. Environmental analyses depicted two geographical groups of

Betim Xhekaj and Ina Hoxha contributed equally to this study.

sand flies in Kosovo, with notable differences between the species. In total, 223 blood meals of five sand fly species were analysed. Of seven identified host species, the predominant blood meal source was observed to be cattle, but the DNA of dogs and humans, among others, was also detected. This study assessed biological as well as ecological factors of sand fly occurrence, which should help better understand and evaluate potential hot spots of disease transmission in Kosovo.

KEYWORDS
Balkan, blood meal, climate, DNA barcoding, *Leishmania*, *Phlebotomus*

INTRODUCTION

Phlebotomine sand flies (Diptera: Psychodidae: Phlebotominae) are small hematophagous insects of high medical and veterinary importance. Of more than 1050 valid described species, 100 species of the genera *Phlebotomus* and *Lutzomyia* are suspected or proven vectors of *Leishmania* spp., the causative agent of leishmaniasis, and many species are also involved in the transmission of various arboviruses in the Old and New World (Galati & Rodrigues, 2023). Leishmaniasis is one of the most important neglected tropical diseases. It is endemic in more than 98 countries and affects approximately 12 million people globally, with annual incidence over 58,000 for visceral leishmaniasis (VL) and more than 220,000 for cutaneous leishmaniasis (CL) (WHO, 2021). In Europe, the disease is endemic in all Mediterranean countries, including those on the Balkan Peninsula (Maia et al., 2023). In addition, increasing attention has recently been given to sand fly-borne viruses, most notably the phleboviruses, and vesiculoviruses, which are also of medical importance as they can cause disease in infected animal and human hosts (Ayhan & Charrel, 2019; Jancarova et al., 2023).

It has been known for decades that *Leishmania* and phleboviruses are endemic in the Balkan countries. However, recent studies have suggested a much higher prevalence and diversity than previously reported (Ayhan & Charrel, 2018; Vaselek, 2021a, 2021b). Moreover, the Balkan region harbours a diverse sand fly fauna that has been recently revised by extensive sampling efforts of the VborNet and VectorNet projects after several decades of neglect (Dvorak et al., 2020).

In the Republic of Kosovo, a landlocked country bordered by Albania, Montenegro, Serbia and North Macedonia, nine sand fly species of two genera have been reported recently (Dvorak et al., 2020; Vaselek et al., 2020). There is growing evidence that some of these species are involved in the local circulation of sand fly-borne pathogens. Serological evidence of phlebovirus infections have been observed in cattle, sheep and humans (Ayhan et al., 2017; Kniha et al., 2019; Venturi et al., 2011). Also, *L. infantum* presence in dogs has been reported recently (Xhekaj et al., 2020; Xhekaj, Hoxha, et al., 2023; Xhekaj, Stefanovska, et al., 2023), and *L. tropica* and *L. infantum* DNA have been detected in sand flies (Vaselek et al., 2020; Xhekaj, Hoxha, et al., 2023). Considering the wide distribution of the two important vector species, *Phlebotomus neglectus* and *Phlebotomus perfiliewi*, in all seven regions of Kosovo, the circulation of phleboviruses and *Leishmania* spp. is highly likely (Vaselek et al., 2020; Xhekaj, Hoxha, et al., 2023).

Although a few studies have been performed recently in Kosovo, biological and ecological factors that might shape sand fly distribution were not considered. In the present study, we attempted to fill these gaps by assessing the current sand fly distribution and diversity, supported by a DNA barcode inventory, performing blood meal analysis to assess host usage and identifying environmental factors associated with sand fly presence in Kosovo.

MATERIALS AND METHODS

Study area

The land area of the Republic of Kosovo is 10,910 km^2, with mountains in the eastern part at the border to Montenegro and in the southern parts at the borders to Albania and North Macedonia. The elevation varies from the lowest of point 297 metres (m) above mean sea level (AMSL) (in White Drin Valley near the border with Albania) to the highest point of 2656 m (Gjeravica). The country is divided into seven districts (Figure 1). The climate is considered moderate continental, and is influenced by the hot air masses that cross the Adriatic Sea into western Kosovo. Animal farming is common throughout the country and dogs are widely present, either feral, kept in private households as pets or as shepherd dogs.

Sand fly trapping

Entomological surveys were conducted from June 30 to September 17, 2022, and from July 5 to August 16, 2023. A total of 46 different locations were surveyed, each for one night; four locations sampled in September 2022 (end of sand fly season) were re-sampled in July 2023. All other locations were sampled only once (Supplementary Table 1). Sampling was done with up to six CDC miniature light traps (John W. Hock Company, Gainesville, FL, USA) per location, resulting in 106 trap nights. The number of placed light traps correlated with the availability of different animal hosts/buildings at the respective location. When possible, traps were set both outdoors and indoors at a given location (Supplementary Table 2). We trapped at 19 sites in Prishtina, 1 in Mitrovica, 14 in Peja, 9 in Prizreni and 3 in Gjakova district (Figure 1). Sampling locations were chosen based on previous sand fly surveys (Dvorak et al., 2020; Vaselek et al., 2020; Xhekaj,

