Genetic diversity of the cardiopulmonary canid nematode Angiostrongylus vasorum within and between rural and urban fox populations

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Abstract: Angiostrongylus vasorum is an emerging parasitic cardiopulmonary nematode of dogs, foxes, and other canids. In dogs, the infection causes respiratory and bleeding disorders along with other clinical signs collectively known as canine angiostrongylosis, while foxes represent an important wildlife reservoir. Despite the spread of A. vasorum across various countries in Europe and the Americas, little is known about the genetic diversity of A. vasorum populations at a local level in a highly endemic area. Thus, in the present study, we investigated the genetic diversity of 323 adult A. vasorum nematodes from 64 foxes living in the canton of Zurich, Switzerland. Among those, 279 worms isolated from 20 foxes were analyzed separately to investigate the genetic diversity of multiple worms within individual foxes. Part of the mitochondrial cytochrome c oxidase subunit I (mtCOI) gene was amplified and sequenced. Overall, 16 mitochondrial haplotypes were identified. The analysis of multiple worms per host revealed 12 haplotypes, with up to 5 different haplotypes in single individuals. Higher haplotype diversity (n = 10) of nematodes from foxes of urban areas than in rural areas (n = 7) was observed, with 5 shared haplotypes. Comparing our data with published GenBank sequences, five haplotypes were found to be unique within the Zurich nematode population. Interestingly, A. vasorum nematodes obtained from foxes in London and Zurich shared the same dominating haplotype. Further studies are needed to clarify if this haplotype has a different pathogenicity that may contribute to its dominance. Our findings show the importance of foxes as a reservoir for genetic parasite recombination and indicate that high fox population densities in urban areas with small and overlapping home ranges allow multiple infection events that lead to high genetic variability of A. vasorum.

DOI: https://doi.org/10.1016/j.meegid.2020.104618

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Infection, Genetics and Evolution 87 (2021) 104618

Contents lists available at ScienceDirect

Infection, Genetics and Evolution

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

Research paper

Genetic diversity of the cardiopulmonary canid nematode *Angiostrongylus vasorum* within and between rural and urban fox populations

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ARTICLE INFO

Keywords:
Angiostrongylus vasorum
Cardiopulmonary nematode
Fox
Genetic variation
Population diversity
Phylogeography

ABSTRACT

Angiostrongylus vasorum is an emerging parasitic cardiopulmonary nematode of dogs, foxes, and other canids. In dogs, the infection causes respiratory and bleeding disorders along with other clinical signs collectively known as canine angiostrongylosis, while foxes represent an important wildlife reservoir. Despite the spread of *A. vasorum* across various countries in Europe and the Americas, little is known about the genetic diversity of *A. vasorum* populations at a local level in a highly endemic area. Thus, in the present study, we investigated the genetic diversity of 323 adult *A. vasorum* nematodes from 64 foxes living in the canton of Zurich, Switzerland. Among those, 279 worms isolated from 20 foxes were analyzed separately to investigate the genetic diversity of multiple worms within individual foxes. Part of the mitochondrial cytochrome *c* oxidase subunit I (mtCOI) gene was amplified and sequenced. Overall, 16 mitochondrial haplotypes were identified. The analysis of multiple worms per host revealed 12 haplotypes, with up to 5 different haplotypes in single individuals. Higher haplotype diversity ($n = 10$) of nematodes from foxes of urban areas than in rural areas ($n = 7$) was observed, with 5 shared haplotypes. Comparing our data with published GenBank sequences, five haplotypes were found to be unique within the Zurich nematode population. Interestingly, *A. vasorum* nematodes obtained from foxes in London and Zurich shared the same dominating haplotype. Further studies are needed to clarify if this haplotype has a different pathogenicity that may contribute to its dominance. Our findings show the importance of foxes as a reservoir for genetic parasite recombination and indicate that high fox population densities in urban areas with small and overlapping home ranges allow multiple infection events that lead to high genetic variability of *A. vasorum*.

1. Introduction

Angiostrongylus vasorum is a cardiopulmonary nematode of dogs (*Canis lupus familiaris*) and wild canids such as red foxes (*Vulpes vulpes*). The adult worm resides in the pulmonary arteries and the heart of definitive canid hosts. The parasite has an indirect life-cycle, where coprophagic snails and slugs act as an intermediate host: *A. vasorum* first-stage larvae (L1) excreted in canid feces develop to infective third-stage larvae (L3) (Guilhon and Bressou, 1960) in gastropods. Dogs and other canids are infected by oral ingestion of infected intermediate hosts; definitive hosts can remain infected and excrete L1 throughout their lifespan (Guilhon and Cens, 1969, 1973). The parasite causes a series of clinical signs in dogs known as canine angiostrongylosis, which have the potential to be fatal (Chapman et al., 2004; Sigrist et al., 2017; Traversa et al., 2008). Dogs frequently present with respiratory signs induced by verminous pneumonia, but also bleeding, neurological, and other unspecific signs (Chapman et al., 2004; Glaus et al., 2016; Koch and Willesen, 2009).

Apparently limited to France until the mid-sixties, epidemiological studies and case reports have shown that in the last decades the parasite is rapidly emerging across Europe, and also present in single areas of East Africa and North and South America. Over the years, *A. vasorum* in canids has geographically spread widely and the number of clinical cases in dogs has increased, supported by enhanced diagnostic tools and disease awareness (Liu et al., 2017; Morgan and Shaw, 2010; Schnyder et al., 2015).

Isolated cases of canine angiostrongylosis were observed in Switzerland for the first time towards the end of the sixties (Wolff et al., 1969). Recent epidemiological studies in dogs and foxes showed that *A. vasorum* is actually present in most parts of the country, and 3% of

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https://doi.org/10.1016/j.meegid.2020.104618
Received 3 August 2020; Received in revised form 24 October 2020; Accepted 2 November 2020
Available online 11 November 2020

dogs are seropositive for antibodies against the parasite (Lurati et al., 2015). The analysis of fox samples dating back 3 decades illustrated the impressive spread of this parasite within the country, exemplary for Europe, and confirmed the fundamental role of foxes as reservoir hosts (Gillis-Germitsch et al., 2020). In foxes, pathological lesions are comparable to the ones of dogs (Poli et al., 1991; Schnyder et al., 2010), however, the description of clinical signs is rare (Philbey and Delgado, 2013; Simpson, 1996). Experimental studies in foxes repeatedly inoculated with infectious L3 demonstrated a high parasite tolerance with increasing worm burdens and L1 excretion (Woolsey et al., 2017). Under natural conditions this implies increasing environmental L1 contamination over time, with infection of the local gastropod population, allowing the establishment of this parasite in definitive fox hosts, with local prevalence above 80% (Gillis-Germitsch et al., 2020). Therefore, foxes do not develop protective immunity, as also supported by the decrease of serological antibodies despite high worm burdens (Gillis-Germitsch et al., 2017a).

Despite numerous prevalence studies on *A. vasorum*, little is known about its population genetics and evolution that may have reflective implications for the epidemiology of angiostrongylosis. The mitochondrial gene cytochrome *c* oxidase subunit I (mtCOI) has previously been considered as a suitable marker for genetic differentiation of Angiostrongylus taxa (Eamsobhana et al., 2010). A partial sequence of the mtCOI marker gene was employed to investigate genetic diversity within *A. vasorum* parasite populations in three studies (Blanch-Lazaro et al., 2018; Jefferies et al., 2009; Jefferies et al., 2010). Jefferies et al. (2009) showed that *A. vasorum* specimens from Europe and South America represent two separate lineages and the divergence of the two was estimated to have happened between 11 and 67 million years ago (Jefferies et al., 2009). Analysis of *A. vasorum* specimens isolated from red foxes, dogs, and coyotes illustrated that the same haplotypes are shared among different hosts living in close geographic localities, indicating potential interspecies transmissions (Jefferies et al., 2010). Genetic analysis of London worm isolates has shown a high number of haplotypes within and between city foxes (Blanch-Lazaro et al., 2018).

