POLAR EVOLUTION: MOLECULAR GENETIC AND PHYSIOLOGICAL PARAMETERS OF ANTARCTIC ARTHROPOD POPULATIONS

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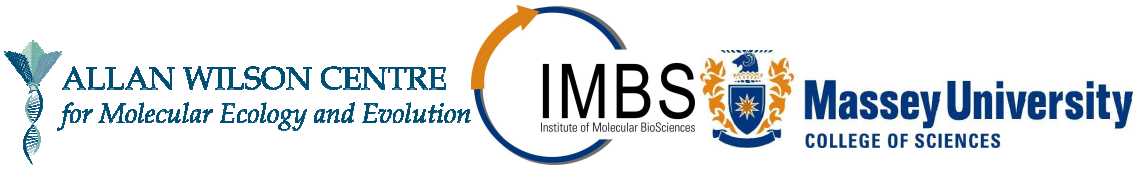

Ice floe off Cape Bird, with Adélie penguins on board; January 2007

To move forward relentlessly in the quest for discovery;
And yet to not pass by the moments of perfect tranquillity

ABSTRACT

This thesis is presented as a collection of research papers synthesising knowledge gained during the period of candidacy. Its underlying focus is the examination of evolution from a variety of perspectives for terrestrial arthropods (springtails) in an Antarctic setting. These perspectives include investigation of the ways in which springtail populations respond both physiologically and genetically to environmental variability over historical and contemporary time-scales. While the physiological and genetic may seem two worlds apart, this thesis recognises that, in reality the two are inextricably linked. Thus, when genetic differentiation between populations of the same species can be demonstrated, physiological differentiation of these populations may also be predicted (and *vice versa*). Therefore, across several locations and springtail species, physiological and genetic parameters of individuals and populations are examined both separately and, where possible, in concert.

The physiological aspect of this thesis focuses on the springtail *Gomphiocephalus hodgsoni* from continental Antarctica. In addition to providing the first metabolic rate data for a continental Antarctic springtail, seasonal variation in metabolic rates is examined across multiple temporal and spatial scales to evaluate the ways in which individuals and populations respond to environmental variability. Metabolic activity in this species is intricately linked to a variety of factors, both intrinsic and extrinsic. These include biological function, temperature profiles in the local microclimate, and body mass and genetic differences among populations.

In the genetically-focused aspect of this thesis, population genetic patterns of *G. hodgsoni* from several continental locations and *Cryptopygus antarcticus antarcticus* from locations across the Antarctica Peninsula are compared. Here, the importance of differing evolutionary histories in influencing patterns of contemporary genetic population structure is highlighted. While both species have been similarly affected genetically by Pleistocene (2 Ma – present) glacial cycling, it is clear that differences in timing of colonisation events and subsequent population expansions have left distinct genetic signatures in each species. In a separate molecular study, phylogenetic analyses are employed to study members of the circum-Antarctic springtail family Isotomidae.

The genetic ancestry among these closely related species is shown to reflect a diverse evolutionary origin in the Miocene (23 – 5 Ma), subsequent to which both vicariant and dispersal processes have been important. Phylogenetic re-constructions tease out the relationships among sister species, and the identification of several genetically distant lineages suggests that a revision of current species designations is required.

Finally, two studies that integrate the physiological and molecular genetic are presented. First, metabolic rate variation across several locations on sub-Antarctic Marion Island in the springtail *Cryptopygus antarcticus travei* is examined. This variation is related to the genetic structure of populations to show that historical and contemporary environmental characteristics have left their trace in the expression of both genetic and physiological variability of these populations. Second, the perceived association between metabolic rate and genetic (mutation) rate is investigated more closely - a sophisticated Bayesian correlation analysis detects that there is an indirect relationship between metabolic rate and underlying species phylogeny in *C. a. travei*.

Thus, the physiological and molecular genetic elements of this thesis test or advance important hypotheses within their own fields, and the integrated approach applied is a new step in interpreting evidence of physiological adaptation in Antarctic species. In its multi-faceted approach to evolutionary studies, this thesis enhances understanding of the current picture of springtail evolution in polar environments.

ACKNOWLEDGEMENTS

No thesis would be possible without the input of supervisors, in guise small and large, positive and negative, valuable and annoying! Thus, I wish to start by thanking them all: David Penny, Mark Stevens, Barbara Holland and Pete Convey. I wish to thank David for taking me on in the first place, supporting me financially over the final 6 months, and mostly for making me think differently because you do. Our discussions were always entertaining, always a little side-tracked and often inspirational. Mark drew me to Massey at the beginning and a lot has taken place over the years since. While it didn't all come out smelling roses, we endured, and I think the final result is something we can both be proud of. Barbara, I leaned on a lot over the final six months of my PhD. I really enjoyed the moments where you took the time to properly teach me – with your help I now feel like I actually know (a little) something about phylogenetics! Finally, Pete kind of copped a handful coming on board about a third of the way in to my PhD. For me, our collaboration has been interesting and educational (is there anything you don't know?) and I really appreciate the time you've taken to answer my questions and evaluate my work as well as be a friend. It was also a special bonus having you out at Cape Bird (where I could bug you 24/7!) – maybe one day I'll get over to your side!

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For both work-related things and otherwise, I thank Renae Pratt, Liat Shavit-Grievink, Andrew Clarke, Gabe Redding, Ian Hogg, Tracey Jones, Charlene Scheepers and Emily Atkinson for their friendship, support and advice at various times throughout.

From the first, I have had a sense of awe and excitement about working in Antarctica and my trips south have nourished this. It is with enthusiasm and privilege that I have pursued my research to date (could I really complain about work when I had penguins, seals and killer whales (Cape Bird) and the Royal Society Ranges (Garwood Valley) as back-drop?) and this has fostered in me a life-long love and commitment for Antarctica. In lieu of this, I thank Ian Hogg for the opportunity of the first trip, and I thank Kelly Tarlton's Underwater World, Sir Robin Irvine and Antarctica New Zealand for providing/administering the student scholarships that permitted me to head South for both the first (during my MSc), and three subsequent (PhD) field seasons. I wish to thank Shulamit Gordon especially, whose dealings with me through the years have been friendly, helpful and supportive. While my work in the field was mostly a solo endeavour, I always had people from other science events around, and I'd like to thank them now for their company. This includes everyone from Marion Island station (2007), the penguin group at Cape Bird (2006/07 and 2007/08) and the Waikato group at Garwood Valley (2008/09). Special thanks to Kerry Barton for making the first Cape Bird season so special, and to Grace Tiao for a memorable New Years in 2009!

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CHAPTER ONE:

THESIS INTRODUCTION

1.1 INTRODUCTORY STATEMENT

Biological diversity exists in the dimensions of form, space and time (Pachepsky et al. 2001). Indeed, we understand the essence of biological diversity as a result of the history of life upon Earth, expressed through change across these dimensions. Examining such change is examining the substance of evolution.

Evolution is the major unifying principle of biology, and evidence of its effects colour all levels of biological organisation, from molecules to populations to ecosystems. Clarifying how evolutionary processes have governed the ways in which populations and species are structured is a key goal of evolutionary biology.

This thesis is presented as a collection of research papers synthesising knowledge gained during the period of candidacy. Its underlying focus is the examination of evolution from a variety of perspectives for terrestrial arthropods (springtails) in an Antarctic setting. These perspectives include investigation of the ways in which springtail populations respond both physiologically and genetically to environmental variability over historical and contemporary time-scales. These responses are then characterised in the context of the underlying mechanisms that may drive genetic and physiological variability. Thus, this thesis has three main themes: (1) the physiological; (2) the (molecular) genetic; and (3) the mechanistic relationship between these.

To honour these themes, the thesis borrows from a number of knowledge platforms. The following sections serve to outline important concepts drawn from these platforms to form the foundation of this work. A description of the factors that come into play when using Antarctica as a research ecosystem is followed by a section on the target taxa (springtails), which outlines the ways in which these organisms cope with 'life in the freezer'. A section on evolution as it relates to this thesis follows, with descriptions based on evolutionary processes (metabolic and genetic) at both the population and individual (i.e. molecular) levels. Having introduced the underlying concepts relevant to the scope of this thesis, the next section serves to explain how the individual chapters that follow employ these concepts in order to address the major themes of the thesis. Finally, a brief passage outlines the contributions of others to the work presented in this thesis.

1.2 CONCEPTS

1.2.1 Antarctica and springtails as templates

1.2.1.1 Antarctica

Antarctica, the southern-most continent, is surrounded by the Antarctic Circumpolar Current and Polar Frontal Zone, which isolate it and its outlying archipelagos geographically, climatically, thermally and oceanographically (Clarke et al. 2005; Barnes et al. 2006). Alongside this isolation, Antarctica experiences a unique set of environmental conditions (Walton 1984; Convey 1996a), including a climate of 'extremes'. Prolonged low temperatures are characteristic in Antarctica; indeed, the lowest recorded temperature in the world (-89.6 ºC) was measured at inland Vostok Station (Convey 1996b). More than ten glacial cycles have dominated the Antarctic landscape over the Pleistocene (~2 My[1]) and 'full' interglacial conditions have persisted over just 10% of the last 2 My (Barnes et al. 2006).

Terrestrial life, a high proportion of which is endemic (Pugh & Convey 2008) is restricted to the small ice-free pockets of accommodating habitat that are partitioned across just ~0.3% of the continent (British Antarctic Survey 2004). Antarctic terrestrial environments are characterised by low primary productivity and biodiversity (Convey 1996b) and have been remarkably stable from a geological perspective over the past few million years (e.g. Lawver & Gahagan 2003; Sugden et al. 2006; see also Convey et al. 2008). Continental Antarctica in particular has a limited thermal energy budget due to short summers with low air temperatures (an upper limit of 10ºC). Organisms there face elevated UV radiation, large daily fluctuations in temperature, very low (< -50°C) winter temperatures, extensive freeze/thaw events, desiccation stress and long periods of low water and nutrient availability (Convey 1996b).

Ecological (i.e. biotic) interactions (e.g. competition, predation) are thought to be relatively unimportant in the simple Antarctic terrestrial ecosystem (Convey 1996b; Hogg et al. 2006). Primary producers are restricted to algae, lower plants (bryophytes) and lichens, and the highest invertebrates in soil food webs are microarthropods (Block

[1] Throughout this thesis "My" refers to 'millions of years' and "Ma" refers to 'millions of years ago'

1984; Virginia & Wall 1999; Convey 2001). Under such circumstances, the impacts of abiotic factors are presumed to dominate (Convey 1996b; Sinclair & Sjursen 2001), but in reality, little is known about the effects of the Antarctic environment (both historical and contemporary) on either physiological or genetic parameters of populations.

However, repeated glacial cycles, physical barriers (e.g. ice sheets and glaciers) and environmental heterogeneity are likely to have had important effects on biological responses over short-, intermediate- and long-term (i.e. evolutionary) time-scales (Sinclair et al. 2003a). In particular, survival of taxa through glacial episodes has only been possible in ice-free refugia (Wise 1967; Hogg & Stevens 2002; Stevens & Hogg 2006; Convey & Stevens 2007). Populations that become restricted to such refugia may undergo divergence in isolation, leading to patterns of both physiological and genetic differentiation, with the ultimate potential result of speciation. Indeed, genetic differentiation within populations over both small (< 1 km) and intermediate (tens to hundreds of kilometres) spatial scales has been reported for several Antarctic terrestrial invertebrates (e.g. Frati et al. 2001; Fanciulli et al. 2001; Stevens & Hogg 2003; McGaughran et al. 2008, 2009a) and physiological diversification among populations has been demonstrated in studies of sub-Antarctic taxa (e.g. Chown et al. 1997; Klok & Chown 2003). Thus, Antarctica provides a 'natural laboratory' in which to study genetic and physiological structure among populations.

1.2.1.2 Springtails

Terrestrial life in Antarctica possesses well-developed stress tolerance abilities for the environmental conditions faced. In particular, invertebrates can experience daily temperature fluctuations of >30ºC, annual fluctuations as high as 80ºC, and frozen environments with long periods of restricted or zero resource availability (Peck et al. 2006). During these periods, invertebrates may spend considerable time inactive and uncoupled from ecosystem processes in a cryptobiotic survival state. Thus, when conditions allow, they must maximise activity, growth, development and progression through their life cycle. In addition, they must obtain sufficient resources within the limited 'summer' growing season (ranging from < 1 to 6 months across the geographically widespread Antarctic habitats) with which to survive the long inactive

winter (Convey 1996b; Worland & Convey 2001). This places a limit on the range of life history strategies available to polar invertebrates, and many species display common 'solutions' to the problems imposed by Antarctic existence. Indeed, Convey (1996b) notes that in such an extreme environment, characteristics of the Antarctic habitat itself may be responsible for the evolution of certain aspects of organism life histories.

In practice, however, survival of terrestrial taxa requires very little morphological specialisation to the Antarctic environment. Indeed, while Antarctic arthropods are generally classified as A-/S- (adversity or stress) selected (Greenslade 1983; Convey 1997) most of the underlying strategies employed are ancestrally characteristic of the taxonomic groups concerned. These include many morphological (e.g. melanism, wing reduction), behavioural (e.g. habitat selection, thermoregulation, activity patterns), ecological (e.g. life cycle extension) and physiological (e.g. freeze tolerance and/or avoidance, elevated metabolic rate, desiccation resistance, supercooling) strategies (Block 1990; Sinclair & Sjursen 2001). In addition, multiple life stages of many Antarctic terrestrial taxa are capable of over-wintering (Convey 1996b; Hayward et al. 2003) and the phenomena of increased life spans and rapid growth rates under short-term favourable conditions have been widely reported in polar invertebrates (see Convey 1997).

The springtail fauna of Antarctica (approximately 25 species; Greenslade 1995), includes a high proportion of endemics. Springtails are often the numerically dominant arthropod of Antarctic terrestrial habitats, and many species have wide (although fractured) distributions. They are often endemic in their respective Antarctic regions (Pugh & Convey 2008) and are restricted to areas of high soil moisture and/or access to water (e.g. lake edges, snow patch edges, moist river beds, glacier foregrounds, vegetated areas) (Kennedy 1993). Where present, springtails generally occur in the soil and vegetation, and on the underside of rocks, which provide shelter from wind and desiccation (Stevens & Hogg 2002). In fact, Antarctic springtails have been referred as model organisms on account of the extent of physiological research that they have been the focus of, and their dominant role in Antarctic terrestrial ecosystems (Sinclair et al. 2003b).

1.2.2 Evolution's raw material: individual variation

1.2.2.1 Metabolic rates

The low thermal energy budget of terrestrial habitats is an important feature which essentially dictates the scope of Antarctic life. Several factors contribute to the energy budget of organisms, including activity, growth and metabolism. In particular, 'metabolism' defines the complex of biochemical reactions that govern the flow of energy and transformation of materials in organisms (Hochachka & Somero 2002). Because it affects the rate of most other organic processes (e.g. rates of survival, growth and reproduction), metabolic rate may be considered a fundamental biological rate (Brown et al. 2004).

In Antarctica, arthropods may employ an opportunistic metabolic strategy that enables exploitation of small thermal increments within microhabitats through elevation of metabolic rates to allow activity at temperatures that would immobilise temperate forms. This is known as metabolic cold adaptation (MCA), whereby an animal living at low temperature is described as cold-adapted if its metabolic rate is greater than that of a comparable temperate species measured at the same temperature (Block 1990). The mechanisms underlying this ability remain to be clarified. However, this hypothesis essentially relates to a special case of spatial variation in the expression of population metabolism in response to environmental differentiation. Such adaptation demonstrates the potential ability of terrestrial Antarctic invertebrates to 'tune in' to the temporal variation in their local microhabitat and respond appropriately (Convey 1996; Worland & Convey 2001).

Optimal utilisation of positive developmental periods as they occur (Worland & Convey 2001) is an important element of Antarctic invertebrate life history strategy, because costs will be incurred if organisms are stimulated to respond inappropriately to misleading environmental cues. Since energy input to different areas of Antarctica varies (exposing resident taxa to different degrees of environmental severity that include lower temperatures and shorter growing seasons at more 'extreme' sites), variable rates of energy turnover across locations are expected (Chown & Storey 2006), and from this, evolutionary effects will likely cascade. Thus, Antarctic terrestrial species are expected to show a coordinated response to environmental selective pressures and studies of such

responses will increase knowledge about the ways in which species are able to persist in the face of environmental change.

1.2.2.2 Genetics

In addition to physiological evolution of life history parameters such as metabolic rate, populations undergo genetic evolution in response to environmental variation over time. Environmental processes may therefore influence the ways in which populations and species are structured genetically. In addition, ecological forces such as isolation, local extinction and recolonisation, and geographical forces (both tectonic and palaeobiological) are important factors that affect the observed genetic variation both within and among natural populations (Harrison & Hastings 1996).

As mentioned earlier, patterns of genetic differentiation (expressed as low levels of connectivity between populations and/or large variability within populations) are common in Antarctic terrestrial invertebrates (e.g. Frati et al. 2001; Fanciulli et al. 2001; Stevens et al. 2007; McGaughran et al. 2008, 2009a). This has been inferred to be a consequence of the effects of Plio-Pleistocene glaciations over the last ~2 My (and longer) on species ranges and population sizes (e.g. Stevens & Hogg 2003). In particular, the fragmentary nature of the Antarctic landscape in conjunction with the perceived limited dispersal abilities of most Antarctic terrestrial taxa, have resulted in common patterns of divergence among populations that have been isolated for some time.

As populations become isolated in space, divergence over time may result in allopatric speciation. For most Antarctic taxa, this is likely an ongoing process. Indeed, potential incipient speciation has been detected in one continental Antarctic springtail (Stevens & Hogg 2003) and the genetic structure of the springtail *Cryptopygus antarcticus antarcticus* around the Southern Hemisphere has recently been suggested as harbouring a number of cryptic species (Stevens et al. 2006). Patterns of regionalisation and differentiation are also evident in the Antarctic marine benthos where evolutionary patterns detected by classical and molecular phylogenetic approaches also require the parallel existence of multiple refugia (e.g. Allcock 2005, Linse et al. 2006; Raupach & Wägele 2006; Lörz et al. 2007).

Despite the potential difficulties of dispersal in many Antarctic terrestrial organisms, re-colonisation events from refugia following glacial periods are also likely to be important components of population structure in Antarctic terrestrial organisms. In particular, dispersal following oceanic and atmospheric currents and occasional successful long-distance dispersal events over land has been detected in springtail populations (e.g. Stevens & Hogg 2002; Hughes et al. 2006; McGaughran et al. 2008).

Thus, genetic studies based on Antarctic taxa can provide insight into evolutionary pathways, including species origins and relationships and connectivity (both historical and contemporary) between populations.

1.2.2.3 Mutation rates

Despite the considerable literature exploring general physiological and genetic patterns and processes, terrestrial invertebrate evolutionary principles in the cold are largely unexplored (but see Clark et al. 2004), and little is known about how evolutionary (mutation) rates vary within and between taxa with environmental/climatic changes (Held 2001).

The role of life history parameters in affecting evolutionary rates both generally and in polar climates gives rise to the 'metabolic rate', 'body temperature' and 'generation time' hypotheses (Ritchie et al. 2004 and references therein). In each of these, the respective parameter is given ultimate responsibility for 'controlling' evolutionary rate (e.g. Martin & Palumbi 1993; Gillooly et al. 2001; Allen et al. 2006). For example, metabolic rate has been mechanistically related to the molecular process of mutation – this effect being mediated by oxygen radicals (highly reactive molecules with free electrons that can damage DNA directly by attacking the sugar-phosphate backbone or nucleotides) (Barja 1999; Cooke et al. 2003). Documented effects of body temperature mostly relate to the consequences of higher temperatures increasing reaction rates through speeding up molecular movement (e.g. Gillooly et al. 2005), or conversely lower temperatures leading to lower efficiency of protein manufacture (Fraser et al. 2002). Finally, a theoretical association between generation time and accumulation of nucleotide substitutions is expected if most substitutions are via replication errors and if there are broadly similar numbers of cell divisions per generation of species: species

with a short generation time will experience similar numbers of cell replications and the same degree of DNA substitution per generation as do long generation time species, but will accumulate a greater number of DNA changes per year (Martin & Palumbi 1993; Mooers & Harvey 1994).

In practice, it is difficult to tease apart the effects of each of these mechanisms. For example, in animals the association between metabolic rate and DNA substitution rate is confounded by correlations between these parameters and body size, generation time, and other physiological life history variables. Thus, current thinking tends to point to an overall 'metabolic theory of ecology' (Gillooly et al. 2001; Brown et al. 2004; West & Brown 2004; Allen et al. 2006). In this theory, two fundamental variables influence the tempo of evolution – the generation time and the mutation rate, and both are direct consequences of biological metabolism. They are governed by the body-size and temperature dependence of mass-specific metabolic rate (Allen et al. 2006). While the mechanistic view behind this theory is a subject of controversy in the literature (e.g. Makarieva et al. 2008), most authors do not refute the evident relationships that exist between metabolic rate, temperature, several other life history variables, and evolutionary rate.

Importantly, much of the empirical work attempting to investigate this issue further has drawn from large inter-specific datasets (e.g. Gillooly et al. 2001, 2007; Brown et al. 2004; West & Brown 2004; Allen et al. 2006). Investigations to find a direct link between metabolic rate and the rate of DNA substitution in polar environments, and from intra-specific platforms, are notably absent. Such work would potentially advance metabolic-based theories of the mutational process by enhancing knowledge about the mechanistic basis by which evolution proceeds.

1.3 FROM CONCEPTS TO PRACTICE

Having introduced the underlying concepts relevant to the scope of this thesis, the following section serves to explain how the individual chapters that follow use these concepts in practice.

1.3.1 Chapter detail

This thesis employs a multi-faceted research strategy encompassing both physiological and molecular genetic approaches to study evolution in Antarctica. Chapter Two investigates spatial and temporal variation in metabolic rates of an Antarctic springtail. Variation in physiological responses over spatial and temporal scales is known to exist in terrestrial Antarctic systems (e.g. Sinclair 2001; Hugo et al. 2004; Adams et al. 2006; Peck et al. 2006; Chown & Convey 2007). However, prior to the work presented in Chapter Two, little was known about metabolic responses to the unpredictable Antarctic environment, particularly with regard to terrestrial biota (although logical deduction allowed the assumption that native species should be able to respond to their local environmental conditions in a timely and efficient manner, e.g. Convey 1996). In fact, metabolic rate had never been investigated in a continental Antarctic springtail. Thus, Chapter Two addresses questions regarding metabolism through examination of seasonal variation in metabolic rates of the continental Antarctic springtail *Gomphiocephalus hodgsoni*. The study results are evaluated in the ecological context of species response to environmental cues in the form of microhabitat variation. This work is published in the *Journal of Insect Physiology* (McGaughran et al. 2009b).

Chapter Three extends the work of Chapter Two by examining metabolic rate variation in *G. hodgsoni* at two further scales: (1) annual (variation across seasons); and (2) spatial (variation within a season across locations). The objective of this work is to explore how differences in physiological attributes among populations may evolve and how they can be affected by environmental factors. Specifically, metabolic measures made in a second season (with a different environmental profile) at continental Cape Bird are examined to expand knowledge about the ways in which organisms respond to temporal variation in environmental conditions. In addition, metabolic rates are reported for *G. hodgsoni* collected from two additional inland locations to examine the influence of spatial variation. This spatial variation is largely influenced by differential environmental characteristics, such that the inland locations experience lower temperatures, potentially drier conditions and shorter growing seasons than does Cape Bird. Such differences may lead to lower rates of energy turnover and greater allocation of resources to survival (hence lower metabolic rates) for the inland populations.

Alternatively, greater metabolic adaptation may mean that individuals are better able to utilise the more limited opportunities of a shorter active season at the inland sites. Thus Chapter Three explores temporal and spatial structure in metabolic rates in the context of underlying ecological, physiological and genetic differences among populations. This work is published in the *Journal of Insect Physiology* (McGaughran et al. 2009c).

In Chapter Four, differences among populations are examined further in the twin evolutionary contexts of molecular and physiological divergence using the springtail *Cryptopygus antarcticus travei* from sub-Antarctic Marion Island. Historical geographic barriers on Marion Island are known to have led to population differentiation from a molecular perspective for many of its inhabitant species (e.g. Mortimer & Jansen van Vuuren 2006; Myburgh et al. 2007). The same environmental characteristics that have influenced this genetic differentiation may also be responsible for sustaining physiological differentiation of populations across this island. Thus, in this chapter, a multi-disciplinary approach is employed that generates metabolic rate and molecular genetic datasets for *C. a. travei* from several populations across the island. This dataset is used to assess metabolic rate structure in the context of underlying molecular genetic structure and systematic environmental variation. The work presented in Chapter Four is submitted to the journal *Polar Biology*.

Chapter Five extends the work of Chapter Four by further examining the possible link between metabolic rate and the tempo of DNA evolution in a theoretical context. In particular, this work investigates increasing evidence for a postulated relationship between physiological parameters and rates of DNA evolution. Portions of the mitochondrial (mtDNA[2]) and metabolic rate datasets developed for *C. a. travei* in Chapter Four are used to examine whether a correlation between mass-specific metabolic rate and root-to-tip (i.e. genetic) distance on a set of mtDNA trees can be detected, and whether population metabolic rates are in any way related to their underlying phylogeny. Thus, the 'metabolic rate hypothesis' of nucleotide mutation is tested using data on relative intra-specific genetic divergence. The work presented in Chapter Five is submitted to the journal *Evolution*.

[2] This abbreviation will be used hereafter to refer to 'mitochondrial DNA'

An appreciation of the fundamental role of genetic processes to the overall evolution of populations underlies Chapters Six and Seven, which each employ molecular genetic approaches to further decipher the evolutionary history of Antarctic springtails. Chapter Six presents parallel phylogeographic studies of the springtails *Cryptopygus antarcticus antarcticus* and *G. hodgsoni* from the Antarctic Peninsula and continental Antarctica, respectively. These species are the most common springtails in their respective regions and are thought to have had similarly long continuous associations with the Antarctic landscape (e.g. Convey & Stevens 2007; Convey et al. 2008). Using information in mtDNA cytochrome *c* oxidase subunit I (*cox1*) and II (*cox2*) genes, this work aims to identify refugia and colonisation routes, and describe population structure and demography separately for *G. hodgsoni* and *C. a. antarcticus*. In addition, population genetic and demographic patterns for these species are compared to determine whether postulated common evolutionary histories in the separate Antarctic regions have resulted in similar genetic signatures across these contemporary populations. This work is published in the *Journal of Biogeography* (McGaughran et al. 2009a).

Finally, Chapter Seven presents a regional phylogenetic analysis of the springtail genus *Cryptopygus* and its close relatives (Isotomidae). Samples from several locations throughout continental-, maritime- and sub-Antarctica are examined at three different genetic loci in order to investigate relationships among species and test hypotheses about species origins. The origin of many Antarctic species remains unclear (e.g. Frati et al. 2000), however species distributions are thought to reflect a varied derivation for many springtails (Frati & Carapelli 1999) that includes both relic and more recent immigrant species (e.g. Wallwork 1973; Greenslade 1995; Marshall & Pugh 1996; Marshall & Coetzee 2000; Pugh & Convey 2000). Chapter Seven examines this evolutionary dichotomy of old and young with a view to establishing the biogeographical pathway of *Cryptopygus* species in Antarctica and elucidating connectivity among locations. In addition to building a more accurate reconstruction of the phylogenetic relationships among these particular Antarctic springtail species, the relative importance of forces responsible for the contemporary distribution of Antarctic

Cryptopygus are evaluated using dispersal-vicariance analysis. The research presented in Chapter Seven is submitted to the journal *Molecular Phylogenetics and Evolution*.

Chapter Eight completes the thesis with a section entitled *Future Research*, which outlines plans to continue some of the work begun here and also suggests future areas of investigation for the respective research fields. This is followed by a thesis summary. Finally, off-prints of two additional (published) research papers completed during the course of the PhD period are included in an Appendix at the end of the thesis. The first (McGaughran et al. 2008), is based on research completed during Masterate study, with further analysis and write-up occurring during the PhD period; the second (Stevens et al. 2007), is based on new research performed during the PhD period.

1.4 CONTRIBUTION OF CO-AUTHORS

Acknowledgements follow the main body of work in each chapter. Beyond this, the work presented here is my own, however it has benefited from contribution of co-authors and this section serves to outline those contributions in detail.

In the case of the physiological work, I was responsible for all project design, funding and field-work. Comments on early project design for Chapter Two were provided by Pete Convey. I performed all analyses for the physiological data, except the GLM analyses in Chapter Four, which were performed by Steven Chown. Gabe Redding provided me with a script to analyse metabolic rate data and also assisted me with equipment design and maintenance (Chapters Two, Three and Four). The co-authors contributed in some form to the writing for Chapters Two, Three and Four, however in all cases, the majority of this work is my own.

I performed all molecular genetic work (Chapters Four to Seven) with a few exceptions: Giulia Torricelli and Mark Stevens performed some of the sequencing in Chapters Six and Seven, respectively; while colleagues from the University of Siena contributed significantly to the *cox2* dataset for *Cryptopygus antarcticus antarcticus* in Chapter Six. Project design for Chapter Five was based on my own ideas, with the expertise of Barbara Holland allowing them to be tested. In particular, Barbara showed me how to use many of the phylogenetic programmes employed, and also walked me

through the analyses in her capacity as co-supervisor. This extended to providing comments and discussion on the manuscript, the final draft of which was also commented on by David Penny. I designed the project in Chapter Six and performed all of the analyses. The co-authors contributed comments on a final version of the manuscript. Initial project design for Chapter Seven was based on contributions from Mark Stevens. All subsequent design and analyses were based on assistance from Barbara Holland and discussions with Matthew Phillips, Jing Wang and David Penny. A final draft of the manuscript was contributed to by the co-authors.

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CHAPTER TWO:
TEMPORAL METABOLIC RATE VARIATION IN A CONTINENTAL ANTARCTIC SPRINGTAIL

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2.1 ABSTRACT

Terrestrial systems in Antarctica are characterised by substantial spatial and temporal variation. However, few studies have addressed the paucity of data on metabolic responses to the unpredictable Antarctic environment, particularly with regard to terrestrial biota. This study measured metabolic rate variation for individual springtails at a continental Antarctic site using a fiber-optic closed respirometry system incorporating a custom-made respiration chamber. Concurrent measures of (behavioural) activity were made via daily pitfall counts.

Metabolic rate of *Gomphiocephalus hodgsoni* measured at constant temperature varied systematically with progression through the austral summer, and was greatest mid-season. This finding of clear intra-seasonal and temperature-independent variation in mass-specific metabolic rate in *G. hodgsoni* is one of very few such reports for a terrestrial invertebrate (and the only such study for Antarctica), and parallels physiological studies in the Antarctic marine environment linking metabolic rate elevation with biological function rather than temperature adaptation *per se*. However, response to temperature at relatively short time-scales is also likely to be an important part of the life history strategy of Antarctic terrestrial invertebrates such as *G. hodgsoni*, which appears capable of both physiologically and behaviourally 'tuning' in to short-term thermal variability to respond appropriately to the local unpredictable Antarctic habitat.

2.2 INTRODUCTION

Terrestrial ecosystems in Antarctica are characterised by substantial spatial and temporal variation at several levels encompassing both the organismal and the ecological (e.g. Sinclair 2001; Hugo et al. 2004; Lawley et al. 2004; Adams et al. 2006; Peck et al. 2006; Chown & Convey 2007). In particular, temporal variation among individuals is strongly linked with phenotypic plasticity, which appears prevalent in Antarctic terrestrial species and may be strongly influenced by environmental unpredictability (Chown & Convey 2007). Indeed, life histories of terrestrial taxa tend

to be dominated by responses to the seasonally variable, 'stressful' environment (Convey 1996; Vernon et al. 1998) in conjunction with the low Antarctic energy budget. Environmental constraints such as large daily microhabitat temperature fluctuations (> 30°C) and long periods of limited resource availability (Peck et al. 2006) are reflected in terrestrial arthropods by slow growth rates and extended life cycles compared to their temperate counterparts (Convey 1996).

Optimum utilisation of positive developmental periods as they occur (Worland & Convey 2001) is another facet of this system because costs will be incurred if organisms are stimulated to respond inappropriately to misleading environmental cues. Thus, Antarctic terrestrial species are expected to show a coordinated response to environmental selective pressures. Sinclair et al. (2003) note that such a response may manifest over three broad scales: daily (short-term), seasonal (intermediate) and evolutionary (long-term). Rapid (daily) response strategies would seem particularly beneficial in the context of the unpredictable Antarctic climate, enabling individuals to track changes in habitat temperature and respond appropriately. This is demonstrated by the maritime Antarctic isotomid springtail *Cryptopygus antarcticus antarcticus*, which has the ability to vary its supercooling point within a matter of hours in response to natural environmental variations in temperature (Worland & Convey 2001). This species also exhibits an evolutionary response developed over the longer term to cope with the short growing season imposed by the Antarctic environment – its metabolic rate is elevated relative to that of comparable temperate relatives (a phenomenon known as 'metabolic cold adaptation' (MCA)) (Dunkle & Strong 1972; Block & Tilbrook 1975, 1978; Block 1990). Over intermediate (seasonal) time-scales it is now largely accepted that, in the thermally stable Antarctic marine environment where MCA has not been identified, systematic seasonal variation in individual metabolic rates is driven by the requirement for respiration products (i.e. ATP) to fuel specific processes such as food digestion or egg maturation (see Clarke 1993, 1998; and Peck et al. 2006 for review).

Unfortunately, variation in metabolic rates over all three time-scales has only rarely been investigated in terrestrial invertebrates (Zinkler 1966; Block & Tilbrook 1975, 1978; Kauri et al. 1975; Block & Young 1978; Testerink 1983; van der Woude &

Joosse 1988), and very few studies have attempted to examine field metabolic rate variation at the intraspecific level for either range-restricted species or invertebrates in general. Metabolic studies of terrestrial Antarctic Collembola are particularly limited to early work, which focused on *C. a. antarcticus* (Block & Tilbrook 1975, 1978), and the metabolism of continental Antarctic arthropods remains unstudied.

This lack of research data may have considerable significance in an evolutionary context, since springtails are likely to have had a continuous (isolated) history within continental Antarctica over time-scales sufficient for development of evolutionary responses to extreme environmental stresses imposed by glaciation processes (Stevens et al. 2006; Convey & Stevens 2007; Convey et al. 2008). The paucity of metabolic investigations in the Antarctic is also unfortunate in that metabolic rates are often related to the overall energy turnover of populations, to the extent that they may be responsible for determining species distribution patterns, abundances and range limits (Novoa et al. 2005; Slabber & Chown 2005). Furthermore, maximum and basal metabolic rates are likely to determine survival in extreme circumstances. In particular, phenotypic plasticity in metabolism may be crucial for maintenance of an individual's energy budget in the face of changing environmental conditions (Novoa et al. 2005).