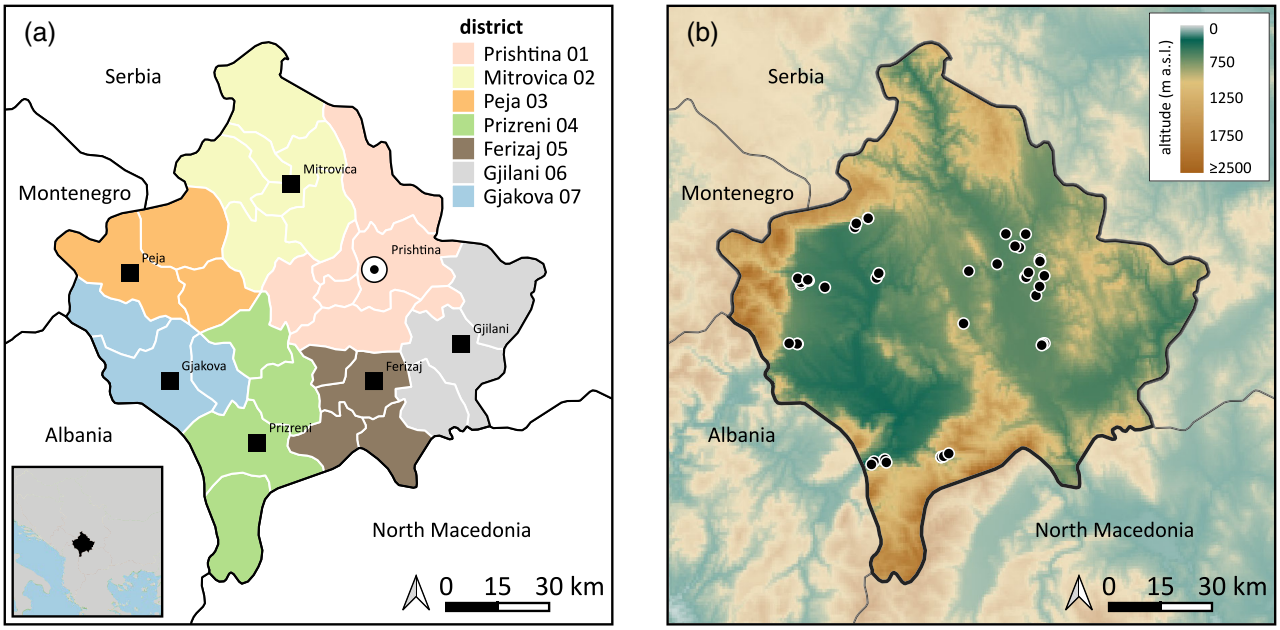

FIGURE 1 Map of the Republic of Kosovo. Seven districts of Kosovo with the capital Prishtina and major cities (a). The geographic position of Kosovo (black) in the Balkan region is given in the lower left corner. Elevation map of Kosovo including all trappings sites of this study (b).

Hoxha, et al., 2023), particularly selecting peri-urban and urban locations in understudied regions, which represented 31 private households with animals as well as five cattle farms, three mixed animal farms, three dog shelters, two chicken farms and two (sylvatic) caves. Traps were given a unique code and specific information was recorded for each trap: precise geographic coordinates (to 3 decimal degrees), habitat type (peri-urban/urban), location of trap (indoors/outdoors) (Supplementary Table 2) and presence of potential hosts in the immediate vicinity (Supplementary Table 3). Traps were set around 1 m above the ground at 1–2 h before sunset (6–8 pm) and collected around sunrise (7–9 am). After collection, the nets were placed immediately in dry ice, transported to the laboratory under cool conditions and stored at −80°C until dissection.

Morphological identification of sand flies

The head and terminal segments of the abdomen of captured specimens were dissected and slide-mounted in CMCP-10 mounting medium (Polysciences, Inc., Warrington, PA, USA). Identification was based on male genitalia, female spermatheca and pharyngeal armature using published keys by Lewis (1982) and Dantas-Torres et al. (2014). The remaining body parts, as well as engorged specimens, were placed in 1.5-mL tubes for molecular analyses.

Nucleic acid isolation

All specimens were individually homogenised in 500 µL Dulbecco's modified Eagle medium (DMEM) supplemented with 20% bovine serum albumin, 1% penicillin/streptomycin, 10 µg/mL gentamicin and 0.25 µg/mL amphotericin B (all from Gibco, Thermo Fisher Scientific). Two metal beads (3 mm diameter) were added to each 2.0-mL tube and specimens were homogenised with a TissueLyser bead mill (QIAGEN GmbH, Hilden, Germany) for 1 min of shaking at 30 Hz. The homogenate was cleared by centrifugation in a 4°C benchtop centrifuge for 5 min at 14,000 rpm. For nucleic acid extraction, homogenates of individual sand flies were pooled by location, sex and species using 20–100 µL of homogenate per pool. A QIAmp® RNeasy mini kit 250 (Qiagen, Hilden, Germany) was used following the manufacturer's protocol, with a final elution in 50 µL. The remaining supernatants were stored at −80°C for prospective RNA-based screenings.

Barcoding and haplotyping

To confirm the morphological identification of sand fly specimens, a barcoding PCR targeting a 658 bp fragment of the cytochrome c oxidase subunit I (COI) gene was performed using the primers LCO1490/HCO2198 following the protocol of Folmer et al. (1994).

To generate barcodes of all sand fly species recently found in Kosovo, we included unsequenced specimens from a previous study by Xhekaj, Hoxha, et al. (2023).

All PCRs were performed using a 2× EmeraldAmp® GT PCR Master Mix (Takara Bio Europe AB, Göteborg, Sweden), as described in Xhekaj, Hoxha, et al. (2023). The samples were sent to Microsynth (Microsynth Austria GmbH, Vienna, Austria) for Sanger sequencing, and the obtained sequences from both strands were aligned with ClustalX 2.1 (Larkin et al., 2007) and edited with GeneDoc 2.7.0 (Nicholas, 1997). The consensus sequences were submitted to the US National Center for Biotechnology Information (NCBI) GenBank sequence database, and compared with reference sequences in the

database using the nucleotide basic local alignment search tool (BLAST), specifically recording the minimum and maximum percent identity for the top 100 BLAST results when query coverage was >86%.

Blood meal analysis

For blood meal analysis, we included specimens from this study and unpublished data collected in the survey by Xhekaj, Hoxha, et al. (2023). Three PCRs were applied for blood meal analysis. First, a PCR amplifying a fragment of the 16S rRNA using the primers L2513/H2714 was applied (Table 1). Samples producing no amplicon were subjected to two more PCRs, namely one targeting the prepronociceptin (PNOC) gene with the primers PNOCF/PNOCR and an avian-specific PCR targeting the cytochrome b (Cytb) gene using the primers L15330AV (L0)/H15551AV (H1) (Table 1). Obtained sequences were compared with reference sequences, as described above.

