Nematode community dynamics in Australian

vertebrates: Impacts of contemporary captive

management practices

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Declaration

The research described in this thesis is original and has not been submitted, in any form, for the award of a higher degree at another university or tertiary institution. I consent to a copy of this thesis being made available through Macquarie University library for consultation, loan and photocopying, subject to the provisions of the copyright act 1968.

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Date

Abstract

Captive management practices may alter host-parasite ecology, driving the emergence of disease in species of commercial or conservation significance. The development of sustainable, integrated techniques for the control of potentially pathogenic taxa requires an understanding of the factors influencing parasite transmission, establishment and intracommunity competition in captive environments. In this study, the community dynamics of parasitic nematodes (suborders; Trichocephalatina and Strongylida) were examined under two captive management regimes; intensively farmed saltwater crocodiles (*Crocodylus porosus*) and non-commercial populations of red kangaroos (*Macropus rufus*). Four aspects of parasite ecology were investigated; genetic diversity within nematode populations, the role of extrinsic environmental factors in mediating nematode-associated helminthosis, the development of high-throughput molecular methods for characterising parasite communities and structuring of nematode assemblages within and between conspecific host populations.

18S ribosomal RNA gene sequences (n=55) were obtained from *Paratrichosoma* sp (nematoda: Trichosomoididae) eggs present in the epidermis of *C. porosus*. The level of genetic diversity distributed within the *Paratrichosoma* sp population was relatively high (241 variable positions in the 1094 bp alignment), suggesting that rates of nucleotide base-pair substitution are accelerated in this genus of nematodes. A significant negative linear relationship (P=0.011, R^2=0.327) was identified between mean monthly rainfall and the monthly incidence of *Paratrichosoma*-associated helminthosis.

The accuracy with which terminal restriction fragment length polymorphism (T-RFLP) analysis, was capable of distinguishing between the constituent taxa of macropodid parasite communities was assessed by comparing sequence data from ~20 species of nematode (suborder Strongylida). Our results demonstrate that, with fluorescent labelling of the forward and reverse terminal restriction fragments (T-RFs) of the ITS+ rDNA region, the

restriction endonuclease *Hinf1* was capable of generating species specific T-RFLP profiles. A notable exception was within the genus *Cloacina*, in which closely related species often shared identical T-RFs. This may be a consequence of the group's comparatively recent evolutionary radiation.

The strongylid nematode communities of *M. rufus* were characterised across seven captive sites, using T-RFLP and Illumina MiSeq next-generation sequencing of the ITS2 locus. The prevalence (P=<0.001) and mean intensity (df=6, F=17.494, P=<0.001) of strongylid nematode infection differed significantly between the sites. Significant levels of parasite community structure were observed (ANOSIM, P = 0.01), with most of the variation being distributed within, rather than between, captive sites. The range of nematode taxa that occured in captive red kangaroos appeared to differ from that of wild conspecifics, with representatives of the genus *Cloacina*, a dominant nematode parasite of the macropodid forestomach, being detected at only two of the seven study sites. This study also provides the first evidence for the presence of the genus *Trichostrongylus* in a macropodid marsupial. Our results demonstrate that contemporary species management practices may exert a profound influence on the structure of parasite communities in captive systems.

Results from this thesis demonstrate a strong anthropogenic influence on the structure of nematode assemblages in captive host populations. Studies of this nature will contribute to the development of more effective captive management practices by elucidating the factors influencing community structure and infection dynamics in a group of parasites that can exert a profound influence on the health and viability of their host populations.

Chapter Description

Chapter 1: General introduction, research objectives and positioning of chapters

I was responsible for reviewing the literature and writing the chapter. Feedback was provided by my primary supervisor, Michelle Power and my co-superviosr, Grant Hose.

Chapter 2: Dermatological conditions of farmed Crocodilians: A review of pathogenic and parasitic agents

I performed the review of literature and wrote the chapter. Direction and feedback were provided by my supervisor Michelle Power, my co-supervisor Grant Hose and Sally Isberg, chief scientist at Darwin Crocodile Farm, NT.

Chapter 3: The application of whole-genome amplification to DNA obtained from degraded and low yield invertebrate samples

Ethanol preserved nematode specimens were obtained from collections held at Berrimah Veterinary Laboratories, NT. I performed all associated lab work including whole genome amplification (WGA), PCR amplification of the 18S rRNA gene and quantitation of template DNA, WGA product and PCR amplicons. I was also responsible for data analysis and statistics. I wrote the manuscript with feedback provided by Michelle Power.

Chapter 4: Genetics and infection dynamics of *Paratrichosoma* sp in farmed saltwater crocodiles (*Crocodylus porosus*)

Saltwater crocodiles displaying symptoms of *Paratrichosoma*-associated helminthosis were euthanised by farm personnel. I performed dissections on-site at Darwin Crocodile Farm, NT, to recover adult nematode specimens and tissue samples containing *Paratrichosoma* sp eggs.

I was responsible for the associated lab work which included microscopy, DNA extractions from both individual nematodes and crocodilian tissue samples, PCR amplification of the nematode 18S rRNA gene and phylogenetic sequence analysis. Microscopy was performed with technical assistance from Nicole Vella, Macquarie University. A sampling regime to monitor patterns of helminthosis among living crocodiles at Darwin Crocodile Farm was designed by myself, with input from Sally Isberg, and implemented by farm personnel. I carried out data analysis and wrote the manuscript with feedback provided by Sally Isberg, Grant Hose and Michelle Power.

Chapter 5: Towards the molecular characterisation of parasitic nematode assemblages: an evaluation of terminal-restriction fragment length polymorphism (T-RFLP) analysis

Frozen nematode specimens were obtained from collections held at the University of Melbourne, VIC, and identified to species level by Ian Beveridge. I carried out DNA extractions, PCR assays of the ITS+ and CO1 loci, phylogenetic sequence analysis and T-RFLP. I analysed the data and wrote the manuscript with feedback provided by my supervisor Michelle Power and my co-supervisor Grant Hose.

Chapter 6: Parasitic nematode communities of the red kangaroo, *Macropus rufus*: richness and structuring in captive systems

I designed the sampling regime for the collection of faecal samples from red kangaroos. Faecal material was collected on-site by keepers and mailed to Macquarie University where I performed faecal egg counts, DNA extractions, PCR amplifications, T-RLP analysis and Illumina MiSeq next-generation sequencing. I was also responsible for all statistics and data analysis. I prepared the manuscript with feedback from my supervisor Michelle Power and my co-supervisor Grant Hose.

Chapter 7: Conclusions

I summarised the major findings of this thesis and outlined implications for future research.

Essential input and feedback was provided by my supervisor Michelle Power and my co-supervisor Grant Hose.

Publications

Lott MJ, Hose GC, Power ML (2015) Parasitic nematode communities of the red kangaroo, *Macropus rufus*: richness and structuring in captive systems. Parasitol. Res. Advance Online Publication. DOI 10.1007/s00436-015-4494-z

Lott MJ, Hose GC, Isberg SR, Power ML (2015) Genetics and infection dynamics of *Paratrichosoma* sp. in farmed saltwater crocodiles (*Crocodylus porosus*). Parasitol. Res. 144: 727-735.

Lott MJ, Hose GC, Power ML (2014) Towards the molecular characterisation of parasitic nematode assemblages: an evaluation of terminal-restriction fragment length polymorphism (T-RFLP) analysis. Exp. Parasitol. 144: 76-83

Lott MJ, Elderidge MDB, Hose GC, Power ML (2012) Nematode community structure in the brush-tailed rock-wallaby, *Petrogale penicillata*: Implications of captive breeding and the translocation of wildlife. Exp Parasitol. 132: 185-192.

Conference Presentations

Lott MJ, Hose GC, Isberg SR, Power ML (2014) Crikey! Crocodile farming & *Paratrichosoma* infection dynamics in Northern Australia; presented at the Annual International Conference of the Wildlife Disease Association, Albuquerque, 27-1 July/August.

Lott MJ, Hose GC, Power ML (2013) A fluorescence-based analysis of macropodid parasite communities: the good, the bad and the ugly; presented at the Wildlife Disease Association Conference, Grampians, 29-4 September/October.

Lott MJ, Hose GC, Power ML (2013) Of macropods and nematodes: A novel technique for investigating host-parasite population dynamics; presented at the World Association for the Advancement of Veterinary Parasitology Conference, Perth, 25-29 August.

Lott MJ, Elderidge MDB, Hose GC, Power ML (2011) Nematode biodiversity in a threatened marsupial: A tool for ecosystem management; presented at the South-eastern Australia Research Symposium, Scotia, 12-14 July.

Lott MJ, Elderidge MDB, Hose GC, Power ML (2010) Nematode biodiversity in a threatened marsupial: A tool for ecosystem management; presented at the International Congress for Parasitology, Melbourne, 15-20 August.

Acknowledgements

It's been nearly eight years since I began my Bachelor of Science at Macquarie University. If somebody had pulled me aside in those early days and told me I was starting on a path that would eventually lead to a PhD, I probably would have laughed at them (and then slowly backed out of the room while avoiding direct eye-contact, because obviously I was dealing with a crazy person). If they'd told me the same thing just two years ago, I would have been only slightly less sceptical. But clearly I owe that crazy clairvoyant an apology because, after almost four years of candidature, the finish line is in site and the list of people I have to thank for that is not short.

To my supervisor, Michelle Power; you're guidance, support and enthusiasm over the years has been invaluable. You were instrumental in convincing me to pursue a career in science and any future success I attain will be due in no small part to the quality of your mentorship. For that, and everything else, you have my sincere and heartfelt thanks. I would also like to extend my gratitude to my co-supervisor, Grant Hose. It is largely thanks to your efforts that I have any understanding of biostatistics and your constant good humour helped make the otherwise tedious number crunching enjoyable.

I'm also indebted to Sally Isberg for the opportunity to develop my project in exciting, crocodile-related directions. Your industry experience, guidance in the field and critical assessment of my written work will always be greatly appreciated. It is not an exaggeration to say that, without your help, there would have been no thesis. To Ian Beveridge, master practitioner of the sadly dying art of nematode morphological taxonomy, who sourced adult nematode specimens for genetic analysis, took the time to assign species-level classifications to them all and asked literally nothing in return. To have worked with a world-renowned expert who remains so approachable and committed to assisting the research of students was a rare privilege and a genuine pleasure.

A pleasant work environment makes all the difference and for that I have to thank my labmates; Amy Asher, Lachlan Byatt, Tiffany Delport, Elke Vermeulen, Koa Webster and, though they've long since moved on to bigger and better things, Cristel Cheung, Louise Crotty and Liette Vandine. The encouragement, technical support and camaraderie you have provided over the years has been nothing short of phenomenal. I would also like to acknowledge my fellow PhD candidates, many of whom I am fortunate enough to count among my closest friends. To Paul Duckett, Peterson Coates, Linda Armbrecht, Adam Wilkins, Francesca Manea, Siobhan Dennison, Peter Schlegel, Jasmin Ruch, Torben Riehl, Peri Bolton, Samuel Andrew, Dalia Rendon-Castaneda and Paolo Momigliano; I'm a better person for having known each and every one of you. Thank you so much for everything you have helped me to achieve.

They say you can choose your friends but not your family. I count myself extremely fortunate then to have the family that I do. To my parents, Graham and Kelly, you remain a source of inspiration and it is to you as much as anyone that I owe the completion of this thesis. Thank you for your constant love and support, nobody could have asked for more. I am also grateful to my brother Joshua and my sister Hannah. I would not be who I am today without the two of you.

Finally, and in no way least, I would like to thank my partner, Elayna Truszewski. If I make it through this thing with my sanity intact I'll have you to thank for it. Your companionship, your support and your love mean the world to me. Thank you for being you.

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1 General introduction and thesis rationale

1.1 The ecological significance of host-parasite relationships

Parasitic helminths are a ubiquitous and cosmopolitan component of ecological communities. The biomass of helminth assemblages frequently rivals or exceeds that of sympatric arthropod and vertebrate species (Kuris et al 2008, Preston et al 2013). Consequently, helminths can exert a significant influence on the topography of food webs by modifying the flow of energy between trophic levels (Amundsen et al 2009, Dunne et al 2013). Helminth parasites also alter the resources available to their hosts; through both direct extraction during feeding and by stimulating redistribution of metabolisable energy to immune defence (Colditz 2008, Gómez and Nichols 2013). Even in the absence of overt pathology, the subsequent impacts on the development, dispersal, survival and reproductive output of host organisms can have profound implications for community structure and function, making helminth parasites a fundamentally important driver of diversity in many ecosystems (Gorrell and Schulte-Hostedde 2008, Schwanz 2008, Robar et al 2010).

Despite their broad ecological significance, the role of helminth parasites in wildlife biology has traditionally been trivialised or ignored (Pedersen and Fenton 2006, Gómez and Nichols 2013). Attitudes towards helminth parasites are often particularly antagonistic when they occur in captive environments, where they are commonly perceived as being innately deleterious to the health or productivity of host populations. Consequently, management practices have often extended no further than attempts to eradicate these parasites in their entirety (Gompper and Williams 1998, Dunn et al 2009, Griffith 2012). Comparatively few investigators have endeavoured to characterise the biotic and abiotic factors influencing helminth community ecology in captive settings. However, to neglect host-parasite population dynamics in captive environments is to overlook a fundamental component of

ecosystem organisation, one that may have unforseen secondary affects for the viability of both animal-based industries and conservation efforts around the world (Marcogliese 2004, Lambden and Johnson 2013).

1.2 Helminth parasites in captive host populations

1.2.1 An industry perspective

Helminths are widely regarded as one of the most economically significant groups of parasites currently affecting livestock production systems (Perry et al 2002, Waller 2006). In Australia, gastrointestinal nematodes have been linked to retarded growth and delayed fertility in sheep and cattle, with subsequent annual losses estimated at up to AU$ 407m (Sackett et al 2006). Nematode-associated helminthosis may also reduce the quality of livestock derived products, such as wool and leather, and in more extreme cases may be associated with heightened mortality in host animals (Dargie 1987, Gulland 1992, Manolis and Webb 2011). Arguably the greatest contemporary issue facing the livestock industry is the problem of anthelmintic resistance (Waller 1999, Waller 2006). The misuse or over-application of broad spectrum anthelmintics in sheep and goats has led to a high prevalence of multi-drug resistance in their nematode parasites, which now threatens the viability of small-ruminant industries in developed and developing countries around the world (Kaplan 2004, Waller 2006). Anthelmintic resistance was long thought to be less common in the gastrointestinal nematode communities of horses and cattle, but a number of studies suggest that the frequency of multi-drug resistance in these helminths has increased dramatically in recent years (Kaplan 2002, Mejía et al 2003, Sutherland and Leathwick 2011, Peregrine et al 2014).

Even when applied correctly, broad spectrum anthelmintic treatments are expensive and limited in their effectiveness (Waller 1999, Waller 2006). Additionally, there is evidence

that anthelmintic treatment regimes alter the dynamics of inter-parasite competition in mixed-community assemblages. The suppression of gastrointestinal nematode populations has been known to cause a transient but substantial increase in the abundance of coccidian parasites, such as *Eimeria*, in some host systems, suggesting dynamic competition between these two taxa (Knowles et al 2013). As coccidiosis can severely compromise the health of young or immunocompromised animals, this reaffirms the importance of maintaining a holistic ecological perspective, in which intra-community interactions between parasites are accounted for, when developing strategies for the management of helminths in captive animal production systems (Daugschies 2005, Sarah et al 2013). Consequently, the development of effective treatment regimes will be contingent on a detailed knowledge of helminth community structure and the factors influencing the transmission and establishment of potentially pathogenic taxa in host species of economic significance.

1.2.2 A conservation perspective

The rapid and continuing loss of global biodiversity in the early 21st century has prompted renewed interest in the development of effective, sustainable approaches to the management of threatened species (Butchart et al 2010, Minasyan et al 2010, Mora and Sale 2011). Captive breeding programs, and the associated translocation of wildlife, provide a powerful tool for conserving genetic diversity by supplementing or re-establishing failing populations in the wild (Griffith et al 1989, Cunningham 1996). The establishment of viable populations in captivity also serves to increase the resilience of threatened fauna to localised environmental disturbances (Griffith et al 1989). However, the risk of disease affects many aspects of captive breeding and reintroduction. The potential for species translocation events to introduce novel parasites into immunologically naïve populations is well documented (Warner 1968, Zeller and Murgue 2001, Kutz et al 2004, Wikelski et al 2004). The negative

effects of helminths and other parasites on contemporary conservation efforts may be further

compounded by low genetic diversity and small population size in many threatened species

(Matocq and Villablanca 2001, Siddle et al 2007, Lee et al 2010).

Captive environments alter the dynamics of host-parasite interaction, with profound

implications for the health of translocated animals. Even when antiparasitic drugs, such as

anthelmintics, are not regularly employed, captive host populations may be maintained at

levels below what is necessary for the maintenance of certain parasite taxa (Poulin 1996,

Lindenfors 2007). As resistance to helminthosis and other parasitic diseases is largely

dependent on exposure, these immunologically naive hosts may be at greater risk of adverse

health effects when translocated and exposed to parasitic helminths endemic to their species

traditional home ranges (Barger et al 1985, Eriksen et al 1992, Cunningham 1996).

Alternatively, increased host population densities in some captive environments may enhance

transmission of certain helminths, altering the composition of parasite communities and

leading to previously benign species exerting pathogenic effects (Keymer 1982). Finally,

captive populations may be infected with helminths that are foreign to the typical range or

species of the host organism (Cunningham 1996). The influence which helminths exert on the

fitness of their hosts suggests that mismanagement of these parasites has the potential to not

only negate the benefits of captive breeding programs but may have an overall negative

impact on global conservation efforts (Viggers et al 1993, Woodford and Rossiter 1993).

Consequently, a mechanistic understanding of the effects which captive management

practices exert on the structure of helminth communities is required to maximise the success

of threatened species recovery programs.

1.3 Integrated management of parasitic helminths

Integrated parasite management encompasses a broad range of strategies which use a combination of chemical and non-chemical techniques to minimise the impacts of parasites on the health and productivity of their hosts (Waller 1999). Integrated approaches to the management of parasitic helminths have proven successful in reducing the risk of anthelmintic resistance in a number of economically significant host species and are increasingly recognised as a financially and environmentally sustainable alternative to the control of helminths in captive systems (Krecek and Waller 2006, Torres-Acosta and Hoste 2008, Woodgate and Besier 2010). Previous studies in tropical locations such as Fiji and the Pacific island of Tongatapu have shown that the concentration of *Haemonchus contortus* and *Trichostrongylus* spp. larvae peaked on pastures approximately one week after contamination, dropping to nearly undetectable levels within four to six weeks (Banks et al 1990, Barger et al 1994). A system of rotational grazing was subsequently developed for their goat hosts which successfully reduced the helminth burdens of livestock to less than half that of conspecifics in adjacent areas. However, as climate can exert a significant influence on the development and persistence of free-living larval stages, rotational grazing schemes must be tailored to fit the unique ecology of helminths in different geographical regions (Barger et al 1994).

   In temperate regions, the high degree of host specificity exhibited by many parasitic helminths has been exploited to develop a grazing management system of parasite control based on interchanging species of livestock (Barger and Southcott 1978, Donald et al 1987). Helminths which are detrimental in one species of host may be less pathogenic, less prolific or fail to establish themselves entirely in another. Studies conducted in northern NSW, Australia have demonstrated that the frequency of drenching can be greatly reduced in production systems where sheep and cattle are alternated at two to six month intervals

(Barger and Southcott 1978, Donald et al 1987). It has been suggested that these practices could also be effective in managing helminth parasites in tropical regions but that grazing intervals may need to be shortened to account for climate-mediated variability in parasite development and transmission (Waller 1999).

The establishment of refugia, a proportion of parasites that are not exposed to chemical treatments, is another method by which selection for anthelmintic resistant alleles in helminth assemblages may be reduced (Kenyon et al 2009). Parasitic helminths typically exhibit an aggregated (right-skewed) distribution within host populations. Consequently, in any given production system, a small subset of hosts can be expected to harbour high helminth burdens, while the majority of individuals will have comparatively few helminths or, in some cases, none at all (Barger 1985). It has been suggested that selection for anthelmintic resistance could be greatly reduced if chemical treatments were directed solely at hosts that were disproportionally responsible for pasture contamination (i.e. those with the highest worm burdens). This approach would mitigate parasite-mediated effects on host productivity, while reducing selection for anthelmintic resistant alleles in the component of the parasite population that infect the untreated animals (Van Wyk et al 1997, Waller 1999). In South Africa, limiting the application of anthelmintics to hosts displaying clinical symptoms of anaemia has proven highly successful in reducing the prevalence of anthelmintic resistance among populations of *H. contortus* parasitising small ruminants. However, this approach is dependent on identifying clinical symptoms of disease associated with particular parasite taxa and on the ability to monitor long term changes in parasite transmission dynamics and community structure (Malan and Van Wyk 1992, Waller 1999).

It is well established that many of the pathogenic affects commonly associated with helminth infection are due to malnutrition and undernutrition. Consequently, it is often possible to reduce mortality and production losses by strategic feed supplementation of

susceptible individuals within host populations (Whitlock et al 1943, Donaldson 1997, Van Houtert 1997). The effectiveness of supplementary feeding techniques can be further enhanced if periods of peak parasite abundance can be anticipated, possibly by identification of environmental correlates of infection (Donaldson 1997, Van Houtert 1997). In addition to improving host nutrition, supplementary feeding may be used to administer biological control agents for helminth parasites. Spores from the nematophagous microfungus *Duddingtonia flagrans* are capable of surviving passage through the gastrointestinal tract of ruminants (Gronvold 1996, Larsen et al 1998). These spores then rapidly germinate in faeces, capturing helminth parasites and reducing the concentration of infective third-stage larvae on pastures. However, due to the possibility of variable effectiveness of biological control agents on different helminth taxa, the ability to rapidly and accurately characterise the species composition of helminth assemblages will improve the success of these treatment strategies by facilitating targeted responses (Gronvold 1996, Larsen et al 1998, Campos et al 2008).

1.4 The need for a mechanistic understanding of parasitic helminth communities

It is clear that a comprehensive understanding of the mechanisms that shape parasitic helminth communities is required to maximise the success of disease management programmes in captive systems (Waller 1999, Waller 2006). A mechanistic understanding of community structuring will make it possible to predict the response of helminths to management strategies specific to particular captive environments (e.g. intensive production or threatened species conservation) and, by extension, to anticipate the impacts on the health of their host organisms (Pedersen and Fenton 2006). Ultimately, this knowledge will be founded on the ability to monitor interactions between the component taxa of mixed-species assemblages, both within individual hosts and across whole host populations, and to identify biotic or abiotic features of the environment which influence these associations. Conversely,

the development of sustainable integrated parasite control measures will be problematic or impossible in captive systems in which parasite community structure and host-parasite population dynamics are poorly understood.

High-throughput molecular techniques, such as the fluorescence-based terminal restriction fragment length polymorphism (T-RFLP) analysis, and massively parallel sequencing platforms, such as Illumina MiSeq, may provide sufficient depth of coverage to not only characterise complex helminth assemblages, but to monitor changes in community dynamics across both time and space (Marsh 1999, Ekblom and Galindo 2011). This would make them invaluable in gaining an understanding of the mechanisms which shape parasite communities in captive systems. However, while the efficacies of these methodologies are well documented in bacteria, protozoa and fungal communities, they have seldom been applied to metagenomics analyses in metazoan parasites (Dickie et al 2002, Wang et al 2004, Lazarevic et al 2009, Tymensen et al 2012, Schmidt et al 2013).

1.5 Research objectives

Native Australian vertebrates may be bred and kept in captivity for a variety of purposes. Crocodilians, such as the saltwater crocodile (*Crocodylus porosus*) are raised under intensive production conditions for their meat and valuable hides (Blake and Loveridge 1975, Thorbjarnarson 1999). Others, including many macropodid marsupials, are of high conservation concern, with numerous threatened species breeding programs having been established around the country (Eldridge 2010). However, there is currently a deficit of knowledge on how the parasitic helminth communities of crocodilians and macropodids respond to captive management practices. Ecological studies which investigate not only the taxonomic composition of helminth assemblages but also identify both spatiotemporal

patterns of distribution and environmental correlates of helminthosis will be required if sustainable integrated parasite management strategies are to be developed.

Crocodilian-helminth community interactions were evaluated in a population of captive saltwater crocodiles due to the aforementioned commercial significance of this species. The red kangaroo, *Macropus rufus*, was selected as a model macropodid species. While not considered a high conservation priority themselves, the well documented and relatively simple parasitic helminth communities of red kangaroos make them an excellent model organism for investigating the effects which captive management regimes may exert on the host-parasite interactions of macropodid marsupials.

The primary aim of this thesis was to increase our understanding of the role contemporary captive management practices play in shaping the helminth communities of native Australian vertebrates. The secondary aim was to demonstrate how molecular techniques may be employed to characterise the epidemiology and structuring of parasite communities in response to both intensive production systems and non-commercial captive breeding programs. This was achieved through four key objectives;

1. Identifying nematodes responsible for disease in commercially farmed saltwater crocodiles and characterising the genetic diversity of their populations.

2. Examining the extent to which extrinsic environmental factors, such as temperature or precipitation, mediate nematode-associated helminthosis in saltwater crocodiles under intensive farming conditions.

3. Developing rapid high-throughput molecular techniques (T-RFLP and Illumina MiSeq) which can be used to characterise and monitor whole parasitic nematode communities in macropodid marsupials.

4. Employing these techniques to investigate the structure and spatio-distribution of nematode assemblages within and between captive red kangaroo host populations.

1.6 Positioning of thesis chapters

Chapter 2: Dermatological conditions of farmed Crocodilians: A review of pathogenic and parasitic agents.

M. J. Lott & G. C. Hose & S. R. Isberg & M. L. Power

Effective management of pathogenic agents which target the crocodilian dermis and epidermis is essential for maintaining the viability of commercial farming operations. This chapter reviews the major viral, bacterial, fungal and helminth taxa associated with skin conditions in intensively farmed species of crocodilian. Best management practices for minimising the impact of these organisms on the production of high quality crocodilian leather products is discussed, with an emphasis on addressing extrinsic factors which influence transmission and pathogenicity. This chapter also highlights gaps in the understanding of the ecology and epidemiology of key parasitic and pathogenic taxa, and proposes future avenues of research to increase our knowledge of skin disease and aetiological agents in captive crocodilian production systems.

Chapter 3: The application of whole-genome amplification to DNA obtained from degraded and low-yield invertebrate samples

M. J. Lott & M. L. Power

The accurate identification of biological specimens, and their associated taxonomy, underpins all ecological research. Establishing robust phylogenetic relationships requires that gene sequence data be validated by morphometric analysis. However, due to a declining morphotaxonomic skill base, studies which seek to compliment genetics with morphological data are increasingly reliant on pre-identified specimens held in museum collections. This can be problematic due to DNA degradation accompanying long term storage in ethanol or formalin-based solutions. This chapter investigates whether it is possible to overcome this

limitation through the application of whole genome amplification to DNA extracted from crocodilian nematodes held in long term (>10 years) ethanol storage.

Addresses objective 1

Chapter 4: Genetics and infection dynamics of *Paratrichosoma* sp. in farmed saltwater crocodiles (*Crocodylus porosus*)

M. J. Lott, G. C. Hose, S. R. Isberg & M. L. Power

Parasitology Research (2015) 144; 727-735

Paratrichosoma-associated helminthosis has been identified in saltwater crocodiles under intensive farming conditions in northern Australia. Developing sustainable integrated management practices requires an understanding of *Paratrichosoma* population genetics and infection dynamics in captive systems. This chapter examines the genetic relationships of a population of *Paratrichosoma* sp. at Darwin Crocodile Farm using 18S rRNA sequence data, and investigates several possible environmental correlates of the incidence and intensity of helminthosis among affected crocodiles.

Addresses objectives 1 and 2

Chapter 5: Towards the molecular characterisation of parasitic nematode assemblages: an evaluation of terminal-restriction fragment length polymorphism (T-RFLP) analysis

M.J. Lott, G.C. Hose and M.L. Power

Experimental Parasitology (2014) 144; 76-83

Understanding the factors which regulate spatiotemporal structuring of parasite assemblages in captive environments requires the development of non-invasive techniques for monitoring entire helminth communities across time and space. This chapter explores the development of a rapid fluorescence-based method, terminal restriction fragment length polymorphism (T-

RFLP) analysis, for characterising parasitic nematode assemblages in macropodid marsupials. The accuracy with which T-RFLP was capable of distinguishing between the constituent taxa of a parasite community was assessed by comparing sequence data from two loci (the ITS+ region of nuclear ribosomal DNA and the mitochondrial CO1) across ~20 species of nematodes (suborder Strongylida).

Addresses objective 3

Chapter 6: Parasitic nematode communities of the red kangaroo, *Macropus rufus*: richness and structuring in captive systems

M.J. Lott, G.C. Hose and M.L. Power

Parasitology Research (2015) DOI 10.1007/s00436-015-4494-z

In this chapter, we employ two high-throughput molecular techniques to characterise parasitic nematode (suborder Strongylida) communities across seven captive populations of red kangaroo, *Macropus rufus*. The first is T-RFLP analysis of the ITS+ region of nuclear ribosomal DNA, while the second is Illumina MiSeq next-generation sequencing of the ITS2 region of nuclear ribosomal DNA. The prevalence, intensity of infection, taxonomic composition and comparative structure of strongylid nematode assemblages was assessed at each study site. The relative efficacy of the two techniques for monitoring helminth community dynamics in captive systems is compared and discussed.

Addresses objective 3 and 4

Chapter 7: Conclusions

This chapter summarises the major findings of the thesis and highlights that contemporary captive management practices are capable of exerting a significant influence on the structure of helminth communities in host species of commercial and conservation significance. The

implications for the viability of host populations in captive systems and the biodiversity of sympatric helminth species are discussed.

Appendix 1

Nematode community structure in the brush-tailed rock-wallaby, *Petrogale penicillata*: Implications of captive breeding and the translocation of wildlife

M.J. Lott, G.C. Hose and M.L. Power

Experimental Parasitology (2012) 132; 185-192

A publication describing research from my Honours year that was prepared and submitted for publication in the second year of my PhD candidature. This study was the first attempt to adapt terminal restriction fragment length polymorphism (T-RFLP) analysis for the characterisation of parasitic helminth communities in macropodid hosts and formed the basis for much of the work performed in the course of my PhD.