Thus, the aim of the current study was to examine whether systematic metabolic rate variation was detectable over time when measured at constant temperature through the austral summer in the endemic continental Antarctic springtail *Gomphiocephalus hodgsoni* Carpenter, 1908 (Collembola: Hypogastruridae). This was made possible through the development of a novel and sensitive technique that permitted measurement of individual animal metabolic rates. A supplementary aim was to evaluate whether patterns of metabolic rate variation could be placed into an ecological context by attributing them to environmental cues at the microhabitat scale, and by comparing them to concurrent measures of behavioural activity (via pitfall traps) on intra-seasonal time-scales.

2.3 METHODS

2.3.1 Location and species

Cape Bird (77º13'S, 166º26'E) is an ice-free area at the northern tip of Ross Island in the Ross Sea Region of Antarctica (Fig. 2.1). Work was carried out from 29 November 2006 to 29 January 2007 (encompassing the bulk of the austral summer at this site) and all animal collections were made under permit in a < 10 m^2 area southeast of Cape Bird research hut (Antarctic Specially Protected Area (ASPA) 116; http://www.era.gs/resources/apa).

The springtail *G. hodgsoni* was used for all analyses in this study. This species, endemic to southern Victoria Land, is a representative of a dominant arthropod group inhabiting ice-free regions throughout Antarctica, and is the only springtail present at this location, where the population is a single evolutionary unit (ESU) (McGaughran et al. 2008). Collections of large (non-juvenile) animals were made by gently sweeping individuals off the underside of rocks into a collection vessel using a very fine paintbrush.

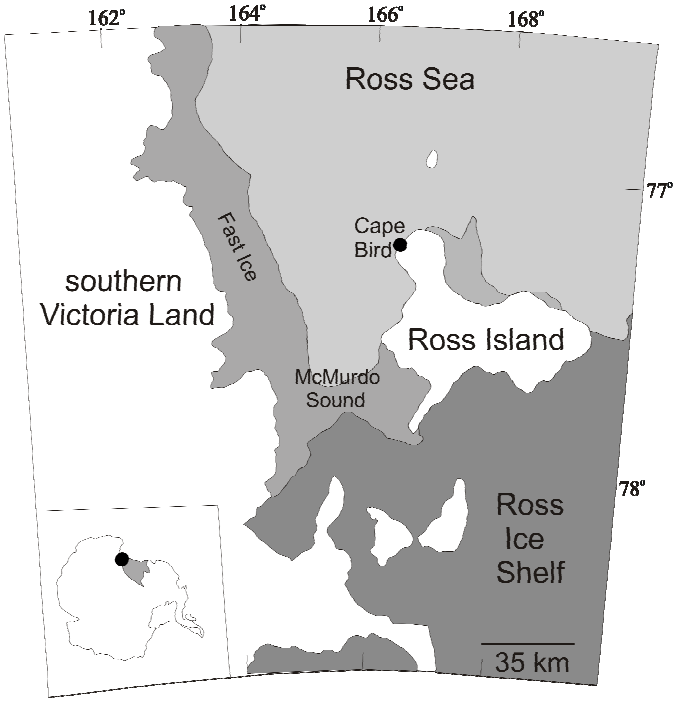

Figure 2.1 Location of Cape Bird in the Ross Sea Region of Antarctica.

2.3.2 Metabolic rate measurements

2.3.2.1 Equipment used and measurement technique

A fiber-optic oxygen sensing system (Ocean Optics Inc., USA) was used to monitor oxygen partial pressure (pO_2) over time for individual animals in a closed respirometry system. The oxygen sensing system was calibrated for multiple temperatures and oxygen percentages by the manufacturer, and was then calibrated before each reading using both the calibration data provided (Ocean Optics) and a single point calibration in air (20.95 vol % oxygen, as recommended by the manufacturer). Following calibration, individual 'field-fresh' animals that had been collected from the underside of rocks between 0900 – 1500 h and then stored for 1-2 hours at the research hut ambient temperature (~6 – 8ºC), were placed in a custom-made 40 µl volume chamber (Fig. 2.2), into which the oxygen probe was inserted. The chamber was sealed with a glass slide pressed firmly against a rubber o-ring. A metal 'lid' was then screwed down over the glass slide to ensure an airtight seal. During a 3 hr period, the partial pressure of oxygen in the chamber was recorded continuously.

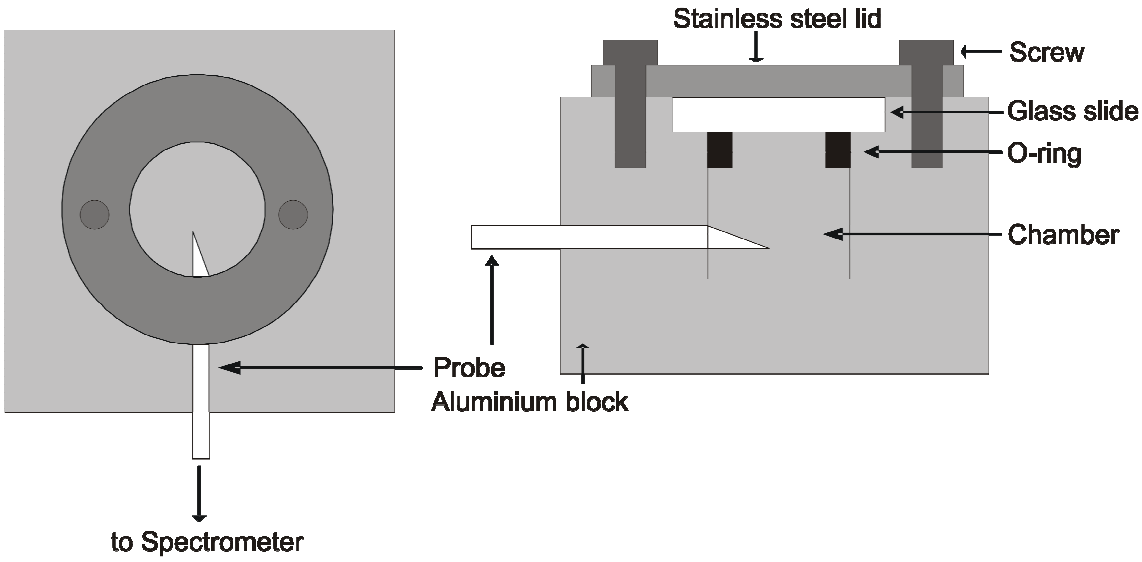

Figure 2.2 Custom-made respiration chamber used to measure metabolic rate of individual *Gomphiocephalus hodgsoni* specimens; left, top view; right, side view.

Temperature, held at 10 ± 0.1°C using a custom-made heating chamber, was monitored concurrently using oxygen sensing software during each measurement run

(OOISENSOR ver. 1.05, OceanOptics Inc., USA). This chamber temperature was somewhat higher than the mean summer microhabitat temperature (~3.5ºC) over the entire two month period at Cape Bird, but well within the range experienced within the species' typical microhabitats (Janetschek 1963; Fitzsimons 1971; Sinclair & Sjursen 2001; Sjursen & Sinclair 2002). Analysis of temperature data during the 0900 – 1500 h collecting times for the 40 days of metabolic rate measurements showed that the mean temperature during this time was 7.4º ± 0.5ºC [S.E.M.].

Upon completion of a run, partial pressure profiles were used to calculate oxygen consumption rates on a per animal basis for each individual, using the observed drop in pO_2 over a given time period in conjunction with the chamber volume. The first ten minutes of each run was discarded prior to this analysis in order to allow for system equilibration and animal adjustment to the measurement chamber. Individual animal mass was used to express corresponding oxygen consumption rates on a mass-specific basis. As accurate mass measurement was not possible at this remote study location, a photographic technique was used to estimate mass using the relationship: $W = 6.1894L^{3.119} \times 10^{-9}$ (after Block & Tilbrook 1975), where W = mass (μg), L = length (μm), as modelled for the similarly sized springtail *C. a. antarcticus*. Photographs of individual springtails on a background grid were taken with a camera kept at a constant height using a fixed tripod. The length of each photographed individual was then measured using software (SCREEN CALIPERS ver. 3.3 [Iconico, Inc. 2006]) calibrated to the background grid. As estimated by this method, adults of *G. hodgsoni* used in this study had a mean live mass of 19.2 ± 1.5 μg [S.E.M.].

2.3.2.2 Equipment sensitivity

Given the small size of *G. hodgsoni*, respiration rates were expected to be near to the resolution limit of the oxygen sensing system (see www.oceanoptics.com). Hence, blank runs (n = 10) were completed following the protocol above except without the addition of an animal to the measurement chamber.

2.3.2.3 Equipment application

The methodology described above was repeatedly applied over a 40 day period (20 December 2006 – 28 January 2007) on a total of 53 field-fresh individuals in order to examine temporal variation of metabolic rates during the austral summer. Field restrictions meant that data could not be collected on all days, or at all times during particular days, hence the number of measurements made per day varied from 0 – 5. Microclimate data was also collected over this time to allow investigation of any relationship(s) between metabolic rate variation and changes in the microclimate (see below).

2.3.3 Pitfall traps

In parallel to measurement of metabolic rates, a measure of behavioural activity throughout the field period was obtained using pitfall trapping. In brief, small plastic traps (n = 10) consisting of a vial (4.5 cm diameter; 6 cm depth) partially filled with mono-ethylene glycol and placed in a PVC sleeve, were sunk into the ground in the same < 10 m^2 area c. 1 m apart on 30 November 2006. These traps were placed within the general vegetated area as described in the ASPA 116 management plan (see References, section 2.6), but without disturbing the sensitive bryophyte vegetation protected by this plan. Traps were checked twice daily (at 0900 and 2100 local time; encompassing the period of direct sunlight at this site for most of the summer) for presence of springtails. Field practicalities dictated that traps were operated for 4 consecutive days, and closed every 5^{th} day. We recognise that activity data collected in this manner may be compromised by systematic progression through different life stages in species with synchronised development. Unfortunately, field developmental data are not available for *G. hodgsoni*, although the general features typical of life cycles of Antarctic terrestrial invertebrates (multi-year life cycles, limited growth opportunity per season, lack of synchronisation, overlapping of generations; Convey 1996) mean that such a developmental pattern is unlikely to apply to this species.

2.3.4 Microhabitat parameters

iButton thermochron and hygrochron data loggers (DS1921, DS1923; Maxim Integrated Products, Sunnyvale CA, USA) were used to monitor temperature (± 0.5ºC) and relative humidity (± 0.6%) every 30 minutes over the study period. Five iButtons were placed in soil under rocks (with surface area < 100 cm^2) representative of those chosen for springtail collection. Replicate temperature (T) and relative humidity (RH) data were then processed to obtain mean maximum (max), minimum (min), mean (α) and range (R) statistics for the day of metabolic rate measurement (D_0) and days 1 (D_{-1}), 3 (D_{-3}) and 5 (D_{-5}) preceding the day of measurement. These latter three measures were made in order to evaluate whether any variation in metabolic rates related to the microclimate was subject to a time lag.

2.3.5 Statistical analyses

All statistical analyses were performed using MINITAB (ver. 14, Minitab Inc., Pennsylvania, United States). Variation in relative metabolic rates was analysed by grouping the data into four 10-day measurement blocks (End December ('ED'; n = 15); Beginning January ('BJ'; n = 14); Mid January ('MJ'; n = 11); and End January ('EJ'; n = 13)). This was necessary in order to provide significant resolution of the metabolic rate variation for the period in question because field restrictions meant that data could not be collected on all days. Following normality tests, ANOVAs, where appropriate with Tukey's *post-hoc* pairwise comparisons, were used to determine whether live mass, metabolic rate and environmental temperature differed significantly between measurement blocks.

Linear least-squares regression analyses were performed to determine the relationships between metabolic rate and microhabitat data (RH, T_{max}, T_{min}, T_{α}, and T_R on D_0, D_{-1}, D_{-3} and D_{-5}). A Spearman's rank correlation was performed by ranking the metabolic rates of each period according to both the time period, and the mean environmental temperature of the period in question.

Numbers of springtails caught daily in all pitfall traps were analysed using ANOVA to examine the degree of temporal variation in behavioural activity. Linear least-

squares regression analyses were performed between daily pitfall counts and daily environmental temperature variables (T_{max}, T_{min}, T_{α}, T_R and RH, on D_0).

2.4 RESULTS

2.4.1 Metabolic rates

2.4.1.1 Equipment sensitivity

Blank measurement runs (n = 10) generated very low estimates of equipment generated 'noise', averaging 0.004 ± 0.002 $\%O_2.hr^{-1}$ [s.d.]. This was much lower than, and significantly different from, rates obtained during runs using individual live animals (mean for all data: 0.179 ± 0.068 $\%O_2.hr^{-1}$ [s.d.] (t_{52} = 18.71; P < 0.001) (Fig. 2.3). Hence, the resolution achieved by this equipment and protocol is appropriate to permit measurement of true rates of metabolism in *G. hodgsoni*.

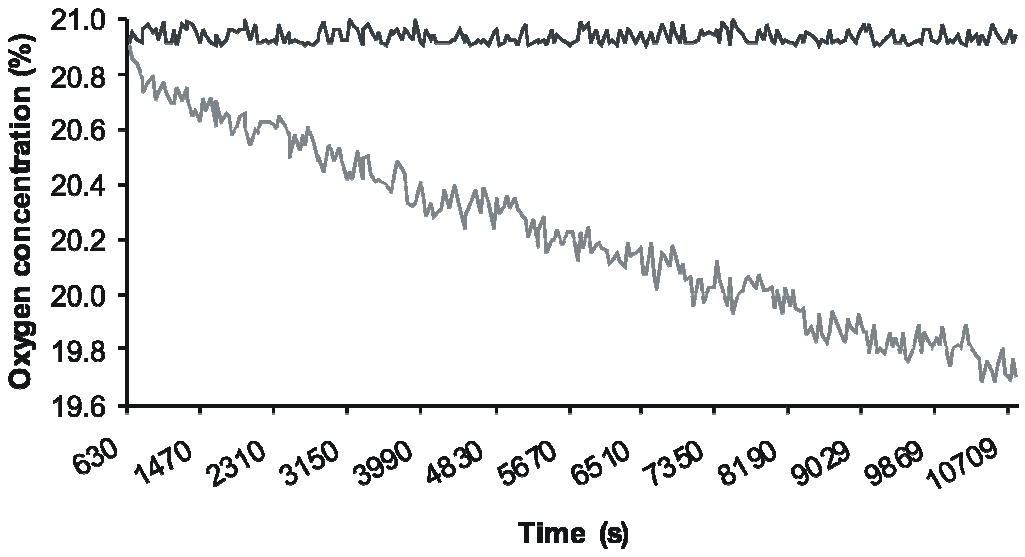

Figure 2.3 Illustrative changes in percentage oxygen over time in the metabolic chamber for a 'blank' run with no animal present (black line) and an experimental run (dark grey line) using an individual of *Gomphiocephalus hodgsoni*. Note that readings start at 630 seconds because the first ~10 minutes of each run was excluded from analysis in order to allow for system equilibration and potential animal adjustment to the measurement chamber.

2.4.1.2 Metabolic rate variation

Mean mass-specific metabolic rates of field-fresh animals measured at 10ºC varied significantly across the different measurement periods (Fig. 2.4), thus an intra-seasonal effect on metabolic rate was apparent ($F_{3,52}$ = 4.09; P = 0.012). In particular, metabolic rates in the ED period were significantly greater than those in the MJ and EJ periods (Tukey's *post hoc* P < 0.05). Body mass of measured animals did not differ significantly across the 10-day data blocks ($F_{3,52}$ = 1.30; P = 0.287), however there were significant differences in the microclimate data obtained during these periods (see below).

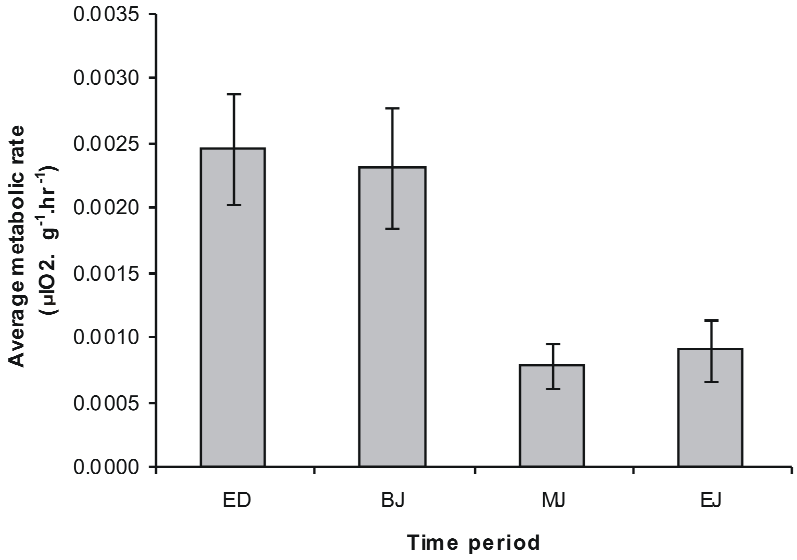

Figure 2.4 Mean metabolic rate (± S.E.M.) of *Gomphiocephalus hodgsoni* individuals over four 10-day blocks ('ED' = end December; 'BJ' = beginning January; 'MJ' = middle January; 'EJ' = end January) in 2006/07.

2.4.1.3 Metabolic rate variation and microclimate variables

Microhabitat (under rock) temperature did not drop below -1.9ºC and was relatively stable (mean 3.5 ± 0.4ºC [S.E.M.]) over the period of recording (Table 2.1). In particular, maximum, minimum and mean temperatures under rocks at the end of January were 3.5ºC, -0.2ºC and 2.2ºC warmer than those measured at the beginning of the study period, respectively. Like temperature, the microclimate data indicated that

relative humidity remained reasonably stable across the season. Despite the general increase in microhabitat temperature as the season progressed, an ANOVA showed that the significant differences across the four 10-day blocks ($F_{3,22} = 4.18$; $P = 0.017$) did not conform to a successive change, as the highest mean temperatures occurred during the MJ period followed by: EJ > BJ > ED (Table 2.1).

Table 2.1 Mean (S.E.M.), maximum, minimum and range of temperature and relative humidity in air, 2 – 3 cm in soil, and under a rock, during the entire period 29 November 2006 – 29 January 2007. Full, 24-hour data for the periods during which metabolic rate analysis took place are also provided for under rock temperature ('ED' = end January; 'BJ' = beginning January; 'MJ' = middle January; 'EJ' = end January; see text).

Variable	Level	Mean	Max	Min	Range
Temperature (ºC)	Air	3.5(0.1)	15.2	-2.9	18.1
	Soil	3.4(0.1)	22.0	-3.0	25.0
	Rock	3.5(0.4)	17.1	-1.9	18.9
	ED	2.1(0.7)	6.5	-0.8	7.3
	BJ	3.6(1.1)	8.9	-0.2	9.1
	MJ	5.9(0.8)	11.9	0.6	11.3
	EJ	5.5(0.7)	12.6	-0.3	12.9
Relative humidity (%)	Air	72.1(0.4)	108.5	26.0	82.5
	Soil	108.6(0.1)	115.8	75.8	40.0
	Rock	114.4(0.4)	121.8	97.1	24.7

Metabolic rate of field-fresh animals and environmental temperature were positively correlated (Spearman's r = 0.297; P = 0.020). Regression analyses identified the strongest significant relationships between metabolic rate and T_{min} on D_0 ($F_{1,18} = 10.87$, $r^2 = 37.6\%$, $\beta = -0.000775$; $P = 0.004$) followed by metabolic rate and T_{min} on D_1 ($F_{1,18} = 9.67$, $r^2 = 34.9\%$, $\beta = -0.000793$; $P = 0.006$) (Table 2.2).

2.4.2 Behavioural activity

2.4.2.1 Pitfall activity variation

Pitfall activity varied significantly across the season and, like metabolic rate, peaked in the ED period when analysed in 10-day study blocks ($F_{5,444} = 4.14$; $P = 0.001$). An additional peak was also recorded for pitfall activity in the EJ period (Tukey's $P < 0.05$) (Fig. 2.5).

Table 2.2 Results of the regression analyses of metabolic rate against various environmental (microhabitat) temperature variables for *Gomphiocephalus hodgsoni*. Upper value in table is the F-test value (df = 1,18 for all F-values), middle value is the regression coefficient (r^2), lower value is the regression slope (β); '*' indicates $P < 0.05$

Time	T_α	T_{max}	T_{min}	T_R
D_0	7.74*	5.87*	10.87*	3.51
	30.10	24.60	37.60	16.30
	-0.000251	-0.000129	-0.000775	-0.000115
D_{-1}	7.62*	7.35*	9.67*	5.08*
	29.70	29.00	34.90	22.00
	-0.000233	-0.000132	-0.000793	-0.000126
D_{-3}	9.24*	7.56*	2.39	6.51*
	33.90	29.60	11.70	26.60
	-0.000268	-0.000150	-0.000455	-0.000155
D_{-5}	3.94	4.40	0.06*	4.60*
	17.90	19.70	0.40	20.40
	-0.000270	-0.000177	-0.000090	-0.000190

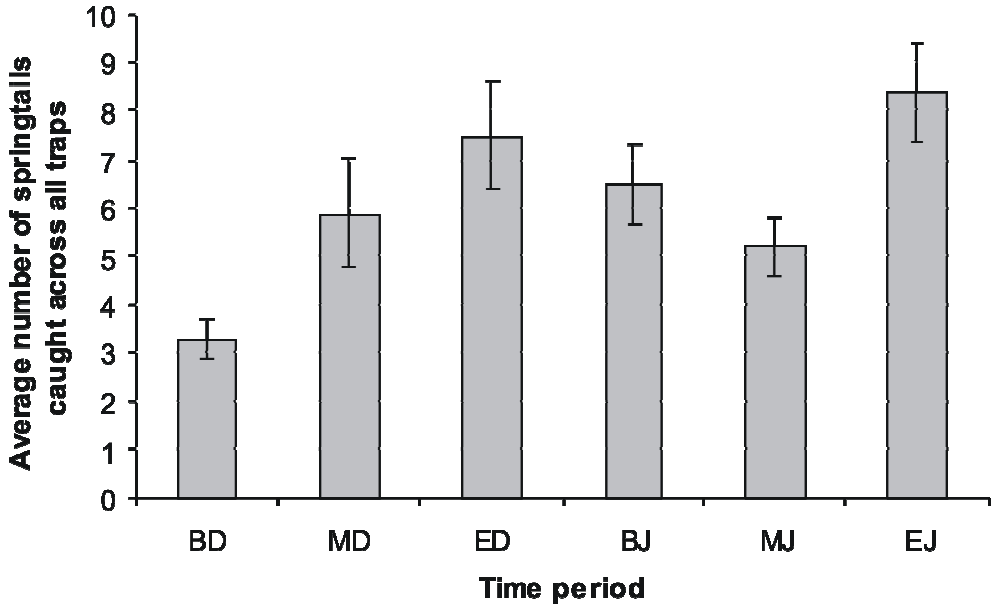

Figure 2.5 Mean pitfall activity (± S.E.M.) of *Gomphiocephalus hodgsoni* individuals over six 10-day blocks ('BD' = beginning December; 'MD' = middle December; 'ED' = end December; 'BJ' = beginning January; 'MJ' = middle January; 'EJ' = end January) in 2006/07.

2.4.2.2 Pitfall activity variation and microclimate variables

When pitfall counts were regressed against mean microclimatic temperature variables for each day of the season, significant relationships were identified for T_{max} ($F_{1,38}$ = 5.55, r^2 = 12.7%, β = 4.50; P = 0.024) and T_R ($F_{1,38}$ = 7.39, r^2 = 16.3%, β = 5.39; P = 0.010).

2.5 DISCUSSION

Metabolic rates for *Gomphiocephalus hodgsoni* (mean obtained across the austral summer from 53 individuals: 0.0017 ± 0.0002 $\mu lO_2.\mu g^{-1}.hr^{-1}$ [S.E.M.]) are comparable to those reported in previous studies of maritime Antarctic springtails. For example, metabolic rates at 10°C for a similar sized isotomid springtail (*C. a. antarcticus*) from the Antarctic Peninsula averaged 0.0015 $\mu lO_2.\mu g^{-1}.hr^{-1}$ (Dunkle & Strong 1972) and studies on temperate springtails of the family Isotomidae (Zinkler 1966) also fall within the range of magnitude of metabolic rates found in this study. Taken in a comparative context, the current study is consistent with earlier findings in support of MCA in Antarctic springtails (e.g. Dunkle & Strong 1972; Block & Tilbrook 1975, 1978).

Metabolic rate variation (measured at constant temperature) was detected on an intra-seasonal timescale in the current study. Specifically, it was apparent that springtails increased or decreased their resting metabolic rate across periods as short as ten days. Seasonal changes in metabolic rate of springtails have been shown previously in a limited number of studies with longer experimental periods than employed here. For example, Testerink (1983) measured mass-specific metabolic rate at 15ºC in the springtail *Orchesella cincta* (Entomobryidae) from the Netherlands, and found that animals in the spring had significantly higher metabolic rates than those in autumn. This was attributed to differences in energy partitioning, whereby the spring population assigned relatively more energy to growth and reproduction while in autumn population energy use was restricted to general maintenance. Conversely, van der Woude and Joosse (1988) found no seasonal variation in mass-specific metabolic rate measured at

15ºC for the temperate springtails *O. cincta* (Entomobryidae)[3] and *Tomocerus minor* (Oncopoduridae) in the Netherlands. Instead, a reduction in metabolic costs during drought and frost was apparent[4]. An additional study by Kauri et al. (1975) reported a winter reduction in mass-specific metabolic rate at 8ºC (after 3 – 10 d acclimation at 10ºC) for two isotomid springtails (*Isotoma viridis* and *Tetracanthella britannica*) in Norway. The common thread among these studies is the impact of environmental conditions upon changes in energy expenditure.

This link was investigated further here, through analysis of the relationship between metabolic rate variation and the corresponding variation in microclimate variables. Metabolic rate of *G. hodgsoni* in the present study responded to microclimate temperature, with increases in T_{min} in particular appearing to play the most significant immediate role (compared to T_{α}, T_{max} and T_R) on increases in metabolic rate. In addition, minimum temperatures on the preceding day may act as a cue for 'setting' metabolic activity levels on the current day, as T_{min} on D_{-1} was also significantly related to metabolic rate. Concurrent measures of pitfall activity showed an additional effect of microclimate variation on activity levels. In this case, T_{max} showed the strongest relationship with pitfall activity, indicating a rapid 'migrational' response of *G. hodgsoni* to high microclimate temperatures as they occur. Thus, *G. hodgsoni* appears to be capable of both physiologically and behaviourally 'tuning' in to short-term thermal variability to respond appropriately to the local unpredictable Antarctic habitat.

However, despite finding a relationship between metabolic rate and local microclimatic parameters, it is clear from the current study that metabolic rate in this species is not simply a direct function of temperature, because the decreases demonstrated during later measurement periods (Table 2.1; Fig. 2.4) occurred while mean microhabitat temperatures had increased. This observation is one of very few

[3] Note added following publication: In fact, these authors did find a reduction in metabolic rate in *O. cincta* over the winter, however this reduction was not large enough to distinguish differences brought about throughout the year as a result of individual moulting

[4] Note added following publication: Droughts and frosts in summer resulted in a reduction of metabolic rates in *O. cincta* in order to reduce metabolic costs during these low-resource periods, thus providing some support for an (intra-seasonal) effect of environmental conditions on metabolic rates

from the polar terrestrial environment that provides comparability with more extensive studies on Antarctic marine species (e.g. Clarke 1991a,b, 1993, 1998; Peck et al. 2006). It provides the first strong implication that, as widely found in the marine environment (Clarke 1993), systematic metabolic rate variation in the terrestrial environment may be related to intrinsic energetic requirements – in this case with elevated metabolic activity coinciding with that portion of the summer season during which important life history functions such as replenishing nutritional status after the depleting Antarctic winter take place (*cf.* Wise & Spain 1967) – rather than only the extrinsic influence of temperature *per se*.

Conclusive proof of this linkage will require a considerable expansion of autecological and ecophysiological work on this and other polar species as, while the restriction of 'short active season' is widely appreciated (see Convey 1996), there remains a general paucity of detailed studies at the species level (Hogg et al. 2006). Further, the possibility of systematic variation in metabolic rate over time within an individual organism in response to specific energetic requirements remains difficult to assess. Metabolic rate elevation alone merely imposes an increased energetic cost on the organism and, in the absence of comparable rate elevation in other key processes, will simply result in an increased rate of resource depletion (Clarke 1991b, 1993; Convey 1996; Chown & Gaston 1999). Hence, an important requirement for the advancement of understanding of metabolic rate variation lies in identifying its functional consequences for terrestrial biota.

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CHAPTER THREE:
TEMPORAL AND SPATIAL METABOLIC RATE VARIATION IN AN ANTARCTIC SPRINGTAIL

The work in this chapter is accepted for publication:

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3.1 ABSTRACT

Spatial and temporal environmental variation in terrestrial Antarctic ecosystems are known to impact species strongly at a local scale, but the ways in which organisms respond (e.g. physiologically, behaviourally) to such variation are poorly understood. Further, very few studies have attempted to assess inter-annual variability of such responses.

Building on previous work demonstrating intraseasonal variation in standard metabolic rate in the springtail *Gomphiocephalus hodgsoni*, we investigated variation in metabolic activity of *G. hodgsoni* across two austral summer periods at Cape Bird, Ross Island. We also examined the influence of spatial variation by comparing metabolic rates of *G. hodgsoni* at Cape Bird with those from two other isolated continental locations within Victoria Land (Garwood and Taylor Valleys).

We found significant differences between metabolic rates across the two years of measurement at Cape Bird. In addition, standard metabolic rates of *G. hodgsoni* obtained from Garwood and Taylor Valleys were significantly higher than those at Cape Bird where habitats are comparable, but environmental characteristics differ (e.g. microclimatic temperatures are higher).

We discuss potential underlying causes of these metabolic rate variation patterns, including those related to differences among individuals (e.g. physiological and genetic differences), locations (e.g. habitat quality and microclimatic regime differences) and populations (e.g. acclimation differences among *G. hodgsoni* populations in the form of metabolic cold adaptation (MCA)).

3.2 INTRODUCTION

Studies examining variation among populations of the same species are crucial for understanding how differences in physiological attributes evolve and how they can be affected by environmental factors (Lardies et al. 2004). The Antarctic terrestrial environment is known for its seasonal variability and the extreme challenges it presents to the organisms that inhabit it (Peck et al. 2006). Over sufficient time scales, the

environmental characteristics of local habitats may drive the evolution of certain aspects of organism life histories (e.g. Southwood 1977, 1988; Convey 1996; Vernon et al. 1998). In particular, adaptive strategies of Antarctic terrestrial taxa are largely dominated by responses of individuals to their immediate environment. These responses include characteristics which maximise activity, growth, development and survival throughout the short (1 – 3 month) active season, in addition to obtaining resources sufficient for surviving the inactive winter (Convey 1996; Worland & Convey 2001).

In terrestrial habitats in Antarctica, microhabitat temperatures can vary widely on both daily and seasonal scales (annual ranges of 50ºC or more are not uncommon), and differences in water availability occur seasonally and between habitats (Davey & Rothery 1996; Convey et al. 2003; Peck et al. 2006). Thus, climatic unpredictability is a consistent feature of the Antarctic terrestrial environment, and differences among individuals across both temporal and spatial scales that generate differential fitness consequences may be expected to be significant over both the short- and long-term (Chown 1993; Davey & Rothery 1996; Kingsolver & Huey 1998). Indeed, variation within individuals over time is characteristic of most physiological traits (Chown 2001) and differences among individuals in a variety of such traits may have profound effects on fitness at both individual and population scales. Despite this, such differences have received scant attention in an ecological context and investigations of intra-specific physiological variation (not to mention intra-seasonal and inter-annual variation) are rare (e.g. Davey & Rothery 1996; Spicer & Gaston 1999; Castañeda et al. 2004; Lardies et al. 2004; but see Lam 1999 and references therein).

Thus, although variation in physiological traits forms the substance of evolutionary physiology, the way in which this variation is partitioned is poorly known (Chown et al. 1999) and temporal intra-specific variation in metabolic rates has only rarely been investigated in terrestrial invertebrates (see McGaughran et al. 2009a and references therein). Also, despite the considerable research effort that has been devoted to studies of the ecophysiology of stress tolerance in arthropods of extreme environments, detailed metabolic studies of terrestrial Antarctic Collembola were, until recently, largely limited to one species from the maritime Antarctic (Chown & Convey 2007).

Recent work evaluating temporal metabolic rate variation in the continental Antarctic springtail *Gomphiocephalus hodgsoni* Carpenter, 1908 (Collembola: Hypogastruridae) demonstrated that standard metabolic rate in this species varied systematically during the short summer activity period, with changes unrelated to diurnal cycling and body mass being apparent over periods as short as 10 days (McGaughran et al. 2009a). Additionally, seasonal changes in metabolic rate of springtails have been shown in a limited number of studies; however, all consist of measurements taken within a one-year period (e.g. Kauri et al. 1975; Testerink 1983; Bennett et al. 1999). Indeed, few ecophysiological studies have extended to replicate work across years (for a notable exception, see Kukal & Duman 1989).

Conversely, several studies of physiological processes other than metabolism have incorporated an 'annual' element and much work has looked at temporal variation over shorter time scales. This includes an 11 year study on water status in arthropods in the maritime Antarctic (Convey et al. 2003) and several reports of immediate (i.e. daily) (e.g. Baust & Lee 1983; Worland & Convey 2001; Sinclair et al. 2003) and seasonal (e.g. Davey & Rothery 1996; van der Merwe et al. 1997) responses of Antarctic arthropods to environmental cues (Chown 2001). In addition, recent work by Hawes et al. (2007) incorporated multiple scales of temporal resolution into an examination of cold-hardening responses in the Antarctic mite *Halozetes belgicae*, finding phenotypic plasticity in cold-hardening ability in the form of seasonal, weekly and hourly acclimatization.