Leishmania DNA screening

The extracts of all individual and pooled females were screened for the presence of *Leishmania* DNA. A sensitive nested-PCR protocol using the primer combinations R221 (5′-GGTTCCTTTCCTGATTTACG-3′)/R332 (5′-GGCCGGTAAAGGCCGAATAG-3′) (van Eys et al., 1992) for the first round and R223 (5′-TCCCATCGCAACCTCGGTT-3′)/R333 (5′-AAAGCGGGCGCGGTGCTG-3′) (Cruz et al., 2002) for the second round was applied. Cycling conditions were as follows: First round: 94°C/5 min; 15 cycles: 94°C/30 s, 53°C/30 s, 72°C/30 s; 72°C/10 min; second round: 94°C/5 min; 32 cycles: 94°C/30 s, 65°C/30 s, 72°C/30 s; 72°C/10 min.

Sand fly mapping and distribution

Sand fly occurrence and diversity indices based on three surveys conducted by CDC light trapping (this study, Vaselek et al., 2020; Xhekaj, Hoxha, et al., 2023) were plotted into maps using Quantum GIS 3.4.11 (QGIS Development Team, 2019). Map data on country, district and municipality borders were taken from Natural Earth (naturalearthdata.com). Elevation data were taken from www.worldclim.org (Fick & Hijmans, 2017).

Environmental factors and their sources

For further analysis, elevation, wind speed and climate-related factors were incorporated into the study. Elevation and wind speed data at the 10 m level were retrieved from the Global Wind Atlas dataset (Davis et al., 2023). Climate data were obtained from the WorldClim dataset, using values from WorldClim version 2.1 for the period 1970–2000 (Fick & Hijmans, 2017). The spatial patterns of environmental factors were displayed in QGIS3.36.0 using Grass-Gis 8.3.1.

Statistical analyses

Due to the small number of sand flies captured in the study, we refrained from statistical analyses comparing sand fly abundance, which is only presented as percentages. Elevation data are presented as mean and standard deviation (SD).

RESULTS

Sand fly trapping and identification

Altogether, 46 locations were surveyed, of which 24 (52.2%) were positive for sand flies (Figure 1). In total, 303 sand flies were caught, of which 117 (38.6%) were males and 186 (61.4%) were females, including 65 engorged specimens. Morphological identification and barcoding revealed six different sand fly species of the genera *Phlebotomus* and *Sergentomyia*. The majority of caught sand flies were *Ph. neglectus* (295, 97.4%), followed by *Ph. tobbi* (4, 1.3%), and only a

TABLE 1 Applied PCR protocols for blood meal analysis.

Target	Primers	Fragment	PCR conditions	Reference
16S rDNA	L2513 (5′-GCCTGTTTACCAAAAACATCAC-3′) H2714 (5′-CTCCATAGGGTCTTCTCGTCTT-3′)	244 bp	94°C/5 min; 35 cycles: 94°C/30 s, 53°C/15 s, 72°C/30 sec; 72°C 10 min	Kitano et al. (2007)
PNOC	PNOCF (5′-GCATCCTTGAGTGTGAAGAGAA-3′) PNOCR (5′-TGCCTCATAAACTCACTGAACC-3′)	330 bp	94°C/5 min; 35 cycles: 95°C/30 s, 57°C/15 s, 72°C/30 sec; 72°C 10 min	Haouas et al. (2007)
Cytb (Avian specific)	L15330AV (5′-GGACAAATATCATTCTGAGG-3′) H15551AV (5′-GGGTGGAATGGGATTTTGTC-3′)	220 bp	94°C/5 min; 35 cycles: 94°C/30 s, 60°C/45 sec, 72°C/1 min; 72°C 10 min	Lee et al. (2008)

single specimen of each of the other species, namely *Ph. perfiliewi*, *Ph. balcanicus*, *Ph. mascittii* and *Se. minuta* (1, 0.3%) (Table 2).

Barcoding

A COI barcode inventory of sand fly species caught in this study and in a previous study by Xhekaj, Hoxha, et al. (2023) was generated. In total, 101 COI sequences of eight species were obtained, with the number varying by captured specimens per district (Table 3). All barcodes showed high BLAST identity with reference sequences, which confirmed the identities of the respective species (Table 3). Only *Ph. alexandri* could not be barcoded as no samples were available from this and the previous study (Xhekaj, Hoxha, et al., 2023).

Sand fly distribution and diversity

For mapping the currently known sand fly distribution, we combined datasets of this study and extracted data from Vaselek et al. (2020) and Xhekaj, Hoxha, et al. (2023). In total, we used 177 data points, of which 118 (66.7%) were positive for sand flies. Based on all three analysed surveys, *Ph. neglectus* showed the widest distribution, present at 102 locations, followed by *Ph. perfiliewi*, found at 38 locations in all seven districts. *Phlebotomus tobbi* and *Ph. simici* were recorded at 12 and 7 locations, respectively, in four different districts. *Sergentomyia minuta* was trapped at seven locations in three districts. All other species (*Ph. balcanicus* at five, *Ph. papatasi* at three, *Ph. alexandri* at three and *Ph. mascittii* at two locations) were trapped in two districts. Sand flies in Kosovo generally can be found in mid-elevation areas (mean: 540 m, SD: ±136 m), but some species, like *Ph. neglectus* and *Ph. mascittii*, were collected in relatively high regions (Table 4, Figure 2).

Most sampled locations were positive for a single sand fly species (78), 25 locations were positive for two species, and 12 locations for three species. At single locations, four and six species were observed in the Prizreni district, respectively, and five species at one location in the Gjakova district (Table 5).

TABLE 2 Caught sand flies by species and sex in Kosovo in 2022 and 2023.

