Population genetic structure of the parasitic nematode *Spirocerca lupi* in South Africa

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Highlights

• Genotyped 9 microsatellites in 130 *Spirocerca lupi* nematodes from three regions.

• *Spirocerca lupi* worms disperse effectively over long distances (>100 km).

• Worms from different hosts amalgamate frequently.

• Inbreeding stems from matings between a few worms in each host.

• Worms from a host are more closely related than worms between hosts.

Graphical Abstract
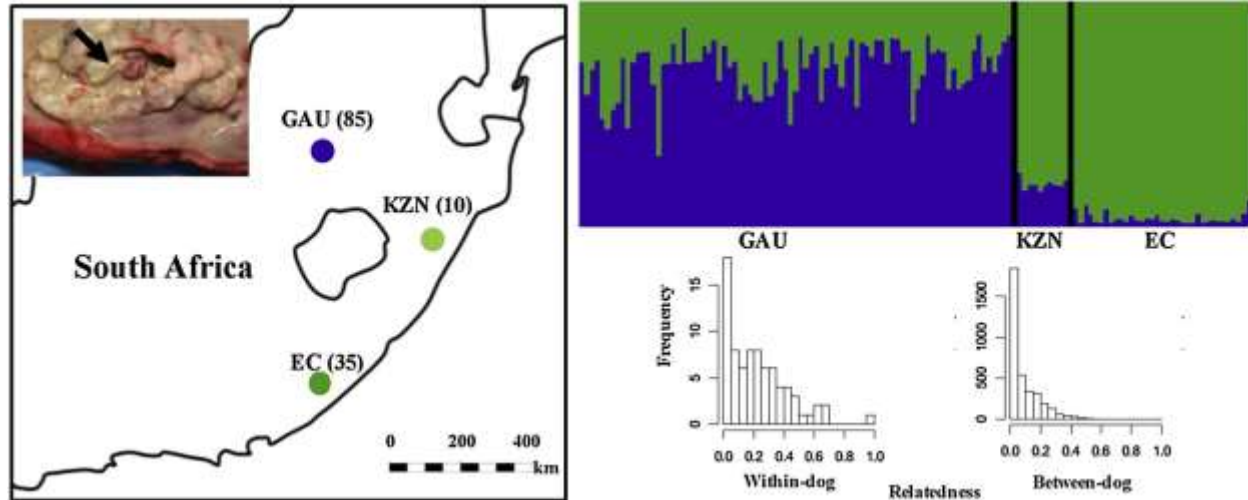
ABSTRACT

Spirocerca lupi is a parasitic nematode of canids and occurs in most tropical and subtropical regions around the world. While its life cycle is well known, insight is lacking about its mating structure within-hosts, genetic variability and long-distance dispersal ability. These characteristics contribute significantly to the dynamics and spread of potential resistance genes, which impacts on the control of *S. lupi*. To evaluate the population structure and infer potential mating behaviour of *S. lupi*, we genotyped 130 samples at nine microsatellite loci from three geographical locations in South Africa, between 600 and 1000 km apart. These loci identified unique individuals with high levels of polymorphism suggesting that these are not newly established *S. lupi* populations in South Africa and that effective population sizes must be large. Population genetic analyses showed that populations are not very distinct, that worms within dogs are more similar to each other than random worms from each population, and that mating is at random within dogs. We can thus infer that the parasite is frequently transported over great distances. Even so, two genetically distinct populations could be identified. Relatedness of

worms within dogs were significantly higher than between dogs and together with F-statistics suggests some non-random transmission of parasites between hosts. While mating is random within a host, parasites from a host are more likely to be related and hence an increase in homozygosity is seen. The implications of this genetic structure on parasite control are considered.

Keywords: canids, gene flow, microsatellites, F-statistics, population structure, relatedness

1. Introduction

The canine oesophageal worm, *Spirocerca lupi*, is a parasitic nematode that infects carnivores, predominately the domestic dog (Bailey, 1972; Soulsby, 1982). It is important because of the poor prognosis in the untreated final host (Oryan et al., 2008; van der Merwe et al., 2008) and because it occurs on all continents except Antarctica (Fitzsimmons, 1960; van der Merwe et al., 2008). Potential increases in prevalence (Mazaki-Tovi et al., 2002; Kok et al., 2011; Aroch et al., 2015) and distribution (Al-Sabi et al., 2014; Giannelli et al., 2014; Wright et al., 2016) add to the potential threat posed by *S. lupi*. Knowledge of *S. lupi*'s life cycle has been exploited to control the parasite (Bailey, 1972; van der Merwe et al., 2008). By augmenting this knowledge with a molecular population genetic approach, we can quantify transmission dynamics of the parasite further and predict how resistance may develop and spread (Criscione et al., 2010; Beesley et al., 2017).

