Chapter 1

Literature review

1.1. INTRODUCTION

Diseases caused by parasites are studied worldwide in order to reduce the risk of infection and prevent the spread of alleles that confer resistance to treatment. The utilisation of molecular biology tools has had important implications for the study of disease processes and particularly, the epidemiology of diseases. Molecular epidemiology is a field that emerged in the 1970s and has evolved rapidly to include genetics as a tool to study the dynamics of a disease in a population. Thus the term 'genetic epidemiology' has been introduced in several studies to describe the potential of studying genes to describe disease processes (Tibayrenc 1998). This allowed scientists to infer evolutionary history from the observed genetic differences among sampled individuals and made it possible to genotype individuals anywhere in the world (Restif 2009).

The genetic structure of a parasitic population can be studied to answer questions pertaining to the dynamics of the population. For instance, the structuring of genetic variation allows us to estimate population processes such as dispersal, mating behaviour, transmission dynamics and the formation of drug resistant genotypes (Criscione *et al.* 2005; Steinauer *et al.* 2010). In epidemiology studies it is used to gain insight into disease progression and to determine how traits for genetic resistance to anti-parasitic agents are spread through the population (Paterson & Viney 2000; Prugnolle *et al.* 2005a).

The genetic variation that is found in populations of individuals is influenced by the biology and environment of the individuals throughout their lifespans. This includes reproductive success, migration, population size, natural selection and historical events (Sunnucks 2000). Population genetic models are applied to investigate patterns between genetic variants and demographic features, which can then be used to infer the biology of an organism (Sunnucks 2000). Individual processes produce effects at the population level, and through the investigation of these processes much insight can be gained on evolutionary systems within a species.

Important questions can be addressed concerning the epidemiology, fine-scale genetic structure and transmission dynamics of a parasitic population using co-dominant genetic markers. Thus, the design and application of microsatellites as genetic markers will be explored and the statistical analysis tools that can be utilised to evaluate the data will also be examined. Parasites are highly diverse in that they have evolved

distinctive lifecycles, modes of reproduction, host species and ecosystems (Criscione *et al.* 2005). Thus integrating all these aspects with information on the genetic structure improves the understanding of parasite epidemiology.

Little is understood about the transmission dynamics of the parasitic nematode *Spirocerca lupi* that causes canine spirocercosis. The incidence of spirocercosis has reportedly increased in recent years in South Africa (personal communication with local veterinarians in the Tshwane Metropole and pathologists from Onderstepoort Veterinary Institute). Thus, details on the nematode's characteristics will be provided so that some insight can be gained. Conventional methods of detection of the nematode lack accuracy and may be risky or life-threatening for an infested animal (Traversa *et al.* 2007). Clinical symptoms of spirocercosis are detected at advanced stages of the disease when treatment is ineffective. Thus, in order to gain better perspective on the spread of spirocercosis, so that control mechanisms can be implemented, more fine-scale genetic analyses need to be conducted.

1.2. GENETIC STRUCTURING OF PARASITIC POPULATIONS

Firstly, some of the terminology will be defined that is commonly used by parasitologists to describe parasitic populations so that the correct usage of terms may be implemented (Margolis *et al.* 1982). An infrapopulation is one that includes all individuals of a species in an individual host at a particular time. The component population refers to all the individuals of a specified life history phase at a particular place and time. Lastly, a suprapopulation includes all developmental phases of a species at a particular place and time (Bush *et al.* 1997).

Co-evolutionary interactions between hosts and parasites can be understood through the characterisation of host and parasite population genetic structure (PGS) (Prugnolle *et al.* 2005a). In parasites, PGS varies from panmixia, i.e. frequent sexual recombination (Criscione *et al.* 2005; Johnson *et al.* 2006) to moderately and highly structured populations (Belanger *et al.* 2011; Redman *et al.* 2008). Parasite PGS has significant consequences for evolution such as host-race formation, adaptation to host defences and the evolution of drug resistance (Blouin *et al.* 1995). To examine the pattern of genetic structure, one must determine whether genetic diversity is distributed within or among populations.

The PGS of parasitic populations is determined mainly by the effective sizes of the populations and the rates of gene flow among them. Large effective population sizes (N_e) are likely to result in high within-population diversity (Blouin *et al.* 1995). Gene flow can be described as the movement of genes between populations (Paterson & Viney 2000) due to the movement of gametes or individuals (Nadler 1995). If the level of gene flow is high among populations, there is great opportunity for the spread of resistant alleles, resulting in resistance to treatment drugs. Repeated treatment with anthelmintic drugs often leads to the selection of

resistance alleles (Bourguinat *et al.* 2008; Wolstenholme *et al.* 2004) and resistance has been documented previously in parasitic nematodes of domesticated ruminants around the world (Blackhall *et al.* 1998a; Coltman *et al.* 2001; Gilleard & Beech 2007; Xu *et al.* 1998).

Effective population size and gene flow depend strongly on the life-cycle patterns and life-histories of both parasites and hosts, thus PGS differs among different nematode species (Blouin *et al.* 1995). Studies on mitochondrial DNA (mtDNA) sequences have shown high levels of diversity for *Haemonchus contortus* and other trichostrongyloid nematodes, sometimes up to ten times higher than that seen in other species (Blouin *et al.* 1995; Hu & Gasser 2006). Similarly, nuclear loci also show high levels of genetic diversity within populations (Blackhall *et al.* 1998a; Blackhall *et al.* 1998b; Hoekstra *et al.* 1997; Otsen *et al.* 2000).

Genetic structuring reflects the number of alleles exchanged between populations, thus impacts the genetic composition of individuals in a population. The exchange of alleles between populations generates polymorphism and may increase the local effective population size by resisting random changes in allele frequencies. Novel genetic variants can be introduced through the processes of recombination, mutation and gene flow (Brandt *et al.* 2007). Gene flow homogenizes allele frequencies at all loci across populations (de Meeûs *et al.* 2007). When gene flow is high in a population, local adaptation is prevented (Brandt *et al.* 2007). It has been noted that when the environment changes through space and time, dispersal becomes increasingly adaptive (Brandt *et al.* 2007). The successful exploitation of hosts is essential for parasite fitness. Thus, selection is expected to be stronger on parasites than on hosts (Brandt *et al.* 2007), allowing parasites to have a greater evolutionary potential to facilitate adaptation to their hosts.

Many parasite species have genetic features that favour the development of resistance (Belanger *et al.* 2011). Rapid rates of nucleotide substitution and large effective population sizes result in high levels of genetic diversity (Blouin *et al.* 1995). Low dispersal rates of either the vector or host may prevent a species from forming a panmictic unit which results in a structured metapopulation. The extent of sub-structuring depends both on the level of dispersal and the effective sizes of local populations. The pattern and degree of substructuring can have profound effects on the response of the parasite to selection and thus on the spread of drug resistance. Nematodes that have shown a population structure with high levels of gene flow have the genetic potential to respond to chemical attacks and the ability to spread resistance alleles (Johnson *et al.* 2006). Selection also plays an important role in the maintenance of resistance alleles (Belanger *et al.* 2011).

A species' geographical distribution is typically more widespread than individual dispersal capacity. In a species that occupies a large geographic area, local differentiation can occur without the presence of any geographic barriers (Belanger *et al.* 2011). Therefore populations are often genetically differentiated through isolation by distance, a term coined by Sewall Wright in 1943 (Slatkin 1993), where populations in close proximity are genetically more similar than more distant populations (Balloux & Lugon-Moulin 2002).

Transmission dynamics and the distribution of parasites among hosts are expected to have a large effect on mating systems and demic structure (Criscione & Blouin 2006). In population genetics, a subpopulation (deme) is generally considered as the smallest level of population structure (Balloux & Lugon-Moulin 2002). A deme is defined as a cohesive genetic unit with recurring generations such that random genetic drift can occur over successive generations (Criscione *et al.* 2005). The constitution of a parasite deme is determined by the levels of genetic diversity, opportunities for local adaptation as well as other evolutionary processes (Charlesworth 2003; Criscione & Blouin 2006).

Infrapopulations should behave as demes if parasite offspring are transmitted collectively from host to host over several generations (Criscione & Blouin 2006). Alternatively, the parasite component population would function as the deme if offspring were well mixed between hosts. Intermixture of offspring occurs when the definitive host consumes many infected intermediate hosts instead of just one. If each of the intermediate hosts contains a diverse number of unique parasite genotypes, the possibility that mating will occur between genetically identical individuals is decreased in the definitive host (Rauch *et al.* 2005). The use of numerous intermediate hosts also promotes high genetic diversity and well-mixed infrapopulations (Criscione & Blouin 2006). Host movement also determines parasite gene flow (Blouin *et al.* 1999; Criscione *et al.* 2005). In a parasite with a complex life cycle it is assumed that gene flow will be determined by the movement of the most mobile host (Prugnolle *et al.* 2005b).

