Phylogeography of the rodent mites *Laelaps giganteus* and *Laelaps muricola* using mitochondrial and nuclear DNA markers: an evolutionary approach to host-parasite interactions

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Some of the contents contained in this thesis (Chapters 2-4) are taken directly from manuscripts submitted or drafted for publication in the primary scientific literature. This resulted in some overlap in content between the chapters.

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Abstract

Laelaps giganteus and *Laelaps muricola* (Mesostigmata; Laelapidae) are widespread and locally abundant mites on small mammals in southern Africa. The large host range and complex life history of these ectoparasites suggest possible intraspecific cryptic diversity in these taxa. The mechanisms responsible for speciation in response to codiversification in parasite-host systems are poorly understood. Similarly, how biogeography, parasite life history, and host vagility influence evolutionary codivergences is at present unknown in mite systems in southern Africa. A comparative phylogeography approach was followed to study the evolution and taxonomy of two mite species and their known host species. The main objectives of the study were to: (1) investigate the evolutionary history and taxonomic status of two southern African Mesotigmatid mites, *L. giganteus* and *L. muricola*, using a multidisciplinary approach including a combination of mitochondrial and nuclear DNA markers and selected morphological characters, (2) apply a comparative phylogenetic framework to *L. giganteus* which is only found on a single rodent genus, *Rhabdomys*, in an attempt to better understand codivergence between parasites and hosts, particularly at the phylogeographic level, and (3) determine whether *L. muricola* with a wide host range, yet similar life history, would show similar phylogeographic patterning to the host specialist *L. giganteus* across southern Africa. To assess the genetic and morphological diversity in *L. giganteus* and *L. muricola*, 228 rodents were collected from eight localities in southern Africa. This sample included nine previously recorded host species and on these, *L. muricola* was predominantly recorded from *Mastomys natalensis* and *Micaelamys namaquensis* while *L. giganteus* was found on *Rhabdomys dilectus* and *Lemniscomys rosalia*. Phylogenetic analyses of mtDNA cytochrome oxidase subunit I (COI) and nuclear ITS1 data strongly supported the recognition of *L. giganteus* and *L. muricola* as distinct species, a scenario

partly supported by sequence data of the Tropomyosin intron. Strong support for evolutionarily distinct lineages within *L. giganteus* was found: *L. giganteus* lineage 1 was confined to *R. dilectus* and *L. giganteus* lineage 2 was confined to *L. rosalia*. These host-specific monophyletic lineages were separated by 9.84% mtDNA sequence divergence and 3.44% nuclear DNA sequence divergence. Since quantitative morphometric analyses were not congruent with these findings, these two lineages more than likely represent cryptic species. Further sampling across southern Africa indicated that *L. giganteus* occurs on four rodent species within the genus *Rhabdomys*. Cytochrome Oxidase I parsimony haplotype networks derived for 262 host and 278 parasite specimens showed marked phylogeographic congruence, which was in part confirmed by analyses of the Tropomyosin (TropoM) intron. Although distance-based cophylogenetic analyses in AXPARAFIT failed to support significant mtDNA codivergences ($P \geq 0.020$), event-based analyses revealed significant cophylogeny between *Rhabdomys* and *L. giganteus* lineages using CORE-PA ($P = 0.046$) and JANE ($P = 0.000$). These findings, in conjunction with the weak congruence previously reported among the permanent ectoparasitic lice *Polyplax* and *Rhabdomys*, suggest that parasite-host intimacy (time spent on the host) is not the main driver of significant codivergence in the study system. Instead the restricted dispersal ability of *L. giganteus* resulted in strong spatial structuring and when this was coupled to an intimate relationship with the host, significant codivergence emerged. Both event-based reconstruction methods also indicated host switching that in some instances could be linked to climate-induced range shifts in the host distribution. When host range shifts occur, the phylogeographic signature of *L. giganteus* is preserved, as the genetic contribution of the dispersing individuals is overwhelmed by the large number of individuals already present in nests within the new environment, a phenomenon described as a parasite "drowning on arrival". Novel phylogeographic insights into the host range of *L. muricola* are also shown, expanding the

contemporary information available on this species in southern Africa. Results show the first evidence of a putative cryptic *L. muricola* lineage on the brown rat, *Rattus norvegicus*, present in South Africa. On native hosts, *L. muricola* indicate a lack of phylogeographic structuring owing to its generalist life style and the unique life history of some of its hosts. *Mastomys coucha* and *M. natalensis* are able to survive in multiple refugia and rapidly expand once favourable conditions set in. The pattern we find in this host generalist confirms that host dispersal is driving the genetic structure in both *L. muricola* and *L. giganteus*.

Opsomming

Die parasitiese myte, *Laelaps giganteus* en *Laelaps muricola* (Mesostigmata; Laelapidae) is wydverspreid en volop op klein soogdiere in suider Afrika. Die groot gasheer spektrum en komplekse lewensgeskiedenis van hierdie ektoparasiete mag aandui dat daar moontlike intraspesifieke kriptiese diversiteit in hierdie taxa is. Die meganismes verantwoordelik vir hierdie patrone en spesiasie met die klem op ko-diversifikasie in parasiet-gasheer stelsels is egter onduidelik op die oomblik. Hoe prosesse soos biogeografie, parasiet lewensgeskiedenis en gasheer verspreiding evolusionêre ko-diversifikasie beinvloed is ook tans heeltemal onbekend in myt biologiesie stelsels in Suid-Afrika. Hier word ‘n vergelykbare filogeografiese benadering tussen die twee mytspesies en hulle bekende gasheerspesies gevolg. Die hoof doelstellings van die studie was om: (1) die evolusionêre geskiedenis en taksonomiese status van twee suider-Afrikaanse Mesostigmata myte, *L. giganteus* en *L. muricola*, te ondersoek deur gebruik te maak van ‘n multi-dissiplinêre benadering wat ‘n kombinasie van mitokondriale DNS (mtDNS), kernDNS merkers en uitgesoekte morfologiese karakters insluit (2) ‘n vergelykbare filogenetise raamwerk tussen *L. giganteus* en *Rhabdomys* te gebruik in ‘n poging om meer duidelikheid te kry oor hoe parasiete met hul gashere op filogeografie vlak ko-diversifiseer, en (3) te bepaal of die ruimtelike genetiese struktuur van *L. muricola*, ‘n myt met ‘n weier gasheerspektrum, ooreenstem met die van *L. giganteus*, ‘n spesie met ‘n nouer gasheerspektrum, in suider Afrika. Om die genetiese en morfologiese diversiteit in *L. giganteus* en *L. muricola* te bepaal is 228 klein soögdiere van agt lokaliteite in Suid-Afrika versamel. Hier was nege van die voorheen geidentifiseerde gashere versamel. *Laelaps muricola* was waargeneem op *Mastomys natalensis* en *Micaelamys namaquensis* terwyl *L. giganteus* slegs op *Rhabdomys dilectus* en *Lemniscomys rosalia* gevind was. Filogenetise analises van die mtDNS (COI) en kernDNS (ITS1) data het

oorweldigende ondersteuning aangedui dat *L. giganteus* en *L. muricola* as twee aparte spesies beskryf moet word terwyl die TropoM kernDNS interon dit deels ondersteun het. Sterk ondersteuning is ook verleen dat twee nuwe afstammelinge binne *L. giganteus* voorkom: *L. giganteus* lyn 1 kom slegs voor op *R. dilectus* terwyl die *L. giganteus* lyn 2 slegs op *L. rosalia* voorkom. Hierdie gasheer spesifieke monofiletiese lyne is ook geskei deur 9.84% mtDNS volgorde bepaling divergensie en 3.44% kernDNS volgorde bepaling divergensie. Siende dat kwantitatiewe morfometriese ontledings nie die genetiese onderskeiding ondersteun het nie verteenwoordig die twee heel waarskynlik kriptiese spesies. Verdere versameling van gashere om die patroon te bevestig het getoon dat *L. giganteus* op vier ander spesies binne die genus *Rhabdomys* voorkom. Sitochroom Oxidase I parsimoniese haplotiepe netwerke wat gebaseer is op 262 gasheer en 278 parasiet individue het aangedui op duidelike filogeografiese ooreenkomste, wat deels bevestig was deur analises van die TropoM intron. Alhoewel afstand-gebaseerde ko-filogenetiese ontledings in AXPARAFIT nie ko-diversifikasie ondersteun nie ($P > 0.02$), het gebeurtenis-gebaseerde ontledings getoon dat beduidende ko-filogenie tussen *Rhabdomys* en *L. giganteus* afstammelinge (CORE-PA: $P = 0.046$ and JANE: $P = 0.00$) bestaan. Hierdie bevindinge en die swak kongruensie wat voorheen vir die permanente ekto-parasitiese luis *Polyplax* op *Rhabdomys* genoteer was is teenstrydig met die voorspelling dat gasheer-parasiet intimiteit (tyd gespandeer op die gasheer) die hoof dryfkrag is van beduidende ko-diversifikasie. Hier stel die bevindinge ook voor dat die filogeografiese sein afgegee deur ‘n parasiet bevolking behou word na nuwe individue aansluit by die lokale bevolking. Hierdie verskynsel word beskryf as die parasiet “verdrink by aankoms”. Nuwe inligting oor die gasheerspektrum van *L. muricola* in suider Afrika is ook gevind. Bewyse word hier gelewer oor ‘n moonlike kriptiese parasiet wat op die indringer bruin rot, *Rattus norvegicus*, voorkom in Suid-Afrika. Verdere resultate dui daarop dat waneer na die natuurlike geshere van *L. muricola* gekyk word, geen filogeografiese

stuktuur voorkom nie. Die patroon mag dalk veroorsaak word deur die feit dat *L. muricola* se gashere in verskeie habitatte kan oorleef en vinnig kan aanteel en versprei wanneer goeie toestande aanbreek.

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Chapter 1

General Introduction

1.1. Phylogeography and parasitism

Phylogeography has been used to describe the spatial geographical distribution of alleles in a species in order to uncover modes of speciation (Avise 2000). Congruent patterns between multiple species can then be used to explain processes and geographical factors that may have influenced the pattern (Avise 2000; Hickerson *et al.* 2010). Population genetic structure across the landscape is primarily determined by the dispersal potential of the species; however other factors such as resource availability, habitat heterogeneity and population densities and dynamics may also play a role leading to genetic structure (Avise 2000; Meyer *et al.* 2009).

Pioneer parasite population genetic and phylogeographic studies investigated mostly single host parasite systems (Nadler *et al.* 1990; Hafner and Nadler 1990; Moran and Baumann 1994). More recently, emphasis has shifted to describe the phylogeographical patterns of multiple parasites in a particular system (Nieberding *et al.* 2004; Štefka and Hypša 2008; Aoki *et al.* 2009; Archie *et al.* 2011; Noureddine *et al.* 2011). Parasites can live on their host (host specific) whilst others utilise multiple hosts (host generalist) (see Mullen and O'Connor 2002 for mite examples). This together with the fact that parasites differ in life history from each other provides unique and interesting hypotheses worth testing. For instance, host specific permanent parasites that spend their entire life on the host (e.g. such as lice), should show a genetic structure more similar to the host (Baumann and Baumann 2005;

McCoy *et al.* 2005; Morelli and Spicer 2007; Light and Reed 2009; du Toit *et al.* 2013*a*) compared to parasite taxa that temporarily attach to one or multiple host species (e.g. ticks, mites and fleas) (Nourreddine *et al.* 2011; Cangi *et al.* 2013). Along the same lines, it is anticipated that the phylogeographic pattern of temporary parasites with free-living stages in the environment outside the host's nest (most ticks and helminths) will differ from temporary taxa with free-living stages within the host's nest (most fleas and mites). In both cases the survival of free-living stages will be affected by the external environmental conditions (Archie *et al.* 2011). Explaining the phylogeographic genetic structure of parasites are thus complex since geographic populations can be structured spatially between microhabitats within individual hosts, between individuals or populations of the same host species and between different host species (DeMeeûs *et al.* 1998; Clayton *et al.* 2003; Noureddine *et al.* 2011). Comparative population genetic studies of parasites can thus help to explain the mechanisms involved in shaping these systems (du Toit *et al.* 2013*a*, *b*; van der Mescht *et al.* 2015*a*, *b*).

It has been suggested that unravelling the genetic structure of parasites, in particular host-specific parasite species, can lead to a better understanding of the hosts' evolutionary trajectory though time (Nieberding and Morand 2006). For instance, a study of host-parasite congruence between bast scale (*Matsucoccus feytaudi*) and pine tree (*Pinus pinaster*) revealed putative refuges used by the host during the Quaternary ice age (Burban *et al.* 1999; Burban and Petit 2003). In the order Rodentia the interaction between the field mouse (*Apodemus sylvaticus*) and its nematode parasite (*Heligmosomoides polygyrus*) also showed glacial refugal areas, and even revealed host migration routes (Nieberding *et al.* 2004, 2005). Phylogeographical investigations of host-specific parasites have also proven their value for uncovering cryptic speciation in host species and host origin. In a study by Pellmyr *et al.*

(1998), which compared the phylogeography of hoary bowlesia (*Bowlesia incana*) and its host-specific parasitic butterfly (*Greya powelli*) found that the plant was introduced to North America much earlier than previously thought. A comparative phylogeographic study of collared lemmings (*Dicrostonyx* spp.) and a host-specific cestode group (*Paranoplocephela arctica* complex) indicated that the lemmings colonized and underwent a secondary dispersal from Beringia to the Canadian Arctic region (Wickström *et al.* 2003). Interestingly, a previous study investigating the host genetic structure alone did not reveal host colonization in this species complex (Ehrich *et al.* 2000).

One of the reasons why parasite genetic structure is useful to infer host biogeography is amongst others the rapid generation time in parasites (Page and Hafner 1996; Nieberding and Morand 2006). Particularly for host-specific parasites, the accelerated generation time of the parasite, limited gene flow between parasites on different hosts, and often small effective population sizes (Ne), lead to a molecular rate of change up to 10 times higher than in their host (Clayton and Johnson 2003; see Huyse *et al.* 2005; Whiteman and Parker 2005 for reviews). Consequently, the possibility of unresolved gene and species trees due to incomplete lineage sorting in the host can be indirectly revealed by study of the parasite (Nieberding and Olivieri 2006; Johnson *et al.* 2003).

1.2.Parasite and host coevolution and host switching

The relationships between hosts and parasites have often been studied due to the potential impact they may have on the health of domestic animals, wildlife and humans (Nieberding and Olivieri 2006). The process where two species (one a parasite and the other a host) impose selective forces on each other over evolutionary time is defined as a form of

coevolution (Nieberding and Olivieri 2006). Thompson (1994) describes this type of coevolution as the process in host-parasite interactions which relatively describes the rate of parasite infectivity versus host resistance. It is also important to note, that the host-parasite interaction at this level can also be seen as an evolutionary arms race. Showing coevolution by making use of similar phylogenetic trees between parasite and host has, however, rarely been shown. Similar branching patterns in the phylogenies can also be interpreted as codivergences, where no reciprocal selection pressures are assumed. Interestingly, although these three terms are interlinked (also see Paterson *et al.* 2001; Nieberding and Olivieri 2006), Page (2003) stated specifically that it is very difficult to explain codivergences without some form of coevolution.

The level of codiversification (codiversification refers to how closely the host and parasite phylogenetic trees match) between host and parasite is determined by how "associated" (association refers to what relationship a parasite shares with its host) a parasite is with its host (Brooks 1979). The degree of congruence can indicate if parallel speciation took place in the case of association by decent (parasite-host associations arise because the host inherited the parasite from its ancestor) (Page 1994; Weiblen and Bush 2002). Incongruence may suggest host switching or lineage sorting or even intra-host speciation (association by colonization) (Brooks and Paterson 2005; Brooks and Hoberg 2007). Artificial congruence may also exist between host and parasite. This pattern may arise as a result of sequential host-switches, where the parasite repeatedly colonise the close relatives of the host and subsequently speciate (Brooks and McLennan 1993).

The evolutionary relationship between host and parasite is therefore heavily dependent on the life history of the parasite. In the case of host-specific parasites (parasite

species only occur on a single host species, as is the case with most anoplurid lice) congruence is more likely (Brown *et al.* 1997; Charleston and Robertson 2002). Multi-host parasites or generalist parasites may show a more complicated pattern as a result of the presence of cryptic species in the parasite itself (Knee *et al.* 2012). The parasite may also fail to speciate while the host undergoes divergence, and incongruence between a host and a generalist parasite may thus be due to incomplete host switching (Johnson *et al.* 2003; Banks and Paterson 2005). To date most studies that have investigated host-parasite interactions have focussed on processes such as co-speciation which are above the species level (see Page 2003 for review). Information regarding parasite genetic structure at the species level however is critically needed to reveal important aspects of parasite ecology and evolution (Nadler *et al.* 1995; Barrett *et al.* 2008; Archie *et al.* 2011).

Two parasite studies in southern Africa have been done, showing the presence of cryptic species in the host-specific louse of *Rhabdomys* (see section 1.5); here limited codivergence was noted between this parasite and any of its hosts (du Toit *et al.* 2013*a*). In addition, a generalist fur flea (*Listropsylla agrippinae*) showed phylogeographic structure congruent with host vicariance in the region while a generalist nest flea (*Chiastopsylla rossi*) showed no congruence and a pattern of interpopulational divergence attributed to a lack of dispersal (van der Mescht *et al.* 2015*a*). Here focussing on mites can add an extra dimension to further explain how life history differences can influence codiversification between hosts and parasites.

1.3. Taxonomy of *Laelaps giganteus* and *Laelaps muricola*

The mesostigmatid mites *Laelaps giganteus* and *L. muricola* (order Acarina) belong to the family Laelapidae (Zumpt 1961). The family is in the super family Dermanyssoidae which has a diverse assemblage of life forms consisting of both free-living and parasitic taxa. Members in the genus *Laelaps*, forming the focus of the present thesis, are parasites of vertebrate hosts (Radovsky 1994). The morphological character that unites the Mesostigmata is the presence of a tritosternum and a stigmata lateral to coxae III and IV. *Lealaps* females have an opisthogenital shield with four pairs of setae on females. In particular, *Lealaps giganteus* (Berlese 1918) has a genital plate (Fig. 1.1A; a) far from the anal shield (Fig. 1.1A; b) relative to other species within *Lealaps*, which is covered with par-anal setae that are in line with the posterior margin of the anal opening (Fig. 1.1A). In contrast, *L. muricola* (Träghardh 1910) has a smooth and fairly wide heart-shaped genital plate extending greatly behind the hind legs (Fig. 1.1B; a) and has small distance from the anal shield (Fig. 1.1B; b). At present, morphological descriptive characters suggest that *L. giganteus* and *L. muricola* are both valid species, although Zumpt (1961) surmised about the possibility of multiple sub-species in a check-list on the Arthropod Parasites of Vertebrates in Africa, south of the Sahara. Zumpt (1961) however, does not provide supporting evidence for this statement and the current diversity is thus not known at present.

Fig. 1.1A: Ventral view of *Laelaps giganteus* with opistogenital plate (a) distant from the anal plate (b). **B.** Ventral view of *Laelaps muricola* with heart-shaped opistogenital plate (a) and has small distance from the anal plate (b).

1.4. Life history and feeding habits of *L. giganteus* and *L. muricola*

Information regarding the life history and feeding habits within the genus *Lealaps* is very limited, with the exception of *Laelaps echidninus* (Mullen and O'Connor 2002). Mites of this genus are commonly found in the nests of their sub-Saharan murid hosts (Zumpt 1961) though it has been suggested that adult stages also occur on the body of the host (Radovsky 1994). Four developmental stages have been recorded in *L. echidninus*. Females that had a blood meal can give birth to live larvae which do not feed. This is followed by two nymphal stages (protonymph and deutronymph) and the adult stage which all feed off the host (Mullen and O'Connor 2002). At least 16 days are required to complete the life cycle, and adults can survive without feeding for up to three months. Mullen and O'Connor (2002) suggest that *L. echidninus* are unable to pierce the skin of their hosts and opportunistically obtain blood meals (which are needed for vitality and reproduction) from damaged body parts of their hosts. In the absence of emperical evidence for *L. giganteus* and *L. muricola* it is assumed that they will follow a similar life cycle.

If the same life history is present in these two mite species we can predict that *L. giganteus* and *L. muricola* will follow a generalist parasitic lifestyle as they are able to feed on broad spectrum of hosts to obtain a blood meal to complete their life cycle. The opportunistic nature of these species is exemplified in the fact that they have been recorded on several rodent species and across a relatively large geographical range (Zumpt 1961). A broad host preference will facilitate dispersal events and contact between different mite populations on various host species (Johnson *et al.* 2003; Banks and Paterson 2005). Based on this it is unlikely that *L. giganteus* and *L. muricola* will share a common evolutionary history with any specific murid host species. A lack of genetic structure, as a result of greater gene flow, is therefore expected in these generalist species.