Transmission of the parasite can be prevented at two points in its life cycle. The life cycle has been summarized by many (Bailey, 1972; Soulsby, 1982; van der Merwe et al., 2008) as follows – eggs of *S. lupi* are released in the faeces of the final host (canine). If these eggs are

ingested by dung beetles that are the intermediate host, the eggs develop to the third larval stage. To continue the life cycle, infected beetles must be eaten by either the final host or a paratenic host (including lizards, poultry, various wild birds and small mammals). If a paratenic host eats the larvae, they will briefly excyst but then re-encyst. If the final host consumes an infected paratenic or intermediate host, larval development continues and is completed. The adult worms live within nodules in the oesophageal wall of the definitive host. Here they mate and females lay eggs in the oesophageal lumen that pass out in the host faeces. The lifecycle can thus be broken by picking up dog faeces, which severs the link provided by dung beetles (van der Merwe et al., 2008) and by not feeding raw remains of potential paratenic hosts to dogs, which eliminates the link that paratenic hosts play (Bailey, 1972).

Any single or combination of the hosts - intermediate, paratenic and final, can disperse the parasite; but how effective this dispersal is, and over what distances they travel are unclear. In a study on *S. lupi* that included samples from a 25km x 15km area, the mitochondrial cox1 gene showed no geographical structure suggesting that dispersal is in excess of 10's of kms (de Waal et al., 2012). To quantify dispersal over longer ranges, samples need to be compared from more distant localities (Ebbs et al., 2016).

Any *S. lupi* population is divided into smaller populations confined to hosts, so-called infrapopulations (Margolis et al., 1982). While worms are not able to disperse by themselves, their hosts' dispersal and feeding behavior will determine the worms' population structure. Infrapopulations can be admixed when a host feeds on more than one infected intermediate or paratenic host or when a beetle consumes infected faeces from more than one infected dog. If in this way, eggs and/or larvae are sufficiently mixed during infection all infrapopulations would form one genetic population. On the other extreme, if eggs and/or larvae from different sources

never mix, then each infrapopulation is a genetic unit called a deme. These are two extremes between which reality lies. As a result, the fraction of genetic variation between and within infrapopulations depends on the amount of mixing (Nadler, 1995; Hedrick, 2000; Paterson et al., 2000; Prugnolle et al., 2002; Théron et al., 2004; Criscione et al., 2005, 2010; Prugnolle et al., 2005a, 2005b; Criscione and Blouin, 2006; Steinauer et al., 2010; de Waal et al. 2012). When there is limited mixing of infrapopulations, genetic drift will cause infrapopulations to become different from one another over time (Wright, 1931). Such genetic drift will increase differences between infrapopulations and will reduce the genetic variation within infrapopulations (Wright, 1931). The impact of genetic drift is more severe when populations are small (Wright, 1931) and *S. lupi* has small infrapopulations. The harmonic mean population size in the intermediate host is only 6 individuals at the most (du Toit et al., 2008; de Waal et al., 2012) and the mode is between 6 and 12 for the final host (Fitzsimmons, 1960). Random mating within the infrapopulation will result in a Hardy-Weinberg equilibrium within the infrapopulation. However, since members of the infrapopulation are related, such random mating will inevitably result in an excess of homozygosity at the population level.

de Waal et al. (2012) inferred frequent admixture would be required to explain the high mitochondrial diversity observed in infrapopulations. Since mitochondria are maternally inherited de Waal et al. (2012) only measured the female population's dynamics and could not reveal the degree of inbreeding in worms. Nuclear microsatellites can overcome these shortcomings of mitochondrial DNA.

Intermediate levels of admixture among small infrapopulations results in a mixture of matings between unrelated individuals on the one side and between relatives on the other (mixed mating system). The consequence of such inbreeding is that even rare recessive alleles can be

homozygous and selection can favour such alleles (Wright, 1921). In the context of parasites, this means that even if resistance alleles are recessive, they can be selected for and increase rapidly (Criscione et al., 2010; Beesley et al., 2017). Co-dominant nuclear markers such as microsatellites can be used to quantify inbreeding as well as relatedness between worms.

To evaluate the population structure over a large spatial scale in South Africa and to provide insight about relatedness between worms between and within hosts, we did a population genetics study of 130 worms from 47 dogs from three populations that are between 600 and 1000 km apart using nine previously developed microsatellites (Reid et al., 2015).