In order to implement management strategies, it is crucial to have reliable estimates of population differentiation in order to understand the extent to which populations are genetically isolated from each other. In small isolated populations, genetic drift is likely to be experienced and deleterious mutations may become fixed, thus affecting the evolutionary potential of the population (Balloux & Lugon-Moulin 2002). It is hypothesised that the population genetic structures of parasites are usually associated with high levels of inbreeding, which reduces genetic diversity and leads to the fixation of alleles (Roos *et al.* 2004), resulting in low infra-population genetic variability. Therefore, parasites from different geographic regions must have a high amount of genetic variation (Otranto *et al.* 2005), assuming a low amount of gene flow between the areas. Unlike parasitic nematodes of plants and of insects, gastrointestinal trichostrongyloid nematodes of domesticated ruminants have been shown to have relatively high levels of diversity and gene flow (Blouin *et al.* 1995). This difference relates predominantly to differences in host movement, parasite biology, population sizes and transmission patterns, which are of epidemiological importance (Hu & Gasser 2006).

1.3. QUANTIFICATION OF GENETIC VARIATION

The utilisation of statistical tools to estimate patterns of gene flow in nematodes and other eukaryotes has advanced the field of population genetics. Wright's F_{ST} (Wright 1965) and Slatkin's R_{ST} (Slatkin 1995) are the most commonly reported statistics used to estimate population genetic structure (Balloux & Lugon-

Moulin 2002) by estimating patterns of genetic differentiation among hosts (Criscione *et al.* 2005). R_{ST} is an analogue of F_{ST} which assumes a stepwise mutation model (Balloux & Lugon-Moulin 2002).

The stepwise mutation model (SMM) was specially designed to reflect the mutation pattern of microsatellite loci more accurately (Balloux & Lugon-Moulin 2002). Novel alleles are created through the gain or a loss of one repeat unit, thus resulting in an expansion or contraction (Bhargava & Fuentes 2010) of the microsatellite. An allele with *i* repeats mutates to either *i*-1 or *i*+1 repeats, with an equal probability of $\mu/2$ (mutation (μ)) in both directions (de Meeûs *et al.* 2007). Alleles of very different sizes will be more distantly related than those of similar sizes since the SMM has a memory of allele size (Balloux & Lugon-Moulin 2002). Under the classical infinite alleles model (IAM) the only two states are "same" and "different", where existing alleles are transformed into new alleles that were previously not present in the population (de Meeûs *et al.* 2007). Under the SMM, however, there is a continuum of different similarities (same size, similar in size, very different in size). The IAM assumes that every mutational event produces a new allele and does not allow for homoplasy (Bhargava & Fuentes 2010). Considering that the SMM allows for homoplasy makes it ideal for microsatellite analysis (Balloux & Lugon-Moulin 2002).

Allelic diversity, observed and expected heterozygosities, violation of Hardy-Weinberg equilibrium (HWE) expectations and genotypic linkage disequilibrium can be determined for the parasitic populations. Deviations from HWE could be due to genetic sub-structuring of the population, caused by admixture or inbreeding. If no linkage disequilibrium is observed between sets of markers upon pairwise analysis, markers can be assumed not to be linked and can be used as independent loci. Analysis of molecular variance (AMOVA) is conducted to test for population differentiation/structure (Excoffier *et al.* 2005) by quantifying genetic variance at three levels defined by *F*-statistics: F_{IS}, F_{ST} and F_{IT} (de Meeûs *et al.* 2007). These parameters are estimated by Weir & Cockerham's (1984) unbiased estimators. The three F-statistics are connected by the following relationship: $(1 - F_{IT}) = (1 - F_{IS})(1 - F_{ST})$ (Nadler 1995).

F_{IS} ($_I$ stands for individual and $_S$ for subpopulations) measures the inbreeding of individuals within infrapopulations (Selkoe & Toonen 2006) that is due to the local non-random union of gametes in a subpopulation (de Meeûs *et al.* 2007). F_{IS} requires measures of observed and expected heterozygosity (Gorton *et al.* 2012). F_{IS} ranges from -1 to 1, with 0 indicating that there is HWE within the subpopulation (Gorton *et al.* 2012).

Negative values of F_{IS} indicate an excess of heterozygotes which can be due to factors such as inbreeding avoidance, small population sizes and asexual reproduction (de Meeûs *et al.* 2007). Positive values indicate an excess of homozygotes compared to HWE, thus allelic frequencies will not be identical (de Meeûs *et al.* 2007). Heterozygote deficit can be produced when the criteria for an ideal population is violated by phenomena such as strong inbreeding (matings between relatives) or selection for or against an allele (Selkoe

& Toonen 2006). Alternatively, two genetically distinct groups may be unknowingly grouped into a single sampling unit by the sampler and analysed as one, either because they co-occur but rarely interbreed, or because the spatial scale chosen for sampling individuals is larger than the true scale of a population (Selkoe & Toonen 2006). This phenomenon is known as the Wahlund effect and may be a common cause of heterozygote deficit in population genetic studies (de Meeûs *et al.* 2007; Gorton *et al.* 2012; Selkoe & Toonen 2006). When samples from the same deme are pooled together, there should be no significant change in F_{IS}, however a significant increase is expected when a sample from a different deme is included in the pool (Balloux & Lugon-Moulin 2002). F_{IS} thus also provides indications of whether we have sampled one or several distinct demes (Balloux & Lugon-Moulin 2002). Both of these causes of heterozygote deficit, inbreeding or the Wahlund effect, should affect all loci, instead of just one or a few.

F_{ST} measures the level of genetic structuring between subpopulations which is a reflection of the level of inbreeding among infrapopulations (Selkoe & Toonen 2006). It is also a measure of the Wahlund effect since it reflects the inbreeding that results from the subdivision of the population into sub-populations that do not freely exchange migrants (de Meeûs *et al.* 2007). A significant F_{ST} may be caused by genetic drift; however this is not always true for parasitic populations. Small sample sizes of parasites per host can cause imprecise/biased estimates of F_{ST}. F_{ST} values may range from 0 to 1, where 0 indicates that no genetic subdivision exists between subpopulations due to equal allele frequencies across all subpopulations, and increasing values indicate the fixation of unique alleles in their respective subpopulations (Gorton *et al.* 2012). A value of 1 indicates that subpopulations are completely independent. This can occur when subpopulations are isolated for a long time span (de Meeûs *et al.* 2007).

F_{IT} is the inbreeding of individuals in the total population which results from both F_{IS} and F_{ST} (de Meeûs *et al.* 2007; Prugnolle *et al.* 2002). Values of F_{IT} may range from -1 to 1, where either all individuals are heterozygous for two of the same alleles or where all individuals are homozygous with at least two alleles (de Meeûs *et al.* 2007).

Isolation between subpopulations (demes) allows differentiation to occur. This allows a species to develop a high level of diversity. If there are *n* demes that are not completely isolated, migration occurs more frequently than mutation (Charlesworth 2003). For such a species, a low F_{ST} is expected for the island or stepping-stone models, which are models of population structure and migration and will not allow for migration pressure to change deme sizes (Nagylaki 2000). When demes are connected, loss of diversity within demes is prevented. The effective size of a deme does not determine diversity unless the deme is cut off from the gene flow (Charlesworth 2003).

Multi-locus genotype data obtained from using unlinked markers is used to infer population structure in the program STRUCTURE (Pritchard *et al.* 2007). Allele frequencies are calculated and used in Bayesian

analysis to determine the number of genetically distinct populations present in a sample (Hubisz *et al.* 2009). The use of the program STRUCTURE has been implemented previously in studies on parasitic nematodes such as *Dirofilaria immitis* (Belanger *et al.* 2011) and *Rotylenchulus reniformis* (Leach *et al.* 2012) to determine the number of population clusters.

1.4. MICROSATELLITES AS GENETIC MARKERS

Epidemiology and population genetic studies require the use of high resolution genetic markers. Genetic markers are used to determine the level of genetic variation within populations. They are derived from regions of the genome of the organism under study. Variations in these gene regions are studied across individuals from different sites (de Meeûs *et al.* 2007). Loci must vary between individuals in order to be informative. For population structure studies, non-coding loci are preferred since they are neutral and are thus not under selection. This makes them more likely to reflect only demographic factors such as population size and dispersal so that parasite spread can be tracked and population history may be inferred (Archie *et al.* 2008).