Species	Male	Female (engorged)	Total (%)
Phlebotomus neglectus	113	182 (65)	295 (97.4%)
Phlebotomus perfiliewi	1	0 (0)	1 (0.3%)
Phlebotomus tobbi	3	1 (0)	4 (1.3%)
Phlebotomus balcanicus	0	1 (0)	1 (0.3%)
Phlebotomus mascittii	0	1 (0)	1 (0.3%)
Sergentomyia minuta	0	1 (0)	1 (0.3%)
Total	117	186 (65)	303

Geographical and environmental analysis

Sand fly presence was associated with regions showing moderate to moderately low mean wind speed values (2.2 and 2.5 m/s^{-1}). Positive trapping sites revealed temperate and continental conditions ranging between 11.0 and 11.8°C average annual mean temperatures. Among the analysed species, *Ph. alexandri* generally inhabits the warmest sites in Kosovo, and *Ph. mascittii* the coldest. Considering precipitation patterns, the average annual precipitation at trapping locations by species indicated relatively humid environments, with average annual precipitation between 706 and 938 mm. Among all species, *Phlebotomus neglectus* was identified to occur at the driest and *Ph. alexandri* was identified to occur at the most humid regions (Supplementary Table 4).

Comparing the occurrence of certain sand fly species and the annual mean temperature patterns in Kosovo, we generally delineate two main eco-geographic clusters of sand fly species in Kosovo. First, *Ph. alexandri*, *Ph. balcanicus*, *Ph. papatasi*, *Ph. simici*, *Ph. tobbi* and *Se. minuta* were present in regions where annual mean temperatures were relatively high, and those species were generally observed absent from mountainous regions (Figure 3a). Second, and on the contrary, *Ph. neglectus* and Ph. *perfiliewi* have a widespread distribution in Kosovo. In western Kosovo, where there is a west-to-east decreasing precipitation gradient, *Ph. neglectus* appeared to tolerate higher annual humidity conditions than *Ph. perfiliewi*, which only appears in the lowland regions of Dukagjin Plain, while *Ph. neglectus* also occupies the easternmost foothills of the Albanian Alps (Figure 3b). *Phlebotomus mascitti* showed a unique eco-geographical pattern.

When comparing the collecting sites according to the number of collected sand fly species, positive trapping sites were generally less windy (mean annual wind speed 0.20–0.24 m/s^{-1}) than negative trapping sites (mean annual wind speed 0.32 m/s^{-1}). The highest density of positive trapping sites was restricted to the relatively wind-shaded plains (Figure 3c). Locations with more diverse sand fly assemblages showed higher annual mean and higher mean temperatures in the coldest quarter (December–February) (Supplementary Table 5).

Blood meal analyses and Leishmania DNA screening

Altogether, we analysed 244 engorged female sand flies. The combination of three host target genes provided successful amplification of 223 blood meals from five sand fly species. All obtained sequences showed >90% query coverage and >99% sequence identity, with reference sequences from GenBank using BLAST. The majority of analysed engorged specimens were *Ph. neglectus* (114, 51.1%) and *Ph. perfiliewi* (102, 45.7%); all others comprised *Ph. simici* (4, 1.8%), *Ph. tobbi* (2, 0.9%) and *Se. minuta* (1, 0.5%). Seven different host species were identified, of which *Bos taurus* (cow) was predominant (169/223, 75.8%) (Table 6).

Phlebotomus perfiliewi was observed to have fed on seven different host species, and *Phlebotomus neglectus* fed on four host species, with humans being the second most common source.

TABLE 3 Generated barcodes, haplotypes and accession numbers of sand flies from Kosovo caught in our study and by Xhekaj, Hoxha, et al. (2023).

Species	Barcodes	Haplotypes	Accession numbers	BLAST identity[a]
Larroussius				
Ph. neglectus	30	11	PP296427–PP296456	97.16% (OL352136) to 100% (KY848830)
Ph. perfiliewi	27	11	PP296457–PP296483	94.83% (KF483665) to 100% (KU519504)
Ph. tobbi	7	4	PP296484–PP296490	97.48% (MN086639) to 100% (OL352107)
Adlerius				
Ph. balcanicus	7	2	PP296491–PP296497	94.01% (MT344027) to 99.84% (MK425636)
Ph. simici	22	3	PP296498–PP296519	95.89% (MT452060) to 100% (MT452051)
Phlebotomus				
Ph. papatasi	2	2	PP296521–PP296522	98.75% (MT074074) to 99.70% (MZ049661)
Transphlebotomus				
Ph. mascittii	1	1	PP296520	99.84% (KX963380) to 100% (MN812830)
Sergentomyia				
Se. minuta	5	3	PP296523–PP296527	93.15% (KJ481117) to 99.47% (KP828551)

TABLE 4 Currently known sand fly presence by location, altitude (m AMSL) and districts based on three analysed surveys (this study; Vaselek et al., 2020; Xhekaj, Hoxha, et al., 2023).

Species	Locations (*n*)	Min to max altitude in m AMSL (mean, sd)	Districts (*n*)
Ph. neglectus	102	137 to 1494 (552, 156)	#1–#7 (7)
Ph. perfiliewi	38	322 to 792 (520, 137)	#1–#7 (7)
Ph. tobbi	12	313 to 789 (503, 161)	#4–#7 (4)
Ph. balcanicus	5	331 to 552 (467, 92)	#4, #7 (2)
Ph. simici	7	322 to 789 (519, 182)	#4–#7 (4)
Ph. papatasi	3	331 to 440 (402, 62)	#3, #4 (2)
Ph. alexandri	3	313 to 545 (433, 116)	#4, #7 (2)
Ph. mascittii	2	545 to 882 (714, 239)	#4, #7 (2)
Se. minuta	7	331 to 611 (466, 105)	#3, #4, #7 (3)

A single analysed blood meal of *Se. minuta* was shown to originate from a human host (Table 6, Figure 4). Chicken DNA in one *Ph. perfiliewi* specimen could only be amplified with the specific avian primers L15330AV/H15551AV. All screened RNA/DNA pools were negative for *Leishmania* DNA.