2. Materials and methods

2.1. Sample collection, DNA extraction and microsatellite amplification

Veterinarians collected *S. lupi* samples from necropsies of naturally infected *Canis familiaris* which had passed away from complications of *S. lupi* infections in three populations in South Africa from 2005 to 2009. The populations are in three different provinces namely, Gauteng (Tshwane Metropole - 85 specimens), Kwa-Zulu Natal (Durban - 10 specimens) and the Eastern Cape (Grahamstown - 35 specimens) (Fig. 1A). In order to quantify within-dog variation, three worms from each dog were genotyped, where possible. We will refer to the three different regions as populations and to worms from different host dogs as infrapopulations.

We extracted DNA from tissue samples not exceeding 25mg from each worm with the Qiagen DNeasy blood and tissue kit (Qiagen, California) following the manufacturers specifications. Nine microsatellite loci were amplified and genotyped in all specimens in a single diagnostic multiplex as described in Reid et al. (2015).
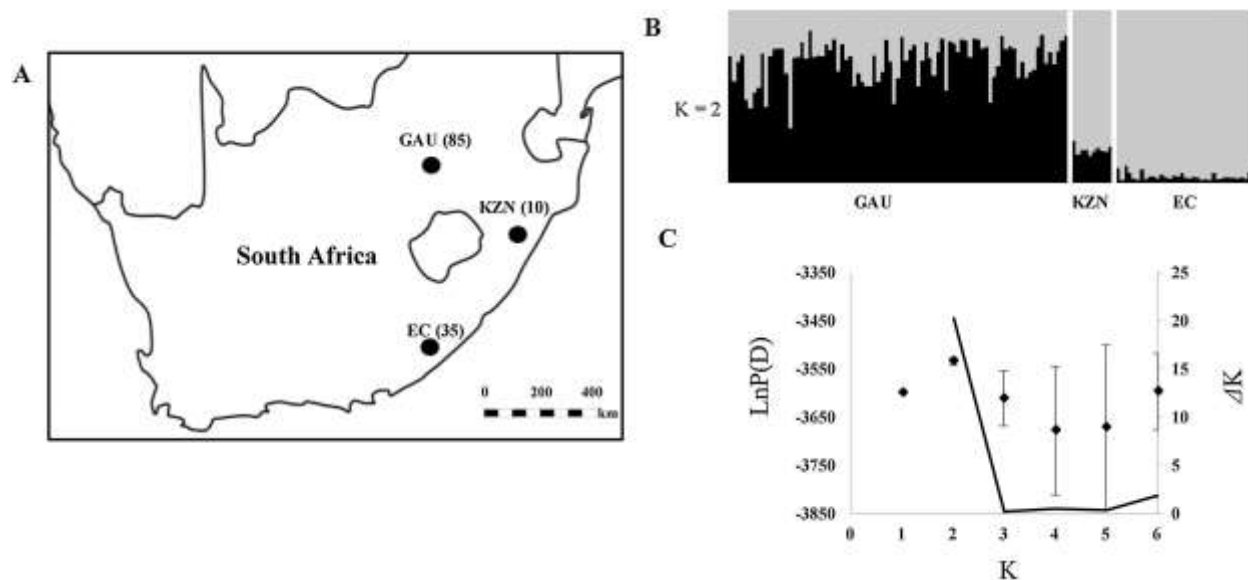
Fig. 1. Sampling locations and geographic structuring of *Spirocerca lupi* from South Africa. A) Map of South Africa indicating the sampling locations and number of worms per population GAU – Tshwane metropole in Gauteng, KZN – Durban in Kwa-Zulu Natal and EC – Grahamstown in the Eastern Cape. B) STRUCTURE plot of K = 2 C) Likelihood (LnP(D), filled circles and error bars) and Delta K (ΔK, solid line) plots.

Microsatellite loci were genotyped on an ABI3500 at the sequencing facility of the University of Pretoria (South Africa). Twenty-two individuals were re-genotyped to estimate scoring error and to confirm the consistency of migration of individual alleles. We scored microsatellite loci using GENEMARKER version 2.4.0 (SoftGenetics, State College, Pennsylvania, USA).

2.2. Data analysis

We calculated several summary statistics in GENETIX version 4.05.2. (Belkhir et al., 1996-2004) and included the number of alleles, expected heterozygosity (H_E), observed heterozygosity (H_O) and Weir and Cockerham's (1984) inbreeding coefficient (F_{IS}) for each population and over all populations. Allelic richness (A_R) was calculated in FSTAT version 1.2

(Goudet, 1995) to compare levels of genetic diversity taking into account the difference in sample sizes.