Microsatellites are co-dominant genetic markers, meaning that each of the two alleles at a locus in an individual can be identified, whereas with dominant inheritance DNA fragments can only be scored as being either absent or present (Sunnucks 2000). These markers are also hyper-variable, abundant, selectively neutral, relatively easy to score and widely dispersed in eukaryotic genomes, making them useful for population genetic studies (de Meeûs *et al.* 2007; Schwenkenbecher & Kaplan 2007). Microsatellites, also known as SSRs (short sequence repeats), are among the most commonly used molecular markers in population and evolutionary biology (Evanno *et al.* 2005; Sunnucks 2000). They are widely used for the following: to evaluate the genetic diversity within species, to identify and test the paternity and relatedness of individuals in a population, to study population structure and gene flow and for gene mapping (Cotti 2008).

These markers consist of short, tandemly repeated DNA sequence motifs that are generally composed of two to four base pairs (e.g. AC, CGT, GATA, etc.). They are found in genomes of both prokaryotes and eukaryotes and are distributed in protein-coding and non-coding regions of the nuclear genome (Tóth *et al.* 2000). However, they tend to occur more frequently in non-coding DNA such as introns and intergenic regions (Cotti 2008). Generally, trinucleotide repeats are predominantly found in exons, whereas repeats consisting of multiples of two, four, and five base pairs are rarely found in genes (Cotti 2008). Microsatellite repeats may be pure, compound, complex and/or interrupted (Buschiazzo & Gemmell 2006), however most loci that are used as markers contain perfect repeats. Loci containing compound and interrupted repeats tend to be less polymorphic (Jarne & Lagoda 1996).

Microsatellites are known to have different phases – birth, growth and death (Molnar *et al.* 2012), which may stretch over tens or hundreds of millions of years (Chambers & MacAvoy 2000). During these phases, microsatellites accumulate mutations that are known to result in high rates of polymorphism (Ellegren 2004). The highly polymorphic nature of microsatellites makes them valuable as genetic markers and since microsatellites are neutral markers, the degree of polymorphism is proportional to the underlying mutation rate (Ellegren 2004). High mutation rates are caused by slippage of the DNA polymerase and proofreading during DNA replication. Slippage of DNA polymerase leads to either the loss of the microsatellite repeat unit or to a very high number of repeats. Polymorphism is thus generated through differences in the number of repeats of the sequence between individuals (de Meeûs *et al.* 2007; Schwenkenbecher & Kaplan 2007; Selkoe & Toonen 2006). A marker with a high mutation rate can answer questions about present day demography or connectivity patterns, or detect changes in the recent past (10-100 generations) (Selkoe & Toonen 2006).

Microsatellite mutation rates vary considerably depending on the locus, number of repeat units (length in base pairs), type of repeat motif , the flanking sequence, the recombination rate, the organism, and possible interruptions in the microsatellite (Bhargava & Fuentes 2010; Schlötterer 2000). The most important factor that seems to affect mutation rate is microsatellite length (Ellegren 2004). It was previously thought that microsatellites with longer repeat units (e.g. tetra-) are likely to be more mutation prone (Balloux & Lugon-Moulin 2002; Schwenkenbecher & Kaplan 2007) and evolve faster than those with shorter repeat units (e.g. di-) (Chambers & MacAvoy 2000). However, recent studies have found that shorter repeat units are unstable, thus allowing more slippage events to occur (Bhargava & Fuentes 2010). Comparisons across species show that microsatellite loci can be conserved over long evolutionary time spans (Bezault *et al.* 2012). It is still not known why microsatellites have been shown to evolve faster in some species compared to others (Stolle *et al.* 2013). It is perhaps a result of biochemical changes to the DNA replication/ repair machinery, or it may represent larger population sizes that have undergone more processes such as drift, mutation, selection, etc. and are influenced by mutations that add or subtract repeat units that create new alleles (Chambers & MacAvoy 2000). It is also possible that the lack of very long microsatellite repeats is caused by selection that maintains microsatellites within a certain size limit (Schlötterer 2000).

General trends that were observed when studying mutation rates of microsatellites in the genome of the soil nematode, *Pristionchus pacificus*, were that on average, large loci have more mutations than smaller ones (Molnar *et al.* 2012). Imperfect loci were also seen to accumulate fewer mutations than perfect loci. It was also found that the mutation process favours the gain of repeat units more than the loss of units, thus having the tendency to lengthen (Ellegren 2004). However, mutation rate in microsatellites varies across loci, between alleles and among species, so what may hold true for one species may not be the same in others (Meglecz *et al.* 2012). It is thus challenging to define key factors that have an effect on microsatellite mechanisms: errors during recombination, unequal crossing-over, polymerase slippage during DNA

replication or repair (Molnar *et al.* 2012). Microsatellites are also known to be hotspots for recombination, so they may act as evolutionary switches that regulate the mutation rate to undergo rapid evolution when a population needs to respond to environmental changes (Bhargava & Fuentes 2010).

The most common dinucleotide repeats found in nematode as well as other eukaryotic genomes are $(CA)_n$ followed by $(AT)_n$, $(GA)_n$ and $(GC)_n$, with GC repeats being uncommon (Ellegren 2004; Johnson *et al.* 2006; Otsen *et al.* 2000; Schwenkenbecher & Kaplan 2007). Because of their higher mutation rates, dinucleotide repeats are mostly targeted as markers. However, trinucleotide repeats are more feasible for population genetics studies because the differences in size between a number of sequences are easier to score (Schwenkenbecher & Kaplan 2007). In plants, the $(AT)_n$ repeat motif was found to be the most common. The contrasting distribution of microsatellite motifs in different genomes indicates that the mechanisms of mutation and repair, of specific motifs, vary amongst organisms or perhaps there is variation in the selective constraints that are involved (Ellegren 2004).

In *Ancylostoma caninum* (Schwenkenbecher & Kaplan 2007) microsatellites of various repeat motifs were found consisting of both G/C and A/T nucleotides. $(AT)_n$ sequences were more abundant than $(GC)_n$ microsatellites, which indicated a low GC-content in the genome, correlating with the results of other studies on nematodes. Among trinucleotide repeats, the following motifs were commonly found, in order of abundance: $(GAA)_n$, $(TAT)_n$, $(ACA)_n$, and $(CTG)_n$. Common dinucleotide repeats found were $(GA)_n$ and $(GT)_n$ repeats, which are also commonly found in other nematode species (Johnson *et al.* 2006; Otsen *et al.* 2000).

Microsatellite markers have only recently been used in studies on parasitic nematodes, even though their potential to be suitable markers is very high. This may be due to the fact that the successful isolation of these markers requires very precise work at each step to obtain a set of primers that can amplify polymorphic microsatellite loci. Throughout the process, loci may potentially be lost and the number of loci that can be used in the end will be a fraction of the original number (Zhan *et al.* 2009). These pitfalls can be avoided by carefully selecting loci during the isolation process. The microsatellite-enriched strategy for microsatellite isolation bears a high risk of producing multiple copies of the same fragments/clones when a large number of microsatellites are isolated (Zane *et al.* 2002). This risk will be minimised by using a set of selection criteria (see Figure 1.1) to eliminate markers that may provide false interpretations of the population structure.