1.5. Hosts of *L. giganteus* and *L. muricola*

The most recent check-list compiled by Zumpt (1961) lists several murid rodents as potential hosts for *L. giganteus* and *L. muricola*. The rodent species include *Rhabdomys pumilio, Lemniscomys griselda, L. striata*, *Mastomys natalensis*, *Dasymys incomtus*, *Pelomys fallax*, *Arvicanthis niloticus*, *Aethomys chrysophilus*, *Micaelamys namaquensis*, *Mylomys cuninghamei*, *Saccostomys campestris*, *Otomys irroratus*, *Parotomys litteldalei* and *Tatera afra*, *Rattus morio* and *R. rattus* (Zumpt 1961). In most cases, these host species do not occur in sympatry across southern Africa (see Skinner and Chimimba 2005 for distribution maps). The four-striped mouse, *Rhabdomys*, has the widest geographic range across southern Africa and occurs in isolated patches in central Africa. The distribution of this host covers extensive areas of Namibia, Botswana, Zimbabwe, Mozambique, Swaziland, Lesotho, South Africa, Tanzania, Kenya, Uganda, the DRC, Angola, Zambia and Malawi (Skinner and Chimimba 2005).

Rhabdomys pumilio has a high overall abundance in natural and urban habitats and has been noted as an economically important pest species, which frequently undergoes population explosions (de Graaff 1981). Within the Western Cape Province of South Africa *R. pumilio* was found to harbour a great diversity of ectoparasites, which include 13 ixodid tick species; 11 mite species (including *L. giganteus*), eight flea species and a single host-specific louse species (*Polyplax arvicanthis*) (Matthee *et al.* 2007).

The taxonomy of the four-striped mouse has been riddled with controversy. Various methods have been used to address relationships within this genus, including morphological data by Roberts (1951) (describing 20 subspecies) and Meester *et al.* (1986) (only recognising seven subspecies of the original 20). Chromosome data have also been employed by Rambau *et al.* (2003) who identified two chromosome races. Based on mtDNA sequences, two species have been described in the genus *R. pumilio*. One occurs in the south-western regions of southern Africa and *R. dilectus* consisting of two subspecies namely *R. dilectus dilectus* and *R. d. chakae*. Both species occur in the central and eastern parts of South Africa (Rambau *et al.* 2003). More recently, DNA sequence data and niche modelling identified three additional clades within the previously described *R. pumilio* (du Toit *et al.* 2012). They have distinct geographic distributions and ecological niche modelling supports a strong correlation between the regional biomes and the distribution of distinct evolutionary lineages (du Toit *et al.* 2012). Based on the results from du Toit *et al.* (2012), four species are recognized in the genus *Rhabdomys* these being *R. dilectus*, *R. pumilio*, *R. intermedius* and *R. bechuanae*.

Other hosts of the two mite species, which have been investigated in a population genetic framework in southern Africa, include the namaqua rock mouse *M. namaquensis*

(Chimimba 2001; Russo *et al.* 2010) and the vlei rat, *O. irroratus* (Taylor *et al.* 2009; Engelbrecht *et al.* 2011). *Micaelomys namaquensis* is a rock-dwelling species that has a large southern interior distribution in southern Africa (Skinner and Chimimba 2005). To date, pronounced genetic structure has been found in rock-living vertebrate species in southern Africa. In particular, the Knersvlakte (a region of extremely low rainfall in the Western Cape Province of South Africa) has been found as a contemporary phylogeographic barrier. More recent studies suggest that the Orange River is also a barrier to gene flow in these taxa (Matthee and Robinson 1996; Bauer 1999; Matthee and Flemming 2002). *Otomys irroratus* has a wide distribution across South Africa, Eastern Zimbabwe, Western Mozambique, Lesotho and Swaziland (Meester *et al.* 1986; Skinner and Chimimba 2005) and prefers areas which are permanently covered with verdant vegetation. Several studies that investigated the population genetic structure of the vlei rat have suggested the presence of two species (separated by a 7% sequence divergence at the mtDNA cytochrome *b* level) within this taxon. It appears that their distribution corresponds to the two major bioclimatic regions (wet eastern side of the country and dryer western side of the country) of South Africa (Taylor *et al.* 2009; Engelbrecht *et al.* 2011). The cross over zone between these two major bioclimatic regions was also identified as a contact zone for the two vlei rat species (Engelbrecht *et al.* 2011).

From the above, specific phylogeographical barriers have been identified as contributors to the species boundaries for several of the potential host species of *L. giganteus* and *L. muricola* in southern Africa. However these barriers to gene flow cannot be seen as the result of disruptive selection pressures since cladogenesis could have resulted from allopatry and genetic drift. Given this pattern of cladogenesis observed in multiple hosts of *L. giganteus* and *L. muricola*, one can hypothesise that multiple taxonomic groups in the two *Laelaps* species can exist. Specifically, given its obligate blood feeding needs and the

presence of multiple taxonomic units in three of its southern African hosts namely *R. pumilio* (Rambau *et al.* 2003; du Toit *et al.* 2012); *O. irroratus* (Taylor *et al.* 2009; Engelbrecht *et al.* 2011) and *M. namaquensis* (Chimimba 2001; Russo *et al.* 2010). Should genetic structure be detected in this generalist parasite, then it seems reasonable to also suggest that similar vicariance factors may have had an impact on cladogenesis for other hosts not sampled. Apart from the biogeographic factors that influence the distribution of the host species, genetic differentiation in the parasite can also occur as a result of differences in host life history (du Toit *et al.* 2013*b*).

1.6. Aims

The main aims and objectives of the study were the following:

1. To investigate the evolutionary history and taxonomic status of two southern African mesotigmatid mites, *L. giganteus* and *L. muricola*, using a multi-disciplinary approach. For the latter aim I used a combination of mitochondrial and nuclear DNA markers and also included selected morphological characters. The objective here was to investigate the relative importance of host range as a contributing factor towards lineage diversification. This was tested by sampling multiple previously described hosts of these parasites at different localities.

2. To apply a comparative phylogenetic framework to *L. giganteus* and *Rhabdomys* in an attempt to better understand codivergence between parasite and host, particularly at the phylogeographic level.

3. Determine whether *L. muricola* with a wider host range but overall similar life history would show similar phylogeographic patterning to *L. giganteus* across southern Africa.

Chapter 2

Evidence of cryptic speciation in mesostigmatid mites from South Africa*

***** Findings from this chapter has been published in *Parasitology 141 (2014) 1322-1332*

2.1. Introduction

Recent molecular studies highlighted the need to re-address parasite taxonomy previously based on traditional morphological approaches (Williams *et al.* 2006; Smith *et al.* 2007; Ståhls and Savolainen 2008; Perkins *et al.* 2011; du Toit *et al.* 2013*a, b*). This holds true especially for small-bodied invertebrates which are often characterized by a slow rate of change in morphological features (Clayton *et al.* 2003; Huyse *et al.* 2005; Whiteman and Parker 2005), and in the case of ectoparasites, speciation may also be subjected to evolutionary processes related to their host (Roy *et al.* 2008, 2010; Perkins *et al.* 2011; du Toit *et al.* 2013*a*, *b*).

Since a comprehensive understanding of cryptic diversity is needed to better understand taxonomy and the processes generating biodiversity, most modern taxonomic studies have a total evidence approach incorporating both morphology and DNA sequencing (Bickford *et al.* 2007; Morelli and Spicer 2007; Detwiler *et al.* 2010; Shäffer *et al.* 2010; Skoracka and Dabert 2010; Apanaskevich *et al.* 2011; Knee *et al.* 2012). Apart from providing new insights into the systematics of the taxa concerned, a thorough phylogenetic analysis can also be used to explore the mechanisms involved in parasite speciation. For example, well-resolved congruent phylogenies of a particular parasite and host system can enable coevolutionary scenarios to be described between the complementary parasite and host lineages (Page 1996; Morelli and Spicer 2007; du Toit *et al.* 2013*a, b*).

The evolutionary history of ectoparasite taxa is complex. They can be structured spatially between microhabitats within individual hosts, between individuals or populations of the same host species, and between different host species (de Meeûs 2000; Clayton *et al.* 2003; Noureddine *et al.* 2011). Differences in several factors, such as life cycle, mode of transmission, interspecific competition and host specificity could result in more pronounced genetic structure in a given parasite species or population (Blouin *et al.* 1995; Nadler 1995; Criscione and Blouin 2004, 2005; Barrett *et al.* 2008; Cangi *et al.* 2013) and different parasite races may even be formed as a result of disruptive selection in the host (Maynard Smith 1966; Bush 1994). However, without sufficient data, the contemporary taxonomy and evolutionary predictions for ectoparasites remain highly speculative.

In the African context, molecular investigations focusing on the taxonomy and coevolution of mammalian ectoparasites are virtually non-existent (but see Cangi *et al.* 2013; du Toit *et al.* 2013*a*, *b*). Pertinent to the focus of the present study, regional investigations on mites of the order Mesostigmata seem to be limited to species surveys only (Hirst 1925; Zumpt 1961; Matthee *et al.* 2007, 2010; Matthee and Ueckermann 2008, 2009; Viljoen *et al.* 2011). Since mite studies conducted elsewhere show pronounced genetic disparity when compared with morphological traits (Morelli and Spicer 2007; Knee *et al.* 2012), it seems reasonable to hypothesize that the current diversity of parasitic mites in southern Africa is also underestimated (also see de León and Nadler 2010; Nadler and de León 2011).

To gain more insights into the evolution and taxonomy of mesostigmatid mites we performed both morphological and molecular investigation on two mite species, *Laelaps giganteus* (Berlese 1918) (Fig. 1.1A) and *Laelaps muricola* (Träghardh 1910) (Fig. 1.1B). The mites belong to the subfamily Laelapinae (Mesostigmata: Laelapidae) and can be

differentiated from other species in the genus by a unique opisthogenital shield with four pairs of setae on adult females (Hirst 1925). Both *Laelaps* species are geographically widespread in sub-Saharan Africa and are reported from multiple rodent species (Hirst 1925; Zumpt 1961). In addition, Zumpt (1961) hypothesized about the possibility of multiple sub-species within *L. giganteus*.

In this study the evolutionary history and taxonomic status of two recognized southern African Mesotigmatid mites, *L. giganteus* and *L. muricola*, was investigated using a combination of partial mitochondrial cytochrome oxidase subunit I (COI), the nuclear internal transcribed spacer 1 (ITS1) and the intron and the intron region of the Tropomyosin (TropoM) gene. Selected morphological characters were also included. The relative importance of host range as a contributing factor towards lineage diversification was tested by sampling multiple previously described hosts of these parasites at eight different localities.

2.2. Materials and Methods

2.2.1. Samples collected

Hirst (1925) and Zumpt (1961) lists the South African hosts of *L. giganteus* and *L. muricola* as being *Rhabdomys dilectus*, *Rhabdomys pumilio*, *Lemniscomys rosalia*, *Mastomys natalensis*, *Dasymys incomtus* (type host for *L. giganteus*), *Aethomys chrysophilus*, *Micaelamys namaquensis*, *Saccostomys campestris*, *Otomys irroratus*, *Parotomys litteldalei*, *Rattus rattus* and *Tatera afra* while several other hosts are recorded for the rest of sub-Saharan Africa (see Zumpt 1961). To achieve maximum host overlap at sampling sites, efforts focused on the eastern side of South Africa (see Skinner and Chimimba 2005 for host

distributions) (See section 1.5 in Chapter 1). Attempts were made to sample as many host species possible (Table 2.1) at eight different collection sites (Fig. 2.1). The mite *Androlaelaps marshalii* (Berlese 1911) collected in this study from *Tatera brantsii* was used as an outgroup for some of the phylogenetic analyses. In instances where *A. marshalii* failed to amplify, the GENBANK sequence of the more distantly related *Dermanyssus apodis* (FM897373.1) was also used as an alternative outgroup.

Table 2.1: Collection localities, host species, total number of individuals per host species and number of host individuals that harboured either *L. giganteus* or *L. muricola.*

Province	Locality	Code	Host species	Total hosts	*L. giganteus*	*L. muricola*
Eastern Cape	Alice	AL	*Micealamys namaquensis*	15	-	14
			Otomys irroratus	8	-	-
			Rattus rattus	2	-	-
			Rhabdomys dilectus	6	-	-
	Hogsback	HB	*Micealamys namaquensis*	4	-	8
			Mus musculus	3	-	-
			Otomys irroratus	2	-	-
			Rhabdomys dilectus	10	2	-
KwaZulu Natal	Vryheid	VH	*Lemniscomys rosalia*	1	1	-
			Mastomys natalensis	5	-	-
			Micealamys namaquensis	3	-	-
			Rhabdomys dilectus	3	-	-
	Inkunzi	IN	*Aethomys chrosophilus*	4	-	-
			Mastomys natalensis	9	-	-
			Otomys irroratus	2	-	-
			Rattus rattus	3	-	-
			Rhabdomys dilectus	9	2	-
Gauteng	Rietvlei	RV	*Mastomys natalensis*	18	-	2
			Rhabdomys dilectus	24	10	-
	Kaalplaas	KP	*Mastomys natalensis*	18	-	7
			Rhabdomys dilectus	30	18	-
			Steatomys pratensis	10	-	-
North West	Zeerust	ZE	*Lemniscomys rosalia*	2	-	-
			Mastomys natalensis	2	-	1
	Mooinooi	MN	*Lemniscomys rosalia*	14	7	-
			Mastomys natalensis	21	-	12

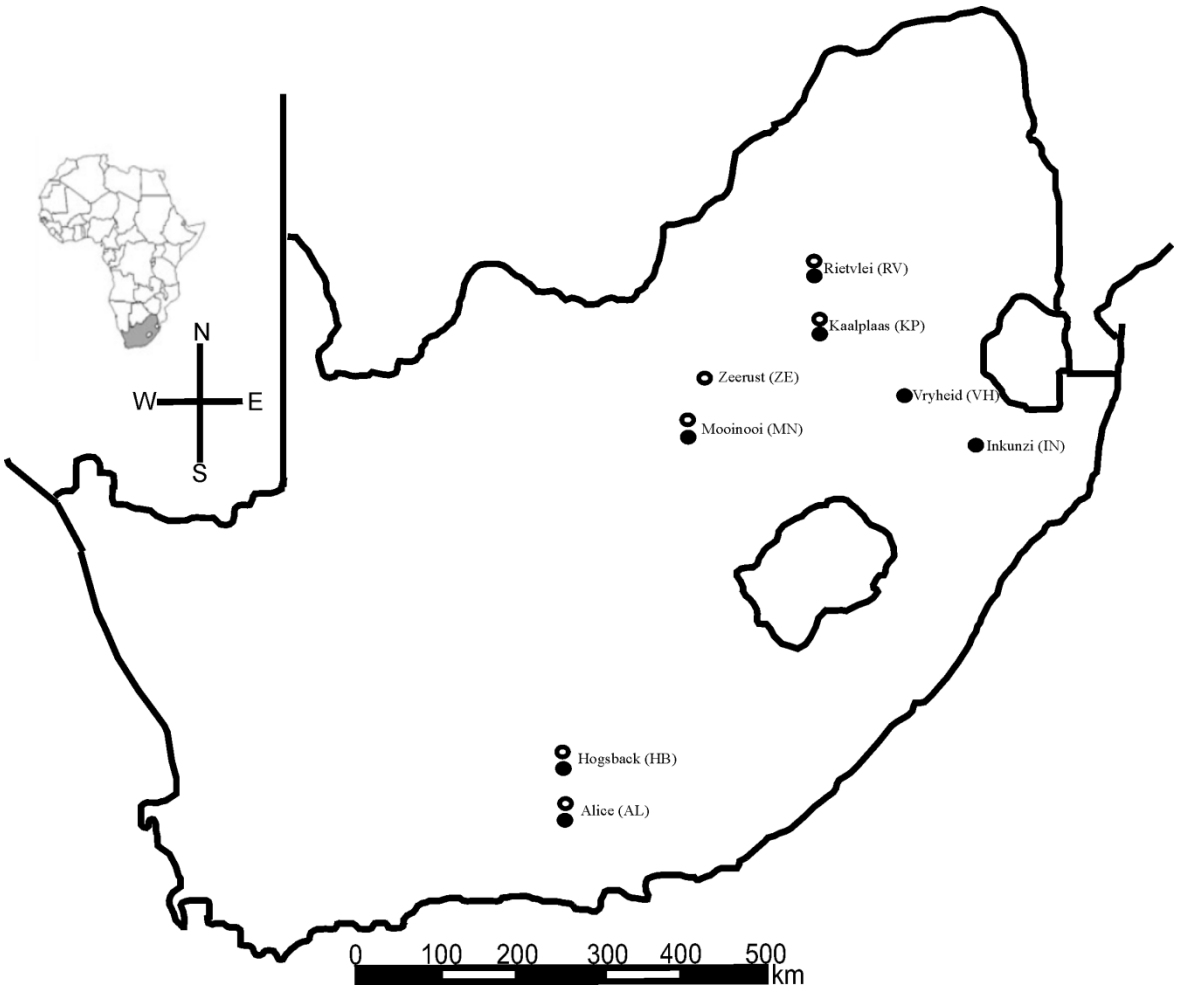

Fig. 2.1: Collection localities in South Africa from where *Laelaps giganteus* (filled circles) and *L. muricola* (open circles) were recorded.

Rodents were trapped using Sherman-type live traps that were set in trap lines (each trap 10 m apart). Trapping was done for a minimum period of 4 days (dependent on the trapping success). Adult hosts were selected for parasite screening and placed in a plastic bag and euthanized using an intra-peritoneal injection with sodium pentobarbitone (200 mg / kg^{-1}) (ethical approval for euthanasia method was granted by Stellenbosch University: SU-ACUM11-00004). Ectoparasites were obtained by brushing the pelage of the host using standard procedures (Ignoffo 1958; Burger *et al.* 2012). To prevent cross contamination between conspecific samples, brushes were cleaned with 100 % ethanol after each use. Cross

contamination among host species was avoided by using separate clean brushes for each host species at each site. Only female mites were selected for inclusion in the genetic analyses as there is a significant female bias on rodent hosts (1 male: 128 females per host; Matthee *et al.* 2007) and females also possess more distinct morphological characters for identification (Matthee and Ueckermann 2009). Mites were preserved in 100 % ethanol for molecular and morphological analysis.

2.2.2. DNA extraction and sequencing

Genomic DNA was extracted using a Macherey-Nagel kit (GmbH and Co.) following the protocol of the manufacturer. Whole animals were placed individually into an ependorf tube with extraction buffer without grinding and digested at 56 °C for a minimum of 3 h during which time individual extraction reactions were mixed every 30 min using a vortex. Following extraction, the remaining exoskeletons of all mites were stored individually and used for the morphological component of the study (see section 2.2.5). Extracted DNA was stored at −20 °C and later thawed for PCR use.

Universal primers LCO1490 and HCO2198 of COI were used to amplify 708 base pairs (bp) of the gene (Folmer *et al.* 1994). Also, two nuclear genes were included in this study namely ITS1, for which 700 bp were amplified using the primers described in Roy *et al.* (2008) and TropoM for which 570 bp were amplified as described in Roy *et al.* (2010).

All PCR reactions were optimized and carried out using 25 µl reaction volumes with a GeneAmp® PCR system 2700 thermal cycler (Applied Biosystems). COI regions were amplified via a ‘cold start’ reaction consisting of a denaturation cycle of 1 min at 95 °C

followed by a 10-cycle loop of 1 min at 95, 45 and 72 °C, respectively. A 30-cycle loop was then followed using the exact same conditions apart from increasing the 45 °C annealing temperature to 59 °C. All reactions were terminated by a final 5 min extension period at 72 °C. PCR conditions for ITS1 and TropoM followed Roy *et al.* (2010) with annealing temperatures of 49 and 54 °C, respectively. After amplifications, 5 µl of the PCR products were visualized on a 1% agarose gel. The remainder of the PCR product was purified with a NucleoFast 96 PCR kit (Macherey-Nagel). Cleaned products were then Sanger sequenced using BigDye Chemistry and analysed with an ABI 3730 XL DNA Analyzer (Applied Biosystems, Inc.).

2.2.3. Sequence processing and alignment

Sequences were authenticated using the BLASTN tool on GENBANK (http://blast.ncbi.nlm.nih.gov/ Blast.cgi) and the mtDNA sequences were also translated to amino acids with the online tool EMBOSS/TRANSEC (www.ebi.ac.uk/Tools/emboss/ transeq/index.html). All correctly identifie as mite sequences were manually aligned and edited using BIOEDIT SEQUENCE ALIGNMENT EDITOR v. 7.0.9 (Hall, 2005). To avoid the inclusion of missing data and ambiguities, 3' and 5' end sections of the sequences were truncated.

2.2.4. Phylogenetic analyses

Phylogenetic relationships among individuals sequenced were firstly inferred using maximum parsimony (MP) in PAUP* v4.0b10 (Swofford 2002). A heuristic search was done following the tree bisection-reconnection branch exchange method (TBR) with all characters

assigned equal weights and unordered. Stability of the nodes on the MP tree was assessed with bootstrapping using 1000 resampling pseudo-replicates and the TBR method. Bootstrap values above 75 % were considered well supported while bootstrap values below 75 % were considered poorly supported (Felsenstein 1985). Using the program JMODELTEST v. 2.1.2 (Darriba *et al.* 2012) and the Akaike information criteria (AIC), the best-fit model of evolution was determined for each gene fragment (Akaike 1973; Nylander 2004). Using the latter as a guide for prior input, Bayesian analysis were performed in MRBAYES v. 3.2 (Ronquist and Huelsenbeck 2003), including five parallel Monte Carlo Markov chains. A total of five million generations were used while the chains were sampled every 100th generation.