The geographic structuring of *S. lupi* between populations was assessed by calculating genetic differentiation (F_{ST}; Weir and Cockerham, 1984) in GENETIX with 200 permutations, as well as Jost's *D* between the sampling locations using SMOGD version 1.2.5 (Crawford, 2010).

We used analysis of molecular variance (AMOVA), as implemented in ARLEQUIN version 3.5.1 (Excoffier and Lischer, 2010), to calculate *F*-statistics with the default locus-by-locus method (see supplementary text for interpretation of *F*-statistics). Four infrapopulations each containing a single worm with one or more loci that failed were excluded. We calculated these statistics at all three levels of hierarchical structure of alleles - individuals, dogs (or infrapopulations) and populations (three localities). The data was recoded in R (R Core Team 2016) as explained in Meirmans (2006) to calculate $F_{SC(max)}$ and $F_{CT(max)}$. These can then be used to calculate corrected measures: $F'_{SC} = F_{SC}/F_{SC(max)}$ and $F'_{CT} = F_{CT}/F_{CT(max)}$.

The correlation between G_{ST} (an F_{ST} analogue) and H_S over loci was calculated using CoDiDi (Wang, 2015) and a Pearson's product-moment correlation in R (R Core Team, 2016). First, we considered the role of mutations at a local level by analysing the two larger populations (Grahamstown and Tshwane Metropole) separately, and taking into account divisions into infrapopulations. Second, we estimated the role of mutations at a larger scale by combining all three populations.

In addition, we used STRUCTURE version 2.3.4 (Pritchard et al., 2000) to assess population structure by using a Bayesian approach to infer the most likely number of populations. We used an admixture model, with sampling locations as prior (Hubisz et al., 2009) and correlated allele frequencies. Runs for $K=1-6$ were carried out each with 2 000 000 Markov

Chain Monte Carlo iterations allowing a burn-in period of 200 000 iterations. We kept default values for all other parameters. The program STRUCTURE HARVESTER (Earl and von Holdt, 2012) was used to apply the Evanno method (ΔK, Evanno et al., 2005) to detect the value of K, the number of genetic groups that best fit the data.

We estimated inbreeding coefficients separately for each of the two larger populations with the trio maximum likelihood method in COANCESTRY (Wang, 2011) to avoid a Wahlund effect. We calculated relatedness between worm pairs from the two populations with larger sample numbers (Tshwane Metropole and Grahamstown). Since there may be a Wahlund effect between these localities, relatedness was calculated for each population separately estimating allele frequencies from the data. We used Wang's (2011) trio maximum likelihood estimate as implemented in COANCESTRY (Wang, 2011) which accounts for inbreeding. We separated relatedness estimates into two categories - between worms from the same dog host (R_w) and between worms from different dog hosts (R_b). To determine if the R_w estimates are significantly higher than R_b, we permuted relatedness values 10 000 times randomly to the same number of within and between dog comparisons to obtain a null distribution of the difference in R (R Core Team, 2016). We compared the actual difference to this null distribution.

3. Results

The diversity estimates are summarized in Table 1 and indicated similar levels of heterozygosity between locations. The average observed heterozygosity was slightly lower than the expected heterozygosity in the largest population, while the small difference in these parameters were in the opposite direction for the two smaller populations. While the number of

Table 1. Summary of standard population genetics analyses for each population of *Spirocerca lupi*.