Appendix 2

Final approval letter from Animal Ethics Committee

1.7 References

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2 Dermatological conditions of farmed Crocodilians: A review of pathogenic and parasitic agents

2.1 Abstract

The control of pathogenic agents that target the crocodilian dermis and epidermis is essential to the long term success of intensive farming operations worldwide. This paper reviews the known viral, bacterial, fungal and helminth taxa associated with skin conditions in commercially significant crocodilian species. Best management practices for minimising the impact of these organisms on production outcomes is discussed, with an emphasis on addressing extrinsic factors which influence transmission and pathogenicity. It is argued that, in the past, reduced immune function arising from inadequate thermal regulation was the leading cause of skin disease in captive crocodilians. Consequently, innovations such as temperature control, coupled with the adoption of more stringent hygiene standards, have greatly reduced the prevalence of many skin conditions in intensively farmed populations. However, despite improvements in animal husbandry and disease management, viral pathogens such as West Nile virus and pox-virus continue to afflict crocodilians in modern captive production systems. This review examines the importance of characterising host-pathogen community dynamics in order to develop sustainable, holistic approaches to disease control in intensively farmed crocodilian species.

2.2 Introduction

The sustainable use of crocodilians has had a significant and positive impact on the conservation status of many previously threatened species (Blake and Loveridge 1975, Bolton and Laufa 1982, Revol 1995). Regulated commercial use has formed the basis for the

management of vulnerable crocodilian populations in more than 40 countries, and continues to generate strong economic incentives for their protection in both developed and developing nations (Thorbjarnarson 1992, Revol 1995, Thorbjarnarson 1998). The rearing of crocodilians under intensive farming conditions is a comparatively recent innovation, driven primarily by the demand for high quality skins used in the creation of luxury fashion goods (Thorbjarnarson 1999). In the Northern Territory alone, the total production value of crocodile farming has been estimated at $21 million annually, with this figure expected to climb as high as $40 million over the next two to three years (Jean 2013). However, increasingly stringent quality standards, driven in part by the global financial crisis, are contributing to a market where only grade one skins are financially viable. A single blemish can lead to a hide being classified as second grade, while additional imperfections can result in a third or fourth grade hide, reducing the value by up to 50% (Buenviaje 1998, Thorbjarnarson 1999). The control of pathogenic and parasitic agents which target the crocodilian dermis and epidermis is therefore imperative to the long term success of commercial farming operations (Thorbjarnarson 1999).

Crocodilian species of economic importance include *Alligator mississippiensis* (USA), *Caiman crocodilus crocodilus* (Colombia, Bolivia), *Caiman crocodilus fuscus* (Colombia), *Caiman latirostris* (Argentina), *Caiman yacare* (Brazil, Argentina, Bolivia, Paraguay), *Crocodylus niloticus* (Africa), *Crocodylus novaeguineae* (Papua New Guinea, Indonesia), *Crocodylus porosus* (Australia, Papua New Guinea, Indonesia, Malaysia, Singapore, Thailand) and *Crocodylus siamensis* (Thailand, Vietnam, Cambodia). Of these, *C. porosus* skins are generally held to be superior due to the lack of osteoderms in the ventral scales and the high number and general regularity of the transverse ventral scale rows (Thorbjarnarson 1999). This review will address the major pathogenic agents – viral, bacterial and parasitological – associated with dermatological conditions in the more

commonly farmed crocodilian species with an emphasis on life-cycle, epidemiology, and current management practices.

2.3 Viruses

2.3.1 Flaviviridae

West Nile Virus (WNV) is a mosquito-borne Flavivirus that has been reported in several crocodilian species, including the commercially significant *A. mississippiensis* and *C. niloticus* (Miller et al 2003, Steinman et al 2003). The virion is a spherical particle (~50 nm in diameter) characterised by a nucleocapsid core surrounded by a lipid bilayer membrane in which the viral envelope and membrane proteins are embedded. Replication occurs in the perinuclear space of the host cell's endoplasmic reticulum (ER) followed by virus assembly within the lumen of the ER. Virions are then transported to the cell membrane through the secretory pathway for exocytosis (Deubel et al 2001).

Avians are widely regarded as the primary reservoir host for WNV, with mosquitoes of various species acting as vectors (Unlu et al 2010). However, there is substantial evidence for the direct transmission of WNV within captive *A. mississippiensis* populations through a faecal-oral route, as high cloacal shedding of WNV virus particles has been detected in infected individuals (Klenk et al 2004). The formation of viremia may also make bloodborne transmission possible, while the ingestion of animals with high viral loads by conspecifics presents another possible source of infection. However, these forms of transmission are unlikely in captive crocodilian populations in developed countries due to stringently enforced hygiene and animal housing regulations. The ability of WNV to maintain itself in crocodilian blood and tissues for prolonged periods of time indicates that these animals may serve as important amplifiers and reservoir hosts for the virus (Klenk et al 2004, Jacobson et al 2005).

Symptoms associated with WNV infection in *A. mississippiensis* include lethargy, loss of motor control, muscle spasms, multiorgan necrosis, mild interstitial pneumonia, glossitis, enteritis meningoencephalomyelitis and, possibly, neurological lesions. Heightened mortality has been associated with WNV outbreaks among captive *A. mississippiensis* populations but not in *C. niloticus* (Miller et al 2003, Steinman et al 2003, Jacobson et al 2005). Young animals in particular seem to be at risk, with an overall mortality rate of 7% (Miller et al 2003, Klenk et al 2004). More recently, evidence has emerged for the association of WNV exposure with the development of lymphohistiocytic proliferative syndrome of alligators (LPSA), a skin condition characterised by the formation of small gross lesions in the ventral scales. A study of forty LPSA positive *A. mississippiensis* demonstrated that WNV was also present in the skin lesions in 97.5% of cases (Nevarez et al 2008).

There are currently no specific treatments or vaccines available for WNV. However, as a potentially zoonotic virus, its control is a high priority for commercial farming operations (Rappole et al 2000, Unlu et al 2010). The elimination or reduction of mosquito populations, the strict quarantine of infected individuals and stringent protocols governing the sterilization and disposal of contaminated equipment or clothing are essential for preventing the spread of the virus. Minimising intrinsic stressors which may compromise normal immune function, such as excessive ambient noise and ineffective temperature control have also been suggested as ways of minimising the pathogenic effects of WNV (Miller et al 2003, Klenk et al 2004, Unlu et al 2010).

2.3.2 Poxviridae

Crocodile and caiman poxviruses have been identified in five commercially significant species; *C. c. crocodilus*, *C. c. fuscus*, *C. yacare*, *C. niloticus* and *C. porosus* (Jacobsen et al 1979, Huchzermeyer et al 1991, Penrith et al 1991, Buenviaje et al 1998, Ramos et al 2002).

Structurally, the virions are brick-shaped with rounded corners, approximately 100-200 nm in diameter and possess a biconcave or 'dumbbell-shaped' central core which is surrounded by two symmetrical proteinaceous lateral bodies (Huchzermeyer et al 2009). A single surface tubule on the outer membrane layer forms continuous helices that wind around the virion. In mature virions (MVs) both the core and the lateral bodies are enclosed by a single lipid bilayer. The less abundant extracellular virions (EVs) are similar in appearance to the MVs but possess a double membrane (Jacobson et al 1979, Pandey et al 1990, Gerdes et al 1991, Huchzermeyer et al 2009). Molecular analysis of crocodile poxviruses in *C. niloticus* suggests that at least two morphologically indistinguishable but genetically and epidemiologically distinct Poxviridae taxa exist within crocodiles (Huchzermeyer et al 2009). Poxviruses are unusual in that the replication and transcription of the viral genome occurs exclusively in the cytoplasm of the host cell. Virions typically exploit glycosaminglycans on the surface of the host cell's plasma membrane to mediate endocytosis (Lefkowitz et al 2006). The outer membrane layers are then lost, releasing the viral core into the cytosol of the host cell and precipitating the expression of enzymes required for the transcription of early genes. The expression of intermediate and late genes follows, triggering the replication of genomic DNA and the production of structural proteins respectively (Gerdes et al 1991, Lefkowitz et al 2006). Progeny virions are assembled in cytoplasmic domains known as viral factories, maturing into intracellular mature virions (IMVs). These IMVs may then be released by cell lysis or acquire a second double membrane and bud as external enveloped virions (EEVs). While taxa specific variation is certain to exist, the conservation of genes associated with replication, virion assembly and virion structure in crocodile poxviruses suggests that they conform to this basic pattern (Afonso et al 2006, Lefkowitz et al 2006, Huchzermeyer et al 2009).

Infection with crocodile poxvirus typically presents as crusty brown or yellow lesions on the head, oral cavity, belly, or ventral and lateral surfaces of the body and tail. It has also been associated with opthalmia, reduced growth rates and developmental abnormalities caused by skin shrinkage. Mortality is typically low unless complicated by opportunistic bacterial or fungal infections (Pandey et al 1990, Huchzermeyer et al 1991, Afonso et al 2006). In *C. niloticus*, infection with "atypical" crocodile poxvirus causes the formation of deeply penetrating skin lesions. Unlike "classical" crocodile pox infection, in which spontaneous recovery is usually observed within several weeks or months, these lesions cause permanent scars or 'pit-holes' (Huchzermeyer et al 2009). Caiman poxvirus is associated with greyish-white lesions on the dorsal scales of the head, body and limbs. Both crocodile and caiman poxviruses appear to be more prevalent among hatchlings and juvenile individuals, with adults typically being asymptomatic (Jacobson et al 1979, Penrith et al 1991, Ramos et al 2002, Afonso et al 2006).

No specific treatments or vaccines are currently available for crocodilian poxviruses. Without knowledge of the direct transference pathways, prevention may be possible through the implementation of strict hygiene measures on affected farms. Pens and rearing units should be cleaned and disinfected regularly and the use of surface water should be avoided. However, it is unlikely that these measures alone will be sufficient to completely eliminate infection (Huchzermeyer et al 2009).

2.4 Bacteria

2.4.1 Dermatophilaceae

Dermatophilus is a Gram-positive, filamentous, facultative anaerobic bacterium that occurs in soil worldwide. It is characterised by two forms; hyphae and motile zoospores. The hyphae stage consists of branching filaments 1-5 µm in diameter that develop into pockets of coccoid

cells by transverse and longitudinal division. The coccoid cells subsequently mature into flagellated ovoid zoospores approximately 0.6-1 µm in diameter (Zaria 1993).

Outbreaks of a *Dermatophilus* species morphologically and biochemically similar to *D. congolensis* have been reported in *A. mississippiensis* and *C. niloticus* and the bacterium has been described as the most common cause of skin disease in farmed *C. porosus* in Australia (Bounds and Normand 1991, Stuart 1993, Buenviaje et al 1998). *Dermatophilus* infection is responsible for the condition dermatophilosis or 'brown spot' disease. As the name suggests, the primary symptoms are the formation of brown or red lesions of varying size, often accompanied by subcutaneous nodules up to 3 cm in diameter. Lesions typically occur on the neck and abdomen of afflicted individuals but may also appear on the tail and feet. The ulcerations are occasionally accompanied by subcutaneous granulomas. In more severe cases extensive skin erosion of up to 5 cm has been observed (Buenviaje et al 1997, Buenviaje et al 1998).

The factors influencing the occurrence and transmission of dermatophilosis among crocodilians are unclear. However, it has been suggested that their aquatic habitat presents a favourable set of conditions for the proliferation of the genus *Dermatophilus* by retarding keratin shedding, leading to the accumulation of biological debris. (Buenviaje et al 1997, Buenviaje et al 1998).

The addition of 1 mg/L copper sulphate to pen water has proven to be an effective treatment for dermatophilosis in crocodilians. Forced emersion for 15 minutes twice a day for a period of nine weeks successfully eliminated dermatophilosis-associated lesions in treated animals, leaving the hides of previously infected animals blemish free (Buenviaje et al 2004). Forced emersion is unlikely to be necessary in contemporary captive production systems, as all commercial farms now possess indoor pen set-ups with artificial control of water temperature. Therefore, most crocodilians will willingly spend a significant proportion of

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their time submerged, particularly in the colder months (NRMMC 2009). Permitting

voluntary emersion also has the advantage of circumventing the risk of exacerbating

infections by reducing stress arising from frequent contact with farm personnel. (Buenviaje et

al 2004). As with other pathogenic agents, the quarantine of infected animals in conjunction

with strict biosafety practices may serve to prevent the spread of *Dermatophilus* bacteria

through captive populations.

2.4.2 Enterobacteriaceae

Serratia fonticola is a Gram-negative, rod-shaped, facultative anaerobic bacterium that has

been recovered from the skin lesions, blood and organs of a single infected *C. niloticus*

(Rossi 1996). The bacteria are motile, peritrichous and are approximately 0.5 µm in width

and 30 µm in length (Gavini et al 1979). Fatal septicaemia, a condition strongly correlated

with lesions of this nature in other reptile species, was also reported in the infected *C.*

niloticus (Rossi 1996). *Serratia fonticola* was identified as the most probable aetiological

agent. Other symptoms associated with *S. fonticola* in the infected crocodile included

pericarditis, the formation of multifocal nodules on the liver and abscesses on the lungs and

spleen (Garcia et al 2008).

As there is currently only a single confirmed case of *S. fonticola* infection in a

crocodilian, the factors governing the transmission of the bacterium remain largely unknown.

However, it has been suggested that *S. fonticola*, a normal component of water flora in many

regions, may become pathogenic when the host animal is immunocompromised (Gavini et al

1979, Garcia et al 2008). Temperature associated stress, arising from the inability to properly

thermoregulate, is known to impair immune function in many reptilian species, including

crocodilians (DeNardo 1996; Pare´ et al 2006). The *S. fonticola* infected *C. niloticus*

originated from an indoor enclosure at a zoo where it was exposed to suboptimal water

temperatures. The maintenance of crocodilians in enclosures that mimic the thermal range of their native environments as closely as possible may prevent opportunistic infection by pathogenic bacterial agents such as *S. fonticola* (Garcia et al 2008).

2.4.3 Mycobacteriaceae

Mycobacterium is a genus of non-flagellated, Gram-positive, aerobic bacteria associated with skin conditions in a range of reptiles, including crocodilians. Morphologically, bacterial cells are either straight or slightly curved rods that range from 0.2-0.6 µm in width and 1-10 µm in length (Conn and Dimmick 1947). Mycobacterial dermatitis has been reported in *C. porosus* although the species responsible was not identified. *Mycobacterium intracellulare* has been reported in *C. latirostris* and at least one case of mixed-species infection with *Mycobacterium szulgai* and *Mycobacterium chelonae* has been reported in *C. c. fuscus* (Buenviaje et al 1998, Slany et al 2010, Kik 2013).

In *C. porosus*, infection with *Mycobacterium* sp presented as skin lesions characterised by red to grey nodules approximately 2-5 mm in diameter on the snout, jaws and conjunctiva and along the ventral scales of the neck and thigh. In some cases well defined granuloma were detected in the dermis. In several more severe cases, *Mycobacterium* sp. infection resulted in localised erosion of the epidermis (Buenviaje et al 1998). *Mycobacterium* sp. infection did not present as skin lesions in *C. latirostris* but was associated with thickening of the intestinal wall and enlargement of the spleen and liver. Disseminated granulomas were also detected in the liver, spleen and intestinal wall (Kik 2013). Pathological effects associated with mixed-species *Mycobacterium* infection in *C. c. fuscus* also included enlargement of the spleen, with complete destruction of the inner structure of the organ, and the formation of granulomas in the liver and lungs (Slany et al 2010). Chronic weight loss and anorexia have been associated with *Mycobacterium* spp.

infection in both caiman species. It has been hypothesised that both the pathogenic response and the distribution of mycobacteria throughout the host tissue varies according to the species of pathogen and reptilian host (Slany et al 2010, Kik 2013).

The source and mode of transmission of *Mycobacterium* spp. in crocodilians is not currently known. However, the high environmental occurrence of *Mycobacterium* spp., coupled with the comparatively low prevalence of infection, suggests that crocodilians may have some innate resistance to these organisms. Symptoms may only present when the individual has been immunocompromised by extrinsic environmental factors (Slany et al 2010). Alternatively, the lack of reported cases of mycobacteriosis in crocodilians may simply reflect the difficulties involved in isolating and identifying the causative agent. Treatment is generally not recommended due to the chronic nature of mycobacteriosis and because the disease is usually well advanced by the time symptoms present (Slany et al 2010, Kik 2013). There is also a high risk of infection to human handlers and other animals. Therefore, exposure to infected animals should be limited, pens should cleaned frequently with water exceeding 60°C and high pressure hoses or sprinkling systems which create aerosols should be avoided (Kik 2013).

2.4.4 Streptococcaceae

Streptococcus agalactiae has been reported in one commercially significant crocodilian species, *C. porosus*. A second *Streptococcus species*, most likely *Streptococcus dysgalactiae* subsp *equisimilis* has been detected in *C. niloticus* (Madsen 1993, Bishop et al 2007). *Streptococcus* are Gram-positive bacteria characterised by their distinctive globular shape. As mitosis proceeds along a single axis they often appear in long chains of cells (Facklam 2002). Pathogenic members of this genus are classified by their haemolytic properties. Alpha-hemolytic species oxidise iron in haemoglobin while Beta-hemolytic species rupture

erythrocytes and produce toxic compounds which interfere with the activity of leukocytes (Lawrence et al 1985, Facklam 2002).

Streptococcus agalactiae, a group B streptococcus responsible for pneumonia, meningitis and sepsis in human infants, has also been associated with necrotizing fasciitis in juvenile *C. porosus* in a single outbreak (Bishop et al 2007). This condition was characterised by inflammation, necrosis and sloughing of ventral scales and soft tissue on the neck, chest and forelimbs. As in Group A streptococcus infection, tissue damage was presumably mediated by virulence factors such as pyrogenic exotoxin. High mortality was observed in infected animals (Kuo et al 1998, Bishop et al 2007).

Multilocus genotyping of *S. agalactiae* isolates provided strong evidence that the streptococcus strain affecting *C. porosus* was of human origin. This highlights the risk of anthropogenic sources of disease to animals housed under intensive farming conditions and the need for strict handling and biosafety procedures to prevent the transfer of potentially pathogenic agents (Bishop et al 2007). In humans, septicaemia and other severe infections associated with *S. agalactiae* are more commonly observed in neonatal, elderly or immunocomporomised individuals (Wong et al 2004). Mitigating extrinsic stressors and limiting contact with at-risk animals such as hatchlings may prevent further outbreaks of *S. agalactiae* associated necrotizing fasciitis. Comparatively little effort has thus far been made to develop preventative treatments due to the rarity of *S. agalactiae* associated necrotizing fasciitis in crocodilians. However, a vaccine derived from the concentrated extracellular products of inert *S. agalactiae* has been trialled in the Nile tilapia, *Oreochromis niloticus*, another poikilothermic organism, with some success (Bishop et al 2007, Pasnik 2005).

2.4.5 Vibrionaceae

Aeromonas hydrophila is a Gram-negative, rod-shaped, non-spore forming, facultative anaerobic bacterium commonly found in fresh or brackish water. It is also a normal component of the intestinal micoflora of some species of crocodilian and has been associated with pathogenicity in the commercially significant species *A. mississippiensis*, *C. niloticus* and *C. porosus*. The bacteria are motile, possessing a polar flagellum, and are typically 1-3 mircometers in length and 0.3-1 μm in width (Gorden et al 1979, Turutoglu et al 2005, Roh et al 2011).

A. *hydrophila* is considered an opportunistic pathogen, with infections typically occurring in response to stress from trapping, handling or injury, disease related immunosuppression, increased water temperature and possibly eutrophication (Shotts et al 1972, Araujo et al 1991, Turutoglu et al 2005, Roh et al 2011). Within crocodilian populations, transmission may occur via exposure to contaminated faecal material, consumption of infected prey species or through contact with external skin lesions (Turutoglu et al 2005, Roh et al 2011).

Fatal septicaemia as a consequence of *A. hydrophila* infection has been reported in both *C. porosus* and *C. niloticus*. Red and brown skin lesions of various sizes were also observed in *C. niloticus.* These lesions were scattered across the abdomen, tail and feet of the infected crocodile and progressed to erosion of the epidermis. Other reported symptoms associated with *A. hydrophila* infection include necrotizing enteritis, intestinal hemorrhage, serositis and pancreatitis (Gorden et al 1979, Turutoglu et al 2005, Roh et al 2011).

The ubiquitousness of *A. hydrophila* in aquatic environments, coupled with its resistance to a wide range of antimicrobial agents, suggests that eradication of the bacterium from commercial farming operations may not be a realistic goal. Consequently, effective management practices will involve mitigating conditions which lead to pathogenicity in

crocodilians. High levels of faecal pollution have been associated with increased abundance

of some *Aeromonas* species (Shotts et al 1972, Araujo et al 1991). Regular removal of animal

waste from pens or enclosures may serve to lower infection rates. Immunosuppression caused

by the inability to properly thermoregulate has also been linked to an increased likelihood of

A. hydrophila infection (Gorden et al 1979, Turutoglu et al 2005, Roh et al 2011). As

crocodilians typically thermoregulate by moving between an aquatic and terrestrial

environment, commercial farms in most countries are now required to house animals in

enclosures that provide access to both, minimising stress and subsequent disease

susceptibility (NRMMC 2009). Other potentially stressful activities, such as handling, should

also be kept to a minimum (Gorden et al 1979, Turutoglu et al 2005, Roh et al 2011).

2.5 Fungi

2.5.1 Nectriaceae

Members of the genus *Fusarium* have been associated with mycotic dermatitis in several

commercially significant crocodilian species. *Fusarium* sp. was identified as a common cause

of skin lesions in farmed *C. porosus* in Australia and *C. c. crocodilus*. A possibly distinct

species, *Fusarium solani* has also been observed in deep tissue mycoses in *C. porosus*

(Buenviaje et al 1994, Buenviaje et al 1998, Jacobson et al 2000).

    Fusarium is a genus of filamentous fungi, commonly found in soil and plant debris.

Typically saprotrophic, they have been associated with a range of diseases in both plants and

animals. Typical features of *F. solani* include elongate monophialides that range from 50-70

μm in length and 2-3μm wide, which bare ovoid microconidia 9-15 μm in length and 2-4 μm

in width. Chlamydospores are typically globular or elliptic, 9-12 μm by 8-10 μm and may

occur singly or in pairs. Fungal hyphae are septate with a relatively uniform diameter of 2.5

to 3 µm and occasional dichotomous branching (Leslie and Summerell 2006, Zhang et al 2006).

Gross signs of infection in crocodilians commonly include a grey, gelatinous appearance to affected skin areas (Buenviaje et al 1994, Buenviaje 1998). These lesions are typically most prevalent on the dorsal skin of the head but may appear anywhere on the body. On a microscopic level, ulceration or erosion of infected skin regions is common and mats of fungal hyphae on the epidermis were often observed to penetrate down to the dermis or muscle (Buenviaje et al 1994, Buenviaje 1998). Acanthosis, spongiosis and the formation of rete ridges were common on the epidermis of infected individuals. In some cases, subcutaneous granuloma persisted after re-epithelisation. It should be noted that it is common to recover multiple fungal taxa from crocodilian skin lesions. Hence these symptoms may not be limited to or caused by *Fusarium* sp. infection alone (Buenviaje et al 1994, Buenviaje 1998).

Pathogenicity of *Fusarium* sp. in most animal species is associated with suppression of normal immune function (Zhang et al 2006). Consequently, the removal or management of extrinsic stressors may serve to prevent infection in intensively farmed crocodilians. Topical agents such as povidone iodine (0.005%) and chlorhexidine have been used to treat superficial mycotic dermatitis in reptiles. Several families of drugs (azoles, polyene macrolide antibiotics, flucytosine, griseofulvin) have also demonstrated potent antifungal properties. However, the efficacy and safety of most of these drugs for crocodilians is largely unknown (Jacobson et al 2000).

2.5.2 Onygenaceae

The *Chrysosporium* anamorph of *Nannizziopsis vriesii* has been associated with fatal mycotic dermatitis in juvenile *C. porosus* (Thomas et al 2002). *Nannizziopsis vriesii* is a

keratinophilic microfungus that is capable of both sexual and asexual reproduction. Only the asexually reproducing anamorph *Chrysosporium* has been demonstrated to cause skin lesions in reptiles. Fungal hyphae are typically between 2 and 4 μm in width. Conidia form on the sides of the branching hyphae or on swollen condidiogenous cells and are single-celled, subglobulose and hyaline, ranging in size from 6-7 μm in length and 3.5-4 μm in width. Arthrocondia are 1.5-2 μm in width and 3 μm in length and typically form large groups in regions of necrotic tissue (Guarro et al 1991, Paré et al 1997).

Pathogenic effects of *Chrysosporium* in *C. porosus* include gross leathery plaque-like lesions, approximately 1-2 cm in length, on the head, jaw, abdomen, limbs, tail and feet of infected individuals. These lesions are composed of necrotic, hypertrophic or hyperplasic epidermal cells. If the fungal growth is not checked, then these cutaneous infections may spread and become fatal (Thomas et al 2002).

It has been suggested that the *Chrysosporium* anamorph of *N. vriesii* colonises crocodilians through cuts or scratches in the epidermis. The tendency of crocodilians in captivity to gather together in clusters or piles provides ideal conditions for the transmission of the erumpent fungal arthrocondia. Extrinsic stressors such as handling, transport, overcrowding and poor temperature control may increase the probability of infection (Thomas et al 2002). There is also evidence that the fungus can persist in the environment for significant lengths of time. Treatment of lesions with the iodine-based antiseptic Betadine, followed by emersion in water containing low levels of formalin, has proven successful in halting the progression of *Chrysosporium* infection. However, complications arising from secondary bacterial infection can reduce the effectiveness of these treatments (Thomas et al 2002).

2.5.3 Trichocomaceae

The *Trichocomaceae* are a cosmopolitan family of saprotrophic fungi. They are a ubiquitous component of soil biota in most regions and are commonly associated with decaying plant matter (Pitt et al 200). Members of this family have also been implicated in a range of diseases affecting both plants and animals. *Aspergillus niger*, *Aspergillus flavus* and *Penicillium oxalicum* have been identified in both superficial and necrotising lesions on the skin and gingiva in *C. porosus* in Australia and C*rocodylus acutus* in Mexico (Buenviaje et al 1994, Buenviaje et al 1998). However, due to the presence of other aetiological agents, it is often unclear whether *Trichocomaceae* species are the primary causative agents of skin disease or if they simply opportunistically colonised the site of infection. The ubiquitous nature of these fungi, coupled with the recovery of *Aspergillus* and *Penicillium* isolates from healthy scales of *A. mississippiensis* may suggest the latter (Jasmin and Baucom 1967, Jacobson et al 2000).

2.6 Helminths

2.6.1 Capillariidae

The Capillariid nematode, *Paratrichosoma crocodylus* is known to parastise *C. porosus* and *C. novaeguineae* in Papua New Guinea. A second, closely related species, *Paratrichosoma recurvum* has been recovered from *Crocodylus moreletii* and possibly C*rocodylus acutus* in Mexico (Ashford and Muller 1978, Spratt 1985, Moravec and Vargas-Vazquez 1997). However, symptoms characteristic of *Paratrichosoma* spp. infection have been detected in seven other species of crocodilians, including the commercially significant *C. niloticus*. The genus has a cosmopolitan distribution, having been reported in such diverse locations as Africa, the Americas, Southern Asia and Australia (Webb and Messel 1977, Webb and

Manolis 1983, Spratt 1985, Whitaker and Andrews 1989, Moravec and Vargas-Vazquez 1997).

Members of the genus *Paratrichosoma* are long, slender nematodes, typically 2-7mm in length and 0.082-0.167mm in width. The cuticle over most of the body is unusually thick and smooth for a Capillariid nematode, while the cloaca also has a thick cuticular lining. Males lack both caudal papillae and a distinct lobe on the posterior end but do possess a well-developed spicule, the anterior of which has a hyaline cover. Other unique features include poorly developed or absent mesenchynal cells at the esophagointestinal junction and a stichosome which fails to reach the intestine (Ashford and Muller 1978, Spratt 1985, Moravec and Vargas-Vazquez 1997). Morphologically, the two described *Paratrichosoma* species are extremely similar. Notable differences include the presence of rough transverse grooves on the spicule of *P. crocodylus* but not on the spicule of *P. recurvum* and protruding polar plugs on the eggs of *P. recurvum* which do not occur on the eggs of *P. crocodylus*. Additionally, mesenchynal cells at the esophagointestinal junction are absent in *P. crocodylus* but present in *P. recurvum*, albeit in an extremely reduced state, while in *P. crocodylus* the posterior end of the ovary exceeds the anterior end of the rectum but does not in *P. recurvum* (Moravec and Vargas-Vazquez 1997).

The primary evidence of *Paratrichosoma* spp. activity in crocodilians is the formation of distinctive serpentine scars on the ventral scales of the lower jaw, chest and abdomen of infected animals. These scars are burrows, created by the movement of mature gravid females through the epidermis, into which they deposit their eggs. The unembryonated eggs develop within these burrows before being released into the environment during the normal shedding of host epithelial cells. No other pathological affects are currently associated with *Paratrichosoma* spp. infection (Ashford and Muller 1978, Moravec and Vargas-Vazquez 1997).

Little is currently known about the life-cycle of *Paratrichosoma* spp., including the mode by which it colonises its crocodilian hosts. It has been suggested that infective larvae may be ingested with stones (gastroliths) which crocodilians consume for ballast or possibly within an intermediate host which is being exploited as a food source. Alternatively, free-living larvae in the surrounding environment may burrow directly into the ventral skin of the crocodilian. This scenario is perhaps less plausible as the thick epidermal layer of crocodilians would likely prevent external penetration (Tellez and Paquet and Durand 2011).

No confirmed treatments for *Paratrichosoma* spp. infection currently exist and the efficacy of broad spectrum anthelmintics remains unknown. Successful management may involve preventing transmission by disrupting key stages of the parasites lifecycle, for example by targeting the hypothetical intermediate host, or through the isolation of infected animals from the general population.

2.7 Conclusion

Many of the fungal and bacterial agents associated with skin conditions in intensively farmed crocodilians appear to be naturally occurring components of the animal's microbiota or else ubiquitous in the surrounding environment (Shotts et al 1972, Araujo et al 1991, Zaria 1993, Jacobson et al 2000, Slany et al 2010). A higher prevalence or severity of infection in hatchling and sub-adult members of the population is also a recurring theme in the literature (Penrith et al 1991, Thomas et al 2002, Bishop et al 2007). Taken together, these elements may indicate that extrinsic stressors are the primary etiological factors contributing to skin disease in commercial farming operations.