Spatial variation in the expression of physiological traits has received comparatively greater attention, driven largely by recognition of the importance of temperature (often *via* its proxy, latitude) in defining population parameters. Temperature is known to affect physiological performance, so its variation likely accounts for an array of features that may define population dynamics (Chown 1993). For example, many studies have found differences in low-temperature capabilities of species over latitudinal gradients (e.g. Hawes & Bale 2007 and references therein) and cold tolerance strategies have been shown to vary between hemispheres (Sinclair & Chown 2005). Further, Lardies et al. (2004) suggest that geographic variation in life

history traits may underlie differences in metabolic rates among individuals from different populations (see also Lardies & Bozinovic 2008).

Metabolic rates have been found to differ between populations or species from habitats differing in latitude, altitude and (correspondingly) temperature (Klok & Chown 2003; Chopelet et al. 2008). In some cases, such differences have been attributed to 'metabolic cold adaptation' (MCA) - a phenomenon where, at the same trial temperature, species from colder locations have elevated metabolism compared with those from warmer locations (see Block 1990). This is potentially an important element of spatial metabolic rate variation and, amongst terrestrial invertebrates, has been reported in beetles, grasshoppers, weevils and several polar microarthropods (see Addo-Bediako et al. 2002 and references therein). However, MCA is not a universal feature of polar (or alpine) species, and there are several published studies of species showing no evidence for its expression (e.g. Lee & Baust 1982; Nylund 1991; Chown 1997).

Here, we examine (i) the magnitude of temporal (intra- and inter-annual) differences in individual metabolic rate for a population of the springtail *G. hodgsoni* at a continental Antarctic site, and (ii) address spatial variation in metabolic rate by comparing metabolic rates of *G. hodgsoni* in isolated populations from comparable habitats at three separate locations in Victoria Land. Using this combined approach, we discuss potential underlying influences of temporal and spatial structure in metabolic rate, including differences among individuals (e.g. physiological and genetic), locations (e.g. environmental) and populations (e.g. acclimation differences corresponding to MCA).

3.3 METHODS

3.3.1 Location

This study extends previous work on the springtail *G. hodgsoni* that took place at Cape Bird (Ross Island, 77º13'S, 166º26'E) (Fig. 3.1) during the period 29 November 2006 – 29 January 2007 (Season One (S1), see McGaughran et al. 2009a). Collections of large (> 1 mm in length (i.e. non-juvenile)) animals were made by gently sweeping

individuals off the underside of small rocks into a collection vessel using a very fine paintbrush. Temporal variation over inter-annual scales was investigated by replicating S1 work between 12 – 23 November 2007 and 7 – 23 January 2008 (Season Two, S2). These dates were defined by Antarctic logistic practicalities, with the intention of covering the maximum possible proportion of the short 'summer' season.

In order to examine spatial variation in metabolic rates, analyses on samples collected from Garwood (78º01'S, 163º55'E) and Taylor Valleys (77º40'S, 163º06'E) (Fig. 3.1), were performed in the Scott Base 'Wet Laboratory' from 23 – 28 January 2008 (S2) (see below). These samples were collected in the same manner as collections made at Cape Bird from comparable habitat (i.e. from the underside of small rocks in areas of snow-melt/alongside streams) to minimize differences among sites as far as possible.

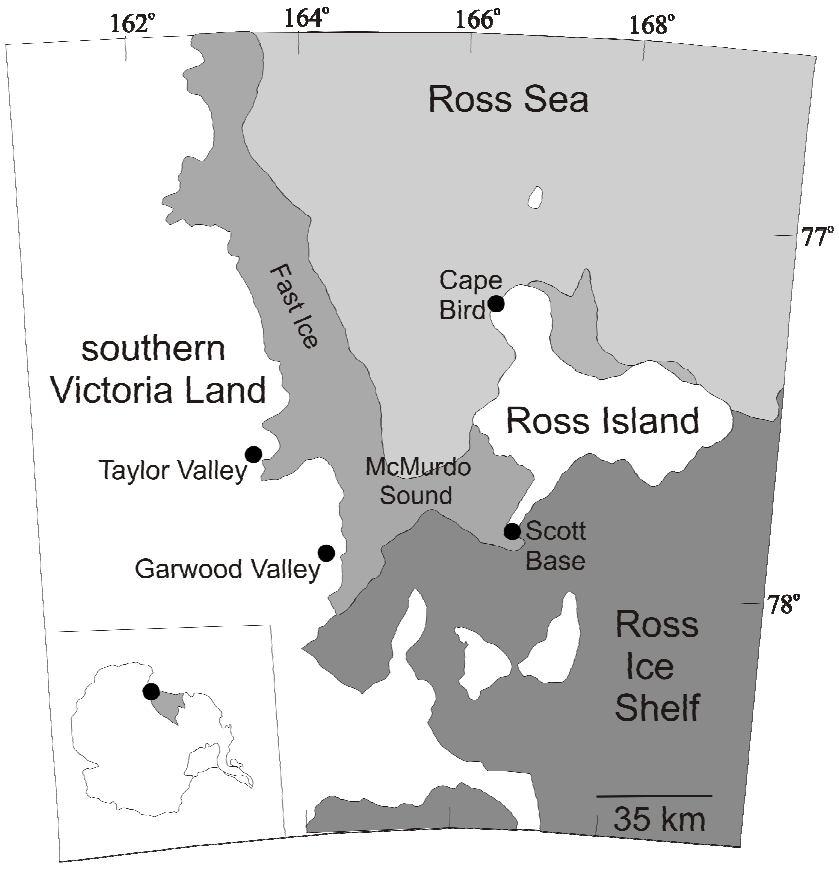

Figure 3.1 Map showing the location of the Ross Dependency and southern Victoria Land in Antarctica (inset), and the geographic locations within this region that are referred to in the text.

3.3.2 Metabolic rates

Standard metabolic rates were measured in S2 following the protocol of McGaughran et al. (2009a) during S1, except with a modified chamber of reduced volume (from 40 µl) which allowed shorter machine run times and consequently, higher sample throughput [tests performed at Massey University showed that metabolic data obtained from New Zealand springtails were not significantly different among the two chambers (data not shown)]. In brief, this protocol employed a fiber-optic oxygen sensing system (Ocean Optics Inc., USA) to monitor oxygen partial pressure (pO_2) over time for individual animals in a closed respirometry system. Following machine calibration, individual animals that had been collected from the underside of rocks between 0900 – 1500 h and then stored for 1-2 hours at the research hut ambient temperature (~ 6 to 8ºC), were placed in a 10 µl volume chamber. During a 2 hr period, where, following the protocol of S1, the temperature was maintained at 10 ± 0.1°C (which is within the range experienced within the species' typical microhabitats; see McGaughran et al. 2009a), the partial pressure of oxygen in the chamber was recorded continuously. Completed partial pressure profiles were used to calculate oxygen consumption rates on a per animal basis for each individual, using the observed drop in pO_2 over a given time period in conjunction with the chamber volume. Because accurate mass measurement was not possible at this remote study location, individual animal mass was calculated using a photographic technique (based on the relationship between weight and length: $W = 6.1894L^{3.119} \times 10^{-9}$, where W = weight (µg), L = length (µm), as modeled for the similar sized maritime Antarctic springtail *Cryptopygus antarcticus* (see Block & Tilbrook 1975)), and the programme Screen Calipers ver. 3.3 (Iconico, Inc. 2006) (see McGaughran et al. 2009a).

Given the small size of the animals in this study, respiration rates were expected to be near the resolution limit of the oxygen sensing system (see www.oceanoptics.com). Hence, blank runs (n = 10) were completed following the protocol above except without the addition of an animal to the measurement chamber, to provide an estimate of the drift or 'noise' of the equipment used (see McGaughran et al. 2009a for quality control information). Trial runs indicated that metabolic rates of individuals measured during the S2 November period were too low to separate from equipment noise. Hence, in this

part of the season, groups of five individuals were pooled for metabolic measurements, with all other experimental details remaining the same. The necessity of using this approach allowed examination of population variation (i.e. comparing November to January in S2) but resulted in a loss of resolution of individual variation.

In addition to metabolic measures performed at Cape Bird in S2, samples collected from Garwood Valley on 20 January 2008 and Taylor Valley on 22 January 2008 (see Fig. 3.1) were returned to Scott Base immediately for storage at 4ºC in containers with soil and stones to replicate field conditions. Samples were subsequently moved to the Scott Base Wet Laboratory and kept at ambient hut temperature (~ 6 to 8ºC) for 1-2 h prior to metabolic rate measurements, which were performed from 23 – 28 January following the methodology described above. The metabolic rate measurements made for Garwood and Taylor Valley samples were then compared to those made at Cape Bird in the period 18 – 21 January.

3.3.3 Microhabitat parameters

McGaughran et al. (2009a) determined that conditions under small surface rocks were most representative of the microhabitat conditions experienced by *G. hodgsoni*. Hence, ten iButton (five thermochron and five hygrochron) data loggers (DS1921, DS1923; Maxim Integrated Products, Sunnyvale CA, USA) were used to monitor temperature (± 0.5ºC) and relative humidity (± 0.6%) in soil under ten separate rocks in the small collection area (< 50 m^2) at Cape Bird, logging every 60 minutes for the entire field period in S2 (i.e. 14 November 2007 – 20 January 2008). All replicate temperature (T) and relative humidity (RH) data were processed to obtain average maximum (T_{max}; RH_{max}), minimum (T_{min}; RH_{min}), mean (T_{α}; RH_{α}) and range (T_R; RH_R) for the day of metabolic rate measurement (D_0) and the day preceding the day of measurement (D_{-1}). In addition temperature data were used from iButtons deployed during S1 at the Cape Bird field site, and all available further data for December 2006, January 2007, December 2007 and January 2008 were downloaded from Automatic Weather Stations for 'air' at Cape Bird (77.22 S, 166.44 E, 38 m a.s.l.; http://uwamrc.ssec.wisc.edu/aws/capebirdmain.html), and for 'air' and 'soil' (0 cm) at Taylor Valley (Lake Fryxell Station; 77.10 S, 163.10 E, 19 m a.s.l.;

http://www.mcmlter.org/queries/met/met_home.jsp), and processed to obtain T_{α}, T_{max}, T_{min} and T_R. Comparative data for Garwood Valley do not exist.

3.3.4 Statistical analyses

All statistical analyses were performed using the program Minitab (ver. 14, Minitab Inc., Pennsylvania, USA). Initial regression-based analyses of the allometric scaling relationships (whereby metabolic rate is predicted to scale with the three-quarter power of body mass; e.g. West et al. 2002) between mass-specific metabolic rate and mass showed considerable variability in the dataset (see Table 3.1), hence all subsequent analyses used whole animal metabolic rates.

Relationships among whole animal metabolic rate and: (1) temporal (i.e. time period); and (2) spatial (location) variability were investigated with univariate tests of significance using a general linear model (GLM). For each test, we used whole animal metabolic rate as the dependent variable and mass as covariate, with time period (both within S2 – i.e. November 2007 vs. January 2008, and between S1 and S2 – i.e. December 2006, January 2007 (S1) vs. November 2007, January 2008 (S2)) and location (Cape Bird, Garwood Valley, Taylor Valley) as additional covariates. In each test, the covariates were analysed separately and also crossed.

T-tests were used to determine differences in temperature and relative humidity regimes between November and January (S2), and between S1 and S2. Linear least-squares regression analyses were also performed on the S2 data to determine the relationships between metabolic rate and these microclimate variables.

3.4 RESULTS

Blank measurement runs generated very low estimates of equipment generated 'noise', equating to 0.0040 ± 0.0012 $\%O_2.hr^{-1}$ [s.d.]. This was much lower than, and significantly different from, rates obtained during runs using individual live animals (mean for all January data: 0.183 ± 0.041 $\%O_2.hr^{-1}$ [s.d.] (t_{54} = 17.58, $P < 0.001$), indicating that the equipment resolution was sufficient to allow measurement of true metabolic rates in this study.

3.4.1 Intra-seasonal variation in metabolic rate, live mass and microclimate

Mean whole animal metabolic rates of 'field fresh' animals measured at 10ºC varied significantly between the November and January measurement periods of S2 ($F_{3,79}$ = 21.51; $P < 0.001$) (Table 3.1, Fig. 3.2 – time periods 3 and 4) and this was unrelated to differences in mass ($F_{3,79}$ = 0.58; P = 0.450) or interactions between mass and time ($F_{3,79}$ = 0.52; P = 0.473). Specifically, metabolic rates in November were significantly lower than those in January (0.0027 ± 0.0015 µl.hr^{-1} [s.d.] and 0.0105 ± 0.0025 µl.hr^{-1} [s.d.], respectively).

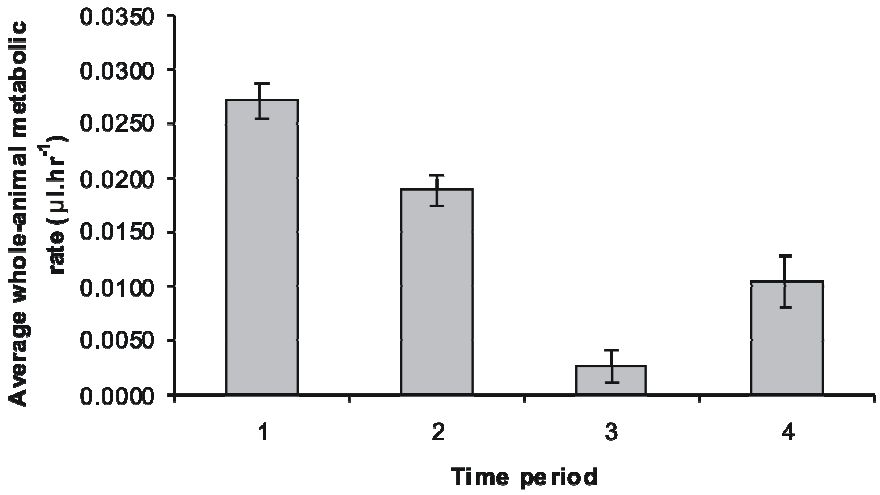

Figure 3.2 Mean metabolic rate (± s.d.) of *Gomphiocephalus hodgsoni* over four time periods across two Antarctic seasons (1 = December 2006; 2 = January 2007; 3 = November 2007; 4 = January 2008) at Cape Bird (Ross Island), Antarctica. Statistical comparison revealed significant differences between all periods ($F_{3,79}$ = 21.51; $P < 0.001$).

Mean microhabitat (under rock) temperatures varied greatly between the two measurement periods of S2 (t_{810} = -14.39; $P < 0.001$). In January the microhabitat temperature did not drop below -1.8ºC and was relatively stable (4.3º ± 0.2ºC [s.d.]), however, maximum, minimum and mean temperatures under rocks for the January period were 3.5ºC, 7.1ºC and 4.3ºC warmer than those measured in the November period, respectively (Table 3.2).

Table 3.1 Metabolic rate (± s.d.) and mass (± s.d.) data, and their allometric relationship, for *Gomphiocephalus hodgsoni* temporally (across four time periods), spatially (across three locations), and for the overall dataset. Regression equations are given in the form y = a + b*x*, where 'y' is ln(metabolic rate) and '*x*' is ln(mass), and 'b' is the scaling exponent.

Dataset	Metabolic rate ($\mu l.hr^{-1}$)	Mass (µg)	Regression equation	F	r^2	*P*
Temporal (Cape Bird)						
December 2006 (S1)	0.0025(0.0016)	14.6(7.5)	y = -2.87 + 0.162*x*	$F_{1,13} = 0.34$	2.6	0.569
January 2007 (S1)	0.0014(0.0014)	21.0(11.2)	y = -3.27 + 0.244*x*	$F_{1,33} = 2.01$	5.7	0.165
November 2007 (S2)	0.0027(0.0015)	24.0(6.3)	y = -3.42 – 0.418*x*	$F_{1,28} = 3.02$	9.7	0.093
January 2008 (S2)	0.0105(0.0025)	30.9(9.4)	y = -2.03 – 0.994*x*	$F_{1,60} = 74.37$	55.3	<0.001
All temporal data	0.0054(0.0046)	24.9(10.7)	y = -2.57 – 0.606*x*	$F_{1,140} = 15.93$	10.2	<0.001
Spatial (S2)						
Cape Bird	0.0098(0.0022)	34.3(7.3)	y = -3.75 + 0.133*x*	$F_{1,12} = 0.13$	1.0	0.729
Taylor Valley	0.0134(0.0067)	18.6(6.8)	y = -3.05 – 0.083*x*	$F_{1,13} = 0.16$	1.2	0.697
Garwood Valley	0.0166(0.0052)	28.0(5.8)	y = -4.33 + 0.698*x*	$F_{1,8} = 1.22$	13.2	0.302
All spatial data	0.0127(0.0055)	27.0(9.6)	y = -2.53 – 0.578*x*	$F_{1,37} = 10.46$	22.0	0.003
Spatial + Temporal	0.0071(0.0057)	25.4(10.4)	y = -2.57 – 0.593*x*	$F_{1,179} = 21.17$	10.6	<0.001

Table 3.2 Air and microclimate (soil) temperature and relative humidity data at Cape Bird and Taylor Valley during relevant time periods. All soil data collected from Cape Bird are from iButtons deployed during S1 and S2, while 'air' data from Cape Bird and all Taylor Valley data are from Automatic Weather Stations (see Methods, section 3.2.3); any gaps in the table indicate periods for which no data are currently available.

Location	Time period	Location	Temperature				Relative Humidity			
			Mean(s.d.)	Max	Min	Range	Mean(s.d.)	Max	Min	Range
Cape Bird	Dec 06	Soil	2.4(1.5)	11.2	-1.9	13.0	114.3(2.6)	118.3	109.0	9.2
		Air	-2.7(2.2)	7.1	-9.6	18.1				
	Jan 07	Soil	4.9(2.3)	17.1	-1.1	18.1	115.1(1.8)	119.7	106.6	13.1
		Air	0.6(1.3)	7.5	-3.6	11.1				
	Nov 07	Soil	0.0(1.5)	12.5	-8.9	21.4	107.7(3.1)	115.8	88.5	27.3
		Air	-7.5(4.7)	7.2	-31.6	38.8				
	Jan 08	Soil	4.3(1.6)	16.0	-1.8	17.8	110.2(0.9)	113.8	104.9	8.9
		Air	-0.9(2.0)	7.2	-11.6	18.8				
Taylor Valley	Dec 06	Soil	-3.9(2.6)	2.6	-10.2	12.9				
		Air	-3.8(2.8)	2.6	-10.2	12.9	63.3(17.2)	97.2	22.5	74.6
	Jan 07	Soil	4.8(1.7)	20.2	-4.2	24.4				
		Air	-0.2(1.2)	5.5	-5.5	11.0	63.6(14.2)	100.0	21.1	78.9
	Nov 07	Soil	-6.9(8.0)	13.1	-26.4	39.5				
		Air	-9.9(7.4)	0.2	-33.3	33.5	69.7(15.2)	100.0	27.7	72.3
	Jan 08	Soil	3.2(5.0)	17.8	-8.3	26.0				
		Air	-1.8(2.2)	3.7	-7.5	11.23				

Relative humidity remained largely constant within periods over S2 and did not drop below 99.9% under rocks in January. However, the November period was distinct from the January period (t_{883} = -10.12; $P < 0.001$) most likely as a result of the comparatively lower November minimum (88.5%) (Table 3.2).

Within S2, regressions of whole animal metabolic rate against temperature and relative humidity variables identified several significant linear relationships. The strongest of these linear relationships for the temperature data were with T_{min} at both D_0 (T = 6.16, r^2 = 71.7%, β = 0.00938; $P < 0.001$) and D_{-1} (T = 6.50, r^2 = 73.8%, β = 0.00954; $P < 0.001$). Conversely the strongest linear relationships for the relative humidity data were found between metabolic rate and RH_{max} at D_0 (T = 6.38, r^2 = 73.1%, β = -0.217; $P < 0.001$) and D_{-1} (T = 4.87, r^2 = 61.3%, β = -0.180; $P < 0.001$) (Table 3.3).

Table 3.3 Results of regression analyses of metabolic rate against various environmental (microhabitat) variables for *Gomphiocephalus hodgsoni*. Abbreviations correspond to temperature and relative humidity mean (T_α; RH_α), maximum (T_{max}; RH_{max}), minimum (T_{min}; RH_{min}), and range (T_R; RH_R) for the day of metabolic rate measurement (D_0) and the day preceding day of measurement (D_{-1}). Upper value in the table is the T-test value, middle value is the regression coefficient (r^2), lower value is the regression slope (β); '*' indicates $P < 0.05$.

Variable	Time	T_α	T_{max}	T_{min}	T_R
Temperature	D_0	5.58*	2.96*	6.16*	-0.57
		67.5	36.9	71.7	2.1
		0.00496	0.00245	0.00938	0.00903
	D_{-1}	4.75*	1.49	6.50*	-1.72
		60.1	12.9	73.8	16.5
		0.00476	0.00409	0.00954	0.01200
		RH_α	RH_{max}	RH_{min}	RH_R
Relative humidity	D_0	4.57*	6.38*	2.70*	-1.07
		58.2	73.1	32.8	7.1
		-0.1380	-0.2170	-0.0531	0.0102
	D_{-1}	4.65*	4.87*	4.34*	-3.17*
		59.1	61.3	55.7	40.1
		-0.1020	-0.1800	-0.0479	0.0127

3.4.2 Inter-seasonal variation in metabolic rate, live mass and microclimate

Variation in metabolic rates between S1 and S2 revealed significant differences associated with time ($F_{3,132} = 12.92$; $P < 0.001$) but not mass ($F_{3,132} = 0.20$; $P = 0.653$) or its interaction with time ($F_{3,132} = 0.26$; $P = 0.613$) (Table 3.1, Fig. 3.2).

The S2 November field period was earlier than and did not overlap with the commencement of fieldwork in S1, hence microclimate data to allow direct comparison between the two seasons are unavailable. Comparison of microclimate temperature data from December 2006 with overlapping data for December 2007 (T_α, T_{max}, T_{min}, and T_R) indicated that the data for the entire months were generally similar, although the first week of December 2006 was significantly different to the same period in 2007 ($t_{14} = 7.22$; $P < 0.001$; $t_{14} = 3.63$; $P = 0.003$; $t_{14} = 5.00$; $P < 0.001$; $t_{14} = 2.05$; $P = 0.060$; for T_α, T_{max}, T_{min}, and T_R; respectively) (Fig. 3.3). In particular, the average temperature was 2.9ºC higher in the first week of December 2006, while the maximum and minimum temperatures for this period were 4.3º and 1.7º warmer in 2006 than in 2007.

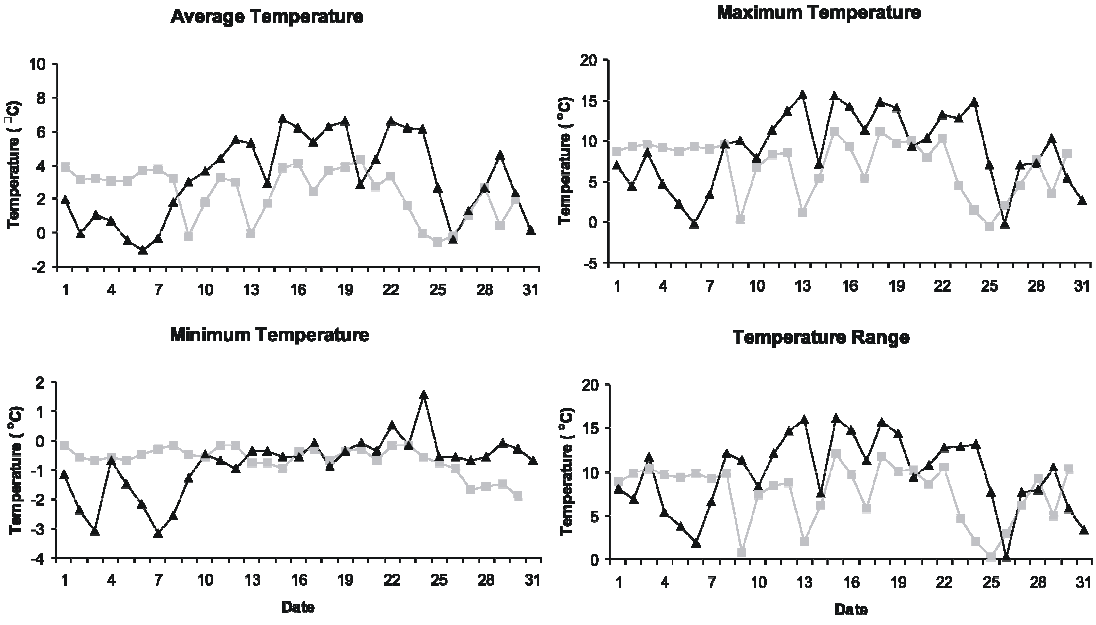

Figure 3.3 Microclimate data comparing mean, maximum, minimum and range of temperature under a rock at Cape Bird (Ross Island), Antarctica in December 2006 (grey squares) and 2007 (black triangles). In particular, note the differences between data collected from 1 – 7 December in each year.

Comparison of relative humidity data between S1 and S2 revealed significant differences between the seasons in all variables (RH_{α}: $t_{98} = 11.38$; $P < 0.001$; RH_{max}: $t_{98} = 14.63$; $P < 0.001$; RH_{min}: $t_{98} = 3.30$; $P = 0.001$; RH_R: $t_{98} = 3.81$; $P < 0.001$), the most notable of which is the low minimum (88.5% RH) reported in the November 2007 period (Table 3.2).

3.4.3 Spatial variation in metabolic rate, live mass and microclimate

Mean whole animal metabolic rate varied significantly at the spatial level and this variation corresponded to mass ($F_{3,37} = 32.13$; $P < 0.001$), location ($F_{3,37} = 4.29$; $P = 0.045$) and the interaction between mass and location ($F_{3,37} = 18.40$; $P < 0.001$) (Table 3.1). In particular, metabolic rates for samples from Taylor Valley (0.0134 ± 0.0067 $\mu l.hr^{-1}$ [s.d.]; n = 15) and Garwood Valley (0.0166 ± 0.0052 $\mu l.hr^{-1}$ [s.d.]; n = 10), were significantly higher than those for samples analysed over a similar time frame at Cape Bird (0.0098 ± 0.0022 $\mu l.hr^{-1}$ [s.d.]; n = 16) (Table 3.1, Fig. 3.4).

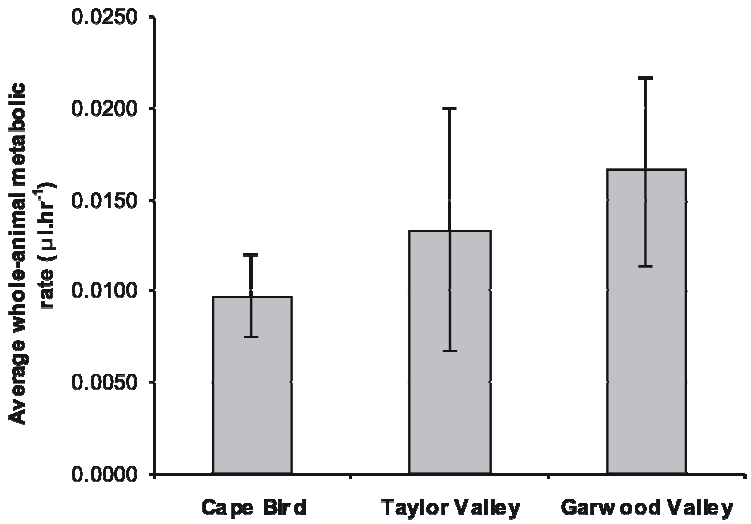

Figure 3.4 Mean metabolic rate (± s.d.) in January 2008 of *Gomphiocephalus hodgsoni* at three locations (Cape Bird, Taylor Valley, Garwood Valley) in the Ross Dependency showing significant metabolic rate differences across locations ($F_{3,37} = 4.29$; $P = 0.045$).

Examination of the limited available microclimate data across sites shows that, in all cases, Taylor Valley displays lower mean and minimum temperatures, and in all but

one case lower maximum temperatures than Cape Bird (Table 3.2). The available relative humidity data corresponds to 'soil' at Cape Bird, and 'air' at Taylor Valley, so a direct comparison of this variable across sites is not possible (Table 3.2). However, if relative humidity variation follows the same pattern as temperature variation (these variables were found to be correlated at Cape Bird in S1; A. McGaughran, unpublished), then the inland valley sites may also experience lower levels of relative humidity than Cape Bird.

3.5 DISCUSSION

We previously reported the first clear evidence of intra-seasonal variation in standard metabolic rate, measured at constant conditions, for a polar terrestrial invertebrate (McGaughran et al. 2009a). Metabolic rates of *G. hodgsoni* measured in the current study (mean *mass-specific* metabolic rates in January 2008 at Cape Bird for 54 individuals: 0.00037 ± 0.00016 $\mu lO_2.\mu g^{-1}.hr^{-1}$ [s.d.]) fall at the lower end of the published range for Antarctic micro-arthropods (0.0003 − 0.0017 $\mu lO_2.\mu g^{-1}.hr^{-1}$ (Block & Tilbrook 1975; McGaughran et al. 2009a)) but again displayed temporal variation, both in the form of intra- and inter-seasonal differences. An important conclusion from data obtained in S1 was that metabolic rate in *G. hodgsoni* varies systematically over time, most likely as a function of demand for the product of respiration (ATP) (McGaughran et al. 2009a). However, superimposed on this, as indicated in the current study, is the influence of microclimate temperature on metabolic rate in terms of both daily variability in minimum temperatures and differing environmental conditions between years.

The significance of temperature in the life of ectotherms is well appreciated for its effects on a diverse array of physiological traits including metabolism (Kingsolver & Huey 1998 and references therein). Its role as a primary cue dictating activity in Antarctic arthropods (in addition to other factors such as feeding and moisture availability) is also acknowledged (Worland et al. 2007). The results of the current study suggest the importance of temperature cues early in the season, which may be capable of setting the precedent for all future activity within that season. In addition,

the extremely low metabolic rates identified in the November period (S2), may help to define the commencement of the active season in biological terms. Indeed, the average soil temperature around this time was 0.0ºC ± 1.5 [s.d.], and above-zero microhabitat temperatures do not become prevalent until December (see Table 3.2). The lowest relative humidity (88.5%) was also logged during November (S2) and, while it is unknown how these measurements translate into hydration states of individuals, desiccation stress has been noted at 98% RH in other soil-dwelling springtails (e.g. Holmstrup et al. 2001; Kaersgaard et al. 2004), while seasonal cycles identified in body water content of maritime Antarctic springtails have been interpreted as a direct signal of changing levels of desiccation stress in their micro-environment (Convey et al. 2003). Thus, the high metabolic rates detected in S1 around the end of December/beginning of January may indicate that springtails are active and replenishing exhausted energy reserves following emergence of all life stages from inactive 'winter' and/or desiccation states at this time (see McGaughran et al. 2009a).

Alongside the roles of temperature and other environmental factors (e.g. relative humidity) in dictating temporal variation in metabolic rates, are their potential effects in driving spatial variation among populations from different locations. However, there are several problems inherent in comparing individuals from different areas because of potential individual and/or population differences in acclimation, activity level, mass, sex, reproductive status, underlying genetic characteristics, hydration state and diet (Lardies et al. 2004).

Of these, body mass is known to be strongly influential on metabolic rates (Hochachka & Somero 2002) and analysis of mass among the three populations of this study showed that this variable was important in driving patterns of spatial metabolic rate variation. However, our analyses showed that variability apparent at the local population level on spatial scales appears to far outweigh any underlying scaling relationship (see Table 3.1), thus spatial variation in metabolic rates is only partly explained by differences in mass.

Indeed, the GLM analyses highlighted location and the interaction between mass and location, as additional significant factors accounting for metabolic rate variation. Location may be considered as a proxy for several factors, including temperature and

relative humidity differences among sites, and the available climate data suggest that Taylor Valley experiences a more 'extreme' climate than Cape Bird (see Table 3.2). While no data are currently available for Garwood Valley, we expect that its climate is likely to parallel that at Taylor Valley given its inland continental location. In practice, the two valley locations experience lower temperatures, potentially lower relative humidity (see Results, section 3.4.3) and a shorter growing season than Cape Bird, thus we might predict lower rates of energy turnover and greater allocation of resources to survival for these populations. However, our data indicate that metabolic rates measured in Taylor and Garwood Valley individuals were significantly higher than those of Cape Bird individuals. This observation is perhaps consistent with greater metabolic adaptation of the Valley populations in the form of expression of MCA, such that individuals at these more 'extreme' sites may be better able to utilize the more limited opportunities of a shorter active season or better withstand temperature and/or desiccation stresses. In the case of potential relative humidity differences among sites, variation in hydration status of individuals has been recently shown to affect metabolic rates in the Antarctic invertebrate *Belgica antarctica* (Benoit et al. 2007).

Finally, differential fitness among populations/locations may also be affected by processes operating at the molecular scale. For example, several studies have demonstrated a significant genetic contribution to variation in individual energy budgets, and heterozygosity is often spatially or temporally correlated with variation in environmental factors and whole-animal physiological processes (Garton 1984; Hawkins et al. 1986; Mitton et al. 1986; Teska et al. 1990; Pujolar et al. 2005). In particular, individuals with greater heterozygosity have been shown to have a greater aerobic scope for activity (Mitton et al. 1986). Previous genetic work has shown that the Cape Bird population of *G. hodgsoni* has very low levels of heterozygosity compared to Taylor Valley (Stevens & Hogg 2003). Furthermore, McGaughran et al. (2008) describe low levels of genetic variability in the mitochondrial gene cytochrome *c* oxidase I at Cape Bird, while samples from Garwood and Taylor Valleys show much greater variability at this locus (McGaughran et al. 2009b). Therefore, differences in genetic variability among populations, if accompanied by differential effects on fitness

(e.g. via adaptability potential), may also be important in structuring spatial metabolic rate variation of populations, such as seen here.

In summary, it is clear that the life history strategy of Antarctic terrestrial arthropods such as *G. hodgsoni* is governed by a matrix of factors (both biotic and abiotic). Over temporal scales, both intrinsic seasonal variation and local microhabitat conditions clearly have strong influences on metabolic rate variation, including potentially setting restrictive limits on activity very early in the season. From a spatial perspective, metabolic rate variation may be caused by differences among populations in mass, energy assimilation and expenditure (i.e. MCA), climatic and habitat differences and/or a combination of other factors including genetic variability and its relationship to fitness. In addition, a suite of other factors that in some cases remain difficult to quantify and/or account for (e.g. diet, developmental stage, sex, thermal history, hydration status) may be important influences of both spatial and temporal patterns of physiological variability. Studies to isolate and quantify these factors are clearly required.