Locus	Total[a]					Tshwane (n=85)					Durban (n=10)					Grahamstown (n=35)				
	N_A	A_R	H_E	H_O	F_{IS}	N_A	A_R	H_E	H_O	F_{IS}	N_A	A_R	H_E	H_O	F_{IS}	N_A	A_R	H_E	H_O	F_{IS}
SL02	9	4.7	0.68	0.65	0.05	9	4.9	0.72	0.68	0.05	3	3	0.56	0.75	-0.29	5	3.9	0.57	0.56	0.03
SL04	6	3.6	0.47	0.47	0.02	6	3.8	0.45	0.43	0.04	4	3.8	0.51	0.44	0.19	4	3.4	0.51	0.54	-0.06
SL06	8	5.4	0.79	0.65	0.19	8	3.6	0.79	0.66	0.18	4	3.6	0.51	0.7	-0.33	7	4.9	0.74	0.62	0.18
SL10	10	4.9	0.73	0.6	0.18	9	3.7	0.76	0.58	0.24	4	3.7	0.42	0.4	0.09	6	4.7	0.66	0.71	-0.05
SL13	9	4.8	0.68	0.73	-0.07	9	5	0.67	0.74	-0.09	5	5	0.66	0.75	-0.06	5	4.5	0.67	0.7	-0.02
SL14	18	7.1	0.83	0.86	-0.03	17	7.8	0.83	0.83	0.01	8	7.8	0.85	1	-0.15	7	4.8	0.72	0.88	-0.21
SL15	13	5.1	0.56	0.47	0.16	12	5.9	0.67	0.57	0.16	4	3.7	0.37	0.44	-0.12	5	2.5	0.21	0.23	-0.06
SL17	9	5.2	0.77	0.63	0.19	9	5.5	0.77	0.69	0.11	5	4.7	0.68	0.5	0.31	4	3.8	0.72	0.52	0.3
SL18	7	5	0.78	0.88	-0.13	7	5.2	0.78	0.87	-0.1	4	3.9	0.66	0.9	-0.33	6	4.6	0.75	0.91	-0.21
All[b]	9.8	-	0.7	0.66	-	9.5	-	0.72	0.67	-	4.5	-	0.58	0.65	-	5.4	-	0.62	0.63	-

N_A: number of alleles; A_R: Allelic Richness, based on 8 samples; H_E: expected heterozygosity; H_O: observed heterozygosity; F_{IS}: inbreeding coefficient.

[a] Total values are calculated per locus over all samples.

[b] The last row indicates the mean values calculated over all the loci. A_R is based on 8 samples.

alleles was the highest for the largest sample, allelic richness indicated similar levels of diversity between populations (Table 1).

Weir and Cockerham's (1984) F_{ST} estimates suggest that while the three populations are genetically distinct, the differences are small. Specifically, between Gauteng and Kwa-Zulu Natal, $F_{ST} = 0.037$ (p < 0.001), between Gauteng and Eastern Cape, $F_{ST} = 0.035$ (p < 0.001) and between Kwa-Zulu Natal and Eastern Cape, $F_{ST} = 0.045$ (p < 0.001). Jost's D estimates suggested similar differences except that the Kwa-Zulu Natal-Eastern Cape comparison was smaller than the other two (Gauteng and Kwa-Zulu Natal 0.043, Gauteng and Eastern Cape 0.044 and between Kwa-Zulu Natal and Eastern Cape 0.029).

While F_{IS} was slightly negative it was not significantly different from zero (Table 2). On the other hand, F_{IT} was significantly larger than zero suggesting that either infrapopulations and/or populations are sufficiently different from one another to cause a Wahlund effect (Table 2). The AMOVA suggests that this is predominantly caused by differences between infrapopulations because F_{SC} and F'_{SC} are respectively substantially larger than F_{CT} and F'_{CT} (both F_{SC} and F_{CT} are significantly larger than zero, Table 2). The correlation between diversity (H_S) and differentiation (G_{ST}) as calculated by CoDiDi (Wang, 2015) showed no significant negative correlations either from worms within dogs in Tshwane metropole (ρ = -0.231, $p = 0.55$) or in Grahamstown ($\rho = 0.019$, $p = 0.96$) or comparing populations ($\rho = 0.118$, $p = 0.76$). The lack of significance suggests that demographic processes outweigh mutation. The corrected estimate of F_{CT}, $F'_{CT} = 0.095$ is three times higher (Table 2). Together, these data suggest that allele frequencies between populations are so similar that long-distance dispersal occurs frequently; that while there is admixture of infrapopulations, it is infrequent enough to render infrapopulation allele frequencies different from one another. As a result, even though worms

mate randomly within hosts, they do not appear to mate randomly within the population and at this level some inbreeding occurs.

Table 2. *F*-statistic estimates from AMOVA, without and with a correction for the maximum estimates based on recoded data different from 0.

F-statistic	Estimate	Corrected estimates
F_{IS}	-0.064	
F_{SC}	**0.100**[a]	$F'_{SC} = 0.265$
F_{CT}	**0.030**	$F'_{CT} = 0.095$
F_{IT}	**0.071**	

[a] bold indicate estimates significantly different from 0.

STRUCTURE identified two clusters, one consisting of the Gauteng samples and the other of the Eastern Cape and Kwa-Zulu Natal samples, with no additional sub-structuring observed (Fig. 1B and Fig. 1C).