The main aim of this study was to investigate the genetic diversity of *A. vasorum* in foxes from the Zurich area. In particular, we intended to gain insights into the genetic diversity of *A. vasorum* nematodes isolated from foxes living in known endemic urban and rural areas and to assess the genetic diversity of multiple worms within single fox hosts. The hypotheses were that high fox densities in urban regions (Deplazes et al., 2004; Wandeler et al., 2003) and subsequent environmental contamination with L1 increase the chance of multiple infection events for urban foxes, which might lead to higher worm burdens and therefore, due to cross-fertilization between different haplotypes (Blanch-Lazaro et al., 2018), to higher genetic diversity of worms isolated from urban foxes both at (1) population and (2) individual level.

2. Materials and methods

2.1. Nematode collection and DNA extraction

A total of 337 individual *A. vasorum* nematodes were collected and identified morphologically post-mortem from the lungs, hearts, and pulmonary arteries of 64 foxes *(Vulpes vulpes)* hunted between 2013 and 2017 in the canton of Zurich (1729 km^2), Switzerland, following a previously described protocol (Gillis-Germitsch et al., 2020). All nematodes were separately washed three times in PBS before stored at −20 °C until use. DNA was extracted using the E.Z.N.A.® Mollusc DNA extraction kit (Norcross, OMEGA, USA) following the manufacturer's protocol. DNA concentrations were measured on NanoDrop One (Thermo Fisher Scientific, Waltham, MA, USA) and stored at −20 °C. Low-quality sequences were discarded and excluded from the analysis; therefore, sequences of 323 specimens were available for further use. With a sample size of 323, haplotypes with a frequency of at least 1% were expected to be identified with a 95% confidence level (Grewe et al., 1993). Among those, 86 specimens were isolated from 9 foxes that were shot within the city of Zurich (urban area), while 193 nematodes were isolated from 11 foxes culled in the rural areas within the canton but outside of the city. In addition, 44 single nematodes were collected from individual foxes living in rural, urban, or semi-rural areas of Zurich (Table 1). The coordinates of the sampling points are represented in Fig. A.1.

2.2. Amplification and sequencing

The extracted DNA was used as a template for PCR amplification of the 710 bp region of the mitochondrial cytochrome oxidase subunit 1 (mtCOI) gene using LCO1490 (5' GGTCAACAAATCATAAAGATATTGG −3′) and HCO2198 (5' TAAACTTCAGGGTGACCAAAAAATCA-3′) – a widely used primer pair for invertebrates (Folmer et al., 1994). PCR assays were performed in a 25 μL final reaction volume that consisted of 12.5 μL multiplex (QIAGEN Multiplex PCR Kit), 3 μL extracted DNA, 2 μL primer mix, and 7.5 μL ddH2O. Previously confirmed genomic DNA along with ddH20 were used as positive and negative controls, respectively. PCR was started with the following conditions: initial denaturation at 95 °C for 15 min followed by 40 cycles of 95 °C for 45 s, 50 °C for 1 min, 72 °C for 1.5 min with 10 min at 72 °C final extension step. PCR products were run on 1.5% agarose gel at 100 V for 40 min and visualized using Gel Red (Biotium, Chemie Brunschwig AG, Basel, Switzerland) under UV illumination. The concentration of PCR products was estimated by comparing band thickness with a known concentration of ladders using ImageJ software (Rueden et al., 2017). PCR products were purified and sequenced at Microsynth AG (Balgach, Switzerland) using the LCO1490 primer. All sequences were manually checked and ambiguous nucleotides were corrected. PCR and sequencing steps were repeated for the unique haplotypes to assure the absence of artificial diversity. The dominant haplotypes of the potentially heterozygous diploid genome of *A. vasorum* were identified using the corresponding quality report chromatogram file when possible. Amplicon sequences were deposited in the GenBank database under the accession numbers MT738959 - MT739281.

2.3. Population analysis

Sequence alignment and trimming were done using MEGA-X software (version 10.1.15) (Kumar et al., 2018). Haplotype networks were generated using a minimum spanning network algorithm of PopART (Population Analysis with Reticulate Trees) (Leigh and Bryant, 2015). Haplotype diversity (Hd) and nucleotide diversity per site (Pi) were performed in DnaSP6 (Version 6.12.03) (Rozas et al., 2017) while fixation (F_{ST}) indices, neutrality tests (Tajima's *D* (Tajima, 1989) and Fu's F_S (Fu, 1997)) were calculated in ARLEQUIN (version 3.5.2.2, Excoffier and Lischer, 2010). Sequences were analyzed by grouping into four datasets in two of which previously published GenBank sequences were added to our dataset to address different questions as follows: group 1 included all sequences of our Zurich *A. vasorum* population ($n = 323$), group 2 consisted of nematodes that were isolated from foxes which were either shot in the city ($n = 86$) or in the countryside ($n = 193$) (Table 1). The same group of the dataset was also used to analyze multiple *A. vasorum* specimens per fox ($n = 279$) from 20 urban and rural foxes of Zurich. Group 3 consisted of all Zurich sequences complemented with 96 published *A. vasorum* mtCOI sequences (accession numbers LT990053–LT990148) from another restricted area in Europe, i.e. London (Blanch-Lazaro et al., 2018) ($n = 419$), and group 4 included all currently available mtCOI sequences of *A. vasorum* (accession numbers GQ982733–GQ982876) isolated from dog, fox and coyote hosts from nine different countries (Blanch-Lazaro et al., 2018; Jefferies et al., 2010).

Table 1
Summary of sampling and genetic diversity parameters of 323 *Angiostrongylus vasorum* specimens obtained from 64 foxes shot in the canton of Zurich, Switzerland. N: Number of sequences obtained from a corresponding number of single *A. vasorum* specimens, H: number of haplotypes, Hd: haplotype diversity, Pi: nucleotide diversity (per site), NA: not applicable.

	Fox origin	Age	Gender	N	H	Hd	Pi	Fu's Fs	Tajima's D
Foxes with multiple nematodes (n = 20)									
Fox_1	Urban	Adult	Male	5	3	0.800	0.00172	−0.475	0.24314
Fox_2	Urban	Adult	Male	12	4	0.561	0.00221	−0.076	−0.81892
Fox_3	Urban	Adult	Female	12	2	0.167	0.00029	−0.476	−1.14053
Fox_4	Rural	Young	Male	8	2	0.536	0.00092	0.866	1.1665
Fox_5	Rural	Young	Female	88	4	0.450	0.00082	−0.577	−0.35909
Fox_6	Rural	Young	Male	10	1	NA	0	NA	NA
Fox_7	Rural	Adult	Female	5	1	NA	0	NA	NA
Fox_8	Urban	Young	Male	3	1	NA	0	NA	NA
Fox_9	Rural	Adult	Female	5	1	NA	0	NA	NA
Fox_10	Urban	Young	Male	6	3	0.733	0.00298	0.758	−0.05722
Fox_11	Urban	Young	Male	4	2	0.5	0.00258	1.716	−0.75445
Fox_12	Urban	Young	Female	13	2	0.154	0.00053	0.362	−1.46801
Fox_13	Urban	Adult	Male	24	5	0.623	0.0018	−0.767	−0.06141
Fox_14	Rural	Adult	Female	8	2	0.536	0.00184	2.083	1.4488
Fox_15	Rural	Adult	Female	19	2	0.199	0.00034	−0.055	−0.56216
Fox_16	Rural	Young	Male	16	3	0.492	0.0009	−0.29	−0.3301
Fox_17	Rural	Adult	Male	14	4	0.582	0.00115	−1.29	−0.886
Fox_18	Urban	Young	Female	7	3	0.667	0.00131	−0.438	−0.27492
Fox_19	Rural	Adult	Male	14	1	NA	0	NA	NA
Fox_20	Rural	Young	Female	6	1	NA	0	NA	NA
	Urban total			86	10	0.602	0.00168	−4.386*	−1.475*
	Rural total			193	7	0.456	0.00092	−2.875	−0.983
	Subtotal			279	12	0.504	0.00116	−7.170*	−1.618*
Foxes with single nematodes (n = 44)									
Single worms				44	13	0.673	0.00214	−8.714*	−1.901*
Summarizing nematodes of all 64 foxes									
Total				323	16	0.528	0.00129	−11.652*	−1.720*