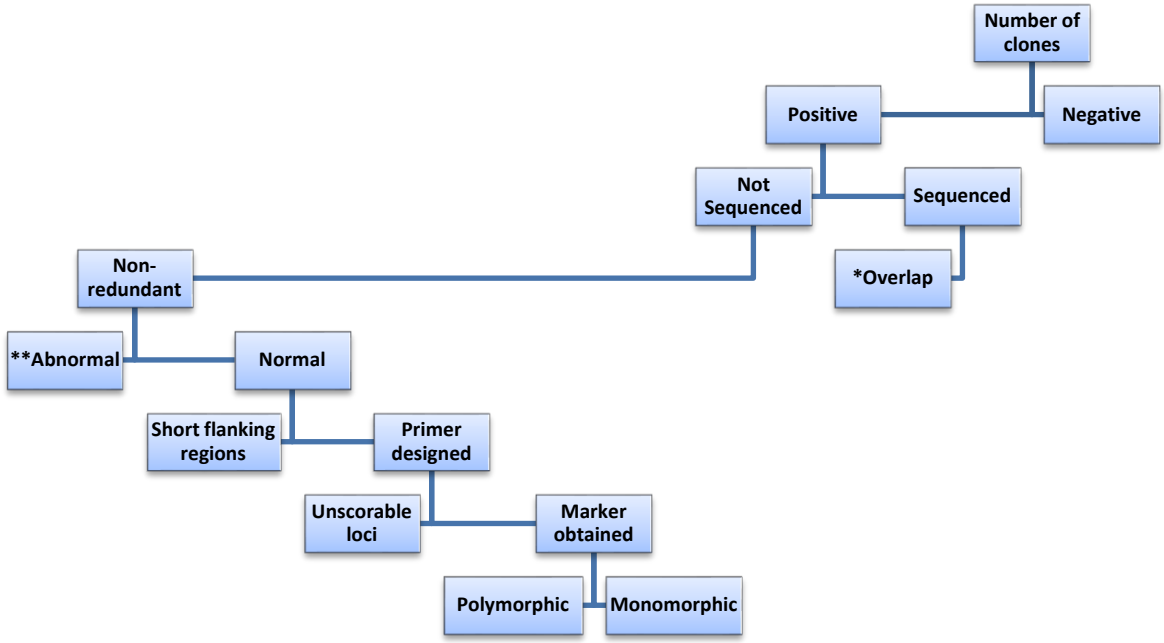

Figure 1.1: Selection criteria for the development of ideal microsatellite markers (Adapted from Zhan *et al.*, 2009). *Overlap refers to sequences that have the same flanking regions and are therefore considered as the same loci. **Abnormal refers to the fact that GC content of flanking regions is low and the length of flanking regions is short.

Firstly, losses can occur when identifying clones containing microsatellite sequences because some sequences do not contain a microsatellite. Sequences that do contain microsatellites are not all unique, therefore some redundancy is likely. Typically, a third of the sequenced clones are lost due to the absence of a unique microsatellite (Cotti 2008). It is also possible to find chimeric sequences, in which one of the microsatellite flanking regions matches that of another clone.

Secondly, the microsatellite flanking regions may not be suitable for primer design. Microsatellites may be located too close to the end of an insert to accommodate primer design in the flanking region, and even when the length of sequence is sufficient, the base composition may be unsuitable. There is no standard cut off length for the minimum number of length of repeats that define a microsatellite (Meglecz *et al.* 2012), however it is essential to design primers for sequences that have at least five repeats in order to avoid the use of proto-microsatellites, which are identified as short intermediate stages that have 3–4 repeat units (Bhargava & Fuentes 2010).

Thirdly, some primer pairs may seem suitable but fail to amplify. The optimization of reaction conditions can improve success and the microsatellite enrichment protocol, developed by Zane *et al.* (2002), eliminates most problems associated with microsatellite marker isolation.

Many of the drawbacks associated with microsatellite markers can be eliminated with careful selection of loci during the isolation process (Selkoe & Toonen 2006). Error rates must be calculated to ensure that genotyping is consistent. This can be done by repeating amplification in a random subset of 10-15% of the total number of samples, and then counting the number of genotypes that differ between the first and second amplification (Selkoe & Toonen 2006).

In order to be useful, markers must amplify microsatellites from most individuals, allow for unambiguous genotyping, be polymorphic and have a relatively low frequency of null alleles in the populations under study (Grillo *et al.* 2006). When conducting microsatellite analysis, inheritance data must be interpreted with caution because the presence of undetected null alleles may interfere with results. Null alleles may be caused by mutations in one or both primer binding sites, which can prevent amplification during PCR. Upon examination of an electrophoresis gel, homozygous individuals for a null allele do not show any band at all, whereas heterozygotes have only one band and therefore mimic a homozygote; thus indicating a heterozygote deficit when population data are analysed (Dakin & Avise 2004). Since there is generally a high degree of genetic heterogeneity in nematodes (Grillo *et al.* 2006; Johnson *et al.* 2006; Roos *et al.* 2004), it is essential that null alleles be detected.

Low quantity or quality of DNA is also known to cause genotyping errors (Pompanon *et al.* 2005) since these conditions favour allelic dropouts and the chances of false alleles being amplified. Allelic dropouts are caused when only one of the two alleles present at a heterozygous locus is amplified, since its denaturation is favoured due to the low GC content (Pompanon *et al.* 2005). It is usually the longer allele in a heterozygote that does not amplify or cannot be detected in the genotype scoring process (Dakin & Avise 2004; Selkoe & Toonen 2006). False alleles are allele-like artefacts that are generated by PCR. When the amount of template DNA is low it is more likely for contaminant DNA molecules to also be amplified (Pompanon *et al.* 2005). It may be necessary to re-extract and re-amplify the DNA. However, if alleles fail to amplify at just one locus while all other loci amplify normally, then the problem is not poor quality DNA; it is likely that the individual is homozygous for a null allele (Selkoe & Toonen 2006). During PCR, at the end of elongation, some types of Taq polymerase tend to add an adenine nucleotide to the 3' end of the newly synthesised strand, which creates an artefactual peak on the trace data, which may also lead to genotyping error (Pompanon *et al.* 2005). Human errors when handling samples or data is the most significant contributor to genotyping error (Pompanon *et al.* 2005).

Erroneous interpretations that may be caused by null alleles may be avoided by redesigning primer pairs for the locus (Dakin & Avise 2004), adjusting PCR conditions (Selkoe & Toonen 2006), avoiding the mutated primer binding site and by examining multiple microsatellite loci, thus reducing the influence of null alleles (Cotti 2008). A statistical software program that estimates the frequency of null alleles for each locus is MICROCHECKER (Van Oosterhout *et al.* 2004). When high accuracy is required in genotyping analyses

(e.g. parentage analysis), even rare null alleles can cause results to be misinterpreted, thus any loci with strong evidence of null alleles should be excluded (Selkoe & Toonen 2006).

In some instances, microsatellites may be conserved across species. Heterologous amplification of any DNA sequence by PCR will depend on the source, characteristics of the genomic library and evolutionary distance of the species sampled (Cotti 2008). Therefore, primers developed for a particular species can be applied in the study of related taxa in order to avoid the laborious and time-consuming process of developing new microsatellites. It is expected that microsatellites are transferable between related species (Zhan *et al.* 2009). It is also important to note that the high mutation rate in microsatellites makes their use across highly divergent taxa practically impossible for reasons that follow (Schwenkenbecher & Kaplan 2007).

Firstly, microsatellite primer sites may not be conserved, i.e. the primers used for one species may not amplify in another species. This is because microsatellites are usually found in noncoding regions where the nucleotide substitution rate is higher than in coding regions (Zane *et al.* 2002). This can be illustrated by a study where the transferability of loci was tested between closely related species. Out of 34 makers developed for *Ancylostoma caninum*, only eight and ten loci amplified respectively in closely related species *Ancylostoma duodenale* and *Necator americanus* (Schwenkenbecher & Kaplan 2007). There is however an advantage of this species-specific nature of microsatellites: it ensures that cross-contamination by a non-target organism is less likely compared to techniques that use universal primers.

Secondly, the high mutation rate increases the chances of homoplasy and it cannot be assumed that two alleles identical in state are identical by descent. Allelic diversity may be underestimated and estimates of gene flow may be inflated when mutation rates are high (Selkoe & Toonen 2006). Two types of homoplasy exist: detectable and undetectable. Detectable homoplasy is often revealed by sequencing alleles. If a point mutation occurs, the size of an allele will remain unchanged, whereas insertions or deletions will create a new allele with the same size as an existing allele. Undetectable homoplasy occurs when identical alleles are shared by two individuals even if they do not share a recent common ancestor (de Meeûs *et al.* 2007). This occurs in the stepwise mutation process. Since the stepwise mutation model predicts a 50% chance of back-mutation, undetectable homoplasy presents frequently when the mutation rate is high, however this can be accounted for during analyses (Selkoe & Toonen 2006).

Furthermore, when using microsatellites, there tend to be very few loci (4-20) available to work with. This increases the probability of having a selection bias acting on one or more loci which may give a misleading impression of the true pattern of change for the whole genome.

In the field of molecular ecology, the general consensus is that in most cases, the more loci studied, the more reliable the resulting data set will be. However, a screening process must be carried out in order to eliminate

loci that can lower both the precision and the accuracy of genetic estimates (Selkoe & Toonen 2006). On the other hand, reducing the number of loci also reduces statistical power and the probability of genome-wide sampling. Twenty markers or less have been used to study the patterns of parasite population changes and gene flow (Grillo *et al.* 2006; Johnson *et al.* 2006; Schwenkenbecher & Kaplan 2007). More than twenty markers are considered necessary for parasitic identification and for linkage analysis and mapping of genes associated with drug resistance (Schwenkenbecher & Kaplan 2007).