Individual inbreeding coefficients (*F*) for the 85 worms from Tshwane metropole and 35 from Grahamstown were similar with means of 0.126 and 0.107 and varied between 0 and 0.646, and 0 and 0.756, respectively (Fig. 2). Note that while some worms are indeed inbred, the majority have very low inbreeding coefficients and the distribution is multimodal (Fig. 2). These observations suggest a mixed mating system.

The mean estimates of relatedness for the 78 R_w and 3492 R_b values in Tshwane metropole were 0.22 and 0.08 respectively, and closely resembled estimates from Grahamstown, 0.21 and 0.12 respectively. Relatedness of worms from the same dog were significantly higher than relatedness of worms from different dogs for both localities (permutation test: $p < 0.0001$; Fig. 3). Relatedness values within dogs varied with modes that may correspond to unrelated individuals, half sibs and full sibs (Fig. 3a & 3b). The relatedness of worms within dogs were

almost twice the coefficient of kinship (or F_{SC}, 0.215 ~ 2x0.100) as expected (Pamilo and

Crozier, 1982).
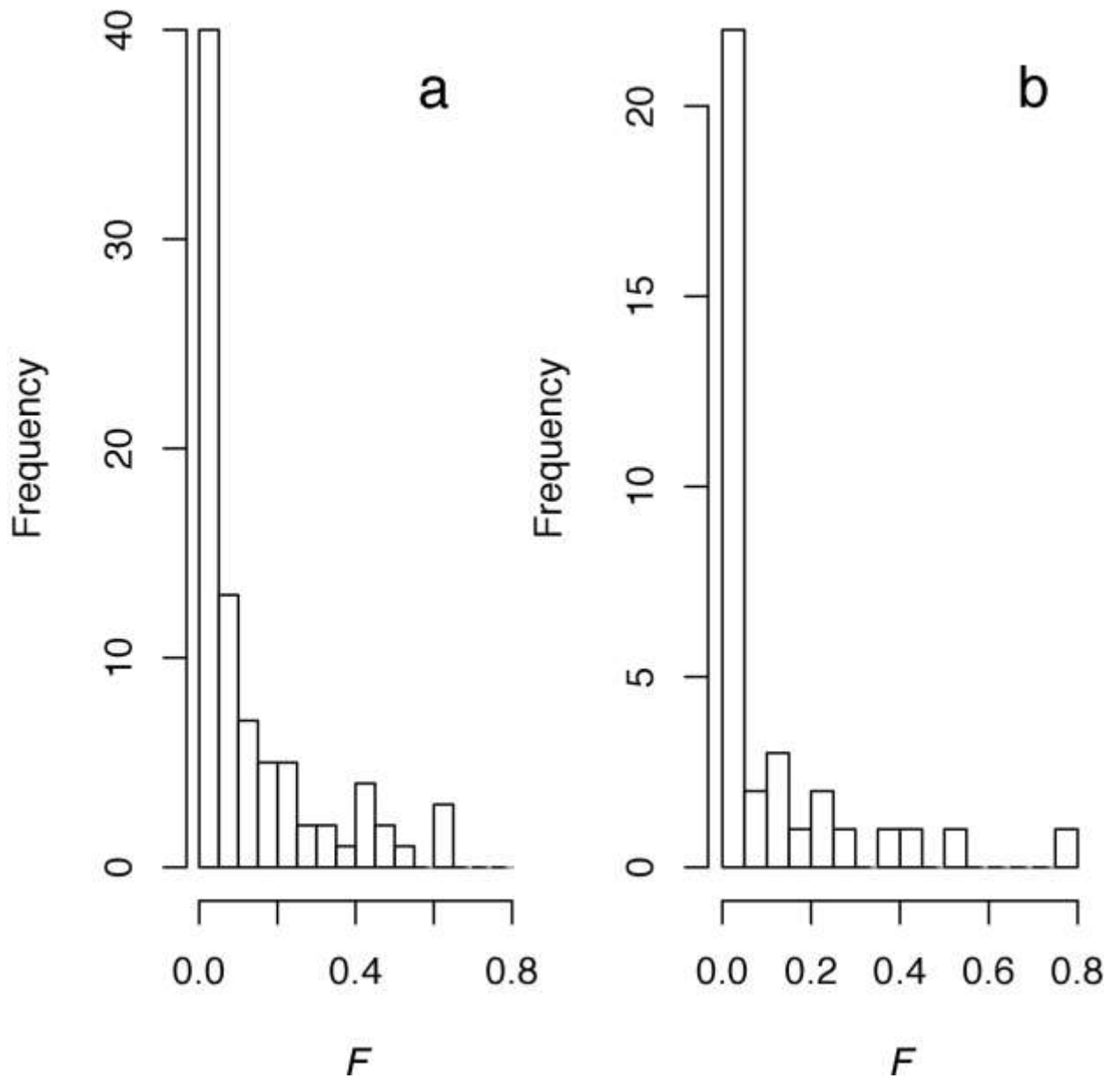
Fig. 2. Individual inbreeding coefficients of *Spirocerca lupi* worms using COANCESTRY for two populations: (a) Gauteng, (b) Eastern Cape.
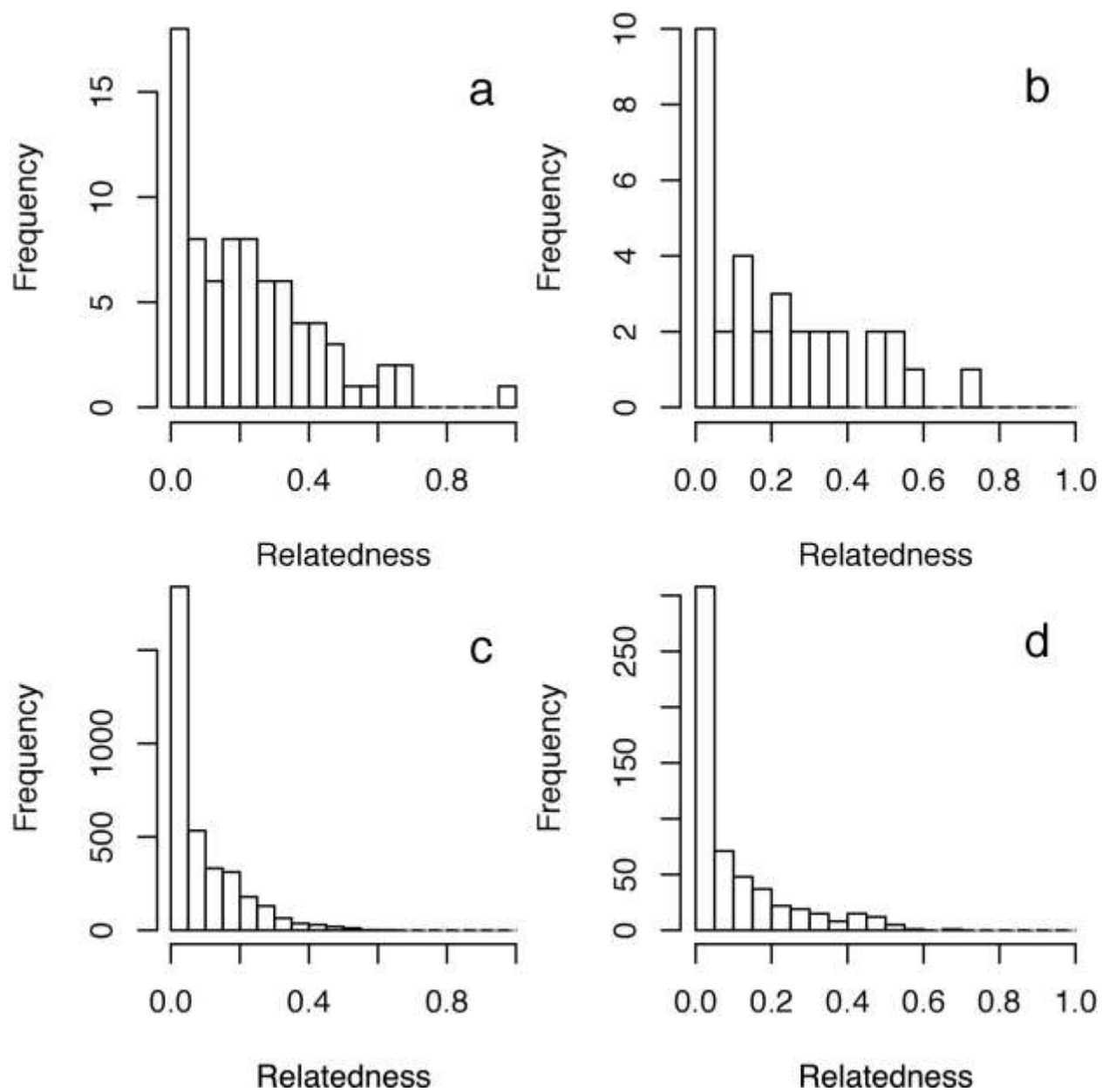
Fig. 3. Relatedness of pairs of *Spirocerca lupi* worms drawn from the same (R_w, a & b) and drawn from different

dogs (R_b, c & d) from Gauteng (a & c) and Eastern Cape (b & d).