The generated samples were summarized with the sump command in MRBAYES to determine statistical stationarity and based on these results 25 % were discarded as burn-in. The PSRF (potential scale reduction factor) value was also used to assess whether the data were adequately sampled (Rambaut and Drummond 2007). The sumt command in MRBAYES was used to obtain statistical support values for the nodes on the trees. Trees were visualized with the program FIGTREE v. 1.2.2 (http://tree.bio.ed.ac.uk/software/gtree/). Nodes with posterior probabilities P<0·95 were considered not significantly supported. After individual analyses, a concatenated dataset was created and analysed using Parsimony (as described above) and Bayesian analyses in a partitioned fashion for each gene fragment (COI, ITS1 and TropoM). The latter Bayesian analysis was run for 22 million generations (until the S.D. of split frequencies were below 0·01) including 58 representative specimens for which all three gene complements were available.

In order to also incorporate population level processes, the individual genes were also analysed in SPLITSTREE v. 4.5 (Huson and Bryant 2006). For each gene, uncorrected P distances were used to draw a neighbour-net network (Bryant and Moulton 2004), using equal angle splits to present the relationships (Dress and Huson 2004).

2.2.5. Morphological analysis

All specimens for which sequences were obtained were mounted in Heinze-PVA medium following the protocol stipulated in Matthee and Ueckermann (2009). Following the key of Evans and Till (1979), 14 morphologically diagnostic characters were recorded (Table 2.2A, B, C) and measured in micrometres using a Zeiss Axioscope Research microscope (Zeiss). *Laelaps giganteus* has a genital plate that is distinctly separate from the anal shield and is narrower relative to other species within the genus (especially *L. muricola*). The venter is inundated with short stout setae. Trochanter I has one spine and five small setae. Coxa I has one spine and one spine-like seta while coxae II–III each has two spines and coxa IV one spine (Hirst 1925). In contrast, *L. muricola* has a smooth and fairly wide heart-shaped genital plate extending greatly behind the hind legs. Hairs on the venter are numerous, thick and longer than those of *L. giganteus* (Hirst 1925). Trochanter I has six small setae, coxae II–III each with one spine and one acute and stout seta and coxa IV with one small spine. Morphological measurements were analysed with a ZEN Imaging Software system (Zeiss). To test for a significant size difference between specimens, 9–10 individuals from each genetic lineage were measured for all characters and the mean was calculated for each. To exclude the effects of missing measurements on the principal component analysis, the data for each measured character group was mean centred. This was done by determining the mean for each character group and deducting those values from each data point in the

character group and dividing the subsequent value by the S.D. of the particular variable. The mean centred data were then normalized in the open source software program GNU Octave (www.gnu.org/software/octave/) in order to assign the same weight to each character. The first component (PC1) gave information on how samples differed from each other while the second component (PC2) showed how variables relate to each other.

Table 2.2A: The morphological characters measured for *Laelaps giganteus* from *Rhabdomys dilectus* in micrometers following the nomenclature of Evans and Till (1979).*

Laelaps giganteus* from *Rhabdomys dilectus													
Morphological characters	**Animal number**												
	Nr. 1	**Nr. 2**	**Nr. 3**	**Nr. 4**	**Nr. 5**	**Nr. 6**	**Nr. 7**	**Nr. 8**	**Nr. 9**	**Nr. 10**	**Average**	**stdDev**	**Size range**
Length of dorsal shield	1325	1294	1324	1300	1406	1235	1228	1248	1191	1271	1282	61.59	1191-1406
Width of dorsal shield	970	1002	984	948	906	901	837	839	872	937	920	58.41	837-1002
Spine on Trochnater I	39	38	40	34	42	35	47	43	42	47	41	4.42	34-47
Spine on Coxa I	60	65	59	58	63	60	65	74	65	68	64	4.85	58-74
Anterior spine on Coxa II	74	69	60	68	61	61	0	69	61	70	59	21.4	60-74
Posterior spine on Coxa II	72	74	64	61	64	66	74	63	71	76	69	5.46	61-76
Anterior spine on Coxa III	73	62	50	68	57	71	62	60	71	70	64	7.44	50-73
Posterior spine on Coxa III	63	70	66	67	65	65	63	64	65	65	65	2.06	63-70
Spine Coxa IV	52	58	55	56	50	53	53	56	51	57	54	2.69	50-58
Distance Sternal setae 1-3	236	238	234	241	226	236	237	232	237	235	235	4.02	226-238
Distance Sternal setae 2-2	248	270	268	251	251	245	247	237	251	243	251	10.39	243-270
Para-anal setae	166	195	182	173	164	176	175	196	180	172	178	10.79	164-196
Post anal seta	256	282	268	256	240	249	244	250	247	250	254	12.43	240-282
Width of genital shield, across 2nd pr of setae	310	298	290	286	298	281	278	292	289	294	292	9.22	278-310

* Zeros in the table indicate that the particular appendage broke during DNA extraction and could not be measured.

Table 2.2B: The morphological characters measured for *Laelaps giganteus* from *Lemniscomys rosalia* in micrometers following the nomenclature of Evans and Till (1979).*

Laelaps giganteus* from *Lemniscomys rosalia												
Morphological characters	**Animal number**											
	Nr. 1	**Nr. 2**	**Nr. 3**	**Nr. 4**	**Nr. 5**	**Nr. 6**	**Nr. 7**	**Nr. 8**	**Nr. 9**	**Average**	**stdDev**	**Size range**
Length of dorsal shield	1286	1211	1257	1131	1104	1189	1214	1264	1241	1211	61.02	1104-1286
Width of dorsal shield	944	842	895	824	879	915	817	947	850	879	49.35	824-947
Spine on Trochnater I	43	38	39	42	37	31	41	39	36	38	3.61	31-43
Spine on Coxa I	68	63	67	66	59	71	76	75	69	68	5.4	59-76
Anterior spine on Coxa II	70	73	71	72	70	65	70	63	70	69	3.24	63-73
Posterior spine on Coxa II	82	78	72	69	63	67	71	78	64	72	6.62	63-82
Anterior spine on Coxa III	65	65	54	62	61	71	70	68	62	64	5.24	54-71
Posterior spine on Coxa III	68	77	65	61	58	67	59	67	63	65	5.77	59-77
Spine Coxa IV	52	57	56	40	41	56	51	44	36	48	7.96	36-57
Distance Sternal setae 1-3	239	238	245	239	232	249	231	238	236	239	5.68	231-239
Distance Sternal setae 2-2	233	228	232	235	218	239	237	226	233	231	6.4	218-239
Para-anal setae	166	175	162	200	184	150	136	168	179	169	18.81	136-200
Post anal seta	247	258	248	251	0	289	0	221	0	168	127.35	221-289
Width of genital shield, across 2nd pr of setae	277	276	286	285	278	303	316	0	320	260	98.97	276-320

* Zeros in the table indicate that the particular appendage broke during DNA extraction and could not be measured.

Table 2.2C: The morphological characters measured for *Laelaps muricola* from *Mastomys natalensis* and *Micaelamys namaquensis* in micrometers following the nomenclature of Evans and Till (1979).*

	Laelaps muricola* from *Mastomys natalensis and Micaelamys namaquensis												
Morphological characters	**Animal number**												
	Nr. 1	**Nr. 2**	**Nr. 3**	**Nr. 4**	**Nr. 5**	**Nr. 6**	**Nr. 7**	**Nr. 8**	**Nr. 9**	**Nr. 10**	**Average**	**stdDev**	**Size range**
Length of dorsal shield	1461	1440	1528	1470	1451	1434	1526	1432	1334	1387	1446	58.1	1334-1528
Width of dorsal shield	980	1004	1028	1009	1021	977	982	923	909	917	975	44	909-1028
Spine on Trochnater I	0	0	0	0	0	0	0	0	0	0	0	0	0
Spine on Coxa I	60	52	53	37	53	44	51	55	48	54	51	6.4	37-60
Anterior spine on Coxa II	0	0	0	0	0	0	0	0	0	0	0	0	0
Posterior spine on Coxa II	66	65	57	65	62	66	60	70	68	53	63	5.2	53-70
Anterior spine on Coxa III	0	0	0	0	0	0	0	0	0	0	0	0	0
Posterior spine on Coxa III	49	46	0	50	56	51	48	52	51	49	45	16.1	46-56
Spine Coxa IV	28	26	22	23	21	26	27	26	29	23	25	2.7	21-29
Distance Sternal setae 1-3	265	272	275	262	278	269	291	269	262	259	270	9.5	259-291
Distance Sternal setae 2-2	336	327	348	339	341	323	334	339	323	324	333	8.7	323-341
Para-anal setae	129	144	139	147	127	129	138	134	146	118	135	9.4	118-147
Post anal seta	191	157	172	0	0	186	205	182	181	174	145	77.3	172-205
Width of genital shield, across 2nd pr of setae	420	425	446	445	432	427	420	419	428	396	426	14.2	396-446

* Zeros in the table indicate that the particular appendage broke during DNA extraction and could not be measured.

2.3. Results

2.3.1. Host and parasite demographics

By sampling eight localities, 228 specimens were collected from 10 different potential host species (Table 2.1). Of the 10 different Muridae host species that were collected only four carried the mites of interest (Table 2.1). The four-striped grass mouse, *R. dilectus* was the most common host collected at all sampling sites (with the exclusion of Zeerust). Based on morphology *L. giganteus* was only recorded from *R. dilectus* and the single-striped grass mouse, *L. rosalia* (Table 2.1). *Laelaps muricola* were collected at six localities from two host species, namely the Southern multimammate mouse, *M. coucha*, and the Namaqua rock mouse, *M. namaquensis* (Table 2.1). The remaining hosts were predominantly infested with mites belonging to the genus *Androlaelaps*. These findings do not deviate significantly from that reported by Matthee *et al.* (2007).

2.3.2. Gene sequence characteristics

Attempts were made to sequence 84 *Laelaps* specimens for the COI locus, ITS1 and TropoM regions. We were successful in obtaining sequences for all specimens for COI and ITS1, but despite numerous attempts only managed to get 58 sequences for the TropoM gene (GENBANK accession numbers: COI: KF805772–KF805856; ITS1: KF805857–KF805940; TropoM: KF505941–KF805998). Sampling data corresponded to the two morphologically recognized species *L. giganteus* (N= 40) and *L. muricola* (N= 44), respectively (Table 2.1). JMODELTEST selected the GTR+G model as the best-fit model of substitution for all three gene fragments. A total of 644 bp were analysed for the COI region and excluding the

outgroup, this resulted in 522 (81·05%) invariant and 105 (16·30%) parsimony informative characters. The ITS1 region presented 468 base pairs, of which 382 (81·62%) were invariant and 47 (10·04%) parsimony informative while TropoM produced 464 useable base pairs, of which 384 (82·75%) were invariant and 44 (9·48%) parsimony informative characters.

2.3.3. Pair-wise divergence and phylogenetic reconstructions

Bayesian and Parsimony analyses of the COI data revealed the existence of at least three monophyletic lineages (Fig. 2.2). The three lineages support the distinction between the morphologically recognized *L. muricola* and *L. giganteus* and furthermore provide strong evidence for the existence of at least two genetic lineages within *L. giganteus* (Fig. 2.2). The three genetic lineages are separated by 9·84–10·51% mtDNA sequence divergence and 3·55–7·72% nuclear DNA divergence (Table 2.3). The distinctions of the three genetic clades are supported by intra-lineage sequence divergences that, apart from TropoM were markedly lower than inter-lineage sequence divergences (Table 2.3).

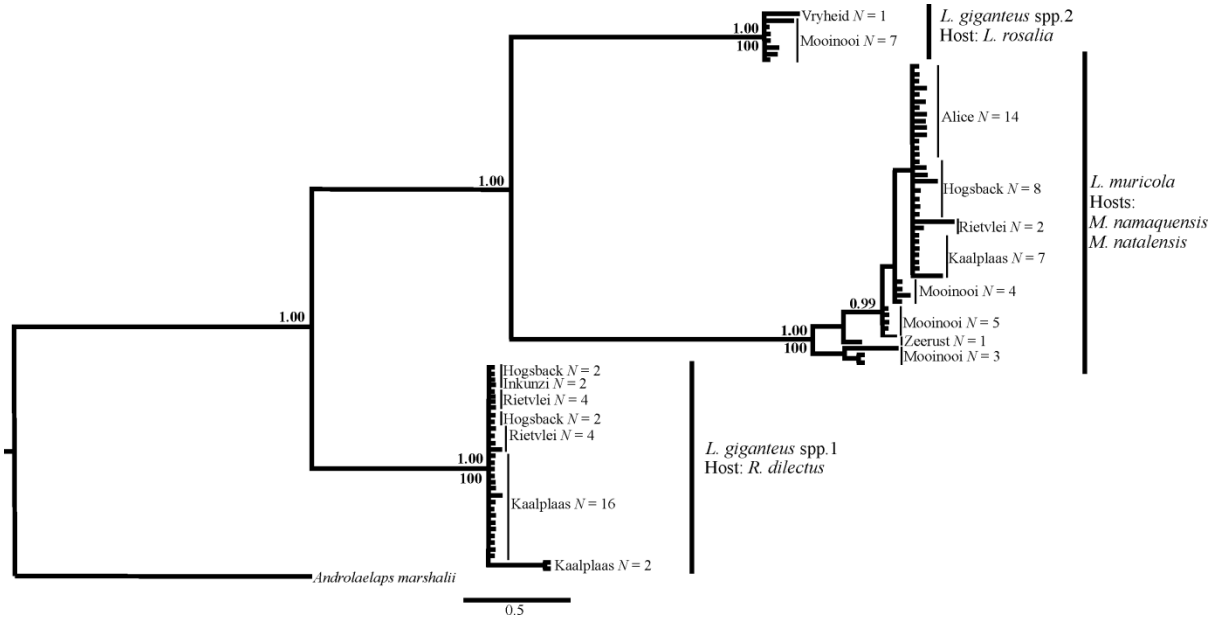

Fig. 2.2: Bayesian phylogeny indicating the three major clades retrieved from the COI dataset. The two cryptic lineages within *L. giganteus* are indicated as *L. giganteus* lineage 1 and *L. giganteus* lineage 2, with *L. muricola* grouped sister to *L. giganteus* lineage 2.

Table 2.3: Pair-wise genetic divergence values within and between the described *Laelaps* lineages.

	COI (% ± SD)	ITS1 (% ± SD)	TropoM (% ± SD)
Sequence divergence between *L. muricola* + *L. giganteus*	10.51 ± 0.43	5.18 ± 1,67	7.72 ± 5.13
Sequence divergence between *L. giganteus* lineage 1 and 2	9.84 ± 0.18	3.55 ± 0.71	3.45 ± 0.39
Sequence diversity within *L. giganteus* lineage 1	0.16 ± 0.35	0.84 ± 1,49	0.25 ± 0.15
Sequence diversity within *L. giganteus* lineage 2	0.38 ± 0.35	0.42 ± 0.69	1.91 ± 1.05
Sequence diversity within *L. muricola*	0.79 ± 0.72	1.56 ± 1.22	5.90 ± 5.20

Parsimony and Bayesian analyses of the ITS1 data consistently support the recognition of the two recognized species, but analyses based on TropoM were unresolved

(data not shown). This result is best illustrated by the Neighbour-net network analyses of the faster-evolving ITS1 data (Fig. 2.3) when compared with the mixed signals obtained for TropoM (Fig. 2.4). Combining the data in a single matrix provided robust support for the two recognized species but due to the TropoM data failed to support the strict monophyly of the two *L. giganteus* lineages (combined analyses not shown).

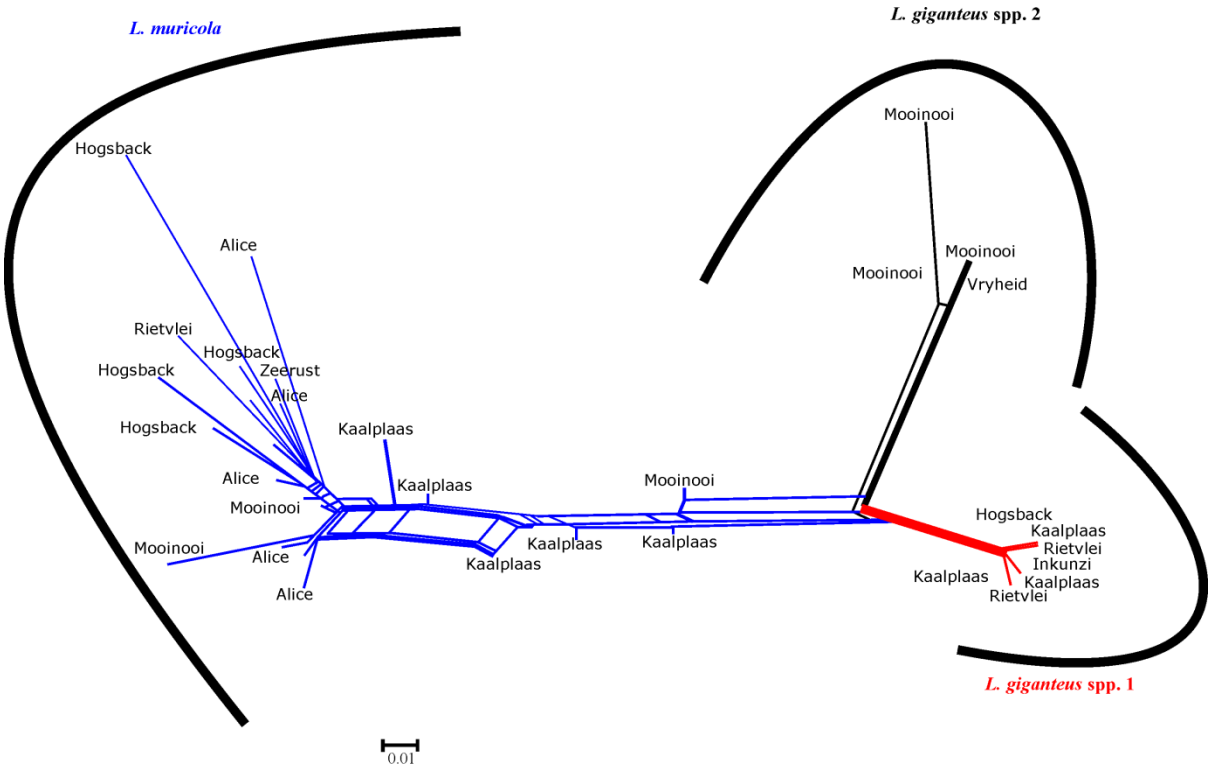

Fig. 2.3: *Laelaps* Neighbour-Net phylogenetic network of the ITS1 dataset indicating the three major groupings recovered in the COI phylogeny (labelled as such). Ambiguous signal and conflicts are indicated by multiple connections.

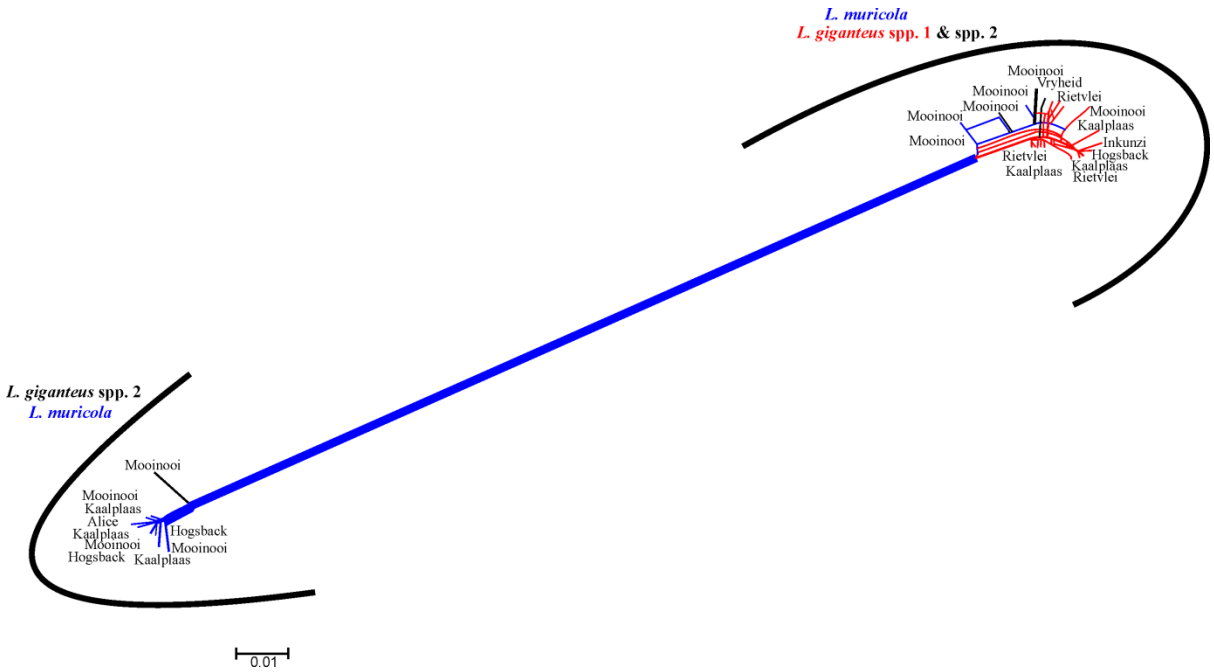

Fig. 2.4: *Laelaps* Neighbour-Net phylogenetic network of the TropoM dataset indicating the three major groupings recovered in the COI phylogeny (labelled as such). Ambiguous signal and conflicts are indicated by multiple connections.