1.5. CHARACTERISTICS OF *SPIROCERCA LUPI*

Spirocerca lupi (order *Spirurida*, family *Thelaziidae*) is an infectious helminth (parasitic worm) that is known to cause spirocercosis in canids, mostly in dogs. This parasite was first detected in 1760 by Morgagni and Courten (Chandrasekharon *et al.* 1958). For over a decade now, *S. lupi* has emerged as a serious concern for the canine population worldwide (Lobetti 2000; Mazaki-Tovi *et al.* 2002; Ranen *et al.* 2004). A study conducted in 2000 revealed a prevalence of 28% in South Africa, with Gauteng (46.3%) and Kwazulu-Natal (35.1%) being the highest areas of incidence (Lobetti 2000). The prevalence of this parasite in its intermediate dung beetle host in the rural and urban areas of Pretoria was shown to be 2.3% and 13.5%, respectively (Du Toit *et al.* 2008). Clinical signs of spirocercosis are not unique and may be confused with other diseases. Diagnostic methods for canine spirocercosis are limited in their efficiency and very little molecular information is available for this nematode.

1.5.1. Life cycle

Adult *S. lupi* worms are usually found in nodules in the wall of a dog's thoracic oesophagus. Larvae are contained in eggs produced by the female worms. The eggs are transported to the oesophageal lumen via an opening in the nodule. The eggs are eventually excreted in the faeces or vomitus of infested dogs (Last & Smith 2007). Coprophagous beetles of the Scarabaeidae family are the intermediate hosts that ingest the faeces of infested dogs that contain the *S. lupi* eggs. It has been noted that these dung beetles prefer omnivore (pig and dog) dung to carnivore dung (Du Toit *et al.* 2008). Once ingested by the beetle, the eggs hatch and develop to infective L3 stage larvae within two months, which encyst within the tissues of the intermediate host. Dogs are infested by ingestion of the coprophagous beetle or paratenic host containing the infective (L3) larvae (Figure 1.2).

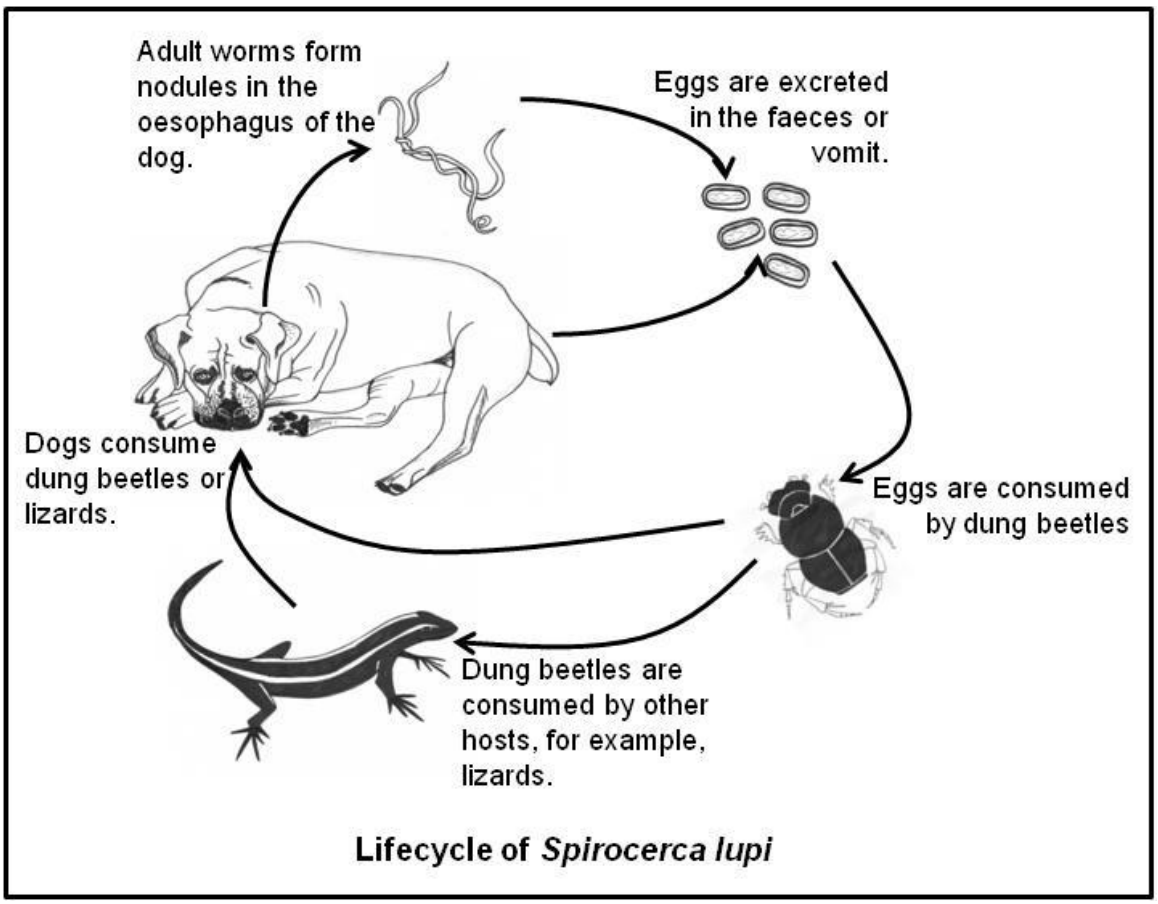

Figure 1.2: Diagram showing the lifecycle of *Spirocerca lupi*. Illustrated by PJ de Waal

Larvae follow a specific migratory route in the definitive host (Van der Merwe *et al.* 2008). Larvae excyst in the stomach and penetrate the gastric mucosa. They migrate along the arteries and then mature in the thoracic aorta, where they develop to L4 stage (Gottlieb *et al.* 2012). Approximately three months after infestation the worm eventually moves to the caudal oesophagus where it reproduces sexually and establishes an opening through which to pass eggs (Mazaki-Tovi *et al.* 2002). A nodule eventually develops around the worm that is located in the oesophageal wall (Bailey 1972).

Females can produce up to three million eggs per day (Bailey 1972). Eggs contain L1 larvae that are passed through the opening in the oesophageal wall to the gastrointestinal tract and finally to the faeces (Gottlieb *et al.* 2012). Adult worms can remain in the host's oesophagus for up to two years. Male and female worms can only be differentiated after 60 days of infestation (Van der Merwe *et al.* 2008). Adult worms are pinkish-red in colour (Bailey 1972) with males up to 54 mm and females up to 80 mm in length (Naem 2004).

Nodules vary between 1 and 4 cm in diameter, and typically contain 3 to 6 worms (Van der Merwe *et al.* 2008). A minimum of one worm and a maximum of twelve worms were found in each nodule from stray dogs in Bangladesh (Das *et al.* 2011), however more than 30 worms can be found in a nodule (Van der Merwe *et al.* 2008).

1.5.2. Prevalence

Incidences of *S. lupi* infestation have been reported worldwide (Figure 1.3). The majority of cases have been reported from regions with a warm climate, i.e. tropical and subtropical regions (Bailey 1972; Mazaki-Tovi *et al.* 2002; Van der Merwe *et al.* 2008). Reports indicate a tendency towards a summer seasonal incidence in South Africa (Lobetti 2000), whereas most cases were diagnosed in winter in Israel (Mazaki-Tovi *et al.* 2002), with these seasons falling during the months of October to March in the two countries. In Israel, it has been suggested that because the vector beetle is more abundant during the warmer months and carries large numbers of larvae (Mazaki-Tovi *et al.* 2002), clinical symptoms only manifest in the colder months when the adult worms reach the oesophagus. Differences in climate conditions affect the seasonality of the intermediate hosts and thus determine the seasonality of the disease. It is also important to note that six months (Lobetti 2000) are required for the development of the parasite, therefore, infestation could have taken place at any time. This is the reason why dogs younger than one year old are at a lower risk of being diagnosed with spirocercosis (Mazaki-Tovi *et al.* 2002).