Immune function is known to be highly temperature dependent in crocodilians (Miller et al 2003, Merchant and Britton 2006, Bishop et al 2007). Inadequate thermal control, and subsequent impairment of normal immune function, has been implicated in

increased pathogenicity of viral, bacterial and fungal agents in the vast majority of recorded outbreaks in captive crocodilian populations (Kik 2003, Miller et al 2003, Klenk et al 2004, Garcia et al 2008, Unlu et al 2010). Increased sensitivity to temperature fluctuations has also been suggested as an explanation for the comparatively higher rate of mortality observed in hatchling and juvenile animals during outbreaks (Miller et al 2003). However, it should be noted that many of the outbreaks of pathogenic microorganisms documented in this review appear to be relatively isolated incidents that occurred in situations where there was a failure to follow best practice for managing crocodilians in captivity (Rossi 1996, Bishop et al 2007). As the crocodilian farming industry has grown, techniques for maintaining the health of populations under intensive production conditions have continued to be refined. In most developed countries, this has led to the establishment of stringent guidelines for hygiene, handling and enclosure design (e.g. temperature control), with the goal of minimising the impacts of extrinsic stresses on the health of farmed crocodilians (IUCN 1971–1990). By preventing or mitigating conditions under which microorganisms become pathogenic, these innovations appear to have been effective at reducing the economic costs of skin disease for commercial farming operations. Outbreaks of bacterial and fungal pathogens may continue to affect facilities in developing countries where minimum standards for the maintenance of crocodilians in intensive production systems may be less heavily enforced or absent (Thorbjarnarson 1992, Thorbjarnarson 1999). Viral pathogens and parasitic helminths also continue to afflict crocodilians in modern captive production systems, despite improvements in animal husbandry and disease management (Nevarez 2008, Huchzermeyer 2009).

In many of the investigated cases, crocodilian skin lesions contained multiple pathogenic organisms. This complicates treatment as the precise etiological agent or agents often cannot be identified with certainty (Buenviaje et al 1994, Jacobson 2000). Furthermore, the ubiquitous or symbiotic nature of many potentially pathogenic organisms may ultimately

make eradication unlikely or even undesirable. The adoption of integrated management practices, which use a combination of chemical and non-chemical techniques to minimise the impacts of pathogens on host productivity, is likely to be a more economically and environmentally sustainable approach (Waller 1999, Waller 2006). In the absence of a mechanistic understanding of the transmission or establishment of metazoan parasites and microorganisms within intensive crocodilian production systems, the most effective control strategies will be those that prevent pathogenicity by managing extrinsic stressors. The form these management practices may take can be broadly divided into two main approaches. The first is to mimic the natural ecology of crocodilians as closely as possible by reducing the density of farmed animals, creating large buffer zones that prevent the spread of disease and ensuring that the industry does not exceed the carrying capacity of the local environment (Blake and Loveridge 1975, Bolton and Laufa 1982, Revol 1995). The obvious problem with this approach is that a low intensity operation of this nature would require a much larger area for a much lower return than more intensive farming systems. The second approach is to isolate commercial systems from the environment in an effort to artificially optimise them. Such a system would involve treatment and recirculation of pen water, artificial temperature control, the use of antibiotics, inoculations, anthelmintics and sterilising agents such as formaldehyde and iodine to treat infections and, possibly, selective breeding for resistance to disease. This approach has the potential to generate very high commercial outputs but also requires a high degree of management (Blake and Loveridge 1975, Bolton and Laufa 1982, Revol 1995, Bostock et al 2010). The limited amount of data currently available on the ecology of potentially pathogenic agents in crocodilians also makes designing effective treatment regimes difficult and existing practices may actively exacerbate the problem (e.g. by selecting for antibiotic resistance). There is a clear need to compare these two approaches in a cost-benefit analysis, in order to identify an economically viable middle ground and to

allow farm holders to make informed decisions regarding the control of pathogenic agents which target crocodilian skin.

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3 The application of whole-genome amplification to DNA obtained from degraded and low yield invertebrate samples

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3.1 Abstract

Molecular-based approaches to the study of invertebrate taxonomy and ecology are often confounded by a lack of high quality genomic DNA. It may be possible to overcome this limitation through the application of whole genome amplification (WGA) to old or degraded samples. The efficacy of two commercially available WGA kits, Genomeplex[TM] and GenomiPhi[TM], was investigated for use on DNA extracted from specimens of parasitic nematodes that had been held in long term (>10 years) ethanol storage. The success of each kit, as inferred from the yield and quality of the WGA treated DNA, was determined by Gel electrophoresis, spectrophotometry and PCR-based screening of the 18S rRNA gene. Both the Genomeplex[TM] and GenomiPhi[TM] WGA kits appeared to significantly increase the amount of genomic DNA, generating microgram quantities from starting concentrations as low as two nanograms. However, the PCR success rate was not improved and DNA sequencing of the WGA-amplified samples was not possible. The high post-WGA DNA yields observed in this study were most likely a consequence of GenomePlex[TM] adapter sequences and random GenomiPhi[TM] hexamers being preferentially amplified over the degraded nematode template sequences. As such, it appears that WGA is an ineffective technique for enhancing DNA quantity when the original sample is from low-yield or degraded material.

3.2 Introduction

The accurate identification of biological specimens underpins all ecological research. DNA-based approaches to helminth taxonomy have led to more robust estimates of biodiversity, while high-throughput sequencing technologies have facilitated increasingly large scale comparisons of community structure and function (Blaxter 2004, Bhadury et al 2006, Ekblom and Galindo 2011, Tanaka et al 2014). However, while a DNA-based taxonomy is promising, it is far from infallible. Differential selection pressure on genetic markers, coupled with phenomenon such as horizontal gene transfer, can lead to situations where the evolutionary history of genes differs from those of the organisms from which they are derived (Yang 1994, Rzhetsky 1995, Dunning Hotopp et al 2007). Consequently, accurately establishing phylogenetic relationships will always require that gene sequence data be validated by morphology-based taxonomy (Willa and Rubinoff 2004).

Faced with a decreasing number of skilled taxonomists, studies which seek to compliment genetics with morphological data are increasingly reliant on pre-identified specimens held in museum and laboratory collections (Coomans 2002, Hopkins and Frekleton 2002). This can be problematic due to DNA degradation accompanying long term storage in ethanol or formalin-based solutions, which may render downstream molecular applications impossible. This issue is further compounded by the comparatively low amount of total DNA and resilient cuticle structure present in many helminth species (McManus et al 1985, Dawkins and Spencer 1989, Seesao et al 2014).

Whole genome amplification (WGA) is one method by which the quality and quantity of template DNA can be improved (Ballantyne et al 2007, Gunn et al 2007, Ahrabi et al 2010). Commercially available WGA kits may be PCR-based or use techniques such as multiple displacement amplification (MDA). PCR-based WGA protocols typically involve the random fragmentation of genomic DNA, followed by the ligation of short adapter

sequences with degenerate primer sites onto the template molecules. The DNA fragments are then amplified using universal primers and a high fidelity DNA polymerase (Pruett et al 2004). In contrast, MDA employs an isothermal amplification method in which random hexamer primers bind to denatured DNA and a novel DNA polymerase, Phi29, exponentially amplifies the target genome with a high degree of accuracy (Dean et al 2002). MDA is widely considered to be superior to PCR-based approaches to WGA as it is able to produce high yield, highly uniform amplification of the entire template genome (Hosono 2003, Handyside 2004). This will only be an issue, however, when the goals of the study require high fidelity, whole genome sequencing. For genetic marker identification or species discrimination through genotyping, it is not clear that either method is inherently superior to the other (Ballantyne et al 2007).

In this study we assessed the suitability of two commercially available WGA kits, the PCR-based GenomePlex[TM] (Sigma, St. Louis, USA) and the MDA-based GenomiPhi[TM] (GE Biosciences, Piscataway, USA), for amplifying genomic DNA extracted from parasitic nematode specimens held in long term ethanol storage. All nematode samples had consistently failed to amplify using standard kit-based DNA extraction and PCR techniques. It was the goal of this study to determine whether either of the WGA kits was capable of increasing the quality and quantity of the nematode specimens DNA to the point that downstream molecular applications, such as PCR and genotyping, could be performed.

3.3 Materials and Methods

3.3.1 Nematode specimens and DNA extraction

Representatives of 9 nematode species were acquired from a collection of ethanol preserved specimens held at Berrimah Veterinary Laboratories (Northern Territory, Australia). These nematodes originated from two species of Australian crocodilians, *Crocodylus porosus* and *Crocodylus johnsoni*, and were collected between 28 and 10 years prior to DNA extraction (Table 3.1). Genomic DNA was recovered from the mid-body region of ethanol preserved nematodes by placing them in a 1.2 mL Eppendorf tube with 20 µL PCR buffer. Samples were ground up using a plastic pestle and incubated at 70 °C for 10 min, after which 2 µL of 10 mg/ml Proteinase K was added to the solution. Samples were then incubated for 3.5 h at 56°C. Twenty microlitres of GeneReleaser (BioVentures, Durham, USA) was added to the digested product and overlaid with 2 drops of sterile paraffin oil. Samples were heated on the high setting of a 750 Watt microwave oven for 6 min, after which they were centrifuged at 13,000 rpm for 2 min. The supernatant was then transferred to clean 0.5 mL Eppendorf tubes (Wimmer et al 2004, Lott et al 2014).

Table 3.1 Species of nematode utilised in this study, including hosts, sample codes, dates and locations of collection.

Nematode Species	No. of Specimens	Host Species	Area of Origin	Collection Date	Sample Reference No.
Dujardinascaris mawsonae	10	*Crocodylus porosus*	Kakadu National Park, QLD	Mar 2003	923, 1-10
Eustrongylides crocodile	2	*Crocodylus porosus*	–	May, 2005	928, 1-2
Gedoelstascaris australiensis	10	*Crocodylus porosus*	–	1994	560, 1-10
Johnstonmawsonia sp.	6	*Crocodylus porosus*	Kakadu National Park, QLD	Jul, 1995	242, 1-6
Micropleura sp.	7	*Crocodylus johnstoni*	Darwin Crocodile Farm, Noonamah, NT	Jun, 1995	029/251, 1-7
Multicaecum agile	10	*Crocodylus porosus*	Kakadu National Park, QLD	1995	217, 1-10
Oswaldofilaria kanbaya	3	*Crocodylus sp*	–	Nov, 2000	573, 1-3
Terranova crocodili	10	*Crocodylus porosus*	Kakadu National Park, QLD	2003	924, 1-10
Goezia sp.	2	*Crocodylus porosus*	Kakadu National Park, QLD	May, 1996	268, 1-2

3.3.2 Whole Genome Amplification

3.3.2.1 GenomePlex^{TM}

GenomePlex^{TM} (Sigma, St. Louis, USA) WGA was performed according to the
manufacturer's instructions. All incubation steps were conducted in a Mastercycler Gradient
thermal cycler (Eppendorf, Hamburg, Germany). One microlitre of $10 \times$ Fragmentation
buffer was added to 1μL of DNA (2-10 ng/ μL) in a 0.5 ml Eppendorf microcentrifuge tube.
The solution was incubated at 94 °C for 4 min and cooled on ice. OmniPlex libraries were
prepared by adding 2 μL of $1\times$ Library Preparation buffer and 1 μL of Library Stabilisation
Solution. Samples were mixed by vortexing and incubated at 95 °C for 5 min, before being
cooled on ice again. One microlitre of Library Preparation Enzyme was added to the samples,
which were vortexed briefly and incubated at 16°C for 20 min, 24°C for 20 min, 37°C for 20
min and 75°C for 5 min. Amplification reactions were performed with 7.5 µl of $10\times$
Amplification Master Mix, 5.0 µl WGA DNA Polymerase and 47.5 µl sterile PCR H$_2$0. A
denaturation step of 95°C for 3 min preceded 14 cycles of 94°C for 15 s and 65°C for 5 min.
A positive control consisting of human genomic DNA and a negative control consisting of
sterile PCR H$_2$O were included with the reactions. GenomePlex^{TM} WGA products were
purified using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) according to
the manufacturer's Spin Protocol. GenomePlex^{TM} WGA reactions were performed in
triplicate for all samples.

3.3.2.2 GenomiPhi^{TM}

GenomiPhi^{TM} (GE Biosciences, Piscataway, USA) WGA was performed according to the
manufacturer's instructions. One microlitre of DNA (2-10 ng/ μL) was added to 9 μL of
Sample buffer. Samples were denatured at 95 °C for 3 min then cooled on ice. Ten
microlitres of the amplification reaction master mix, consisting of 9 μL Reaction buffer and 1

µL Phi29 enzyme mix, were added to the samples and incubated at 30°C for 3 h. The Phi29

DNA polymerase enzyme was heat inactivated by incubating the samples at 65°C for 10 min.

A positive control consisting of human genomic DNA and a negative control consisting of

sterile PCR H$_2$0 were included with the reactions. GenomiPhi^{TM} WGA reactions were

performed in triplicate for all samples.

3.3.3 Quantitation of genomic DNA and Statistical Analyses

Agrose gel electrophoresis was used to confirm the presence of DNA in the WGA products.

The concentration of DNA in both the original extractions and the WGA products generated

from each nematode specimen were quantitated by fluorescence spectrophotometry using a

Nanodrop1 ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, USA) at 340

nm. As the data were non-parametric, Wilcoxon signed-rank tests were used to determine if

there was a significant difference between the average DNA concentration of each of the

preserved nematode species before and after WGA amplification. Wilcoxon signed-rank tests

were also used to test whether the GenomePlex^{TM} and GenomiPhi^{TM} kits generated

significantly different final yields of genomic DNA. A posthoc Bonferroni correction was

applied to the Wilcoxon signed-rank test results in order to correct for multiple comparisons.

All statistical analyses were performed in Minitab 17 (State College, Pennsylvania, USA).

3.3.4 Gene specific PCR

A ~1 kb region of the nematode 18S ribosomal RNA gene was targeted using the general

Eukaryotic primers EukF (5'- AACCTGGTTGATCCTGCCAG -3') and EukR (5'-

CCTTCTGCAGGTTCACCTAC -3') and the nematode specific primer set Nem_18S_F (5'-

CGCGAATRGCTCATTACAACAGG-3') and Nem_18S_R (5'-

GGGCGGTATCTGATCGCC-3') (Medlin et al 1988, Floyd et al 2005). Both PCRs were

performed in volumes of 25 μL, with each reaction consisting of 12.5 μL Gotaq Green

Mastermix, 0.2 μM of each primer, 9.5 μL PCR H2O and 2 μL of template DNA. A

denaturation step of 94°C for 5 min preceded 35 cycles of 94°C for 30 s, 54°C for 30 s and

72°C for 1 min. This was followed by a final extension at 72°C for 10 min. PCR products

were resolved by electrophoresis on 2% agarose gels and purified using the QIAquick PCR

Purification Kit according to the manufacturer's Spin Protocol (QIAGEN, Hilden, Germany).

Sequencing was performed on a 3730XL DNA analyser at the Macrogen sequencing facility

(Macrogen Inc., Seoul, South Korea).

3.4 Results

Both the GenomiPhi[TM] and Genomeplex[TM] WGA kits appeared to significantly increase the

concentration of genomic DNA for all of the ethanol-preserved nematode specimens (Table 2

and Figure 1). The average final DNA yield generated by the Genomeplex[TM] treatment was

shown to be significantly higher than the average amount produced by the GenomiPhi[TM]

treatment in three nematode species after Bonferroni correction (P=0.017); *Terranova*

crocodili (W=55, P=0.0027), *Eustrongylides crocodile* (W=55, P=0.0027) and *Multicaecum*

agile (W=43, P=0.015). For all remaining species, there was no significant difference in the

DNA yield produced by either of the WGA protocols. Both the electrophoretic gel profile and

spectrophotometric analysis revealed a high yield of DNA present in the no template water

controls. While the human DNA positive control was successfully amplified by PCR using

the generic Eukaryotic primer set EukF and EukR, none of the nematode specimens could be

genotyped. Nematode template DNA from all study specimens consistently failed to amplify,

despite the increase in DNA yield detected after treatment with WGA protocols.

Table 3.2 Total average DNA yields before and after treatment with each of the

commercially available WGA kits.

Nematode Species	Original DNA Yield (ng/μl)	GenomePlex™		GenomiPhi™		GenomePlex™ vs GenomiPhi™
		DNA Yield (ng/μl)	Significance	DNA Yield (ng/μl)	Significance	
Dujardinascaris mawsonae	2.5	954.2	W=-55, P=0.0027	918.2	W=-55, P=0.0027	W=21, P= 0.1492
Eustrongylides crocodile	2.1	950.3	W=-55, P=0.0027	650.3	W=-55, P=0.0027	W=55, P=0.0027
Gedoelstascaris australiensis	4.0	985.4	W=-55, P=0.0027	922.2	W=-55, P=0.0027	W=39, P= 0.025
Johnstonmawsonia sp.	2.2	1011.3	W=-55, P=0.0027	910.8	W=-55, P=0.0027	W=7 P=0.3707
Micropleura sp.	3.3	976.8	W=-55, P=0.0027	891.4	W=-55, P=0.0027	W=-9 P=0.3336
Multicaecum agile	9.3	982.5	W=-55, P=0.0027	913.1	W=-55, P=0.0027	W=43, P= 0.015
Oswaldofilaria kanbaya	2.7	971.7	W=-55, P=0.0027	937.3	W=-55, P=0.0027	W=4 P=0.4286
Terranova crocodili	4.7	950.3	W=-55, P=0.0027	793.7	W=-55, P=0.0027	W=55, P= 0.0027
Goezia sp.	3.3	954.8	W=-55, P=0.0027	984.2	W=-55, P=0.0027	W=-1 P= 0.488
Human Control	17.2	851.2	W=-55, P=0.0027	944.7	W=-55, P=0.0027	W=-1 P=0.488
H$_2$0 Control	0.0	912.7	W=-55, P=0.0027	916.4	W=-55, P=0.0027	W=-15 P=0.2297
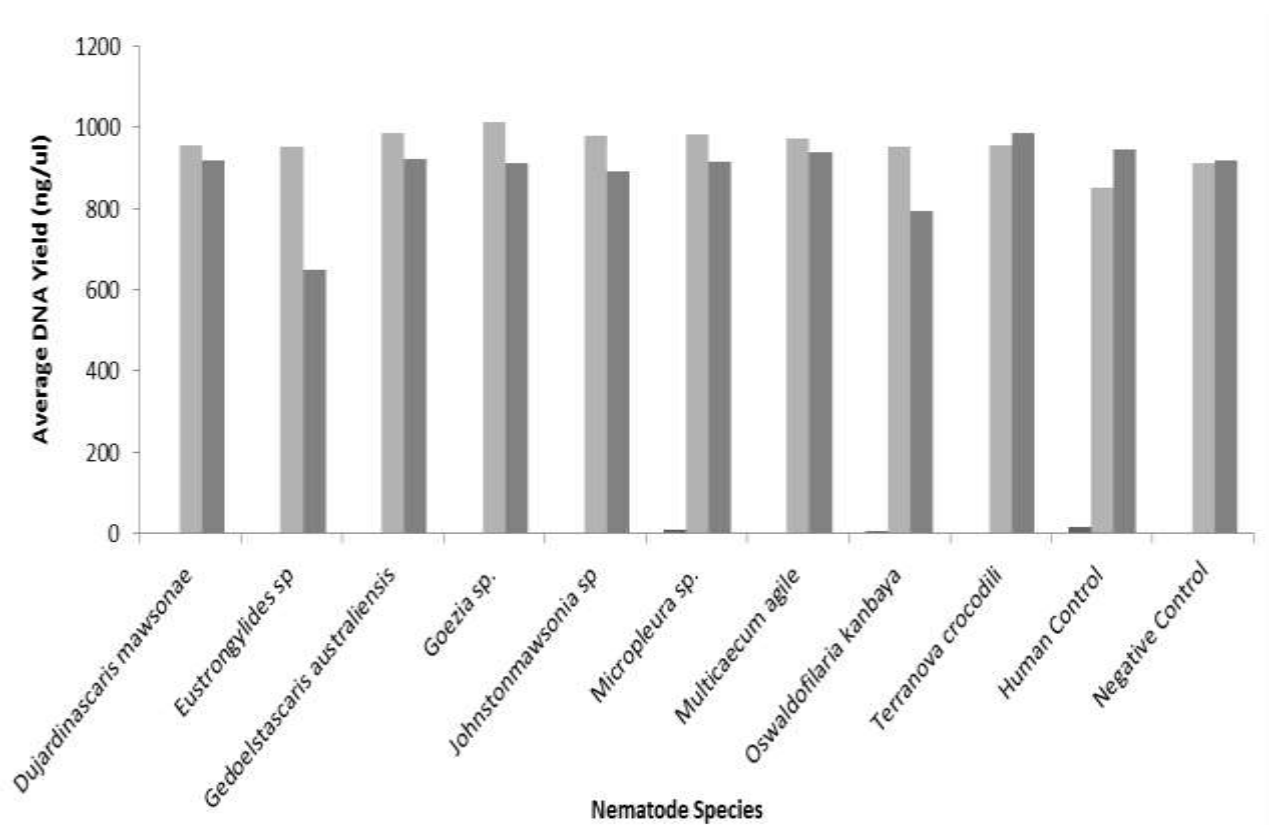
Figure 3.1 Average DNA yield for each nematode species before and after treatment with both of the WGA protocols. DNA yields from GenomePlex^TM amplified samples are shown in pale grey on the left while GenomiPhi^TM treated samples are shown in dark grey on the right.

3.5 Discussion

Genotyping of helminth specimens from old or degraded template DNA is frequently unsuccessful (McManus et al 1985, Dawkins and Spencer 1989, Seesao et al 2014). This study investigated the potential of WGA to improve the quantity and quality of genomic DNA extracted from various species of parasitic nematodes, thereby increasing the success rate of downstream molecular applications. A significant increase in total DNA yield was detected in samples treated with the GenomePlex[TM] and GenomiPhi[TM] WGA kits. However, the success rate of gene specific PCR was not improved. This is consistent with the findings of several previous studies in which reduced genotyping success, false allele generation and allelic dropout were consistently associated with low quality DNA samples (Lee et al 2006, Gunn et al 2007). As both WGA protocols preferentially amplify unsheared DNA, it is highly probable that the long term storage of the study specimens in an ethanol solution has irreparably damaged their genomic DNA. The high DNA yields observed in WGA treated samples in this study are most likely the result of the GenomePlex[TM] adapter sequences and the random GenomiPhi[TM] hexamers being amplified instead of the degraded nematode sequences (Holbrook et al 2005). This would also explain the high concentration of DNA detected in the H_2O controls, despite the lack of template. Taken collectively, these data indicate that the efficacy of WGA cannot be interpreted from spectrophotometry-based methods alone but require gene-specific PCR screening to determine if downstream applications, such as genotyping are viable.

The average final DNA yield in four species of nematode was significantly higher in the GenomePlex[TM] treated samples than in the GenomiPhi[TM] treated samples. Why this would be the case in only this subset of specimens is unclear, but could be due to the presence of inhibitors which differentially affect the PCR-based and MDA-based amplification protocols (Huggett et al 2008). The phenomenon may also be partly statistical

in nature. The unavoidably small sample size employed in this study could have led to a type-II error, as there may have been insufficient power to detect significant variation between the median of the paired differences and zero (Belknap et al 1996). Thus the disparity in final DNA yield between GenomePlex[TM] and GenomiPhi[TM] treated samples may have existed in a greater number of specimens but was only detected in species for which a larger number of individuals could be analysed.

This study has demonstrated that the commercially available WGA kits, GenomiPhi[TM] and GenomePlex[TM], are unlikely to be suitable for the amplification of genomic DNA from old and/or degraded invertebrate specimens. In situations where the nature of the research precludes the use of freshly obtained samples, it is imperative that specimens be stored under conditions which will maintain the viability of DNA for downstream molecular applications, such as $-80^{o}C$ refrigeration, where possible.

3.6 Acknowledgements

3.7 References

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4 Genetics and infection dynamics of *Paratrichosoma* sp. in farmed saltwater crocodiles (*Crocodylus porosus*)

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4.1 Abstract

Paratrichosoma-associated helminthosis has been identified in saltwater crocodiles under intensive farming conditions. The development of sustainable integrated management practices is dependent on a detailed understanding of *Paratrichosoma* spp. population genetics and infection dynamics. This study investigated the genetic relationships of *Paratrichosoma* sp. in a population of commercially farmed saltwater crocodiles, *Crocodylus porosus*, in northern Australia. 18S ribosomal RNA gene sequence data were obtained from *Paratrichosoma* sp. eggs present in the epidermis of infected animals. A high level of genetic diversity was distributed within the *Paratrichosoma* sp. population (241 variable positions in the 1094 bp alignment), indicating an accelerated rate of nucleotide base pair substitutions in this genus of nematodes. Several possible environmental correlates of the incidence and intensity of helminthosis, including season, rainfall and mean monthly temperature, were investigated by visual inspection of crocodile skins. Stepwise logistic regression revealed a significant negative linear relationship (P=0.011, R-sq=32.69%) between mean monthly rainfall and the incidence of monthly *Paratrichosoma*-associated helminthosis. Variation in the severity of *Paratrichosoma*-associated helminthosis could not be explained by any of the independent environmental variables included within an ordinal regression analysis. The large genetic diversity in these nematodes indicates a high probability of anthelmintic resistant alleles occurring in the population. We discuss how the spread of these alleles may be mitigated by adopting targeted treatment protocols.

4.2 Introduction

The intensive husbandry of crocodilians has been instrumental in reducing pressure placed on wild populations by unregulated hunting and has created strong commercial incentives to develop sustainable practices for the production of crocodilian-derived wildlife products (Blake and Loveridge 1975; Bolton and Laufa 1982; Revol 1995). Unlike most industrial livestock operations, the majority of the total product value in crocodilian farming is derived from the sale of export-quality skins, used in the manufacture of luxury fashion goods (Buenviaje et al. 1998). As even minor blemishes can drastically reduce the value of a hide, the management of pathogenic agents which target the crocodilian dermis and epidermis is critical to the long term viability of commercial ranching and farming operations around the world (Thorbjarnarson 1999; Manolis and Webb 2011).

Crocodile farmers in Australia report a skin defect characterised by a series of serpentine scars on the ventral scales of the lower jaw, chest and abdomen. These distinctive lesions are consistent with those caused by *Paratrichosoma*, a cosmopolitan genus of nematodes believed to parasitise at least eight species of crocodilians across Africa, the Americas, Southern Asia and Australasia (Moravec and Vargas-Vazquez 1998; Tellez and Paquet-Durand 2011). However, beyond observation of the associated helminthosis, there are currently no studies which have confirmed the presence of *Paratrichosoma* spp. in Australian crocodiles (Buenviaje et al 1998). The genus *Paratrichosoma* is represented by two described species; *Paratrichosoma crocodylus* and *Paratrichosoma recurvum*. These species can be distinguished from other capillarid nematodes by a thick body cuticle, a thick cuticular lining around the cloaca, the absence of a distinct caudal lobe or papilla in males, absent or poorly developed mesenchynal cells at the esophagointestinal junction and a stichosome that does not reach the intestine (Ashford and Muller 1978; Moravec and Vargas-Vazquez 1998, Spratt 1985). Though morphologically similar, *P. crocodylus* can be differentiated from *P.*

recurvum by the complete absence of mesenchynal cells at the esophagointestinal junction, rough transverse superficial grooves on the spicule and an ovary that exceeds the anterior end of the rectum in females (Ashford and Muller 1978, Spratt 1985). Conversely, in *P. recurvum*, reduced mesenchynal cells are present at the esophagointestinal junction, there are no rough transverse grooves on the spicule and the ovary does not exceed the anterior end of the rectum. The eggs of *P. recurvum* also display protruding polar plugs absent in the eggs of *P. crocodylus* (Moravec and Vargas-Vazquez 1998). Both *Paratrichosoma* species are comparatively large for capillarid nematodes, with the males being on average one third to one half as long as females (Ashford and Muller 1978, Moravec and Vargas-Vazquez 1998). Information regarding the life-cycle, transmission dynamics and infection rates of *Paratrichosoma* is extremely limited at present, though it has been established that gravid females deposit their eggs in the serpentine burrows they create in the host's epidermis, after which they are deposited into the environment via the normal shedding of the host's epithelial cells (Moravec and Vargas-Vazquez 1998; Moravec 2001). Whether *Paratrichosoma* has a direct or indirect lifecycle and the mode by which it colonises the definitive crocodilian host is likewise unknown, although oral ingestion has been suggested as the most plausible route (Manolis and Webb 2011; Tellez and Paquet-Durand 2011).

Any attempt to develop sustainable, integrated approaches to the control of *Paratrichosoma*-associated helminthosis in intensively farmed crocodilians is problematic, due to the currently limited information available on host-parasite interactions in these species. An understanding of both genetic structuring within *Paratrichosoma* sp. assemblages and the role that extrinsic environmental factors play in influencing transmission and infection intensity within host populations is essential if effective management practices are to be implemented. To that end, this study investigated the genetic relationships of parasitic nematode species, with a particular emphasis on *Paratrichosoma* sp., present in a population

of saltwater crocodiles (*Crocodylus porosus*) under intensive farming conditions in a tropical region of northern Australia. The incidence and intensity of *Paratrichosoma*-associated helminthosis among the farmed crocodiles were also examined in an effort to identify environmental correlates of infection, such as temperature and rainfall.

4.3 Materials and Methods

4.3.1 Ethics Statement

This study was approved by the Macquarie University Animal Ethics Committee (permit number: 2012/038) and was performed with permission from the Northern Territory Government (scientific licence number: SL101075). All animal husbandry was in strict accordance with federally-approved guidelines defined by the "Code of Practice on the humane treatment of wild and farmed Australian crocodiles".

4.3.2 Parasite monitoring and tissue collection

Between February 2013 and July 2014, a total of 8,250 saltwater crocodiles held in individual pens at Darwin Crocodile Farm (Northern Territory, Australia) were examined at six week intervals by farm personnel for signs of *Paratrichosoma* sp. infection. Individual pens were arranged in blocks of 50, with each block sharing a continuous water supply (Isberg and Shilton 2013). All crocodiles included in the study had been held in individual pens for a minimum of six months prior to their initial examination and were approximately three years of age, in accordance with the normal operating practices of the farm. A categorical scale was devised to estimate infection intensity with values 1-4 as follows; (1) Low: <10 scales with nematode tracks, scars are faint/largely healed over, (2) Moderate: ~10-20 scales with nematode tracks, scars may be faint or clearly visible, (3) High: ~20-30 scales with nematode tracks, scars are pronounced and clearly visible, (4) Very high: >30 scales with nematodes

tacks, scars may be fresh and/or clearly visible. Four crocodiles identified as having high parasite burdens were euthanised to recover adult nematode specimens and crocodilian epidermal tissue containing *Paratrichosoma* sp. eggs. At necropsy, pieces of crocodile abdominal skin displaying the characteristic scarring associated with *Paratrichosoma* activity were removed from the hide and shredded under a dissecting microscope. The stomach and intestines were opened and any nematodes removed and counted. The liver, heart, lungs and kidneys were also examined for parasitic helminths. Faecal samples and gut secretions were screened for nematode eggs. All adult nematodes and tissue samples were washed in physiological saline and stored in 70% ethanol prior to DNA extraction.