DISCUSSION

This study comprehensively updated and mapped the currently known sand fly distribution in Kosovo. In addition, we obtained COI barcodes for eight of nine endemic species and conducted the first blood meal analysis for sand flies in Kosovo that comprised more than 220 engorged specimens of five species. We also provided the first insights into environmental preferences of the present species.

The Balkan region harbours a diverse sand fly fauna. However, up-to-date knowledge of its species composition, ecological and trophic preferences remains incomplete due to several decades of limited vector biology research. In addition, involvement of the endemic sand fly fauna in local transmission cycles of sand fly-borne pathogens has been rarely assessed. Only recently, a study by Dvorak et al. (2020) assessed sand flies in eight Balkan countries comparing historical data as well as active field surveillance. In his study, they found that *Phlebotomus neglectus* was the most widely distributed species, being present in all eight Balkan countries. This was also observed in our study, as more than 97% of the collections were *Ph. neglectus*. Combining our survey and those by Vaselek et al. (2020) and Xhekaj, Hoxha, et al. (2023), it is evident that *Ph. neglectus* exhibits the widest distribution in Kosovo, being reported from all seven districts of the country and found at 102 of 118 locations positive for sand fly presence. While *Ph. perfiliewi* was also found in all districts, it was only observed at 38 locations.

Our results delineate two main eco-geographic clusters of sand fly species in Kosovo. The first cluster is mainly restricted to the south-western part of Kosovo and, to a lesser extent, to the south-eastern part of the country. These are relatively warm and wind-

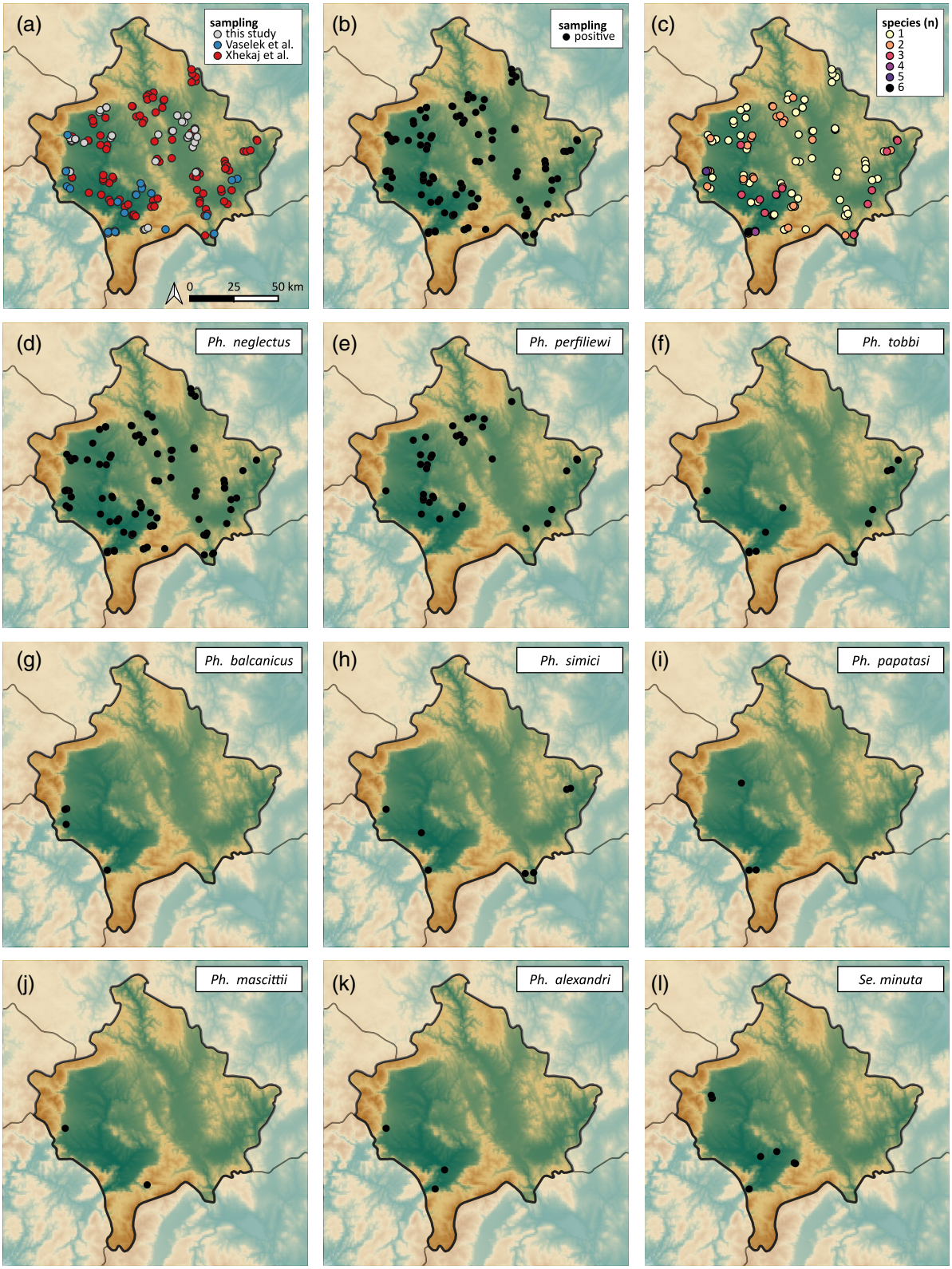

FIGURE 2 Mapped sand fly distribution by species in Kosovo based on three surveys (this study; Vaselek et al., 2020; Xhekaj, Hoxha, et al., 2023). Sampling sites by survey (a), sampling sites positive for sand flies (b), number of species present by location (c) and individual distribution maps for all nine sand fly species (d–l).

shaded intra-mountainous valleys. These regions (mainly the warmest in south-western parts) also harbour locations where multiple species are present, even though the presence of more than three species at the same location was rare. The other cluster, consisting of *Ph. neglectus* and *Ph. perfiliewi*, seems to comprise species tolerating higher fluctuations of environmental parameters within the studied

TABLE 5 Number of species present at sampled locations by district based on data of three different surveys (this study; Vaselek et al., 2020; Xhekaj, Hoxha, et al., 2023).