* Significant p-value ($p < 0.05$).

3. Results

As advanced, 14 out of 337 sequences were discarded due to their low quality, leaving 323 obtained sequences originating from adult *A. vasorum* specimens, collected from 64 foxes. Each sequence comprised 582 nucleotides of the mtCOI gene. The data were analyzed in groups as defined above (section 2.3).

3.1. Group 1: Overall genetic diversity of *A. vasorum* specimens obtained from foxes within the canton of Zurich

The analysis of the mitochondrial mtCOI gene of 323 *A. vasorum* specimens revealed 16 polymorphic sites defining 16 single nucleotide polymorphisms (SNP) haplotypes. The minimum-spanning haplotype network for the 323 sequences is shown in Fig. 1. Each of the 64 foxes had 1–5 different haplotypes, with a haplotype diversity value of up to 0.8. Among 20 foxes with multiple nematodes analyzed, six harbored worms of only one haplotype. The neutrality tests (Tajima's *D* and Fu's Fs) reflect the size of genetic changes: zero values indicate that the population is not evolving in term of genetic diversity, negative values correlate with the number of occurring mutations, while positive values indicate that genetic diversity is decreasing. Both Tajima's *D* (−11.652) and Fu's Fs (−1.720) tests were negative and significant ($p < 0.05$) for the total Zurich population (Table 1). The 16 haplotypes and their variable nucleotide positions are presented in Table 2. Eleven of the 16 variable sites were parsimony-informative sites while five were singleton sites.

Overall frequencies of haplotypes are indicated in Table 3. One haplotype dominated by enclosing 67.2% (217/323) of all sequences, while the second and third most common haplotypes were found in 38 and 22 of total sampled specimens, respectively. Five haplotypes were each represented by one single sequence (Table 3).

3.2. Group 2: Comparison of rural and urban isolates from Zurich foxes

In total, 279 sequences were included in this subset of analysis, 86 from nematode sequences of 9 city foxes and 193 from specimens of 11 rural foxes. Combined, 12 haplotypes were detected. Of 279 sequences 192 (68.2%) belonged to one haplotype, while five haplotypes were represented by a single sequence each and had therefore a frequency of 0.4% (Fig. 2 and Table 3). The diversity of *A. vasorum* isolates within individual fox hosts is illustrated in Fig. 3. Fourteen out of 20 foxes harbored nematodes presenting more than one haplotype of *A. vasorum*. Five was the maximum number of haplotypes identified in a single (urban) fox (Table 1). Considered separately, five out of seven haplotypes that were detected in rural isolates were also found in urban isolates. In the latter ones, in addition to the five common haplotypes, five unique haplotypes were found. Five out of 11 rural foxes had nematodes of only one haplotype while only one fox out of nine urban foxes harbored specimens of a single haplotype. Both nucleotide and haplotype diversities were higher in urban isolates than in those of rural isolates (Table 1). The student's *t*-test was used to compare the values of the means of haplotype and nucleotide diversities of the two populations: the difference was significant, with *p*-values of 0.05 and 0.01 for haplotype and nucleotide diversity, respectively. The Fu's Fs and Tajima's *D* tests of neutrality were negative for both urban and rural isolates; however, the values were only significant ($p < 0.05$) for the urban population (Table 1). Pairwise F_{ST} analysis, where a F_{ST} value of 0 indicates that the two compared populations have completely overlapping haplotypes while a F_{ST} value of 1 indicates the opposite (i.e. entirely non-overlapping sets of haplotypes), between rural and urban isolates was performed: this value was 0.018 and revealed a significant level ($p < 0.05$) of differentiation between two subpopulations (Table A.1).

Furthermore, fox age did not affect the haplotype diversity (p-value = 0.46), while gender did by trend, with male foxes having higher haplotype diversity than female foxes (p-value = 0.065), although not as