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CHAPTER FOUR:
METABOLIC RATE, GENETIC AND MICROCLIMATE VARIATION AMONG SPRINGTAIL POPULATIONS FROM SUB-ANTARCTIC MARION ISLAND

The work in this chapter is submitted for publication:

McGaughran, A., Convey, P., Stevens, M.I., Chown, S.L. Metabolic rate, genetic and microclimate variation among springtail populations from sub-Antarctic Marion Island. *Polar Biology*, submitted 29 August 2009.

4.1 ABSTRACT

We investigated the ways in which populations express spatial heterogeneity in the twin evolutionary contexts of molecular and physiological differentiation.

Measurement of metabolic rates (made at constant temperature) of individuals of the springtail *Cryptopygus antarcticus travei* from six geographically distinct populations on sub-Antarctic Marion Island were supplemented with mtDNA (*cox1*) haplotype analysis to examine in parallel both physiological and genetic variation of distinct populations.

We found evidence of genetic structure among populations and a general indication of long term isolation with limited gene flow. While we found support for an overall pattern of metabolic rate structure among populations from different geographic locations on the island, we were unable to demonstrate a coherent common pattern between this and genetic variation.

However, spatial structure in metabolic rate variation was strongly related to the extent of variability in microclimate among sites, and also showed some indication of a phylogeographic signal. Thus, over the relatively short timescale of Marion Island's history (< 1 million years), the periodic geographic barriers that have influenced population differentiation from a molecular perspective may also have resulted in some physiological differentiation of populations.

4.2 INTRODUCTION

Spatial variation in the environment is known to affect species in various ways, and subsequently to elicit a variety of responses. In particular, differences in energy assimilation and expenditure are important in shaping population structure and dynamics (e.g. Convey 1998; Kozłowski et al. 2004; Lardies et al. 2004). In polar environments, physiological processes that influence energy assimilation and use, and their variation in response to environmental conditions, are likely to have a significant influence on the evolution of life history strategies (Convey 1996a; Clarke 1998; Chown & Convey 2007), especially since diversity is low and extreme conditions are either

common or unpredictable (Fischer 1960; Southwood 1978, 1988; Greenslade 1983; Peck et al. 2006). Indeed, adaptive strategies of Antarctic terrestrial taxa are largely dominated by responses of individuals to their immediate environment, and include characteristics which maximise activity, growth, development and survival (Convey 1996a,b; Worland et al. 2000; Worland & Convey 2001; Sinclair & Chown 2003; Sinclair et al. 2003; Hawes et al. 2006; Lee et al. 2006). Under such circumstances, physiological variation among individuals and populations that generates differences in fitness may be significant.

Nonetheless, such differences have received scant attention in an ecological context in the region (but see e.g. Davey & Rothery 1996; Convey 1998; Klok & Chown 2003, 2005). Investigation of intraspecific variation in metabolic energy supply in particular would prove beneficial in this context because metabolic rate (generally measured indirectly as VO_2) is essentially a measure of the energetic cost of living, its function being to provide the ATP that fuels all processes within the organism (Clarke 1993, 1998). Thus, metabolic efficiency likely has a significant influence on organism fitness (Kozłowski et al. 2004; Lardies et al. 2004; Lardies & Bozinovic 2008).

Several studies from the polar regions have shown that metabolic rates differ between populations or species from habitats differing in latitude, altitude and (correspondingly) temperature (e.g. Block & Young 1978; Young & Block 1980; Strømme 1986; Sømme et al. 1989; Chown 1997; Chown et al. 1997). In some cases, such differences have been attributed to 'metabolic cold adaptation' (MCA) - a phenomenon where, at the same trial temperature, species from colder locations have elevated metabolism compared with those from warm locations (see Block 1990). However, differential fitness among populations may also be influenced by processes operating at the molecular scale.

Elsewhere, several studies have demonstrated a genetic contribution to variation in individual energy budgets, and heterozygosity is often spatially or temporally correlated with variation in environmental factors and whole-animal physiological processes (Garton 1984; Hawkins et al. 1986; Mitton et al. 1986; Teska et al. 1990; Pujolar et al. 2005). Individuals with greater heterozygosity have also been shown to have a greater aerobic scope for activity (Mitton et al. 1986). Another element of molecular work also

considers the role of life history parameters such as metabolic rate, body size, and generation time in affecting evolutionary rates. In practice, it is difficult to truly define the role of individual parameters, however, current thinking points to an overall 'metabolic theory of ecology' (Gillooly et al. 2001; Brown et al. 2004; West & Brown 2004; Allen et al. 2006) which supposes that generation time and mutation rate (both direct consequences of biological metabolism) are governed by the body-size and temperature dependence of mass-specific metabolic rate (Allen et al. 2006). While the mechanistic view behind this theory is a subject of controversy in the literature (e.g. Makarieva et al. 2008), most authors do not refute the evident relationships that exist between metabolic rate, temperature, several other life history variables, and evolutionary rate. Thus a variety of factors including functional (ATP-demand, MCA) and genetic, may influence contemporary patterns of population metabolic rate structure.

Recently, evidence of regionalisation and molecular divergence within terrestrial arthropod species has been identified across sub-Antarctic Marion Island (Mortimer & Jansen van Vuuren 2006; Myburgh et al. 2007). Moreover, evidence is accumulating that substantial spatial differences in microclimate across the island are not only associated with elevational change or with changes in soil depth, but also with spatial position both at the island-wide and local scales (Boelhouwers et al. 2003; Hugo et al. 2006; McGeoch et al. 2008; Nyakatya & McGeoch 2008). The acclimatisation conditions resulting from local geographic variation in climatic regimes across Marion Island therefore provide a natural framework allowing investigation of evolutionary physiological responses to climate variation. Of particular interest here are differences related to current environmental factors and those resulting from large-scale variation in environmental conditions over longer time-scales (e.g. as caused by volcanism, glaciation).

In this study, we focus on the springtail *Cryptopygus antarcticus travei* Déharveng, 1981 (Collembola, Isotomidae), to examine the extent of spatial metabolic rate variation in a sub-Antarctic ectotherm. This subspecies of the widely distributed nominate species *C. antarcticus* is endemic to Marion Island, a geologically young (< 1 Ma; McDougall et al. 2001) island subjected over its short history to subdivision by major

cycles of glaciation and periods of substantial volcanic activity (see Myburgh et al. 2007 and references therein). This has resulted in genetic differentiation of populations of indigenous terrestrial arthropods, including *C. a. travei* (Mortimer & Jansen van Vuuren 2006; Myburgh et al. 2007), which appears to have been an early coloniser in the island's history (Stevens et al. 2006).

Specifically, we investigated whether the long-term persistence of *C. a. travei* on Marion Island, as well as allowing clear genetic differentiation of geographically discrete populations (see Myburgh et al. 2007), has also led to differentiation of metabolic rates, possibly via differential environmental regimes across the island. We did this by adding significantly to the molecular dataset currently available for *C. a. travei* to examine genetic characteristics of Marion Island populations of this species. In conjunction with this work, we obtained complementary data on metabolic rates (measured at constant temperature) for a subset of individuals from six populations in order to examine metabolic rate structuring among populations. Using this combined genetic and physiological dataset, we determined whether spatial structure in metabolic rates can usefully be interpreted in the context of parallel underlying genetic structure and systematic environmental variation.

4.3 METHODS

4.3.1 Location and sample collection

Marion Island (46º54'S, 37º55'E) and Prince Edward Island form an isolated archipelago in the Indian Ocean sector of the Southern Ocean (Fig. 4.1). Around 400 km^2 in size, the island rises to an ice plateau (1200 m a.s.l.) at its centre. Its climate is cool, wet and windy, with limited seasonal temperature variation and high annual precipitation (Chown & Froneman 2008). Thus, low and high temperatures (in the context of local ranges of variability) can occur at any stage of the year, and warm periods in winter and/or cold periods in summer are not uncommon (Deere & Chown 2006). Given its oceanic location, the interplay between stability and unpredictability in terms of temperature variation is likely to have been a feature throughout the history of Marion Island (see Boelhouwers et al. 2008).

The springtail *C. a. travei* is found across the island, often associated with mosses and the alga *Prasiola crispa* Menegh. Lightf. Collections of adult animals (and subsequent analyses) were made from six geographic regions (Trypot Beach (TR), Cape Davis (CD), Katedraalkrans (KA), Swartkop Point (SW), Kildalkey Bay (KD), and Mixed Pickle (MP)) (Fig. 4.1; Table 4.1) on Marion Island between 14 April and 5 May 2007. After collection, samples were kept outside the laboratory for a minimum of one day and a maximum of three days in order to maintain near-natural field conditions. Individuals were then moved to plastic vials containing a moist Plaster-of-Paris base and moss shoots as a food source (the moss habitat also includes epiphytic algae, likely to be the primary diet of this species, cf. Worland & Lukesova 2001), and stored at 10° ± 0.5°C in a Sanyo MIR incubator (Sanyo, Loughborough, UK) (12:12 L:D) for 24 h prior to metabolic rate measurement.

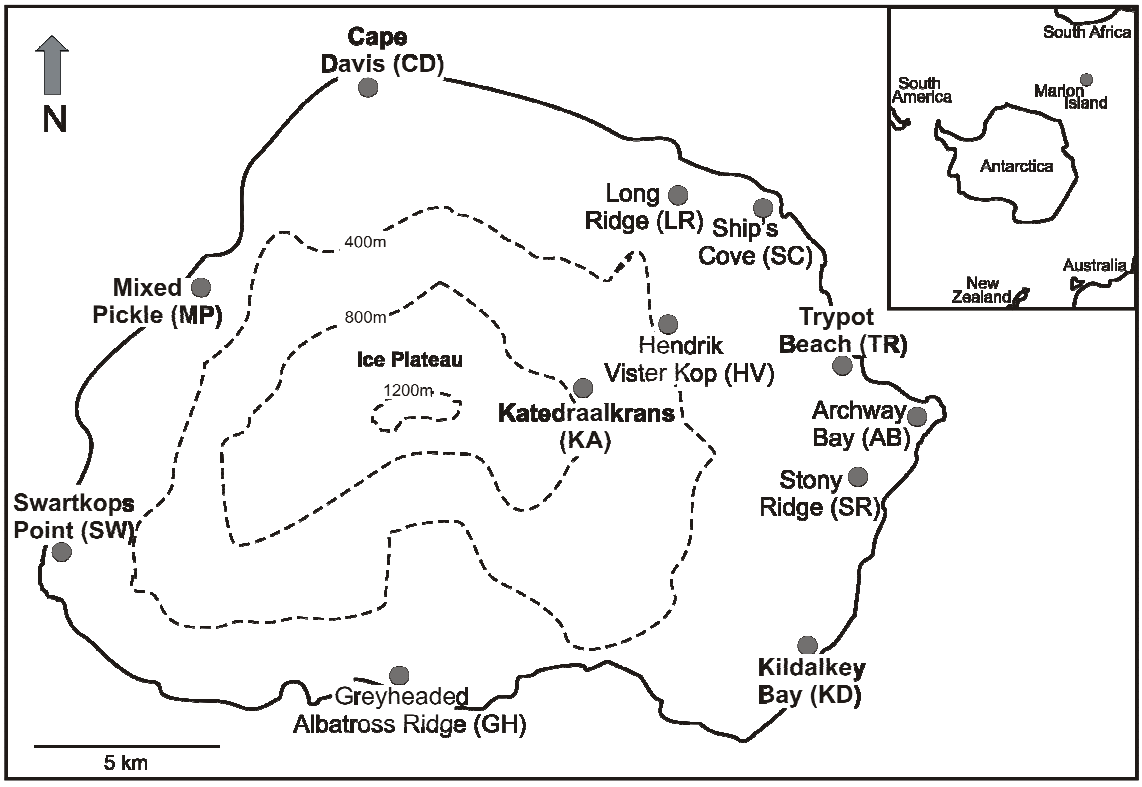

Figure 4.1 Map showing the geographic locations on Marion Island referred to in the text. Locations in bold type indicate those for which collected individuals underwent both metabolic rate and genetic analyses (see Methods; section 4.3.1). Additional locations represent sampled populations from Myburgh et al. (2007) (see Methods, section 4.3.3). Inset: Marion Island's location in the Indian Ocean.

4.3.2 Population metabolic rate structure

The method employed to measure rates of oxygen consumption followed that of McGaughran et al. (2009). In brief, a fiber-optic oxygen sensing system (Ocean Optics Inc., USA) was used to monitor oxygen partial pressure (pO_2) over time for individual animals in a closed respirometry system calibrated (by the manufacturer) for multiple temperatures and oxygen concentrations. Before each rate measurement was made, a single point calibration in air (20.95 vol % oxygen) was completed as per manufacturer's instructions.

Following calibration, individual animals that had been starved for 24 h were placed in a custom-made 40 µl chamber (see McGaughran et al. 2009), into which the oxygen probe was inserted. During a 3 h period, temperature and pO_2 in the chamber were recorded continuously using oxygen sensing software (OOISENSOR ver. 1.05, OceanOptics Inc., USA). Temperature during runs was held at 10º ± 0.1°C using a Sable Systems PTC-1 cabinet (Sable Systems, Las Vegas, USA). This measurement temperature was slightly higher than the average summer microhabitat temperature later measured at Marion Island (see Results, section 4.4.4), however, was selected primarily for comparability to existing metabolic rate work on springtails in continental Antarctica (e.g. McGaughran et al. 2009). Partial pressure profiles were used to calculate oxygen consumption rates on a per animal basis for each individual using the observed drop in pO_2 over a given time period in conjunction with chamber volume and gas density calculations using the ideal gas law. An estimate of individual animal mass was used to express corresponding oxygen consumption rates on a mass-specific basis. Photographs of individual springtails were measured using image analysis software (LEICA APPLICATION SUITE, Leica Microsystems, South Africa) and mass was estimated using the relationship: $W = 6.1894L^{3.119} \times 10^{-9}$ (after Block & Tilbrook 1975), where W = mass (µg), L = length (µm), as modelled for the nominate subspecies *C. a. antarcticus* on maritime Antarctic Signy Island.

While measurements of activity were not possible, all metabolic runs showed constant rates of oxygen percentage decrease over time, therefore we consider that the measurements obtained are a reasonable estimate of standard metabolic rate. Given the small size of the animals in this study, respiration rates were expected to be near the

resolution limit of the oxygen sensing system (see www.oceanoptics.com). Hence, blank runs (n = 10) were completed following the protocol above except without the addition of an animal to the measurement chamber, and a mean baseline was calculated and subtracted from each metabolic rate measurement.

Table 4.1 Location where samples of *Cryptopygus antarcticus travei* were collected during April 2007 from Marion Island (in italics); and of samples from Myburgh et al. (2007).

Location	Latitude	Longitude	Altitude (m)
Trypot Beach (TR)	46º53'05" S	37º52'05" E	13
Swartkop Point (SW)	46º55'28" S	37º35'44" E	57
Kildalkey Bay (KD)	46º58'01" S	37º31'10" E	19
Ship's Cove (SC)	46º51'14" S	37º50'30" E	30
Hendrik Vister Kop (HV)	46º53'12" S	37º48'49" E	282
Katedraalkrans (KA)	46º53'54" S	37º46'29" E	768
Long Ridge (LR)	46º52'55" S	37º47'11" E	515
Archway Bay (AB)	46º53'56" S	37º53'42" E	39
Greyheaded Albatross Ridge (GH)	46º57'43" S	37º42'31" E	84
Mixed Pickle (MP)	46º52'20" S	37º38'21" E	50
Cape Davis (CD)	46º49'41" S	37º42'14" E	63
Stony Ridge (SR)	46º55'03" S	37º51'31" E	162

4.3.3 Population genetic structure

Following metabolic rate analysis, total DNA was extracted from individuals via a 'salting-out' procedure (Sunnucks & Hales 1996). Upon extraction, a 710 bp fragment of the mitochondrial cytochrome *c* oxidase (*cox1*) gene was amplified using the primers, cycling conditions, and purification methods described in McGaughran et al. (2008), and sequenced on a capillary ABI3730 genetic analyser (Applied Biosystems Inc., Foster City, CA) at the Allan Wilson Centre Genome Service, Massey University. In addition to the 51 sequences obtained in this manner, a further 62 sequences incorporating six extra populations (Ship's Cove (SC), Hendrik Vister Kop (HV), Long Ridge (LR), Archway Bay (AB), Greyheaded Albatross Ridge (GH), Stony Ridge (SR); Fig. 4.1, Table 4.1) from Myburgh et al. (2007) were used to estimate a haplotype network in the programme TCS (ver. 1.21; Clement et al. 2000) using a connection limit of 95%. An additional haplotype network including only individuals for which

metabolic rates were obtained was also generated. Individual metabolic rates were arbitrarily grouped into three categories corresponding to 'low' (< 0.0010 $\mu lO_2.\mu g^{-1}.hr^{-1}$), 'medium' ($0.0010 < x < 0.0020$ $\mu lO_2.\mu g^{-1}.hr^{-1}$), and 'high' (> 0.0020 $\mu lO_2.\mu g^{-1}.hr^{-1}$), and mapped onto this second network to examine the relationship between the partitioning of metabolic rate and molecular genetic structure among populations of *C. a. travei*. In both networks, missing data in the alignment (which can distort networks; Joly et al. 2007) were not included, and loops were resolved using the criteria suggested by Crandall and Templeton (1993).

The programme ARLEQUIN (ver. 2.000; Schneider et al. 2000) was used to explore genetic characteristics of the distinct populations. Specifically, we obtained measures of haplotype (h) and nucleotide (π) diversity indices (Nei 1987) separately for each population, and pairwise differentiation (φ-st values) between populations using simple p-distances. Finally, analysis of molecular variance (AMOVA; Excoffier et al. 1992) was used to measure the extent to which genetic variance could be assigned to the hierarchical structure of population organisation for the six populations with both genetic and physiological data available (testing both with no group structure, and with group structure according to a possible north-east/south-west break based on metabolic results (see below): Group 1: 'Cape Davis, Mixed Pickle, Swartkop Point', Group 2: 'Katedraalkrans, Kildalkey Bay, Trypot Beach'), with statistical significance of variance components tested with 16,000 permutations.

4.3.4 Microclimate measurements

To determine whether the microclimate differed among locations, iButton thermochron data loggers (DS1921; Maxim Integrated Products, Sunnyvale CA, USA) were installed by other investigators in April 2008, and used to monitor temperature (± 0.5ºC) at five of the six sites every 60 minutes. iButtons were placed just below the soil surface for locations CD, MP, KA and SW and at 20cm below the soil surface for TR. Temperature data were processed to obtain mean daily maximum, minimum, mean and range for each population.

4.3.5 Statistical analyses

Statistical analyses were performed using MINITAB (ver. 14; Minitab Inc., Pennsylvania, United States). ANOVA was used to determine if live mass or metabolic rate differed significantly among populations. A Spearman's rank correlation was used to test for any direct relationship between metabolic rate and mean population mass. The same analysis was also used to test for correlation between metabolic rate and mean microclimate measures for each population.

In addition, we investigated the relationships among metabolic rate, genetic haplotype and geographic location using univariate tests of significance and a general linear model (GLM). Initially, we used metabolic rate as the dependent variable and tested for significance using mass as covariate and location as the categorical factor. This was then repeated using haplotype as the categorical factor. Finally, a GLM analysis was performed where location and haplotype were crossed. These tests were based on the simplified *a priori* assumption that a functional relationship would exist between *cox1* haplotype and metabolic rate. The premise underlying this was based on the relationship between metabolism, oxidative stress and DNA mutation, whereby findings of increased metabolic rate correlate to findings of increased oxidative damage (through generation of DNA-damaging metabolic by-products such as free radicals) and increased rates of DNA mutation (e.g. Sohal et al. 1990; Adelman et al. 1998; Barja 1999; Cooke et al. 2003)[5].

4.4 RESULTS

4.4.1 Population metabolic rate structure

Blank measurement runs generated very low estimates of equipment-generated 'noise', averaging 0.0012 $\mu lO_2.hr^{-1}$, which equates to 0.000077 $\mu lO_2.\mu g^{-1}.hr^{-1}$ (2 s.f.) using the mean live mass of 17.7 µg. This was much lower than, and significantly different from rates obtained during runs using individual live animals (mean of all data = 0.0019 ± 0.0002 $\mu lO_2.\mu g^{-1}.hr^{-1}$ [S.E.M.] (n = 51)) ($t_{52} = 18.71$; $P < 0.001$). Hence, the equipment

[5] See also Chapter Five.

resolution was sufficient to allow measurement of true rates of metabolism for the size of animals in this study.

Live mass estimates differed significantly between the six populations ($F_{5,43}$ = 3.35, P = 0.012), with the TR population showing the highest mean mass (26.1 ± 4.6 µg [S.E.M.]), followed by SW > KD > CD > KA > MP (Table 4.2). Comparison of metabolic rates also revealed significant differences between these populations ($F_{5,43}$ = 3.00; P = 0.021). In particular, the SW population showed the highest mean metabolic rate (0.0029 ± 0.005 $\mu lO_2.\mu g^{-1}.hr^{-1}$ [S.E.M.]), followed by: MP > CD > KD > KA > TR (Fig. 4.2; Table 4.2).

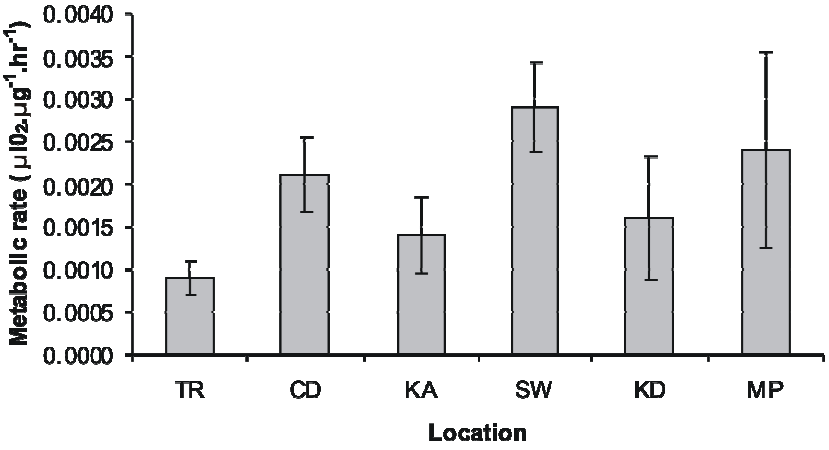

Figure 4.2 Mean metabolic rate (± S.E.M.) of *Cryptopygus antarcticus travei* from six populations ('TR' = Trypot Beach, 'CD' = Cape Davis; 'KA' = Katedraalkrans; 'SW' = Swartkop Point; 'KD' = Kildalkey Bay; 'MP' = Mixed Pickle) across sub-Antarctic Marion Island.

This corresponds roughly to a north-east/south-west metabolic rate break among populations, with the highest metabolic rates on the south-western side of the island, and the lowest on the north-eastern side (see Fig. 4.1). Metabolic structure among populations was confirmed by the GLM analyses, which revealed that metabolic rates of the SW population were significantly different from the other populations ($F_{5,42}$ = 8.196; P < 0.001), with mass non-significant ($F_{5,42}$ = 0.531; P = 0.470). In addition, the Spearman's correlation analysis showed that the differences in metabolic rates among populations were not correlated to mass differences (r = -0.257; P = 0.623).

Table 4.2 Mean live mass(S.E.M) and metabolic rate(S.E.M) (with baseline subtracted) for the six geographic locations on Marion Island, from which *Cryptopygus antarcticus travei* samples were collected and analysed. Results of the ANOVA for each variable are given in the last line of the table (see Methods, section 4.3.5).

Location	n	Mean live mass (µg)	Mean metabolic rate ($\mu lO_2.\mu g^{-1}.hr^{-1}$)
Trypot Beach (TR)	14	26.1(4.6)	0.0009(0.0002)
Cape Davis (CD)	9	11.2(1.8)	0.0021(0.0003)
Katedraalkrans (KA)	9	11.1(1.6)	0.0014(0.0005)
Swartkop Point (SW)	11	22.6(3.3)	0.0029(0.0005)
Kildalkey Bay (KD)	4	14.4(3.8)	0.0016(0.0007)
Mixed Pickle (MP)	4	8.2(2.6)	0.0024(0.0011)
		$F_{5,43} = 3.35$, $P = 0.012$	$F_{5,43} = 3.00$; $P = 0.021$

4.4.2 Population genetic structure

The network displayed in Figure 4.3 shows relationships among the 39 unique haplotypes (30 singletons), obtained from the complete data-set (469 bp; GenBank Accession no.s: GQ848918:GQ848956) of 113 sequences of *C. a. travei*. A single haplotype (H1 – found in 53 individuals) dominates the network, with the majority of specimens connected by one to three steps to this haplotype. Given this, we putatively assign potential 'ancestral' status to this haplotype. A single path in the lower half of the network connects a more 'derived' set of haplotypes that are separated from the core by a greater number of mutational steps (Fig. 4.3). Overall, the configuration of this network conforms closely to that of the 610 bp network presented in Myburgh et al. (2007).

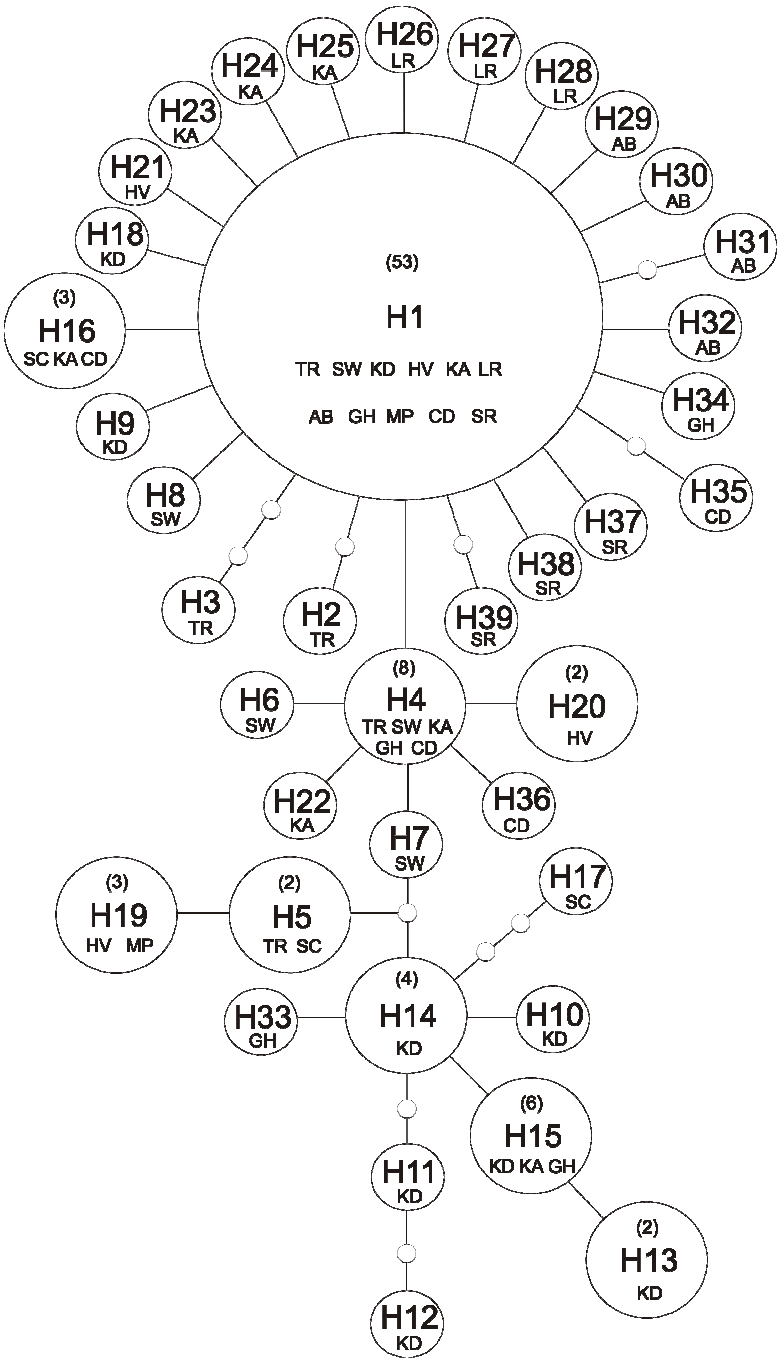

Figure 4.3 Haplotype network showing the nucleotide substitution relationships among 39 haplotypes (113 individuals) of *Cryptopygus antarcticus travei* from Marion Island. For each haplotype, the central label is the haplotype code, the upper value in parentheses is the haplotype frequency (when > 1), and lower label is the population code (see text and Table 4.1). Each line represents one mutational step, while '○' indicates an inferred or missing haplotype.

Haplotype-sharing among *C. a. travei* populations was common, with 39 haplotypes shared among 113 individuals and six (or 15%) of the total haplotypes

present in more than one population/location. The most common haplotype (H1), was present in all populations except SC, while the number of haplotypes present in each population was relatively high (e.g. eight haplotypes were present in both the KD and KA populations). Unique haplotypes were present in all populations except MP, and in particular, six unique haplotypes were present in the KD population (Table 4.3). Of all populations, KA showed the greatest number of genetic relationships to other populations with four of its eight haplotypes shared with other populations.

Table 4.3 Population statistics and genetic characteristics of sampled locations for *Cryptopygus antarcticus travei*: n, number of individuals; H, number of haplotypes; P, number of polymorphic sites; h, haplotype diversity; π, nucleotide diversity. Genetic population codes correspond to those used in Table 4.1.

Genetic population	n	H	P	h(s.d.)	π(s.d.)	Distribution of haplotypes within populations
TR	15	5	9	0.562(0.143)	0.003(0.002)	H1, H2, H3, H4, H5
SW	20	5	3	0.505(0.126)	0.001(0.001)	H1, H4, H6, H7, H8
KD	15	8	10	0.895(0.053)	0.006(0.004)	H1, H9, H10, H11, H12, H13, H14, H15
SC	3	3	7	1.000(0.272)	0.009(0.008)	H5, H16, H17
HV	9	5	8	0.861(0.087)	0.006(0.004)	H1, H18, H19, H20, H21
KA	13	8	9	0.808(0.113)	0.004(0.002)	H1, H4, H15, H16, H22, H23, H24, H25
LR	4	4	3	1.000(0.177)	0.003(0.003)	H1, H26, H27, H28
AB	6	5	5	0.933(0.122)	0.004(0.003)	H1, H29, H30, H31, H32
GH	7	5	7	0.905(0.103)	0.007(0.004)	H1, H4, H15, H33, H34
MP	4	2	5	0.500(0.265)	0.005(0.004)	H1, H19
CD	11	5	5	0.618(0.164)	0.002(0.002)	H1, H4, H16, H35, H36
SR	6	4	4	0.800(0.172)	0.003(0.002)	H1, H37, H38, H39

Haplotype diversity (h) for *C. a. travei* (excluding locations where n < 4), ranged from 0.505 – 0.933 and was highest (h = 0.933 ± 0.122 [s.d.]) in the AB population (Table 4.3). Measures of nucleotide diversity (π) were highest in the GH population (π = 0.007 ± 0.004 [s.d.]). Several φ-st values were large and significant – a general indication of long-term isolation and low gene flow between locations (Table 4.4). The similarity of KA to other populations (see above) was indicated by comparatively low φ-st values (ranging from 0.006 – 0.417 between KA and other populations; Table 4.4).

AMOVA analysis revealed high levels of significant genetic structure among the subset of six populations (Table 4.5). In particular, ~72% of variation was apportioned within populations. When group structure was assigned to populations for each species (see Methods, section 4.3.3), the amount of variation among groups and among populations within groups was similar (10 – 20%), with most variation (~68%) again apportioned within populations (Table 4.5). Thus, variation within populations outweighs any significant support for a north-east/south-west genetic break among these six populations.

4.4.3 Metabolic rate and genetic structure

The relationship between patterns of metabolic rate and genetic structure is illustrated in Figure 4.4. While individuals with the central haplotype (H1) represent all populations and all metabolic rate ranks (see Fig. 4.4 legend), several of the haplotypes from individuals with high and medium metabolic ranks were more 'derived' genetically (i.e. they are one or more mutational step(s) away from the central haplotype). Conversely, individuals with a low metabolic rank have haplotype designations that include the central (putatively 'ancestral') haplotype 'H1', and several haplotypes that are within 1 – 3 mutational steps from this haplotype. The only exception to this pattern involves two haplotypes that represent individuals with 'low' metabolic ranks (H5 and H14) in the lower part of the network. Thus, Figure 4.4 appears to provide some support for an overall similarity between population genetic and metabolic rate structure among populations.

Table 4.4 Pairwise population φ_st values based on 516bp of COI for 113 *Cryptopygus antarcticus travei* specimens. Numbers in italics denote comparisons for which $P < 0.05$; average pairwise differences within localities are indicated in the diagonal

Population	TR	SW	KD	SC	HV	KA	LR	AB	GH	MP	CD	SR
Trypot Beach (TR)	0.132											
Swartkop Point (SW)	0.021	0.194										
Kildalkey Bay (KD)	*0.488*	*0.559*	0.374									
Ship's Cove (SC)	*0.472*	*0.635*	0.100	0.330								
Hendrik Vister Kop (HV)	0.050	0.111	*0.278*	0.144	0.103							
Katedraalkrans (KA)	0.027	0.006	*0.417*	*0.385*	0.016	0.108						
Long Ridge (LR)	0.026	0.153	*0.473*	*0.397*	0.071	0.017	0.128					
Archway Bay (AB)	0.072	*0.174*	*0.491*	*0.429*	0.118	0.065	0.028	0.162				
Greyheaded Albatross Ridge (GH)	*0.206*	*0.304*	0.065	0.013	0.012	0.116	0.193	*0.233*	0.154			
Mixed Pickle (MP)	0.043	0.072	*0.251*	0.076	0.163	0.085	0.000	0.044	0.090	0.078		
Cape Davis (CD)	0.035	0.009	*0.497*	*0.512*	0.070	0.034	0.046	*0.082*	0.226	*0.004*	0.140	
Stony Ridge (SR)	0.017	0.090	*0.495*	*0.465*	0.100	0.019	0.006	0.040	0.233	0.030	0.025	0.138

However, the GLM analyses were unable to corroborate this suggestive pattern; when haplotype was used as the categorical factor, both haplotype ($F_{2,45}$ = 0.619; *P* = 0.543) and mass ($F_{2,45}$ = 1.942; *P* = 0.170) were non-significant. Additionally, the GLM analysis where location and haplotype were crossed gave a non-significant result ($F_{4,36}$ = 0.619; *P* = 0.652).