4. Discussion

In combination, the data suggests a genetically variable population with effective dispersal over long ranges (100's of km). While worms from different dogs are significantly different from one another, admixture and dispersal must occur frequently. The facts that worms from some infrapopulations are related whereas others are not, and that some worms are inbred whereas others are not, is consistent with the idea that *S. lupi* has small infrapopulations that undergo frequent admixture.

The high variation observed here at nuclear markers in combination with mitochondrial data (de Waal et al., 2012) suggests a persistent and large population. The first archaeological evidence of domestic dogs in southern Africa dates back to some 1500 years ago (Greyling et al., 2004) and our data do not contradict the view that *S. lupi* may have been part of this association from the very beginning.

The similarity between populations may reflect the mutational process rather than high levels of migration, but the CoDiDi analyses cast doubt on this conclusion. F'_{CT} is substantially higher than F_{CT}, but suggests 7.5 migrants per worm generation (= (1 - 0.095)/(4 x 0.03); Meirmans and Hedrick 2011). While this estimate relies on unrealistic assumptions (Whitlock and McCauley 1999), the implication is high gene flow between populations. Given that the infrapopulation sizes are between 6 and 12 worms (Fitzsimmons, 1960; du Toit 2008), it means that at least one infected host needs to migrate between these localities per worm generation. Similar high gene flow was observed for *Fasciola hepatica* (Beesley et al., 2017) where hosts are routinely moved over large distances. Since parasite gene flow relies on host movement (Criscione et al., 2005; Ebbs et al., 2016), the implication is that dogs and/or beetles are mobile.

STRUCTURE analyses and Jost's *D* supports a view of low differentiation between populations. These findings show that if a new mutation conferring drug resistance arose it will rapidly spread over distances of 100's of kilometers.

The tussle between genetic drift, which reduces infrapopulation genetic variation, and admixture, which has the opposite effect, suggests frequent admixture but not panmixia. A lack of panmixia at a population level is reflected in the significant value of F_{SC} (= 0.100) that measures the differentiation between infrapopulation within a population. De Waal et al.'s (2012) estimate of φ_{SC} (equivalent of F_{SC}) = 0.272 for a mitochondrial gene translates to 0.068 (=0.272/4) for nuclear genes. It also suggests significant structure at the level of infrapopulations. This lack of panmixia can stem from either hosts being sampled from isolated subpopulations or from clumped transmission of infrapopulations. A lack of local population structure (de Waal et al. 2012) and low F_{CT} between populations suggest that isolated subpopulations do not exist and that F_{SC} stems from clumped transmission of infrapopulations. The fact that within host relatedness is higher than between host relatedness further supports a view that infrapopulations are not one panmictic entity. The infrapopulations of *S. lupi* admix less than those of several other parasites (Criscione and Blouin 2006; Criscione et al., 2010; Johnson et al., 2006; Beesley et al., 2017). While populations are not panmictic, frequent admixture will ensure that new resistant mutations can effectively spread locally.

The AMOVA estimate of F_{IS} that is not different from zero (Table 2) suggesting that mating is at random within the final host. However, it is not the case at the population level since mating is restricted to worms from the same dog host. This conclusion is clear from individual inbreeding coefficient estimates with averages of 0.07 and 0.126, which straddles the F_{SC} estimate of 0.100. The life cycle and multimodal nature of individual inbreeding values (Fig. 3)

suggest a mixture of in- and outbreeding. Hence, heterozygosity at microsatellite loci reflects inbreeding history (Greeff et al., 2009). This level of inbreeding means that if a recessive resistant allele arises, it can be homozygous even when rare and can increase due to selection (Wright, 1921; Criscione et al., 2010; Beesley et al., 2017).

Similar to some internal parasites (Criscione et al. 2007; Steinauer, 2009), *S. lupi* shows no indication of a preference for, or an aversion to inbreeding. While this observation is not surprising for parasites that are restricted to find mating opportunities within a host, it is certainly not universal (Beltran et al., 2008; Beesley et al., 2017). As is expected for internal parasites (Steinauer, 2009), *S. lupi* did not suffer from inbreeding depression.

5. **Conclusion**

Our study has shown that selection can favour recessive resistance alleles, and that resistance alleles can spread effectively over short and long ranges.

Supplementary material

Supplementary data S1: Excel file of data.

Supplementary text: The use and meaning of F-statistics

Data accessibility

All genotyped microsatellite data is available in the supplementary material

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