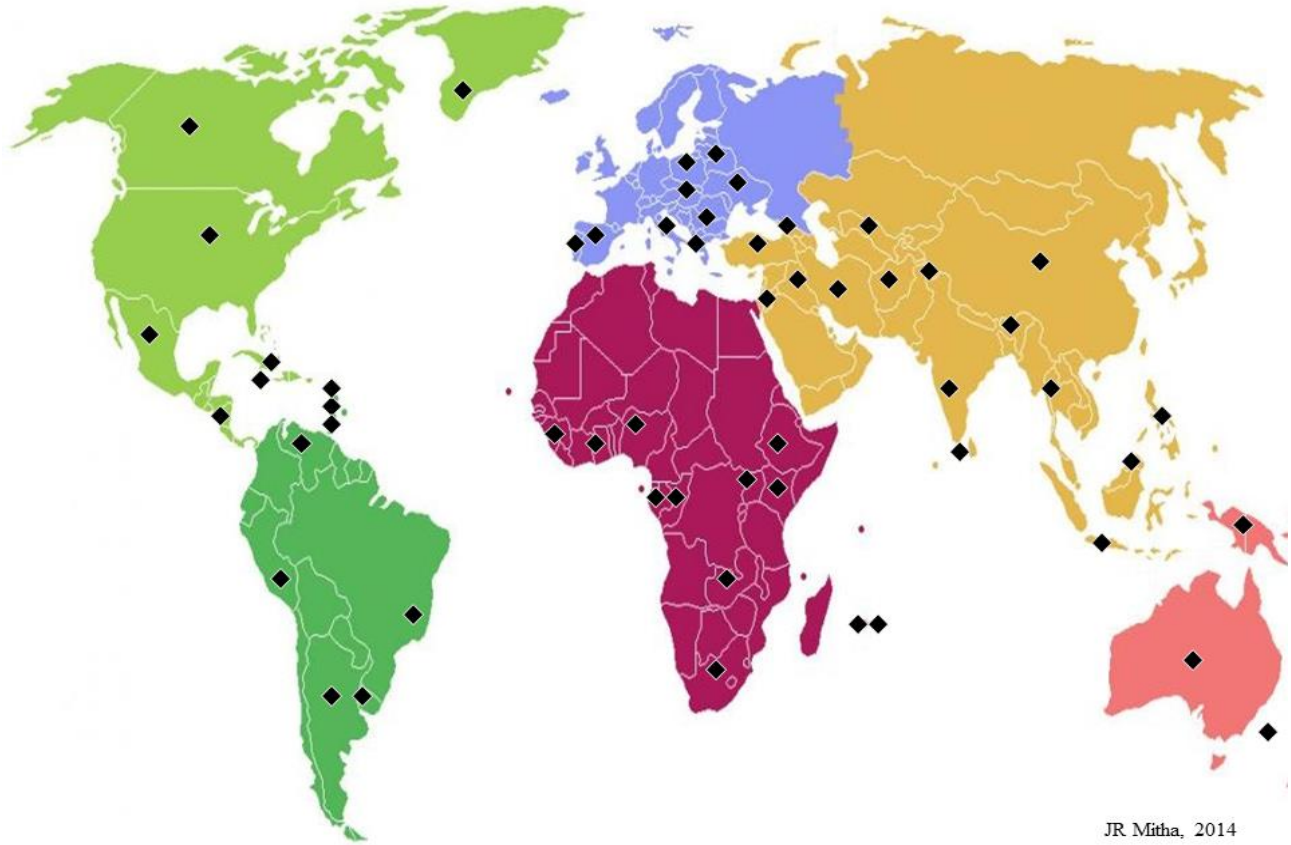

Figure 1.3: Reported worldwide incidences of *Spirocerca lupi*.

Most studies conducted thus far have found that spirocercosis has an increased incidence in urban areas (Du Toit *et al.* 2008; Mazaki-Tovi *et al.* 2002), although others have found that rural areas have a higher incidence (Minnaar *et al.* 2002). In a study by Minnaar *et al.* (2002), *S. lupi* was found in 13% of dogs examined in a peri-urban resource-limited community in Bloemfontein (Free State, South Africa). Access to veterinary services in this area is limited and dogs in this area are rarely dewormed. It is assumed that dogs in rural areas would be more susceptible to infestation because of a lack of available treatment and diagnostic facilities; however prevalence varies in different areas and depends on factors such as the degree of rural development, utilisation of pesticides, efforts at disease control and changing nutritional habits (Van der Merwe *et al.* 2008). Areas with the lowest dung beetle species diversity have been shown to have the highest prevalence of infestation (Du Toit *et al.* 2008). Due to the life cycle of *S. lupi*, prevalence of infestation is expected to increase in an enzootic area until preventative control measures are initiated. The rate of increase would depend on the population densities of definitive, intermediate and transport hosts (Bailey 1963).

1.5.3. Host characteristics

The beetle *Onthophagus sellatus* is the main intermediate host of *S. lupi* in Israel (Gottlieb *et al.* 2011; Mazaki-Tovi *et al.* 2002). Beetles from South Africa, also of the genus *Onthophagus* were found to be infested with *S. lupi* (Du Toit *et al.* 2012; Du Toit *et al.* 2008), which suggests that this genus is the preferred intermediate host for optimal larval development and for transmission to paratenic and definitive hosts (Kok *et al.* 2011).

Paratenic hosts such as rodents, hedgehogs, lizards (Anantaraman & Sen 1966), birds and rabbits can also ingest the beetles and become infested with the encysted larvae (Bailey 1963). Paratenic hosts are those in which a parasite does not undergo changes in its development but is maintained for long periods until reaching a definitive host. The infestation spreads to carnivores that are likely to kill and ingest these paratenic hosts as prey. Although the worm is found mostly in domestic dogs (*Canis lupus familiaris*), there have been reports of incidences in animals such as the domestic cat (Sowemimo 2012), bobcat (Stone & Pence 1978), coyote, fox, jackal, jaguar, lynx, snow leopard, wolf (Bailey 1972), bush dog (*Speothos venaticus*) (Rinas *et al.* 2009) and raccoon (Popiolek *et al.* 2011).

A specific age does not predispose dogs to infestation with *S. lupi* (Mazaki-Tovi *et al.* 2002; Minnaar *et al.* 2002), however due to the life-cycle of the nematode, infested dogs under six months of age have not yet developed oesophageal disease and clinical symptoms to be diagnosed with spirocercosis. Studies have found that larger breeds of dogs, e.g. German shepherds and Labrador retrievers, are more prone to infestation (Lobetti 2000), since they are likely to spend more time outdoors (Dvir *et al.* 2010; Mazaki-Tovi *et al.* 2002). No sex bias is observed in infested hosts (Chikweto *et al.* 2012; Das *et al.* 2011), however

females are associated with spirocercosis-associated oesophageal lesions that may undergo malignant transformation (Dvir *et al.* 2008; Ranen *et al.* 2004).

1.5.4. Symptoms

Common clinical symptoms that are found in dogs infested with *S. lupi* are vomiting, regurgitation, weight loss, salivation, dysphagia (difficulty in swallowing) and odynophagia (painful swallowing) (Last & Smith 2007). Odynophagia and regurgitation (Berry 2000) occur when the formation of an oesophageal mass causes an obstruction that prevents the passage of solid or semi-solid food (Bailey 1963). These symptoms are exacerbated as nodules increase in size and number, and dogs may even become emaciated because they are unable to swallow (Kok *et al.* 2011). However, these symptoms do not always manifest in infested dogs, and can therefore not be used for diagnostic purposes.

Symptoms also vary greatly and depend on the stage of the disease, aberrant migrations and possible complications (Van der Merwe *et al.* 2008). Aberrant migration of the larvae may cause damage to the aorta which results in aneurysms (Van der Merwe *et al.* 2008). Aortic rupture may cause the sudden death of a dog (Bailey 1963; Rinas *et al.* 2009). Fever and dyspnoea (shortness of breath) has also been reported by veterinarians (Lobetti 2000). Dyspnoea and cough is caused by obstruction of the airway by the nodule (Mazaki-Tovi *et al.* 2002). The development of spondylitis (inflammation of the vertebrae) (Bailey 1963) varies from 33%- 87% of cases, as detected in dogs from Israel (Mazaki-Tovi *et al.* 2002; Ranen *et al.* 2004).

Inflammation and the chronic loss of blood from an ulcerated nodule causes anaemia. Mild anaemia was found in approximately 50% of cases (Mazaki-Tovi *et al.* 2002; Ranen *et al.* 2004). The most common haematologic abnormality found was leukocytosis (elevated white blood cell count) (82%) (Ranen *et al.* 2004). Biochemical analyses have revealed elevated levels of serum creatine kinase, as well as other enzymes such as alkaline phosphatase, amylase and lactate dehydrogenase in infested dogs (Mazaki-Tovi *et al.* 2002; Ranen *et al.* 2004).