	Locations positive for n species						
District	**1**	**2**	**3**	**4**	**5**	**6**	**Total**
Prishtina 01	17	1	0	0	0	0	18 (15%)
Mitrovica 02	6	6	0	0	0	0	12 (10%)
Peja 03	15	4	1	0	0	0	20 (17%)
Prizreni 04	16	6	3	1	0	1	27 (23%)
Ferizaj 05	7	1	1	0	0	0	9 (8%)
Gjilani 06	8	1	4	0	0	0	13 (11%)
Gjakova 07	9	6	3	0	1	0	19 (16%)
Total	78 (66%)	25 (21%)	12 (10%)	1 (1%)	1 (1%)	1 (1%)	**118 (100%)**

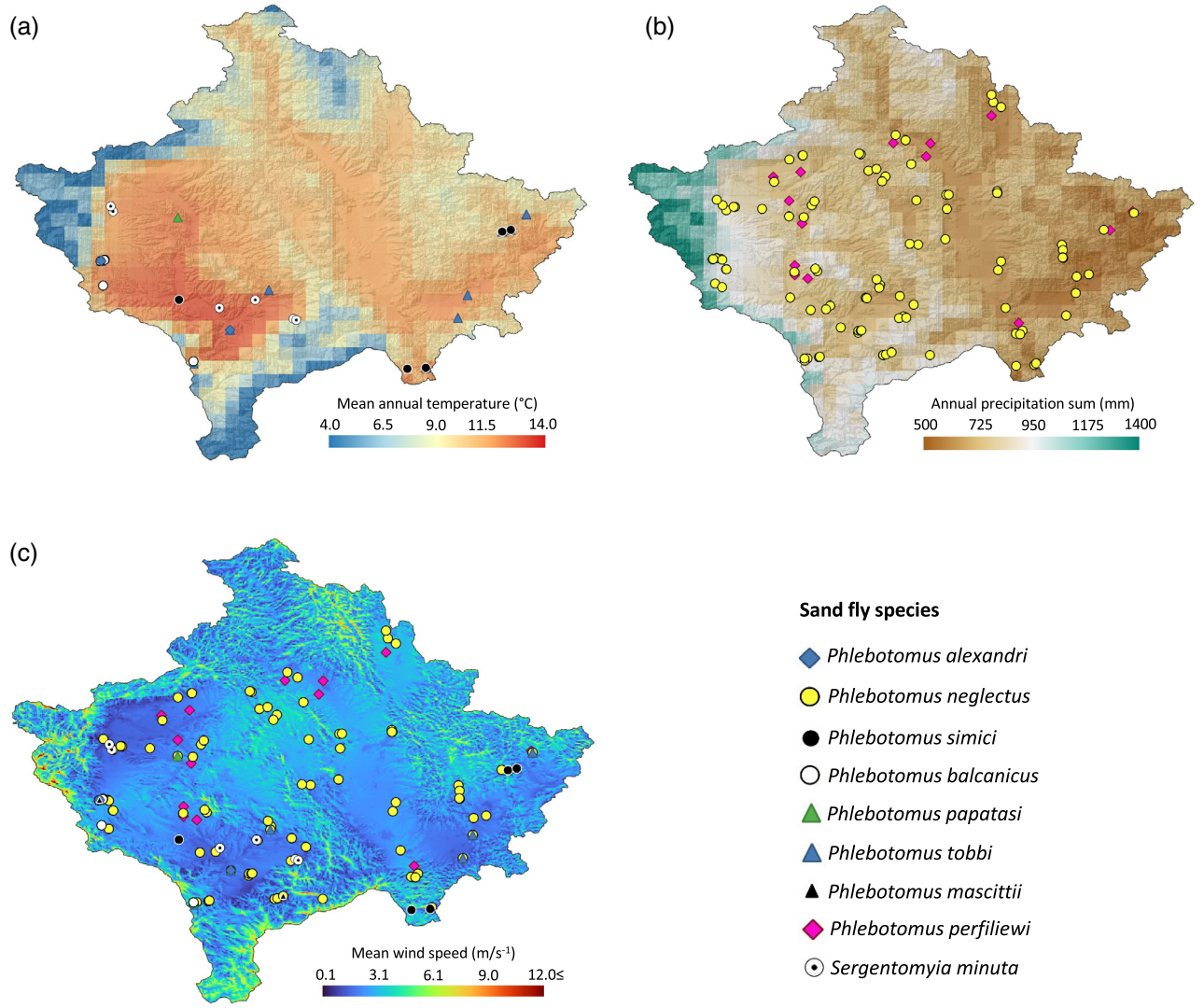

FIGURE 3 Mapped environmental factors combined with sand fly presence based on the currently known distribution. Mean annual temperature map with sand fly species (*Ph. alexandri*, *Ph. balcanicus*, *Ph. papatasi*, *Ph. simici*, *Ph. tobbi* and *Se. minuta*) associated with warmer regions of Kosovo (a), average annual precipitation map with the distribution of the predominant species *Ph. neglectus* and *Ph. perfiliewi* (b), mean wind speed map showing all nine sand fly species (c).

TABLE 6 Analysed blood meals by sand fly species caught during two surveys in the years 2022 and 2023 (this study and Xhekaj, Hoxha, et al., 2023). Blood meals: Cow (*Bos taurus*), dog (*Canis lupus familiaris*), goat (*Capra hircus*), chicken (*Gallus gallus*), human (*Homo sapiens*), European hare (*Lepus europaeus*) and sheep (*Ovis aries*).