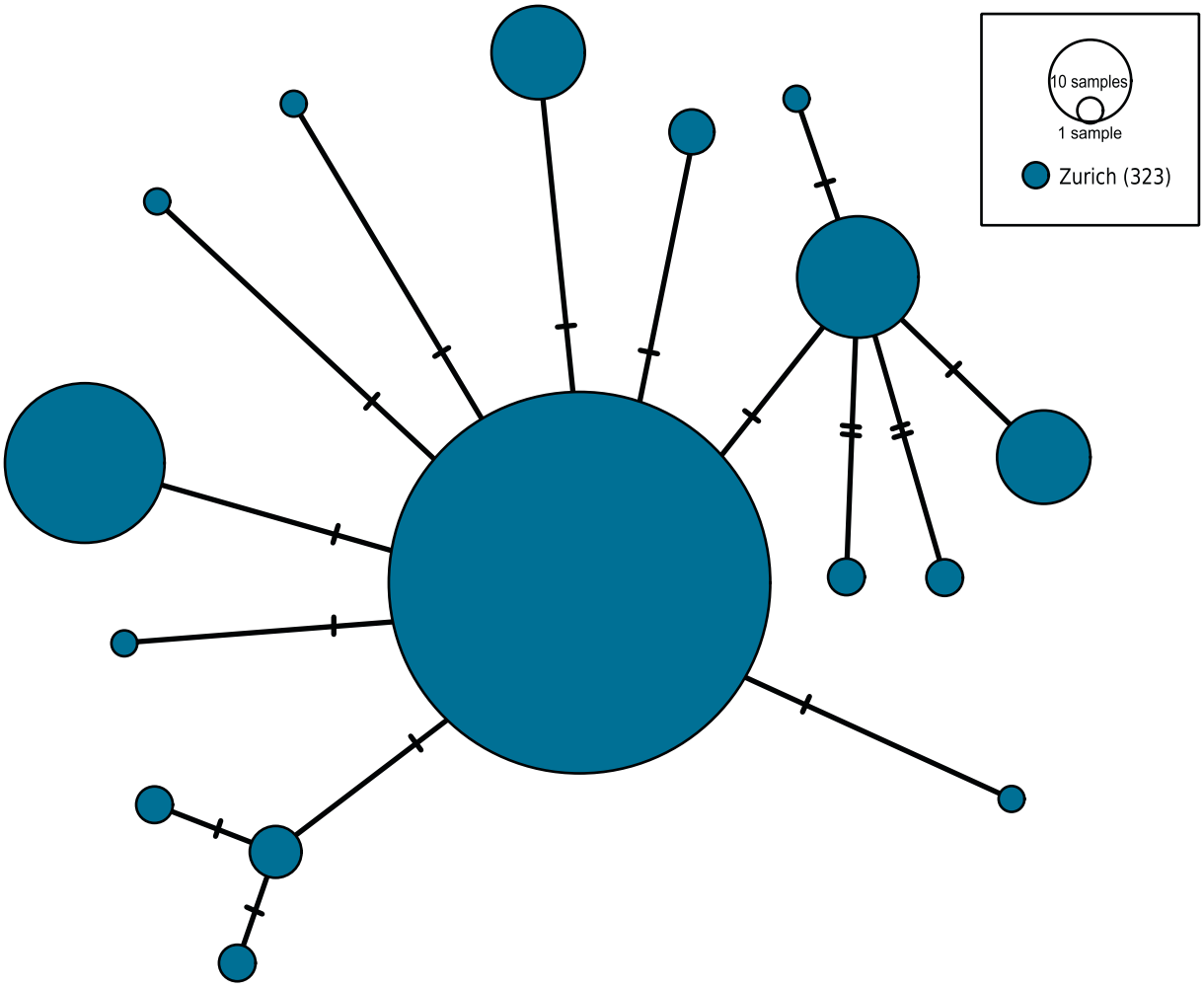

Fig. 1. Minimum-spanning haplotype network for mitochondrial cytochrome *c* oxidase subunit I (mtCOI) sequences of 323 adult *Angiostrongylus vasorum* nematodes isolated from 64 Zurich foxes. Each circle represents one haplotype, and the diameter of the circle is proportional to the number of individuals with the same haplotype. The length of the branches is arbitrary. Lines on the branches show the number of mutations between two haplotypes.

Table 2
Haplotypes and their variable nucleotide positions in the mtCOI gene sequence of 323 *Angiostrongylus vasorum* nematodes collected from 64 foxes in the canton of Zurich.

	Nucleotide position															
	019	046	130	190	203	268	322	325	364	367	412	496	523	526	538	558
Hap_1	A	T	T	A	T	G	T	T	C	T	T	A	A	G	A	A
Hap_2	A	C	T	A	T	G	T	T	C	T	T	A	A	G	A	A
Hap_3	A	T	T	A	T	G	T	T	C	T	T	A	A	A	A	A
Hap_4	A	T	C	A	T	G	T	T	T	T	T	A	G	G	A	A
Hap_5	A	T	T	A	T	A	T	T	C	T	T	A	A	G	A	A
Hap_6	A	T	T	A	T	G	T	T	C	T	T	A	A	G	A	G
Hap_7	A	T	T	A	T	G	T	T	T	T	T	A	A	G	A	A
Hap_8	A	T	T	G	T	G	T	T	T	T	T	A	A	G	A	A
Hap_9	G	T	T	A	T	G	C	T	C	T	T	A	A	G	A	A
Hap_10	A	T	T	A	C	G	T	T	T	T	C	A	A	G	A	A
Hap_11	A	T	T	A	T	G	C	T	C	T	T	A	A	G	A	A
Hap_12	A	T	T	A	T	G	C	T	C	T	T	A	G	G	A	A
Hap_13	A	T	T	A	T	G	T	T	T	T	T	A	A	G	G	A
Hap_14	A	T	T	A	T	G	T	T	C	T	T	G	A	G	A	A
Hap_15	A	T	T	A	T	G	T	C	C	T	T	A	A	G	A	A
Hap_16	A	T	T	A	T	G	T	T	C	C	T	A	A	G	A	A

Table 3
Haplotype numbers (N) and frequencies (F) of *Angiostrongylus vasorum* nemataodes collected from foxes in Zurich.

	Zurich all		MultiWorm		SingleWorm		Rural		Urban	
Haplotypes	N	F (%)	N	F (%)	N	F (%)	N	F (%)	N	F (%)
Hap_1	217	67.18	192	68.82	25	56.82	139	72.02	53	61.63
Hap_2	13	4.02	10	3.58	3	6.82	5	2.59	5	5.81
Hap_3	38	11.76	35	12.54	3	6.82	27	13.99	8	9.30
Hap_4	2	0.62	1	0.36	1	2.27	0	0.00	1	1.16
Hap_5	1	0.31	1	0.36	0	0.00	0	0.00	1	1.16
Hap_6	1	0.31	1	0.36	0	0.00	1	0.52	0	0.00
Hap_7	22	6.81	20	7.17	2	4.55	16	8.29	4	4.65
Hap_8	13	4.02	11	3.94	2	4.55	3	1.55	8	9.30
Hap_9	2	0.62	1	0.36	1	2.27	0	0.00	1	1.16
Hap_10	2	0.62	1	0.36	1	2.27	0	0.00	1	1.16
Hap_11	4	1.24	4	1.43	0	0.00	0	0.00	4	4.65
Hap_12	2	0.62	0	0.00	2	4.55	0	0.00	0	0.00
Hap_13	1	0.31	0	0.00	1	2.27	0	0.00	0	0.00
Hap_14	1	0.31	0	0.00	1	2.27	0	0.00	0	0.00
Hap_15	3	0.93	2	0.72	1	2.27	2	1.04	0	0.00
Hap_16	1	0.31	0	0.00	1	2.27	0	0.00	0	0.00
Nematodes, total	323		279		44		193		86	
Fox total	64		20		44		11		9	