4.3.3 DNA extraction

The extraction of genomic DNA from adult nematode specimens was accomplished by placing the mid-body region in a 1.2 mL Eppendorf tube containing 20 μL PCR buffer. Samples were ground up using a plastic pestle and incubated at 70°C for 10 min. Two microlitres of 10 mg/mL Proteinase K was then added to the solution which was incubated at 56°C for 3.5 h. Twenty microlitres of GeneReleaser (BioVentures, Durham, USA) overlaid with 2 drops of sterile paraffin oil, was added to the digested product which was then heated on the high setting of a 750 Watt microwave oven for 6 min. Samples were centrifuged at 13,000 rpm for 2 min and the supernatant transferred to clean 0.5 ml Eppendorf tubes (Wimmer et al. 2004; Lott et al. 2014). Mixed-community DNA, including that of both the parasitic nematode *Paratrichosoma* sp. and the crocodilian host, was extracted using the DNeasy Blood & Tissue Kit according to the manufacturer's instructions (Qiagen, Hilden, Germany).

4.3.4 PCR amplification

Amplification of a ~1 kb region on the nematode 18S ribosomal RNA gene was accomplished using the primer set Nem_18S_F (5'-CGCGAATRGCTCATTACAACAGG-3') and Nem_18S_R (5'-GGGCGGTATCTGATCGCC-3') (Floyd et al. 2005). PCRs were performed in volumes of 25 μL which consisted of 12.5 μL Gotaq Green Mastermix, 0.2 μM of each primer, 9.5 μL PCR H2O and 2 μL of template DNA. An initial denaturation step of 94°C for 5 min preceded 35 cycles of 94°C for 30 s, 54°C for 30 s and 72°C for 1 min. This was followed by a final extension at 72°C for 10 min. PCR products were resolved by electrophoresis on 2% agarose gels and purified using the QIAquick PCR Purification Kit according to the manufacturer's Spin Protocol (Qiagen, Hilden, Germany).

4.3.5 *Paratrichosoma* 18S clone library

Cloning was performed to obtain individual Paratrichosoma 18S sequences from mixed genome PCR product. Clone sequences were sourced from *Paratrichosoma* sp. eggs in a single, continuous cutaneous burrow from each crocodile specimen. Amplicons were ligated into pGEM T-Easy plasmid vectors (Promega, Madison, USA), transformed into competent One Shot TOP10 *Escherichia coli* cells (Invitrogen, Carlsbad, California) and cultured on LB agar plates (Oxoid, Adelaide, Australia) containing 40 mg X-gal/mL (Bioline, London, UK), 100 mg ampicillin/mL (Sigma–Aldrich, St Louis, USA) and 1 mL IPTG (Bioline, London, UK). Recombinant plasmids were identified by PCR based screening and purified using the QIAprep Miniprep Kit according to the manufacturer's instructions for the Spin Protocol (Qiagen, Hilden, Germany). Sequencing was performed on a 37303130 × 1 DNA analyser at the Macrogen sequencing facility (Macrogen Inc., Seoul, South Korea). All 18S sequences generated in the course of this study have been deposited in GenBank (accession numbers: KP115942-KP116016).

4.3.6 Phylogenetic analysis of nematode 18S sequences

Nematode 18S sequences were examined for ambiguities and assembled into contigs using the program GENEIOUS Pro version 7.1.5 (Biomatters Ltd., Auckland, New Zealand). Sequence data from 18 orders and 32 species of nematode were obtained from GenBank and used as a reference by which the phylogenetic relationships of our study species could be inferred. Alignment was by ClustalW employing the default parameters. The evolutionary model which best fit the unpartitioned 18S sequence data was evaluated in jModelTest 2.1.4 using the Bayesian Information Criterion (BIC) (Darriba et al. 2012). Phylogenies were inferred by Bayesian analysis in MrBayes version 3.2.2 (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003). The Hasegawa-Kishino-Yano (HKY) model of nucleotide substitution was employed with all sites being drawn from a Gamma distribution (Hasegawa et al. 1985). Four Markov chains were run for two million generations with sampling every hundred generations. A 50% majority rule consensus tree was constructed from 15000 post burn in trees. The analyses were run twice using different random starting trees in order to determine the congruence of the likelihood values and posterior clade probabilities. Posterior probabilities >0.95 were considered high support. The nematomorph *Gordius aquaticus* was used as an outgroup (accession number: X80223.1).

4.3.7 Statistical analysis of *Paratrichosoma*-associated helminthosis rates

An ordinal logistic regression with backwards stepwise selection was used to evaluate the relationship between extrinsic environmental conditions and the intensity of *Paratrichosoma* associated- helminthosis. All climate data were sourced from the weather station located at Elizabeth Valley, NT (12.64° S, 131.08° E). The model was fitted to the data using the four categories of infection intensity as the dependent variable. The individual pen block in which

helminthosis was detected, the pen from which the infected individual originated, the mean rainfall level for each month, the mean low and high temperatures for each month and the season in which infection was first detected were included in the model as independent variables. Individual pen, pen of origin and season were modelled as categorical variables, while the log transformed rainfall and temperature data were modelled as continuous variables. A stepwise logistic regression analysis was used to identify environmental predictors of incidence, defined here as the proportion of new *Paratrichosoma* infections each month per 8,250 crocodiles. The model included mean monthly rainfall and the mean high and low temperatures for each month as continuous independent variables and season, pen of origin and individual pen as categorical independent variables. For all regression analyses, the association between a dependent and independent variable was accepted as significant when the P-value was less than 0.05. All statistical analyses were performed using the MINITAB 17 statistical software package (State College, Pennsylvania, USA).

4.4 Results

4.4.1 18S sequence analysis

A total of 55 clone sequences, 4 adult nematode specimens and 16 L4 stage larvae were recovered from the crocodiles during the course of this study. The adult and larval specimens occurred exclusively in the peritoneal cavity and were all identified as belonging to the species *Micropleura australiensis* using the BLAST search algorithm. *Paratrichosoma* sp. eggs recovered from epidermal burrows were bioperculate and thickshelled, averaging 0.078 mm in length and 0.027 mm in width. Eggs displayed protruding polar plugs characteristic of *P. recurvum* (Figure 4.1A-C). No nematode eggs or larvae were identified in gut mucosal or faecal samples. The length of the amplified 18S RNA gene fragment was 1,083-1,094 bp for the clone sequences and 890 bp for all representatives of *M. australiensis*. No nucleotide

variation was detected among the *M. australiensis* specimens. A total of 241 (22%) variable

positions were identified in the clone alignment. These consisted of 97 purine transitions, 55

pyrimidine transitions, 70 transversions, 9 multiple mutational changes and 14

insertion/deletion events. The clone sequences formed a monophyletic clade with the other

representatives of the order Trichocephalida with a high level of support (0.99) (Figure 4.2).

This topology is consistent with the clones having originated from the genus *Paratrichosoma*.

The *Paratrichosoma* sp. clone sequences appear to form two major clades, with Clade 1

being the more basal of the two. However, it is not possible to draw a strong conclusion on

the basis of the currently available 18S rDNA data as the node joining Clades 1 and 2 is

supported by a relatively low posterior probability value (0.79). Clade 2 was composed of

numerous sub-clades with levels of support which ranged from very high (1.00) to low

(0.57). The relative positions of *Paratrichosoma* clone sequences within the phylogeny did

not appear to be related to the identity of the crocodile specimen from which the eggs and

mixed genome DNA was sourced.
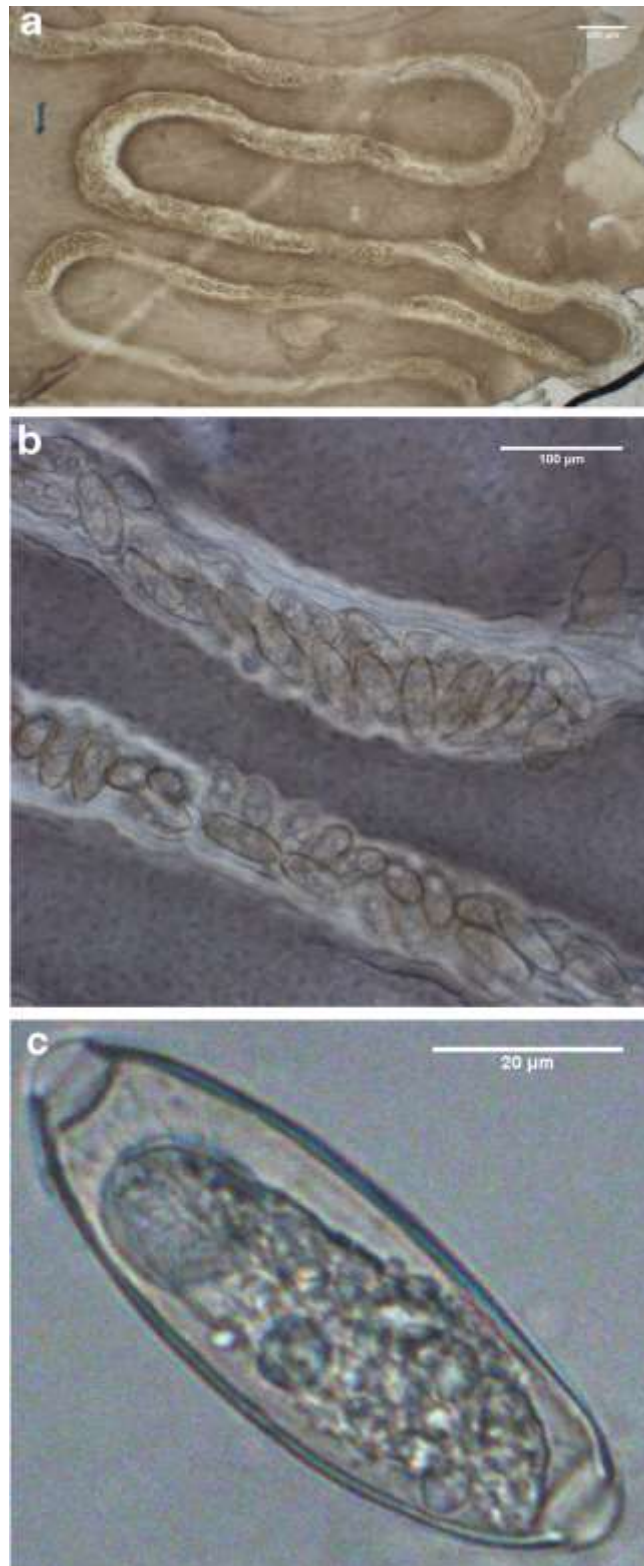
Figure 4.1A-C Eggs of *Paratrichosoma* sp. recovered from a crocodile's abdominal scales.

A Serpentine tunnels in the epidermis, characteristic of *Paratrichosoma* activity. **B**

Unembryonated *Paratrichosoma* sp. eggs present in an epidermal tunnel. **C** Unembryonated

Paratrichosoma sp. egg showing protruding polar plugs characteristic of *P. recurvum*.
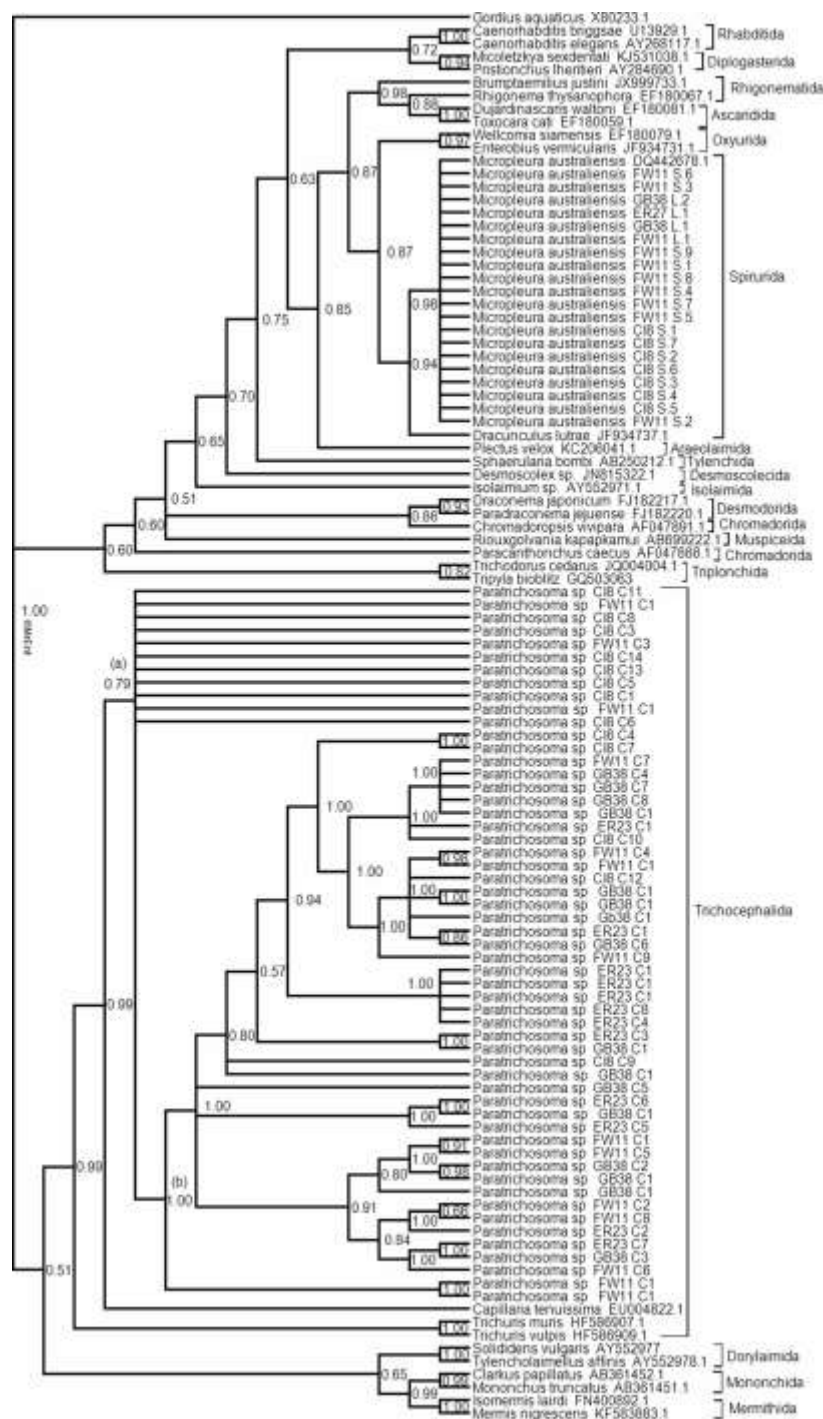
Figure 4.2 Majority rule consensus phylogenetic tree constructed from 15000 post burn-in

trees under Bayesian inference for nematode 18S sequences. Posterior probability values

greater than 50% are shown for the interior branches. Vertical bars on the right depict the

order to which each species belongs. The two major clades of *Paratrichosoma* sp. clones are

denoted by the letters (a) and (b). Accession numbers for reference sequences are shown in

brackets. *Gordius acquaitucs* was used as an outgroup.

4.4.2 Factors influencing intensity and incidence of *Paratrichosoma*-associated helminthosis

Clinical signs of *Paratrichosoma* sp. infection were identified in 108 crocodiles (1.3%) over the 19 months of data collection. The majority (73.41%) of animals that developed symptoms of *Paratrichosoma* infection did so within the first 267 days of being relocated to individual pens (Figure 4.3). No significant association was found between the intensity of helminthosis and any of the independent environmental variables included in the ordinal regression analysis. The backward stepwise logistic regression model identified a significant negative relationship between mean monthly rainfall and the incidence of *Paratrichosoma*-associated helminthosis (P=0.01 R-sq=37.2%; Figure 4.4). The monthly incidence of *Paratrichosoma*-associated helminthosis relative to rainfall is shown in Figure 4.5.
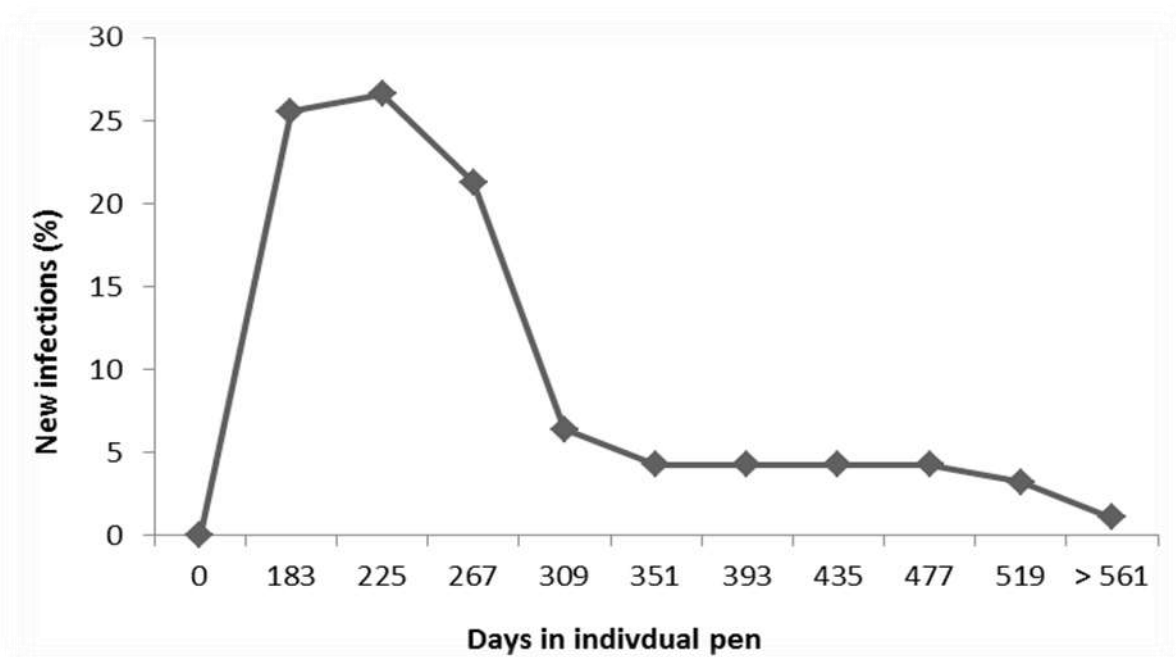
Figure 4.3 The percentage of infected crocodiles which first displayed signs of *Paratrichosoma*-associated helminthosis at each scheduled examination. Examinations were performed every 42 days (six weeks) after the first 183 days (six months). Time point zero represents the day on which crocodiles were relocated to individual pens.

97
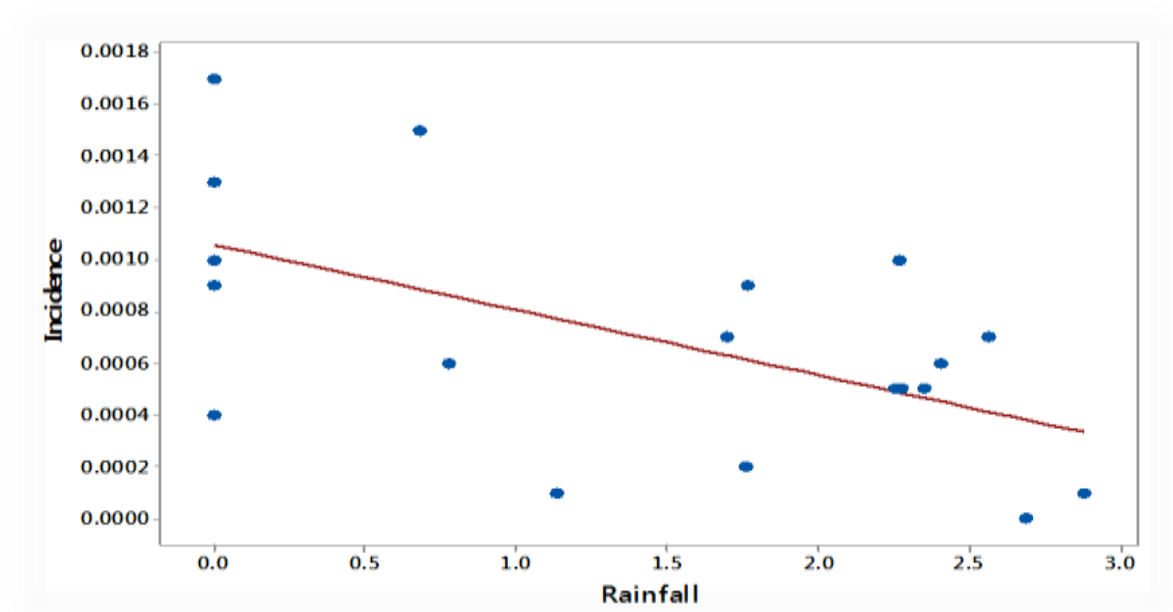
Figure 4.4 Fitted plot line showing the negative linear relationship relationship between monthly incidence of *Paratrichosoma* sp. infection and log transformed mean monthly rainfall data. The regression equation is, Incidence = 0.001058 - 0.000251 Rainfall.
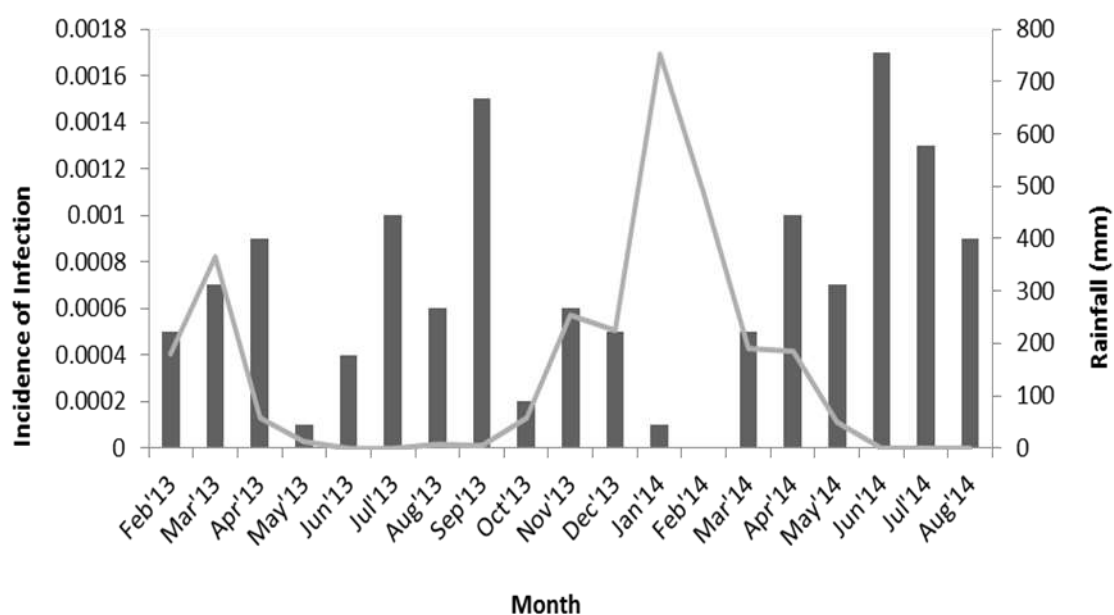
Figure 4.5 Monthly incidence of *Paratrichosoma* sp. infection at Darwin Crocodile Farm, NT, Australia. The bar graph depicts the proportion of infected animals per total population of 8,250 on the primary X-axis, while mean monthly rainfall is depicted as a line graph on the secondary X-axis.

4.5 Discussion

Considerable genetic variation was found within the *Paratrichosoma* sp. populations of commercially farmed saltwater crocodiles. By comparison, the ribosomal rDNA sequences of *M. australiensis*, the only other nematode species recovered during the course of this study, were highly conserved. Low intraspecific variation is the primary reason for the widespread use of the 18S ribosomal RNA gene in nematode taxonomy (Floyd et al 2002; Hasegawa et al. 2009). There are two possible explanations for the unusually high level of sequence diversity observed in *Paratrichosoma* sp. First, the unique biology or ecology of *Paratrichosoma* sp. may have led to an increased rate of nucleotide base pair substitutions or, second, the *Paratrichosoma* community at Darwin Crocodile Farm could be represented by a complex of closely related but genetically distinct species or sub-species.

Exposure to free radicals, such as reactive oxygen species, is known to accelerate nucleotide substitution in metazoans (Holterman et al. 2006). Parasites in particular are frequently exposed to high levels of free radicals, which are released by their host as part of the immune response. Reactive oxygen species are commonly associated with chronic inflammation, where they are involved in the propagation of proinflammatory and growth-stimulatory signals (Hensley et al. 2000; Holterman et al. 2006). It is reasonable to assume that the act of burrowing through the host's epidermis to create the tunnels in which *Paratrichosoma* deposit their eggs, coupled with the long term persistence of adult specimens in the deeper tissues of the skin, would produce a more robust immune response than the comparatively non-destructive endoparasitic nematodes which inhabit the body cavity or gastrointestinal tract. The accelerated rate of nucleotide base-pair substitutions observed in *Paratrichosoma* could therefore be a consequence of prolonged exposure to high levels of reactive oxygen species, arising from chronic inflammation of the crocodilian dermis and epidermis. Rapid accumulation of DNA replication errors has also been documented in nematode species with

shorter generation times (Holterman et al. 2006). However, the current lack of information regarding the lifecycle of *Paratrichosoma* makes it impossible to determine the extent to which generation length may influence genetic diversity at this time.

Alternatively, the high level of *Paratrichosoma* 18S sequence variation observed here may be indicative of a mixed-species infection. The application of molecular techniques to the taxonomy of parasitic nematodes has revealed an abundance of genetically distinct species within morphologically indistinguishable taxa. Furthermore, these cryptic species often occur across relatively limited spatial scales, leading to considerable regional diversity in parasite communities (Chilton et al. 1995; Chilton et al. 2009; Kanzaki et al. 2012). The captive bred and ranched juvenile crocodiles at Darwin Crocodile Farm are occasionally supplemented by wild crocodiles removed from Darwin Harbour and the surrounding area. As crocodiles are capable of dispersing large distances, up to 600 km in some cases, it is not impossible that some of these animals may have originated from isolated areas with genetically distinct *Paratrichosoma* species or subspecies which were subsequently introduced into the farm population (Campbell et al. 2010). This explanation is perhaps less likely, given the relative lack of structuring among the *Paratrichosoma* clones. The phylogeny constructed from 18S sequence data did not contain well supported major clades which could realistically be interpreted as species or regional population divisions. Strong genetic differentiation between parasite populations is also typically a trait of taxa with limited dispersal ability. By comparison, the high level of genetic diversity distributed within the *Paratrichosoma* population is characteristic of parasites with large effective population sizes and hosts which range across much larger geographical regions (Kaplan 2002; McCoy et al. 2003; Louhi et al. 2010).

Mean monthly rainfall was the only significant environmental correlate of infection. The negative linear relationship observed between rainfall and the incidence of

Paratrichosoma-associated helminthosis is unusual among parasitic helminths (Fabiyi 1985; Huffman et al. 1997; Sithithaworn 1997; Stromberg 1997). It is possible that the shedding of eggs in *Paratrichosoma* sp. has evolved to coincide with periods of low rainfall when rivers, ponds and other bodies of water are typically smaller, increasing the population density of the definitive crocodilian host as well as any potential intermediate host organisms such as fish or aquatic arthropods. Excessive rainfall may also remove infective third-stage larvae from paddocks and shallow aquatic environments or flush out the breeding sites of intermediate hosts, leading to reduced rates of infection (Huffman 1997; Stromberg 1997).

All cases of *Paratrichosoma*-associated helminthosis developed after crocodiles had been moved to individual pens. However, it is very likely that the initial contact with *Paratrichosoma* sp. occurred prior to the host's relocation, as prolonged exposure to infected individuals through a continuous water supply did not appear to increase the likelihood of other crocodiles developing symptoms. The delay between initial infection and the onset of helminthosis may have been due to *Paratrichosoma* sp. larvae resuming development after a period of hypobiosis (Gibbs 1986). Alternatively, *Paratrichosoma* sp. larvae may have a limited ability to disperse through aquatic environments or require an intermediate host which was not present in the individual pens to complete their lifecycle.

Taken collectively, these data suggest that the management of *Paratrichosoma* spp. outbreaks through the application of anthelmintic drugs should be approached with extreme caution. The large genetic diversity observed in these parasites, and the high gene flow it implies, indicates a considerable opportunity for the development and spread of anthelmintic resistant alleles (Kaplan 2002; Louhi et al. 2010). Targeted treatments, in which anthelmintics are selectively applied to animals with the highest risk of developing Paratrichosoma-associated helminthosis, could be one way to forestall the spread of anthelmintic resistance (Kenyon et al. 2009). The treatment of crocodiles isolated in

individual pens within the first six months of relocation, particularly when that period coincides with times of expected low rainfall, may be the most sustainable approach. This will minimize damage caused to valuable crocodile hides at a time when helminthosis appears to be most severe while preventing anthelmintic resistant alleles from becoming fixed in the population due to the apparent inability of *Paratrichosoma* sp to propagate under individual pen conditions. *Paratrichosoma*-associated helminthosis is a growing concern, with current data suggesting low prevalence in Northern Australian crocodilian production systems. As such, farm and ranch operators are presented with a unique opportunity to implement ecologically and economically sustainable management practices from the outset. However, there is a clear need for further studies of *Paratrichosoma* population and infection dynamics. The collection of long term data sets across multiple years and geographical regions will further the identification of environmental correlates of helminthosis and assist in the development of sustainable integrated management practices.

4.6 Acknowledgements

4.7 References

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5 Towards the molecular characterisation of parasitic nematode assemblages: an evaluation of terminal-restriction fragment length polymorphism (T-RFLP) analysis

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5.1 Abstract

Identifying factors which regulate temporal and regional structuring within parasite assemblages requires the development of non-invasive techniques which facilitate both the rapid discrimination of individual parasites and the capacity to monitor entire parasite communities across time and space. To this end, we have developed and evaluated a rapid fluorescence-based method, terminal restriction fragment length polymorphism (T-RFLP) analysis, for the characterisation of parasitic nematode assemblages in macropodid marsupials. The accuracy with which T-RFLP was capable of distinguishing between the constituent taxa of a parasite community was assessed by comparing sequence data from two loci (the ITS+ region of nuclear ribosomal DNA and the mitochondrial CO1) across ~20 species of nematodes (suborder Strongylida). Our results demonstrate that with fluorescent labelling of the forward and reverse terminal restriction fragments (T-RFs) of the ITS+ region, the restriction enzyme *Hinf1* was capable of generating species specific T-RFLP profiles. A notable exception was within the genus *Cloacina*, in which closely related species often shared identical T-RFs. This may be a consequence of the group's comparatively recent evolutionary radiation. While the CO1 displayed higher sequence diversity than the ITS+, the subsequent T-RFLP profiles were taxonomically inconsistent and could not be used to further differentiate species within *Cloacina*. Additionally, several of the ITS+ derived T-RFLP profiles exhibited unexpected secondary peaks, possibly as a consequence of the restriction enzymes inability to cleave partially single stranded amplicons. These data suggest that the question of T-RFLPs utility in monitoring parasite communities cannot be addressed without considering the ecology and unique evolutionary history of the constituent taxa.