It was previously reported that infestation with *S. lupi* can lead to the development of an oesophageal granuloma which can undergo malignant transformation to form a sarcoma (Ribelin & Bailey 2006), which can metastasize to the lungs. However later studies show that nodules are incorrectly referred to as granulomas (Van der Merwe *et al.* 2008), since the use of the term is histologically incorrect. Neoplastic transformation was reported in 26% of cases in South Africa, although the exact mechanism by which it occurs is unknown (Mazaki-Tovi *et al.* 2002; Ranen *et al.* 2004). It was found that the vast majority of cases occur typically in the caudal oesophagus.

1.5.5. Diagnosis

Unfortunately most cases of spirocercosis are only diagnosed in the advanced stages of the disease, since clinical symptoms and adult worms are absent in early stages (Gottlieb *et al.* 2012). Several prognostic and diagnostic procedures have been developed, with varying degrees of sensitivity.

Radiography is used initially to detect a mass in the caudal oesophagus (Dvir *et al.* 2010). This technique lacks sensitivity and specificity for aetiological diagnosis and its findings often vary (Traversa *et al.* 2007). It is also difficult to differentiate between *S. lupi* nodules and neoplasms from other opaque soft-tissue masses (Traversa *et al.* 2008). Nodules may also be small and therefore not visible on the radiograph (Dvir *et al.* 2010).

Endoscopy is an invasive technique that allows surgeons to determine the exact location and size of the oesophageal mass. It is both sensitive and specific and can differentiate between nodules and neoplasms, but not between tumours and granulomas (Mazaki-Tovi *et al.* 2002; Ranen *et al.* 2004). Endoscopic findings vary depending on the progression of the disease (Van der Merwe *et al.* 2008). Endoscopic biopsy can be used to diagnose the nature of the mass, however, can be misleading because of false negative results (Ranen *et al.* 2004). Endoscopy has been found to be more sensitive than radiography in detecting oesophageal masses (Mazaki-Tovi *et al.* 2002). Patients must be restrained or sedated in order to undergo radiography and general anaesthesia is required to carry out endoscopy. Besides being expensive and time-consuming, these procedures may be risky or life-threatening for an animal (Traversa *et al.* 2007).

The faecal flotation technique lacks accuracy for a number of reasons. Firstly, passage of eggs occurs for a short period in the lifespan of a worm and is unpredictable. Eggs will only be detected when adult worms, found in oesophageal nodules, shed eggs (Mazaki-Tovi *et al.* 2002). Secondly, only once the nodule matures and there is an opening, will the female enter the oesophageal lumen, which allows the passage of eggs into the faeces. Thirdly, eggs can only be detected with flotation techniques using sugar and salt solutions, which require special laboratory techniques (Van der Merwe *et al.* 2008). Eggs are also relatively small and have higher specific gravities compared to the other nematodes' eggs (Dvir *et al.* 2010). False negative results may be obtained with the faecal flotation technique because some flotation fluids have higher specific gravities. Flotation fluids with higher specific gravities tend to crystallise rapidly which may cause the helminth eggs to disintegrate (Minnaar *et al.* 2002). The faecal flotation must be repeated when negative results are obtained in order to improve diagnostic accuracy (Mazaki-Tovi *et al.* 2002), however repeated faecal flotations are more labour intensive. Faecal flotation sensitivity can be increased by using a modified sugar flotation technique (Markovics & Medinski 1996; Mazaki-Tovi *et al.* 2002). Recent studies have found that using a $NaNO_3$ (SG 1.22) solution with a modified centrifugal flotation method provides higher

sensitivity when examining faeces since high egg counts can be obtained (Christie *et al.* 2011). This method is also easy to perform and inexpensive.

Effective diagnosis, treatment and control of spirocercosis rely on accurate identification of the parasite in the host species. Since clinical symptoms in host species are not sufficient for the specific detection of infestation with these nematodes, molecular approaches using PCR-based methods have proven to be a valuable tool to overcome this limitation. PCR-based assays are proven to be more sensitive in detecting and identifying parasitic infestation. Diagnostic accuracy can be optimised by combining faecal flotation with a PCR-based assay that uses faeces or mucous from the host and targets a specific *S. lupi* gene (Traversa *et al.* 2007). The high sensitivity of the PCR technique permits the specific amplification of genes or gene fragments from small amounts of material such as tiny, individual nematodes and their different life-cycle stages (Hu & Gasser 2006). Sensitivity and specificity values can be obtained at 100%. The molecular approach also yields a higher prevalence of positive results. The major advantage of this approach over traditional molecular methods is that PCR synthesizes millions of copies of the target DNA which are available for subsequent analysis. PCR yields *S. lupi* specific amplicons regardless of co-infection with other parasites (Traversa *et al.* 2008). Early diagnosis of *S. lupi* infestations could greatly improve the prognosis of an infested dog. A PCR diagnostic test could be a fast and simple method to detect *S. lupi* within the faeces of a dog suspected of having spirocercosis. Since *S. lupi* is not the only parasitic nematode found in the canine gut, the PCR test must be very specific. This technique is yet to be optimised to be used as a method of detection, however when the presence of *S. lupi* larvae can be detected by such a simple and non-invasive test, it would be possible to reduce the effects of spirocercosis in infested dogs. There are also prospects for using bacterial symbionts in the detection of *S. lupi* (Gottlieb *et al.* 2012).

1.5.6. Treatment

A number of different drugs have been used to treat or prevent the progression of spirocercosis, but none have been effective in killing both adult and larval stages of the nematode, without producing side-effects in the host (Van der Merwe *et al.* 2008). An efficient as well as cost-effective treatment against both adult and larval stages is yet to be developed to control the disease (Kok *et al.* 2011). Treatment is more effective if the disease is diagnosed early and if there are no complications (Lobetti 2000). The most commonly used anti-helminthics include the macrocyclic lactones ivermectin and doramectin.

Doramectin was found to be the most effective drug in killing adult worms effectively and decreasing egg shedding while eliminating oesophageal lesions (Berry 2000; Lobetti 2011). In South Africa, this drug was successfully applied in the treatment of small, smooth nodules (Van der Merwe *et al.* 2008). Milbemycin oxime, administered at regular intervals, was found to be effective against pre-adult stages of the nematode

(Kok *et al.* 2011). A monthly treatment with imidacloprid 10%/ moxidectin 2.5% spot-on (Advocate®) has been shown to have preventative effects on puppies aged 2-4 months (Le Sueur *et al.* 2010).

Surgery is ineffective in treating infested dogs. Three out of nine cases of dogs, where neoplastically transformed nodules were removed surgically, did not recover and died within three days, due to complications (Ranen *et al.* 2004). There are a number of causes for the high complication rates in oesophageal surgery, which include excessive tension at the suture line, lack of serosa (outermost layer of the oesophagus that anchors the oesophagus), constant motion at the suture site, passage of undigested food or saliva over the suture site, segmental blood supply and lack of omentum (a double layer of peritoneum that covers internal organs) (Van der Merwe *et al.* 2008). However, it was found that partial oesophagectomy is a safe and effective technique for the removal of sarcomas of the distal thoracic oesophagus. Dogs that underwent this procedure recovered rapidly and enjoyed a good quality of life for several months (Ranen *et al.* 2004).

No significant conclusions could be drawn on the effectiveness of chemotherapy in the treatment of spirocercosis-associated oesophageal sarcomas (Ranen *et al.* 2004; Van der Merwe *et al.* 2008), however this form of treatment produced severe adverse reactions in two cases (Ranen *et al.* 2004) and was subsequently terminated.

It may be worthwhile to apply treatment regimens that are found to be effective in other nematodes that are similar to *S. lupi*. A study that compared common Spirurida worms by constructing a phylogenetic tree based on c*ox1* sequences, found that the canine heartworm *Dirofilaria immitis* clustered with *S. lupi* haplotypes (Traversa *et al.* 2007). The infection produced by *D. immitis* is similar to spirocercosis, however it was concluded that the many differences underlying these two parasitic infections may make the application of similar treatment protocols ineffective (Van der Merwe *et al.* 2008).

1.5.7. Prevention strategies

Veterinarians as well as pet owners should be informed about dung management (Last & Smith 2007). It was observed that farms with poor sanitation had a high percentage of infestation with *S. lupi* (Bailey 1963). Thus faeces need to be disposed of in an appropriate manner to reduce environmental contamination by eggs, which will in turn reduce the number of infested beetles. Dogs should be prevented from hunting, scavenging and eating uncooked viscera (Bailey 1972). Control mechanisms should be in place to decrease egg shedding from infested animals (Van der Merwe *et al.* 2008).