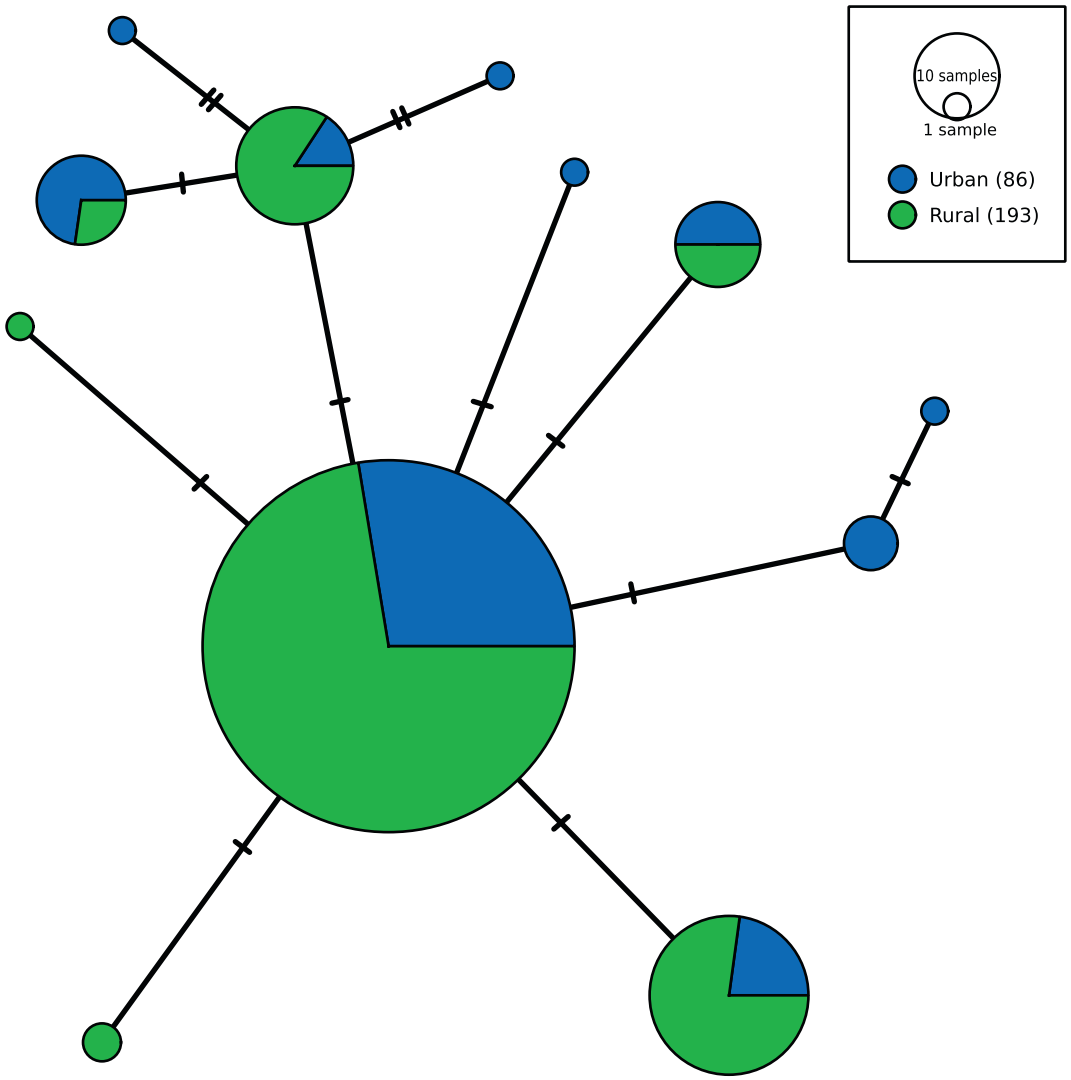

Fig. 2. Haplotype network from partial mtCOI sequences of multiple adult *Angiostrongylus vasorum* nematodes from Zurich foxes that were either shot in rural (green, 11 foxes, 193 worms) or urban (blue, 9 foxes, 86 worms) areas (Table 1). The diameter of the circle is proportional to the number of individuals with the same haplotype. The number of lines on branches shows the number of mutations between two haplotypes, while the length of branches is arbitrary.

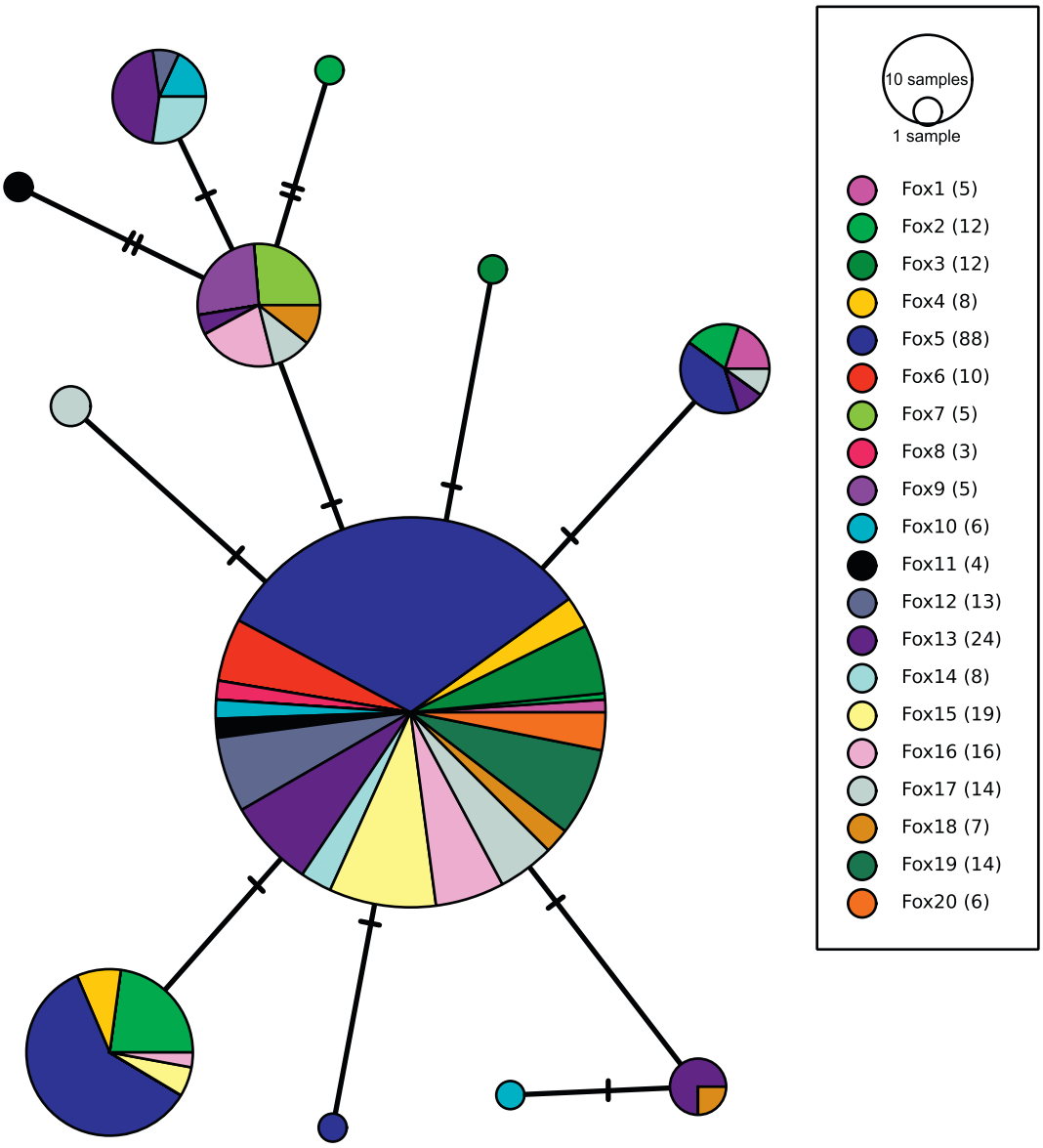

Fig. 3. Haplotype network analysis using mtCOI sequences of multiple adult *Angiostrongylus vasorum* nematodes from individual foxes in Zurich ($n = 20$). Each color represents a different fox isolate; the diameter of the circle indicates the number of mtCOI sequences belonging to the same haplotype. The number of lines on the network branches indicates the number of mutations between two haplotypes. The number of worms isolated from each fox given in parenthesis.

much as locality, i.e. urban vs. rural (Fig. A.2).

3.3. Group 3: Comparison of A. vasorum nematodes obtained from foxes of two local areas within Europe

This group consisted of 323 and 96 sequences that were isolated from two local areas in Europe, i.e. the canton of Zurich and the Greater London urban area, respectively. The 323 sequences from Zurich were trimmed to 554 bases to adjust with the 96 Greater London sequences. This analysis resulted in overall 37 haplotypes of which 15 isolates were found in Zurich and 26 in London. Four haplotypes were common in both sites and the dominating haplotype for Zurich and London was the same (Fig. 4). The second most common haplotype was also shared between the London and Zurich nematode populations. Pairwise F_{ST} comparison showed a significant level of differentiation ($F_{ST} = 0.139$) between the two populations (Table A.1).