5.2 Introduction

Parasites exert a profound influence over the population dynamics of their host species. In addition to the risk of disease, potential consequences of infection include the modification of normal host behaviour, lower reproductive outputs, reduced rates of growth and development, the alteration of sex ratios within the infected population and heightened mortality arising from changes to both intraspecific and interspecific competition (Minchella and Scott, 1991; Terry et al., 1998; Hakkarainen et al., 2006; Miura et al., 2006; Lefèvre et al., 2009). By comparison, the factors which regulate temporal and regional structuring within parasite communities remain relatively understudied (Poulin, 2001; Arneberg, 2002; Aussavy et al., 2011).

The examination of parasite community structure has traditionally been dependent on morphology-based approaches, which are often confounded by cryptic species and a declining taxonomic skill-base (Coomans 2002, Hopkins and Frekleton 2002). Molecular tools, such as cloning and Sanger sequencing, have overcome some of these limitations, but suffer from a relatively low throughput which limits their utility in characterising assemblages of parasites which may consist of hundreds or thousands of individuals (Fierer et al, 2007; Warnecke and Hugenholtz, 2007; Nadler and Pérez Ponce de León; 2011).

Advances in technologies applicable to metagenomics have rendered the wholesale comparison of parasite communities possible, both within and between host populations. Terminal restriction fragment length polymorphism (T-RFLP) analysis is a fluorescence-based fingerprinting technique which has been used to investigate the structure, diversity and dynamics of free-living microbial communities as well as assemblages of both protozoan and metazoan parasites (Liu et al., 1997; Dunbar et al., 2000; Wang et al., 2004; Waldron et al., 2009; Lott et al., 2012). Comparably sensitive to 'next-generation' technologies such as 454-pyrotag sequencing, T-RFLP has proven a rapid, repeatable and highly cost effective

approach to community-level analysis (Blackwood et al., 2003; Schütte et al., 2008; Pilloni et al., 2012).

T-RFLP exploits polymorphisms in restriction enzyme recognition sites on PCR amplicons to generate DNA fragments of varying sizes. Sequence variation is visualised in the form of peaks on an electropherogram, with each peak corresponding to a unique terminal restriction fragment (T-RF) attached to a fluorescently labelled primer. Subsequent analyses of community composition have often been founded on the assumption that each peak variant corresponds to a discrete taxonomic unit (species, genus etc.), yet this is not always the case. Richness or diversity estimates derived from T-RFLP may not accurately reflect the true composition of a species assemblage, thereby introducing biases into comparisons of community structure (Dickie and Fitzjohn, 2007; Hartmann and Widmer, 2008). This limitation may be addressed by employing a modified methodology termed 'data-base T-RFLP', in which taxon identity is established by comparing the T-RFLP peak profile of a sample to a database of known T-RF variants. Richness and/or diversity can then be interpreted from the number of identified taxa as opposed to the number of unique peaks (Dickie et al., 2002; Zhou and Hogetsu, 2002).

Clone libraries provide a means to establish the identity of a subset of species within a parasite assemblage of interest (Moeseneder et al., 2001; Wang et al., 2004; Jin et al., 2010; Scheublin et al., 2010).   However, a consequence of target gene variability between different parasite taxa is that the percentage similarities of input sequences will not necessarily be indicative of true taxonomic relationships, only their relative similarity to the nearest database matches (DeSalle et al., 2005; Gemeinholzer et al., 2006; Ferri et al., 2009). Ideally sequence data should be linked to other taxonomically informative factors, such as morphology, for a definitive interpretation of species identity (Moeseneder et al., 2001; DeSalle et al., 2005; Ferri et al., 2009).

In this study, we assessed the suitability of data-base T-RFLP as a methodology for characterising assemblages of bursate nematodes (suborder Strongylida) which had previously been identified by morphological means. The variability of restriction enzyme recognition sites on a region of ribosomal DNA encompassing the internal transcribed spacers (ITS-1 and ITS-2) and the 5.8S rRNA gene was compared to a segment of the mitochondrial cytochrome oxidase subunit 1 (CO1) to determine which was more efficacious for T-RFLP. ITS sequences are more commonly used in nematode taxonomy as these loci display high interspecies diversity but are typically conserved within members of the same species (Powers et al 1997, Gasser and Newton 2000, Blouin 2002). However, it was hypothesised that the CO1 derived T-RF peak profiles might prove more effective for discriminating between closely related nematode taxa due to the comparatively higher rate of base-pair substitutions known to occur in the nematode mitochondrial genome (Denver 2000, Blouin 2002). Our goal was to determine whether, through a combination of gene marker and restriction enzyme choice, T-RFLP was capable of generating taxon specific T-RF peak profiles at either the species or genus level.

5.3 Methods

5.3.1 Sample acquisition and DNA extraction

Representatives of 18-20 parasitic nematode species (suborder Strongylida) were acquired from a collection of frozen nematodes (-80°C) held at the University of Melbourne. These specimens originated from various species of macropodid marsupial obtained between 22 years and less than 12 months previously. Nematodes were identified using the morphological characters of the head and tail, which were removed, cleared in lactophenol, and deposited in the South Australian Museum (SAM), Adelaide for future reference (Table 5.1). Genomic DNA was extracted from the mid-body region of individual nematodes by

placing them in 1.2 mL Eppendorf tubes containing 20 μL PCR buffer and grinding them up with a plastic pestle. This process was followed by an initial incubation step of 70°C for 10 min, after which 2 μL of 10 mg/mL Proteinase K was added to the solution. Samples were then subjected to a second incubation step of 56°C for 3.5 h. The digested product was further treated with 20 μL GeneReleaser (BioVentures, Durham, USA) overlaid with 2 drops of sterile paraffin oil. Samples were heated on the high setting of a 750 Watt microwave oven for 6 min, after which they were centrifuged at 13,000 rpm for 2 min and the supernatant transferred to clean 0.5 ml Eppendorf tubes (Wimmer et al 2004).

Table 5.1 Species of nematode examined, including hosts, sample codes, dates and locations of collection of voucher specimens.

Nematode Species	No.of Speciemens	Host Species	Area of Origin	Collection Date	Sample Codes	SAM Reference No.
Cloacina caenis	3	*Petrogale purpureicollis*	Breakaway Creek, Mt Isa, Qld	Jun, 1997	15H1.5-7	46730
Cloacina ernabella	1	*Petrogale purpureicollis*	Leichhardt River, Mt Isa, Qld	Oct, 1998	15K2.2	46719
Cloacina hydriformis	1	*Macropus rufus*	Mulga Park Station, NT	Jul, 2011	39G1.3	46717
Cloacina liebigi	3	*Macropus rufus*	54-57 km N Bourke, NSW	Jun, 1996	WP13.5,6,8	46713
Cloacina parva	1	*Petrogale purpureicollis*	Breakaway Creek, Mt Isa, Qld	Jun, 1997	15H1.3	46729
Cloacina pearsoni	7	*Petrogale assimilis*	Hervey's Range, Townsville, Qld	–	3G24.4-10	46731
Cloacina petrogale	1	*Petrogale purpureicollis*	Breakaway Creek, Mt Isa, Qld	Jun, 1997	15H1.1	46727
[a] *Cloacina robertsi (a)*	5	*Petrogale assimilis*	Hervey's Range, Townsville, Qld	–	3G22.1,3,4,5,6	46721
"	3	"	Magnetic Island, Qld	Sep, 1989	AQ2.2-4	46724
[a] *Cloacina robertsi (b)*	4	*Petrogale persephone*	Flame-tree Hill, Proserpine, Qld	May, 1991	PC3.1-4	46725
"	3	"	Mt Lucas, Strathdickie, Qld	Jul, 1991	PG4.2,3,5	46720
[a] *Cloacina robertsi (c)*	2	*Petrogale inornata*	Kelsey Creek Rd, Proserpine, Qld	Nov, 1992	3J6.3,5	46723
Cloacina similis	7	*Petrogale herberti*	Mt Sebastopol, Qld	Nov, 2001	22W5.1,3,4,5,7,8,9	46726
Filarinema dissimile	1	*Macropus rufus*	Kalgoorlie, WA	Dec, 2001	23M2.1	46716
Filarinema flagrifer	3	*Macropus rufus*	Calooma Station, Qld	Sep, 1995	9W2.1-3	46715
Labiosimplex longispicularis	1	*Macropus rufus*	Wallerberdina Station, SA	Jun, 1989	AB11.1	46711
"	3	*Macropus rufus*	Kalgoorlie, WA	Dec, 2001	23N3.1,4,6	46712
Papillostrongylus barbatus	4	*Macropus rufus*	64 km S Charleville, Qld	Jul, 1992	WT6.1-4	46706
Pharyngostrongylus lambda	1	*Macropus rufus*	46 km N Cunnamulla, Qld	Jul, 1993	LI18.2	46714
[a]*Rugopharynx zeta (a)*	1	*Petrogale herberti*	Mt Sebastopol, Qld	Nov, 2001	22W4.1	46728
[a] *Rugopharynx zeta (b)*	5	*Petrogale penicillata*	–	Mar, 2012	R03.2,3,4,6,9	N/A
Wallabinema cobbi	1	*Macropus rufus*	Werribee Park Zoo, Vic	May, 1995	8M1.6	46709
Zoniolaimus latebrosus	1	*Macropus rufus*	64 km S Charleville, Qld	Jul, 1992	WT1.2	46707

[a] Denotes specimens that may represent components of a species complex.

5.3.2 PCR amplification and sequencing of the ITS+ region

Amplification of strongylid rDNA was accomplished using a semi-nested PCR protocol. The primary reactions employed the primer set NC16 (forward: 5'-AGTTCAATCGCAATGGCTT-3') & NC2 (reverse: 5'-TTAGTTTCTTTTCCTCCGCT-3') (Gasser et al., 1993, Chilton et al., 2003). All PCRs were performed in volumes of 50 μL consisting of 25 μL Gotaq Green Mastermix (Promega, Madison, USA), 0.4μM of each primer, 21 μL PCR H_2O and 2 μL template DNA. An initial denaturation step of 94°C for 3 min preceded 35 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 1 min, followed by a final extension of 72°C for 5 min. The secondary reactions employed the primers 241 (forward: 5'- AAAGGAATTCAAGTCGTAACAAGGTTTCCGTAGG-3') and NC2 with undiluted amplicons of the primary reactions as template (Zarlenga et al., 1998). All other conditions were identical to those of the primary reactions. PCR products were resolved by electrophoresis on 2% agarose gels and purified using the QIAquick PCR Purification Kit according to the manufacturer's Spin Protocol (QIAGEN, Hilden, Germany). Sequencing was performed with the primers 241 and NC2 and ABI PRISM BigDye™ terminator 3.0 cycle sequencing kit using an ABI Prism 3130 × 1 genetic analyser (Applied Biosystems, Melbourne, Australia).

5.3.3 PCR amplification and sequencing of CO1 subunit

Amplification of a subunit of the Cytochrome oxidase 1 from the Strongylid mitochondrial genome was also accomplished using a semi-nested PCR protocol. The primer set Ss47F (forward: 5'-CCTTTACATTTTGCTGGTTTAC-3') and CO1-P2 (reverse: 5'-ACTACAAAATAAGTATCATG-3') was utilised in the primary reactions (Hu et al., 2003). PCRs were performed in 25 μL volumes consisting of 12.5 μL Gotaq Green Mastermix, 0.2 μM of each primer, 9.5 μL PCR H_2O and 2.5 μL template DNA. An initial denaturation step

of 94°C for 3 min preceded 40 cycles of 94°C for 45 s, 50°C for 45 s and 72°C for 2 min, followed by a final extension of 72°C for 5 min. The secondary reactions employed the primer CO1-P1 (forward: 5'-TGATTTTTTGGTCATCCTGA-3') and CO1-P2 with amplicons of the primary reactions (diluted 1:10 with PCR H_2O) as template (Wang et al 2007). All other conditions were identical to those of the primary reactions. PCR products were resolved, purified and sequenced in the same manner as described in section 2.2.

5.3.4 Phylogenetic analysis of ITS+ and CO1 sequences

The ITS+ and CO1 sequence data were examined for ambiguities and assembled into contigs using GENEIOUS Pro version 5.4.6 (Biomatters Ltd., Auckland, New Zealand). Alignment was by ClustalW employing the default parameters. Partitions were chosen a priori for the ITS+ sequences according to gene identity (ITS1, 5.8S, ITS2) and the best fit substitution model for each region evaluated in jModelTest 2.1.4 using the Bayesian Information Criterion (BIC) (Darriba et al 2012). A single best fit substitution model was also identified for the unpartitioned CO1 sequence data. Phylogenies were inferred by Bayesian analysis in MrBayes version 3.2.2 (Huelsenbeck and Ronquist 2001, Ronquist and Huelsenbeck 2003). The SYM+G, Jukes Cantor (JC) and HKY+G models of nucleotide substitution were applied to the ITS1, 5.8S and ITS2 sequence data respectively. A general time reversible (GTR) model with a proportion of the sites invariable and the rest drawn from a gamma distribution with four rate categories was applied to the CO1 sequence data. For both phylogenies, four Markov chains were run for a million generations with sampling every hundred generations. A 50% majority rule consensus tree was constructed from 7500 post burn in trees. The analyses were run twice using different random starting trees in order to determine the congruence of the likelihood values and posterior clade probabilities. The rhabditid nematode

Heterorhabditis sp. was used as an outgroup for both the ITS+ (accession number: GU130179) and CO1 (accession number: DQ643800.1) derived phylogenies.

5.3.5 T-RFLP

For T-RFLP, PCRs were repeated using fluorescently labelled primers. The ITS+ PCRs employed the primer set 241-FAM and NC2-PET, 5' end labelled with 6-carboxyfluorescein (6-FAM) and PET respectively, while the CO1 PCRs were accomplished using the primers CO1-P1-FAM and CO1-P2-PET. The custom oligonucleotides employed in this study were synthesised by Sigma–Aldrich (St Louis, USA) and Applied Biosystems (Melbourne, Australia). All PCRs were conducted in 50 μL reaction volumes consisting of 39.4 μL PCR water, 5 μL 10X PCR buffer, 200 lM dNTP, 1 μL DMSO, 0.6 μL AccuTaq-la DNA polymerase (Sigma–Aldrich St Louis, USA), 2 μL target DNA and 0.1 μM of each primer. Cycling conditions and the methods by which PCR product was purified were identical to those previously described. Sequence data were used to model the positions of binding sites for 200 commercially available restriction endonucleases. Of these, *Hinf1* and *Bsr1* (New England Biolabs) were selected for application to the ITS+ and CO1 respectively, as they were predicted to provide the highest level resolution of taxonomic groups for those gene regions. Restriction digest reactions were performed according to the manufacturer's instructions. Ten μL of PCR product was incubated for 2 hrs at 37°C with 10 U of restriction endonuclease, 2.5 μL of 10× NEbuffer 4 and PCR water up to a final volume of 25 μL. Fluorescently labelled fragments were detected using an ABI Prism 3130 × 1 genetic analyser (Applied Biosystems, Melbourne, Australia) in genescan mode (8.5 kV; 40-s injection; 60 °C for 100 min) employing a G5 filter. GeneScan software 4.0 (Applied Biosystems) was used to analyse T-RF sizes. Samples for which the observed forward and reverse T-RF sizes did not differ by more than 3 bp were ruled as being indistinguishable by T-RFLP.

5.3.6 Cloning of the ITS+ Region

Cloning was performed in order to assess whether sequence diversity in rDNA gene copies influenced T-RF variation within nematode species. Amplicons were ligated into pGEM T-Easy plasmid vectors (Promega, Madison, USA), transformed into competent One Shot TOP10 E. coli cells (Invitrogen, Carlsbad, California) and cultured on LB agar plates (Oxoid, Adelaide, Australia) containing 40 mg X-gal/mL (Bioline, London, UK), 100 mg ampicillin/mL (Sigma–Aldrich, St Louis, USA) and 1 mL IPTG (Bioline, London, UK). Recombinant plasmids were identified by PCR based screening and purified using the QIAprep Miniprep Kit according to the manufacturer's instructions for the Spin Protocol (QIAGEN, Hilden, Germany).

5.4 Results

5.4.1 Analysis of ITS+ rDNA sequence diversity and derived T-RFLP peak profiles

High quality ITS+ sequence data was successfully recovered from 62 of the strongylid nematode specimens, representing two superfamilies**,** eight genera and 18-20 species. The total length of the amplified region, encompassing the ITS1, 5.8S rRNA gene and the ITS2, ranged from 873-920 bp in length, with a pairwise percent similarity score of 88.9% across all study taxa. The ITS+ derived phylogeny supported established taxonomic relationships between most of our study species. The sole exception was *C. hydriformis*, which was grouped outside the clade containing the rest of the Cloacinid species (Figure 5.1). Using the restriction endonuclease *Hinf1,* 13 distinct T-RF peak profiles were generated (Table 5.2). Of these 12 were species specific, with 5-7 species from the genus *Cloacina* sharing identical forward and reverse T-RF sizes (Table 2). The T-RFLP traces of five nematode species (*C. hydriformis*, *C. similis*, *L. longispicularis, P. barbatus* and *Z. latebrosus*) each displayed an additional major peak that could not be reconciled with the position of enzyme binding sites

within the associated rDNA sequence. These peaks occurred at lengths of 362 bp, 351 bp,

355 bp, 368 bp, and 361 bp respectively.
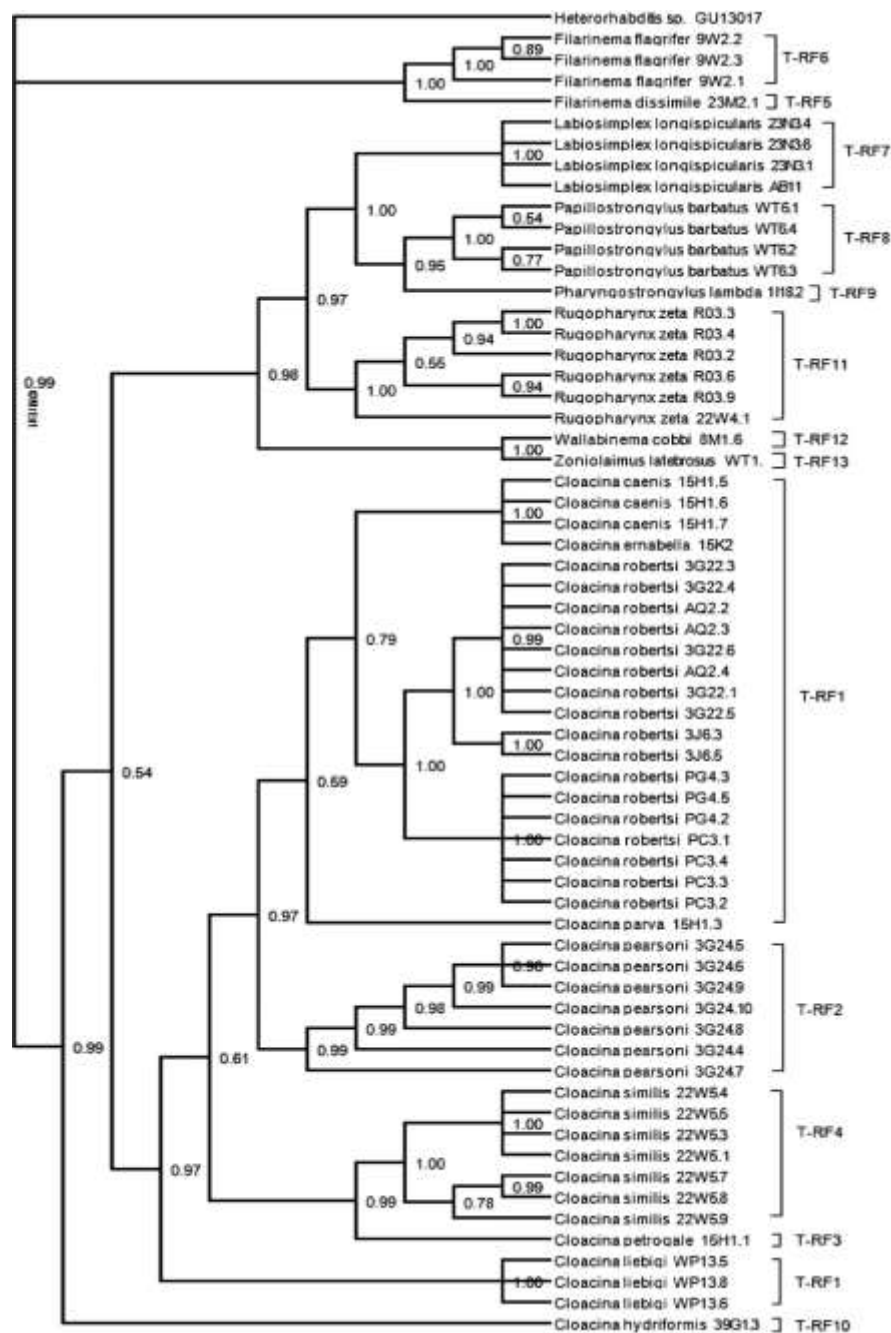
Figure 5.1 Majority rule consensus phylogenetic tree constructed from 7500 post burn-in

trees under Bayesian inference for the ITS+ region. Posterior probability values greater than

50% are shown for the interior branches. Vertical bars on the right depict T-RF defined

operational taxonomic units. Accession numbers for reference strains are shown in brackets.

Heterorhabditis sp. was used as an outgroup.

Table 5.2 Forward and reverse T-RFs generated from the ITS+ and CO1 regions using the restriction enzymes *Hinf1* and *Bsr1*.

Species Name	T-RF Group (ITS+)	Expected T-RF Sizes (*Hinf1*)		Observed T-RF Sizes (*Hinf1*)		T-RF Group (CO1)	Expected T-RF Sizes (*Bsr1*)		Observed T-RF Sizes (*Bsr1*)	
		5'	3'	5'	3'		5'	3'	5'	3'
Cloacina caenis	T-RF 1	354	86	350	83	T-RF 1	85	331	82	332
Cloacina ernabella	T-RF 1	354	86	350	83	T-RF 2	247	169	249	170
Cloacina hydriformis	T-RF 10	156	429	151	423 (362)[a]	N/A[c]	–	–	–	–
Cloacina liebigi	T-RF 1	354	87	350	84	T-RF 3	416	416	418	413
Cloacina parva	T-RF 1	354	86	353	85	T-RF 4	361	55	362	57
Cloacina pearsoni	T-RF 2	153	87	148	83	T-RF 5	85	55	82	57
Cloacina petrogale	T-RF 3	457	419	454	351	T-RF 1	85	331	82	332
Cloacina robertsi (a)	T-RF 1	354	86	349	83	T-RF 2/T-RF 3[b]	247/416	169/416	249/418	170/413
Cloacina robertsi (b)	T-RF 1	354	86	351	83	T-RF 3	416	416	418	413
Cloacina robertsi (c)	T-RF 1	354	85	350	83	T-RF 2	247	169	250	170
Cloacina similis	T-RF 4	422	417	419	410 (349)[a]	T-RF 6/T-RF 7/T-RF 3[b]	111/247/416	305/55/416	109/250/418	306/57/413
Filarinema dissimile	T-RF 5	444	233	442	234	T-RF 6	110	305	109	306
Filarinema flagrifer	T-RF 6	218	215	213	212	T-RF 1	85	331	82	332
Labiosimplex longispicularis	T-RF 7	410	421	410	416 (355)[a]	T-RF 5/T-RF 8[b]	85/247	55/55	82/250	56/56
Papillostrongylus barbatus	T-RF 8	188	435	184	429 (368)[a]	T-RF 3/T-RF 11[b]	416/111	416/305	418/108	413/306
Pharyngostrongylus lambda	T-RF 9	413	218	411	214	T-RF 9	247	169	244	175
Rugopharynx zeta (a)	T-RF 11	358	87	355	84	N/A[c]	–	–	–	–
Rugopharynx zeta (b)	T-RF 11	358	87	355	84	T-RF 2/T-RF 10[b]	247/217	169/169	249/218	171/171
Wallabinema cobbi	T-RF 12	356	420	354	414	T-RF 3	415	415	418	413
Zoniolaimus latebrosus	T-RF 13	458	438	454	422 (361)[a]	T-RF 2	247	169	249	170

[a] Species for which an additional major peak was observed on the T-RFLP electropherogram
[b] Species which exhibited multiple T-RF groups
[c] Species/samples for which the CO1 region failed to amplify

5.4.2 Analysis of CO1 subunit mitochondrial sequence diversity and derived T-RFLP peak profiles

Sequence data for the CO1 was obtained from 54 nematode specimens which could be attributed to two superfamilies, eight genera and 16-18 species. The amplified region encompassed a 415-416 bp long segment of the CO1 with an overall pairwise percent similarity score of 87.9% across all study taxa. The phylogeny constructed from mitochondrial CO1 sequence data did not reflect the taxonomic relationships previously established by morphological and ITS+ sequence data. The restriction endonuclease *Bsr1* generated 11 distinct T-RF peak profiles which were also inconsistent with established taxonomy (Table 2 and Fig 2). Identical peak profiles were observed in several representatives across two different nematode families; the Dromaeostrongylidae and Cloacinidae. Conversely, in some cases multiple peak profiles could be attributed to a single species as in *L. longispicularis, C. similis*, *C. robertsi*, *R. zeta and P. barbatus*.
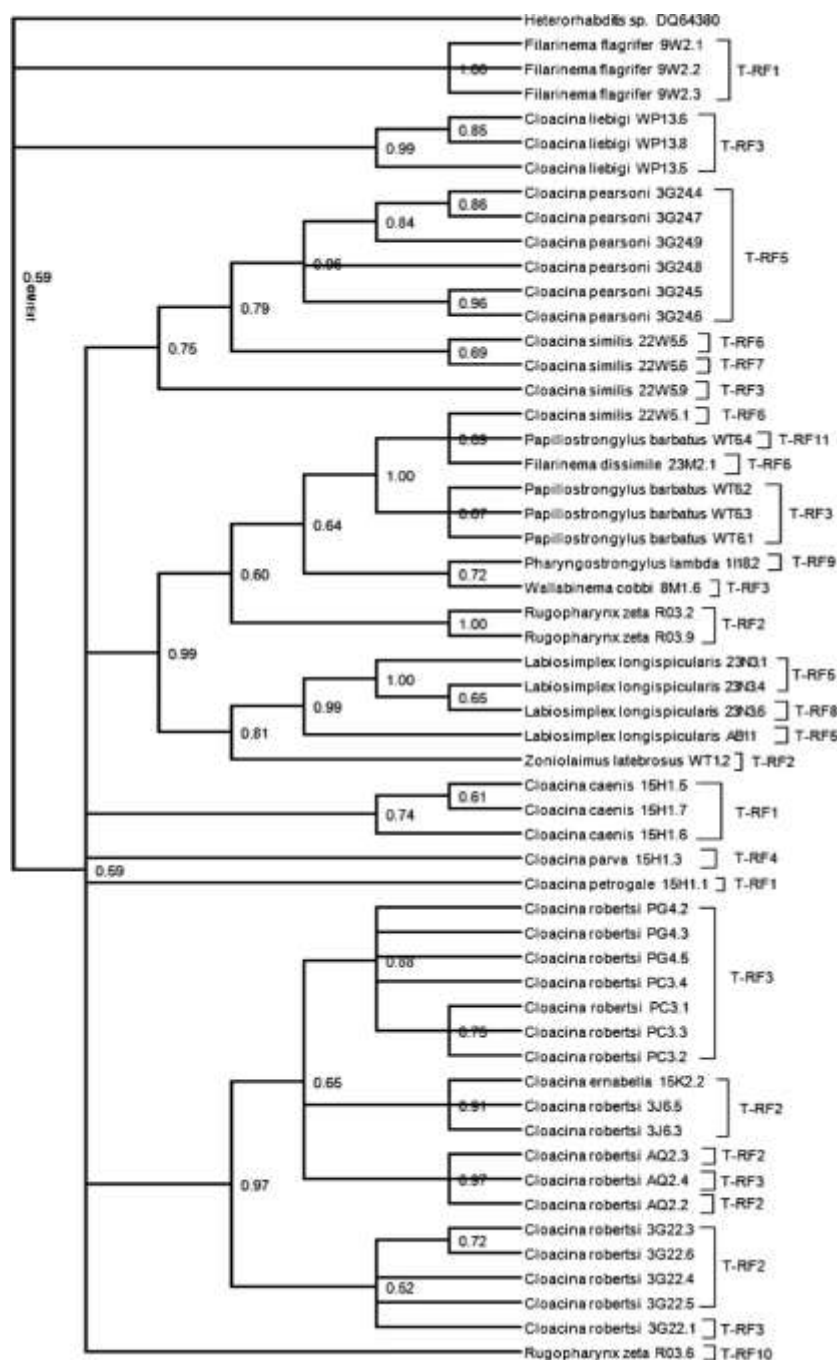
Figure 5.2 Majority rule consensus phylogenetic tree constructed from 7500 post burn-in trees under Bayesian inference for the CO1. Posterior probability values greater than 50% are shown for the interior branches. Vertical bars on the right depict T-RF defined operational taxonomic units. Accession numbers for reference strains are shown in brackets. *Heterorhabditis sp.* was used as an outgroup.

5.4.3 Analysis of Strongylid ITS+ clone isolates

Clone isolates of the ITS+ region were obtained from five nematode species; *C. hydriformis*, *C. similis*, *L. longispicularis, P. barbatus and Z. latebrosus*. T-RFLP traces were generated for 5 clones from each of these representative specimens. Three distinct T-RFs were associated with all clone specimens for *C. hydriformis* (151 bp, 362 bp, 423 bp), *C. similis* (420 bp, 352 bp 412 bp,), *L. longispicularis* (410 bp, 356 bp 417 bp,) and *Z. latebrosus* (454 bp, 361 bp, 422 bp). Three distinct T-RFs (187 bp, 370 bp, 430 bp) were associated with three of the five *P. barbatus* clones, with the remaining two clones displaying only two T-RFs (188 bp and 371 bp). High quality ITS+ sequence data was successfully recovered from 19 of the clone isolates, including representatives from *C. similis*, *L. longispicularis, P. barbatus* and *Z. latebrosus*. In all cases, the positions of the restriction enzyme binding sites within clone sequences were identical to those derived from the original genomic DNA.