Table 4.5 Percentage (%) of variation of molecular variance attributed to levels of hierarchical population structure for *Cryptopygus antarcticus travei* (mtDNA *cox1* sequences), with significance (*P*-value) in parentheses. Test 1 is with no group structure enforced in the AMOVA analysis; Test 2 is with group structure enforced according to a proposed north-east/south-west break (see Methods, section 4.3.3)

Test	Source of Variation	df	Sum of Squares	Variance Components	Percentage of Variation
1	Among populations	5	21.619	0.41253 Va	27.88(< 0.001)
	Within populations	43	45.892	1.06725 Vb	72.12(< 0.001)
	Total	48	67.510	1.47978	
	F_{ST}: 0.27878				
2	Among groups	1	7.906	0.19172 Va	12.28(0.286)
	Among populations within groups	4	13.712	0.30190 Vb	19.34(0.008)
	Within populations	43	45.892	1.06725 Vc	63.38(0.002)
	Total	48	67.510	1.56087	
	F_{SC}: 0.22050; F_{ST}: 0.31625; F_{CT}: 0.12283				

4.4.4 Microclimate measurements

The microclimate data showed that the CD, SW and MP populations (all from the west-coast; Fig. 4.1), were all relatively similar in their temperature mean, maxima, minima and range profiles, however differences between these populations and KA and TR were apparent (Table 4.6). In particular, the high altitude site (KA) had lower temperatures than the other low altitude (coastal) sites and had a lower range of temperature variability. The TR population had the highest mean and minimum temperature of all sites and also the lowest range, however these differences were for data collected 20 cm below the soil surface, which would be expected to experience a lower range of variability and are of limited comparative value with the other sites (Table 4.6).

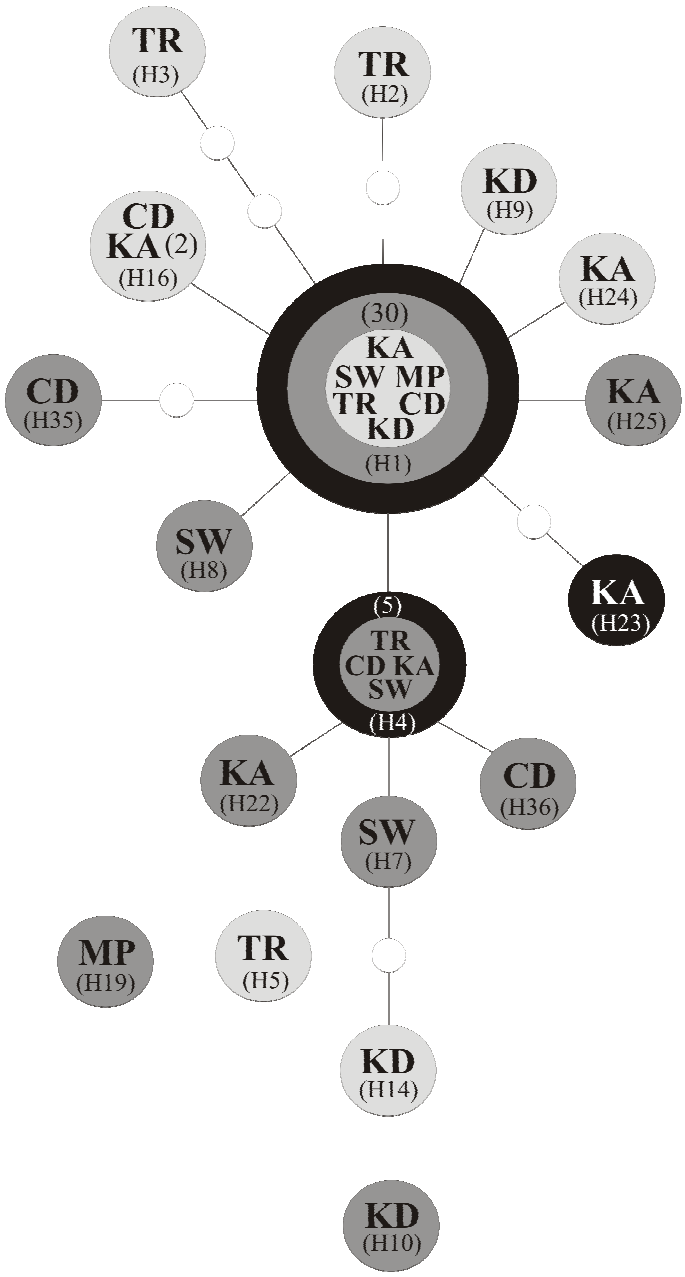

Figure 4.4 A haplotype network showing only those individuals for which a metabolic rate is known. The shading indicates whether individuals with that haplotype have low (< 0.0010 $\mu lO_2.\mu g^{-1}.hr^{-1}$; light grey), medium (0.0010 < *x* < 0.0050 $\mu lO_2.\mu g^{-1}.hr^{-1}$; darker grey) or high (> 0.0050 $\mu lO_2.\mu g^{-1}.hr^{-1}$; black) metabolic rates. Labels within haplotypes refer to population codes (see text and Table 4.1) and numbers in parentheses indicate haplotype frequency (when > 1).

Table 4.6 Microclimate temperature parameters (ºC), including mean, maximum, minimum, and range for five geographic locations on Marion Island 2008 (TR = Trypot Beach; SW = Swartkop Point; KA = Katedraalkrans; MP = Mixed Pickle; CD = Cape Davis) for various periods throughout 01 April - 14 May 2008.

Location	Period of data collection	Mean	Maximum	Minimum	Range
TR*	06.04.08 – 18.04.08	6.9	7.7	6.3	1.4
SW	14.04.08 – 14.05.08	5.0	9.3	2.0	7.1
KA	01.04.08 – 15.04.08	2.0	3.2	1.1	2.0
MP	12.04.08 – 12.05.08	5.2	9.3	2.2	7.1
CD	12.04.08 – 12.05.08	5.0	8.0	2.3	5.8

*NB: these data were collected at 20 cm below the soil surface; all other data was collected from just beneath the soil surface

4.4.5 Metabolic rate and microclimate

The Spearman's rank correlation analysis showed a strongly significant relationship between mean metabolic rate and temperature range ($r = 0.972$; $P = 0.006$). However, the Spearman's correlations with mean and minimum temperature were non-significant ($r = -0.107$ ($P = 0.865$) and $r = -0.400$ ($P = 0.505$), respectively).

4.5 DISCUSSION

Local and regional environmental variability have been found to underlie spatial variation of a range of (non-metabolic) factors that are significant in determining organism life histories and wider scale biological distributions. For example, current patterns of biodiversity across the Antarctic continent are thought to be a response to geographic variation in environmental conditions over time (Peck et al. 2006). Investment in reproduction is greater in northern (sub-Antarctic) populations of the mite *Alaskozetes antarcticus* than in southern (maritime Antarctic) populations (Convey 1998) and, in plants, the balance between sexual or asexual modes of reproduction and dispersal changes with progression into more extreme environmental conditions (Convey 1996a,c; Adams et al. 2006).

Such spatial assortment corresponding to the landscape may also be prevalent at fine scales. In the current study, metabolic rates of *C. a. travei* were found to vary

significantly among discrete populations on Marion Island. In particular, populations from the western side of the island (SW, MP, CD) had higher mean metabolic rates than populations located centrally (KA) or on the eastern side of the island (KD, TR) (Fig. 4.4). A north-east/south-west divide in population genetic structure, and the importance of central grey lava outcrops as refugia, have been proposed for arthropod species on the island (Mortimer & Jansen van Vuuren 2006; Myburgh et al. 2007), and may also be influential factors in the current study. However, while metabolic rate variation among populations was apparent, our analyses indicated that neither genetic haplotype nor location was responsible for its patterns. Indeed, variation in physiological parameters may be influenced by a variety of factors, of which location and genetic haplotype may be viewed as proxies for differentiation brought about by variation in (1) local environmental (microclimate) factors (i.e. location) and (2) historical environmental processes (which may be teased out via genetic inference). Nonetheless, it is intriguing that an east-west signal in temperature mean and variability has been detected on the island (Nyakatya & McGeoch 2008), and that some historical and population genetic evidence also exists for a substantial north-east/south-west geological divide (Mortimer & Jansen van Vuuren 2006; Myburgh et al. 2007; Boelhouwers et al. 2008). Thus, closer examination of both microclimate and genetic data may yield further information about any spatial structuring among populations.

Contemporary climate conditions have been described along an altitudinal gradient for Marion Island, where the mean annual temperature experienced by invertebrate fauna was approximately 6ºC in lowland areas of the island, and 3ºC at 750 m a.s.l (Chown et al. 1997). Investigating the consequences of this temperature gradient for biota, Chown et al. (1997) found that metabolic rates of weevil species (Coleoptera) were generally greater in high-altitude populations, providing an example of metabolic cold adaptation. A further study focusing on thermotolerance variation in Marion Island weevil species found significant intraspecific differences across a climatic gradient (Klok & Chown 2003). The present study suggests that in addition to such altitudinally-related variation, other forms of spatial variation in physiological variables might also exist and should be investigated.

In general, temperature clearly impacts all physiological and biochemical processes, and thus plays a central role within a large component of animal life histories (Nespolo et al. 2003). However, as other environmental variables can interact and/or covary with temperature, it is often difficult to separate the causal effects of temperature alone (Addo-Bediako et al. 2002). Nevertheless, mass-specific metabolic rates obtained in the current study account for body size (the primary determinant of resting metabolic rate in interspecific comparisons among invertebrates alongside temperature; Gillooly et al. 2001; Makarieva et al. 2005), and individual mass, while different among populations, did not explain metabolic rate differences. Thus, a strong contender for a contemporary determinant of the patterns observed in this study is likely to be ambient temperature variation across microhabitats in the different regions of Marion Island.

Indeed, while the microclimate data obtained in the current study contained only a small amount of overlap between populations, it did provide an indication that the temperature of springtail habitats differs among locations on Marion Island (see Table 4.4). In particular, KA and TR were found to have significantly different temperature regimes than the other locations. Specifically, KA was subject to much lower temperatures and a lower range of variability, which is not surprising given its high altitude (768 m a.s.l), while TR differed from the other coastal locations by having the highest mean and minimum, and lowest range of temperatures. It is interesting that KA differed in temperature profile from the other locations and also had one of the lowest mean metabolic rates. It is possible that for this site, low temperatures at high altitude place a restriction on organism activity. Conversely, the SW, MP and CD populations all had similar (high) temperature mean and maxima, and a comparatively high degree of variability (i.e. a high temperature range), and these populations had the highest metabolic rates in this study. The Spearman's correlation analysis found support for a very strong relationship between mean metabolic rate and microclimate temperature range, thus the higher environmental variability of these coastal sites may have resulted in an enhanced ability of local populations to take advantage of higher temperatures when they occur.

In addition to the demonstrated importance of the modern microclimate, historical environmental factors are likely to have played a role in directing population structure

(Avise 2000) on Marion Island. Indeed, clear genetic differentiation of populations at fine scales appears to be a feature of Antarctic systems (e.g. Fanciulli et al. 2001; Stevens et al. 2007; McGaughran et al. 2008) that extends to the sub-Antarctic environment (e.g. Mortimer & Jansen van Vuuren 2006; Myburgh et al. 2007). In particular, the combined effects of periodic volcanism on Marion Island and of Pleistocene glaciation have led to a pattern of inter-population divergence for several terrestrial arthropods (Mortimer & Jansen van Vuuren 2006; Myburgh et al. 2007).

The data obtained in the current study provide further support for the genetic differentiation of populations of *C. a. travei*, with up to 72% of variation apportioned within populations in the AMOVA analyses. Of the twelve populations studied, unique haplotypes were present in eleven populations (all except MP) – a pattern consistent with divergence in isolation. Conversely, high haplotype sharing among the 113 individuals, in addition to specific genetic relationships shown at the population level (e.g. KA shared four haplotypes with other populations; GH, TR and CD each shared three haplotypes with other populations) suggests the presence of a 'global pool' of genetic diversity, from which extant populations are likely sourced. Indeed, the star-like structure of the network presented in Figure 4 indicates a population expansion in the recent genetic history of *C. a. travei* (see Slatkin and Hudson 1991), and a high-altitude refugial population such as could have been provided by KA, may have played a role in this.

The mean metabolic rate structure among populations showed a pattern of: SW > MP > CD > KD > KA > TR. Although TR and KA populations had the lowest mean metabolic rates, individual members of these populations also have 'high' and 'medium' metabolic rates. When examined in concert with the haplotype distribution, the KD population is very distinct from the other populations (with six unique haplotypes), but still shares two haplotypes (H1 and H15) with KA. The remaining populations for which metabolic rate data exist also share haplotypes (e.g. H1 and H4) with KA. Thus it is possible that the KA population represents a refugial source population from which current populations have spread and become structured (both genetically and physiologically).

The collective results of this study suggest that the conditions that have influenced molecular structuring of populations of *C. a. travei* may also have resulted in some degree of differentiation in physiological parameters between populations over time. In addition, differences between populations are likely to reflect variation in local environments, in particular that of temperature variability (range) across the island in different microhabitats. We suggest that responses to both historical factors and contemporary environmental variability have likely played a significant role in defining physiological and genetic structure among populations of *C. a. travei* on Marion Island. Further development of this hypothesis requires comparative studies on species from Marion Island and beyond, to enable a greater understanding of the roles contemporary and historical environments play in shaping population parameters. In addition, further development of the 'metabolic theory of ecology' would benefit from future studies designed to find empirical evidence for a functional relationship between DNA haplotype and metabolic rate.

4.6 ACKNOWLEDGEMENTS

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CHAPTER FIVE:
USING PHYLOGENIES IN ECOLOGY: THE EFFECTS OF METABOLIC RATE ON DNA VARIABILITY AT THE INTRASPECIFIC LEVEL

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5.1 ABSTRACT

There is increasing evidence for a postulated link between physiology and rates of DNA evolution. In particular, metabolic rate, body size and generation time are suggested to play important roles in the rate of nucleotide substitution through their effects on DNA damage and replication frequency.

Here, we examine the relationship between metabolic rate and the tempo of mtDNA (*cox1*) evolution by addressing rates of microevolutionary change at the within-species level in a pre-existing genetic and metabolic rate dataset for the springtail *Cryptopygus antarcticus travei* from sub-Antarctic Marion Island. We test the metabolic rate hypothesis, whereby rates of mtDNA evolution are postulated to be set primarily by mutation pathways mediated by mutagenic by-products of respiration. Using data on relative intra-specific genetic divergence, we examine whether mass-specific metabolic rate is correlated with root-to-tip distance on a set of mtDNA trees, and whether metabolic rates are in any way related to these underlying trees.

Using Bayesian analyses and a novel application of the comparative phylogenetic method, we did not find significant evidence that contemporary metabolic rates directly correlate with the distance from the tree root to the tree tip (i.e. with mutation rate) once the underlying phylogeny is accounted for. However, we did find significant evidence that metabolic rate is dependent on the underlying mtDNA tree, or in other words, lineages with related mtDNA also have similar metabolic rates. We anticipate that future analyses that apply this methodology to datasets with longer sequences or more taxa may have greater power and genetic variability to detect a significant direct correlation between metabolic rate and mutation rate. We conclude with suggestions for possible future analyses that may extend the preliminary approach applied here, in particular highlighting ways to potentially tease apart oxidative stress effects at the mtDNA from the effects of population size and/or selection coefficients operating on the molecular evolutionary rate.

5.2 INTRODUCTION

The rate of molecular evolution is known to vary in three predominant ways (changes in: mutation rate, population size, and selection coefficients; Bromham & Penny 2003), but in practice, the specific causes of rate signature are difficult to isolate (Mooers & Harvey 1994; Mindell et al. 1996). Recent interest has focused on the contribution of nucleotide mutation rate to the rate of molecular evolution. While general trends of this rate variation can be attributed to differences in repair equipment among taxa (Bromham & Penny 2003), multiple variables are expected to affect mutation rate itself.

Mutations arise through unrepaired errors accrued during DNA replication and other damage-causing processes. However, the mutation rate may also be influenced by the life history of a species (Spradling et al. 2001; Bromham & Penny 2003). Thus, factors including body size, generation time, and metabolic rate may play important roles in determining the evolutionary rate of taxa through their effects on the mutation rate. Recently, mutation rates have been analysed indirectly using these biological variables with the intention of developing an all-encompassing theory to describe the patterns and processes of evolutionary rates for a range of taxa (Martin & Palumbi 1993; Mooers & Harvey 1994; Bleiweiss 1998; Gillooly et al. 2001; 2005, 2007; Allen et al. 2006; Wright et al. 2006). According to proposed theories, animal taxa with large body sizes, long generation times, and low mass-specific metabolic rates should have a slower mutation rate (Nunn & Stanley 1998; Barja 1999; Gissi et al. 2000; Estabrook et al. 2007). This is consistent with observations that ectotherms have lower evolutionary rates than endotherms and that small vertebrates with high metabolic rates have higher substitution rates than large vertebrates with lower metabolic rates (Bromham & Penny 2003).

Metabolic rate and generation time (both correlated with body size) may affect mutation rates by altering the mean residence times of nucleotides, such that these would tend to be shorter in small, short-lived and metabolically active species (Bowen et al. 1993). In particular, normal cellular metabolism is well established as a source of reactive oxygen radicals — harmful by-products that account for the background levels of oxidative DNA damage detected in normal tissue (Cooke et al. 2003). In healthy

organisms a small but significant part of respiratory activity generates such radicals (e.g. hydroxide: OH^-), which are capable of modifying several types of macromolecules, including DNA (Cooke et al. 2003). Antioxidants eliminate many of these radicals, however the remaining fraction can cause significant damage. This is generally expected to occur near the sites of radical generation, since the most reactive radicals are poor diffusers (Barja 1999). Thus mtDNA (the site of respiration) is likely to be a prime target for oxygen radical-caused damage, and indeed has a higher rate of molecular evolution than nuclear genes in animals (Bromham & Penny 2003).

Mitochondria are able to repair at least five different types of DNA damage (Barja 1999). However, the repair of mtDNA oxidative damage has been reported as a relatively error-prone process (Souza-Pinto et al. 1999; cited in Barja 1999). In relation to a 'metabolic rate hypothesis', rates of mtDNA evolution are postulated to be set primarily by mutation pathways mediated by mutagenic by-products of respiration. Thus, if reactive oxygen radicals have a mutagenic effect on DNA, then taxa with higher metabolic rates should generate higher concentrations of mutagens and sustain more DNA damage. Indeed, empirical studies have demonstrated that species with higher metabolic rates experience higher rates of reactive oxygen radical production (e.g. Sohal et al. 1990) and higher rates of oxidative DNA damage (Adelman et al. 1988; Cortopassi et al. 1992; Rand 1994).

Interest in metabolic rate for its influence on nucleotide rate variation among populations was stimulated by Martin and Palumbi (1993) and Rand (1994) who documented effects of body size, temperature and correlated variables, including generation time and metabolic rate, on DNA substitution rate across various animal species. More recent work has supported these findings (e.g. Allen et al. 2006; Wright et al. 2006). The mass-specific metabolic rate model of Gillooly et al. (2001) used thermodynamic equations to relate temperature and body size to metabolic rate and Gillooly et al. (2005) demonstrated that body mass, temperature and metabolic rate explain a significant fraction of the variance in nucleotide substitution rates in a broad sample of organisms. Most recently, Gillooly et al. (2007) showed that rates of protein evolution are largely controlled by mutation rates, which in turn are strongly influenced by individual metabolic rate.

A limited number of studies have addressed this question through empirical work (e.g. Avise et al. 1992; Adachi et al. 1993; Martin & Palumbi 1993; Rand 1994; Bleiweiss 1998; Nunn & Stanley 1998; Martin 1999). However, studies of this type have run into inherent difficulties because the effects of different variables on evolutionary rates are hard to tease apart. Divergent groups of taxa usually differ in many respects (e.g. nucleotide generation time, G + C content, various life history traits), making it difficult to isolate single factors acting on DNA evolution. Therefore investigations of rate heterogeneity at an intra-specific level may be helpful, through their avoidance of these particular confounding factors (Zhang & Ryder 1995).

Indeed, few studies to date have examined intraspecific levels of rate heterogeneity. Notwithstanding the difficulties of teasing apart population size and selection effects on rates of evolution (see above), this is most likely due to the relatively small number of mutational events that occur between closely related samples, making it difficult to achieve a statistically significant number of nucleotide mutations between sequences. Choice of a relatively fast-evolving mtDNA gene may go some way towards overcoming this.

Felsenstein (1985) also pointed out that comparative studies must account for the hierarchically structured phylogeny that underlies all species when assessing whether one physiological variable is correlated with another. This is because treating species as the units of analysis in a comparative study assumes that the traits under investigation evolved independently in each individual. Owing to the phylogenetic structure of the data, however, the species will share some portion of the path lengths leading from the root to the tips of a phylogenetic tree, and for closely related species, this may be most of the path length. As a consequence, if taxa with a certain physiological trait are all closely related, they will tend to have a low genetic distance to each other regardless of their trait status (Pagel 1998). Thus, ignoring the underlying coalescent history of taxa will almost certainly bias estimates of any correlation. Fortunately, there are techniques available which can account for the underlying phylogeny when examining hypotheses about trait data (see Pagel 1998).

Here, attempt to use such a method to examine the relationship between metabolic rate and mtDNA (*cox1*) mutation rate. We use mtDNA because, although data on DNA

repair in non-model organisms are scarce, there is evidence that DNA repair efficiency varies in natural populations and may thus be influenced by natural selection (Woodruff et al. 1984; Mason et al. 1985; Nothel 1987; Martin 1999; Sniegowski et al. 2000; Lanfear et al. 2007). It is also recognised that molecular evolution can be highly variable within and among taxonomic groups, at least for mtDNA (Martin 1995). Further, evolutionary lability in metabolic traits including metabolic rate (and by inference, DNA damage rate) mean that variation among individuals will exist at the intra-specific level. Collectively, variation in mtDNA may therefore be used to explore the intraspecific relationships between metabolic rate and the rate of DNA mutation (as opposed to the rate of nucleotide substitution, which is a population-level measurement involving the incorporation of somatic mutations into the germ line).

We explore this by making use of an existing metabolic rate and mtDNA dataset that forms part of a broader project (McGaughran et al. 2008, 2009), to examine the relationship between metabolic rate and mtDNA (*cox1*) mutation rate in the springtail *Cryptopygus antarcticus travei* Déharveng, 1981 (Collembola, Isotomidae) from sub-Antarctic Marion Island. We base our work on the simplified *a priori* assumption that a functional relationship exists between *cox1* haplotype and metabolic rate. The premise underlying this is based on the relationship between metabolism, oxidative stress and DNA mutation outlined in detail above, whereby findings of increased metabolic rate correlate to findings of increased oxidative damage (through generation of DNA-damaging metabolic by-products such as free radicals) and increased rates of DNA mutation (e.g. Sohal et al. 1990; Adelman et al. 1998; Barja 1999; Cooke et al. 2003). Specifically, we test the 'metabolic rate hypothesis' using data on relative intra-specific genetic divergence to determine whether: (1) the trait (mass-specific metabolic rate) is correlated with mtDNA genetic distance (i.e. mutation rate) among individuals; and (2) whether this trait is dependent on the underlying tree.

5.3 METHODS

5.3.1 Location and sample collection

Marion Island (46º54'S, 37º55'E) forms part of an isolated archipelago in the Indian Ocean sector of the Southern Ocean. The springtail *Cryptopygus antarcticus travei* was collected from six locations across the island (Fig. 5.1) during a 3-week period in April 2007.

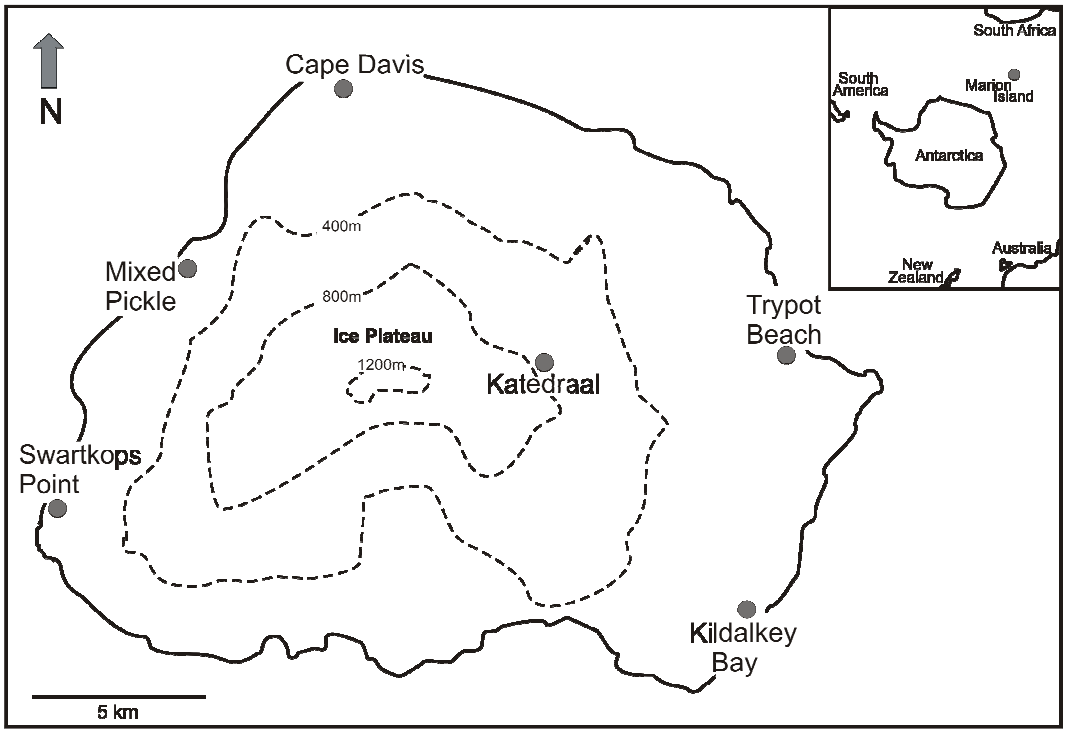

Figure 5.1 Map showing the geographic locations on Marion Island referred to in the text. Inset: Marion Island's location in the Indian Ocean.

5.3.2 Metabolic rate measurements

The method employed to measure rates of oxygen consumption followed that of McGaughran et al. (2009). In brief, a fiber-optic oxygen sensing system (Ocean Optics Inc., Dunedin, FL) was used to monitor oxygen partial pressure (pO_2) over time for individual animals in a closed respirometry system calibrated by the manufacturer for multiple temperatures and oxygen percentages. Upon completion of a run, partial pressure profiles were used to calculate oxygen consumption rates for each individual,

and an estimate of individual animal mass was used to express corresponding oxygen consumption rates on a mass-specific basis (see McGaughran et al. 2009 for further information, including quality control).

5.3.3 DNA extraction, amplification and sequencing

Mitochondrial DNA cytochrome *c* oxidase I (*cox1*) sequences were obtained from all individuals for which a metabolic rate was measured (n = 45). Extraction, thermal cycling and sequencing conditions are outlined in McGaughran et al. (2008).

5.3.4 Haplotype network analysis

TCS ver. 1.21 (Clement et al. 2000) was used to estimate a haplotype network using the statistical parsimony algorithm of Templeton et al. (1992) and a connection limit of 95%. Metabolic rate data was grouped arbitrarily into three roughly equal-sized categories corresponding to 'low' (< 0.0010 $\mu lO_2.\mu g^{-1}.hr^{-1}$), 'medium' (0.0010 < *x* < 0.0020 $\mu lO_2.\mu g^{-1}.hr^{-1}$), and 'high' (> 0.0020 $\mu lO_2.\mu g^{-1}.hr^{-1}$) and mapped onto this network to give a graphical representation of any relationship between mutation rate and metabolic rate.

5.3.5 Combined metabolic rate and DNA mutation rate analyses

To explore whether metabolic rate is correlated with evolutionary rate (distance from the ancestral haplotype), we took two approaches. The first was based on the haplotype network directly; we calculated two test statistics that were designed to measure (1) if similar haplotypes had similar metabolic rates, and (2) if haplotypes that were further from the putative root of the network had higher metabolic rates. The significance of these two test statistics was assessed using a randomisation test. The second approach was to perform correlation analysis on a set of trees generated using Bayesian analyses in the programme BAYESTRAITS ver. 1.0 (Pagel 1999; available from www.evolution.rdg.ac.uk). These approaches are outlined in more detail below.

5.3.5.1 Randomisation tests

The first test statistic, T_1, was the sum, over all pairs of haplotypes whose distance in the haplotype network was less than k, of the absolute value of the difference in metabolic rate: $T_1(k) = \sum_i \sum_{j \in N_k(i)} |m(i) - m(j)|$; where an individual j is in the neighbourhood $N_k(i)$ of an individual i if the distance in the haplotype network between i and j is less than k, and $m(i)$ is the metabolic rate of individual i.

The second test statistic, T_2, was the sum, over all directed edges (u,v) in the haplotype network, of the average metabolic rate of individuals at node v minus the average metabolic rate of all individuals at node u.

The null distribution of each of these test statistics was determined by reassigning the metabolic rates to individuals at random without replacement (i.e. shuffling the metabolic rates) and recalculating the test statistic 1000 times. The most prevalent haplotype was used as the putative root of the network, and all edges were directed away from the root. For each test statistic we report a p-value, which is the number of times out of the 1000 randomisations that the value of the test statistic was higher than for the real (un-shuffled) metabolic rates.

5.3.5.2 BAYESTRAITS (correlation) analysis

5.3.5.2.1 Background to BAYESTRAITS

BAYESTRAITS implements two models of continuous trait evolution: the standard constant-variance random walk model (Model A), in which the given trait evolves randomly (i.e. with no overall tendency to increase or decrease) along a phylogenetic tree; and a directional random walk model (Model B), in which the trait tends to either increase or decrease along the tree, leading to the expectation that mutational steps from the root will be correlated with the trait value (Pagel 1999).

In the standard BAYESTRAITS approach Model A and Model B are compared in order to test the hypothesis that tips further from the root have different average trait values from tips nearer the root due to directional selection (Pagel 1999). Here, we use BAYESTRAITS in a novel way, however, the hypotheses tested produce the same patterns: if there is a causal link between mass-specific metabolic rate and mutation rate

then metabolic rates will be higher for tips that are further from the presumed root of the tree (Model B pattern), whereas if there is no direct correlation between metabolic rate and mutation rate there should be no trend of higher metabolic rates for tips that are further from the presumed root of the tree (Model A pattern).

In the analyses presented here, we determine significance of results based on the use of Bayes Factors as described in the BAYESTRAITS manual (Pagel 1999; www.evolution.rdg.ac.uk). The use of Bayes Factors applies logic similar to that used in likelihood ratio tests, except the marginal likelihoods of two models are compared rather than their maximum likelihoods. The marginal likelihood (approximated by the harmonic mean of the maximum likelihoods in BAYESTRAITS when the Markov chain has run for a sufficient number of iterations; see BAYESTRAITS manual) of a model is the integral of the model likelihoods over all values of the models parameters and over all tree results. Thus, to compare two models, the Bayes Factor 'test statistic' is: 2(log[harmonic mean(better model)] – log[harmonic mean(worse model)]; any positive value favours the dependent model, but conventionally, a ratio > 2 is taken as 'positive' evidence, > 5 is 'strong' and > 10 is 'very strong' evidence for support of one model over the other (Pagel 1999; www.evolution.rdg.ac.uk).

5.3.5.2.2 Analysis using BAYESTRAITS

We initially ran MODELTEST ver. 3.7 (Posada & Crandall 1998) in PAUP* on the *cox1* dataset to determine the best model of evolution for subsequent Bayesian analyses – both the hierarchical Likelihood Ratio Test (hLRT) and Akaike Information Criterion (AIC) returned the HKY model. We then used the programme BAYESPHYLOGENIES to generate a Bayesian phylogeny estimation over 100,000,000 iterations, sampling every 100,000th tree (Pf = 100,000) using the HKY model. We reviewed the resulting files in TRACER ver. 1.4.1 (Rambaut & Drummond 2007) to check convergence and proceeded with a tree file (with 10% burn-in discarded) of 900 trees, to BAYESTRAITS analysis.

All our analyses used the sub-programme 'CONTINUOUS' within the software package BAYESTRAITS. We felt that the most appropriate approach here was to use the programme in MCMC mode (vs. ML mode) on a set of trees generated by Bayesian analyses. Such trees come from the posterior distribution, i.e. they are sampled in

proportion to their likelihood given the sequence alignment data and the model of sequence evolution; this means that running BAYESTRAITS in MCMC mode is effectively averaging over the tree estimate or treating it as a nuisance parameter.

As we required rooted, fully-resolved trees, we assigned an arbitrary individual bearing the most common haplotype (see Fig. 5.2) to the outgroup and forced the analysis to retain zero length branches (using the pset collapse=no command in PAUP*) rather than collapsing branches. We note that this approach may affect the comparison of Model A and B in BAYESTRAITS as an incorrect root node may hamper the detection of trait evolution, hence we repeated our analyses assigning another arbitrary individual bearing the most common haplotype to the root node and checked for concordance of results.

First, we used BAYESTRAITS (in MCMC mode) to examine the correlation between the root-to-tip distance (i.e. mutation rate) of our phylogenetic estimates and the trait (mass-specific metabolic rate) under both the Random Walk (Model A) and Directional (Model B) models of trait evolution. Initial runs showed that the MCMC chain was not mixing well; this was solved by scaling the metabolic rate dataset up by a factor of 1,000, and by lowering the ratedev parameter to 0.002 (Mark Pagel, personal communication). Second, we performed additional analyses under Model A to investigate whether metabolic rate was dependent on the underlying tree. This was assessed by calculating the Bayes Factor for a comparison between a model in which the lambda (λ) parameter was freely estimated versus a model in which it was set to 0 (the latter corresponds to variation in the trait being entirely independent of the underlying phylogeny).

5.4 RESULTS

5.4.1 Haplotype network analysis

There were a total of 16 unique haplotypes for the *cox1* dataset and the maximum number of mutational steps between these was nine (Fig. 5.2). The haploytpe network showed a pattern of one most common (i.e. presumed 'ancestral') haplotype (n = 27) from which several singletons (and one haplotype with n = 4) were derived.

Of the 45 individuals, 17, 11 and 17 corresponded to 'low', 'medium' and 'high' metabolic rate categories, respectively. The ancestral haplotype had a mixture of individuals with 'low', 'medium' and 'high' metabolic rates, however a general trend where more derived haplotypes (i.e. those with a greater number of mutational steps from the proposed ancestral haplotype) have higher metabolic rates was evident. In particular, 11 of the 15 derived haplotypes were from individuals with 'medium' or 'high' metabolic rates. Thus there appears to be some support for an overall pattern of metabolic rate structure coinciding with genetic structure.