3.4. Group 4: Multivariate comparison

This final dataset comprehended all 562 publicly available partial mtCOI sequences that were isolated from dogs, foxes and coyotes necropsied across eight different European countries as well as Canada. Overall 48 haplotypes were identified (Fig. 5, Tab. A.2). Almost half ($n = 269$, 47.8%) of the sequences belonged to one haplotype. This dominant haplotype included isolates from animals coming from Switzerland (218 foxes), UK (43 foxes, one dog), Denmark (three foxes, two dogs), and the Netherlands (two dogs). The second most common haplotype ($n = 81$, 14.4%) was present in six out of the nine sampled countries including specimens from dogs, foxes, and coyotes (Fig. 5, Tab. A.2). The difference between isolates of the Zurich fox population and all other 17 subpopulations (F_{ST} value, Table A.1) was significant, except for the dog population of the Netherlands (low number of samples).

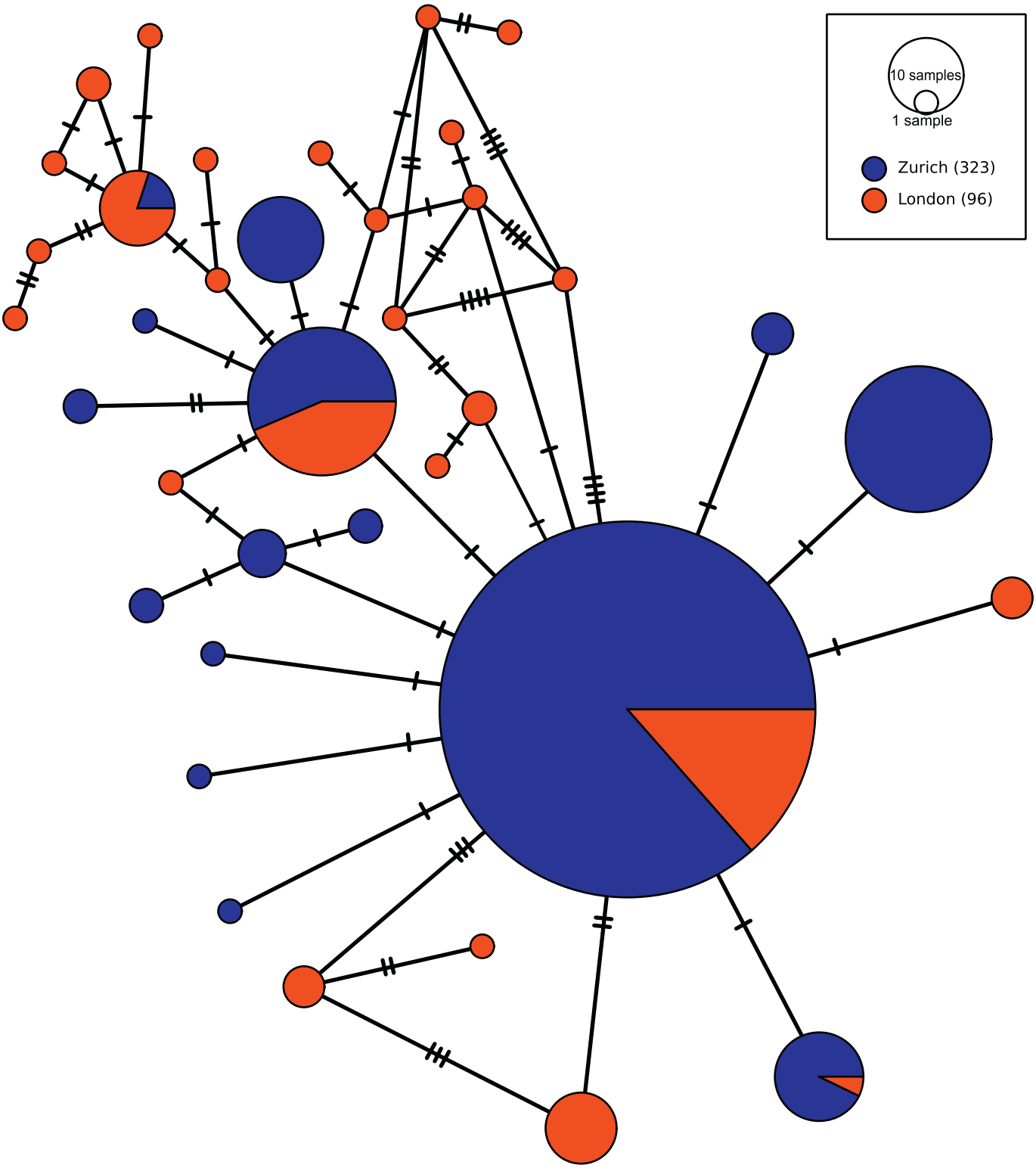

Fig. 4. Haplotype network analysis of *A. vasorum* specimens collected from foxes from Zurich (dark blue, $n = 323$) together with published London isolates (red, $n = 96$, accession numbers LT990053-LT990148) using partial mtCOI sequences. Each circle represents an individual haplotype. The diameter of a circle corresponds to the number of worms belonging to this particular haplotype. The lines on the network branches correspond to the mutation numbers between two haplotypes.

4. Discussion

In this study, the genetic analysis of a high number ($n = 323$) of *A. vasorum* nematodes recovered from foxes shot in a limited but highly endemic region of Switzerland and comprising urban, semirural and rural areas revealed high genetic diversity. We also observed high genetic diversity across multiple worms isolated from individual foxes and identified new haplotypes unique to the Zurich *A. vasorum* population. For the first time, we describe higher genetic diversity in isolates obtained from urban foxes compared to isolates recovered from rural foxes.