Coprophagous beetles can be examined to detect which type of beetles carry this infectious nematode. *Onthophagus* is the best known genus of coprophagous beetles involved in the *S. lupi* life cycle (Du Toit *et*

al. 2008). Since dogs from endemic areas are continuously exposed to infective larvae, a minimum serum concentration required to inhibit or kill *S. lupi* adults and larvae, as well as the maximum treatment interval required to maintain this level must be determined if a treatment is to be implemented (Van der Merwe *et al.* 2008).

Sequencing the mitochondrial genomes of other nematode species, e.g. *Dirofilaria immitis, Onchocerca volvulus, Brugia malayi* and *Strongyloides stercoralis* (Hu & Gasser 2006), has proven to be informative and has recently been conducted for *S. lupi* (Liu *et al.* 2013). Targeted approaches, such as PCR, which screen for particular genes of major functional significance, are also advantageous and effective. A wide range of fundamental biological aspects can be explored, such as developmental regulation and switching, and host-parasite interactions (Gasser & Newton 2000). Novel approaches for controlling parasites, for example, by blocking signaling pathways, can only be implemented if the molecular interactions between host and parasite are understood (Gasser & Newton 2000).

1.5.8. Spirocerca lupi genetics

To date, very few molecular studies have been conducted on the nematode worm, *S. lupi*. Current methods of detection have proven to be non-specific, expensive, time-consuming and risky or life-threatening for an affected animal (Traversa *et al.* 2007). A retrospective analysis of patients presenting with *S. lupi* infection at the Onderstepoort Veterinary Faculty (University of Pretoria) indicates that the reported incidence of the disease has increased significantly in South Africa since the year 2000 (personal communication). Studies to determine the genetic variation of the parasitic population should thus be conducted in order to implement successful control programs and prevent the dispersal of drug resistance alleles.

Studies on the population genetics of *S. lupi* have been limited. The *cox1* gene was isolated and characterised to develop diagnostic primers to diagnose the disease at an earlier stage when the lesions are yet to be induced (Traversa *et al.* 2007). However, the *cox1* gene was found to be highly conserved between Spirurida nematodes and was therefore not specific for *S. lupi* (Traversa *et al.* 2007). The *cox1* gene was also used as a mitochondrial genetic marker to study the sequence variability and population genetics in *S. lupi*. Low genetic variation was found among *S. lupi* individuals from different geographical areas. Two haplotypes were identified in samples from Africa, Asia and Europe (Traversa *et al.* 2007). This study also found that the *S. lupi* population is highly structured between the different geographical regions, with very little genetic diversity within a geographical area (Traversa *et al.* 2007).

A more recent study, however, also targeting the *cox1* gene (De Waal *et al.* 2012), identified eleven haplotypes in Tshwane Metropole (South Africa) alone, which indicates a high amount of genetic variation in *S. lupi* individuals within the same geographical area for Southern Africa. Most of the genetic variation

found could be explained by differences between worms sharing the same host. Additional data regarding the amount of genetic variation is required to resolve this conflict of whether or not there is in fact high genetic diversity within the *S. lupi* population. It was also found that, contrary to the suspected increase in the size of the *S. lupi* population, this parasitic population has been stable for a long period of time (De Waal *et al.* 2012). Thus, it seems that recently, more incidents of spirocercosis have been reported than in previous years.

In order to study parasitic population genetics, a variety of molecular markers have been developed. Of these markers, those developed from mitochondrial DNA (mtDNA) have been used very often. The complete mitochondrial genome of *S. lupi* has been sequenced recently, and is 13, 780 bp in length (Figure 1.4) (Liu *et al.* 2013). An identical gene arrangement was found to that of the nematodes *Thelazia callipaeda* (Thelaziidae) and *Setaria digitata* (Onchocercidae). This genome will aid in the development of mtDNA markers for future studies on *S. lupi*. Although these markers will provide valuable information, their use is limited in that they are maternally inherited and represent a single locus. mtDNA may also not be neutral, since it is affected by demographic, geographic as well as other factors (de Meeûs *et al.* 2007). Thus microsatellite markers will provide a higher resolution since they are biparentally inherited and will provide more information regarding the population structure and diversity of parasitic nematodes.

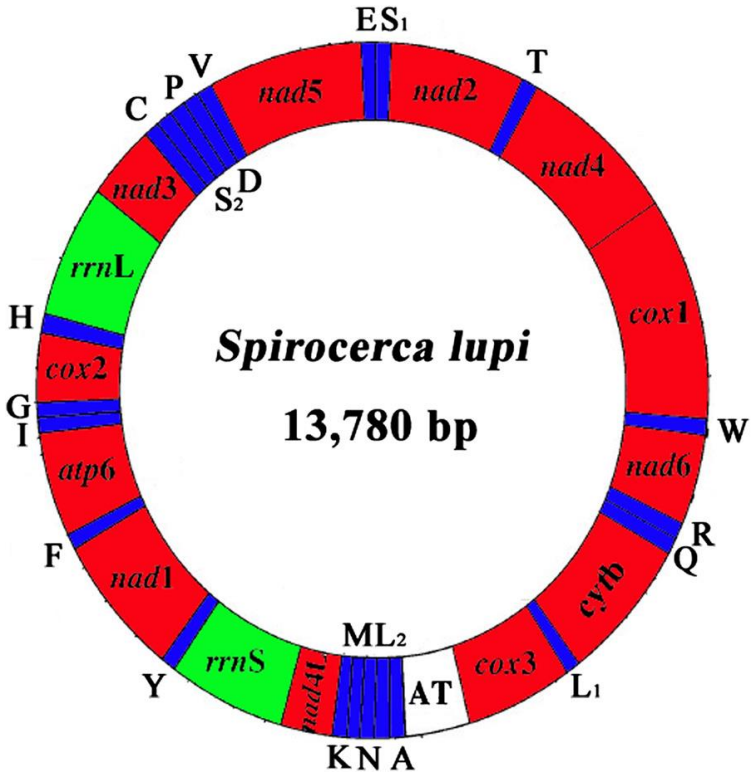

Figure 1.4: The arrangement of the full mitochondrial genome of *Spirocerca lupi* (Liu *et al.* 2013)

1.6. AIM AND OBJECTIVES

In order to implement management strategies, it is important to understand the population dynamics of a parasitic population so that the spread of drug resistance alleles can be prevented or delayed, even before drug resistance begins to emerge in a parasite population. It is also beneficial to know how parasite genes are distributed within and between host populations and how forces such as gene flow, genetic drift and selection are influencing this distribution (Johnson *et al.* 2006).

Polymorphic microsatellite loci have the potential to reveal population genetic diversity and will thus be developed for this purpose. Microsatellites have been proven useful in other studies with parasitic nematodes (Grillo *et al.* 2006; Otsen *et al.* 2000; Schwenkenbecher & Kaplan 2007) and have a broad range of applications. Microsatellites are known to be more sensitive in detecting subtle population structure in species with high levels of gene flow. Microsatellite markers have not been published before for the study of population genetic diversity in *S. lupi*. Therefore, this investigation will characterise polymorphic microsatellite loci for ongoing and subsequent population genetic studies. Population genetic analyses will be performed on *S. lupi* individuals from various locations in South Africa, where an increased number of cases of spirocercosis have been reported. Analysis of the genetic differentiation within and between hosts will reveal genetic structuring of the *S. lupi* population.

Transferability to a species related to the one from which the microsatellites are obtained is sometimes feasible (Zhan *et al.* 2009). Hence, the microsatellites developed for *S. lupi* will be tested on other species in the family or closely related to the family, *Thelaziidae*. Once developed, the microsatellite marker sequences can be submitted to the GenBank database for utilisation in further research.

This research forms part of a larger programme concerned with investigating the population structure, genetic variation and epidemiology of *S. lupi* in South Africa. In the long term, the knowledge gained from this and future work is expected to allow pet owners to reduce the risk of infection of their dogs and also allow veterinarians to understand the risk of their patients contracting this disease. The molecular information gained from studies on *S. lupi* would expand the knowledge base of this little studied, yet important nematode and serve as a foundation for further studies in ecology, epidemiology, diagnosis and treatment of canine spirocercosis.

Main aim:

To assess population genetic variation in the parasitic nematode *Spirocerca lupi* in primary hosts from South Africa

Research questions:

1. What is the spatial genetic variation of *Spirocerca lupi* in South Africa?
2. How does genetic variation compare within and between definitive hosts?

Research objectives:

1. To develop a set of polymorphic microsatellite markers for *Spirocerca lupi.*
2. To determine population genetic variation among primary hosts in South Africa.

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