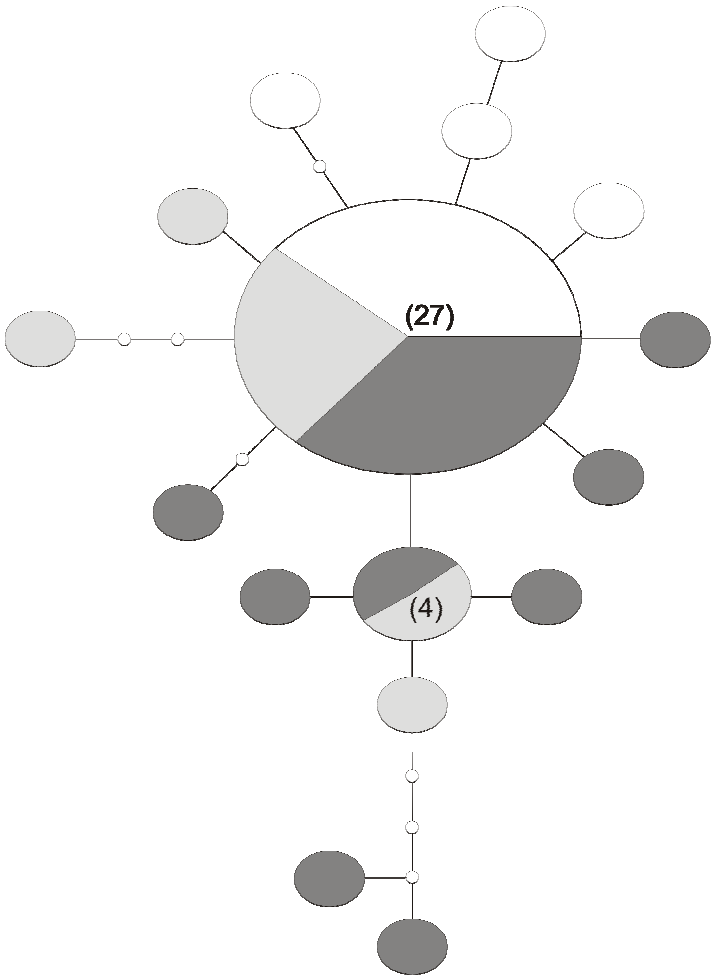

Figure 5.2 Haplotype network for the mtDNA *cox1* dataset for *Cryptopygus antarcticus travei* from Marion Island. Shading indicates whether individuals with that haplotype have low (< 0.010 $\mu lO_2.\mu g^{-1}.hr^{-1}$; white), medium (0.0010 < *x* < 0.0020 $\mu lO_2.\mu g^{-1}.hr^{-1}$; light grey) or high (> 0.0020 $\mu lO_2.\mu g^{-1}.hr^{-1}$; dark grey) metabolic rates. Numbers in parentheses indicate haplotype frequency (when > 1).

5.4.2 Combined metabolic rate and DNA mutation rate analyses

5.4.2.1 Randomisation test analyses

The first test statistic we evaluated, $T_1(0)$, measures the similarity in metabolic rates of individuals with identical haplotypes. The *p*-value for this test was 0.061 meaning that individuals that share the same haplotype have more similar metabolic rates than expected by chance at a 10% level of significance. The *p*-value for $T_1(1)$, which measures the similarity in metabolic rates of individuals that differ by at least one mutational step, was 0.208, which means that these individuals do not have metabolic rates that are more similar than expected by chance at a 10% level of significance.

The second test statistic, T_2, measures whether there is a directional change in metabolic rates as we move away from the putative root of the haplotype network. This test was not significant ($P = 0.871$).

5.4.2.2 BAYESTRAITS analyses

The 900 trees from the post burn-in posterior distribution of the BAYESPHYLOGENIES analysis are summarised as a consensus tree in Figure 5.3. The lack of resolved relationships in Figure 5.3 (in particular the largest polytomy includes the 27 taxa that form the main group in the haplotype network) is expected given the haplotype network (Fig. 5.2) and highlights the importance of averaging over different possible coalescent histories (i.e. using MCMC mode in BAYESTRAITS). The branch lengths shown are means (i.e. the length of a given branch is the average value of that branch length taken over all the trees it appears in); they indicate that there is variation in the root to tip distances when the root is chosen to be an arbitrary taxon from the main haplotype group (Fig. 5.3).

For the first analysis run in BAYESTRAITS (comparing Model A to Model B), we found that, while Model B had a slightly better marginal likelihood, the evidence to support this was not strong (i.e. Bayes Factor < 2) (Table 5.1). This means our analyses are unable to reject a model in which metabolic rate is not correlated with greater genetic distance to the ancestral node.

However, in our subsequent BAYESTRAITS analysis examining the lambda parameter within Model A, we found a significant difference (Bayes Factor > 10)

between the model where λ was estimated and the model where λ = 0 (Table 5.1). This means there is strong evidence that λ ≠ 0, i.e. that metabolic rate is dependant on the underlying mtDNA tree.

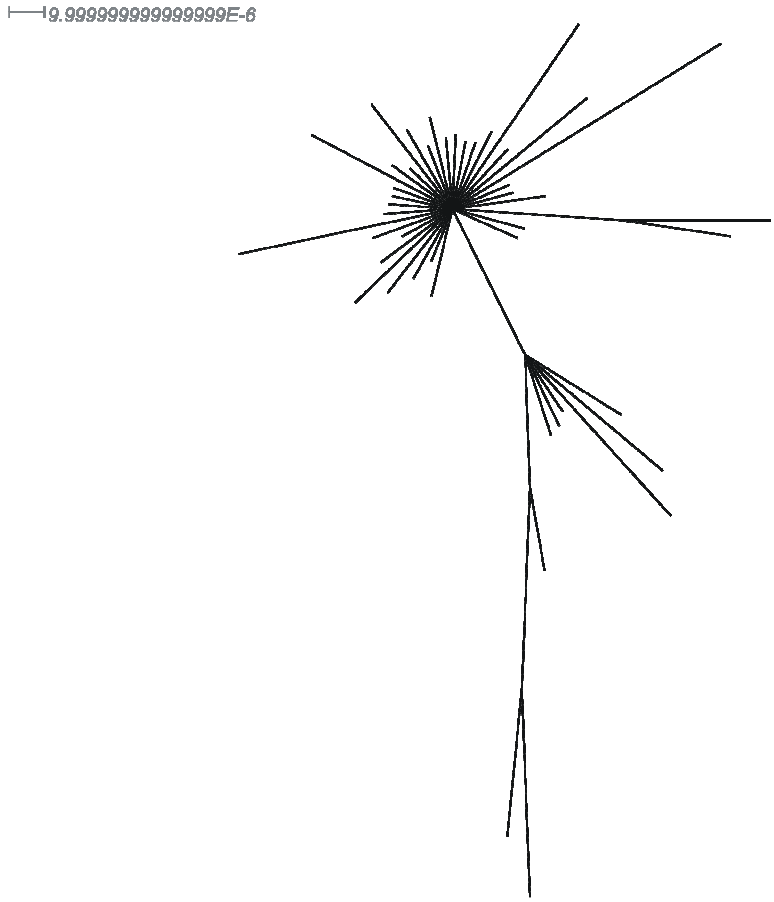

Figure 5.3 Majority-rule consensus tree of the 900 post-burnin trees from the BAYESPHYLOGENIES analysis. Branch lengths are averages over the length of the branch in the trees in which it appeared.

Table 5.1 Harmonic mean of log likelihood values of metabolic rate and DNA mutation rate correlation tests (see Methods, section 5.3.5.2). All tests were performed in BAYESTRAITS on the springtail *Cryptopygus antarcticus travei* from sub-Antarctic Marion Island.

Model	Harmonic mean of log likelihood values	Bayes Factor
A	-77.4104	0.8476
B	-76.5628	
A(λ=est)	-77.0837	21.9678
A(λ=0)	-99.0515	

5.5 DISCUSSION

Metabolic rate, body size, generation time and effective population size vary through evolutionary time. These changes likely introduce substantial noise to comparisons of physiology and life-history parameters with rate of evolution. Despite this, several researchers have reported observing correlations between rate of evolution and other factors (e.g. Martin & Palumbi 1993; Adachi & Hasegawa 1996; Li et al. 1996). This includes work that looked at evolutionary rates in two lineages of Hawaiian *Drosophila* species and showed that population-level phenomena can be important in understanding evolution at the molecular level (DeSalle & Templeton 1988, see also the work of Zhang & Ryder 1995). Metabolic factors were also highlighted as important correlates (if not actual determinants) of variation in evolutionary rates of hummingbirds, whose metabolism may be great enough to alter substitution rates at the level of the nuclear genome (Bleiweiss 1998). Slow mitochondrial evolution in turtles has been shown to potentially correlate with generation time and/or metabolic rate (Avise et al. 1992) and further studies supporting the metabolic rate hypothesis include the work of Cortopassi et al. (1992) on aging in human tissues, a rate assessment performed by Bowen et al. (1993) for marine turtles, and Nunn and Stanley's (1998) study of tube-nosed seabird evolution.

In contrast, several studies have failed to find support for the metabolic rate hypothesis (e.g. Adachi et al. 1993; Bromham et al. 1996; Mindell et al. 1996; Spradling et al. 2001), or have conversely supported body temperature (e.g. Mindell et al. 1996), generation time (e.g. Mooers & Harvey 1994), speciation (e.g. Mindell et al. 1989; Barraclough et al. 1996), or some combination of these factors (e.g. Bousquet et al. 1992). Most recently, a study on the New Zealand tuatara noted the highest rate of molecular change recorded in a vertebrate, but contrastingly slow tempos of metabolism and growth, and a long generation time for this reptile (Hay et al. 2008).

The current study is among the first to examine this clearly complex relationship at the intra-specific level, and began with what appeared to be a degree of support for the proposed relationship between metabolic rate and DNA mutation rate. This support was in the graphical form of a haplotype network onto which metabolic rates were mapped.

However, the results of our analyses that tested for a direct correlation between these variables while accounting for the underlying phylogeny were unable to strongly corroborate this initially promising pattern in *C. a. travei*. In other words, metabolic rate does not appear to directly increase or decrease with evolutionary distance of individuals from the 'ancestral' root of the tree. Conversely, subsequent analysis within a BAYESTRAITS lambda framework provided strong evidence (Bayes factor > 10) for the hypothesis that metabolic rate is dependant on the underlying tree. Thus, we conclude that, while we find no strong evidence to suggest that metabolic rate and mutation rate (when measured as the root to tip path length) are correlated directly, there is an indirect relationship between these two variables, such that lineages with related mtDNA also have similar metabolic rates.

Testing for a direct correlation between DNA mutation rate and metabolic rate as well as several other life history traits is rendered difficult for several reasons (Spradling et al. 2001). For example, we cannot assume that relative rates of oxygen radical production in gonad cells are effectively measured by whole body metabolic rate estimates (Mindell et al. 1996), and although evolutionary rates reflect the evolutionary history of each lineage, life history variables are measured in the present time and retain little information about the way they may have changed over time across a lineage (Gissi et al. 2000). In addition, metabolic rate is clearly not the only causative force leading to DNA damage (Martin 1995) and a variety of factors are likely to contribute to the likelihood of any mutations becoming fixed in the population (e.g. protein function, purifying selection, population size) (Mindell & Thacker 1996).

In relation to *C. a. travei* from Marion Island, it is certainly possible that differences among individuals relate to different selective pressures among different populations. In particular, populations from the western side of the island (SW, MP, CD) have higher mean metabolic rates than populations located on the eastern side of the island (KA, KD, TR) (Fig. 5.1; McGaughran et al. submitted manuscript; see Chapter Four) and this may suggest underlying environmental differences among locations. Such variation over sufficient timescales may initiate selection processes in certain loci.

Finally, the mtDNA *cox1* gene may not be ideal for testing these relationships and the sequence lengths employed here (516 bp) may also be insufficient. Indeed, close inspection of the BAYESPHYLOGENIES tree file generated in this study revealed that many different coalescent histories are compatible with the data (as expected given the haplotype network; Fig 5.2), and this could obscure any association between mutation rate and metabolic rate. Unfortunately, this limitation, which essentially relates to a poor phylogenetic signal in our dataset, lies at the heart of the intra-specific approach and is likely to represent the most severe hindrance to studies of this type.

Although we stand by the *a priori* assumption underlying our research (that a functional relationship exists between mtDNA haplotype and metabolic rate based on the relationship between metabolism, oxidative stress and DNA mutation; see Introduction), we must acknowledge the limits of our approach in determining between the effects of metabolic rate and other important factors which cause variation in evolutionary rate (i.e. population size and/or selection coefficients operating at the molecular level). With this in mind, we intend our results to be interpreted as a 'first step' in the analysis of this complicated issue to expand the metabolic theory of ecology, and wish for them to ultimately serve as a proxy for future studies which are able to tease apart the various confounding factors.

In particular, we recommend that future work include further analysis following our methodological approach and using phylogenetically independent contrasts (PICs). For example, closely related sister taxa with differing life history variables such as generation time, body size, colonisation histories, population size, potential selection coefficients and of course, metabolic rates would provide fertile ground for future study of this issue. Examinations at the intra-specific level using larger datasets and including multiple genetic loci (including nuclear genes) are also envisaged as steps to further enlarge our preliminary demonstration. These latter suggestions in particular would aid the process by giving greater power (i.e. phylogenetic signal) to statistical tests such as the ones performed here. Indeed, we strongly recommend that analyses of this type be repeated as larger datasets become available, and expect that recent advances in DNA sequencing technology allowing higher throughput (e.g. of complete mtDNA genomes) are likely to make such studies feasible in the near future. Finally, our work highlights

the need for development of new statistical approaches that seek to accommodate coalescent processes which could potentially drive variation in branch lengths independently of any underlying variation in mutation rate. We look forward to such advances and the contribution they may make to the generation of general conclusions concerning sources of rate heterogeneity.

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CHAPTER SIX:

CONTRASTING PHYLOGEOGRAPHIC PATTERNS FOR SPRINGTAILS REFLECT DIFFERENT EVOLUTIONARY HISTORIES BETWEEN THE ANTARCTIC PENINSULA AND CONTINENTAL ANTARCTICA

The work in this chapter is published:

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6.1 ABSTRACT

We examined genetic structure among populations and regions for the springtails *Cryptopygus antarcticus antarcticus* and *Gomphiocephalus hodgsoni* (Collembola) from the Antarctic Peninsula and continent, respectively. Samples were collected from 24 and 28 locations across the Antarctic Peninsula and southern Victoria Land regions for *C. a. antarcticus* and *G. hodgsoni*, respectively. We used population genetic, demographic and nested clade analyses based on mtDNA (*cox1* and *cox2*) to identify potential historical refugia and subsequent colonisation routes, and to examine population growth/expansion and relative ages of population divergence.

Both species were found to have population structures compatible with the presence of historical glacial refugia on Pleistocene (2 Ma – present) time-scales, followed by postglacial expansion generating contemporary geographically isolated populations. However, *G. hodgsoni* populations were characterised by a fragmented pattern with several 'phylogroups' (likely 'ancestral' haplotypes present in high frequency), indicating a strong ancestral presence among present-day populations. Conversely, *C. a. antarcticus* had an excess of rare haplotypes with a much reduced volume of 'ancestral' haplotypes, possibly indicating historical founder/bottleneck events and subsequent widespread expansion.

We infer that these differences reflect distinct evolutionary histories in each locality despite the resident species having similar life history characteristics. We suggest that this has predominantly been influenced by variation in the success of colonisation events as a result of intrinsic historical glaciological differences between the Antarctic Peninsula and continental environments.

6.2 INTRODUCTION

Species' genomes contain resolvable indications of past events. In particular, patterns of genetic variation within and among populations contain information about the origin and demography of a species. For example, measures of haplotype and nucleotide diversity can be used to identify population origins and colonisation routes, because

intraspecific diversity should decline away from source populations and/or refugia (Avise 1994; Hewitt 1996). The relationship between genetic and geographic distances can be evaluated (e.g. by a Mantel test; Mantel 1967) and simple estimates of the number of differences between DNA sequences (e.g. p-distances) can be used to infer ages of population and species divergence events through employment of a molecular clock.

Furthermore, the application of modern coalescent theory allows inferences to be made about a species' demographic history, such as past and present population size and ages of population divergence and growth (Kuhner et al. 1998; Schneider & Excoffier 1999). This is because episodes of population decline cause decreases in genetic diversity while episodes of growth cause retention of (rare) alleles or haplotypes that would otherwise be lost (Harpending 1994). Demographic analyses are particularly important in the analysis of contemporary patterns of population structure because the effects of past geological events on the distribution and abundance of species are known to be significant (e.g. Hewitt 1996). In particular, range expansion and colonisation followed by demographic expansion is a common result of glacial cycling and it is possible to find signals of these processes in contemporary populations.

In regions of glaciation, bottleneck events and subsequent population expansions are thought to be common, and Antarctica provides an ideal location for examining demographic hypotheses. Glacial events are important in structuring the genetic diversity of Antarctic terrestrial arthropods (e.g. Frati et al. 2001; Allegrucci et al. 2006; McGaughran et al. 2008) and other biota (see Rogers 2007). Thus, studies focusing on population structure are also well-suited to the Antarctic realm. Specifically, fragmentation of populations and isolation in widely dispersed snow- and ice-free areas (e.g. nunataks, coastal regions, inland dry valleys) that are known to have persisted during glacial maxima (e.g. Convey & Stevens 2007; Convey et al. 2008) are likely to have been important mechanisms of population structuring for Antarctic biota (Rogers 2007), both on more recent Pleistocene and longer-term Miocene and Pliocene (23 – 2 Ma) time-scales.

While snow and ice-free areas have been extremely important in terms of species persistence in Antarctica, they are similarly important over contemporary time-scales,

where terrestrial habitats essentially consist of widely dispersed habitats that are ecologically equivalent to islands surrounded by areas unsuitable for habitation (Beyer & Boelter 2002; Bergstrom et al. 2006). This, combined with the limited ability of terrestrial Antarctic arthropods to withstand extended periods of desiccation (e.g. Convey 1996) results in limited dispersal opportunities and contributes to the disjunct nature of contemporary populations. Accordingly, physical, ecological and geological barriers isolate populations presently, and have also been significant factors over the substantial evolutionary history of Antarctica's endemic terrestrial species (Stevens et al. 2006).

It is now clear that persistent ice-free areas have allowed large elements of the contemporary Antarctic terrestrial biota to have had a long continuous and isolated history within the region (e.g. Convey & Stevens 2007; Convey et al. 2008) and endemic arthropods including several species of springtails and mites may be considered Antarctica's 'relict' fauna (Stevens et al. 2006; Pugh & Convey 2008). In addition, certain regions of Antarctica may be viewed as distinct from each other on account of a variety of differing ecological and climatic factors. For example, an ancient biological and geological boundary (the 'Gressitt Line') exists between the Antarctic Peninsula and continental Antarctica, across which very few terrestrial invertebrate species are shared (Chown & Convey 2007). Furthermore, separate areas within the continent also possess high levels of regional endemism, suggesting that they too are of dissimilar (ancient) origins (Pugh & Convey 2008).

Despite this, Antarctic terrestrial taxa as a general rule employ similar 'adversity selected' life history strategies (typically involving features such as long life cycles, large investment in survival traits, low reproductive output, limited dispersal abilities) to cope with the challenges of the Antarctic environment (Convey 1996). Research to date has shown taxa to have similar patterns of population sub-structuring and differentiation among widely dispersed localities (e.g. Fanciulli et al. 2001; Frati et al. 2001; Stevens et al. 2007; McGaughran et al. 2008), although in reality this is based on studies from one geographical region of the continent (Victoria Land). To redress this, we used the springtails *Cryptopygus antarcticus antarcticus* Willem, 1901 and *Gomphiocephalus hodgsoni* Carpenter, 1908, indigenous to the Antarctic Peninsula and the continent,

respectively, to examine whether the hypothesis of similar patterns of population genetic structure and demography is applicable for these species at the largest scale across the entire Antarctic continent.

These endemic species (*G. hodgsoni*) and subspecies (*C. a. antarcticus*) are the most common springtails in many habitats in their respective regions and are candidates for having similarly long continuous associations with the Antarctic landscape (Convey & Stevens 2007; Convey et al. 2008). Using sequence information from mtDNA cytochrome *c* oxidase subunit I (*cox1*) and subunit II (*cox2*) genes, we aimed to identify refugia and colonisation routes, and describe population structure and demography separately for *G. hodgsoni* and *C. a. antarcticus*. In addition, we compared the patterns seen for each species, reflecting specifically on whether postulated common evolutionary histories of these separate Antarctic regions have resulted in similar genetic signatures across their contemporary populations.

6.3 METHODS

6.3.1 Study areas, species and sample collection

Southern Victoria Land (Fig. 6.1) is a large continental area dissected longitudinally by the Transantarctic Mountains. Within the Ross Dependency, Ross Island is linked to the continent via the permanent Ross Ice Shelf and fast ice adjoins coastal areas for most (often all) of any given year. The geomorphology of the region, and especially of the Dry Valleys of southern Victoria Land supports a record of ice retreat from late Miocene maxima around 10-12 Ma (Sugden et al. 2006). Low rates of precipitation combined with typically very low atmospheric humidity result in a large element of this area being an ablation zone, thus vast areas are permanently free of ice and snow cover, and have been for an extended (multi-million year) time period (Lawver & Gahagan 2003).

In contrast, the Antarctic Peninsula is a long narrow mountain chain that does not have a land connection to the Antarctic continent. However, it is largely covered by snow and ice that merges with the West Antarctic Ice Sheet at its southern extremity. It supports ~120,000 km^2 of grounded ice and has an elevation ranging from 1,000 to >

2,000 m. The west coast of the peninsula is home to numerous deep fjords, islands and small ice shelves, while the east coast is characterised by more extensive ice shelves. Much of the Antarctic Peninsula (Graham Land) hosts an alpine glacier system, which develops in areas further south (Palmer Land, Ellsworth Land) into a continuous ice sheet connecting the peninsula to the continent (Fig. 6.1). This north–south partition sets up strong climatic gradients and the peninsula as a whole experiences precipitation 3–4 times greater than other parts of Antarctica (Barker & Camerlenghi 2002; Smellie et al. 2006).

The species *Gomphiocephalus hodgsoni* (Collembola: Hypogastruridae) and *Cryptopygus antarcticus antarcticus* (Collembola: Isotomidae) are the most common springtails in their respective regions, and were used for all analyses in this study. These springtails are small (< 2 mm in length) and are restricted to ice-free areas of high soil moisture and/or access to water (e.g. lake edges, snow patch edges, moist stream beds) where they inhabit the soil beneath rocks.

Collections were made from the underside of small rocks using an aspirator across a total of 28 locations throughout southern Victoria Land for *G. hodgsoni*, and using Tullgren-type extractions of appropriate substrata at 24 locations throughout the peninsula and associated South Shetland Island archipelago (King George Island) for *C. a. antarcticus* (Fig. 6.1; see Appendix 6.1).

6.3.2 DNA extraction, amplification and sequencing

Total DNA was extracted from specimens following either a ‘salting out’ protocol (Sunnucks & Hales 1996), or using a DNeasy tissue extraction kit (Qiagen, Hilden, Germany). Upon extraction, fragments of the mitochondrial cytochrome *c* oxidase subunit I (*cox1*) and subunit II (*cox2*) genes were amplified for *cox1* using the universal primers LCO1490 (5’-ggtcaacaaatcataaagatattgga-3’) and HCO2198 (5’-taaacttcagggtgaccaaaaaatca-3’) (Folmer et al. 1994) for both species, and for *cox2*, using COIIam (5’-aatatggcagattagtgca-3’) and COIIbm (5’-gtttaagagaccagtactt-3’) (Frati et al. 2001) for *G. hodgsoni* and CA-COXI-53J (5’-ttaatataacctttttccccccaac-3’) and CA-COXII-817N (5’-aaaacatccgcagcagaaattaaag-3’) for *C. a. antarcticus* (specifically designed based on the complete mtDNA sequence of this species; Carapelli et al. 2008).

Amplifications for each specimen used a 10 µl reaction volume containing 1 µl of extracted DNA (unquantified), 1x PCR buffer (Roche, Penzberg, Germany) 2.2 mM $MgCl_2$, 0.2 mM of each dNTP (Boehringer-Mannheim, Mannheim, Germany), 1.0 µM of each primer, and 0.5 U of Red Hot DNA polymerase (Thermo Scientific, Waltham, Massachusetts, United Kingdom).

The thermal cycling conditions for *cox1* were: 94ºC for 1 min followed by five cycles of denaturation and polymerase amplification (94ºC for 1 min, 45ºC for 1.5 min, 1 min at 68ºC) followed by 35 cycles of 94ºC for 1 min, 51ºC for 1.5 min and 1 min at 68ºC, followed by 5 min at 72ºC; and for *cox2* were: initial denaturation at 94ºC for 2 min followed by 35 cycles of denaturation and polymerase amplification (94ºC for 1 min, 50ºC for 1 min and 2 min at 68ºC), followed by 5 min at 72ºC. All reaction products were purified using SAP/EXO (USB Corp., Cleveland, OH, USA). Sequencing used forward and/or reverse primers and was performed directly on a capillary ABI3730 Genetic Analyser (Applied Biosystems Inc., Foster City, CA, USA) at the Allan Wilson Centre Genome Service, Massey University.

6.3.3 Haplotype analysis

Individual sequences were verified as being derived from the relevant taxa using the GenBank BLASTn algorithm and edited using CONTIGEXPRESS (VECTOR NTI ADVANCE ver. 10.3.0, Invitrogen Corporation, Carlsbad, CA, USA). In addition to the new samples extracted for this study, we also included several published sequences of *G. hodgsoni* (see Appendix 6.2 for all GenBank Accession numbers) in all of our analyses. All sequences were aligned using CLUSTALW as implemented in MEGA ver. 4 (Tamura et al. 2007), which was also used to compute uncorrected p-distances between unique haplotypes.

6.3.4 Population structure analysis

ARLEQUIN ver. 3.11 (Excoffier et al. 2005) was used to explore genetic characteristics of populations and to test for the presence of population structure for each species separately. For each gene, we computed haplotype (h) and nucleotide (π) diversity

indices (Nei 1987), and the mean number of pairwise differences (θ), and segregating sites θ(S) at the total, region and population levels.

Analysis of molecular variance (AMOVA; Excoffier et al. 1992) was used to measure the extent to which genetic variance could be assigned to the hierarchical structure of population organisation (testing both with no implied structure, and with structure according to regions: 'Granite Harbour', 'Dry Valleys', 'Southern Dry Valleys', and 'Ross Island' for *G. hodgsoni*; 'Northern', 'Central' and 'Southern' for *C. a. antarcticus* (see Fig. 6.1; Appendix 6.1)), with statistical significance of variance components tested with 16,000 permutations. Pair-wise differences between haplotypes (φ-st values) were calculated using simple distances and these were used to look for significant relationships between population genetic distance (φ-st) and geographic distance (Mantel 1967). Exact tests for population differentiation based on haplotype frequencies (Raymond & Rousset 1995) were also performed.

6.3.5 Demographic analysis

Selective neutrality among populations and genes was tested with significance evaluated over 10,000 replicates, using Tajima's D (Tajima 1989) and Fu's Fs (Fu 1997), as implemented in ARLEQUIN. Mismatch distribution (MMD) analysis was also performed in ARLEQUIN to infer recent demographic changes by plotting the frequency of number of pairwise differences observed compared to that expected under a sudden expansion model (Schneider & Excoffier 1999). In such a plot, a unimodal distribution is taken as support for a population expansion (Rogers & Harpending 1992). Raggedness (*R*) indices (Harpending 1994) were also computed as part of the MMD analysis and were used to assess the significance of the fit of the MMD distribution to that of an expanding population, where a non-significant, low *R* index infers population expansion. In addition, differences between expected and observed mismatch patterns were tested by calculating the sum of the squared deviations (SSD) (Rogers & Harpending 1992) and its significance based on 10,000 replications. A significant SSD *P* value is interpreted here as departure from the estimated demographic model of population expansion.

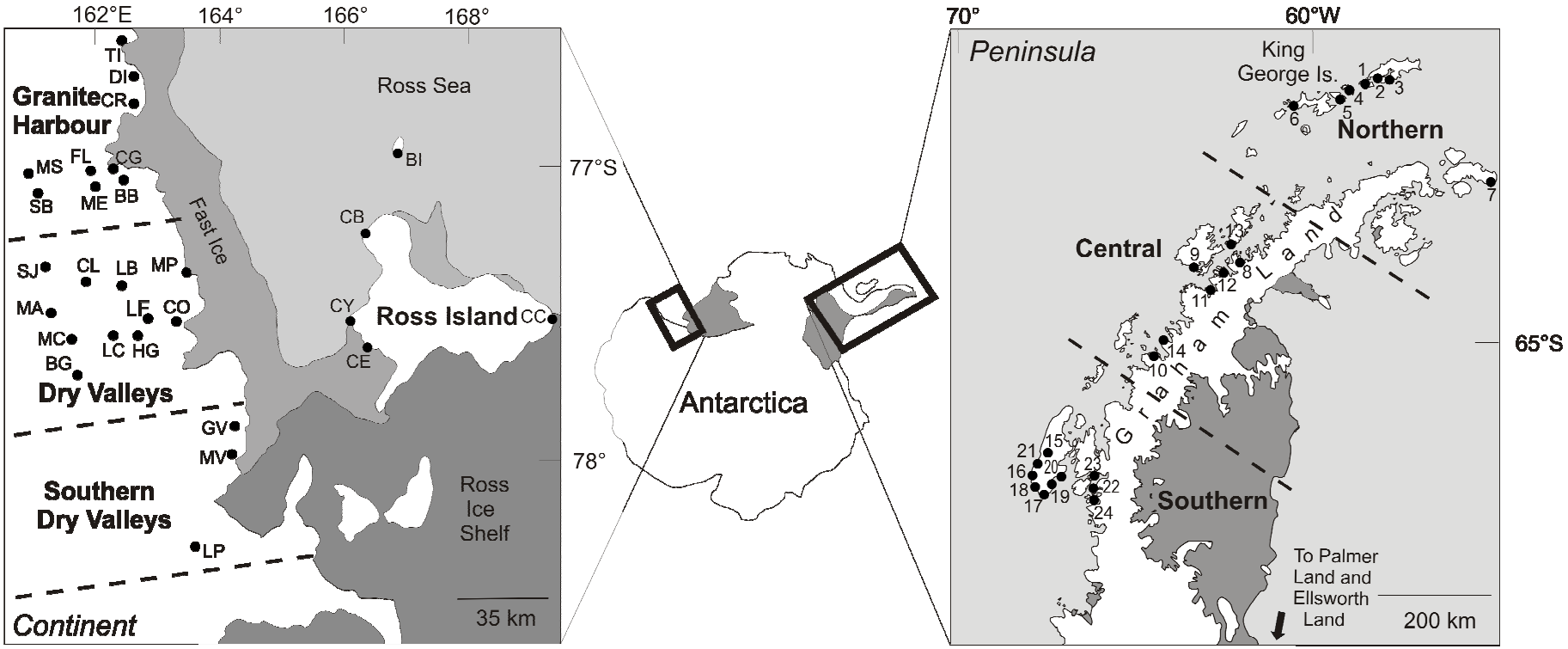

Figure 6.1 Map of Antarctic continental and Peninsula regions, and the populations referred to in the main text and associated tables (see Methods, section 6.3.1; and Appendix 6.1 for further information, including population codes and GPS coordinates).

The MMD analysis can also be used to obtain estimates of the population demographic parameters tau (τ) and theta (Θ) at pre-expansion (Θ_0) and post-expansion (Θ_1) time points. We subsequently used estimates of τ to date the onset of any population expansions using the equation: $t = \tau/2\mu$ where t is the absolute time in years and μ is the mutation rate per locus per generation (Rogers & Harpending 1992). This analysis assumed a generation time of three years for *C. a. antarcticus* (see Convey 1992) and five years for *G. hodgsoni* (based on a shorter active season at continental sites, see Stevens & Hogg 2006 and McGaughran et al. 2008); and a divergence rate of 1.5 – 2.3% per million years for both genes and species (based on an arthropod strict molecular clock conservative calibration derived from comparisons between geological and molecular data; e.g. Brower 1994; Juan et al. 1996; Quek et al. 2004[6]).

Because methods based on pairwise differences and test statistics do not make use of all the information contained in the data, we also used maximum likelihood-based approaches to estimate the times and strengths of demographic events for the overall datasets for each species. The programme FLUCTUATE (Kuhner et al. 1998) was used to make simultaneous estimates of present day Θ ($4N_e\mu$) and the scaled population growth parameter *g*, assuming an exponential model of growth. Estimates of the growth rate *g* are known to be biased upwards (Kuhner et al. 1998). However, positive values of *g* indicate population growth and negative values indicate decline. Here, we consider mean *g* minus 3 s.d. > 0 to be significant evidence of population growth (Lessa et al. 2003). The parameters for the simulations included a transition to transversion ratio of 10.0, empirical base frequencies, two rate categories (1 and 10; probabilities 0.95 and 0.05, respectively), Watterson's estimate (Watterson 1975) for initial Θ and various values of initial *g* (see Kuhner 2003). Runs used ten short chains of 20 increments and 500 steps and five long chains of 20 increments and 100,000 steps and multiple runs were performed to check sufficiency of chain length.

We also estimated diversification time between regions for each species with a coalescent-based model that generates non-equilibrium estimates of divergence time independent of gene migration. The programme MDIV (Nielsen & Wakeley 2001) uses

[6] See Chapter Seven, section 7.3.5 for further information.

a Monte Carlo Markov Chain approach to generate maximum likelihood estimates of Θ; T, the divergence time between two populations scaled by population size; M, the gene migration rate between the two populations, and TMRCA, the time to the most recent common ancestor. We assumed uniform priors and set maximum values for T and M of 10 and 3, respectively. To allow for the possibility of multiple mutations at the same nucleotide site, we used the HKY model (Hasegawa et al. 1985). For each regional comparison, we ran two Markov chains of 5,000,000 cycles with different random seeds, each preceded by a burn-in period of 500,000 cycles. Estimates of T were converted to divergence time in years using $t = T\Theta/2\mu$; where T and Θ are estimated by the programme and μ is the mutation rate per locus per generation (for which we used the same values given above for the MMD analysis).