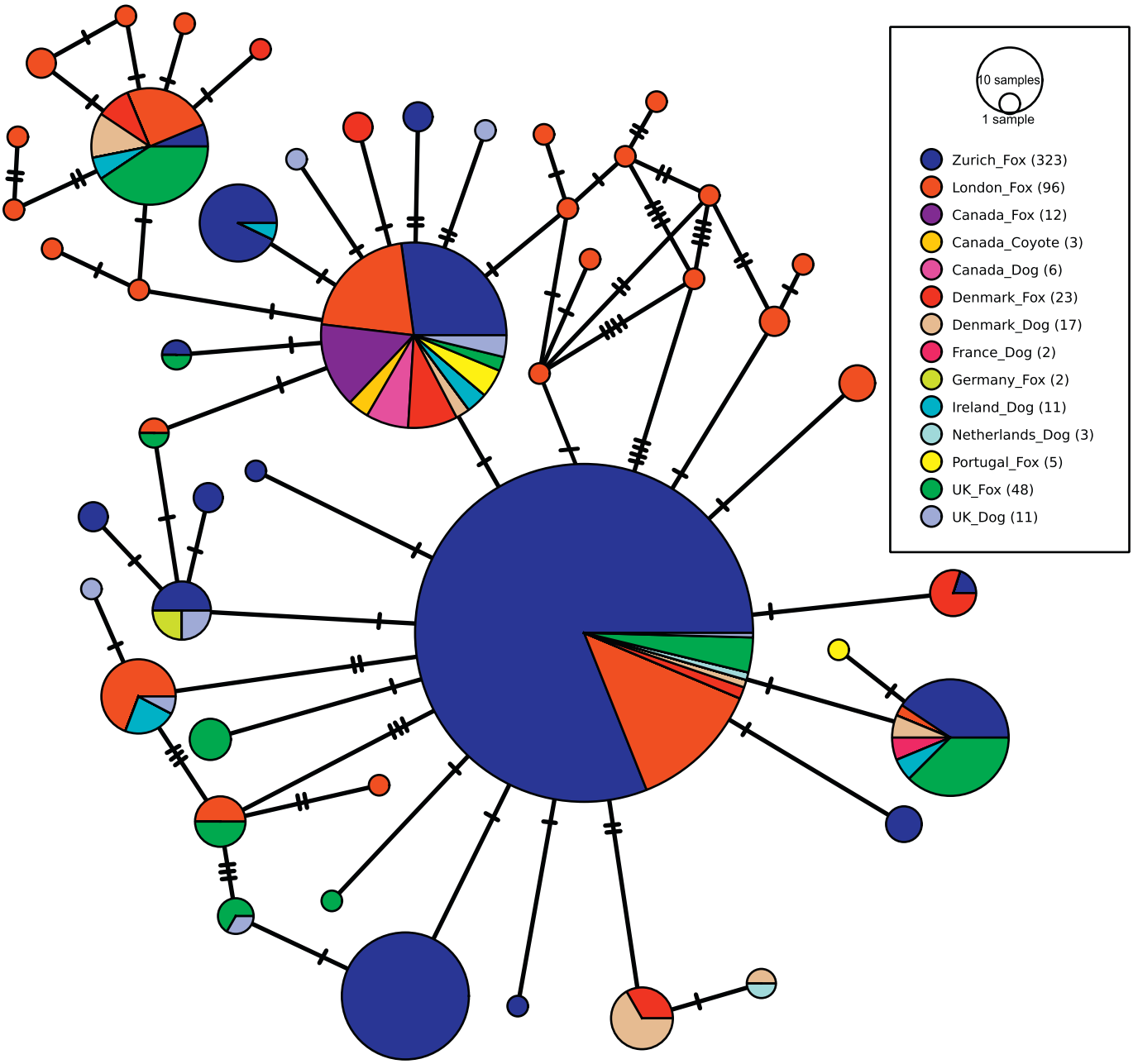

Fig. 5. Haplotype network from partial mtCOI sequences of adult *Angiostrongylus vasorum* specimens from 323 foxes shot in Zurich, along with published sequences (accession numbers LT990053–LT990148 and GQ982733–GQ982876) from Europe and Canada hosted by foxes, dogs, and coyotes ($n = 562$). The diameter of the circle is proportional to the haplotype frequency. Colors indicate in which area the host was shot. The number of lines on the network branches represents the number of mutations between two connected haplotypes.

Significant changes in the Swiss fox populations occurred between 1970 and 1990 when fox populations recovered and increased almost 4-fold after a decrease due to an epidemic of rabies and the establishment of a rabies-vaccination program (Breitenmoser et al., 2000; Gloor et al., 2001; Schweiger et al., 2007). As a consequence of the increase of the Swiss fox population, an increase of the prevalence of the zoonotic fox tapeworm *Echinococcus multilocularis* in foxes and the according intermediate hosts (Hofer et al., 2000), but also in humans (Schweiger et al., 2007) was determined. In parallel, cases of angiostrongylosis in a dog kennel in Zurich (Wolff et al., 1969) and seroprevalence studies in foxes demonstrated the presence of *A. vasorum* in the country since the 1960s and seroprevalence of 2.4% in the 1980s. Higher *A. vasorum* prevalence rates in foxes (representing the most diffused wild carnivore in Europe) than in dogs, as previously reviewed (Koch and Willesen, 2009), are confirmed for Switzerland (Gillis-Germitsch et al., 2020; Lurati et al., 2015; Sigrist et al., 2017). In agreement with the reports of increasing *A. vasorum* prevalence in Danish (Al-Sabi et al., 2014; Saeed et al., 2006) and British (Morgan et al., 2008; Taylor et al., 2015) fox populations, an impressive increase was observed in Swiss foxes, reaching a prevalence of more than 80% (Gillis-Germitsch et al., 2020). These latter data refer to the same highly endemic study area from where the here analyzed nematodes originate. We also showed that the prevalence and worm burdens of *A. vasorum* in foxes significantly increased within only five years in recent times.

Apparently, the intrinsic ability of *A. vasorum* for genetic recombination is high. Observing 16 haplotypes in nematodes of foxes of a geographically limited area such as the canton of Zurich (1729 km^2) provides further evidence of foxes as a reservoir of *A. vasorum*,

considerably contributing to the establishment and spread of the parasite in Switzerland, and playing a fundamental role in its genetics. Also, we found up to 126 specimens of *A. vasorum* in single foxes (Gillis-Germitsch et al., 2020). This allowed us to perform genetic analyses of multiple worms per fox: we overall analyzed 276 *A. vasorum* specimens obtained from 20 foxes with multiple worms (2–88 specimens per fox) among which we observed 12 haplotypes. One haplotype was however dominant, being present in 18/20 foxes and 192/279 (68.8%) of the worms. Contemporaneously, 6/20 animals harbored worms of a single haplotype, and the maximum number of haplotypes per fox was five. Remarkably, a fox from which 88 worms were isolated had only 4 haplotypes suggesting sampling depth per fox was sufficient.

From the biological and ecological points of view, favorable circumstances for high genetic diversity are given by a substantial host population contributing to the geographic spread of the parasite and, importantly, by high worm numbers within a single host. This, in turn, is only possible in hosts that are not (regularly) treated with anthelmintics (like for instance in wildlife reservoirs such as foxes), and where immunological reactions do not eliminate the infection or lead to self-limitation. Zurich city (88 km^2) is a highly populated urban area in which the fox population increased significantly (Deplazes et al., 2004). Large fox populations in cities also lead to shared territories among foxes: habitats of urban foxes are much smaller than geographic ranges of rural foxes (Gloor et al., 2001) due to a higher food abundance in cities. Concerning *A. vasorum*, as advanced, this may lead to multiple infection events, and, therefore, to higher worm burdens and higher reproductive activity of the worms. This facilitates cross-fertilization between different haplotypes, thus potentially leading to higher genetic diversity in a subsequently infected *A. vasorum* host. Our findings showed that individual foxes from urban areas had more *A. vasorum* haplotype diversity than individual rural fox hosts. F_{ST} pairwise comparison of these two populations showed a low but significant level of differentiation, suggesting constraints imposed on gene flow between urban and rural red foxes. The highly negative and significant values of Tajima's *D* and Fu's Fs neutrality tests for the urban population, being lower and not significant for the rural fox population, indicate higher genetic diversity than expected for the urban fox isolates. This was valid for young and adult foxes as well, confirming that, once infected at young age, foxes remain infected due to the lack of onset of immunity in adult animals. In contrast, higher genetic diversity in male foxes may reflect a higher prevalence of *A. vasorum* that has been previously observed in Zurich foxes and for which several reasons have been speculated (Gillis-Germitsch et al., 2020).

The role of gastropod intermediate hosts in contributing to genetic variation of *A. vasorum* is unknown. Interestingly, in the same animals and study period as for *A. vasorum*, two other lungworms of foxes, *Crenosoma vulpis* and *Capillaria aerophila,* did not increase in prevalence and worm burdens (Gillis-Germitsch et al., 2020), indicating lower parasite replication, despite a comparable life cycle with common intermediate hosts (*C. vulpis*). The reasons for these differences are speculative, but they may imply intrinsic lower genetic variation of these parasites, correlated to lower reproduction and fecundity of the parasites and/or a more efficient immunological reaction of the host.