2.3.4 Morphological analysis

Morphological measurements of N = 29 mites originating respectively from each of the three clades (Fig. 2.5a, 2.5b) showed strong morphological differentiation between *L. giganteus* and *L. muricola* but show a large overlap in range sizes for all morphological characters that were measured for the two *L. giganteus* lineages (Table 2.2a; Fig. 2.5a). A test of explained variance showed that more than 95% of the data was described by the first and second principal components. Comparing only the samples from the two genetic lineages in *L. giganteus* showed that no single component described the data; adding support to the finding that these two lineages are possibly cryptic species (Fig. 2.5b).

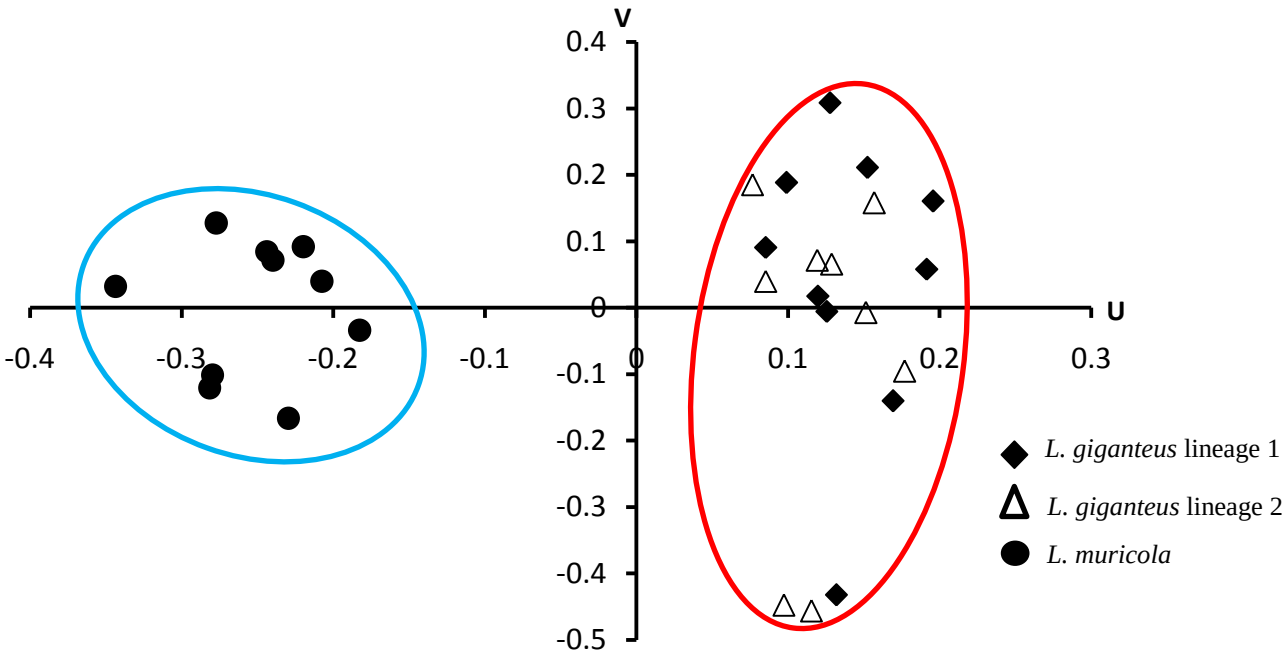

Fig. 2.5a: Principal component analysis of morphological characteristics recorded for the three *Laelaps* lineages. *Laelaps giganteus* lineage 1 (*N*= 10) (diamond shapes) and *L. giganteus* lineage 2 (*N*= 9) (open triangles) and *L. muricola* (*N*= 10) (filled circles).

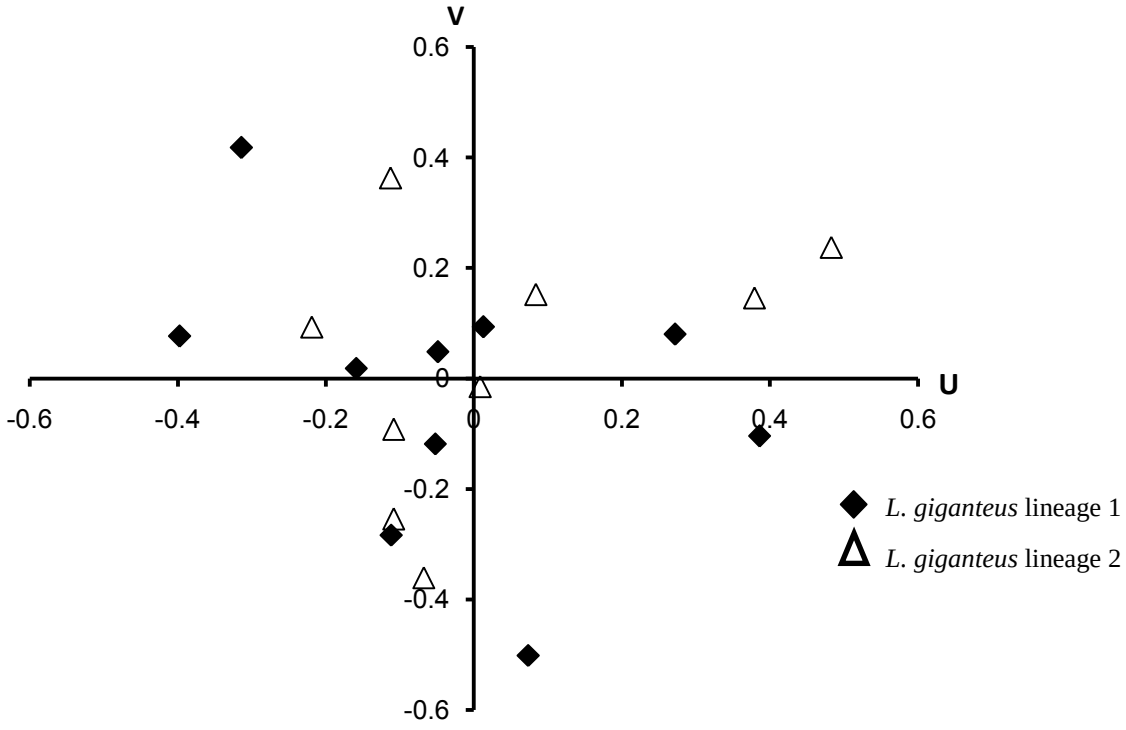

Fig. 2.5b: Principal component analysis of morphological characteristics recorded for two *L. giganteus* lineages. *Laelaps giganteus* lineage 1 (diamond shapes) and *L. giganteus* lineage 2 (open triangles).

2.4. Discussion

The current study provides: (i) novel genetic data to support the currently recognized *L. giganteus* and *L. muricola* as distinct species; (ii) new insights into host range of *L. giganteus* and *L. muricola* in South Africa; and (iii) the first published genetic evidence for cryptic speciation in a mesostigmatid mite occurring in southern Africa. In concert, these findings allow for new insights into the taxonomy and evolution of *L. giganteus* and *L. muricola*. Broadly this study also contributes towards the global need for more investigations examining parasite biodiversity (de León and Nadler 2010).

The marked genetic differentiation between the two recognized species based on mtDNA and nuclear DNA data confirm the original morphological distinction between the two *Laelaps* species (Hirst 1925). With the exception of some evidence (paraphyletic clustering for the TropoM dataset), phylogenetic analyses of all remaining datasets (together with the morphological measurements) support this taxonomic division. Contrary to published findings (Hirst 1925; Zumpt 1961), these two species also seem to be ecologically differentiated based on host preferences. *Laelaps giganteus* was absent on eight of the 10 possible host species but instead was only recorded on Arvicanthini rodents, while *L. muricola* seems to be more of a rodent generalist but was never found on *Rhabdomys*. This absence of *L. muricola* on *Rhabdomys* is seemingly not seasonally influenced (see Matthee *et al.* 2007) and the host specificity is furthermore also in agreement with previous diversity studies based on much larger sample sizes (Matthee *et al.* 2010; Froeschke *et al.* 2013).

The most prominent finding of this study was the discovery of genetically differentiated clades within *L. giganteus* that are morphologically similar. These two lineages form well-supported monophyletic clades when two (COI and ITS1) of the three gene trees are considered and these lineages are separated by a mtDNA sequence divergence value of 9·84% (10·51% separate *L. giganteus* and *L. muricola*; it is also comparable to species level distinctions in other mesostigmatid mites; Roy *et al.* 2008, 2010; Knee *et al.* 2012). Several factors can be put forward as to why the third genetic dataset (TropoM) did not recover the same monophyletic conclusions. Among these, the possible retention of ancestral polymorphisms cannot be discarded as a potential explanation and it is also possible that hybridization between individuals of the two species, and/or individuals belonging to the two *L. giganteus* lineages, could have resulted in some allele sharing at some loci (Ballard and Whitlock 2004; Felsenstein 2004; Maddison and Knowles 2006; Degnan and Rosenberg

2009; du Toit *et al.* 2013*b*). It is prudent that more genetic data are needed before a firm conclusion can be reached to explain the conflict in the TropoM data.

A concrete taxonomic revision is not possible at this stage since the samples reviewed in this study cover only a small area of the overall distribution of the formerly described *L. giganteus* (Hirst 1925; Zumpt 1961). A second confounding difficulty with a revised taxonomy is that the type specimen for *L. giganteus* was described from *Dasymys incomptus* which was collected in Pulima, Ghana, West Africa (Zumpt 1961). Unfortunately, despite several attempts to sample *D. incomptus* locally, no host specimens could be retrieved in the present study. If the genetic pattern obtained for *Laelaps* can be seen as indicative for the entire species, then it is quite likely that *L. giganteus* sampled on *D. incomptus* may in fact also represent a distinct cryptic lineage, and this lineage will then have priority in name. What makes a proper taxonomic assessment also problematic is the fact that only the range of *R. dilectus* was sampled in the present study (which spans the mesic eastern side of South Africa; Skinner and Chimimba 2005; Mucina and Rutherford 2006). Previous studies indicate that *L. giganteus* is commonly found on *R. pumilio* also (occurring along the xeric western regions of South Africa; Matthee *et al.* 2007) but at least four distinct *Rhabdomys* species exist in the region (du Toit *et al.* 2012). Several sibling species have also been described in *D. incomptus* based on chromosomal rearrangements (Volobouev *et al.* 2000). Given the narrow host range observed for *L. giganteus* in the present study, it is quite plausible that *L. giganteus* (*senso stricto*) may harbour significantly more cryptic diversity than is currently recognized. In turn the pattern presented in this study is also consistent with global trends suggesting that mites previously described as one species often harbour multiple cryptic lineages tightly linked to a primary host (Morelli and Spicer 2007; Roy *et al.* 2008; Shäffer *et al.* 2010; Skoracka and Dabert 2010; Martin *et al.* 2010; Knee *et al.* 2012).

In the present study we find strong evidence to suggest that host evolution played some role in the evolution of these ectoparasites since both *L. giganteus* lineages seem to follow a lifestyle reminiscent of a host-specific parasite (the two clades also showed strong host exclusivity independent of geography). This holds despite the fact that 10 different host species of this parasite were collected in partial sympatry. Unfortunately, our taxonomic sampling of hosts is not sufficient to make strong coevolutionary conclusions but it is interesting to note that the average mtDNA and nuclear DNA sequence distances suggest a closer relationship between the two *L. giganteus* lineages. Along these lines, the hosts of the two *L. giganteus* lineages (*R. dilectus* and *L. rosalia*) detected in this study are also phylogenetically closely related in the 'Arvicanthini' group of Muroid rodents (Watts and Baverstock 1995; Ducroz *et al.* 2001; Steppan *et al.* 2005). Some molecular evidence also suggests that the type host of *L. giganteus, D. incomptus*, is basal to the Arvicanthini rodents (Ducroz *et al.* 2001). Contrasting to the pattern observed in *L. giganteus*, *L. muricola* was recorded on *M. coucha* (grass/plain dwelling rodent) and *M. namaquensis* (preferring rocky habitats). In support of the more generalist lifestyle, no interspecific genetic structure was recorded in this species despite the fact that one of its preferred hosts, *M. namaquensis*, has also been shown to contain at least two divergent genetic clades in the region covered by our study (Chimimba 2001; Russo *et al.* 2010).

Although our findings contribute significantly towards a better understanding of the biology of Laelapinae mites, our study highlights the need for more fine-scale sampling across a larger geographic region. Pertinent to such a study would be to include *D. incomptus* and also the four ecologically differentiated *Rhabdomys* species (du Toit *et al.* 2012), since if the species specificity of *L. giganteus* holds, several more undetected lineages may exist.

Chapter 3

Limited dispersal contributes to significant phylogenetic congruence between ectoparasitic mites, *Laelaps*, and their rodent hosts, *Rhabdomys**

* Findings from this chapter is currently accepted in *Molecular Ecology* submission number: MEC-15-1241

3.1. Introduction

The longstanding obligate relationship among parasites and their hosts have enabled researchers to explore several cophylogenetic scenarios. For example, it is believed that host-specific parasites will show complete to partial phylogenetic congruence with their hosts (Hafner and Nadler 1990; Moran and Baumann 1994; Thomas *et al.* 1996; Haukisalami *et al.* 2001; du Toit *et al.* 2013*a*), and incongruence is predicted in systems where parasites are host-generalists (Page and Hafner 1996; Charlston and Robertson 2002; Weiblen and Bush 2002; Huyse and Volckaert 2005). Although host-specificity will promote congruent phylogenies between parasites and hosts, events such as host-switching, failure to diverge and intra-host speciation (parasite duplication) will promote incongruence (Hafner and Nadler 1990; Ronquest and Nylin 1990; Moran and Baumann 1994; Page and Hafner 1996; Banks and Paterson 2005). Phylogenetic tracking is not only dependent on host associations but is also correlated with other population variables including host life history traits and biogeography (Hafner and Nadler 1988; Thomas *et al.* 1996; Nieberding *et al.* 2004; Nieberding and Oliveri 2007; Nieberding *et al.* 2008; van der Mescht *et al.* 2015). Interestingly, it has been hypothesised that in some instances, parasites have the potential to illuminate previously unknown aspects of host evolutionary history (Hafner and Nadler 1988;

Thomas *et al.* 1996) and can thus act as biological magnifying glasses (Nieberding *et al.* 2004).

Hypotheses explaining the mechanisms underlying parasite-host codiversification (the parallel divergence of ecologically associated lineages within two distinct phylogenies; Page 2003) are however mostly based on studies that investigated relationships above the species level (Page 1996; Althoff *et al.* 2014). In the absence of sufficient comparative studies at the population level, several important aspects of parasite evolution and ecology remain largely unexplored (Nadler *et al.* 1995; Barrett *et al.* 2008; Archie and Ezenwa 2011). To provide additional data that can be used to advance insight into the mechanisms involved in parasite-host codiversification at the population level, we applied a phylogeographic and cophylogenetic framework, using a host-specific mite, *Laelaps giganteus senso stricto* (Acari; suborder Mesostigmata) (Berlese 1918) which occurs on the four-striped mouse genus *Rhabdomys* within southern Africa (Matthee *et al.* 2007) This was done in light of the fact that in Chapter 2 we found two lineages of *L. giganeus*, one of which is restricted to *R. dilectus* and the other restricted to *Lemniscomys rosalia*.

The host, *Rhabdomys*, is a widespread predominantly southern African rodent that is dependent on nests for thermoregulation (Couture 1980). They excavate burrows up to 50cm deep and after a gestation period of round 25 days give birth to approximately six young in breeding chambers or nests (Skinner and Chimimba 2005). The small-scale dispersal of *Rhabdomys* is variable since the species is socially flexible (more solitary in the mesic east and more social in the arid western regions of the range; Rymer and Pillay 2013). Their social behaviour can, however, change based on the availability of mating opportunities and food resources (Schoepf and Schradin 2012). At the larger geographic scale, local adaptations to

vegetation types pose some restrictions to dispersal and this led to the recognition of four parapatrically distributed species within *Rhabdomys* (*Rhabdomys pumilio*, *R. intermedius*, *R. bechuanae* and *R. dilectus*; du Toit *et al.* 2012; Meynard *et al.* 2012). Potential assortative mate preference and post-zygotic reproductive barriers between some of the species (*R. pumilio* and *R. dilectus*, Stippel 2009) likely facilitate narrow contact zones between the species (Meynard *et al.* 2012; du Toit *et al.* 2012; Dufour *et al.* 2015).

Members of the parasitic mite genus *Laelaps* complete the majority of their life cycle in the nest of the host (Radovsky 1994), while adult female mites occur mostly on the host (Radovsky 1994; Martins-Hatano *et al.* 2002). Adult males (and other life stages) remain primarily in the nests of hosts explaining their near absence from the fur of host individuals (Martins-Hatano *et al.* 2002). For *L. giganteus*, Matthee *et al.* (2007) documented a sex ratio of one male to 128 females on *Rhabdomys*. Although this more than likely reflect female sex biased dispersal, male gametes can disperse with females who can store sperm for life (Radovsky 1994). Since the dispersal of *L. giganteus* seems entirely dependent on members of the genus *Rhabdomys* (Chapter 2), it seems reasonable to predict that some level of codivergence is to be expected in this study system. If so, we predict that at least four divergent *L. giganteus* lineages may be present throughout the range of *Rhabdomys* (Rambau *et al.* 2003; du Toit *et al.* 2012).

Owing to the high level of specificity of *L. giganteus* to its host genus *Rhabdomys* (Chapter 2), the present study also provides the opportunity for a novel comparison to infer the effect of differences in parasite life history (e.g. time spent on the host and mode of transmission) on codiversification within parasite-host systems. This can be accomplished by comparing our findings to the outcomes of a recent study on the taxon-specific sucking louse

(genus *Polyplax*) that also occur on *Rhabdomys* within the same geographic region (du Toit *et al.* 2013*a*). Despite *Polyplax* being a permanent parasite (complete their entire life cycle on the host), only partial genealogical congruence across the landscape was described (du Toit *et al.* 2013*a*) and the authors attributed their findings to the interplay among parasite traits (host specificity), host-related factors (the vagility and social behaviour of *Rhabdomys*) and the biogeography of the region (du Toit *et al.* 2013*a*). Since the host traits and biogeographic history of the region remain constant between these two studies, potential differences among the patterns observed can most likely be attributed to differences in parasite-specific life history traits.

There are several differences among the life history traits of *L. giganteus* and *Polyplax* (du Toit *et al.* 2013*a*). In contrast to *Polyplax*, which is a permanent parasite, only adult *Laelaps* (and mostly females) spend time on the host while feeding (females can survive for up three months without a blood meal from the host; Mullen and O'Connor 2002). In addition, *Polyplax* has also been found to be more prevalent and abundant on *R. pumilio* compared to *Laelaps* (prevalence of 59% vs. 38% and mean abundance 9.42 (±1.33) vs. 1.54 (±0.19), respectively; Matthee *et al.* 2007). The shorter time spent on the host, as well as the lower prevalence and abundance, will all act in concert to reduce the dispersal potential of *Laelaps* when compared to *Polyplax*. The lower abundance and prevalence of *Laelaps* will also place more impediments on host switching in the narrow zones of contact detected in *Rhabdomys* (du Toit *et al.* 2012). We thus predict that *Laelaps* may show more genetic structuring across the landscape as a result of reduced dispersal potential when compared to *Polyplax*. The lower ability to disperse and less opportunity for host switches may, in contrast to *Polyplax*, also lead to a stronger signal of codiversification among *L. giganteus* and its *Rhabdomys* hosts. However, as mentioned above, significant cophylogenetic signal has most

often been retrieved in systems where parasites occur permanently on the host (such as ectoparasitic lice, Hafner and Page 1995; Light and Hafner 2008). The non-permanency of *L. giganteus* on *Rhabdomys* may therefore also detract from significant phylogenetic tracking by increasing the likelihood of sorting events such as parasite extinction and missing the boat (Page and Hafner 1996; Charlston and Robertson 2002; Weiblen and Bush 2002; Huyse and Volckaert 2005; Macleod *et al.* 2010).

The aim of this study is to apply a comparative phylogenetic framework to a taxon-specific mite species and its rodent host genus in an attempt to better understand codivergence between parasites and hosts, particularly at the phylogeographic level. By making use of comprehensive geographic sampling, comparative data from a previous study using a different parasite species (du Toit *et al.* 2013*a*,*b*), and including regions of predicted range overlap (Ganem *et al*. 2012; du Toit *et al.* 2012; Dufour *et al*. 2015) and a known contact zone between *Rhabdomys* species (du Toit *et al.* 2012), the present study contributes valuable data toward understanding the influence of parasite life history on coevolutionary processes. As predicted, the vagility of the parasitic mite studied herein is directly influenced by host movement across the landscape. When compared to the permanent ectoparasitic lice (showing only partial cophylogenetic congruence with the host), the predominantly nest bound mites show significant congruent cophylogeny with the host. We argue that the nest bound nature and more limited dispersal potential in the mite resulted in less opportunities for host switching and this in turn resulted in more congruent phylogenetic patterns between *Laelaps* and *Rhabdomys*.