6.3.6 Nested clade analysis

We used TCS ver. 1.21 (Clement et al. 2000) to estimate a haplotype network for each gene separately using the statistical parsimony algorithm of Templeton et al. (1992). A connection limit of 95% was used for all networks and predictions from coalescent theory were applied to resolve ambiguous loops (Crandall & Templeton 1993; Crandall et al. 1994; Posada & Crandall 2001). Populations were specified by their GPS coordinates and sample sizes, and then defined into a nested structure following the nesting rules described in Crandall (1996). Using this structure, nested geographical distance analyses were performed using GEODIS ver. 2.5 (Posada et al. 2000) to obtain a measure of how any one particular clade was distributed geographically compared to its closest evolutionary sister clades. Clades with statistically significant values of Dc (the average distance of an individual from the geographic centre of its clade), Dn (the average geographic distribution of a clade relative to other clades in the same nesting category) and/or *I–T* (the distance between interior and tip clades within their higher-level nesting category) were assessed using Templeton's inference key (http://darwin.uvigo.es/software/geodis.html; accessed December 15, 2008), allowing investigation of which factor(s) (e.g. restricted gene flow, past fragmentation, range expansion), may have contributed to significant spatial association among haplotypes.

6.4 RESULTS

6.4.1 Haplotype analysis

To match the existing *cox1* dataset, we used 471 bp of unambiguous alignment (no insertions or deletions) from a total of 289 individuals for *cox1*, and 733 bp of unambiguous alignment from 191 individuals for *cox2* for *G. hodgsoni* in all analyses (see Appendix 6.2). There were 45 unique *cox1* haplotypes ranging in divergence from 0.2 – 2.5% and 58 unique *cox2* haplotypes (0 – 2.9% divergence) for *G. hodgsoni*. The number of haplotypes per location ranged from 1 to 7 (mean: 3.2 and 4.2 for *cox1* and *cox2*, respectively) and 57% of populations for *cox1* and 95% for *cox2* had at least one unique haplotype (Table 6.1). Of the total haplotypes, a limited number were shared among populations for both *cox1* (~29%) and *cox2* (21%). For both genes just over half of this sharing was within regions, while sharing among regions was mostly between Granite Harbour and the Dry Valleys. For *cox1*, sharing between regions also occurred for the Southern Dry Valley and Ross Island regions, however Ross Island was isolated (i.e. shared no haplotypes) from the other regions for *cox2* (Fig. 6.2a).

The networks for both genes for *G. hodgsoni* showed a similar pattern of multiple phylogroups, each with a central, most frequent and widespread (i.e. 'ancestral') haplotype, from which other related haplotypes derive. For *cox1*, H1 was the most common haplotype and this was found in 48 individuals from Granite Harbour, Dry Valley and Southern Dry Valley regions. Connected to H1 were several other proposed 'ancestral' haplotypes: a Dry Valley group (including Marble Point), and two Ross Island groups, which themselves link to a Granite Harbour/Dry Valley group (Fig. 6.2a).

For *cox2*, the most common haplotype (H28; n = 23) was present in the Dry Valleys only. The more central H1 phylogroup (n = 21) included individuals from Granite Harbour and Dry Valley regions, and this group was connected to a Dry Valley group (including a few Southern Dry Valley individuals), which then itself connected to a Dry Valley/Ross Island group (Fig. 6.2b).

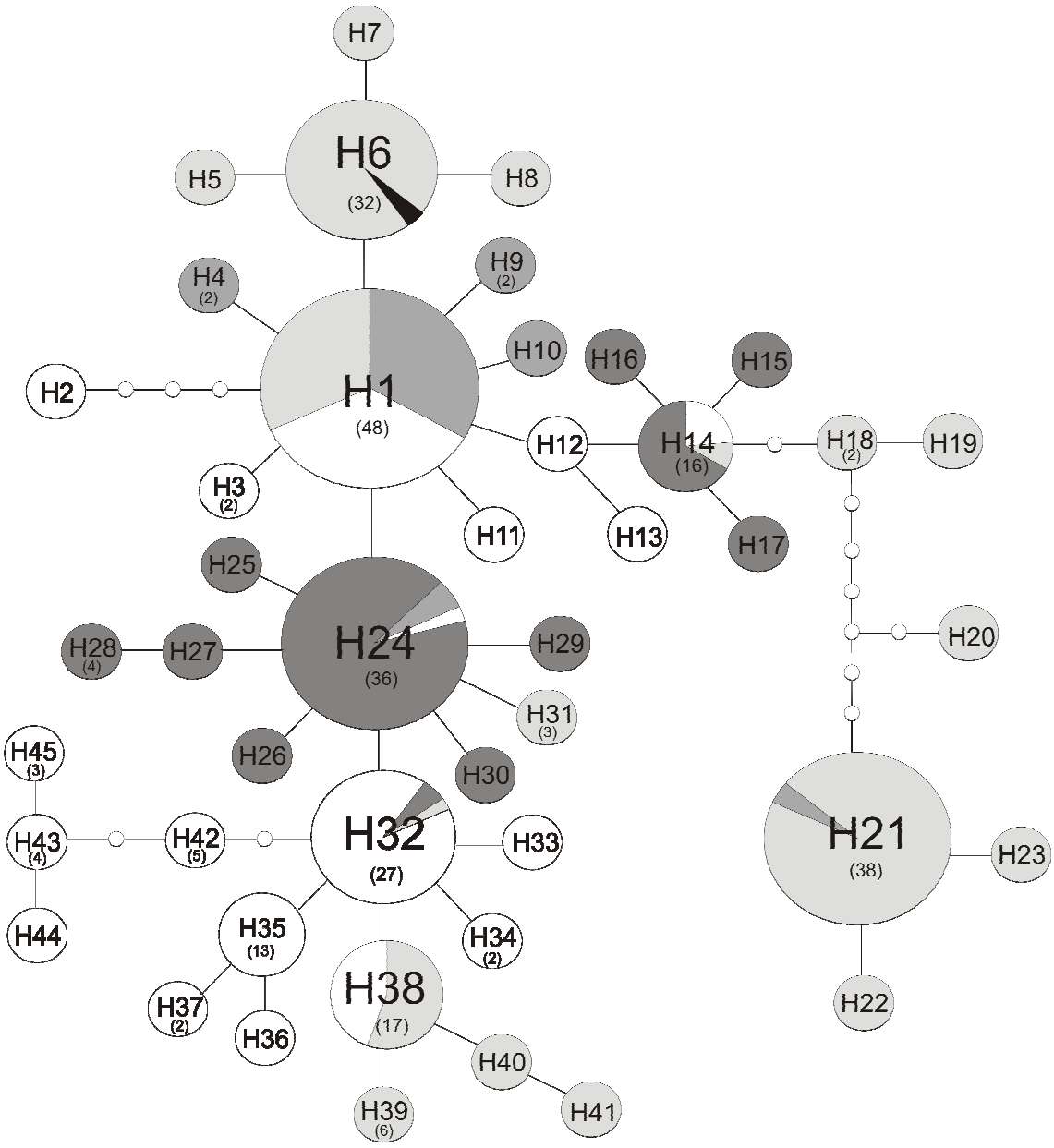

Figure 6.2(a) Haplotype network for *Gomphiocephalus hodgsoni* mtDNA networks for *cox1*. Shading indicates regions according to: white (Granite Harbour) (e.g. H35), light grey (Dry Valleys) (e.g. H39), darker grey (Southern Dry Valleys) (e.g. H9), darkest grey (Ross Island) (e.g. H28) and black (Marble Point) (e.g. H6); '○' indicates mutational steps (missing haplotypes). Haplotype frequencies > 1 are indicated in parentheses and for haplotypes where n > 9, the size of the haplotype is relatively proportional to its frequency.

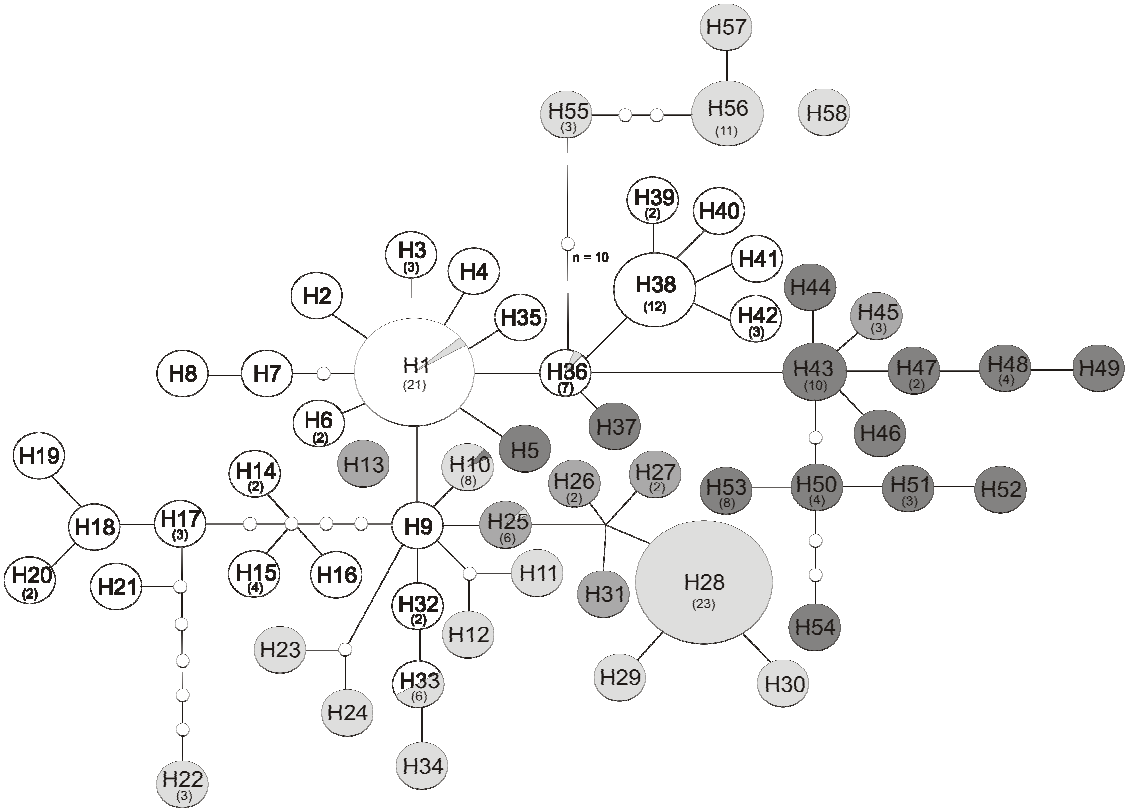

Figure 6.2(b) Haplotype network for *Gomphiocephalus hodgsoni* mtDNA networks for *cox2*. Shading indicates regions according to: white (Granite Harbour) (e.g. H2), light grey (Dry Valleys) (e.g. H28), darker grey (Southern Dry Valleys) (e.g. H26), darkest grey (Ross Island) (e.g. H5); '○' indicates mutational steps (missing haplotypes). Haplotype frequencies > 1 are indicated in parentheses and for haplotypes where n > 9, the size of the haplotype is relatively proportional to its frequency.

For *C. a. antarcticus*, we used 618 bp from 139 individuals for *cox1* and 669 bp from 240 individuals for *cox2* in all analyses (see Appendix 6.3). Haplotype analysis for *C. a. antarcticus* found 89 unique *cox1* haplotypes ranging in sequence divergence from 0 to 9.2%, with the most divergent haplotypes (H29 and H30) found in the Northern region (Population 6; see Appendix 6.1) – removing these gave a divergence range of 0 – 3.7%. For *cox2*, there were 73 unique haplotypes ranging in sequence divergence from 0 to 3.3%. The number of haplotypes per population ranged from 1 to 14 (mean: 6.9 and 4.6 for *cox1* and *cox2*, respectively) and unique haplotypes were present in all populations except 5 and 16 for *cox2* (Table 6.1). As for *G. hodgsoni*, a very limited number of haplotypes were shared among populations for both *cox1* (~11%) and *cox2* (~19%). For both genes, the most common type of sharing was within regions, with only 15-20% of the sharing among regions. Sharing among regions involved Central and Southern populations only for *cox1*, while one instance of sharing between Northern and Southern populations (H9) and Central and Southern populations (H72) was detected for *cox2* (Fig. 6.3).

Network structure for both genes for *C. a. antarcticus* showed similar patterns of relationships among regions. In both cases, an 'upper' Central + Southern group connects to a 'middle' Northern group, which then connects to a 'lower' Southern group. In the *cox1* network, this lower group also contains several Central individuals, while in the *cox2* network several Northern individuals are present in this lower group (Figs 6.3a,b). For *cox1*, H1 was the most common haplotype (n = 13), and was found in Populations 10 (Central), 17 and 18 (Southern), with Populations 4 and 5 holding the central position among Northern haplotypes (Fig. 6.3a). For *cox2*, the most common haplotypes (H33 and H73; n = 21) consisted of individuals from Southern (H73) and Southern and Central (H33) populations (Fig. 6.3b), while individuals from Populations 3, 4, and 5 had the most common Northern haplotype (H1; n = 16).

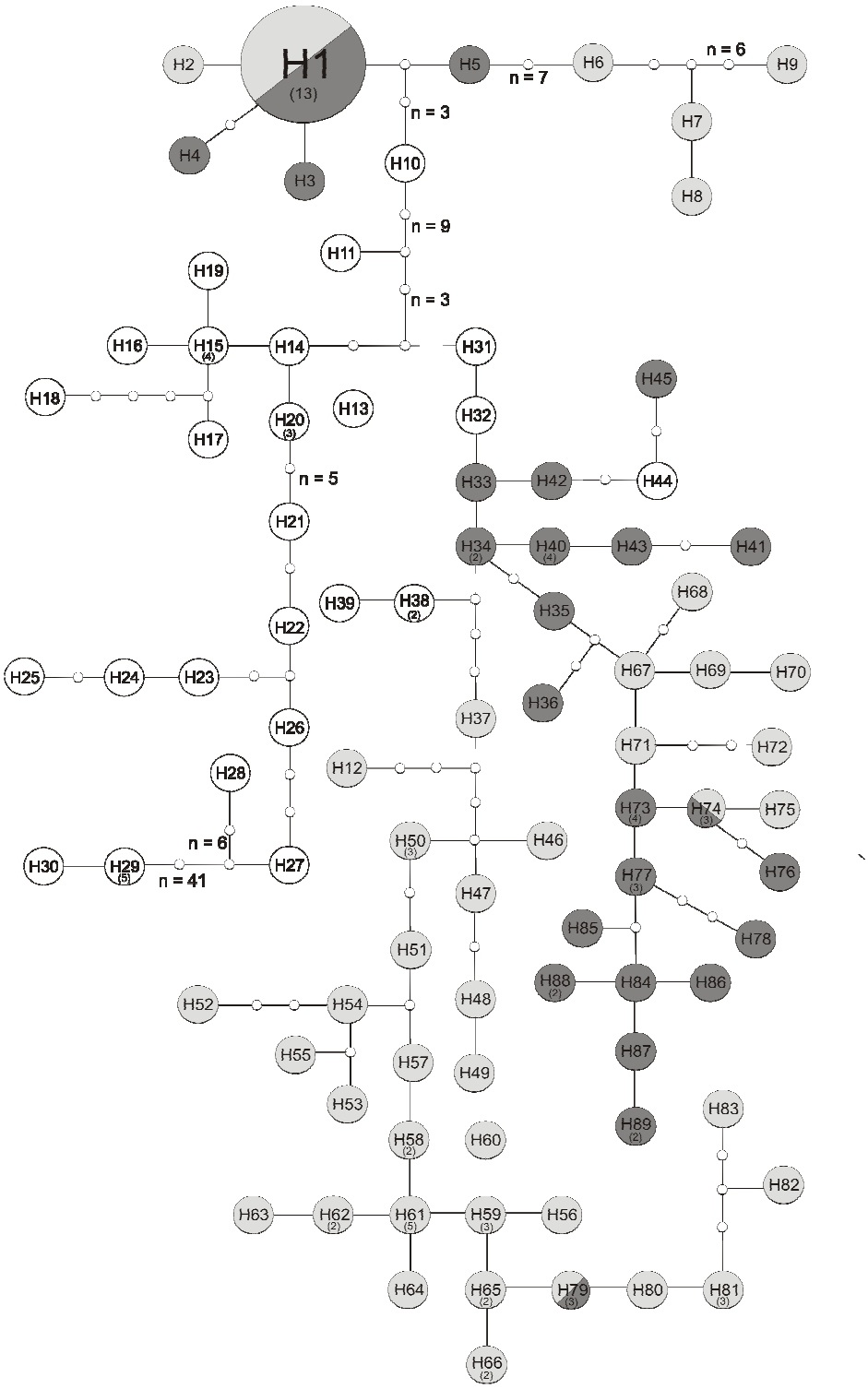

Figure 6.3(a) Haplotype network for *Cryptopygus antarcticus antarcticus* mtDNA networks for *cox1*. Shading indicates regions according to: white (Northern), light grey (Central), darker grey (Southern); '○' indicates mutational steps (missing haplotypes). Haplotype frequencies > 1 are indicated in parentheses and for haplotypes where n > 9, the size of the haplotype is relatively proportional to its frequency. The number of mutational steps between haplotypes is given as n = x in several cases.

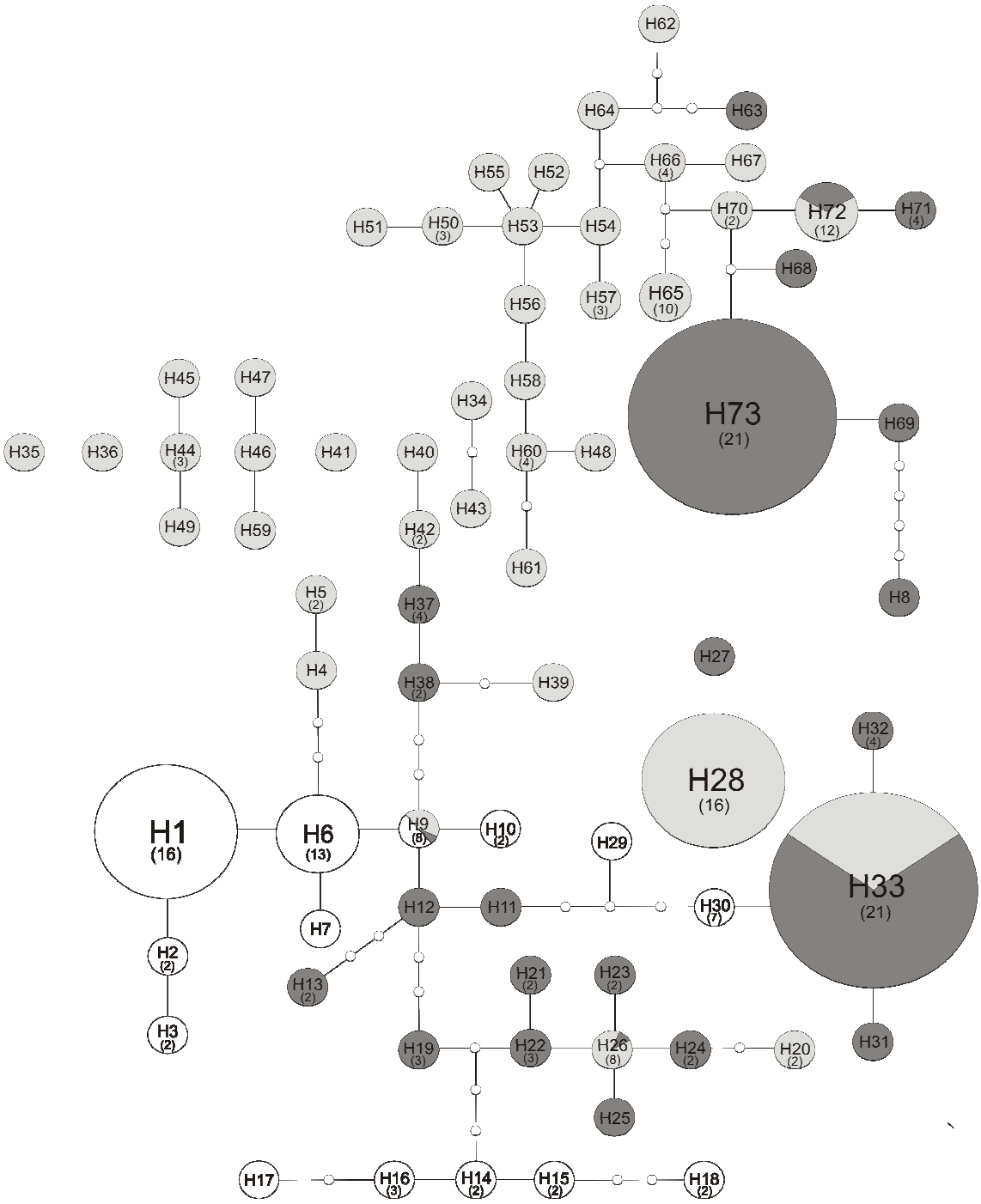

Figure 6.3(b) Haplotype network for *Cryptopygus antarcticus antarcticus* mtDNA networks for *cox2*. Shading indicates regions according to: white (Northern), light grey (Central), darker grey (Southern); '○' indicates mutational steps (missing haplotypes). Haplotype frequencies > 1 are indicated in parentheses and for haplotypes where n > 9, the size of the haplotype is relatively proportional to its frequency.

6.4.2 Population structure analysis

Haplotype diversity (h) in *G. hodgsoni* populations ranged from 0 – 1 (mean: 0.499) for *cox1* and from 0 – 0.927 (mean: 0.644) for *cox2*. Regionally, the Dry Valleys harboured the highest degree of haplotype diversity and Ross Island, the lowest, for both genes. Measures of nucleotide diversity (π) were low for most populations and showed a similar pattern to haplotype diversity, with the highest values found in the Dry Valley populations (0.004 and 0.007 for *cox1* and *cox2*, respectively) (Table 6.1).

For *C. a. antarcticus*, h ranged from 0.491 to 1.000 (mean: 0.894) for *cox1* with most values exceeding 0.900, and from 0.000 to 0.953 (mean: 0.637) for *cox2*. Regionally, h was slightly higher in the Northern populations (h = 0.923 compared to h = 0.880 and h = 0.886 for Central and Southern regions, respectively) for *cox1*, and was similar among regions for *cox2* (h = 0.652, 0.655 and 0.616 for Northern, Central and Southern regions, respectively). In contrast to the pattern found for *G. hodgsoni*, π was comparatively high, ranging from 0.004 to 0.040 for *cox1* and from 0 to 0.014 for *cox2*. Like h, π was higher in the Northern region (π = 0.018) compared with the Central (π = 0.009) and Southern (π = 0.012) regions for *cox1* and was similar among regions (π = 0.005-0.006) for *cox2* (Table 6.2).

AMOVA analysis revealed high levels of genetic structure for both species and both genes (Table 6.3). In particular, slightly more variation was generally found among than within populations. When group structure was assigned to populations for each species (see Methods, section 6.3.4), the amount of variation among populations within groups and within populations was similar, with significant amounts of variation also apportioned to the 'among regions' level (9.70 – 24.8% depending on the species/gene) (Table 6.3).

Further support for population structure came from the Mantel tests, which showed significant correlations between geographic and genetic distance for *G. hodgsoni* (*cox1*: r = 0.245, P = 0.010; *cox2*: r = 0.319, P = 0.013) and *C. a. antarcticus* (*cox1*: r = 0.299, P = 0.011) and a correlation at the 90% level for *C. a. antarcticus* for *cox2* (r = 0.118; P = 0.067). Global differentiation tests found significant differentiation in both genes for *G. hodgsoni* and in *cox2* for *C. a. antarcticus* (P < 0.001), with differentiation detected at the 90% level for *C. a. antarcticus* for *cox1* (P = 0.087).

Table 6.1 Population statistics and genetic characteristics of sampled locations in southern Victoria Land for *Gomphiocephalus hodgsoni* mtDNA *cox1* and *cox2* sequences: n, number of individuals; x, number of haplotypes; h, haplotype diversity; π, nucleotide diversity; θ, mean number of pairwise differences; θ(S) mean number of segregating sites; haplotypes shared among populations are indicated in italics; population codes are as referred to in Fig. 6.1.

Genetic Population populations	n	x	h(s.d.)	π(s.d.)	θ (s.d.)	θ(S)(s.d.)	Distribution of haplotypes within
cox1							
Granite Harbour							
TR	8	2	0.536(0.123)	0.003(0.003)	1.607(1.060)	1.157(0.781)	H42, H45
DI	5	1	0.000(0.000)	0.000(0.000)	0.000(0.000)	0.000(0.000)	*H14*
CR	5	2	0.400(0.237)	0.001(0.001)	0.400(0.435)	0.480(0.480)	H43, H44
FL	8	3	0.679(0.122)	0.002(0.002)	0.786(0.633)	0.771(0.593)	*H32*, *H35*, *H37*
BB	15	6	0.762(0.081)	0.002(0.002)	1.067(0.746)	1.538(0.848)	*H32*, *H34*, *H35*, H36, *H37*, *H38*
CG	14	4	0.714(0.079)	0.002(0.002)	0.934(0.682)	0.943(0.614)	*H24*, *H32*, *H35*, *H38*
ME	13	5	0.539(0.161)	0.001(0.001)	0.615(0.518)	1.289(0.768)	*H24*, *H32*, H33, *H34*, *H35*
MS	13	5	0.628(0.143)	0.003(0.002)	1.333(0.883)	2.256(1.156)	*H1*, H2, H3, H12, H13
SB	8	2	0.250(0.180)	0.001(0.001)	0.250(0.311)	0.386(0.386)	*H1*, H11
Mean	9.9	3.3	0.501(0.247)	0.002(0.001)	0.777(0.519)	0.980(0.678)	
Dry Valleys							
SJ	13	6	0.833(0.082)	0.005(0.004)	2.590(1.482)	2.256(1.156)	*H1*, *H14*, H18, *H31*, *H38*, *H39*
CL	6	3	0.733(0.155)	0.003(0.003)	1.600(1.095)	1.752(1.128)	*H31*, *H38*, *H39*
MA	6	6	1.000(0.096)	0.009(0.006)	4.133(2.391)	4.380(2.388)	*H1*, H19, *H31*, *H32*, H40, H41
MC	3	2	0.667(0.314)	0.003(0.003)	1.333(1.098)	1.333(1.098)	*H1*, *H39*
LB	6	1	0.000(0.000)	0.000(0.000)	0.000(0.000)	0.000(0.000)	*H38*
CO	3	1	0.000(0.000)	0.000(0.000)	0.000(0.000)	0.000(0.000)	*H6*
LF	33	7	0.625(0.068)	0.009(0.005)	4.076(2.085)	2.957(1.202)	*H1*, H5, *H6*, H8, *H21*, H22, H23
LC	1	1	1.000(0.000)	0.000(0.000)	0.000(0.000)	0.000(0.000)	*H21*
HG	28	5	0.725(0.043)	0.009(0.005)	4.071(2.093)	2.570(1.110)	*H1*, *H6*, H7, *H14*, *H21*
BG	16	2	0.125(0.106)	0.001(0.001)	0.625(0.517)	1.507(0.826)	H20, *H21*

Table 6.1 (contd)

Mean	11.5	3.4	0.571(0.387)	0.004(0.004)	1.843(1.756)	1.676(1.440)	
Marble Point	3	2	0.000(0.000)	0.000(0.000)	0.000(0.000)	0.000(0.000)	*H6*
Southern Dry Valleys							
GV	13	5	0.756(0.097)	0.004(0.003)	1.897(1.155)	3.222(1.531)	*H1*, H9, H10, *H21*, *H24*
MV	9	1	0.000(0.000)	0.000(0.000)	0.000(0.000)	0.000(0.000)	*H1*
LP	4	2	0.667(0.204)	0.001(0.002)	0.667(0.626)	0.545(0.545)	*H1*, H4
Mean	8.7	2.7	0.474(0.413)	0.002(0.002)	0.855(0.962)	1.256(1.725)	
Ross Island							
BI	12	4	0.455(0.170)	0.001(0.001)	0.500(0.456)	0.993(0.652)	*H14*, H15, H16, H17
CB	14	5	0.659(0.123)	0.002(0.002)	0.934(0.682)	1.572(0.872)	*H24*, H25, H26, H29, *H32*
CC	19	3	0.292(0.127)	0.001(0.001)	0.503(0.447)	0.858(0.550)	*H24*, *H28*, *H32*
CY	8	2	0.250(0.180)	0.001(0.001)	0.250(0.311)	0.386(0.386)	*H24*, H30
CE	3	2	0.667(0.314)	0.001(0.002)	0.667(0.667)	0.667(0.667)	H27, *H28*
Mean	11.2	3.2	0.465(0.197)	0.001(0.000)	0.571(0.252)	0.895(0.442)	
cox2							
Granite Harbour							
TR	8	2	0.250(0.180)	0.000(0.000)	0.250(0.311)	0.386(0.386)	*H1*, H2
DI	5	1	0.000(0.000)	0.000(0.000)	0.000(0.000)	0.000(0.000)	*H36*
CR	5	2	0.430(0.237)	0.001(0.001)	0.400(0.435)	0.480(0.480)	*H1*, H35
FL	8	5	0.857(0.108)	0.003(0.002)	2.357(1.433)	2.314(1.308)	*H1*, H3, H7, *H38*, *H42*
BB	15	7	0.819(0.082)	0.003(0.002)	2.267(1.318)	3.384(1.546)	*H1*, H6, H8, *H33*, *H38*, *H39*, *H42*
CG	8	6	0.927(0.084)	0.003(0.002)	2.464(1.486)	2.314(1.308)	*H1*, H9, H32, *H33*, *H36*, *H42*
ME	12	6	0.682(0.148)	0.002(0.001)	1.273(0.859)	1.987(1.067)	*H1*, H4, *H38*, *H39*, H40, H41
MS	8	5	0.893(0.086)	0.003(0.002)	2.536(1.521)	2.314(1.308)	H14, *H17*, H18, H19, H20
SB	7	4	0.714(0.181)	0.003(0.002)	2.381(1.468)	2.449(1.412)	H15, H16, *H17*, H21
Mean	8.4	4.2	0.619(0.323)	0.002(0.001)	1.548(1.070)	1.736(1.157)	
Dry Valleys							
SJ	6	4	0.800(0.172)	0.005(0.003)	3.733(2.189)	3.503(1.972)	*H1*, H22, H23, *H33*
CL	5	4	0.900(0.161)	0.004(0.003)	2.800(1.769)	2.880(1.758)	*H10*, H24, *H33*, H34
LF	26	7	0.563(0.108)	0.010(0.006)	7.514(3.626)	5.765(2.159)	H11, H25, *H28*, H29, *H56*, H57, H58
HG	16	5	0.767(0.066)	0.010(0.006)	7.508(3.702)	6.329(2.586)	*H10*, *H28*, H30, *H36*, *H56*
BG	8	3	0.679(0.122)	0.007(0.004)	4.964(2.700)	6.171(2.989)	H12, H55, *H56*

Table 6.1 (contd)

Mean	12.2	4.6	0.742(0.127)	0.007(0.002)	5.304(2.156)	4.177(1.967)	
Southern Dry Valleys							
GV	10	6	0.889(0.075)	0.004(0.002)	2.778(1.602)	2.829(1.459)	*H10*, H13, *H25*, H26, H31, H45
MV	6	2	0.533(0.172)	0.001(0.001)	0.533(0.508)	0.438(0.438)	*H25*, H27
Mean	8	4	0.711(0.252)	0.003(0.002)	1.656(1.587)	1.634(1.691)	
Ross Island							
BI	10	6	0.844(0.103)	0.002(0.002)	1.689(1.077)	1.767(1.014)	H5, H37, *H43*, H47, H48. H49
CB	8	4	0.786(0.113)	0.002(0.002)	1.571(1.042)	1.928(1.136)	*H50*, H51, H52, H54
CC	12	3	0.318(0.164)	0.000(0.001)	0.333(0.356)	0.662(0.501)	*H43*, H44, H46
CY	9	2	0.222(0.166)	0.000(0.000)	0.222(0.288)	0.368(0.368)	*H50*, H53
Mean	9.8	3.8	0.543(0.318)	0.001(0.001)	0.954(0.784)	1.181(0.781)	

Table 6.2 Population statistics and genetic characteristics of sampled locations across the Antarctic Peninsula for *Cryptopygus antarcticus antarcticus* mtDNA *cox1* and *cox2* sequences: n, number of individuals; x, number of haplotypes; h, haplotype diversity; π, nucleotide diversity; θ, mean number of pairwise differences; θ(S) mean number of segregating sites; haplotypes shared among populations are indicated in italics; population codes are as referred to in Fig. 6.1.