Previous studies on genetic analysis of *A. vasorum* had extensively used mtCOI allowing the comparison of our findings to the already published sequences of Europe and Atlantic Canada (Blanch-Lazaro et al., 2018; Jefferies et al., 2010). In particular, Blanch-Lazaro et al. (2018) also showed high genetic diversity and newly observed haplotypes ($n = 26$, obtained from 96 nematodes of 59 foxes) in a geographically limited area in England (Greater London urban area) comprehending 1569 km^2. The higher haplotype diversity (Hd: 0.831) in the London area compared to the Zurich region (Hd: 0.524) might be explained by the sampling of the London worm isolates from only urban foxes, while the canton of Zurich also comprehends large semi-urban and rural areas. This, therefore, supports the hypothesis that urban fox isolates have higher genetic diversity due to the higher population density of definitive hosts. Intriguingly, the comparison of the two datasets revealed that the dominant haplotype is shared between these two study areas, suggesting a common origin. Whether this dominant haplotype presents any difference in pathogenicity (i.e. lower pathogenicity allowing rather the survival of the affected animals) compared to other haplotypes that could have contributed to its dominancy remains to be clarified. As previously observed, a high number of genetically different *A. vasorum* worms in foxes can lead to further genetic recombination, allowing rapid evolution of the parasite (Blanch-Lazaro et al., 2018). The genetic analysis of *A. vasorum*, and possibly also of other nematodes, within a specific endemic and limited area may not only furnish phylogenetic information but may also be used as an indicator of changing prevalence and parasite burdens in the definitive hosts. However, studies that correlate the infection with genetically different isolates with divergent pathogenicity are lacking. Single genetic lineages of *A. vasorum* may have more advantages over others, as recently observed by Cervena et al. (2019) with the related parasite *Angiostrongylus cantonensis*. These authors showed the importance of genetic components for the spread of *A. cantonensis*: from the uniformity of strains outside of original endemic areas they concluded that only some genetic lineages were able to emerge and become invasive outside South East Asia (Cervena et al., 2019).

Joint network analysis using published sequences of Jefferies et al. (2010) and our data also showed that the same haplotypes were shared among different carnivores including foxes, dogs and coyotes from different regions. This confirms the low host species specificity of *A. vasorum* that also includes less frequent definitive hosts, and supports interspecies transmission events (Bertelsen et al., 2010; De Liberato et al., 2017; Gillis-Germitsch et al., 2017b). The interspecies transmission between domestic dogs and wild canids has been evidenced by previous genetic analyses, confirming that the same parasite is circulating in both populations within overlapping endemic areas (Jefferies et al., 2009; Jefferies et al., 2010).

In parallel to fox prevalence, cases of *A. vasorum* infection in dogs have increased in recent years in several European countries such as Denmark (Willesen et al., 2009), Great Britain (Kirk et al., 2014), Italy (Guardone et al., 2013) and Switzerland (Sigrist et al., 2017). The increasing and close presence of foxes in our immediate and recreational surroundings pose a risk of infection for domestic dogs due to continuous exposure to fox parasites, including *A. vasorum* (Deplazes et al., 2004; Otranto et al., 2015). Habitats of foxes and dogs are frequently shared (Gloor et al., 2001); this increases transmission events and the risk of infection for dogs. Especially in the study area, the canton of Zurich, overlapping and dense fox habitats lead to high infection pressure for dogs. As a consequence, increasing cases of canine angiostrongylosis have been observed at the Small Animal Hospital (Glaus et al., 2016; Sigrist et al., 2017) and the Diagnostic Unit of the Institute of Parasitology (M. Schnyder, personal communication) of the Vetsuisse Faculty in Zurich. This is of relevance, as the parasite can have a strong impact on a dog's health.

Several genomic markers have been used to investigate the genetic diversity of *A. vasorum* populations and comparable metastrongylid populations in carnivores. The two mainly used genomic loci are the second internal transcribed spacer (ITS-2) region and the mtCOI gene (Blanch-Lazaro et al., 2018; Jefferies et al., 2009; Traversa et al., 2017). DNA sequences of mitochondrial genes usually present higher variability than nuclear ribosomal DNA and are therefore considered more efficient for studying populations genetics of parasitic nematodes of the same species (Blouin, 2002). This made the mtCOI gene an ideal candidate to study genetic diversity among geographically limited *A. vasorum* populations, where higher similarity is expected among isolates (Gasser et al., 2012; Hu et al., 2004; Jefferies et al., 2010).

Our study could have benefitted from additional genetic analysis using *A. vasorum* isolates from domestic dogs. Our attempts to get mtCOI gene sequences of collected L1 worms of dogs produced errors (data not shown) and could therefore not be further used for analysis. Problems

were attributed to bacterial and fungal contamination of the samples, their age as well as difficulties of washing a single larva properly. Therefore, only *A. vasorum* adult worms could be used, i.e. from foxes, because getting adults from naturally infected dogs is highly challenging.

Furthermore, markers such as ITS-2 have been used to analyze the genetic diversity among geographically wider hence presumably genetically more distantly related *A. vasorum* populations and were, therefore, able to separate two distinct clades of *A. vasorum* (Jefferies et al., 2009) and to distinguish between isolates from different countries (Jefferies et al., 2010). Due to its internal repetitive regions, ITS-2 sequencing requires prior cloning, making it laborious to obtain sequences for a high number of specimens. In our small scale experiment with ITS-2 of 33 (23 L1 from dog hosts and 10 L1 from fox hosts) sequences of *A. vasorum* samples originating from different cantons in Switzerland were compared to each other and analyzed (Schnetzler, 2019). These results had shown a very high number of haplotypes ($h = 15$) with a haplotype diversity value of 0.676. While only one haplotype was found to be shared between two different hosts, there was no pattern in sequences between the two. In this study, we opted to obtain sequences from as many worms as possible to detect rare haplotypes, making mtCOI the most suitable marker. Importantly, using different genetic markers contemporaneously may yield conflicting phylogenetic groups (Kumar et al., 2012) and different haplotype networks (Jefferies et al., 2010). Therefore, it would be important to use several genetic markers in the future, especially when the sampling regions are expanded. Currently, genome sequences of *A. vasorum* are missing. When the genome will become available, it may be utilized for the discovery and development of novel microsatellite markers as a better alternative for population genetic studies of different host isolates.

5. Conclusions

Our work provides evidence of the high genetic diversity in *A. vasorum* populations obtained from foxes in a geographically limited area: we found a high haplotype diversity and present new haplotypes that have not been identified before.

The findings support the potential for multiple infection events and identify foxes as a highly relevant reservoir for genetic exchanges among the parasites. In particular, our analyses revealed a higher haplotype diversity in urban foxes compared to rural fox isolates, with higher intra-individual variability among urban foxes. Urban areas offer abundant and easily accessible food availability and safety from interspecific competitions and hunters (due to strict hunting regulation near residential areas), facilitating higher fox densities in a smaller home range, and therefore higher rates of genetic exchange in parasites of urban foxes. The urban fox phenomenon and habitats suitable for intermediate hosts additionally overlapping i.e. with recreational areas where dogs are walked (Deplazes et al., 2004) are supposed to substantially contribute to the infection of dogs. However, more studies are needed especially with dog isolates to better understand the interspecies transmission of *A. vasorum* parasites and the emergence of novel haplotypes. Importantly, it appears that the spread and establishment of this clinically relevant parasite for dogs cannot be halted. Therefore, knowledge of occurrence, biology, and epidemiology of *A. vasorum* are fundamental.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.meegid.2020.104618.

Funding

Funding was partially provided by Bayer Animal Health GmbH, Leverkusen, Germany.

Ethical standards

Ethical standards were fulfilled.

Declaration of Competing Interest

The authors declare no competing interests.

Acknowledgments

We would like to acknowledge Annina R. Guggisberg, Philipp A. Kronenberg, Deborah Joekel, and Yasemin Yaman for helping with the fox and lung dissections, Cristian A. Alvarez Rojas for support with the fox necropsies and cloning procedures.

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