	Blood meal host							
Sand fly species	Cow	Dog	Goat	Chicken	Human	Hare	Sheep	Total (%)
Ph. neglectus	76	4	0	0	18	0	16	114 (51.1%)
Ph. perfiliewi	88	3	3	1	2	1	4	102 (45.7%)
Ph. simici	3	0	0	0	0	0	1	4 (1.8%)
Ph. tobbi	2	0	0	0	0	0	0	2 (0.9%)
Se. minuta	0	0	0	0	1	0	0	1 (0.5%)
Total	169	7	3	1	21	1	21	223 (100%)

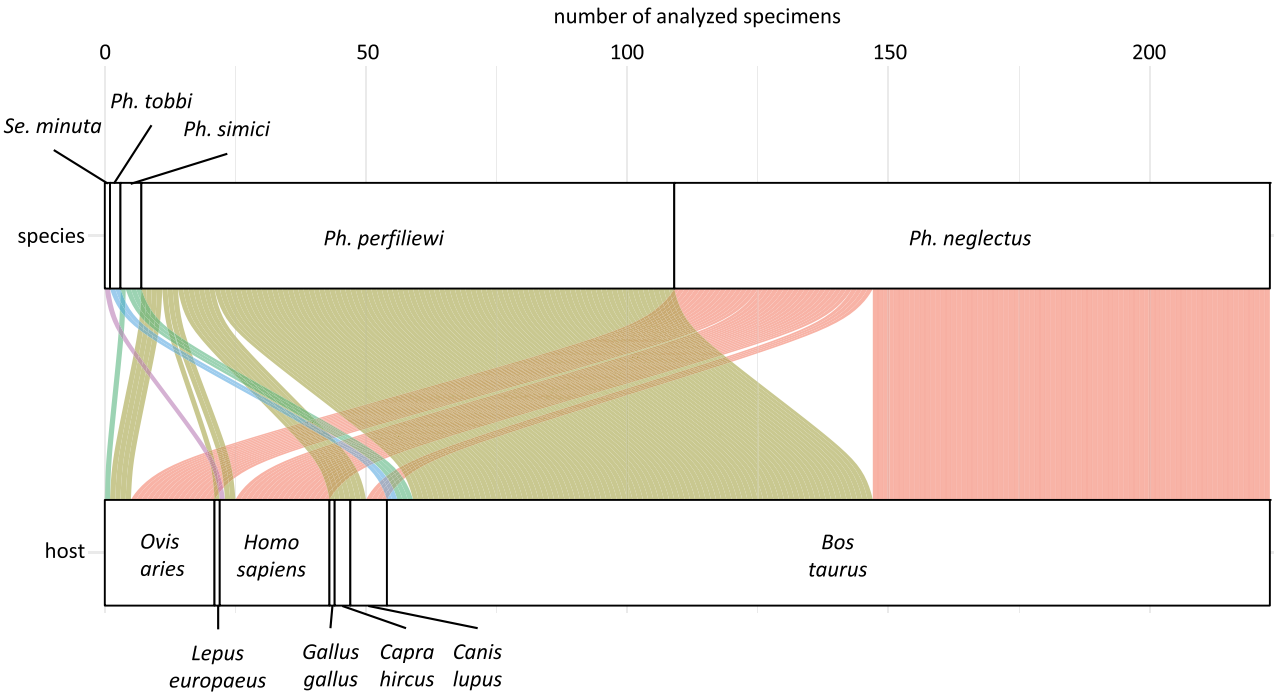

FIGURE 4 Alluvial plot to display identified blood meal hosts by sand fly species. The upper row of the plot shows the sand fly species and indicates the number analysed. The lower row shows the detected blood meal host species. Coloured lines represent different sand fly species.

region compared with other species. Noteworthy, *Phlebotomus mascittii* was found to be present at the coldest trapping site, which is not surprising as this species is assumed to be adapted to cooler climates, taking its Central European distribution into account (Kniha et al., 2023).

We also concluded that wind speed can have a strong negative limiting factor for regional sand fly distribution. However, further investigations are needed to show a difference between the effects of altitude and wind speed, as windy conditions are generally specific to regions of higher altitude where annual mean temperatures are also low and humidity is high. Typically, those factors are disadvantageous for sand flies, and activity usually decreases with increasing wind speed (Carta et al., 2020; Gálvez et al., 2010). However, local tolerances to higher wind speeds can be observed at indoor trapping sites such as animal barns or sheds (Kniha et al., 2021).

While surveyed sites in Kosovo predominantly represented peri-urban or urban locations such as animal barns, dog shelters or private households with animals, we also sampled two small horizontal caves: one in Drenas (Prishtina district) and one in Vermice (Prizren district). In both, we found few *Ph. neglectus* specimens, indicating that this species is not restricted to human dwellings and can inhabit other microhabitats. *Phlebotomus neglectus* was also shown to be cave-dwelling in Romania (Cazan et al., 2021) and the Greek island Crete (Dvořák et al., 2020) and was trapped in hyrax (Hyracoidea) caves in Palestine (Sawalha et al., 2017). This finding highlights the necessity to include also natural localities in future sand fly field surveys to fully understand the ecology of studied sand fly species, which may be partially biased by trappings performed mostly at domestic and peridomestic sites that are most relevant from the perspective of the epidemiology of sand fly-borne diseases.

Noteworthy, due to the absolute rareness of *Ph. alexandri* and *Ph. mascittii* in Kosovo, we sampled three locations in the village Junik (Gjakova district), where both species have been observed to be present in 2014 (Vaselek et al., 2020). However, we only found *Ph. neglectus*, *Ph. perfiliewi* and *Ph. balcanicus*. While we trapped a single *Ph. mascittii* specimen at another location in the Prizren district, we did not trap any specimen of *Ph. alexandri*. *Phlebotomus mascittii* has also been found in very low numbers in other Balkan countries such as Serbia or Bosnia and Herzegovina (Kniha et al., 2023; Vaselek et al., 2017), and its abundance is low even in countries where it represents the only or vastly predominant species such as Germany, Austria, Switzerland and Slovakia (Dvořák et al., 2016; Kniha, Dvořák, et al., 2020; Kniha, Walochnik, et al., 2020; Oerther et al., 2020; Schaffner et al., 2023).