3.2. Materials and Methods

3.2.1. Taxon sampling

To obtain a more comprehensive host representation (particularly for *R. dilectus*), we extended the sampling area of du Toit *et al.* (2012, 2013*b*) and Chapter 2 by adding 12 new localities. Thus parasite and host data were obtained from a total of 25 localities across southern Africa (Table 3.1; Fig. 3.1). Methods for trapping and handling of animals followed the protocol outlined in Chapter 2 (Ethical Clearance by Stellenbosch University: SU-ACUM11-00004). Mites and host tissue samples were preserved in 100% ethanol.

Table 3.1: Localities, host species collected, total number of host specimens sequenced and the number of *L. giganteus* specimens sequenced for COI and TropoM.

***Laelaps giganteus* sampled and localities**			**Cytochrome Oxidase I *L. giganteus***	**Tropomyosin *L. giganteus***
Locations	**Host collected at site**	***N* host sequences**	**644bp**	**534bp**
Loeriesfontein (LF)	*R. intermedius*	*12	3	1
Laingsburg (LB)	*R. intermedius*	*7	5	3
Beaufort West (BW)	*R. intermedius*	*15	7	0
Anysberg (AB)	*R. pumilio*	2	2	12
Oudtshoorn (OH)	*R. pumilio*	*27	11	1
Vanrhynsdorp (VR)	*R. pumilio*	*23	9	0
Stellenbosch (SE)	*R. pumilio*	*12	2	2
Springbok (SB)	*R. pumilio*	*28	10	3
Richtersveld (RV)	*R. pumilio*	*27	11	4
Dronfield (DF)	*R. bechuane*	2	24	10
Rooipoort (RP)	*R. bechuane*	*15	12	0
Mariental (MT)	*R. bechuane*	*4	4	1
Keetmanshoop (KH)	*R. bechuane*	*12	1	0
Windhoek (WH)	*R. bechuane*	*17	15	11
Bethulie (BE)	*R. d. chakae*	1	3	0
Hogsback (HB)	*R. d .chakae*	3	11	11
Alice (AL)	*R. d. chakae*	1	22	0
Fort Beaufort (FB)	*R. d. chakae/R. pumilio*	*/**24	15	0
East London (EL)	*R. d. chakae*	6	15	0
Chelmsford (CH)	*R. d. chakae*	10	22	16
Inkunzi lodge (IN)	*R. d. chakae*	3	18	20
Oribi gorge (OG)	*R. d. chakae*	2	11	1
Vernon Crookes (VC)	*R. d. chakae*	3	14	7
Rietvlei (RT)	*R. d. dilectus/R. d. chakae*	2	12	10
Kaalplaas (KP)	*R. d. dilectus/R. d. chakae*	4	19	5
25 localities		262	278	118

first sequenced in *(du Toit et al. 2012); ** (du Toit et al. 2013*b*)

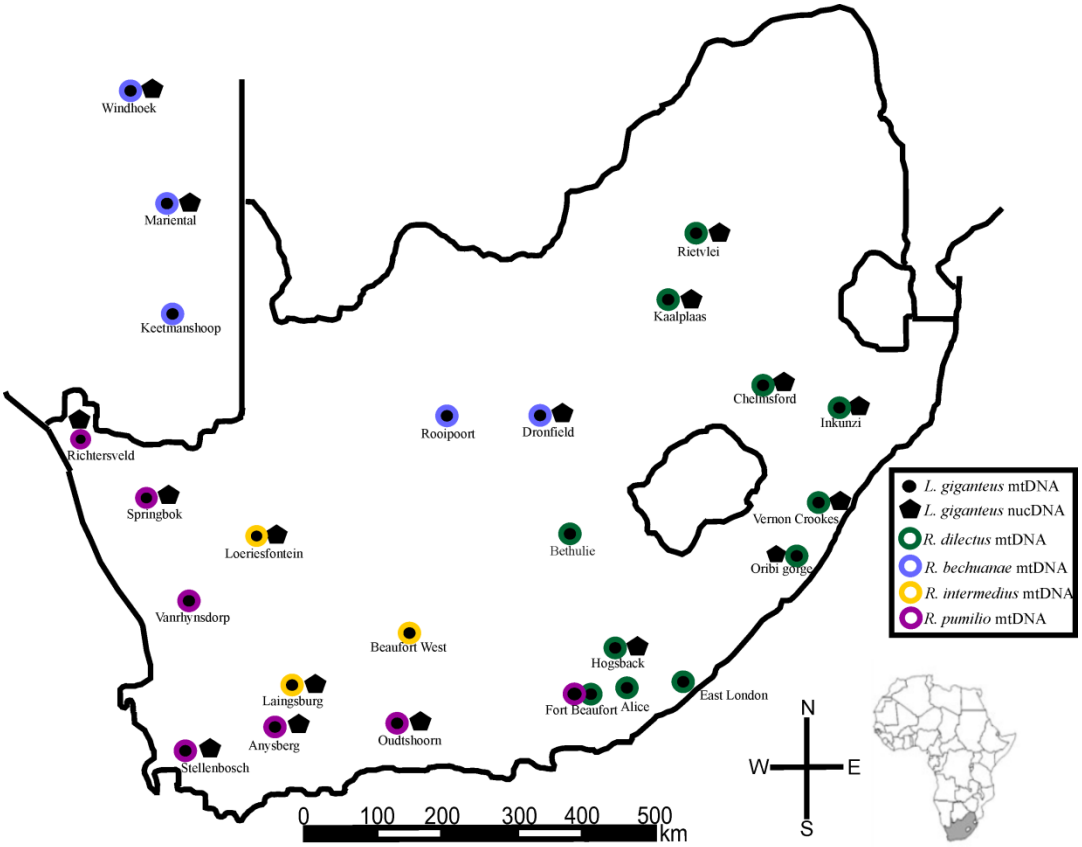

Fig. 3.1: Locality records from across southern Africa where *L. giganteus* and *Rhabdomys* were sampled. Filled circles indicate where mtDNA was amplified for host and parasite, whilst pentagons indicate where nucDNA was amplified for the parasite.

3.2.2: Molecular techniques and data analysis

Whole genomic DNA was extracted from parasite and host specimens using the techniques outlined in du Toit *et al.* (2012) and Chapter 2 section 2.2.2. Amplification and sequencing of the mitochondrial cytochrome oxidase subunit I (COI) and the nuclear intron of the Tropomyosin gene (TropoM) of *L. giganteus* was performed using the primers from Folmer *et al.* (1994) and Roy *et al*. (2010), respectively. PCR reactions were performed in 25 µl reactions containing millipore water, 3.5 µl of 25 mM $MgCl_2$, 3 µl of 10X Mg^{2+}-free buffer, 0.5 µl of a 10 mM dNTP solution and 0.5 µl (10 mM) of the respective primer pairs,

0.2 µl of *Taq* polymerase and 2.5 – 4 µl of template DNA. In each instance the volume of water was adjusted to obtain 25 µl reaction volumes. All PCR reactions for the different markers followed the same temperature cycles as outlined in Chapter 2.

Authenticity of the sequences was putatively established using the BLASTN tool on GENBANK (NCBI BLAST) and mtDNA sequences were translated to the corresponding putative amino acids to confirm functionality using EMBOSS/TRANSEQ (EMBL – European Bioinformatics Institute). Sequences were then edited and manually aligned using BIOEDIT SEQUENCE ALIGNMENT EDITOR v. 7.0.9 (Hall 2005). Missing data and ambiguities were excluded by trimming 3' and 5' end sections of the sequences. For the TropoM nuclear gene, alleles were determined using PHASE v. 2.1.1 (Stephens *et al.* 2001; Stephens and Scheet 2005). The PHASE analysis was performed for 100 000 generations with a burn-in of 10 000 generations. The analysis was considered resolved when probability values of 0.9 or higher were retrieved (Stephens *et al.* 2001). All subsequent analyses were performed on the resulting allelic data.

3.2.3: Genetic relationships

Genetic relationships among mitochondrial and nuclear DNA sequences were established by building networks to a 95% probability of parsimony in TCS v. 1.21 (Clement *et al.* 2000). Networks were refined to conform to the most parsimonious state following Templeton *et al.* (1992). Bayesian analysis of population structure was conducted in BAPS v 6.0 (Corander *et al*. 2008), using all parasite individuals. The non-spatial mixture model for linked loci (Corander and Tang 2007) was implemented in conjunction with the codon linkage model and a series K values from 1 to 25 (maximum number of sampled localities)

each replicated five times. To gain further insight into higher level gene clustering, Bayesian analyses were conducted using MRBAYES v 3.2 (Ronquist and Huelsenbeck 2003). Datasets were divided by codon and each partition subjected to its own optimal prior model of substitution, unlinked across partitions. The best-fit model of nucleotide substitutions was determined for each gene fragment using the Akaike Information Criterion (Akaike 1973; Nylander 2004) in JMODELTEST v 2.1.2 (Darriba *et al.* 2012). Four parallel Monte Carlo Markov chains were run for five million generations and sampled every 500 generations. Stationary was determined by the differences in split frequencies. All trees that form part of the burn-in phase were removed prior to the determination of posterior probabilities.

Haplotype and nucleotide diversity indices were calculated in DNASP v 5.10.01 (Librado and Rozas 2009) and genetic differentiation among the groups identified from the mtDNA haplotype networks was investigated by calculating Φ_{ST} values in ARLEQUIN v 3.5.1.2 (Excoffier *et al*. 1992; Excoffier and Lischer 2010). Corrected sequence divergence values, using optimal models, were calculated using PAUP* v 4.0 b10 (Swofford 2002).

3.2.4. Cophylogeny

Cophylogeny between *Rhabdomys* and *L. giganteus* was investigated using a distance-based approach applied to a dataset consisting of all parasite and corresponding host mtDNA haplotypes as well as a reduced dataset comprising one randomly selected parasite haplotype per sampled locality and the haplotype of its host at that locality (Table 3.1). Within the documented contact zone (Fort Beaufort; see Chapter 1) we included all representative host haplotypes, where parasite haplotypes originated from multiple host

species. Likewise, at Rietvlei and Kaalplaas (Fig. 3.1) both recognized subspecies of *R. dilectus* in the present study (*R. dilectus dilectus* and *R. d. chakae*; Rambau *et al*. 2003), one host belonging to each subspecies and its corresponding parasite were included. Reciprocal parasite and host Bayesian phylogenetic trees were constructed for the respective mtDNA datasets in MRBAYES v. 3.2 (Ronquist and Huelsenbeck 2003). Datasets were divided by codon and each partition subjected to its own model of substitution, unlinked across partitions. Five parallel Monte Carlo Markov chains were run for five million generations and sampled every 1000 generations. After statistical stationarity was reached, 25% of the data were discarded as burn-in using the sump command in MRBAYES v. 3.2. The summarized samples were then reviewed by assessing the convergence parameters in TRACER v. 1.5 (Rambaut and Drummond 2007). Trees were visualized with FIGTREE v. 1.2.2 (http://tree.bio.ed.ac.uk/software/gtree/). Patristic distance matrices calculated from the branch lengths of these parasite and host phylogenetic trees were used as input into AXPARAFIT (Stamatakis *et al.* 2007) and executed in COPYCAT v. 2.02 to improve running time efficiency (Meier-Kolthoff *et al.* 2007). Significant cophylogeny was investigated by comparing 10 000 permutations to the null hypothesis that evolution between parasite and host is independent.

As a comparison to the distance analysis, event-based cophylogenetic analyses were also conducted. Since these analyses pose significant computational time constraints, event-based cophylogeny reconstruction was conducted on the reduced dataset only, using the previously generated host and parasite Bayesian trees as input. Event-based cophylogeny analyses attempt to identify the most plausible scenario to explain the parasite-host pattern observed by assigning costs to four possible biological phenomena (codivergence; duplication; host switching and lineage sorting events) and reconciling the parasite and host

topologies while minimizing the overall cost (de Vienne *et al.* 2007; Merkle *et al.* 2010; Keller-Schmidt *et al.* 2012; Drinkwater and Charlston 2014). A signal cophylogeny would be indicated by a high number of codivergence events in a resulting reconstruction while duplication, host switching and sorting events (failure to diverge, parasite extinction and sampling errors; Keller-Schmidt *et al.* 2011) will detract from a significant cophylogeny signal. Due to the inherent complexity, several programs have been designed in an attempt to untangle the cophylogeny problem (reviewed in Keller-Schmidt *et al.* 2012; de Vienne *et al.* 2013; Drinkwater and Charlston 2014).

The first approach that we followed, as implemented in CORE-PA v. 0.3a (Merkle *et al.* 2010), is advantageous since it does not require a priori cost assignment (Conow *et al.* 2010). Assigning a priori costs is problematic from a biological perspective, especially since it has been shown that cophylogeny reconstructions can be highly variable depending on the cost scheme employed (Keller-Schmidt *et al.* 2012; du Toit *et al.* 2013*b*). CORE-PA v. 0.3a also ignores the host node order to allow the best host switch cost scheme to be recovered, which is a drawback since this may lead to chronologically invalid solutions (Conow *et al.* 2010; de Vienne *et al.* 2013). We implemented 100 000 random cost set permutations with the resulting reconstructions being ranked according to their quality scores, which indicates how well each reconstruction fits the particular cost scheme used (see Dilcher *et al.* 2011; Rosenblueth *et al.* 2013; du Toit *et al.* 2013*a*). Statistical significance was assessed with 1000 random parasite-host associations.

A second approach to disentangling the cophylogeny problem is to fix the order of the nodes on the host tree, thus not allowing chronologically invalid solutions which can occur in programs such as CORE-PA v. 0.3a (Keller-Schmidt *et al.* 2012, Drinkwater and Charlston

2014). The program JANE v. 4 uses this approach; which allows for the codivergence to be plotted to the parent node unambiguously with other events (such as duplications and host switches) subsequently, being mapped to daughter nodes (Conow *et al.* 2010; Keller-Schmidt *et al.* 2012; Drinkwater and Charlston 2014). Since an a priori cost scheme must be specified in this program, the VERTEX cost model with the default cost scheme was first implemented and the genetic algorithm was run for 500 generations with a population size of 300, whilst statistical significance was established by 1000 random tip mapping permutations (Mendlová *et al.* 2012; du Toit *et al.* 2013*b*).

3.3. Results

3.3.1. Data characteristics

Sampling at 25 localities yielded 262 host specimens representative of all four *Rhabdomys* species (Table 3.1; Fig. 3.1). A total of 278 *L. giganteus* specimens were collected from these samples and successfully amplified for a 644 bp section of the mtDNA COI gene (Table 3.1; Fig. 3.1). Despite numerous attempts, PCR and sequencing of the TropoM intron (534 bp) only yielded success for 118 specimens resulting in a data set comprising 246 phased alleles. The GTR+G model was indicated as the best model of substitution for both gene fragments.

3.3.2. Population level genetic relationships

Bayesian analyses of the geographically expanded mtDNA dataset for *Rhabdomys* confirmed the existence of four geographically distinct species (Fig. 3.2). The mtDNA

dataset of *L. giganteus* revealed 103 haplotypes (Genbank nr. KU166401-KU166672; http://dx.doi.org/10.5061/dryad.838f6), which corresponded to six phylogroups separated by at least eleven mutational steps in the statistical parsimony network using the default 95 % confidence interval (Fig. 3.3A; Fig. 3.4). Relaxing the confidence interval to 90 % yielded five distinct haplogroups as a result of the geographical proximate clades e and f connecting (Fig. 3.4). Apart from a single cluster (d, Fig. 3.3 B) the monophyly of all the haplogroups were supported by the Bayesian analysis (Fig. 3.3 B). The BAPS analysis indicated eight clusters that corresponded well to the haplogroups found by the TCS network (as indicated in Fig. 3.3 C). The only discrepancies between the two analyses are confined to additional substructure within Haplogroups a and d (Fig. 3.3C).

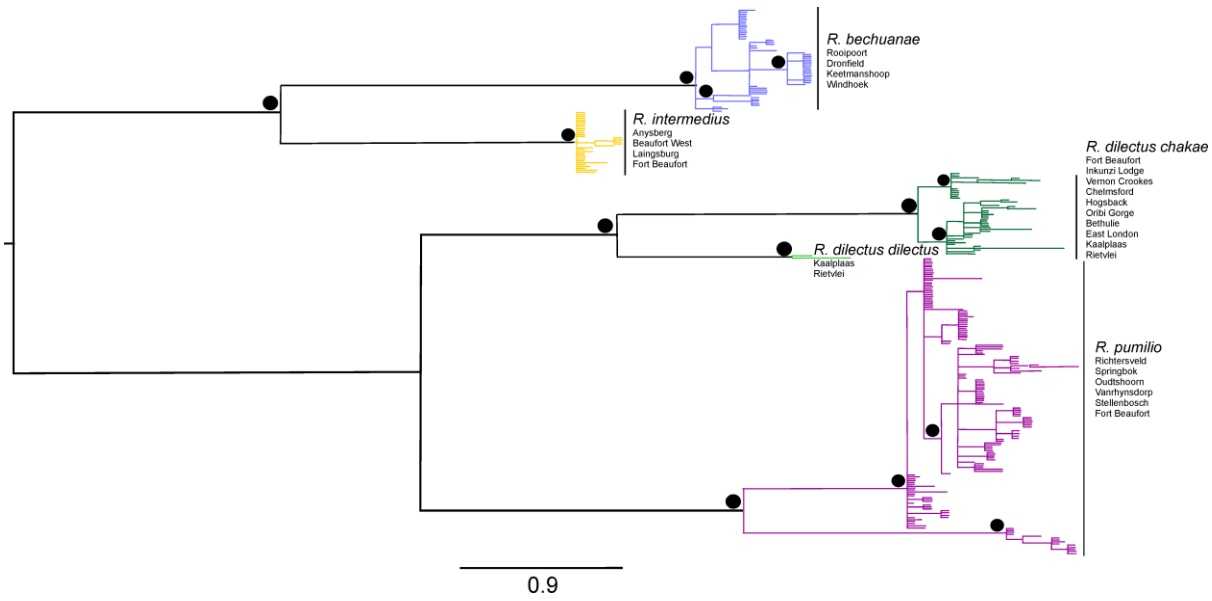

Fig. 3.2: Bayesian inference of the four *Rhabdomys* species in southern Africa. Posterior probabilities of ≥0.95 is indicated by solid circles and open circles show support at ≥0.90. These clades correspond to the host species and the phylogenetic patterning described by du Toit *et al.* (2012) for *Rhabdomys.*

When the individuals comprising the six genetic lineages of *L. giganteus* were examined on the basis of host association, there was a fair amount of congruence between parasite and host genealogical structure, a pattern also reflected by the bayesian analyses (Fig. 3.3B). Incongruences were mainly limited to the contact zone at Fort Beaufort (FB), where all *R. pumilio* hosts carried parasites that more than likely originated from *R. dilectus* hosts (Fig. 3.3C). Genetic admixture was also evident between *R. intermedius* and *R. pumilio* (Fig. 3.3A; clade b and d) and instances of haplotype sharing among the parasites from *R. dilectus* and *R. bechuanae* occuring at Dronfield (DF), Rooipoort (RP) and Chelmsford (CH). Importantly, no host contact was detected at these localities, implying a complete switch between parasite and host lineage.

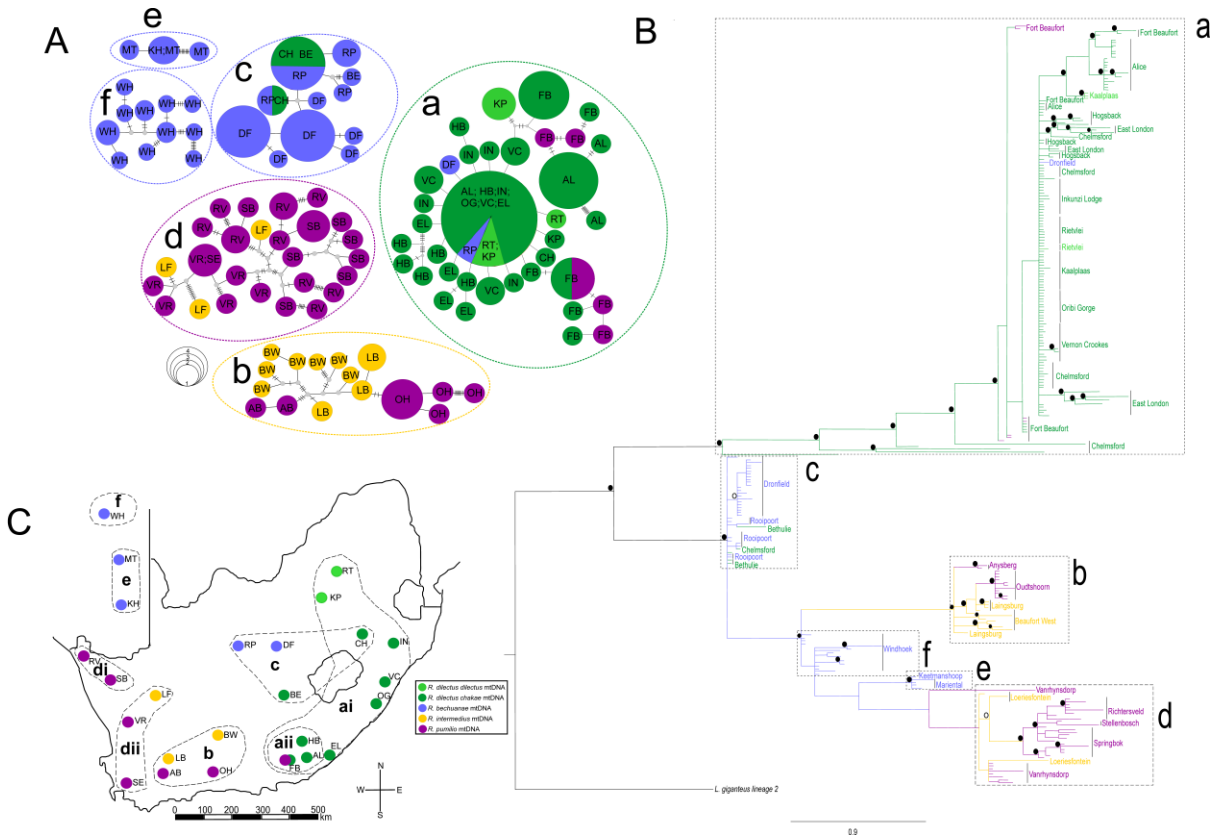

Fig. 3.3A: Cytochrome Oxidase subunit I (COI) statistical parsimony network of *L. giganteus* lineages. Genetic clusters are coloured according to host species (inset). Circles indicate a particular haplotype with the size indicating relative frequency. Abbreviations of locality names correspond to Fig. 1 **B:** Bayesian phylogenetic reconstruction of *L. giganteus* mtDNA COI data. Branches are coloured according to host (inset). Solid circles indicate *Pp*-values ≥0.95 while open cicles indicate *Pp*-values ≥0.90. Clades a-f correspond to those of the haplotype network **C:** Sampling sites of *L. giganteus* across southern Africa. Colours correspond to the *Rhabdomys* hosts (inset) and the eight BAPS groups recovered (ai, aii, b, c, di, dii, e, f) are indicated by dotted lines. Numbering of groups (and subgroups i and ii) correspond to those in A and B.