5.5 Discussion

By applying the restriction enzyme *Hinf1* to the ITS+ rDNA region, we were able to generate species specific T-RFLP profiles for the majority of study taxa. The only exceptions were within the genus *Cloacina*, in which *C. robertsi*, *C. caenis*, *C. ernabella*, *C. parva* and *C. liebigi* were found to share identical forward and reverse T-RF patterns. The obvious implication is that T-RFLP based analyses are likely to underestimate the richness and/or diversity of communities in which multiple species of *Cloacina* co-occur. However, species-specific T-RF peak profiles were obtained for nematodes belonging to other genera, suggesting that our inability to differentiate species within this genus may reflect an idiosyncrasy in the structure of the *Cloacina* ITS+ region as opposed to a limitation of T-RFLP itself.

The high degree of host specificity observed amongst most *Cloacina* species suggests an extensive co-evolutionary relationship with marsupials of the sub-family Macropodinae (Beveridge and Chilton 2001; Beveridge et al., 2002, Chilton et al., 2011). In particular, the genus *Petrogale,* which constituted one of the major host groups for this study, is believed to have undergone a period of rapid speciation between three and six million years ago (Eldridge and Close 1993; Campeau-Péloquin et al., 2001). It is therefore highly probable that their *Cloacina* parasites underwent a period of simultaneous co-evolution (Beveridge and Chilton 2001; Beveridge et al., 2002). As such, the relative conservation of restriction enzyme binding sites within the ITS+ region in some members of this genus may simply reflect a comparatively recent evolutionary radiation. This interpretation is supported by the topology of the ITS+ derived phylogeny, in which *Cloacina* species sharing identical T-RF peak profiles were, with the exception of *C. liebigi*, observed to form a single clade. The exclusion of *C. liebigi* from this clade, despite the apparently identical distribution of enzyme binding sites within the sequence, may be indicative of variable rates of evolution at different loci within the ITS+ gene region (Yang, 1994; Rzhetsky, 1995). These findings would suggest that T-RFLP cannot be relied upon to differentiate parasites belonging to evolutionarily 'young' lineages.

The percentage of identical pair-wise residuals identified in the CO1 alignment, which included all study species, was slightly lower than the ITS+ region. This provides support for the assumption that nematode mtDNA sequences accumulate base pair substitutions more rapidly than ribosomal DNA (Powers et al., 1997; Blouin, 2002). However, the CO1 derived T-RF peak profiles were often highly conserved, with specimens representing different superfamilies exhibiting identical patterns. Conversely, several distinct peak profiles were produced by a number of specimens belonging to a single species. These data suggest that

CO1 derived peak profiles display very low taxonomic fidelity and that, consequently, the CO1 subunit is unsuitable for application to T-RFLP based analyses.

Prominent secondary peaks, that could not be reconciled with the positions of enzyme binding sites on DNA sequences, were observed in the ITS+ derived T-RFLP profiles of several nematode species. These 'pseudo-peaks' were highly reproducible and could not be accounted for by sequence heterogeneity among rDNA gene copies, as the clone sequences at least should have displayed predictable forward and reverse T-RFs. It is possible that these pseudo-peaks were caused by single stranded amplicons in the same region as the terminal restriction site. Restriction enzymes exploit double-stranded structures of two fold rotational symmetry (canonical structures) to bind and cleave DNA. It has therefore been suggested that some restriction enzymes may be incapable of properly cleaving single stranded DNA due to the instability of the associated canonical structures (Nishigaki et al., 1985). If some amplicons were partially single stranded, then the inability of the restriction enzyme to properly cleave binding sites within the single stranded region may have lead to the detection of an additional binding site downstream of the expected terminal restriction site. This may also explain why only a limited number of species exhibited these 'pseudo-peaks, as secondary restriction sites will only be detected by T-RFLP analysis when the primary binding site is not part of a stable canonical structure (Egert and Friedrich, 2003).
Our results demonstrate that database T-RFLP is likely to be an effective technique for discriminating between closely related species within assemblages of metazoan parasites. It should be noted, however, that this is not necessarily synonymous with generating accurate representations of community diversity. The propensity of T-RFLP to overlook less abundant taxa is well documented and highlights the need for further *in situ* studies employing multiple complementary techniques, such as clone libraries and next-generation

sequencing (NGS) to validate the accuracy of T-RFLP derived datasets (Marsh, 1999; Dickie and Fitzjohn, 2007; Hartmann and Widmer, 2008; Hudson, 2008).

The application of T-RFLP to more sophisticated analyses of community diversity is likely to yield additional technical problems. Previous studies have often interpreted the intensity of fluorescence, or peak height, of a T-RF as an indicator of its relative abundance within the sample (Blackwood et al., 2003; Lueders and Friedrich 2003; Sánchez et al., 2006; Schütte et al., 2008). However, it is important to note that this approach is only capable of providing quantitative data for the gene marker variants themselves. Gene copy-number variation, a structural anomaly whereby the number of copies of particular DNA sequence or sequences within a cell is abnormal for a representative of its taxa, is known to be a major component of genetic diversity in nematodes (Maydan, 2010). As such, variation in fluorescence intensity amongst the various T-RFs may reflect differences in rDNA gene copy numbers between the constituent taxa of a parasite community, as opposed to indicating their true abundance. Attempting to limit analyses to taxa which share similar rDNA repeat numbers may be one way to circumvent this limitation (Medinger, 2010).

The ability to generate semi-quantitative, species specific molecular fingerprints of parasite assemblies, coupled with T-RFLPs tendency to oversimplify the richness of such assemblages, suggests that this technique may be best applied as a rapid and cost effective platform for the screening of exotic taxa within established parasite communities. The chief advantages of this approach lie in the relative simplicity of analyses, which can be performed without the considerable data processing and storage space requirements of most next-generation sequencing technologies, while yielding comparable resolution of community structure (Zhang et al., 2011; Pilloni et al., 2012). Ultimately, it appears that the characterisation of parasite communities by database T-RFLP will only be possible with a

comprehensive understanding of the unique genetic composition and evolutionary history of the constituent taxa.

5.6 Acknowledgements

5.7 References

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6 Parasitic nematode communities of the red kangaroo, *Macropus rufus*: richness and structuring in captive systems

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6.1 Abstract

Captive management practices have the potential to drastically alter pre-existing host-parasite relationships. This can have profound implications for the health and productivity of threatened species in captivity, even in the absence of clinical symptoms of disease. Maximising the success of captive breeding programs requires a detailed knowledge of anthropogenic influences on the structure of parasite assemblages in captive systems. In this study, we employed two high-throughput molecular techniques to characterise the parasitic nematode (suborder Strongylida) communities of the red kangaroo, *Macropus rufus*, across seven captive sites. The first was terminal restriction fragment length polymorphism (T-RFLP) analysis of a region of rDNA encompassing the internal transcribed spacers 1 (ITS1), the 5.8S rRNA gene and the internal transcribed spacer 2 (ITS2). The second was Illumina MiSeq next-generation sequencing of the ITS2 region. The prevalence, intensity of infection, taxonomic composition and comparative structure of strongylid nematode assemblages was assessed at each location. Prevalence (P=<0.001) and mean infection intensity (df=6, F=17.494, P=<0.001) differed significantly between the seven captive sites. Significant levels of parasite community structure were observed (ANOSIM, P = 0.01), with most of the variation being distributed within, rather than between, captive sites. The range of nematode taxa that occured in captive red kangaroos appeared to differ from that of wild conspecifics, with representatives of the genus *Cloacina*, a dominant nematode parasite of the macropodid forestomach, being detected at only two of the seven study sites. This study also provides the first evidence for the presence of the genus *Trichostrongylus* in a macropodid marsupial. Our results demonstrate that contemporary species management practices may exert a profound influence on the structure of parasite communities in captive systems.

Keywords: Next-generation sequencing, T-RFLP, Macropodidae, captive breeding, Strongylid nematodes

6.2 Introduction

Parasite mediated-effects are increasingly recognised as important drivers of ecosystem functioning. It has been demonstrated that parasitic organisms exert a profound influence on the structure of food webs, the flow of energy between trophic levels and the dynamics of both interspecific and intraspecific competition (Huxham et al 1995, Torchin et al 2001, Marcogliese 2002, Thompson et al 2005). The subsequent impacts on host reproduction, development, dispersal and survival make parasitism an important mediator of community structure and a major contributor to the evolutionary processes that underlie biodiversity (Lafferty 2003; Mitchell and Power 2003, Ferrer and Negro 2004, Lively et al 2004, Schwanz 2008). However, despite their ecological significance, the factors that regulate temporal and regional structuring of parasite assemblages remain poorly understood. The extent to which anthropogenic induced modification of ecosystem functioning shapes host-parasite interactions is particularly unclear (Lyles and Dobson 1993, Hudson et al 2006).

Contemporary species management strategies such as captive breeding, reintroduction and translocation have the potential to drastically alter pre-existing host-parasite community dynamics (Woodford and Rossiter 1993, Cunningham 1996, Fèvre et al 2006). Given the profound influence parasites exert on host ecology, this may have significant implications for the health and productivity of captive or translocated populations, even in the absence of clinical symptoms of disease (Gómez and Nichols 2013). Quantifying anthropogenic effects on parasite population and community structure is therefore imperative for maintaining viable host populations in captivity and maximising the success of threatened species breeding programs (Woodford and Rossiter 1993, Cunningham 1996, Fèvre et al 2006).

High-throughput, non-invasive molecular techniques, such as the fluorescence-based terminal restriction fragment length polymorphism (T-RFLP) analysis or massively parallel sequencing platforms, such as 454 pyrosequencing and Illumina, have the potential to model

large scale parasite community dynamics across both time and space (Liu et al 1999, Metzker 2010). Studies which have employed T-RFLP or next-generation sequencing to analyse structure, diversity and function in free-living or symbiotic bacteria, protozoa and fungal communities are increasingly common in the literature (Dickie et al 2002, Wang et al 2004, Lazarevic et al 2009, Tymensen et al 2012, Schmidt et al 2013). However, thus far, relatively few investigators have applied these technologies to the evaluation of metazoan endoparasite assemblages (Lott et al 2012, Lott et al 2014).

In this study, we used T-RFLP and Illumina MiSeq next-generation sequencing of the internal transcribed spacer (ITS2) region to characterise the structure of parasitic nematode communities (suborder Strongylida) inhabiting the gastrointestinal tract of the red kangaroo (*Macropus rufus*), a large macropodid marsupial ubiquitous across much of mainland Australia. The family Macropodidae encompasses 50 extant Australian species, seven of which are currently listed as endangered or critically endangered by the IUCN. A further 13 species are listed as vulnerable or near threatened. Consequently, numerous captive breeding programs have been implemented around the country (Eldridge 2010). While the red kangaroo is considered to be of least concern, its well documented and relatively simple strongylid nematode communities make it an excellent model organism for investigating the effects of captive environments on the structure of parasite assemblages in a host taxon of conservation significance (Mykytowycz 1964, Arundel et al 1979). Previous studies across multiple regions have identified the genera *Cloacina*, *Rugopharynx* and *Zoniolaimus* as being among the dominant nematode (suborder Strongylida) parasites of the red kangaroo stomach. The genera *Labiosimplex*, *Filarinema*, *Papillostrongylus*, *Hypodontus*, *Macropostrongyloides* have also been documented in geographically isolated populations, suggesting that they are a common component of the red kangaroo's gastrointestinal fauna (Mykytowycz 1964, Arundel et al 1979).

The goal of this study was three-fold; to determine the prevalence and intensity of strongylid nematode infections in captive red kangaroos, to evaluate the structure of parasitic nematode communities among proximate and geographically isolated macropodid hosts, and to assess whether the taxonomic composition of strongylid nematode communities in captive red kangaroos was consistent with that of wild conspecifics. The ITS2 was selected as a taxonomic marker due to the high interspecific sequence variation, and comparatively low intraspecies sequence variation, associated with this region of rDNA in bursate nematodes (Hoste et al 1993, Chilton 2004). Additionally, there is no evidence of sex-specific or lifecycle stage-specific variability in the ITS2 sequences of strongylids, making this region useful for metagenomic comparisons of species richness (Campbell et al 1995, Stevenson et al 1995, Chilton 2004). Finally, the relative abundances of ITS2-derived operational taxonomic units (OTUs) as recovered by T-RFLP and Illumina MiSeq were compared to determine the potential of each methodology for characterising complex metazoan parasite assemblages.

6.3 Methods

6.3.1 Sampling locations

Between February and March 2014, fresh faecal samples were obtained from seven sites around Australia at which red kangaroos were held in captivity (Table 1). To avoid cross-contamination, an effort was made to collect faeces from individual animals shortly after defecation. Where this was not practical, discrete piles of faecal pellets were targeted to ensure that each sample originated from a single red kangaroo specimen. Faeces were stored at 4 ^{o}C within 8-12 hours of collection to retard the development of strongylid nematode eggs. A total of 60 faecal samples were analysed in the course of this study.

Table 6.1 Locations from which red kangaroo faecal samples were collected, including state, longitude, latitude and host population size and the no. of faecal samples collected at each location.

Site	State	Longitude	Latitude	Population Size	Faecal Samples
Adelaide Zoo	South Australia	34.9142° S	138.6058° E	2	2
Cairns Tropical Zoo	Queensland	16.7581° S	145.6627° E	1	5
Cleland Wildlife Park	South Australia	34.9500° S	138.6958° E	28	22
Healesville Sanctuary	Victoria	37.6822° S	145.5316° E	9	9
Lone Pine Koala Sanctuary	Queensland	27.5329° S	152.9693° E	13	13
Perth Zoo	Western Australia	31.9759° S	115.8530° E	6	5
Taronga Zoo	New South Wales	33.8433° S	151.2411° E	4	4

6.3.2 Parasite screening and DNA extraction

A modified version of the McMaster egg counting technique was used to estimate the number of strongylid eggs per gram (EPG) of faecal material (Henriksen and Aggaard, 1976; Lott et al 2012). Samples were examined with a compound microscope at 100× magnification. The EPG for each sample was calculated using an average of two counts. Faecal egg counts were not taken from samples in which large numbers of larvae were observed to have hatched. Genomic DNA was extracted from strongylid-positive faecal samples using the ISOLATE Faecal DNA Kit as described in the manufacturer's protocols (Bioline, London, UK).

6.3.3 T-RFLP

A semi-nested PCR protocol was used to amplify a region of strongylid rDNA encompassing the internal transcribed spacer 1, the 5.8S rRNA gene and the internal transcribed spacer 2. Primary reactions employed the primer set NC16 (forward: 5'-

AGTTCAATCGCAATGGCTT-3') and NC2 (reverse: 5'-TTAGTTTCTTTTCCTCCGCT-3') (Gasser et al 1993; Chilton et al 2003). Secondary reactions were accomplished using the primer set 241-FAM (forward: 5'-AAAGGAATTCAAGTCGTAACAAGGTTTCCGTAGG-3') and NC2-Pet, 5' end labelled with 6-carboxyfluorescein (6-FAM) and PET respectively (Zarlenga et al 1998). Custom oligonucleotides were synthesised by Sigma–Aldrich (St Louis, USA) and Applied Biosystems (Melbourne, Australia). All PCRs were performed in 50 µL reaction volumes consisting of 5 µL 10 × PCR buffer, 200 µM dNTP, 1 µL DMSO, 0.6 µL AccuTaq-la DNA polymerase (Sigma–Aldrich, St Louis, USA), 0.1 µM of each primer, 39.4 µL PCR water and 2 µL target DNA. An initial denaturation step of 94 $^{\circ}$C for 3 min preceded 35 cycles of 94 $^{\circ}$C for 30s, 55 $^{\circ}$C for 30s and 72 $^{\circ}$C for 1 min, followed by a final extension of 72 $^{\circ}$C for 5 min. Electrophoresis on 2% agrose gels was used to resolve PCR products, which were then purified using the QIAquick PCR purification kit according to the manufacturer's spin protocol (QIAGEN, Hilden, Germany).

Fluorescently labelled PCR product was digested using the restriction endonuclease *HinfI* according to the manufactures instructions (New England Biolabs). Restriction digest reactions consisted of 10 µL PCR product, 10 U restriction endonuclease, 2.5 µL of 10 × NEbuffer 4 and PCR water up to a final volume of 25 µL. Incubations were performed for 2 h at 37 $^{\circ}$C. Fluorescently labelled fragments were detected using an ABI3730×l capillary sequencer in genescan mode at the Macrogen sequencing facility (Macrogen Inc., Seoul, South Korea). T-RFLP data were formatted for downstream analysis using the T-RFLP EXpedited (T-REX) online tool (Culman et al 2009). True peaks were defined as those with an area greater than three standard deviations from the mean, calculated by dividing the area of each peak by the total area of all peaks within a sample and assuming a mean of zero. T-RFs were aligned using a clustering threshold of 2 bp. An attempt to establish taxonomic

identity of T-RFs was made by comparing the variants observed in this study to a database of known macropodid strongylid T-RFs (Lott et al 2014).

6.3.4 Illumina MiSeq

The primers NC13 (forward: 5'- ATCGATGAAGAACGAGC – 3') and NC2 were redesigned to include overhang adapter sequences compatible with Illumina index and sequencing adapters (Newton et al 1998). Amplification of a ~400 bp region of strongylid rDNA, encompassing the ITS2 and flanking sequences, was accomplished using 12.5 µL of KAPA HotStart ReadyMix (KAPABiosystems, Wilmington, USA) 0.2 µM of each primer and 2.5 µL of template DNA in a final reaction volume of 25 µL. An initial denaturation step of $95^{o}C$ for 3 mins preceded 25 cycles of $95^{o}C$ for 30s, $55^{o}C$ for 30s and $72^{o}C$, followed by a final extension of $72^{o}C$ for 5 mins. Amplicons were visualized on 2% agrose gels and purified using the QIAquick PCR Purification Kit according to the manufacturer's Spin Protocol (QIAGEN, Hilden, Germany). The concentration of DNA in each sample was standardised to 10 ng/µL by diluting purified amplicons with PCR H_20. Genomic libraries were prepared using the Nextera XT index kit (Illumina, San Diego, USA). Sequencing was performed in 300 bp paired-end reads on an Illumina MiSeq sequencing platform at the Ramaciotti Centre for Gene Function Analysis, Sydney, Australia.

Reads were trimmed to remove 5' and 3' end adapter sequences, terminal nucleotides (33 nucleotides from the 5' end and 34 from the 3' end) and low quality bases (minimum quality score of 30) using SeqMan NGen (DNASTAR, Madison, USA). Contigs were created by de novo assembly of paired-end reads using a minimum match percentage of 99%. Low frequency contigs (< less than 100 sequences) and all reads of less than 100 nucleotides were removed from the final analysis. Sequence contigs were imported into MOTHUR for assignment into OTUs (Schloss et al 2009). Unique sequences were aligned to a reference

alignment and a preclustering algorithm was used to denoise the data. Sequences were chimera checked with UCHIME (Edgar et al 2011). Pairwise sequence distances were calculated with no penalisation for end gaps and OTUs were clustered using the furthest neighbour algorithm with a minimum identity threshold of 97%. A representative sequence from each OTU was identified and homology queries were submitted to the NCBI database using the BLAST search algorithm. The nucleotide sequence dataset generated in the course of the study was deposited in the NCBI sequence read archive (SRA) under the project accession number PRJNA274870.

6.3.5 Statistical analysis

A Fisher's exact test was performed to compare the prevalence of strongylid nematodes across the seven study sites. Differences in mean infection intensity (as assessed from faecal egg burdens) were analysed using log transformed EPG counts in a one-way ANOVA with post-hoc tukey tests. The total number of discrete T-RFs generated by T-RFLP and the number of OTUs generated by Illumina MiSeq for each sample at each captive site were compared using unpaired Student's t-tests. Groupings within nematode community structure across the various study sites were analysed using hierarchical clustering. Similarity profile (SIMPROF) permutation analysis was used to test the significance of the clusters and the relative similarity of parasite assemblages between the study sites was determined using an analysis of similarity (ANOSIM). For clustering and ANOSIM, both T-RFLP and MiSeq data were converted to binary form (presence-absence) and analysed using the Jaccard similarity coefficient. All multivariate analyses were performed using PRIMER Version 6.1.11 (Plymouth Marine Laboratories). A significance level (α) of 0.05 was used for all statistical tests.

6.4 Results

6.4.1 Abundance of strongylid nematodes

The prevalence of strongylid nematodes differed between the seven sites at which red kangaroo faecal samples were collected (P=<0.001) (Fig 1a). There was also a significant difference in mean infection intensity between the study sites (df=6, F=17.494, P=<0.001). Adelaide Zoo, Healesville Sanctuary and Lone Pine Koala Sanctuary all had significantly higher mean EPG counts than Cairns Tropical Zoo, Cleland Wildlife Park and Taronga Zoo (P <0.05; Tukey's Test). Perth Zoo had a significantly lower mean EPG count than Lone Pine Koala Sanctuary but was not significantly different from the other five sites (P <0.05; Tukey's Test) (Fig 1b).

a)

b)
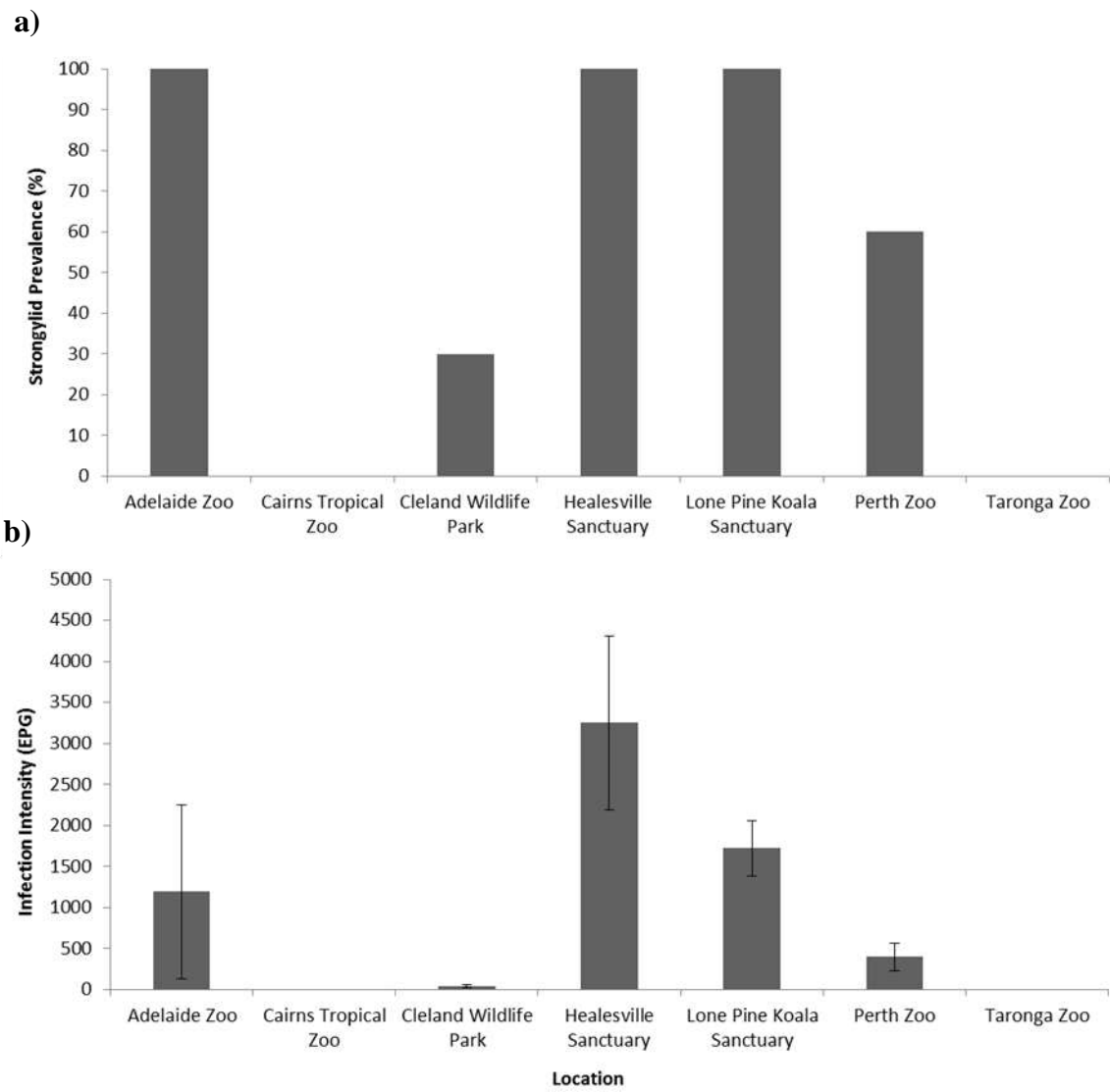
Figure 6.1 Parasite abundance in seven captive red kangaroo populations; (a) The prevalence

of macropodid stronglyids and (b) mean EPG for each site. Bars show standard error.

6.4.2 Characterisation of principal OTUs

6.4.2.1 T-RFLP

The restriction endonuclease HinfI produced 26 distinct 5' end T-RFs that ranged from 101 bp to 492 bp in length. Fourteen 3' end T-RFs were identified with a length of 86-415 bp. As none of the T-RFs generated in this study could be reconciled with the peak sizes of previously defined OTUs, T-RFLP-based comparisons of strongylid community structure were limited to analysis of the more varied forward restriction fragment (Lott et al 2014).

6.4.2.2 Illumina MiSeq

A total of 1,444,613 strongylid ITS2 sequence reads were obtained after quality control. Raw reads were assembled into 679 contigs, with an average length of 246 bp. The mean number of reads per sample was 51,593 with a range of 41,771-74,535. A minimum identity threshold of 93% identified 51 OTUs. The majority of OTUs identified in this study belonged to the nematode family Cloacinidae (35 or 68.63%), with the remainder being placed in the families Chabertiidae (13 or 25.49%), Trichostrongylidae (1 or 1.96%) and Dromaeostrongylidae (1 or 1.96%). A single OTU could not be assigned to a taxonomic rank lower than suborder. Specific genera that could be identified included *Cloacina, Labiosimplex, Papillostrongylus, Rugopharynx, Wallabinema* and *Zoniolaimus*.

6.4.2.3 OTU richness; T-RFLP vs Illumina MiSeq

The number of distinct OTUs identified by Illumina Miseq per sample was significantly lower than the number of discrete T-RFs identified by T-RFLP at two sites; Lone Pine Koala Sanctuary (t=3.52, df=24, P=<0.001) and Perth Zoo (t=3.54, df=4, P=0.02). No significant difference was detected between the mean number of OTUs and T-RFs per sample at

Adelaide Zoo (t=0, df=1, P=1) Cleland Wildlife Park (t=3.78, df=2, P=0.06) or Healesville Sanctuary (t=0, df=12, P=1).

6.4.3 Analysis of strongylid nematode community structure

6.4.3.1 T-RFLP

Hierarchical cluster analysis indicated two major groups of samples diverging at a similarity level of 30.8% (Fig 2a). Cluster 1 was limited to representatives from Lone Pine Koala Sanctuary. Cluster 2 could be further subdivided into three main subgroups which diverged at a similarity level of 38.5% and 50%. The group designated 2a contained a single sample which originated from Lone Pine Koala Sanctuary. Group 2b contained all the nematode positive samples from Cleland Wildlife Park, while group 2c was composed of all the nematode positive samples from Healesville Sanctuary and Perth Zoo, both of the samples from Adelaide Zoo and a single sample from Lone Pine Koala Sanctuary. There was a significant difference in the composition of the strongylid nematode communities across the study sites (ANOSIM, P = 0.01). Nevertheless, while there was some evidence of site fidelity among samples, the dendrogram suggested considerable overlap in the structure of nematode communities between some of the red kangaroo host groups.

6.4.3.2 Illumina MiSeq

Hierarchical cluster analysis revealed three major groups of samples (Fig 2b). Cluster 1 was composed of five samples from Lone Pine Koala Sanctuary. Cluster 2 encompassed all samples from Adelaide Zoo, Perth Zoo and Cleland Wildlife Park, in addition to four samples from Healesville Sanctuary. Cluster 3 constituted the remainder of the samples from both Healesville Sanctuary and from Lone Pine Koala Sanctuary. Cluster 1 could be further subdivided into two main subgroupings that diverged at 8%. Cluster 2 was also divided into

two major subgroupings, diverging at a similarity level of 10%. Group 2a was composed of six samples (two from Adelaide Zoo, one from Perth Zoo and three from Cleland Wildlife Park), while Group 2b consisted of the remaining two nematode positive samples from Perth Zoo and four samples from Healesville Sanctuary. Cluster 3 diverged into two major subgroups at a similarity level of 8%. Group 3a contained only two samples, both from Healesville Sanctuary. Group 3b was composed of eight samples from Lone Pine Koala Sanctuary and a single sample from Healesville Sanctuary. There was a significant difference in the composition of the strongylid nematode communities across the study sites (ANOSIM, P=0.01). However, while there was some evidence of site fidelity in the dendrogram, considerable overlap was also observed in the community structure of samples between some locations.

a.

b.
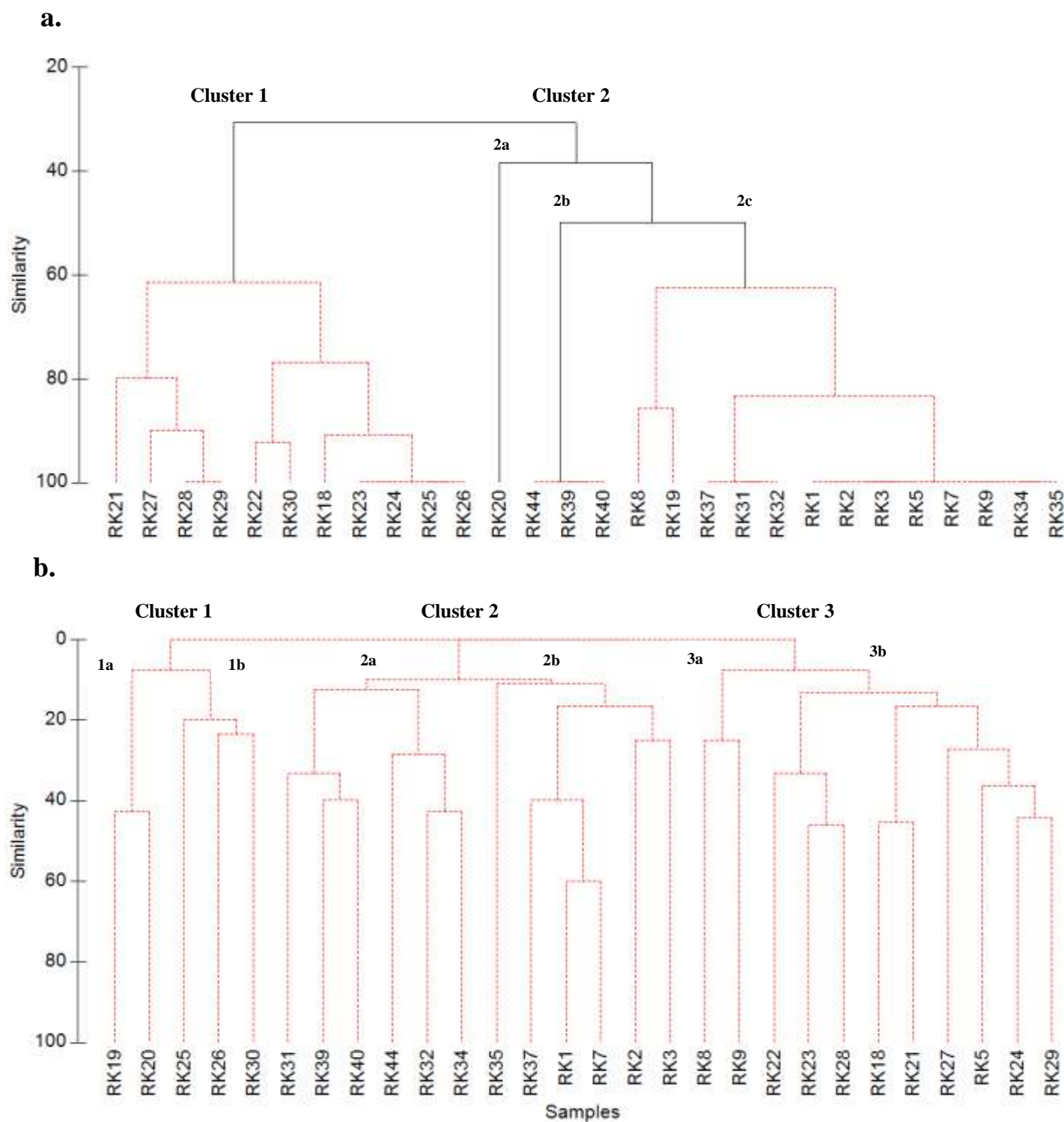
Figure 6.2 Hierarchical dendrogram depicting group average clustering of Jaccard indices of similarity based on non-transformed OTU prevalence data at five of the seven study locations. Clusters of samples connected by dotted lines are significantly different from other clusters (SIMPROF test, $p < 0.05$). (a) T-RFLP data (b) Illumina MiSeq data.