In contrast, *Ph. alexandri*, a proven vector of *Leishmania donovani*, is endemic over a large geographic area that comprises Greece, Turkey, the Middle East, North African countries and the southern coast of Spain, highlighting its connectivity with climatically warmer regions (Maroli et al., 2013). To date, only sporadic records from North Macedonia, Bulgaria, Kosovo and Serbia have been reported, the latter marking the northernmost European distribution. However, no molecular data or barcodes exist for specimens originating from those countries (Dvorak et al., 2020; Vaselek et al., 2019, 2020). Unfortunately, *Ph. alexandri* could neither be trapped in the frame of this study nor by Xhekaj, Hoxha, et al. (2023). As Kasap et al. (2019) identified two distinct lineages of *Ph. alexandri* in Turkey, potentially representing two cryptic species, molecular data from Balkan countries would help to understand its origin, population structure and potential spread.

Considering the broad distribution and abundance of *Ph. neglectus* and *Ph. perfiliewi*, these two species are of primary interest as vectors of *L. infantum* and phleboviruses in Kosovo. While we did not detect *Leishmania* DNA in our samples, Xhekaj, Hoxha, et al. (2023) reported the amplification of *L. infantum* DNA from both species and Vaselek et al. (2020) detected *L. tropica* DNA in a *Ph. neglectus* specimen from the same region in south-western Kosovo. Both species are confirmed vectors of *L. infantum*, and *Ph. neglectus* is the principal vector in neighbouring Albania (Velo et al., 2017). Despite not being the primary blood meal host species, we detected that *Ph. neglectus* and *Ph. perfiliewi* were feeding on dogs and humans, which would facilitate the spillover of *L. infantum*. This is highlighted by Xhekaj et al. (2020); Xhekaj, Stefanovska, et al. (2023), who reported canine *L. infantum* presence in six of seven districts of Kosovo. Local prevalence rates were as high as 21.6% in the south-western part of the country, where we showed the highest mean temperatures and highest sand fly diversity in our study. Also, 12.5% seropositivity was detected in Austrian soldiers deployed in Kosovo without a prior mission in any other potentially endemic region (Kniha, Dvořák, et al., 2020; Kniha, Walochnik, et al., 2020). Given that the major blood meal source in our study was cattle, the potential role of this host species in the transmission of *L. infantum* should be addressed. Generally, livestock are reported to have contact with *Leishmania* in endemic areas but seem to be of low importance as reservoir hosts for species of the *L. donovani*/*infantum* complex (Alam et al., 2011; Paixão-Marques et al., 2019; Rezaei et al., 2022). However, the presence of cattle and thus optimal breeding sites and permanent host availability might positively influence the abundance of the respective disease vector (Svobodová et al., 2009).

On the other hand, Ayhan et al. (2017) found high rates of neutralising antibodies against TOSV and SFSV in cattle and sheep from western and south-western Kosovo. Concurrent sand fly surveys in the analysed areas always identified *Phlebotomus major* (= *Ph. neglectus* in Kosovo) as the predominant species. We observed cattle as the main blood meal hosts, followed by sheep and humans for *Ph. neglectus* and *Ph. perfiliewi*. Thus, circulation of phleboviruses between these two sand fly species and livestock seems evident and might boost phlebovirus transmission between livestock and humans in areas of circulation, although sand flies themselves are considered to be the reservoir hosts (Moriconi et al., 2017). Noteworthy, Austrian soldiers showed anti-IgG and anti-IgM antibodies against phleboviruses after returning from missions in Kosovo (Kniha et al., 2019), which underlines the circulation in the country.

CONCLUSIONS

Our results provide detailed insights into the biological and ecological aspects of sand fly occurrence in Kosovo, a southern Balkan country with an endemic circulation of *Leishmania infantum* and phleboviruses. The acquired data should help to better understand the sand fly distribution in the country and highlight hotspots of sand fly presence and, thus, potential disease transmission in the country.

AUTHOR CONTRIBUTIONS

Betim Xhekaj: Data curation; formal analysis; investigation; methodology; writing – review and editing. **Ina Hoxha:** Investigation; methodology; writing – review and editing; formal analysis; data curation. **Katharina Platzgummer:** Data curation; formal analysis; writing – review and editing; investigation. **Jovana Stefanovska:** Project administration; supervision; writing – review and editing. **Vít Dvořák:** Data curation; formal analysis; validation; writing – review and editing. **Markus Milchram:** Data curation; formal analysis; investigation; writing – review and editing. **Adelheid G. Obwaller:** Writing – review and editing; funding acquisition; project administration; supervision. **Wolfgang Poeppl:** Funding acquisition; writing – review and editing; project administration; supervision. **Nesade Muja-Bajraktari:** Writing – review and editing; methodology. **Julia Walochnik:** Funding acquisition; investigation; resources; writing – review and editing; supervision. **Attila J. Trájer:** Conceptualization; data curation; formal analysis; validation; visualization; writing – review and editing; writing – original draft. **Kurtesh Sherifi:** Project administration; supervision; writing – review and editing. **Aleksandar Cvetkovikj:** Project administration; supervision; writing – review and editing. **Edwin Kniha:** Data curation; formal analysis; funding acquisition; investigation; methodology; project

administration; resources; writing – original draft; validation; writing – review and editing.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no competing interest.

DATA AVAILABILITY STATEMENT

All data are included in the article and Supplementary Material is available in a public repository under the DOI: 10.5061/dryad.qv9s4mwpp.

INFORMED CONSENT STATEMENT

Verbal consent was obtained from all homeowners to trap at their properties.

ORCID

Aleksandar Cvetkovikj https://orcid.org/0000-0002-6582-7020
Edwin Kniha https://orcid.org/0000-0002-6875-7056

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Table S1. Information on number of trap sites, locality and date.

Table S2. Information on habitat type and use, trap placement and success.

Table S3. Information on host presence at sampled locations.

Table S4. The mean environmental values at the collection sites of sand fly species.

Table S5. Minimum and maximum climatic values related to the trapping sites. Ns = number of species at the location, nl = number of positive locations.

Data S1: Structured Reflexivity Statement.

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