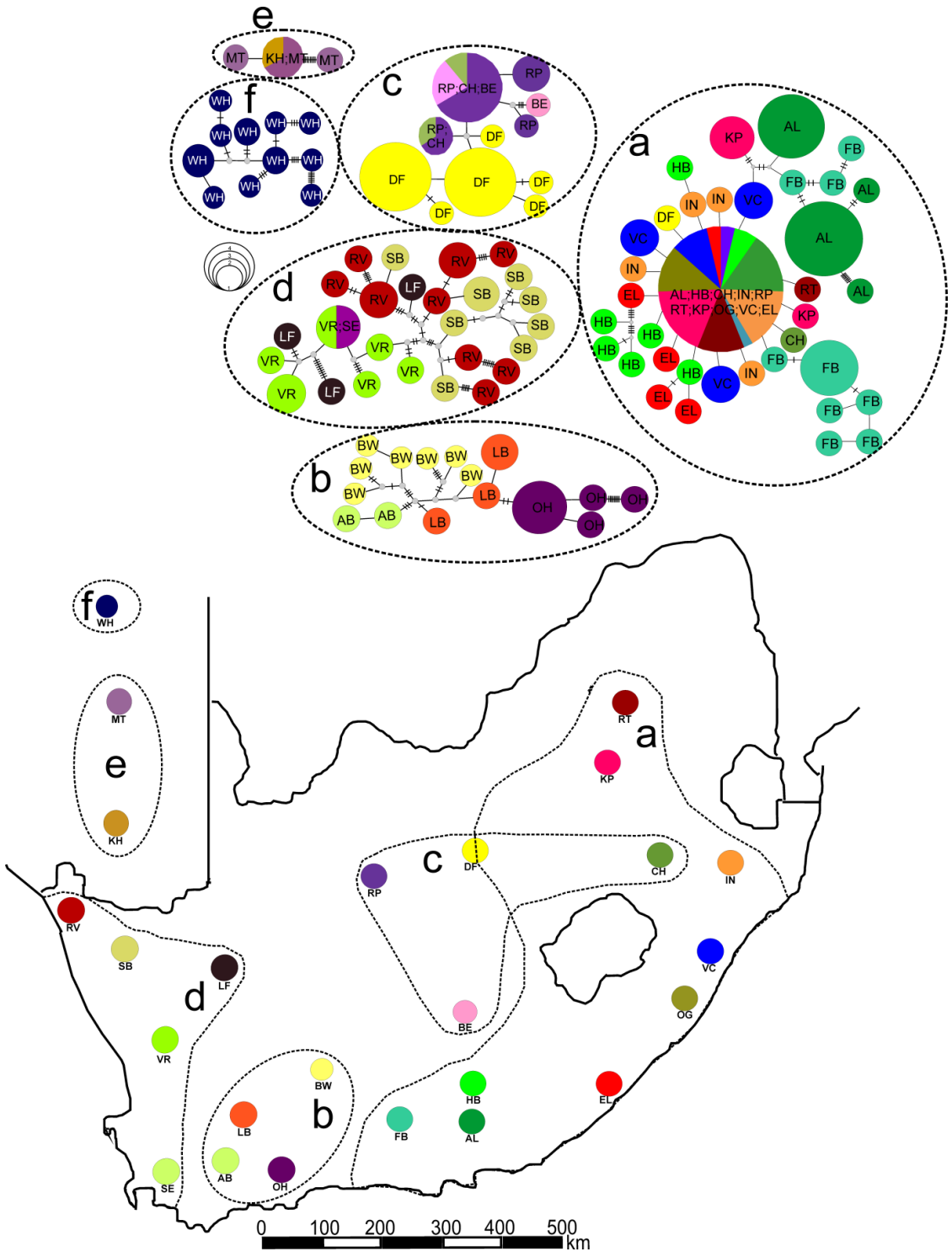

Fig. 3.4: Haplotype network of 278 COI mtDNA *L. giganteus* haplotypes at the 95% confidence interval. Each colour represents a sampling locality and letter codes inside circles correspond to the locality name in Fig. 3.1.

Analysis of the nuclear allelic data resulted in 99 distinct haplotypes (Genbank nr. KU166849-KU166947), which were all connected in a single parsimony network (Fig. 3.5A). Contrary to the mtDNA data, the only apparent host-associated geographic pattern is the clear differentiation among the parasite haplotypes originating from *R. dilectus* and the haplotypes originating from the other *Rhabdomys* species (Fig. 3.5A). The bayesian analyses (Fig. 3.5B) also supported this pattern, indicating the absence of clear differentiation between parasite haplotypes occurring on *R. bechuane*, *R. intermedius* and *R. pumilio* (Fig. 3.5A, B).

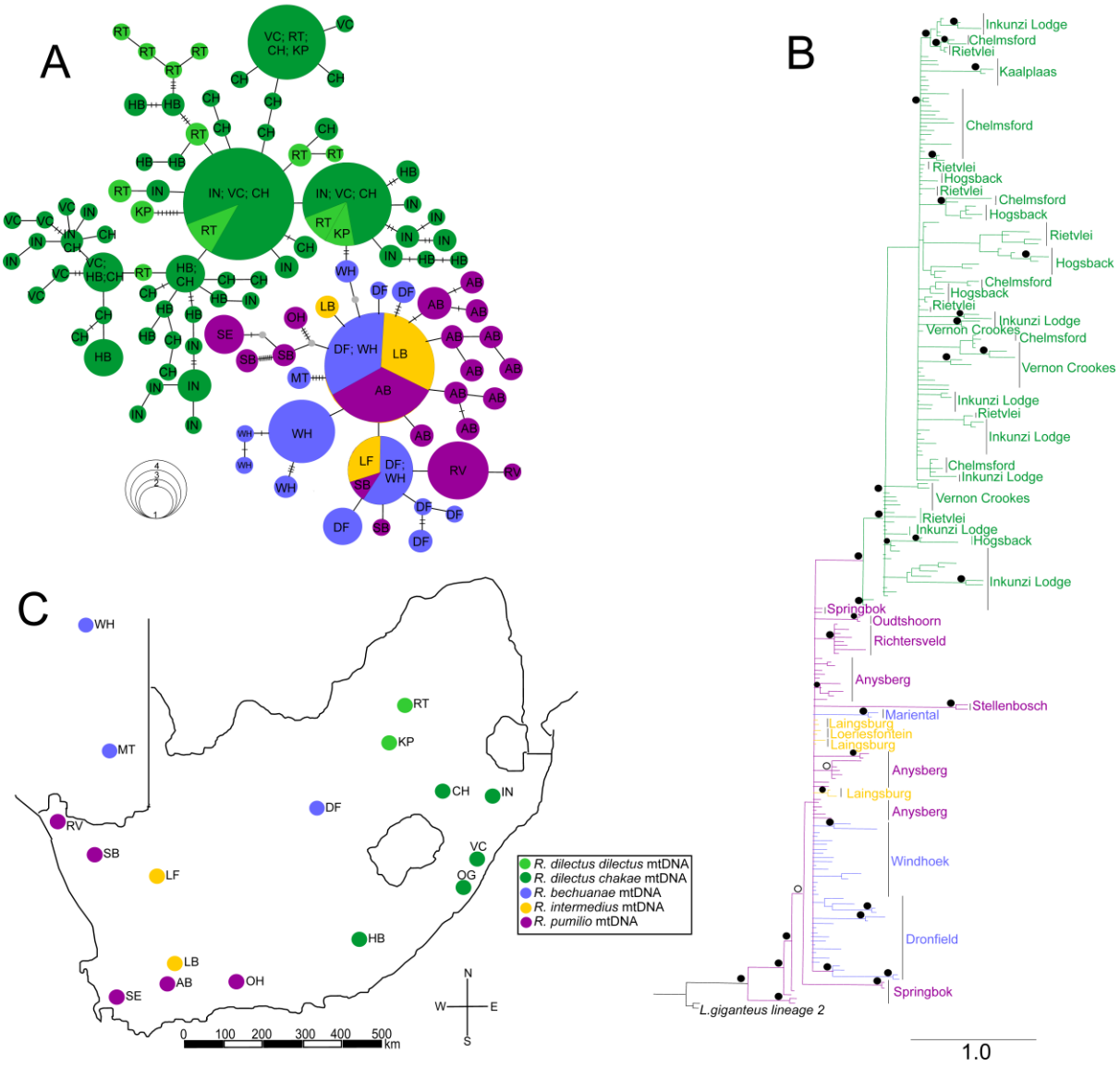

Fig. 3.5A: Tropomyosin (TropoM) statistical parsimony network of *L. giganteus* lineages. Genetic clusters are coloured according to host species (inset). Circles indicate a particular haplotype with the size indicating relative frequency. Abbreviations of locality names correspond to Fig. 1 **B:** Bayesian phylogenetic reconstruction of *L. giganteus* TropoM data. Branches are coloured according to host (inset). Solid circles indicate *Pp*-values ≥0.95 while open cicles indicate *Pp*-values ≥0.90. **C:** Sampling sites of *L. giganteus* across southern Africa. Colours correspond to the *Rhabdomys* hosts (inset).

When grouped according to host species, within-host mtDNA sequence diversity ranged from 0.67% for mites that occur on *R. dilectus* to 2.24% for mites that occur on *R. bechuanae* (Table 3.2). Among-host sequence divergences were, however, much higher and ranged from 4.79% between mites on *R. pumilio* and *R. bechuane* to 9.98% between mites on *R. pumilio* and *R. dilectus* (Table 3.2). Nuclear DNA sequence diversity and divergence values among the mtDNA-defined clades were much lower than those obtained for the mtDNA data, but showed a broadly similar trend when intraspecific and interspecific values were compared (Table 3.2). Analyses of Molecular Variance based on the host-defined parasite groups suggested significant mtDNA population differentiation between the parasite assemblages and again showed *R. dilectus* to be genetically most divergent among the four species studied (Table 3.3). This pattern was similarly reflected by the Φ_{ST} values among the various groups at the nuclear level (Table 3.3).

Table 3.2: Pair-wise genetic divergences between and within the *Laelaps* lineages described at the mtDNA level.

	Net sequence diversity CO1 (% ± SD)	**Net sequence diversity TropoM (% ± SD)**
Within *L. giganteus* on *R. pumilio*	1.55 ± 0.025	1.59 ± 0.009
Within *L. giganteus* on *R. bechuane*	2.24 ± 0.007	0.46 ± 0.001
Within *L. giganteus* on *R. intermedius*	1.24 ± 0.011	0.99 ± 0.005
Within *L. giganteus* on *R. dilectus*	0.67 ± 0.019	0.80 ± 0.002
Between *R. pumilio*_LGI and *R. dilectus*_LGI	9.98 ± 0.008	2.07 ± 0.006
Between *R. bechuane*_LGI and *R. dilectus*_LGI	9.20 ± 0.012	1.70 ± 0.004
Between *R. intermedius*_LGI and *R. dilectus*_LGI	9.59 ± 0.007	2.52 ± 0.002
Between *R. pumilio*_LGI and *R. bechuane*_LGI	4.79 ± 0.005	0.92 ± 0.008
Between *R. pumilio*_LGI and *R. intermedius*_LGI	6.70 ± 0.007	1.74 ± 0.026
Between *R. bechuane*_LGI and *R. intermedius*_LGI	5.39 ± 0.005	1.49 ± 0.026

Table 3.3: Pair-wise Φ_{ST} values between the four *Laelaps giganteus* (LGI) lineages corresponding to the four species in *Rhabdomys*. Values above the diagonal indicate nuclear Pair-wise Φ_{ST} values, whilst below the diagonal line indicates mtDNA Pair-wise Φ_{ST}.

Pairwise *Φst* values				
	*R.bechuane*_LGI	*R.dilectus*_LGI	*R.intermedius*_LGI	*R.pumilio*_LGI
*R.bechuane*_LGI	-	0.51*	0.15*	0.07*
*R.dilectus*_LGI	0.84*	-	0.60*	0.53*
*R.intermedius*_LGI	0.69*	0.88*	-	0.04*
*R.pumilio*_LGI	0.58*	0.86*	0.79*	-

* *P*-value < 0.05

3.3.3. Cophylogeny between mite and host

The observed mtDNA congruence among the *L. giganteus* evolutionary lineages and the four *Rhabdomys* species was not supported by AXPARAFIT, which indicated a non-significant relationship between parasite and host evolutionary histories. No significant correlation was detected between the mtDNA haplotypes for either the reduced (N = 28 haplotypes; P = 0.41) or full dataset comparisons (N = 103 haplotypes; P = 0.04). Although the P-value retrieved for the full dataset is significant at the 0.05% level, it is non-significant within the confines of the program, which measures significance at 0.02% (Stamatakis *et al.* 2007; Meier-Kolthoff *et al.* 2007).

The CORE-PA analysis retrieved a significant signal of cophylogeny between *Rhabdomys* and *L. giganteus* (P = 0.046). The best reconstruction (Fig. 3.6), with a quality score of 0.031 and a total cost of 8.152, consisted of nine codivergences (cost: 0.24), 32 sorting events (cost: 0.06), three host switches (cost: 0.54) and 15 duplications (cost: 0.14). This reconstruction, however, had chronologically invalid host switches, which is known to result from the software not fixing the host node order (Conow *et al.* 2010; de Vienne *et al.* 2013; Fig. 3.6). Irrespective of the timing of host switches, it is evident that switching events occurred among *R. bechuanae* and *R. dilectus* as well as *R. pumilio* and *R. intermedius*, which makes biological sense with regards to the shared *L. giganteus* haplotypes among host species in the former (Fig. 3.3A c; b) and the genetic admixture among parasite phylogroups predominantly associated with particular host species in the latter (Fig. 3.3A b, d; b). Results from JANE using the default cost scheme (co-divergence = 0, duplication = 1, duplication and host switch = 2, sorting event = 1, failure to diverge = 1) also revealed significant codivergence between host and parasite trees (P-value = 0.026). A total of 100

reconstructions with the identical total cost of 26 was retrieved, all indicating 18 co-divergence events, zero duplication, eight losses, zero failures to diverge events and nine host switches. The optimal cost scheme of the best reconstruction obtained in CORE-PA was subsequently also implemented in JANE as a control. Since JANE handles failure to diverge and sorting separately, the cost scheme from CORE-PA was slightly adjusted as follows: co-divergence: 24; sorting: 6; host switch: 54 and duplications: 14; with failure to diverge set to twice as high as the cost of host switches at 108 in order to nullify the effect of this evolutionary event on the analysis. Results from this analysis once again indicated highly significant cophylogeny between the four *Rhabdomys* species and *L. giganteus* (*P*-value = 0.00), with 100 reconstructions (total cost = 24) consisting of 18 co-divergences, 7 duplications, 2 host switches and 35 losses. Unfortunately, JANE does not provide a metric such as the quality score of CORE-PA to evaluate which among the various solutions with the same total cost is best. Irrespective of this, all solutions retrieved from both JANE analyses indicate hosts switches among the same host groups as shown in CORE-PA, with the only difference being the timing of host switching events (data not shown). Thus, independent of the software or cost scheme used, significant codivergence among *L. giganteus* and *Rhabdomys* with host switching events among particular *Rhabdomys* species was consistently retrieved.

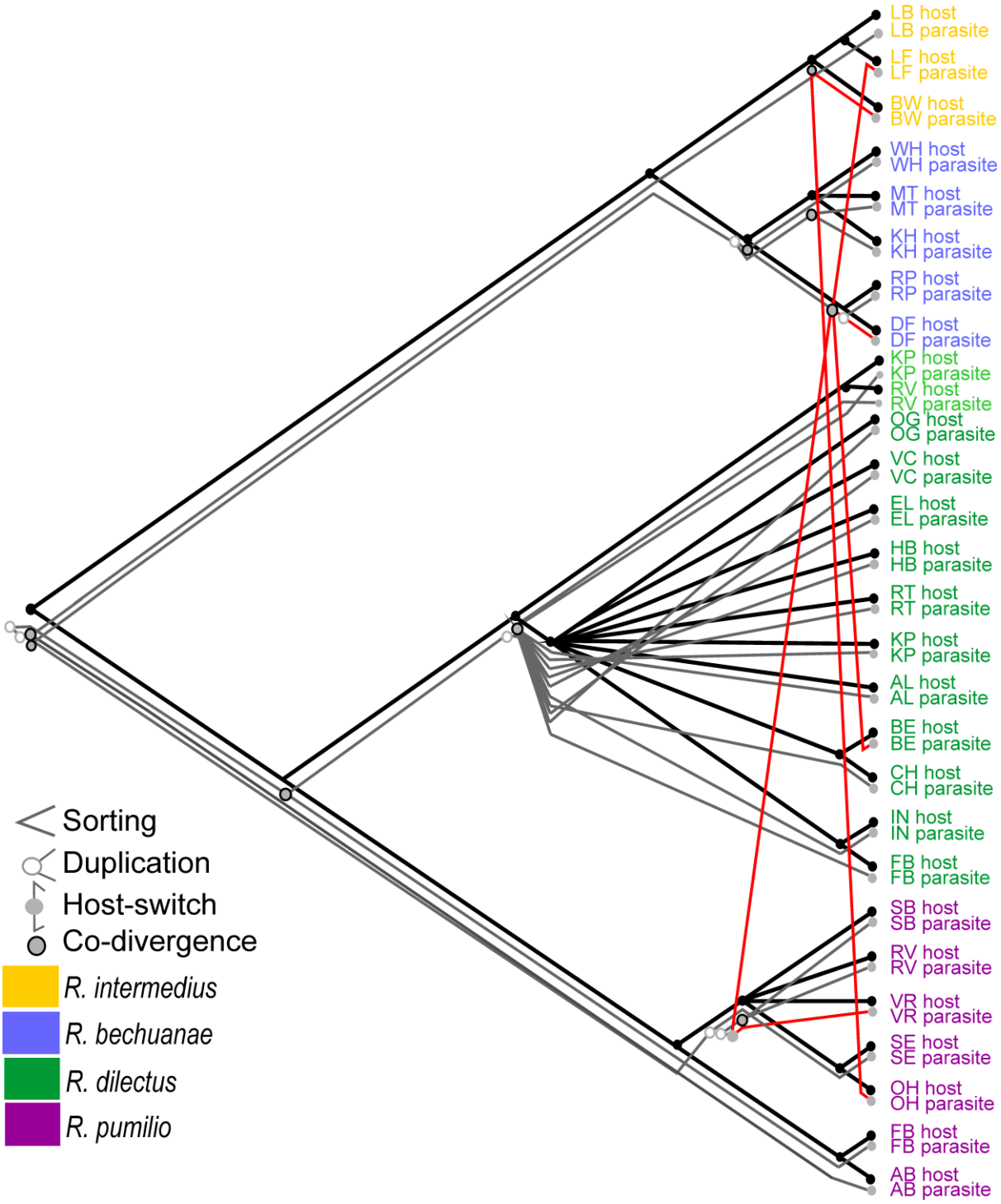

Fig. 3.6: The best reconstruction according to the cost-model fit of coevolutionary history of *L. giganteus* and *Rhabdomys* as proposed by CORE-PA v0.3a. Host switches are indicated by red lines.

3.4. Discussion

The present study provides evidence for the existence of at least six distinct genetic clades within the parasitic mite, *L. giganteus* (Berlese 1918). Haplotype networks, Bayesian trees, sequence diversity and levels of population differentiation at the mtDNA and nucDNA level suggest that several of the *L. giganteus* lineages occurring on the different *Rhabdomys* species have independent evolutionary trajectories (Tables 3.2 and 3.3). In fact, the most divergent host-associated parasite lineage is separated by an average mtDNA sequence distance of 9.98% (±0.008%). This value is only slightly lower than that previously detected between two species in the genus *Laelaps*, *L. giganteus* and *L. muricola* (differing by 10.51% ±0.43%) and in the same range than that detected between the two morphologically cryptic *L. giganteus* species found on two different Arvicanthini host species, *R. dilectus* and *Lemniscomys rosalia* (separated by 9.84% ± 0.18%; Engelbrecht *et al.* 2014).