6.5 Discussion

The prevalence and intensity of strongylid nematode infection varied significantly across the seven study sites. Furthermore, strongylid eggs were entirely absent from the faeces of red kangaroos at both Cairns Tropical Zoo and Taronga Zoo. The differing abundances of parasitic nematodes at these seven locations can be partially explained by the lack of a standardised anthelmintic regime for macropodids within captive systems. Animals which are treated more intensively can naturally be expected to exhibit lower faecal egg counts than untreated conspecifics. However, biotic factors, such as high host density, which are known to enhance transmission rates in wild populations, may also influence the prevalence and intensity of nematode infection in captive environments (Leathwick et al 2008). The speed at which helminth parasites are able to recolonise treated hosts is largely dependent on the density of infective larvae in an enclosure. Consequently, a larger number of hosts, shedding a comparatively greater total number of eggs, can be expected to increase the in-refugia population of free-living nematodes, leading to a higher probability of re-infection. Thus the ability of nematode assemblages to resist perturbations, and their subsequent abundance in captive environments, may also be strongly correlated with the number of hosts at a given site (Brunsdon 1980, Leathwick et al 2008). However, the analysis of density dependent effects on parasites in captive systems is frequently confounded by the lack of sufficiently large macropodid populations from which statistically meaningful comparisons can be drawn.

The distribution of Illumina MiSeq derived OTUs among the faecal samples indicated that the structure of strongylid nematode assemblages in captive red kangaroos was typically not site specific. However, evidence for broader geographical patterns in the composition of parasite communities was uncovered. Samples which originated from eastern Australia were largely distinct from those that were obtained from locations in Western and South Australia. A notable exception was Healesville Sanctuary (VIC), where an overlap was observed in

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nematode community structure with Perth Zoo (WA), Adelaide Zoo (SA) and Cleland Wildlife Park (SA). This was most likely a consequence of host translocation events homogenising parasite assemblages at these sites. The most recent of these translocations involved the individual designated B30393 (sample no. RK3), which was relocated to Healesville Sanctuary (VIC) from Cleland Wildlife Park (SA) approximately 15 months prior to sample collection. At the time of this study, the translocated animal retained a parasite community similar in composition to conspecifics from Western and South Australia. These data suggest that, while host-translocation events may exert an influence on the broader structure of parasite assemblages within macropodid populations, the effects on the gastrointestinal helminth fauna of individual hosts may be more limited, particularly over shorter time periods. Parasitic nematode communities were also characterised by a high degree of variation between the individual hosts at each site. The ability of certain strongylid taxa to establish themselves in particular red kangaroos may have been enhanced or confounded by factors such as sex, age, body size, previous exposure, variable host immunity and patterns of intraspecific interaction within host groups, even on the comparatively limited scale afforded by captivity (Arneberg et al 1998, Raffel et al 2011, Nunn et al 2003, Turner and Getz 2010).

Analysis of ITS2 derived OTUs suggested that the range of nematode taxa that occur in captive red kangaroo populations may differ from those of wild conspecifics. Sequences corresponding to representatives of the genus *Cloacina* were detected in eight individuals from Lone Pine Koala Park and a single individual from Adelaide Zoo. This is unexpected as *Cloacina* constitute one of the dominant nematode parasites of the macropodid forestomach and have been recovered in high numbers from free-ranging red kangaroo specimens across multiple regions (Mykytowycz 1964, Arundel et al 1979). It is unclear why this genus of nematodes is less prevalent in captive systems, but it may be due to the altered dynamics of

parasite transmission, establishment or intracommunity competition in these environments. Alternatively, given the strong effect that season and age-related host immune responses can exert on the shedding of eggs, it is possible that *Cloacina* was simply not present in faeces at the time when samples were being collected (Turner and Getz 2010). Ribosomal DNA sequences corresponding to the Trichostrongylid nematode *Trichostrongylus retortaeformis* were also detected in a single individual from Healesville Sanctuary. *Trichostrongylus* has been reported in trichosurid possums in Australia and New Zealand but has never before been documented in macropodid marsupials (Stankiewicz et al 1996a, Stankiewicz et al 1996b). It is possible that the kangaroo in question was infected by contact with livestock or rabbits, the natural hosts of *T. retortaeformis* (Audebert et al 2002). The lack of infection among other animals in the Healesville population suggests that the transmission rate of this nematode to red kangaroos is not high.

The level of population structure revealed by T-RFLP analysis was substantially lower than that implied by Illumina MiSeq, with the former failing to resolve many of the fine scale relationships between samples that were observed using data generated by the next-generation sequencing platform. Additionally, it was not possible to match T-RFs recovered from red kangaroo faecal samples in this study to previously described T-RF variants recovered from macropodid strongylids. As T-RF size cannot be used to reconstruct phylogenetic relationships, T-RFLP clearly requires a much more extensive database than next-generation sequencing platforms to be useful for the assignment of OTUs to specific taxonomic grades (Lott et al 2014). Taken together, these results indicate that, while T-RFLP may be suitable for monitoring general trends in parasite assemblages of known taxonomic composition, high-throughput sequencing platforms such as Illumina MiSeq are a superior alternative for modelling complex dynamics of multi-parasite community structure across time and space.

This study identified a strong anthropogenic effect on the structure of parasitic nematode assemblages in captive red kangaroo populations. While there is currently no evidence that strongylid nematodes induce pathogenic effects in macropodids, the cost of high worm burdens may manifest as trade-offs in other significant aspects of life-history, particularly when immunologically naïve hosts are exposed to novel parasites through translocation events (Woodford and Rossiter 1993, Cunningham 1996, Fèvre et al 2006) The highly interconnected nature of host-parasite ecology indicates that parasite communities must be managed as diligently as any other aspect of macropodid biology if threatened species recovery programs are to be successful.

6.6 Acknowledgements and Compliance Statement

Formal ethics approval was not required, as there was no contact between any of the authors and the red kangaroos from which faecal samples were sourced. However, the study reported herein was performed in full compliance with current local laws and regulations pertaining to the ethical treatment of animals.

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7 Conclusions

The degree to which contemporary captive management practices affect the structure and diversity of parasitic helminth assemblages in native Australian vertebrates is poorly understood. The development of sustainable, integrated techniques for parasite control requires a mechanistic understanding of the factors which influence the transmission, establishment and pathogenicity of helminths in host-species of commercial and conservation significance. This knowledge will be founded on the ability to characterise the influence of environmental factors on host-parasite community interactions within captive systems, and necessitates the development of cost-effective, high-throughput molecular techniques for monitoring spatiotemporal structuring among parasitic helminth assemblages.

In this thesis, I have investigated the taxonomic composition, epidemiology and spatial structure of parasitic nematode communities in both intensive production systems and non-commercial captive breeding programs. My primary research objectives (section 1.5) were 1. to characterise the nematode taxon responsible for skin disease in intensively farmed saltwater crocodiles, 2. to identify environmental correlates of crocodilian helminthosis in captive systems, 3. to evaluate the efficacy of two high-throughput molecular techniques (T-RFLP and Illumina MiSeq) for monitoring spatial structuring of parasitic nematode communities in macropodid marsupials, and 4. to employ these molecular techniques to investigate the composition and spatio-distribution of nematode assemblages among captive red kangaroo populations. These objectives were achieved.

My primary research outcomes were the characterisation of high levels of genetic diversity in *Paratrichosoma* sp., the primary nematode taxon responsible for skin disease in commercially farmed saltwater crocodiles (objective 1, chpt 4), the identification of precipitation as a significant correlate of *Paratrichosoma*-associated helminthosis in intensive

prodcution systems (objective 2, chpt 4), and the provision of a comprehensive analysis of nematode community structure between and within captive red kangaroo populations using T-RFLP and the Illumina MiSeq sequencing platform (objectives 3 and 4, chpt 5 and 6).

The study presented in this thesis was the first to apply high-throughput next-generation sequencing technology to the analysis of complex metazoan parasite assemblages in a marsupial host. This approach has not only revealed patterns of fine scale community structuring across multiple geographical regions, but indicated that the majority of variation in the parasitic helminth assemblages of macropodid marsupials was distributed within, rather than between captive sites. It was further demonstrated that the species composition of nematode communities in captive red kangaroos, differed considerably from those of wild conspecifics.

These findings indicate that contemporary management practices have the potential to exert a significant impact on the structure of parasite assemblages in captive systems, with profound implications for both the health and productivity of hosts, and the biodiversity of symbiotic helminth species.

7.1 Mechanisms of helminth community interaction: findings and directions for future research

The unique, community-level interactions of sympatric helminth taxa can dramatically affect the response of parasite assemblages to management strategies that are imposed on host populations (Pedersen and Fenton 2006). Therefore, a mechanistic understanding of the biotic and abiotic factors which shape spatial and temporal structuring among helminth communities is essential if we are to accurately predict either the behaviour of parasite assemblages within captive systems, or their response to treatment regimes (Thieltges et al 2008, Johnson and Thieltges 2010). We have identified a significant, negative association

between rainfall and the incidence of helminthosis in crocodilians, while analysis of Illumina MiSeq derived data suggests that host-translocation events may serve to homogenise the parasite communities of macropodid marsupials across broader geographical areas. Despite this, it was shown that variation in helminth community structure in captive red kangaroos was typically not site specific. Collectively, these patterns may indicate that biotic factors operating at the level of individual hosts within captive environments (such as sex, age and patterns of intrapopulation interaction) may exert a greater influence on the composition of macropodid helminth assemblages than macroecological factors (Arneberg et al 1998, Arneberg 2002).

The taxonomic composition of macropodid strongylid communities in captive red kangaroo populations differed considerably from those reported in wild conspecifics (Mykytowycz 1964, Arundel et al 1979). These findings suggest that captive management practices may drastically alter the gastrointestinal helminth fauna of marsupials by changing aspects of host ecology which influence the transmission and establishment of symbiotic helminth taxa (Esch et al 1990, Nansen and Roepstorff 1999). However, the factors influencing the species composition of macropodid helminth communities in captive environments remain unclear. This thesis used population-level data from host organisms to model dynamics of parasite community interaction. Consequently, while we have successfully identified variables which are likely to influence patterns in helminth community dynamics at broader, macroecological scales, I am currently unable to determine the mechanisms which shape helminth community structure at the level of single hosts (e.g. variable immunity, associations between host animals, inter-parasite competition) (Lindenfors et al 2007, Turner and Getz 2010). The ability to accurately predict the effects of captive systems on the spatiotemporal distribution of parasite communities, and the subsequent implications for the fitness of hosts, will require a more nuanced understanding of

the fine scale processes which govern the interaction of component species within individual hosts. Large-scale field experiments which test the response of helminth assemblages to perturbations common to captive environments, such as the introduction of novel parasites into established communities by host translocation events or the removal of certain taxa through intensive treatment regimes, are a logical avenue for future research efforts (Lyles and Dobson 1993, Cunningham 1996). While studies of this nature will necessitate an extraordinary level of cooperation between researchers and captive breeding facilities, they will ultimately be far more successful than simple observational studies at providing insight into intra-community competition between co-infecting helminth species in captive systems.

7.2 High throughput sequencing: a novel tool for characterising parasitic helminth assemblages

Research programmes which seek to monitor the response of complex parasitic helminth assemblages to host management strategies employed in captive systems will depend on the development of cost-effective, high-throughput molecular techniques. We have demonstrated that the Illumina MiSeq next-generation sequencing platform is a highly effective tool for characterising the strongylid nematode communities of macropodid marsupials across spatial scales, providing significantly greater taxonomic depth than other high-throughput molecular methods, such as T-RFLP. A next-generation sequencing approach to helminth metagenomics is likely to be equally effective at measuring temporal changes in community dynamics. However, in order to maximise the success of future metagenomic studies, great care must be taken to ensure that the analysis pipeline is optimised for the composition and biology of the target community (Bragg and Tyson 2014).

Applying next-generation sequencing platforms to the analysis of metazoan parasite assemblages introduces a number of addtional complications that will not occur in studies of

bacteria or other single celled organisms. Gene copy-number variation, a structural anomaly in which a metazoan organism has an atypical number of copies of a gene for a member of its species, may lead to some taxa being overrepresented in community-level analyses, confounding diversity estimate (Medinger et al 2010). As copy number variation is known to be a significant contributor to genetic diversity in many helminths, we limited our analysis to an examination of the comparative taxonomic richness of parasitic nematode assemblages (Maydan et al 2010). However, even this approach is not without its potential pitfalls.

The ITS2 region of the nuclear ribosomal DNA cistron is one of the most widely utilised phylogenetic markers (Song et al 2012). These sequences have been repeatedly demonstrated to show species-level discrimination, and can also be used to resolve higher taxonomic ranks due to the presence of conserved secondary structures employed in rRNA processing (Nei and Rooney 2005). However, in the typical Eukaryotic genome, hundreds or thousands of copies of the ITS2 can exist in tandem. Sequence variation between these copies may lead to metagenomic surveys overestimating the richness of parasite assemblages if intragenomic variants are misidentified as discrete taxonomic groups (Hoy and Rodriguez 2013, Arif et al 2014). The use of operational taxonomic units (OTUs) as the basic unit of comparison may circumvent this issue. By clustering sequences together using a predetermined similarity threshold (in this case, 0.03), ITS2 variants can be distributed into phylogenetically informative OTUs, facilitating comparisons of helminth community structure across captive host populations (Bragg and Tyson 2014).

An additional complication of applying the Illumina MiSeq platform to the analysis of parasite communities in macropodids is that the comparatively short read lengths can be a barrier to sequence assembly. This may have limited our ability to resolve the taxonomic identities of the constituent helminth fauna of an assemblage (Ghyselinck et al 2013, Bragg and Tyson 2014). These issues are likely to be resolved as next-generation sequencing

technologies continue to be refined, with longer read lengths becoming possible and more efficient techniques for analysing the resultant data being developed (Dohm et al 2007, Zerbino and Birney 2008, Schloss et al 2009). The ability to accurately characterise the constituent taxa of a helminth community will be essential for quantifying the effects of contemporary captive management practices on the structure and diversity of parasite assemblages. Studies which characterise intragenomic ITS2 diversity in strongylid nematodes will contribute to improved metagenomic surveys by clarifying the level of similarity which is required for differentiating between discrete ecological entities and noise arising from intragenomic diversity.

Next-generation sequencing technologies are also likely to play an important role in the identification and conservation of functionally important genetic information in parasitic helminth populations and communities (Ekblom and Galindo 2011). Prior to the development of high-throughput sequencing techniques, conservation genetics was typically limited to studies of small numbers of neutral molecular markers to determine variation or heterozygosity (Ouborg et al 2010). However, next-generation sequencing platforms, such as Illumina MiSeq, may facilitate the simultaneous analysis of hundreds of genes. This will enable identification of genes associated with important phenotypic characteristics within parasite populations, allowing researchers and policy makers to determine taxonomic units of conservation interest based on ecologically relevant loci (Hedrick 2004, Ekblom et al 2010). The identification of a wider range of biomarker genes will also be an important resource for researchers investigating the genetic basis for the physiological responses of parasitic helminths to captive management systems.

7.3 Implications for parasite management and conservation

Parasitism constitutes the single most common trophic strategy on the planet. The helminth parasites of vertebrates alone are believed to be approximately twice as speciose as their hosts (Poulin and Morand 2000, de Meeûs and Renaud 2002, Dobson et al 2008). Therefore, to overlook the conservation of parasitic helminths is to neglect a fundamental component of global biodiversity, one that exerts a significant influence on the evolutionary processes that drive the organisation and function of many ecosystems (Marcogliese 2004). Despite this, the investigation of host-parasite relationships in wildlife and livestock has focused almost exclusively on identifying factors which drive the emergence of disease or otherwise interfere with host development or productivity (McLeod 1995, Riley et al 2004, Pedersen et al 2007, Hamer et al 2012). Comparatively little attention has been given to the implications of current species management strategies for the biodiversity of parasitic helminths themselves (Gómez and Nichols 2013).

In addition to our primary research outcomes, I have also generated data which can be used to infer the effects of system-level management on the conservation status of parasitic-helminth communities. Nematodes of the genus *Cloacina* were extremely rare in captive red kangaroo populations, despite their ubiquity in free ranging wild populations (Mykytowycz 1964, Arundel et al 1979). This demonstrates that conservation strategies optimised for host-organisms are not necessarily equally effective for maintaining the viability of parasite populations. Cloacinid nematodes are a major component of the gastrointestinal fauna of macropodid marsupials, including many threatened species that are the focus of ongoing conservation efforts (Beveridge et al 2002, Eldridge 2010). In addition to being susceptible to the same ecological threats that affect free living species (e.g. habitat loss, pollution, climate change) our findings suggest that captive management practices can also actively contribute to a loss of biodiversity in parasite communities, even when active control

measures have not been implemented (Dunn et al 2009). Thus far, there have been no attempts to affect recovery or management plans devised specifically for parasitic helminths (Gómez and Nichols 2013). However, the first step in developing actionable conservation targets is the identification of vulnerable taxa. This in turn will require a greater understanding of the ecological and epidemiological factors which drive the transmission and establishment of parasitic helminths. The use of high-throughput molecular techniques, such as next-generation sequencing platforms, to characterise helminth assemblages in captive host populations will not only contribute to our understanding of the role played by parasitic helminths in captive systems, but will also be a significant step toward the integration of parasites into existing host conservation management strategies.

The results from this thesis demonstrate that contemporary captive management practices exert a significant influence on the structure and taxonomic composition of helminth communities in native Australian vertebrates. As parasitic helminths are capable of exerting wide ranging effects on the health and viablitity of their host populations, maximising the success of both commercial farming operations and threatened species breeding programs will require the active management of host-parasite community interactions in captive systems (Pedersen and Fenton 2006). The implementation of sustainable, integrated parasite management strategies will be made possible through studies which provide information on the processes that drive the dynamics of helminth assemblages at both macroecological scales and at the level of individual hosts. Consequently, the research documented in this thesis will directly contribute to the conservation and sustainable commercial utlilsation of Australia's unique wildlife.

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Appendix 1 Nematode community structure in the brush-tailed rock-wallaby, *Petrogale penicillata*: implications of captive breeding and the translocation of wildlife

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1.1 Abstract

Despite an increasing appreciation of the disease risks associated with wild-life translocations, the effects which captive breeding programs exert on parasite communities remain understudied. This may be attributed, in part, to the current lack of rapid and cost-effective techniques for comparing parasite assemblages between host populations. Terminal restriction fragment length polymorphism (T-RFLP) analysis of the rDNA region encompassing the internal transcribed spacers (ITS-1 and ITS-2) and 5.8S rRNA gene was used to characterise bursate nematode communities (suborder Strongylida) across two captive and two non-captive colonies of the threatened brush-tailed rock-wallaby, *Petrogale penicillata.* A clone library was constructed and a restriction enzyme selected to differentiate the predominant operational taxonomic units (OTUs) by the unique peak profiles they generated. The prevalence, intensity of infection and comparative structure of strongylid assemblages was evaluated for each of the host colonies. Compared to wild conspecifics, captive wallabies exhibited a reduced prevalence of infection and significantly lower faecal egg counts. T-RFLP revealed that a high proportion of the OTUs co-occurred across three of the four study locations. Despite this, the composition of strongylid assemblages was significantly different between the colonies, even when host translocation events had occurred. These results suggest that captive breeding programs may exert a profound impact on parasitic helminth assemblages. Developing efficient techniques for characterising community dynamics in potentially pathogenic organisms is critical to the long term success of species recovery efforts worldwide.

1.2 Introduction

Captive breeding provides a powerful tool for the conservation of threatened fauna.

Translocation events, in which captive-reared animals are used to supplement or re-establish

failing wild populations, ward against low levels of genetic diversity and reduce a species

susceptibility to localised environmental disturbances (Griffith et al 1989). As techniques for

the breeding and reintroduction of threatened species continue to be refined, these practices

are becoming an increasingly integral component of conservation management around the

world (Snyder et al 1996). However, while their value is well established, the long term

ecological impacts of captive breeding programs, and the associated translocation of wild-

life, remain largely unexplored.

Most animals are known to coexist with a complex community of protozoa, bacteria,

parasitic arthropods and helminths (Woodford 2000). Consequently, wild-life translocations

present a three-fold risk of disease; 1) heightened mortality or decreased productivity in

essential life history traits following the exposure of immunologically naïve individuals to

novel parasites and/or pathogens, 2) normally benign infections becoming more aggressive

due to dissimilar environmental conditions or the stress associated with introduction into a

new area, and 3) alterations to the existing host-parasite dynamics of other species sharing the

translocated animals range (Woodford and Rossiter 1993, Cunningham 1996, Fèvre et al

2006). If not managed, the disease risks of wild-life translocations have the potential to

drastically reduce the effectiveness of recovery efforts involving captive breeding

(Cunningham 1996).

Assessing the health impacts of host-translocation events on populations of threatened

fauna requires the ability to accurately model changes to parasite assemblages. However, the

techniques commonly employed by existing studies have proven largely inadequate for this

task. Morphological approaches are complicated by the presence of cryptic species, which

frequently lead to analyses underestimating the diversity of parasitic communities, while the harvesting of mature endoparasites necessitates the death of the host (Hung et al 1999, Anderson and Trueman 2000, Chilton 2004, Tixier et al 2006, Chilton et al 2009). Molecular techniques for identifying parasites have allowed for fingerprinting approaches in which assemblages can be characterised in a non-destructive way. However, older technologies such as molecular cloning and Sanger sequencing remain comparatively expensive, time consuming and limited in their ability to reveal fine-scale trends across host populations (Blouin 2002, Blaxter 2004).

Terminal restriction fragment length polymorphism (T-RFLP) is a fluorescence-based technique that has been applied to the analysis of microbial community structure in a range of environments (Bruce 1997, Liu et al 1997, Dunbar et al 2000, Moeseneder et al 2001, Wang et al 2004, Waldron et al 2009). Polymorphisms in the position of recognition sites on PCR amplified sequences are used to create DNA fragments of varying sizes by digestion with a restriction enzyme. Fluorescently labelled primers, and their associated terminal restriction fragment (T-RF), can be detected using capillary electrophoresis in conjunction with an automated DNA sequencer (Marsh 1999, Blackwood 2003). The chief advantages of T-RFLP are the high throughput of analyses compared to traditional metagenomic sequencing approaches, its sensitivity and its capacity to provide quantitative data on each T-RF, including its size and relative abundance within a sample (Blackwood et al 2003, Schütte et al 2008).

In this study, T-RFLP was evaluated as a tool for characterising communities of bursate nematodes (suborder Strongylida) inhabiting the gastrointestinal tract of the brush-tailed rock-wallaby (*Petrogale penicillata*), a medium-sized macropodid marsupial native to eastern and south-eastern Australia. The species is currently listed as 'Near Threatened' by the IUCN with fewer than 30,000 individuals estimated to remain in the wild (DECC 2008,

Taggart et al 2008). It was predicted that the translocation of captive-reared animals into established colonies would precipitate a restructuring of their nematode assemblages, measurable as distinct suites of parasites shared by geographically isolated populations. Also investigated was the relationship between a captive/wild setting and the prevalence and intensity of infection by macropodid strongylids. This is the first study to apply T-RFLP to the analysis of helminth community dynamics in a mammalian host.

1.3 Materials and methods

1.3.1. Study sites

Fresh faecal material (estimated to be no more than 12 hours old at the time of collection) was sourced from brush-tailed rock-wallaby colonies at four sites. Captive samples were obtained from Waterfall Springs Wildlife Sanctuary and Mount Rothwell Biodiversity Interpretation Centre. Wild samples were harvested at Square Top Mountain and Mount Uringery. Both sites are situated within the Warrumbungles National Park in north-western NSW but are considered isolated. The colony at Square Top Mountain has been supplemented with animals from Waterfall Springs on three occasions; April 2009, February 2010 and March 2010. A total of 200 faecal samples were collected across all four localities between February and August 2010. To prevent eggs from hatching, all faeces were stored at 4°C prior to processing.

1.3.2. Screening for parasites

The number of stronglyid eggs per gram (EPG) of sample was estimated using a modified version of the McMaster method (Henriksen & Aagaard 1976). Faecal material (0.5 – 1 g) was weighed into a flat-bottomed polypropylene specimen tube, to which 10 mL of 56%

Zinc-sulfate flotation solution was added. A wooden tongue depressor was then used to break up the faecal pellets. The resultant solution was further disrupted by vortexing, before being passed through a faecal parasite concentrator (FPC) strainer with 0.6 x 0.6 mm openings (Evergreen Scientific, Los Angeles, California). Samples were scanned on a compound microscope using 100X magnification with the EPG for each being calculated from an average of two counts. Faecal egg counts were not taken from samples in which large numbers of hatched larvae were observed.

1.3.3. DNA extraction and PCR amplification

Genomic DNA was extracted from faecal samples using the ISOLATE Faecal DNA Kit as described in the manufacturer's protocols (Bioline, London, UK). Amplification of strongylid rDNA was accomplished using the primer set NC16 (forward: 5'-AGTTCAATCGCAATGGCTT- 3') and NC2 (reverse: 5'-TTAGTTTCTTTTCCTCCGCT-3') (Gasser et al 1993, Chilton et al 2003). In addition to flanking sequences, the targeted region included the ITS-1, the 5.8S rRNA gene and the ITS-2, collectively referred to here as the ITS+. (Chilton et al 2009). The reaction mixture for all PCRs consisted of 25 µl Gotaq Green Mastermix (Promega, Madison, USA), 0.2 µM of each primer, 2 µL template DNA and 21 µL PCR water for a final volume of 50 µL. Amplifications were performed in a Mastercycler Gradient thermal cycler (Eppendorf, Hamburg, Germany). An initial denaturation step of 94°C for 3 min preceded 35 cycles of 94°C for 30 s, 55°C for 30 s and 72 °C for 1 min, followed by a final extension of 72°C for 5 min. All reactions included a negative control containing only PCR water and a positive control consisting of strongylid (Cloacinidae) DNA. PCR products were resolved by electrophoresis on 2% agarose gels and amplicons found to contain fragments of the appropriate size (~ 900 bp) were purified using

the QIAquick PCR Purification Kit according to the manufacturer's Spin Protocol (QIAGEN, Hilden, Germany).

1.3.4. ITS+ rDNA clone library

Amplicons were ligated into pGEM T-Easy plasmid vectors (Promega, Madison, USA), transformed into competent One Shot TOP10 *E. coli* cells (Invitrogen, Carlsbad, California) and cultured on LB agar plates (Oxoid, Adelaide, Australia) containing 40 mg X-gal/mL (Bioline, London, UK), mg 100 ampicillin/mL (Sigma-Aldrich, St Louis, USA) and 1mL IPTG (Bioline, London, UK). Recombinant plasmids were identified by PCR based screening and purified using the QIAprep Miniprep Kit according to the manufacturer's instructions for the Spin Protocol (QIAGEN, Hilden, Germany). The ITS+ region was sequenced using an ABI Prism 3130x1 genetic analyser (Applied Biosystems, Melbourne, Australia) at the Macquarie University Sequencing Facility. Sequencing reactions were performed with the universal primers M13F and M13R (Messing, 1983) and ABI PRISM BigDye™ terminator 3.0 cycle sequencing kit (Applied Biosystems). The ITS+ clone sequences generated in the course of this study have been deposited in GenBank (accession numbers JX155298 - J X155346).

1.3.5. Phylogenetic analysis of ITS+ region clones

ITS+ sequence data were examined for ambiguities and assembled into contigs using the program GENEIOUS Pro version 5.4.6 (Biomatters Ltd, Auckland, New Zealand). Homology queries were submitted to GenBank using the BLAST algorithm. Sequences were aligned with GENEIOUS alignment. Alignment gaps were treated as missing values in all analyses. A phylogeny was inferred using the Neighbour-joining method with genetic distances calculated by the Tamura-Nei parameter model (Tamura and Nei 1993). The

rhabditid nematode *Heterorhabditis sp.* (accession number: GU130179) was used as an

outgroup. The relative support for clades within the constructed phylogenies was ascertained

by bootstrap analyses (1000 replicates).