The mtDNA, partly supported by the nucDNA data, indicate a high level of haplotypic diversity coupled to a fair amount of divergence among sampling localities throughout the range (Fig. 3.3, 3.4, 3.5 and Table 3.3). This pattern we see here is consistent with the hypothesis that *L. giganteus* is restricted in their dispersal across their range. We propose that the dispersal of *L. giganteus* individuals is constrained due to several life history traits. Apart from being host-specific (as suggested Chapter 2), and spending part of its life cycle off the host (Mullen and O'Connor 2002), *L. giganteus* also has a relative low mean infestation rate (1-3 mites per infected *R. pumilio*; Matthee *et al*. 2007), while females are most often the dispersal stage with males remaining in the nest (Radovsky 1994). As a result, gene flow among *L. giganteus* sampled localities is probably female biased and male gene exchange among localities is predominantly dependent on nest sharing among host

individuals. In concert, these traits would all contribute towards low genetic connectivity among distant localities.

The congruence between the geographic genetic structure of the parasite and host, evident from the haplotype networks, suggest that some level of codiversification has occurred. This is confirmed by the significant codivergence retrieved from the cophylogeny analyses. Although the results from AXPARAFIT did not show any significant cophylogeny between host and parasite at the micro- and macroevolutionary scales, event-based reconstructions from both CORE-PA and JANE showed significant codivergence.

The two event-based methods of cophylogeny reconstruction further suggest several host switches, a pattern which is supported by the mtDNA haplotype network (Fig. 3.3A). Importantly, with the exception of Chelmsford (CH), all host switching events involved localities occurring along known or predicted zones of contact/overlap among host species at Bethulie (BE), Dronfield (DF), Rooipoort (RP), Oudshoorn (OH), Anysberg (AB), Loeriesfontein (LF), and Fort Beaufort (FB) (*cf.* du Toit *et al.* 2012 for predicted ranges). It is interesting to note that at the majority of these localities, complete switching has occurred between parasite and host (Fig. 3.3). For example, at Oudtshoorn (OH) and Anysberg (AB), all hosts sampled belong to *R. pumilio* while all mite haplotypes cluster closer to those occurring on *R. intermedius*. The same switches are true for Loeriesfontein (LF), Fort Beaufort (FB) and Bethulie (BE). The majority of host switching occurred between *R. pumilio* and *R. intermedius* (Fig. 3.6). Interestingly, host switching appears to be much more limited between *R. dilectus* and *R. bechuanae*, a pattern also partly confirmed by nuclear DNA analyses (Fig. 3.5). Since a similar scenario has also been reported for the permanent

parasitic lice species of *Rhabdomys* (*Polyplax*; du Toit *et al.* 2013*a*) this may potentially point to more extensive range overlap or contact between *R. pumilio* and *R. intermedius.*

Recent habitat suitability predictions for the various *Rhabdomys* species (Ganem *et al*. 2012; du Toit *et al.* 2012; Dufour *et al*. 2015) support the probable shifting of host ranges during cyclic climatic changes (also see Whiteman and Parker 2005; Poulin and Keeney 2007). Host switching as a consequence of range shifts and the transient overlap among divergent host species is supported by the fact that the predicted regions of range shifts is markedly congruent with predicted host switching events (Fig. 3.6) as well as regions where phylogenetic incongruence was found between *L. giganteus* and *Rhabdomys* (Fig. 3.3).

What makes our study particularly interesting is the counter-intuitive finding of significant congruence and phylogenetic tracking between the more temporary parasitic mite (part of the life cycle is spent in the nest of the host, Mullen and O'Connor 2002) and the host *Rhabdomys*, as opposed to the weak congruence observed for the permanent parasitic lice, *Polyplax* (du Toit *et al.* 2013*b*). Furthermore, the non-permanent *L. giganteus* shows more pronounced spatial genetic structuring, with a higher level of intraspecific haplotypic diversity within clades and among localities (also see above), when compared to the two permanent parasitic lice (*Polyplax*, du Toit *et al.* 2013*b*). In fact, with the exception of Chelmsford (CH) and Dronfield (DF), haplotypes belonging to the six different geographic clades of *L. giganteus* are all monophyletic (Fig. 3.4).

Since both host traits and the biogeographic history of the region are consistent between the two studies, the reason for the observed differences is most likely a combination of factors relating to the life history of the parasite taxa. Host specificity is an important

factor in predicting whether congruence will be observed within parasite-host systems (see Archie *et al.* 2011 and Noureddine *et al.* 2011 for examples). Host specificity is defined in terms of the range of host species that can be exploited by a parasite, which is determined by the biogeographic and evolutionary history of parasite and host (Poulin and Keeney 2007). Despite being specific to *Rhabdomys*, *L. giganteus* sampled from different *Rhabdomys* species share haplotypes. This points to the possibility that specialization of the mite to the various hosts has either not been completed or the mite maintains its ability to utilise multiple resources (see Agosta *et al.* 2010) and as such host specificity cannot be invoked as an explanation for the observed phylogenetic congruence.

Limited dispersal ability enforces congruence by leading to fewer host switching opportunities (for example: Blouin *et al.* 1995; Nadler 1995; Jerome and Ford 2002; Johnson *et al.* 2003; Johnson and Clayton 2004; Whiteman and Parker 2005). The more pronounced spatial genetic structuring within *L. giganteus* suggests that the parasite that spends more time in the nest is more limited in its ability to disperse across the landscape, and between hosts, when compared to host-facilitated dispersal by permanent parasites (Criscione and Blouin 2004). Furthermore, at the microgeographic scale in regions where species ranges overlap, there seems to be resource partitioning with host species preferring different habitat types, as documented for *R. dilectus* and *R. bechuanae* (Dufour *et al.* 2015). If this holds, it is reasonable to suggest that the different *Rhabdomys* species do not share nests in regions of overlap. Given the above, it is plausible that the significant phylogenetic tracking recorded in *L. giganteus* may be explained by fewer opportunities for host switching when compared to the permanent ectoparasite *Polyplax*. *Laelaps giganteus* infests 38% of the host population with a mean abundance of 1.54 (±0.19) about 2-3 individuals per host whilst *Polyplax* infests 56.13% of the host population with a mean abundance of 9.42 (±1.33) per host, therefore

Polyplax has a higher chance of dispersal when compared to *L. giganteus* (Matthee *et al.* 2007; Matthee and Krasnov 2009; S Matthee unpublished data).

Finding more significant phylogenetic tracking within a non-permanent parasite, however, appears counter-intuitive since reduced presence on the host can increase the likelihood of sorting events (Page & Hafner 1996; Charlston & Robertson 2002; Weiblen & Bush 2002; Huyse & Volckaert 2005). When host ranges shift, parasites may fail to accompany their hosts on the voyage leading to the phenomenon of missing the boat (Paterson & Gray 1997; Paterson *et al.* 1999) and even if parasites do accompany their hosts to the new habitat, they may drown on arrival (Macleod *et al*. 2010; Paterson *et al*. 2003) whereby the new arrivals fail to establish. Furthermore, even if the mites were to successfully overcome these hurdles, their genetic signature would most likely be overwhelmed by those already present in the new habitat. Such scenarios are plausible for *L. giganteus* due to its lower on-host abundance and prevalence (see above; Matthee *et al.* 2007). While these stochastic events would usually detract from finding congruence, in this study system it could do the opposite by limiting the extent of genetic exchange as a result of host switching during host range shifts. This stands in contrast to *Polyplax* lice, which are more abundant and occur permanently on their hosts and thus have more opportunities for host switching during interactions among divergent host species (du Toit *et al*. 2013a). The limited incidence of complete or partial host switches observed within *L. giganteus* is thus probably the result of the interaction between these stochastic events, coupled to the extent and frequency of range shifts in the different host species.

The present study provides definitive proof of significant codiversification between *L. giganteus* and the four *Rhabdomys* species. Complete to partial phylogenetic tracking often

points to host specialization (Hafner and Nadler 1990; Moran and Baumann 1994; Thomas *et al.* 1996; Haukisalami *et al.* 2001; du Toit *et al.* 2013*a*). The observed pattern herein can thus be used as further evidence in support of the idea that the *L. giganteus* species complex is in fact host specialists on *Rhabdomys* (Chapter 2). Furthermore, the data also supports the validity of the ecological differentiation between three of the four recently described *Rhabdomys* species (du Toit *et al.* 2012). The significant codiversification pattern found between *Rhabdomys* and *L. giganteus* does not appear to be the result of host adaptation, but is rather reinforced by the non-permanency of *L. giganteus* on its hosts. This through the reduced dispersal potential and parasites, "drowned-on-arrival", acts in concert to reduce host switching opportunities. Finally, our results suggest that we have uncovered the first example of the biological "magnifying-glass-effect" within southern Africa, where parasites illuminate previously unknown aspects of host evolutionary history (Hafner *et al.* 1988; Thomas *et al.* 1996; Nieberding *et al.* 2004). Here the predicted host switches and the phylogeographic incongruence occurring in the parapatric regions of *Rhabdomys* distributions likely point to previously undetected recent shifts in host distribution.

Chapter 4

The effect of host evolutionary history on the phylogeographic structure of a mesostigmatid rodent mite *Laelaps muricola**

**Prepared for submission to Parasitology*

4.1. Introduction

Parasite population genetic structure can be used to document genetic variability within species (see Noureddine *et al.* 2011). Population genetic studies are often needed to resolve the taxonomy of parasite species plagued with cryptic lineages (Huyse *et al.* 2005; Detwiler *et al.* 2010; Knee *et al.* 2012). Apart from making more accurate assessments on parasite biodiversity, the population genetic structure of species can also be used to infer the dispersal potential of the parasite. This is often vitally important to know in order to explain the mechanisms responsible for speciation (de Meeûs 2000; Criscione *et al.* 2005). In the case of parasites, studies such as these can also be used to predict the spread of diseases transmitted by ectoparasites (Miles *et al.* 2003; de Leòn and Nadler 2010). Although the majority of studies addressing phylogeography of ectoparasites are limited to European, American and Asian countries (Williams *et al.* 2006; Martin *et al.* 2010; Shäffer *et al.* 2010; Knee *et al.* 2012 for example). As a result recent studies in southern Africa have taken a similar focus (Cangi *et al.* 2013; du Toit *et al.* 2013*a*, *b*; Van der Mescht *et al.* 2015*a*, *b*; Chapter 3).

The parasitic mite *Laelaps muricola* (Träghardh, 1910) belongs to the subfamily Laelapinae (Mesostigmata: Laelapidae) and can be differentiated from other species in the

genus by a heart shaped genital shield extending far beyond the hind legs (Hirst 1925). Species within the Laelapinae are primarily associated with small mammals and more specifically their respective nests, but adult stages (females and males) also occur on the body of the host (Radovsky 1994; Mullen and O'Connor 2002). *Laelaps muricola*, the focus of the present study, has been recorded on multiple Murid host species across its distribution in southern African (Zumpt 1961). Unlike the congeneric species *L. giganteus*, no additional information such as abundance on the host and sex ratio is available. However, a recent study that focussed on mesostigmatid diversity on rodents in South Africa and Namibia, recorded *L. muricola* on two murid hosts namely *Mastomys coucha* (grass/plain dwelling rodent) and *Micaelamys namaquensis* (preferring rocky habitats) (Chapter 2). In Chapter 2, we provided evidence that *L. muricola* is a generalist parasite since no inter-specific genetic differentiation was recorded between specimens originating from the two different host species (Chapter 2).

Despite being reported to be a generalist ectoparasite (Zumpt 1961), *L. muricola* was never recorded on the generalist and widespread rodent genus *Rhabdomys* that also occurs in the region. On *Rhabdomys*, the species is replaced by *L. giganteus* and coevolutionary analyses in this system show significant codivergence between host and parasite (Chapter 3). This poses an appealing scenario where two mite species with very similar life histories (Radovsky 1994; Mullen and O'Connor 2002) occur on different hosts with markedly different levels of gene flow (du Toit *et al.* 2012; Sands *et al.* 2015). In the case of *L. giganteus* and *Rhabdomys*, the host is geographically structured with virtually no evidence of gene flow amongst the four geographic regions as described in Chapter 3. In the case of *L. muricola*, one of the hosts, *Micaelamys namaquensis* show similar vicariance patterns to *Rhabdomys* (Chimimba 2001; Russo *et al.* 2010) while the other two, *M. coucha* and *M. natalensis* show extensive gene flow across the same geographic region (Colangelo *et al.*

2013; Sands *et al.* 2015). These findings allow for a phylogeographic comparison between the two *Laelaps* species in an attempt to explain the influence of host movement on the phylogeographic structure of ectoparasitic mites occupying the same geographic area.

Because parasite gene flow is heavily dependent on host movement (Blouin *et al.* 1995; McCoy *et al.* 2003; Crisione and Blouin 2004; Chapter 3) we hypothesize that *L. muricola* may utilize *Mastomys* to disperse and will thus show very limited phylogeographic structure when compared to *L. giganteus.* However, it is also evident that parasitic mites such as *L. giganteus* can be "drowned-on-arrival" in the new environment during host movement (Paterson *et al*. 2003; Chapter 3), and for this reason it is predicted that geographically isolated mite populations will show at least some level of differentiation between sampling sites. However, at this stage no publications are available on *L. muricola*, aside from descriptive publications by Hirst (1925) and Zumpt (1961). Therefore this study will help establish some of the baseline information which may be usefull in future studies.

4.2. Materials and Methods

4.2.1. Taxon sampling

Laelaps muricola individuals were obtained from rodent hosts collected from 15 localities across southern Africa (Table 4.1; Fig. 4.1). Trapping and handling of animals followed the same techniques as outlined in (Chapter 2) with authorization by Stellenbosch University ethics committee (SU-ACUM11-00004). Host individuals were generally identified with field guides, however in instances where this was not possible and in

particular to differentiate between *M. coucha* and *M. natalensis* small tissue samples were sequenced for molecular identification (all material was confirmed in Sands *et al.* 2015). Mite specimens were preserved in 100% ethanol for molecular analysis.

Table 4.1: Collection localities, host species, total number of individuals per host species, number of host individuals that harboured *L. muricola* and the number of specimens sequenced for the two gene fragments.

Province	Locations	GPS	*L. muricola* hosts	COI	TropoM
Northern Cape	Drie Susters	S31.88222; E23.09051	*M. namaquensis*	2	0
	Rooipoort	S28.38147; E24.16475	*M. namaquensis*	9	17
KwaZulu Natal	Albert Falls	S29.46543; E30.40464	*M. natalensis*	9	13
	Vryheid Hill	S27.75377; E30.79897	*M. natalensis*	4	0
	Oribi Gorge	S30.73083; E30.27333	*M. natalensis*	5	1
	Vernon Crookes	S30.26805; E30.59350	*M. natalensis*	4	4
Eastern Cape	Hogsback	S32.59344; E26.92463	*M. natalensis*	19	6
	Alice	S32.79083; E26.84547	*M. natalensis, M. namaquensis*	(7) (8)	1
	East London	S33.00544; E27.70269	*M. namaquensis*	5	0
North West	Mooinooi	S25.47756; E27.33184	*M. coucha*	12	8
	Zeerust	S25.12929; E26.15805	*M. coucha*	2	0
Gauteng	Rietvlei	S26.09174; E25.35824	*M. coucha*	8	0
	Kaalplaas	S25.63489; E28.16717	*M.coucha*	11	6
Western Cape	Tygervalley	S33.86792; E18.59654	*Rattus norvegicus*	11	0
Namibia	Etosha Pan	S19.35569; E15.93871	*M. namaquensis*	3	0
Total				119	56

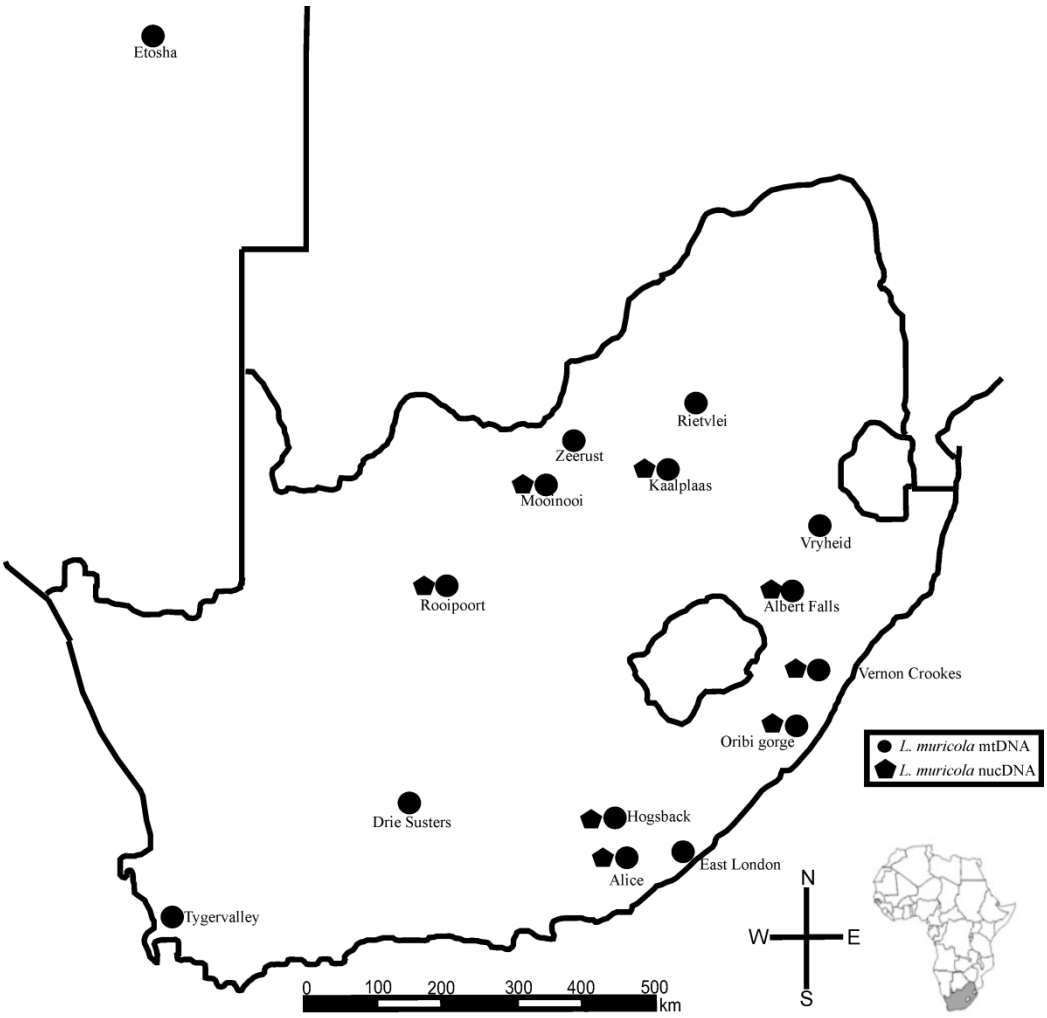

Fig. 4.1: Collection localities in South Africa and Namibia where *Laelaps muricola* (filled circles) were recorded and mtDNA were obtained. Filled pentagons indicate localities with nucDNA sequence representation.

4.2.2. Molecular techniques and data analysis

The genomic DNA of each parasite specimen was isolated using the techniques outlined in Chapter 2. Universal primers described in Folmer *et al.* (1994) were used to amplify and sequence partial segments of the mitochondrial cytochrome oxidase subunit I (COI). To amplify the nuclear intron Tropomyosin (TropoM) the primers from Roy *et al.* (2010) were used. PCR reactions were performed in 25 µl reactions containing millipore

water, 3.5 µl of 25 mM $MgCl_2$, 3 µl of 10X Mg^{2+}-free buffer, 0.5 µl of a 10 mM dNTP solution and 0.5 µl (10 mM) of the respective primer pairs, 0.2 µl of *Taq* polymerase and 2.5 – 4 µl of template DNA. In each instance the volume of water was adjusted to obtain 25 µl reaction volumes. All PCR reactions for the different markers followed the same temperature cycles as outlined in Chapter 2, section 2.2.2.

To check the functionality of the sequence reads, the NCBI GENBANK BLASTN tool was used and all coding regions were translated to putative amino acids to verify functionality with EMBOSS/TRANSEQ (EMBL – European Bioinformatics Institute). BIOEDIT v. 7.0.9 (Hall 2005) was then used to edit and manually align sequences. The nuclear intron sequences of TropoM sequences were then sorted into different alleles using PHASE v. 2.1.1 (Stevens *et al.* 2001; Stephens and Scheet 2005) as implemented in DNASP v. 5.10.1 (Rozas *et al.* 2010). PHASE analysis was performed for 100 000 generations with a burn-in of 10 000 generations. A probability value of 0.9 or higher was used as a reference to indicate a resolved state (Stevens *et al.* 2001). All subsequent analyses were performed on the resulting allelic data.

4.2.3. Genetic relationships and phylogenetic analysis

The unique haplotypes (h), haplotype diversity (H_d), nucleotide diversity (π), pairwise nucleotide differences (k) and number of polymorphic sites (S) were calculated for both gene fragments across all the sampling sites using DNASP v. 5.10.1 (Rozas *et al.* 2010). An AMOVA in ARLEQUIN v. 3.5.1.2 (Excoffier *et al.* 1992) was then used to estimate the genetic divergence (fixation index (Φ_{ST}) between sampling localities. In order to select the

appropriate sequence evolutionary model was the AIC and JMODELTEST v. 3.7 was used (Posada and Crandall 1998).

A haplotype network was constructed using TCS v. 1.21 (Clement *et al.* 2000) firstly to reflect the haplotype diversity within and between the sampled localities. The same network was then coloured to illustrate how individuals from different hosts are related. Isolation by distance was tested using a Mantel test (Mantel 1967) through ALLELES IN SPACE v. 1.0 (Miller 2005).

Higher order phylogenetic relationships were explored with the aid of Bayesian and maximum parsimony trees. The GTR+I+G model was selected as the best-fit model of substitution for both gene fragments and as such was incorporated in the Bayesian analysis performed in MRBAYES v. 3.2 (Ronquist and Huelsenbeck 2003). The data was subsequently partitioned up by codon and each partition subjected to its own model of substitution, unlinked across partitions. Five MCMC chains were run for two million generations and sampled every 1000 generations. Using the sump command in MRBAYES, 25 % of the data were discarded as burn-in. The summarized samples were then reviewed by assessing the convergence parameters and that ESS values above 200 were obtained in TRACER v. 1.5 (Rambaut and Drummond 2007). The consensus tree was then visualized with the program FIGTREE v. 1.2.2 (http://tree.bio.ed.ac.uk/software/gtree/). Nodes with posterior probabilities (pP) ≤ 0.95 were considered not significantly supported.

4.3. Results

4.3.1. Host and parasite range

Expanding here on the sampling done in Chapter 2 and 3, *L. muricola* were collected at eight additional localities across southern Africa and from four host species, namely the Southern multimammate mouse (*Mastomys coucha*), Natal multimammate mouse (*Mastomys natalensis*), Namaqua rock mouse (*Micaelamys namaquensis*) and the Brown rat (*Rattus norvegicus*) (the latter host was confirmed through sequencing of a COI fragment, data not shown) (Table 4.1; Fig. 4.1). A total of N = 119 specimens were collected from these host species (Table 4.1).

4.3.2. Gene sequence characteristics

We were able to successfully amplify all 119 samples for COI however only 56 samples were successfully sequenced for the TropoM gene despite numerous attempts (Genbank accession numbers: COI: KU166673-KU166792; TropoM: KU166793-KU166848). Two sequences of *Androlaelaps marshalli* (Genbank accession number: KF805856; KF805857) were generated used as outgroup for the phylogenetic analysis. Of the possible 708 bp, only 644 bp of the mtDNA COI gene fragment could be used for analysis (due to trimming at the ends and to avoid any missing data). A high haplotype diversity was found (H_d = 0.980) with a low nucleotide diversity π = 0.029 (pair-wise nucleotide differences k = 19.215) and S = 117 polymorphic sites were identified. We amplified 534 bp of the TropoM gene. Similar to the mtDNA pattern a high haplotype diversity was found (H_d

= 0.985) with a low nucleotide diversity π = 0.027 (pairwise nucleotide differences k = 14.403).

4.3.3. Pairwise genetic divergence and phylogenetic reconstructions

The average pairwise divergence at the mtDNA level between nearly all the *L. muricola* specimens irrespective of host species were very low (2.58% ±0.04) despite the significant geographical distance between sampling localities. One exception to the latter was observed. This corresponded to the specimens collected from *R. norvegicus* that differ by 8.13% ±0.048 from the other *L. muricola* found on native rodents in the region (Table 4.2). When drawing comparisons between *L. muricola* individuals found on *M. coucha*, *M. natalensis* and *M. namaquensis* to each other respectively, no group differed by more that 2.60% ±0.04 (which overlaps with intraspecific values on each host) (Table 4.2). Similar to the mtDNA results, an overlap of sequence divergence values was also found in the TropoM gene, suggesting no genetic differentiation between parasites collected from different host specimens (Table 4.2). Bayesian analysis of the COI dataset also supports the monophyly of two distinct *L. muricola* lineages (Fig. 4.2). The first lineage included all the individuals obtained from *R. norvegicus*; within this clade three well supported sub-groups were found which reflects the elevated sequence divergence among individuals found on this single host specimen. The second lineage constitutes all the individuals from the other three host specimens sampled across the geographic range (Fig. 4.2). Owing to the two lineages differing by more than 8% they were treated as unique evolutionary units (see Chapter 2 and 3 for discussions on divergence values among recognized species in mites). Pair-wise Φ_{ST} values for both markers strongly pointed towards no genetic differentiation between the different *L. muricola* specimens sampled from the three different host species (Table 4.3).

Furthermore no significant evidence for isolation by distance with a R^2 equalling 0.01, P = 0.68 was observed.

Table 4.2: Pairwise genetic divergence values within and between the different *L. muricola* mites from different hosts.

	Net sequence diversity - CO1 (% ± SD)	Net sequence diversity - TropoM (% ± SD)
Within *L. muricola* on *M. namaquensis*	2.38 ± 0.032	0.93 ± 0.003
Within *L. muricola* on *M. natalensis*	2.61 ± 0.048	2.11 ± 0.020
Within *L. muricola* on *M. coucha*	0.79 ± 0.007	1.12 ± 0.013
Within *L. muricola* on *R. norvegicus*	2.77 ± 0.014	*
Between *M. coucha* and *M. natalensis*	2.05 ± 0.026	1.02 ± 0.014
Between *M. coucha* and *R. norvegicus*	8.13 ± 0.048	*
Between *M. namaquensis* and *M. natalensis*	2.56 ± 0.041	1.72 ± 0.016

* Indicates that the TropoM for the *R. norvegicus*, *L. muricola* is not available to draw a comparison

Table 4.3: Pairwise Φ_{ST} values between the three *Laelaps muricola* groups corresponding to the three host species. Values above the diagonal indicate nuclear Pairwise Φ_{ST} values; whilst below the diagonal line indicates mtDNA Pairwise Φ_{ST}.

Pairwise *Φst* values			
	M. namaquensis	*M. coucha*	*M. natalensis*
M. namaquensis	-	0.04*	0.00*
M. coucha	0.07*	-	0.00*
M. natalensis	0.01*	0.11*	-

* P-value < 0.05

Fig. 4.2: Bayesian phylogeny indicating the two major clades retrieved from the COI dataset. The two cryptic lineages within *L. muricola* are indicated as lineage 1 and lineage 2.

TCS analysis based on the COI haplotypes broadly connected all the haplotypes sampled from native rodents into one network with limited haplotype sharing and a large number of private haplotypes throughout the range (Fig. 4.3). The haplotypes from *R. norvegicus* however did not connect to any haplotypes from the other hosts (Fig. 4.3). As depicted in the tree-based analyses, the haplotypes from *R. norvegicus* again form three different sub-groups and haplotypes within these sub-groups are also separated by a large number of mutational steps (Fig. 4.3 and Fig. 4.4). Furthermore, when representing the network according to host the haplotypes from *M. natalensis* seem to be the widest spread

across southern Africa; sharing haplotypes with both *M. coucha* and *M. namaquensis* (Fig. 4.4).

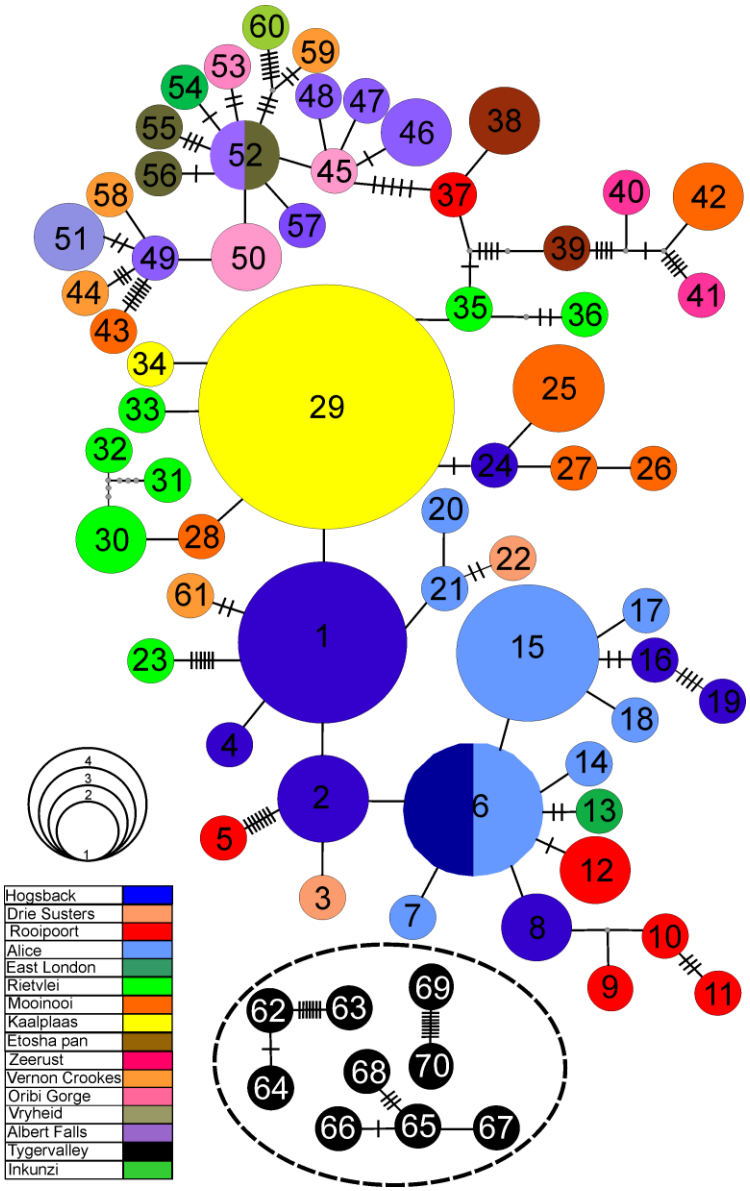

Fig. 4.3: Cytochrome Oxidase subunit I (COI) statistical parsimony network of *L. muricola* lineages. Circles indicate a particular haplotype with the size indicating the relative frequency of the haplotype.

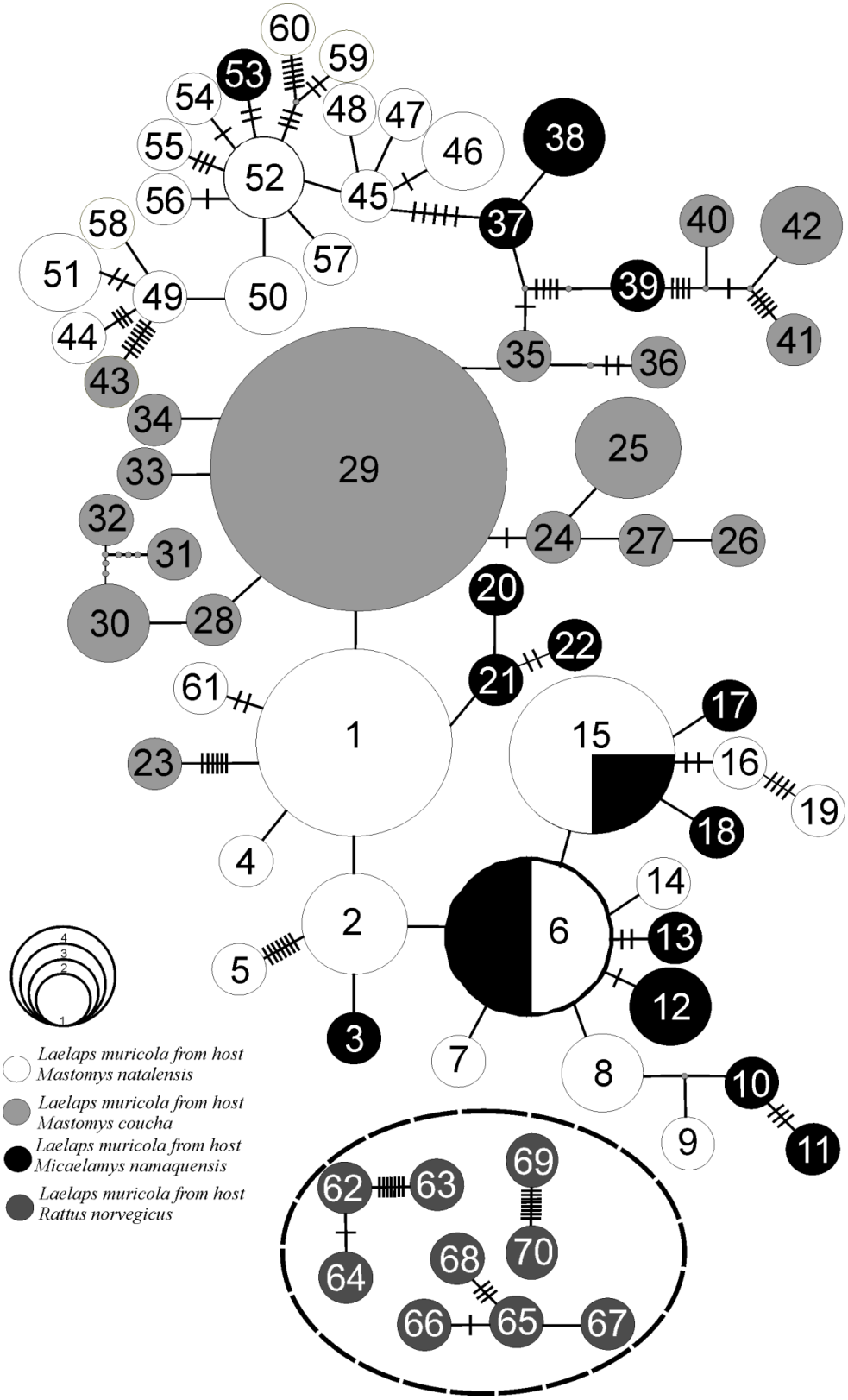

Fig. 4.4: The relative distribution of the haplotypes in *L. muricola* coloured according to host. Circles indicate a particular haplotype with the size indicating the relative frequency of the haplotype.

4.4. Discussion

Here the phylogeography and host range of *L. muricola* are provided, expanding the contemporary information available on this parasitic species of southern Africa (du Toit *et al.* 2013*b*; van der Mescht *et al.* 2015*a*, *b*; Chapter 2). Previous results showed that *L. muricola* is a generalist parasite occurring on multiple rodent species (Chapter 2). These findings are confirmed here by extensive sampling across southern Africa and a lack of genetic support for distinct genetic lineages on each of the host species.

Although the lineage of *L. muricola* occurring on the three native hosts showed very little genetic differentiation among haplotypes, a second divergent lineage of *L. muricola* was found on *R. norvegicus*. The sequence divergence between these two lineages (lineage 1 and 2) is almost equal to the levels found between other recognised mite species in the genus *Laelaps* (9.84% between two cryptic *L. giganteus* species; 10.51% separates *L. giganteus* from *L. muricola*, Chapter 2 and 3) and other mesostigmatid mites (Roy *et al.* 2008, 2010; Knee *et al.* 2012), indicating the presence of a putative cryptic lineage. More comprehensive sampling of *R. norvegicus* and a thorough morphological investigation is needed to confirm or refute the species status of this lineage found on the non-native rat species.

The phylogeographic pattern and levels of divergence between sampling sites are in line with the predicted expectation of generalist ectoparasites (Brown *et al.* 1997; Baer *et al.* 2004; Archie *et al.* 2011; Noureddine *et al.* 2011). *Laelaps muricola* showed multiple host infestations coupled with low genetic differentiation among distant sampling sites and in particular for the specimens on *M. natalensis*, *M. coucha* and *M. namaquensis* (see Noureddine *et al.* 2011; van der Mescht *et al.* 2015*a*, *b* for examples).

Dispersal is an important determining factor in the distribution of species which in turn gives rise to the genetic structure observed in species. Parasites, however, rely predominantly on the host for their dispersal (Vaughn and Taylor 2000) and *L. muricola* is no exception (see Mullen and O'Connor 2002). It is thus proposed that the pattern we observe here is heavily dependent on host dynamics in the region (Cangi *et al.* 2013; du Toit *et al.* 2013*a*, *b*; Chapter 3).

In a recent study Sands *et al.* (2015) illustrated that one of the host genera of *L. muricola*, *Mastomys* was able to survive in multiple refugia during the last glacial maximum and were not strongly affected by the vicariant barriers to gene flow. The authors also showed that certain geographic areas had high levels of unique haplotypes despite the fact that broad scale haplotype sharing was also evident. It was proposed that this host pattern was caused by the habitat resilience of the two *Mastomys* species which are able to rapidly expand once favourable conditions set in and in particular because these species have a high intrinsic propagation rate (Coetzee 1975; Jackson and van Aarde *et al.* 2004; Sands *et al.* 2015).

In *L. muricola*, a large number of private haplotypes characterised the different hosts, yet very few mutational steps accumulated between haplotypes belonging to the different host groups (Fig. 4.4). Therefore, due to the close phylogenetic relationship between the two *Mastomys* species and taking into consideration the phylogeographic patterns of the host (Colangelo *et al.* 2013; Sands *et al.* 2015) the following might explain our data for *L. muricola*.

The private haplotypes on different hosts are maintained because the two plains-dwelling hosts *M. natalensis* and *M. coucha* are partly separated based on ecological

constraints (Sands *et al*. 2015). *Micaelamys namaquensis*, on the other hand is a rock dweller and should have less contact with *Mastomys* species overall. This however, is not sufficient to cause codivergence, since sufficient infrequent contacts among the host species may allow *L. muricola* to propagate and disperse among species (Fig. 4.4). This is supported by the low pairwise Φ_{ST} values and the resultant polytomies in the tree (Fig. 4.2). Further support for frequent movement of *L.* muricola is the lack of isolation by distance between sampling localities. Despite the fact that one of the hosts species, *M. namaquensis*, show a highly structured phylogeographic pattern similar to *Rhabdomys* (Russo *et al.* (2010) for *M. namaquensis* and du Toit *et al.* (2012) for *R. pumilio*), the evidence of extensive range contractions and expansions in *Mastomys* through the recent past (Sands *et al.* 2015) may have facilitated the absence of any geographic genetic structure in *L. muricola.*

Interestingly, the host generalist pattern found here is in contrast to what has been found for the more host specific *L. giganteus* (sister to *L. muricola*) occurring on species within the genus *Rhabdomys* (see Chapter 3). The major difference here being that codivergence between host and parasite was due to a lack of host movement (significant geographically associated vicariance) (see du Toit *et al.* 2012; Chapter 3) leading to pronounced genetic structuring between populations and in many instances also between sampling sites. The strong host tracking pattern we find in *L. giganteus* is therefore not only a consequence of being more host specific, but also a consequence of restriction to host movement among the four *Rhabdomys* species.

In contrast to the absence of geographic structure among *L. muricola* samples on the endemic rodents in South Africa, the genetically distinct lineage on *R. norvegicus* pose an out of the ordinary picture. *Rattus norvegicus* is an invasive species associated with the coastal

regions of South Africa with multiple introductions reported for the species (Skinner and Chimimba 2005; Bastos *et al.* 2011). Although *L. muricola* is reported to be present on *Rattus* species in north African countries, only one *R. norvegicus* individual was caught and found to have *L. muricola*. Remarkable also was the significant genetic variation present among the *L. muricola* individuals on this single animal. In addition, the sequence diversity within the population sampled on *R. norvegicus* was approximately as high as the sequence diversity for all *L. muricola* sampled on all hosts throughout the entire range in southern Africa (2.77% ±0.014). These results therefore suggest that the mites from *R. norvegicus* more than likely do not originate from South Africa and may also suggest that *L. muricola* on *R. norvegicus* may represent novel cryptic diversity.

Chapter 5

Concluding remarks

This study explored the evolutionary history and taxonomic status of two southern African mesotigmatid mites, *L. giganteus* and *L. muricola*, using a multi-disciplinary approach that included a combination of mitochondrial- and nuclear DNA markers and selected morphological characters. This was done to investigate the relative importance of host range as a contributing factor towards lineage diversification in a parasite.

This study provides: (i) novel genetic data to support the currently recognized *L. giganteus* and *L. muricola* as distinct species; (ii) new insights into host range of *L. giganteus* and *L. muricola* in South Africa; and (iii) the first published genetic evidence for cryptic speciation in a mesostigmatid mite occurring in southern Africa. In concert, these findings allow for new insights into the taxonomy and evolution of *L. giganteus* and *L. muricola*. Further evidence is also provided in support of the idea that the *L. giganteus* species complex is host specialists on *Rhabdomys*. Definitive proof of significant codiversification between *L. giganteus* and the four *Rhabdomys* species is also provided, whilst *L. muricola* is confirmed to be a host generalist. Lastly this study provides the first putative evidence of a mesostigmatid mite displaying invasive behaviour in South Africa via the invasive brown rat, *Rattus norvegicus*. Overall, this study highlights the critical need for investigations examining parasite biodiversity and emphasises the rich mostly unexplored parasite biodiversity in southern Africa. This study also highlights the importance of host life history in shaping the phylogeographic pattens of two closely related parasitic mites.

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