1.3.6. T-RFLP for the characterisation of operational taxonomic units

Amplification of clones for T-RFLP was accomplished using the primers NC16-FAM and

NC2-PET, 5' end labelled with 6-carboxyfluorescein (6-FAM) and PET respectively. Custom

oligonucleotides were synthesised by Sigma-Aldrich (St Louis, USA) and Applied

Biosystems (Melbourne, Australia). PCRs were conducted in 50 µL reaction volumes

consisting of 39.4 µL PCR water, 5 µL 10 × PCR buffer, 200 µM dNTP, 1 µL DMSO, 0.6 µL

AccuTaq-la DNA polymerase (Sigma-Aldrich St Louis, USA), 2 µL target DNA and 0.1 µM

of each primer. Cycling conditions and the methods by which PCR product was purified were

identical to those previously described.   The digestion of fluorescently labelled PCR product

was accomplished using the following restriction endonucleases; *BfaI*, *HinfI* and *NlaIV* (New

England Biolabs). All reactions were performed according to the manufacturer's

recommendations and included 10 µl PCR product, 10 U of restriction endonuclease, 2.5 µL

of 10 × NEbuffer 4 and PCR water up to a final volume of 25 µL. The *NlaIV* digestions

included the additional component of BSA (New England Biolabs) at a concentration of 100

µg/ml. Incubations were performed at 37°C for 2 hr. Fluorescently labelled fragments were

detected using an ABI Prism 3130 × 1 genetic analyser (Applied Biosystems, Melbourne,

Australia) in genescan mode (8.5 Kv; 40-s injection; 60 °C for 100 min) employing a G5

filter. GeneScan software 4.0 (Applied Biosystems) was used to analyse T-RF sizes.

Operational taxonomic units (OTUs) were defined as distinct peak profiles shared by groups

of clones. Samples in which the size of the forward and reverse T-RFs differed from

representative clones by no more than ± 2 bp were considered to be part of the same OTU.

1.3.7. T-RFLP for the comparison of community composition

T-RFLP was applied to mixed community DNA samples in order to compare the structure of strongylid assemblages across the four host populations. The enzyme *HinfI* (New England Biolabs) was selected based on our earlier analyses. Reaction conditions for PCRs and restriction digests were identical to those previously described. Only peaks corresponding to identified OTUs were considered in the analysis of community structure. An OTU was considered present if both peaks had a match within the sample at no more than 3 bp error. Peaks not corresponding to any previously identified OTUs were disregarded in the final analysis.

1.3.8. Statistical analyses

A Chi-square test was performed to compare the prevalence of strongylid nematode eggs across the four study colonies. Variation in mean infection intensity was analysed using log transformed data in a general linear model nested ANOVA, where the EPG estimates for each site were nested within the captive/wild factor. For both tests the significance level (α) was 0.05. Parasite assemblage data were analysed using Hierarchical clustering to distinguish groupings within population structure, while similarity profile (SIMPROF) permutation analysis was employed to test the significance of the clusters. The relative similarity of parasite assemblages between colonies was assessed using an analysis of similarity (ANOSIM). For clustering and ANOSIM, data were converted into binary form (presence-absence) and analysed using the Jaccard similarity coefficient. All multivariate analyses were performed using PRIMER Version 6.1.11 (Plymouth Marine Laboratories) and a significance level (α) of 0.05.

1.4 Results

1.4.1. Abundance of strongylid nematodes

The prevalence of strongylid nematode eggs differed between the four study colonies ($X^2 = 60.97$, $p < 0.0001$) and was higher at the two wild sites (figure 1*a*). Average infection intensity was found to be significantly higher among the wild colonies ($p = 0.049$). There was also a significant difference between sites nested within the captive and non-captive groups ($p = <0.000$) (figure 1*b*).
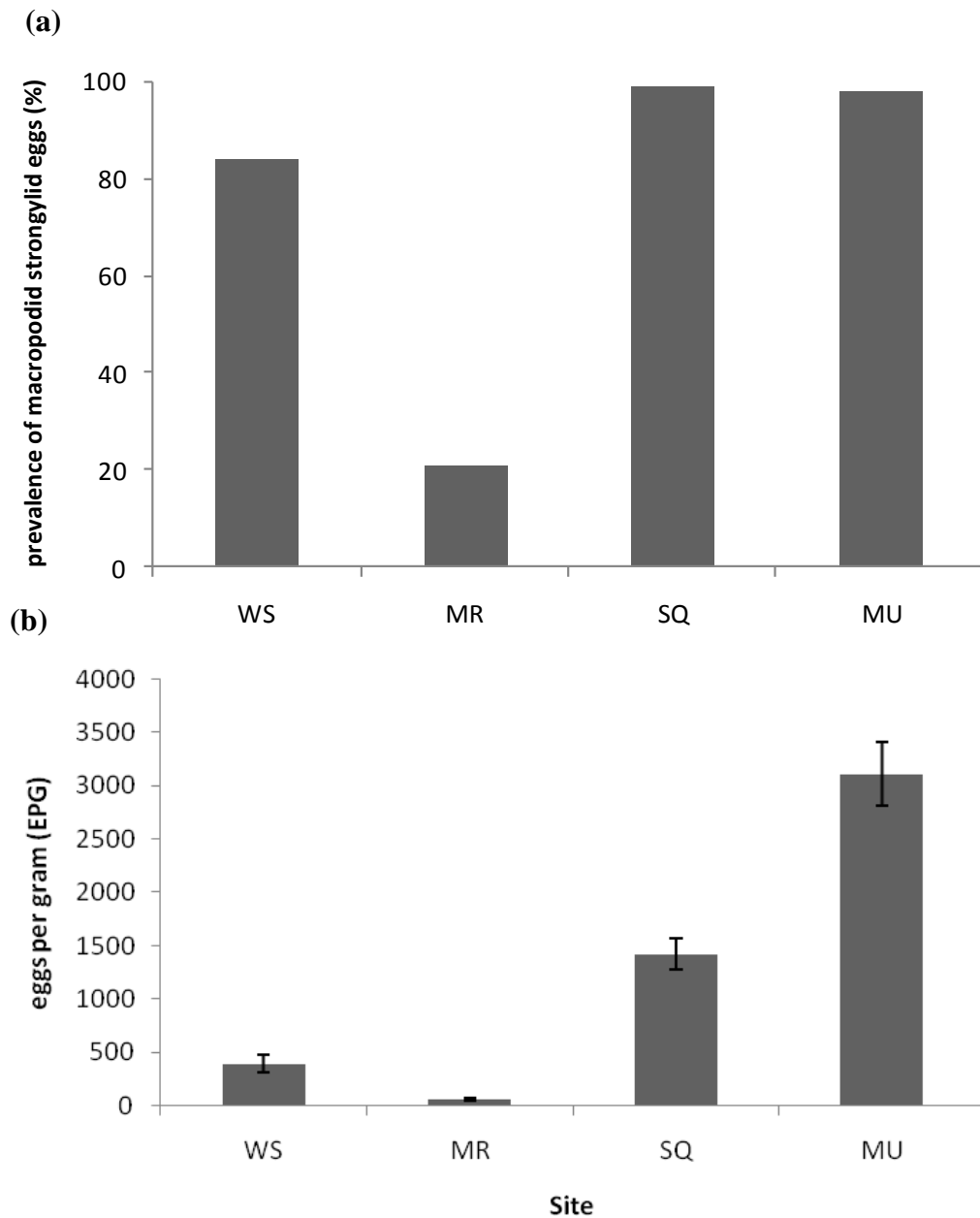
Figure 1.1 Parasite abundance in four populations of brush-tailed rock-wallabies; Waterfall

Springs (WS), Mt Rothwell (MR), Square Top Mountain (SQ) and Mount Uringery (MU).

(*a*) The prevalence of macropodid strongylid eggs and (*b*) mean EPG for each site. Bars show

standard error.

1.4.2. Analysis of ITS+ rDNA sequences and characterisation of principal OTUs

Forty-nine clones were isolated and analysed. The length of the ITS+ region ranged from 894-926 bp in length, including flanking sequences and the 5.8S rRNA gene. The number of OTUs varied depending on the restriction enzyme used (table 1). *HinfI* produced more distinct T-RF peak profiles (n=12) than either *BfaI* (n=10) or *NlaIV* (n=7) although fluorescent labelling of both the forward and reverse primers still proved necessary in order to adequately distinguish between the OTUs. The taxonomic discrimination of T-RFLP was assessed by comparing the phylogenetic relationship of the *HinfI* defined OTUs to that of the ITS+ clone sequences, which could be assigned to one of two nematode families; Trichostrongylidae and Cloacinidae (figure 2). The majority of OTUs were found to conform to distinct clusters of closely related clones.

Table 1.1 Sequence identity and T-RF lengths generated from ITS+ rDNA clones using each of the three restriction enzymes. Sample codes designate the site and sample from which representative clones were drawn.

Restriction enzyme	Observed (predicted) T-RF lengths (bp)		Closest database match	Percentage similarity
	5'	3'		
HinfI				
WS252 C1	197 (201)	430 (434)	*Spiculopteragia asymmetrica/quadrispiculata*	85.40%
WS253 C18	196 (201)	83 (86)	*Cloacina caenis*	86.00%
WS256 C1	197 (201)	233 (235)	*Spiculopteragia asymmetrica/quadrispiculata*	85.70%
WS257 C3	372 (374/375)	83 (86)	*Cloacina robertsi*	95.90%
SQ299 C3	374 (377)	408 (414)	*Labiosimplex australis*	90.20%
SQ299 C5	376 (377/379)	84 (87)	*Labiosimplex australis*	90.20%
MU359 C2 [a]	370 (374)	202 (218)	*Cloacina pearsoni*	95.70%
MU360 C3	371 (374)	202 (206)	*Cloacina pearsoni*	95.60%
MU361 C2	429 (432)	84 (87)	*Labiosimplex australis*	92.70%
MU364 C9	140 (145)	407 (414)	*Labiosimplex australis*	92.50%
MU364 C10	428 (432)	408 (414)	*Labiosimplex australis*	92.90%
MR466 C4	373 (376)	422 (428)	*Cloacina cloelia*	92.20%
MR481 C1	364 (366)	185 (188)	*Cloacina hydriformis*	99.10%
BfaI				
WS252 C1	116 (122)	405 (408)	*Spiculopteragia asymmetrica/quadrispiculata*	85.40%
WS252 C17	250 (254)	208 (210)	*Labiosimplex australis*	91.50%
WS253 C18	116 (122)	126 (129)	*Cloacina caenis*	86.00%
WS257 C3	113 (118/119)	126 (129)	*Cloacina robertsi*	95.90%
SQ299 C5	455 (459)	383 (388)	*Labiosimplex australis*	90.20%
MU359 C2 [a]	114 (119)	126 (141)	*Cloacina pearsoni*	95.70%
MU359 C6	450 (455)	126 (129)	*Cloacina caenis*	95.30%
MU361 C10	454 (458/460)	207 (209/210)	*Cloacina cloelia*	91.80%
MU364 C9	114 (118/199)	206 (210)	*Labiosimplex australis*	92.90%
MR469 C3	287 (290)	102 (104)	*Cloacina hydriformis*	98.70%
MR489 C4	381 (384)	220 (223)	*Cloacina cloelia*	91.40%
NlaIV				
WS252 C1	87 (94)	826 (832)	*Spiculopteragia asymmetrica/quadrispiculata*	85.40%
WS252 C16	86 (91/92)	795 (803/805)	*Labiosimplex australis*	91.50%
WS256 C4	86 (92)	180 (181)	*Cloacina pearsoni*	95.70%
MU361 C2	86 (93)	667 (674/677)	*Labiosimplex australis*	92.70%
MR466 C4	86 (92)	809 (814)	*Cloacina cloelia*	92.20%
MU359 C2	86 (92)	180 (193)	*Cloacina pearsoni*	95.70%
MU359 C4	86 (92)	496 (503)	*Cloacina caenis*	96.80%
MR469 C1	–	–	*Cloacina hydriformis*	98.70%

[a] Denotes samples which were re-assigned to different OTUs based on observed T-RF sizes.
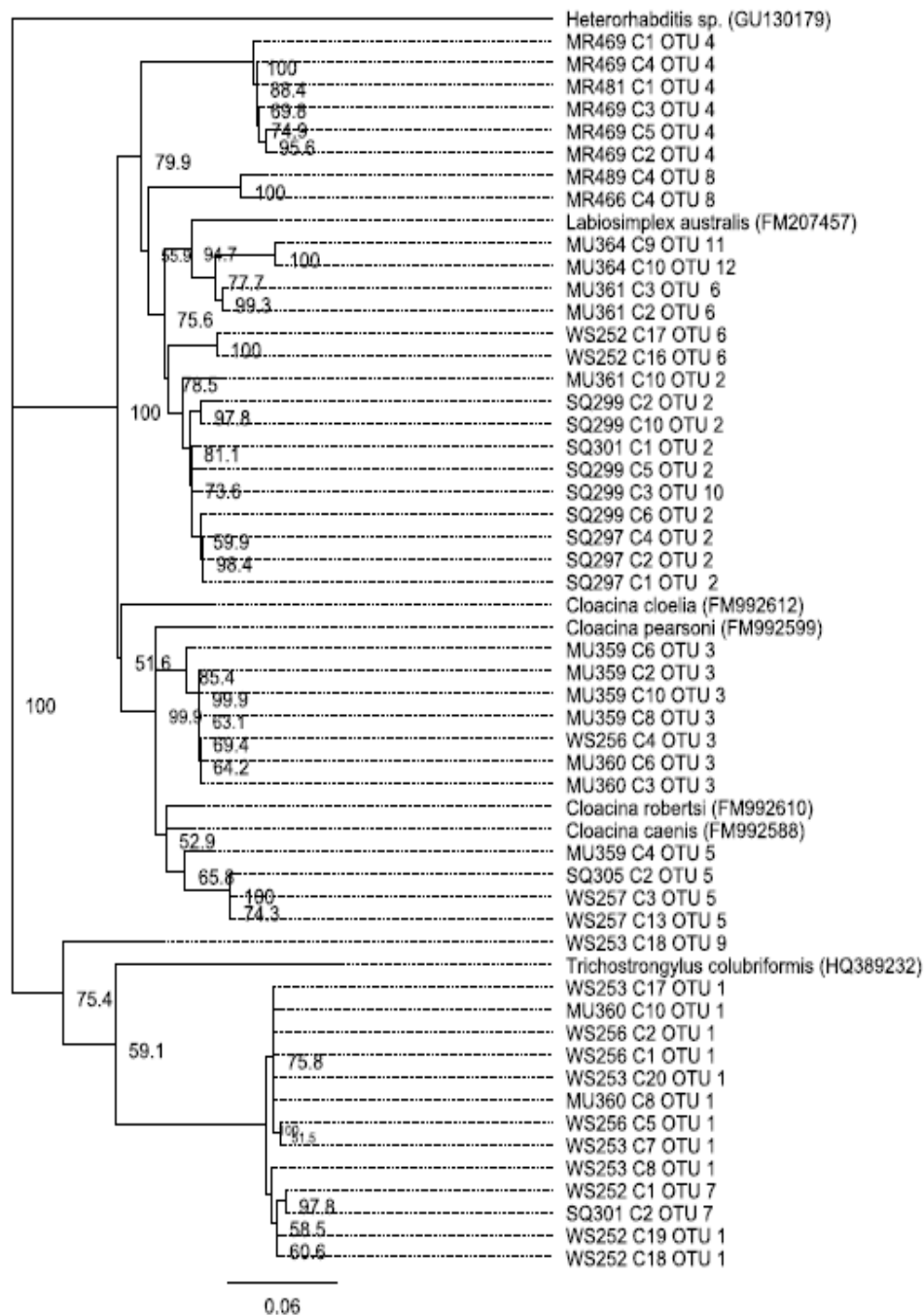
Figure 1.2 Phylogenetic tree constructed from ITS+ sequences derived from macropodid strongylid assemblages at four different locations. The tree was constructed with GENEIOUS Pro version 5.4.4. The Bootstrap values are based on 1000 replications and only values higher than 50% are displayed. The scale bar represents substitutions per nucleotide position. Accession numbers for reference strains are shown in brackets. *Heterorhabditis* sp. was used as an outgroup. Sample codes designate the site, sample and sequential order of representative clones.

1.4.2.1 Cloacinidae

Nine OTUs were assigned to this clade (75%), with the majority of sequences being most

closely related to members of genus *Labiosimplex* (Table 1 & Figure 2). Abundant T-RF

peak profiles corresponding to this group were OTU2 (376/84 bp) and OTU6 (429/84 bp).

Rarer variations were OTU10 (374/408 bp), OTU11 (140/407 bp) and OTU12 (428/408 bp),

each represented by a single clone sequence. OTU4 (364/185 bp) and OTU8 (373/422 bp)

formed separate clusters which did not correspond to any of the reference taxa. The

remaining Cloacinidae sequences were more closely allied with genus *Cloacina*. The

predominant peak profile was OTU3 though OTU5 was also found to be moderately

common, with sizes of 371/202 bp and 372/83 bp respectively.

1.4.2.2 Trichostrongylidae

Four OTUs were placed in this group. However, the assignment of more specific taxonomic

labels was rendered impossible by the lack of homologous sequences currently available in

publically accessible databases (Table 1). The majority of clones in this clade (~79%) were

represented by a single peak profile, OTU1 (197/233 bp). The two sequences constituting

OTU7 (197/430 bp) formed a separate node within the OTU1 cluster. The final T-RF peak

profile, OTU9 (196/83 bp) was represented by a single clone sequence which was positioned

externally to the OTU1/7 clade (figure 2).

1.4.3. Comparison of bursate nematode communities across sites

Hierarchical cluster analysis revealed two major groups of samples diverging at a similarity

level of 27% (Figure 3). Cluster 1 constituted all samples collected from the Warrumbungle's

region, in addition to the majority of those harvested at Waterfall Springs Wildlife Sanctuary.

Cluster 2 was limited to representatives from Mount Rothwell Biodiversity Interpretation

Centre and the remainder of the samples obtained at Waterfall Springs. Cluster 1 could be further divided into 3 main subgroupings which diverged at a similarity level of 39% and 44%. The group designated *1a* was comprised of only five samples (two from Mount Square Top and three from Mount Uringery) while group *1b* constituted the single largest subcluster on the dendrogram, representing the majority of samples from both Mount Square Top and Waterfall Springs with a further five representatives from Mount Uringery. Group *1c* was composed predominantly of samples harvested at Mount Uringery with two representatives from Mount Square Top and a single sample originating from Waterfall Springs. Considerable overlap was observed in the community structure of samples between locations, with little site fidelity evident in the dendrogram. Nevertheless, there was a significant difference in the composition of the strongylid between each location (ANOSIM, P=0.046).
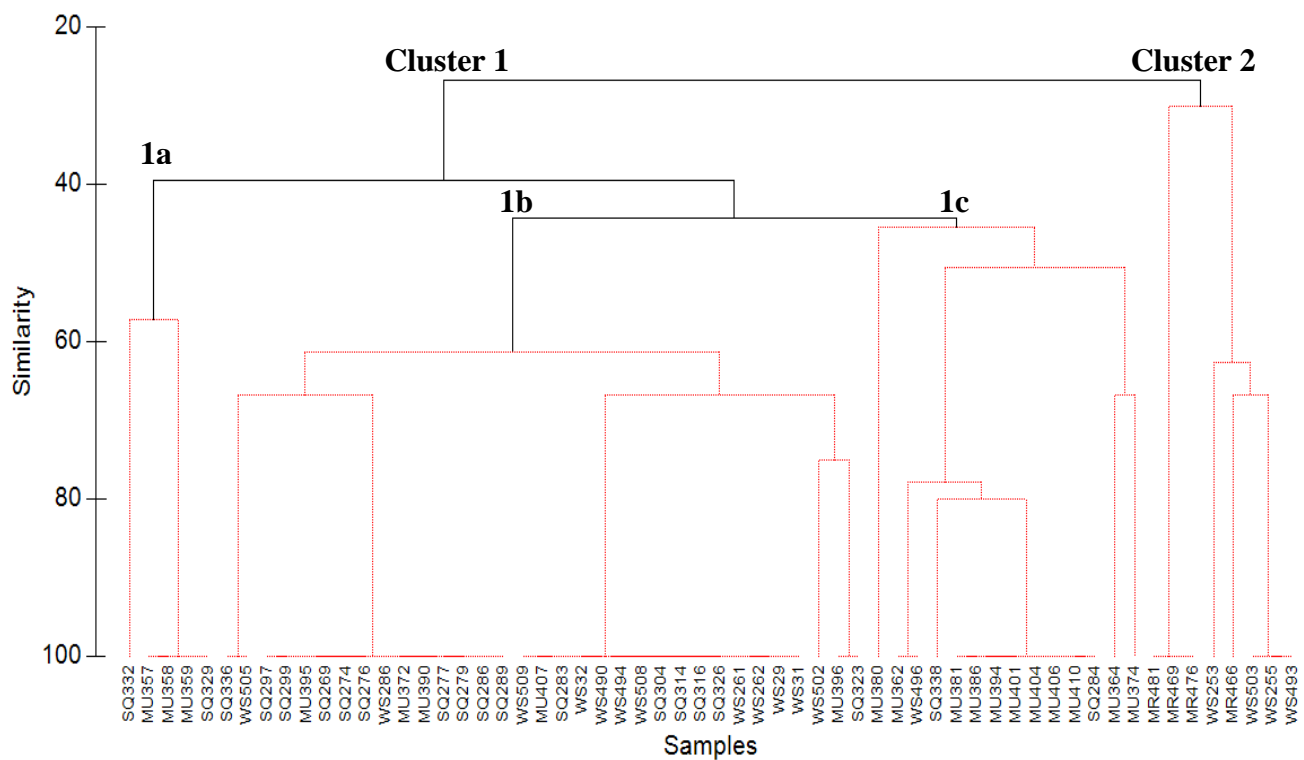
Figure 1.3 Hierarchical dendrogram depicting group average clustering of Jaccard indices of similarity based on non-transformed OTU prevalence data at four study localities. Clusters of samples connected by dotted lines are significantly different from other clusters (SIMPROF test, p<0.05).

1.5 Discussion

The prevalence and intensity of infection by strongylid nematodes varied significantly between captive and wild populations of brush-tailed rock-wallabies. The altered ecological conditions which invariably arise in an artificial environment may be exerting a confounding effect on the typical interaction between these parasites and their hosts. Most captive management programs seek to optimise all aspects of species ecology, including nutritional intake, access to shelter and control of any potential predator or competitor species. Consequently, the physical condition of captive specimens is likely to exceed that of wild conspecifics. This in turn may be facilitating a more robust immune response against parasitic helminths (Koolhaas and Oortmerssen 1987, Sheldon and Verhulst 1996, Ezenwa 2004). The segregation of captive animals among separate pens or enclosures is also likely to retard the rate at which nematodes are able to encounter and colonise new individuals, while supplementary feeding may discourage grazing and thus reduce the likelihood of potential hosts ingesting infective L3 stage larvae (Smales 1977, Arneberg et al 1998, Anderson 2000, Scott and Sutherland 2009).

Environmental factors do not offer the only explanation for variation in macropodid strongylid abundance. The establishment of captive populations outside of a species current distribution is likely to prevent colonisation by typical helminth fauna, particularly if founder animals are treated with anthelmintics prior to translocation. The comparatively low EPG observed among animals at the Mt Rothwell site may be a consequence of opportunistic infection by incidental strongylid taxa (Holmes 1987, Petney and Andrews 1998). It should be noted that the terms 'captive' and 'wild' do not denote two discrete categories of environment but rather encompass a continuum of ecological conditions. As such, any variation arising in the abundance of parasitic helminth communities is likely to be a

consequence of the complex interaction of a range of factors, the exact nature of which will vary considerably on a site by site basis.

Our initial hypothesis was that host translocation events would precipitate a restructuring of the nematode assemblages associated with a particular location. Furthermore, it was predicted that these changes would be measurable as distinct suites of parasites shared by geographically isolated populations. This was not supported by the data. While a considerable degree of overlap was observed in the range of OTUs occurring at Waterfall Springs, Mount Uringery and Mount Square Top, the convergences at these sites do not seem to be explainable by the movements of Brush-tailed Rock-wallabies. The Mount Uringery colony has never been directly supplemented with animals from Waterfall Springs and the relatively limited dispersal capability of the species makes it unlikely that individuals could have moved between the two sites in the Warrumbungles (Hazlitt et al 2006, Piggott et al 2006). Furthermore, the overall similarity in the composition of parasite assemblages at Waterfall Springs and Mount Square Top was found to be negligible, despite these being the only sites in which population exchange is known to have occurred. The strongylid communities of Mount Rothwell were found to be particularly distinctive, possessing several OTUs which did not occur at any of the other localities. This is further evidence that these individuals may have been colonised by local nematode taxa, atypical for brush-tailed rock-wallabies. It is currently unclear whether these results are indicative of shared pools of parasites among geographically proximate populations or if they simply reflect a limitation in our methodology. As our analysis disregarded T-RFs which could not be correlated with any of the clone OTUs it is highly probable that we have overlooked some of the diversity present within any given host population. This would naturally create a bias towards the detection of the most common taxa across each of the sites which may have served to homogenise our results. It is possible that more clones will need to be analysed in order to generate a

comprehensive database of the OTUs unique to each location. Furthermore, while the *Hinf1* defined OTUs showed phylogenetic relationships consistent with the clone sequences from which they were derived, it was also observed that identical T-RFs were often produced by relatively loosely clustered groups of clones. A single OTU is therefore likely to represent several different strongylid taxa, resulting in subsequent T-RFLP analyses underestimating the complexity of nematode communities at each location. While previous studies have also recognised this possibility, it does not necessarily reflect a limitation in the technique itself. The choice of restriction enzyme is known to greatly influence the diversity of the T-RFs which appear in a given profile and will therefore be an important factor in determining how finely constituent taxa can be differentiated. Employing more than one enzyme may also prove necessary in order to adequately distinguish between the significant taxonomic groups in a sample (Klamer et al 2002, Liu et al 1997, Wang et al 2004).

Assessing the viability of T-RFLP as a technique for elucidating fine-scale trends in population structure requires careful consideration of the sequence to be used as a marker. In this study the ribosomal ITS+ region was selected due to the high interspecies variability known to exist at the ITS-1 and ITS-2 loci (Gasser & Newton 2000, Powers et al 1997). However, mitochondrial DNA (mtDNA) may prove to be a more effective means of discriminating between closely related nematode taxa due to the comparatively greater speed at which mutations accumulate in these sequences (Blouin et al 1997, Blouin 2002, Denver et al 2000).

T-RFLP analysis was unable to identify the full range of OTUs known to exist within a sample. The reasons for this are unclear as the prevalence of the omitted OTUs among the clone sequences suggests that they did not necessarily correspond to numerically inferior taxa and that their absence is also unlikely to be a consequence of biases introduced during cell lysis or PCR amplification (Liu et al 1997). If the technique is incapable of characterising

major elements of an assemblage then its application in measuring changes to the community dynamics of parasitic helminths is necessarily limited. In this respect, next-generation DNA sequencing (NGS) technologies may provide a superior platform for the analysis of complex parasitc nematode assemblages. Preliminary studies employing barcoded amplicon Illumina sequencing have demonstrated its capacity to provide highly detailed taxonomic profiles which can be used to monitor bacterial communities (Bokulich et al 2012). 16S amplicon 454 pyrosequencing represents another cost effective methodology for characterising microbial or parasite diversity within a given host population (Ishak et al 2011). However, it has been suggested that, in at least some taxa, T-RFLP is capable of more accurate genus-and-species-level taxonomic discrimination than NGS approaches (Bokulich et al 2012). Thus it is probable that T-RFLP may be used in conjunction with NGS tools to detect low-frequency OTUs, facilitating detailed structural comparisons between parasite communities.

A significant anthropogenic influence was identified on the structure of strongylid nematode communities inhabiting brush-tailed rock-wallabies. Future avenues of enquiry include more fully characterising the diversity present amongst these highly complex parasitic assemblages and in elucidating the exact dynamics by which contemporary species management practices impact upon it. While our results suggest that T-RFLP may be refined to monitor broader trends in helminth community ecology, it currently appears unlikely that the technique will be capable of generating a comprehensive profile of an assemblage. Whether this will be sufficient to identify what may prove to be a comparatively subtle taxonomic reconfiguration of helminth communities following host translocation events has yet to be determined. The ability to characterise population dynamics in a potentially pathogenic group of organisms will facilitate the development of more successful captive breeding programs and contribute to the conservation of unique and threatened elements of biodiversity in a global context.

1.6 Acknowledgements

1.7 References

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Appendix 2 Final approval from Animal Ethics Committee

ANIMAL RESEARCH AUTHORITY (ARA)

MACQUARIE UNIVERSITY

AEC Reference No.: 2012/038-4   <u>Date of Expiry:</u> 30 September 2015

Full Approval Duration: 01 October 2012 to 30 September 2015 (36 Months)

This ARA remains in force until the Date of Expiry (unless suspended, cancelled or surrendered) **and will only be renewed upon receipt of a satisfactory Progress Report before expiry (see Approval email for submission details).**

Principal Investigator:
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Sally Isberg   0427 427 489

Other people participating:
Margaret Byrne   0415 993 783
Kate Coughlan   0438 015 902

In case of emergency, please contact:
the Principal Investigator / Associate Investigator named above
or Animal Welfare Officer: 9850 7758 / 0439 497 383

The above-named are authorised by MACQUARIE UNIVERSITY ANIMAL ETHICS COMMITTEE to conduct the following research:

<u>Title of the project:</u> **Host parasite interactions in crocodiles in Northern Australia**

<u>Purpose:</u> 4 – Research: human or animal biology

<u>Aims:</u>   1. To assemble a comparative genetic database of helminth species known to parasitize crocodiles in Australia
     2. To identify nematode taxa present in infected crocodiles at Darwin Crocodile Farm
     3. To gather data on the prevalence of nematode infections within the host population

<u>Surgical Procedures category:</u> 4 – Minor surgery with recovery

All procedures must be performed as per the AEC-approved protocol, unless stated otherwise by the AEC and/or AWO.

Maximum numbers approved (for the Full Approval Duration):

Species	Strain	Age	Total	Supplier/Source
30 (Other reptiles)	N/A	2 years+	5000	Porosus Pty Ltd (Darwin Crocodile Farm)
		TOTAL	5000	

Location of research:

Location	Full street address
Darwin Crocodile Farm	Stuart Highway, NT, Australia

Amendments approved by the AEC since initial approval:
1. Addition of Margaret Byrne and Kate Coughlan (Executive approved April 2013, ratified by AEC April 2013)

Conditions of Approval: N/A

Being animal research carried out in accordance with the Code of Practice for a recognised research purpose and in connection with animals (other than exempt animals) that have been obtained from the holder of an animal suppliers licence.

_____
Professor Mark Connor (Chair, Animal Ethics Committee)   Approval Date: 16 October 2014