Genetic Population	n	x	h(s.d.)	π(s.d.)	θ (s.d.)	θ(S)(s.d.)	Distribution of haplotypes within populations
cox1							
Northern							
3	8	8	1.000(0.063)	0.010(0.006)	6.321(3.355)	6.556(3.155)	H21, H22, H23, H24, H25, H26, H27, H28
4	10	9	0.978(0.054)	0.017(0.010)	10.800(5.374)	8.484(3.759)	H6, H7, H8, H11, *H15*, H16, H18, *H20*, H44
5	10	8	0.957(0.059)	0.004(0.003)	2.489(1.464)	2.474(1.312)	H13, H14, *H15*, H17, H19, *H20*, H31, H32
6	10	5	0.756(0.130)	0.040(0.022)	24.511(11.784)	17.674(7.459)	H10, H29, H30, H38, H39
Mean	9.5	7.5	0.923(0.113)	0.018(0.016)	11.030(9.608)	8.797(6.427)	
Central							
7	1	1	1.000(0.000)	0.000(0.000)	0.000(0.000)	0.000(0.000)	H9
9	10	9	0.978(0.054)	0.006(0.004)	3.778(2.078)	3.181(1.605)	H46, *H50*, H51, H57, *H58*, *H61*, H63, H64, H68
10	11	4	0.491(0.175)	0.011(0.006)	6.509(3.335)	6.828(3.018)	*H1*, H2, *H74*, H75
11	12	9	0.939(0.058)	0.005(0.003)	3.136(1.747)	3.643(1.722)	*H50*, H52, H53, H54, H55, *H58*, *H61*, H62, *H65*
12	12	9	0.939(0.058)	0.005(0.003)	3.091(1.723)	4.305(1.980)	H12, H59, H60, *H61*, *H65*, H66, H67, H70, *H79*
13	10	8	0.933(0.077)	0.006(0.004)	4.000(2.183)	4.242(2.040)	H37, H56, H69, H71, H72, *H79*, H80, H81
Mean	9.3	6.7	0.880(0.192)	0.006(0.004)	3.419(2.092)	3.700(2.209)	
Southern							
14	5	5	1.000(0.127)	0.019(0.012)	11.800(6.456)	10.080(5.361)	H47, H48, H49, H82, H83
16	11	9	0.964(0.051)	0.007(0.004)	4.182(2.250)	3.756(1.801)	*H34*, H35, H36, H42, H84, H86, H87, H88, H89
17	10	7	0.867(0.107)	0.020(0.011)	12.311(6.081)	9.191(4.044)	*H1*, H3, H5, *H73*, H78, *H79*, H85
18	10	5	0.844(0.080)	0.009(0.005)	5.311(2.801)	7.777(3.473)	*H1*, *H73*, *H74*, H76, H77
19	10	7	0.867(0.107)	0.011(0.006)	6.578(3.396)	9.191(4.044)	H4, H33, *H34*, H40, H41, H43, H45
Mean	9.2	6.6	0.908(0.069)	0.013(0.006)	8.036(3.770)	7.999(2.511)	

Table 6.2 (contd)

cox2							
Northern							
1	10	3	0.622(0.138)	0.005(0.003)	3.556(1.972)	3.181(1.605)	*H6*, H10, H18
2	10	5	0.867(0.071)	0.006(0.004)	4.200(2.277)	3.888(1.895	H2, H3, *H6*, H7, H14
3	10	4	0.800(0.076)	0.007(0.004)	4.667(2.497)	3.535(1.750)	*H1*, *H6*, *H9*, H16
4	10	5	0.756(0.130)	0.007(0.004)	4.889(2.602)	3.888(1.895)	*H1*, *H6*, *H9*, H15, H17
5	10	2	0.357(0.159)	0.001(0.001)	3.556(0.375)	0.353(0.353)	*H1*, *H6*
6	10	3	0.511(0.164)	0.005(0.003)	3.533(1.962)	3.181(1.605)	*H26*, H29, H30
Mean	10	3.7	0.652(0.193)	0.006(0.001)	4.067(0.610)	3.004(1.337)	
Central							
8	10	1	0.000(0.000)	0.000(0.000)	0.000(0.000)	0.000(0.000)	H65
9	21	14	0.952(0.028)	0.007(0.004)	4.819(2.450)	3.335(1.431)	H4, H5, H36, H41, H44, H45, H46, H47, H49, H50, H51, H54, H57, H59
10	10	2	0.356(0.159)	0.001(0.001)	0.356(0.375)	0.353(0.353)	H70, *H72*
11	14	11	0.934(0.061)	0.004(0.003)	2.945(1.640)	3.181(1.605)	H34, H35, H40, H43, H48, H52, H53, H55, H58, H60, H61
12	10	6	0.844(0.103)	0.007(0.004)	4.667(2.497)	1.060(0.704)	*H37*, *H38*, *H42*, H56, H64, H66
13	10	6	0.844(0.103)	0.008(0.005)	5.644(2.958)	3.181(1.605)	*H37*, *H38*, H39, *H42*, H62, H67
Mean	12.5	6.7	0.655(0.389)	0.005(0.003)	3.072(2.410)	1.852(1.552)	
Southern							
15	10	6	0.844(0.103)	0.004(0.002)	2.400(1.422)	0.353(0.353)	*H9*, *H20*, *H22*, H24, H25, *H26*
16	10	2	0.200(0.154)	0.001(0.001)	0.600(0.519)	6.623(2.876)	*H9*, *H28*
17	10	5	0.867(0.071)	0.006(0.003)	3.733(2.057)	3.888(1.895)	H13, H19, *H20*, H21, *H22*
18	10	2	0.356(0.159)	0.001(0.001)	0.356(0.375)	5.462(2.479)	*H32*, *H33*
19	12	6	0.803(0.096)	0.007(0.004)	4.864(2.551)	7.423(3.330)	*H9*, H11, *H26*, *H28*, *H33*, H68
20	10	6	0.844(0.103)	0.005(0.003)	3.467(1.930)	0.993(0.652)	H12, *H26*, H27, *H28*, H31, *H33*
21	11	3	0.727(0.068)	0.011(0.006)	7.055(3.589)	4.242(2.040)	*H33*, H71, *H72*
22	10	4	0.822(0.072)	0.014(0.008)	9.489(4.761)	3.145(1.478)	H23, *H32*, *H33*, *H73*
23	12	2	0.167(0.134)	0.001(0.001)	0.500(0.456)	3.535(1.750)	H69, *H73*
24	10	4	0.533(0.180)	0.006(0.004)	4.022(2.193)	4.242(2.040)	H8, *H9*, H63, *H73*
Mean	10.5	4	0.616(0.280)	0.006(0.004)	3.649(2.962)	3.991(2.215)	

Most φ-st values were large and significant for both species (*G. hodgsoni*: *cox1* mean: 0.609, *cox2*: 0.520; *C. a. antarcticus*: *cox1* mean: 0.498; *cox2*: 0.562), indicating limited gene flow among populations. At the regional level, φ-st values were highest in Ross Island and lowest in the Dry Valleys for both genes for *G. hodgsoni*, while in *C. a. antarcticus* φ-st was similar among regions.

Table 6.3 Percentage (%) of variation of molecular variance attributed to various levels of hierarchical population structure for *Gomphiocephalus hodgsoni* and *Cryptopygus antarcticus antarcticus* (mtDNA *cox1* and *cox2* sequences), with significance (*P*-value) in parentheses. In each case, Test 1 is with no structure enforced in the AMOVA analysis; Test 2 is with structure enforced according to regions (see Methods, section 6.3.4)

Species	Test	Among Groups	Among populations within groups	Within populations
G. hodgsoni	1(*cox1*)		58.34(< 0.001)	41.66
	2(*cox1*)	22.31(0.014)	37.71(< 0.001)	39.98(< 0.001)
	1(*cox2*)		48.60(< 0.001)	51.40
	2(*cox2*)	16.71(0.002)	33.81(< 0.001)	49.47(< 0.001)
C. a. antarcticus	1(*cox1*)		53.18(< 0.001)	48.82
	2(*cox1*)	9.70(0.046)	44.84(< 0.001)	45.46(< 0.001)
	1(*cox2*)		60.65(< 0.001)	39.35(< 0.001)
	2(*cox2*)	24.82(< 0.001)	38.94(< 0.001)	36.23(0.002)

6.4.3 Demographic analysis

The Tajima's D tests predominantly detected no departures from neutrality (i.e. population expansions) for populations of both species. However, Fu's Fs test takes a different approach (D uses information on mutation frequency, Fs on haplotype distribution) of analysis and has been shown under simulation to be the more powerful test of the two (Ramos-Onsins & Rozas 2002). This latter test indicated several significant departures from neutrality for *G. hodgsoni*, including populations from Granite Harbour, Dry Valleys and Ross Island for *cox1* and Granite Harbour and Ross Island for *cox2*. For *C. a. antarcticus* various populations from all three regions showed significant departures from neutrality for *cox1*, as did populations 8 (Fs = -4.86; P = 0.012) and 20 (Fs = -6.45; $P < 0.001$) for *cox2*.

Results of the MMD analysis indicated expansion (in the form of unimodal distributions) for several populations for both species, as well as non-significant SSD

statistics and raggedness indices. Regional analyses indicated expansions for Granite Harbour, Southern Dry Valleys and Ross Island for *cox1* and for Southern Dry Valleys and Ross Island for *cox2* in *G. hodgsoni* (Table 6.4). For *C. a. antarcticus*, both genes showed the same pattern of bimodal distributions (i.e. no expansion) for the Northern region, and unimodal (expansion) for the Central and Southern regions. Finally, MMD analysis for the pooled datasets showed unimodal distributions for both genes in both species (Table 6.4). This was further supported by the results of the FLUCTUATE analysis, which showed significantly positive *g* (growth) values for all datasets (*G. hodgsoni*: *cox1 g* = 653 ± 89 [s.d.], *cox2 g* = 451 ± 115; *C. a. antarcticus cox1 g* = 86 ± 17, *cox2 g* = 640 ± 76).

Dating of population expansions using the population demographic parameter (τ) led to mean estimates ranging from 29,976 – 58,664 yrs for *G. hodgsoni* and 125,286 – 322,714 yrs (with an upper limit of 231,451 yrs if Population 6 is excluded) for *C. a. antarcticus*. Thus, population growth is concluded to have occurred over Pleistocene time-scales. Regional estimates of expansion dates were highest for the Dry Valleys for both genes for *G. hodgsoni*, with the other regional expansion estimates of similar age for *cox1* and Ross Island population growth occurring around one-quarter of the time of the Dry Valley region for *cox2*. For *C. a. antarcticus*, all regional estimates were of similar age for *cox2*, while the Northern region yielded the oldest estimate of population expansion for *cox1* (Table 6.4).

Results of the MDIV analysis estimated the divergence of the Dry Valley and Granite Harbour regions as older than Ross Island and the Southern Dry Valleys by a factor of ~2 for *G. hodgsoni* (*cox1*) and estimated divergence among all regions similarly for *cox2*. Estimates of divergence between regions for *C. a. antarcticus* placed Northern and Central regions in the same age category for both genes, with the Southern region dated approximately one-quarter (*cox1*) or three-quarters (*cox2*) younger.

Table 6.4 Results of Mismatch Distribution analysis (see Methods, section 6.3.5) for *Gomphiocephalus hodgsoni* and *Cryptopygus antarcticus antarcticus* mtDNA *cox1* and *cox2* genes: τ, tau; $T_{1.5\%}$, estimated age of expansion using 1.5% divergence per My; $T_{2.3\%}$, estimated age of expansion using 2.3% divergence per My; θ_0, theta ($4N_e\mu$) pre-expansion; θ_1, theta post-expansion; SSD, sum of squared deviations (* = $P < 0.05$); R, raggedness index; population codes are as referred to in Fig. 6.1.

Region	Pop. code	τ	$T_{1.5\%}$	$T_{2.3\%}$	θ_0	θ_1	SSD	*R*	Modality
G. hodgsoni									
cox1									
Granite Harbour	TI	4.006	113,404	73,959	0.008	3	0.273*	0.790	bimodal
	CR	0.579	16,391	10,690	0.000	174	4.000	0.200	unimodal
	FL	1.017	28,790	18,776	0.000	2,333	0.049	0.290	unimodal
	BB	1.243	35,188	22,948	0.000	2,071	0.024	0.175	unimodal
	CG	1.188	33,631	21,933	0.000	1,553	0.018	0.167	unimodal
	ME	0.769	21,769	14,197	0.000	9	0.015	0.154	bimodal
	MS	0.373	10,559	6,886	0.962	6	0.009	0.061	bimodal
	SB	0.324	9,172	5,982	0.000	5	0.001	0.313	bimodal
	Mean	1.187	33,613	21,921	0.121	769	0.588	0.269	unimodal
Dry Valleys	SJ	3.373	95,485	62,273	0.000	11	0.033	0.122	bimodal
	CL	4.377	123,907	80,809	0.007	2	0.074	0.240	bimodal
	MA	2.617	74,084	48,315	1.908	3,595	0.060	0.204	unimodal
	MC	2.287	64,742	42,223	0.001	11	0.265	1.000	bimodal
	LF	8.969	253,900	165,587	0.012	2	0.164*	0.246	bimodal
	HG	8.910	252,229	164,497	0.006	5	0.105*	0.126	bimodal
	BG	3.000	84,926	55,386	0.049	0	0.023	0.797	bimodal
	Mean	4.790	135,610	88,441	0.283	518	0.091	0.391	bimodal
S. Dry Valleys	GV	1.137	32,187	20,991	0.000	2,012	0.038*	0.182	unimodal
	LP	1.055	29,866	19,478	0.000	1,997	0.090	0.556	unimodal
	Mean	1.096	31,026	20,234	0.000	2,005	0.090	0.369	unimodal
Ross Island	BI	0.624	17,665	11,520	0.000	408	0.009	0.153	unimodal
	CB	1.033	29,243	19,071	0.000	2,394	0.006	0.100	unimodal
	CC	3.041	86,086	56,143	0.125	0	0.029	0.417	bimodal
	CY	0.324	9,172	5,982	0.000	5	0.001	0.313	bimodal
	CE	1.055	29,866	19,478	0.000	1,997	0.090	0.556	unimodal

Table 6.4 (contd)

	Mean	1.215	34,406	22,439	0.025	961	0.027	0.308	unimodal
cox2									
Granite Harbour	TI	0.324	5,894	3,844	0.000	5	0.001	0.313	bimodal
	CR	0.579	10,532	6,869	0.000	174	0.007	0.200	bimodal
	FL	3.186	57,954	37,796	0.000	12	0.041	0.122	bimodal
	BB	2.254	41,000	26,739	0.000	1,365	0.010	0.064	unimodal
	ME	1.279	23,265	15,173	0.426	3	0.006	0.047	bimodal
	MS	2.953	53,715	335,032	0.000	12	0.030	0.108	bimodal
	SB	4.442	80,800	52,696	0.004	5	0.089	0.308	bimodal
	Mean	2.145	39,023	25,450	0.061	225	0.026	0.166	bimodal
Dry Valleys	SJ	5.788	105,284	68,664	0.004	25	0.114	0.307	bimodal
	CL	3.896	70,869	46,219	0.002	19	0.065	0.190	bimodal
	LF	6.000	109,141	71,179	0.875	1	0.101	0.200	bimodal
	HG	3.820	69,486	45,317	5.782	6	0.102	0.186	bimodal
	BG	4.043	73,543	47,963	0.000	3	0.208*	0.534	bimodal
	Mean	4.709	85,664	55,868	1.333	11	0.096	0.283	bimodal
S. Dry Valleys	GV	3.727	67,794	44,214	0.000	3,279	0.071*	0.215	unimodal
	MV	0.831	15,116	9,858	0.000	1,661	0.030	0.289	unimodal
	Mean	2.279	41,455	27,036	0.000	2,470	0.030	0.252	unimodal
Ross Island	BI	1.867	33,961	22,148	0.000	1,915	0.005	0.062	unimodal
	CB	1.322	24,047	15,683	0.000	1,512	0.030	0.157	unimodal
	CC	0.411	7,476	4,876	0.000	18	0.003	0.227	bimodal
	CY	0.294	5,348	3,488	0.000	2	0.001	0.358	bimodal
	Mean	0.974	17,708	11,549	0.000	862	0.010	0.201	unimodal
C. a. antarcticus									
cox1									
Northern	3	2.619	94,180	61,422	0.000	99,999	0.099	0.075	unimodal
	4	5.330	191,661	124,996	11.832	28	0.029	0.028	bimodal
	5	2.719	97,762	63,758	0.000	99,999	0.008	0.060	unimodal
	6	44.164	1,588,064	1,035,694	0.002	3	0.255*	0.373	bimodal
	Mean	13.708	492,916	321,467	2.958	50,007	0.045	0.134	bimodal
Central	9	4.072	146,432	95,499	0.002	99,999	0.022	0.083	unimodal
	10	0.000	0	0	0.000	3,414	0.316*	0.189	unimodal

Table 6.4 (contd)

	11	1.918	68,967	44,978	0.000	99,999	0.058	0.057	unimodal
	12	2.188	78,659	51,299	0.000	99,999	0.068	0.231	unimodal
	13	2.605	93,688	61,101	2.296	21	0.009	0.033	bimodal
	14	20.697	744,238	485,373	0.000	34	0.071	0.120	bimodal
	Mean	5.247	188,664	123,042	0.383	50,578	0.046	0.119	unimodal
Southern	16	6.410	230,498	150,325	0.002	9	0.023	0.046	bimodal
	17	23.471	843,966	550,413	0.004	25	0.047	0.050	bimodal
	18	1.531	55,061	35,909	0.000	99,999	0.025	0.080	unimodal
	19	0.465	16,715	10,901	0.000	99,999	0.297*	0.047	unimodal
	Mean	7.969	286,560	186,887	0.001	50,008	0.032	0.056	unimodal
cox2									
Northern	1	9.600	318,884	207,968	0.002	2	0.153	0.443	bimodal
	2	1.500	49,826	32,495	0.000	99,999	0.060	0.076	unimodal
	3	10.300	342,136	223,132	0.002	8	0.162	0.324	bimodal
	4	11.400	378,675	246,962	0.000	5	0.069	0.143	bimodal
	5	0.500	16,609	10,832	0.000	99,999	0.004	0.210	unimodal
	6	0.000	0	0	0.000	99,999	0.384*	0.485	unimodal
	Mean	6.660	221,226	144,278	0.001	50,002	0.090	0.280	bimodal
Central	9	6.300	209,268	136,479	0.002	12	0.010	0.020	unimodal
	10	0.500	16,609	10,832	0.000	99,999	0.004	0.210	bimodal
	11	2.900	96,330	62,824	0.000	99,999	0.007	0.046	bimodal
	12	8.400	279,023	181,972	0.000	10	0.025	0.056	unimodal
	13	10.500	348,779	227,465	0.002	10	0.037	0.072	unimodal
	Mean	5.720	190,002	123,914	0.001	40,006	0.016	0.081	unimodal
Southern	15	1.500	49,826	32,495	0.000	99,999	0.028	0.097	unimodal
	16	3.000	99,651	64,990	0.000	0	0.057*	0.720	bimodal
	17	7.900	262,415	171,140	0.002	6	0.029	0.066	bimodal
	18	0.500	16,609	10,832	0.000	99,999	0.004	0.210	unimodal
	19	0.000	0	0	0.000	99,999	0.764*	0.194	unimodal
	20	1.800	59,791	38,994	1.612	8	0.028	0.054	bimodal
	21	15.700	521,508	340,114	0.000	2	0.143	0.180	bimodal
	22	16.300	541,438	353,112	0.002	20	0.094*	0.145	bimodal
	23	3.000	99,651	64,990	0.000	0	0.040	0.750	bimodal

Table 6.4 (contd)

	24	0.000	0	0	0.000	99,999	0.366*	0.311	unimodal
	Mean	4.970	165,089	107,667	0.162	40,003	0.045	0.273	unimodal

Estimates of the time when genes last shared a common ancestor (i.e. TMRCA) were regionally highest in the Dry Valley region for *G. hodgsoni* and the Northern region for *C. a. antarcticus* in all cases.

6.4.4 Nested clade analysis

The nested clade analysis (NCA) indicated a significant geographical association for a number of the nested clades for both species and genes. Analysis of suitable clades for *G. hodgsoni* with Templeton's inference key for both genes inferred contiguous range expansion or allopatric speciation at the oldest temporal scales, while these inferences (along with restricted gene flow with isolation by distance) also predominated over medium time-scales. Recent patterns were best explained by a similar pattern of restricted gene flow with isolation by distance and allopatric fragmentation. The inferred processes for *C. a. antarcticus* were similar to those found for *G. hodgsoni*. However, several inferences of long distance dispersal and/or colonisation also characterised this analysis.

6.5 DISCUSSION

We found evidence for strong genetic structure in both *G. hodgsoni* and *C. a. antarcticus*. In particular, 71 – 79% and 81 – 89% of haplotypes occurred in only one population for *G. hodgsoni* and *C. a. antarcticus*, respectively, depending on the gene employed. Almost every population for both species had (several) unique haplotypes and there was very little haplotype sharing among populations. What sharing did occur was predominantly within regions, such that populations for both species were clearly isolated from each other at both local and regional scales. This was also evident from the significant global differentiation tests among haplotypes and from the Φ-st values (which were largely strong and significant). Finally, the AMOVA found support for significant partitioning of variation within and among populations, and among regions, which the Mantel test indicated was significantly related to geographic distance.

Differentiation over very small spatial scales (< 1 km) has been shown among highly isolated populations in all of the (few) studies examining population structure in

terrestrial Antarctica (all studies to date are restricted to springtails and mites in regions of continental Victoria Land) (Fanciulli et al. 2001; Frati et al. 2001; Stevens et al. 2007; McGaughran et al. 2008). Such patterns are largely the result of repeated fragmentation of previously widespread ancestral populations into small compartmentalised units (refugia) during glaciation. These units then remained isolated even as conditions ameliorated, due to the intrinsic properties of the organisms (e.g. limited desiccation tolerance) coupled with distinct local and regional barriers to connectivity in the Antarctic landscape.

In the current study, it seems likely that the most ancient refuge for *G. hodgsoni* encompasses the unique Dry Valley region, which has been free of permanent ice since late Miocene maxima (Lawver & Gahagan 2003; Sugden et al. 2006). Previous work had identified the Dry Valley area as refugial (see McGaughran et al. 2008), but the addition of several new sequences from individuals around Granite Harbour in the current study highlight the importance of this more northerly region as an additional refuge in Pleistocene times. In particular, proposed ancestral (i.e. most frequent and widespread) haplotypes are found in both the Dry Valleys and Granite Harbour and many of the haplotypes shared among regions are found at these locations. It also seems likely that the source of colonists to the Ross Island region was the Dry Valleys, since there are haplotypes shared among these locations and haplotype (and nucleotide) diversity decreases from the Dry Valleys (i.e. the source) to Ross Island. In addition, Ross Island shares no haplotypes outside of its region for *cox2*, and has the highest φ-st value (with the lowest in the Dry Valleys), suggesting a founder event led to its colonisation, following which it has likely served as its own refuge and undergone divergence in isolation (or loss of the Dry Valley haplotypes elsewhere).

For *C. a. antarcticus*, the Northern region (Populations 3, 4 and 5) is likely to be the most ancestral refuge on the Antarctic Peninsula. Measures of haplotype and nucleotide diversity and φ-st values were relatively similar among regions; however haplotypes from the Northern region were located centrally on the haplotype networks, and provided links to Central and Southern haplotypes. Relative age estimates also indicated that populations in the Northern region were oldest in most cases. Finally, there was no contemporary sharing of haplotypes between the Northern and other

regions for *cox1* and only one instance of such sharing for *cox2*. This indicates that early (Pleistocene) long-distance dispersal events may have been important for initial colonisation of the species southwards along the peninsula and highlights the importance of multiple subsequent refugia through time in the Central and Southern regions. The NCA inferences are consistent with this hypothesis, with similar overall patterns found for both species and long distance colonisation and/or dispersal events featuring for *C. a. antarcticus*. Such stochastic colonisation processes are also likely to have largely influenced species distributions in other Antarctic arthropod (e.g. Fanciulli et al. 2001), tardigrade (e.g. Sohlenius & Boström 2006) and lichen (e.g. Romeike et al. 2002) taxa, although these have not yet been the focus of studies using the molecular analyses employed here. Certainly, there is evidence of Pleistocene (time-scales up to 2 Ma) dispersal of springtails (*Cryptopygus* sp.) among the sub-Antarctic islands based on mtDNA (*cox1*) sequence data (Stevens et al. 2006; see also Hughes et al. 2006).

Significant Fs values, unimodal MMD distributions and the results of the FLUCTUATE analyses all suggest that population growth has been an important feature of the demographic history of both species. Expansions were suggested for all regions for both species except for the Dry Valleys (*G. hodgsoni*) and the Northern region of the peninsula (*C. a. antarcticus*). Meanwhile, estimates of the age of population expansions were oldest in the Dry Valley and Northern regions for *G. hodgsoni* and *C. a. antarcticus*, respectively, and for *G. hodgsoni* were mainly youngest in the postulated most recently colonised location (Ross Island), although the majority of age estimates corresponded to Pleistocene time-scales. The dependence of these estimates on specified mutation rates[7] and generation time makes them potentially error-prone. However, both the MDIV and TMRCA results confirm this pattern and the time-scale of differentiation among both regions is clear.

Thus, both species showed strong genetic subdivision and a pattern of multiple glacial refugia with ancient (Pleistocene) postglacial expansion and subsequent growth in isolated populations. However, the expression of these patterns was quite distinct for each species. For example, there were up to two times as many haplotypes per location

[7] See Chapter Seven, section 7.3.5 for further information regarding the uncertainty of the employed molecular clock.

for *C. a. antarcticus* compared to *G. hodgsoni*, and *C. a. antarcticus* had much higher haplotype and nucleotide diversity as well as a greater excess of rare haplotypes relative to *G. hodgsoni*. This latter pattern is strongly indicative of range expansion, which results in the replacement of ancestral haplotypes with novel derived haplotypes (Rowe et al. 2004).

Both species also showed evidence for population expansion. However, for *C. a. antarcticus* this appears to have been of such magnitude as to partially obliterate genetic ancestry (via replacement) in isolated populations. This process would have been aided by multiple early (Pleistocene) founding events on the Antarctic Peninsula which would have caused a decline in ancestral genetic diversity. Indeed, an earlier colonisation event preceding population expansion would explain the earlier estimated ages of population expansion found in the coalescent-based analyses for *C. a. antarcticus* (see Table 6.4). In contrast, life on the continent (for *G. hodgsoni*) appears to impose greater restrictions such that a relative lack of early successful founding events and a more conservative growth rate of populations have meant that contemporary genetic patterns remain more representative of ancient roots.

Sequence divergence calculations based on a molecular clock imply a longer evolutionary period for *C. a. antarcticus* populations. However, these estimates are likely to be biased given the perceived higher mutation rate of *C. a. antarcticus*. The more severe climate and shorter growing season of continental Antarctic locations within the distributional range of *G. hodgsoni* place greater restrictions on the life history of this species; thus generation times in *G. hodgsoni* are likely to be at least two-fold longer than in *C. a. antarcticus* (Convey 1992; McGaughran et al. 2008). Furthermore, *C. a. antarcticus* occupies a diverse range of habitats in comparison to *G. hodgsoni* which may account for the apparent greater success of long-distance colonisation events on the Antarctic Peninsula.

Stevens & Hogg (2006) and McGaughran *et al.* (2008) reported differentiation of the mite *Stereotydeus mollis* in southern Victoria Land that was 7-8 times greater than the differentiation reported for co-distributed individuals of *G. hodgsoni*, despite the two species having similar evolutionary histories. If generation times (or activity levels) differ between *G. hodgsoni* and *S. mollis*, then *S. mollis* may be living life at an

accelerated pace compared to *G. hodgsoni*, and this may also explain the patterns on the peninsula. Certainly, aspects of life histories specific to separate species cannot be discounted in demographic and other evolutionary analyses (e.g. Janko *et al*., 2007). Finally, the divergence values themselves will be strongly inflated by the high p-distance values of haplotypes from Population 6 for *C. a. antarcticus* (*cox1*). In fact, these individuals may represent cryptic species or the beginnings of microspeciation processes, as has been shown using a multivariate morphological analysis for the Antarctic mite genus *Maudheimia* (Marshall & Coetzee 2000).

In summary, we found a common pattern of isolated populations and strong genetic structure in two Antarctic springtails. The general trend of expansion indicates that most populations are not yet at mutation/drift equilibrium. In addition, the importance of historical dispersal/colonisation events during the Pleistocene is most evident in the peninsula species, where multiple founder events and/or bottlenecks and subsequent divergence in isolation appear to have resulted in a distinct genetic pattern.

Future work should employ a multi-locus approach to more adequately address the possibility that selection, not population history, has generated the genetic patterns seen here, since mutation, drift and selection operate independently on unlinked loci (Knowles 2004). Studies focusing on species from additional Antarctic locations would also be beneficial for further assessment of how regional differences may have influenced distinct evolutionary histories in local taxa.

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6.8 APPENDICES

Appendix 6.1 Geographic regions and populations referred to in the text for the springtails *Gomphiocephalus hodgsoni* and *Cryptopygus antarcticus antarcticus* from southern Victoria Land and the Antarctic Peninsula, respectively. Population codes are as referred to in Table 6.1 and Figure 6.1.

Region	Population	Pop. code	Latitude (S)	Longitude
G. hodgsoni				
Granite Harbour	Cape Ross	CR	76°44'	162°58' (E)
	Tripp Island	TI	76°07'	162°25' (E)
	Depot Island	DI	76°42'	162°58' (E)
	Flatiron	FL	77°00'	162°25' (E)
	Cape Geology	CG	77°01'	162°34' (E)
	Botany Bay	BB	77°00'	162°39' (E)
	Mt. England	ME	77°02'	162°28' (E)
	Mt. Seuss	MS	77°02'	161°44' (E)
	Sperm Bluff	SB	77°05'	161°43' (E)
Dry Valleys	St. John's Range	SJ	77°20'	161°54' (E)
	Mautrino Peak	MA	77°31'	161°85' (E)
	Mt. Cerberus	MC	77°42'	161°95' (E)
	Clark Glacier	CL	77°25'	162°07' (E)
	Lake Brownworth	LB	77°27'	162°43' (E)
	Howard Glacier	HG	77°40'	163°06' (E)
	Borns Glacier	BG	77°46'	162°02' (E)
	Lake Fryxell	LF	77°38'	163°13' (E)
	Commonwealth Glacier	CO	77°37'	163°24' (E)
	Lake Chad	LC	77°39'	162°46' (E)
Marble Point	Marble Point	MP	77°26'	163°50' (E)
Southern Dry Valleys	Garwood Valley	GV	78°01'	163°55' (E)
	Miers Valley	MV	78°06'	163°46' (E)
	Lake Penny	LP	78°19'	163°24' (E)
Ross Island	Cape Bird	CB	77°13'	166°27' (E)
	Cape Crozier	CC	77°28'	169°12' (E)
	Cape Royds	CY	77°34'	166°10' (E)
	Cape Evans	CE	77°38'	166°27' (E)
	Beaufort Island	BI	76°56'	166°55' (E)
C. a. antarcticus				
Northern	Thomas Pt., King George Island (1)	1	62°10'	58°28' (W)
	Thomas Pt., King George Island (2)	2	62°10'	58°28' (W)
	Potter Cove, King George Island	3	62°14'	58°39' (W)
	Harmony Pt., Nelson Island	4	62º19'	59º10' (W)
	Coppermine Peninsula, Robert Island	5	62°23'	59°42' (W)
	Byers Peninsula, Livingston Island	6	62º40'	61º13' (W)
	Paulet Island	7	63°35'	55°46' (W)
Central	Danco Island	8	64°44'	62°36' (W)
	Port Lockroy, Goudier Island	9	64°49'	63°29' (W)
	Propsect Point, Graham Land	10	66°01'	65°49' (W)
	Paradise Harbour	11	64°51'	62°54' (W)
	East Coast Lemaire	12	64°49'	62°57' (W)
	Terrada Point	13	64°23'	62°14' (W)
	Petermann Island	14	65°10'	64°10' (W)

Appendix 6.1 (contd)

Southern	Detaille Island	15	66°52'	66°48' (W)
	Mackay Point, Adelaide Island	16	67°32'	68°05' (W)
	Killingbeck Island	17	67°34'	68°25' (W)
	Reptile Ridge, Adelaide Island	18	67°33'	68°98' (W)
	Rothera, Adelaide Island	19	67°34'	68°08' (W)
	North Pt., Rothera, Adelaide Island	20	67°35'	68°04' (W)
	Stork Ridge, Adelaide Island	21	67°24'	68°10' (W)
	Anchorage Island	22	67°38'	67°28' (W)
	Lagoon Island	23	67°37'	67°22' (W)
	Col Lakes, Horseshoe Island	24	67°49'	67°18' (W)

Appendix 6.2 List of GenBank Accession numbers for the unique haplotypes generated in this study from *cox1* and *cox2* coding regions for the springtails *Gomphiocephalus hodgsoni* and *Cryptopygus antarcticus antarcticus*, including 12 published *cox1* sequences for *G. hodgsoni* (indicated by '*').

Haplotype code	GenBank Accession Number	
	cox1	*cox2*
G. hodgsoni		
H1	GQ215236	GQ215269
H2	GQ215237	GQ215270
H3	GQ215238	GQ215271
H4	AY294572*	GQ215272
H5	GQ215239	GQ215273
H6	AY294575*	GQ215274
H7	GQ215240	GQ215275
H8	GQ215241	GQ215276
H9	AY294571*	GQ215277
H10	GQ215242	GQ215278
H11	GQ215243	GQ215279
H12	GQ215244	GQ215280
H13	GQ215245	GQ215281
H14	AY294564*	GQ215282
H15	GQ215246	GQ215283
H16	GQ215247	GQ215284
H17	GQ215248	GQ215285
H18	GQ215249	GQ215286
H19	DQ305360*	GQ215287
H20	GQ215250	GQ215288
H21	AY294605*	GQ215289
H22	GQ215251	GQ215290
H23	GQ215252	GQ215291
H24	AY294584*	GQ215292
H25	AY294583*	GQ215293
H26	GQ215253	GQ215294
H27	GQ215254	GQ215295
H28	AY294591*	GQ215296
H29	GQ215255	GQ215297
H30	GQ215256	GQ215298
H31	GQ215257	GQ215299
H32	DQ305357*	GQ215300
H33	GQ215258	GQ215301
H34	GQ215259	GQ215302
H35	GQ215260	GQ215303
H36	GQ215261	GQ215304
H37	GQ215262	GQ215305
H38	DQ305356*	GQ215306
H39	DQ305359*	GQ215307
H40	GQ215263	GQ215308
H41	GQ215264	GQ215309
H42	GQ215265	GQ215310
H43	GQ215266	GQ215311
H44	GQ215267	GQ215312
H45	GQ215268	GQ215313

Appendix 6.2 (contd)

H46		GQ215314
H47		GQ215315
H48		GQ215316
H49		GQ215317
H50		GQ215318
H51		GQ215319
H52		GQ215320
H53		GQ215321
H54		GQ215322
H55		GQ215323
H56		GQ215324
H57		GQ215325
H58		GQ215326
C. a. antarcticus		
H1	GQ215400	GQ215327
H2	GQ215401	GQ215328
H3	GQ215402	GQ215329
H4	GQ215403	GQ215330
H5	GQ215404	GQ215331
H6	GQ215405	GQ215332
H7	GQ215406	GQ215333
H8	GQ215407	GQ215334
H9	GQ215408	GQ215335
H10	GQ215409	GQ215336
H11	GQ215410	GQ215337
H12	GQ215411	GQ215338
H13	GQ215412	GQ215339
H14	GQ215413	GQ215340
H15	GQ215414	GQ215341
H16	GQ215415	GQ215342
H17	GQ215416	GQ215343
H18	GQ215417	GQ215344
H19	GQ215418	GQ215345
H20	GQ215419	GQ215346
H21	GQ215420	GQ215347
H22	GQ215421	GQ215348
H23	GQ215422	GQ215349
H24	GQ215423	GQ215350
H25	GQ215424	GQ215351
H26	GQ215425	GQ215352
H27	GQ215426	GQ215353
H28	GQ215427	GQ215354
H29	GQ215428	GQ215355
H30	GQ215429	GQ215356
H31	GQ215430	GQ215357
H32	GQ215431	GQ215358
H33	GQ215432	GQ215359
H34	GQ215433	GQ215360
H35	GQ215434	GQ215361
H36	GQ215435	GQ215362
H37	GQ215436	GQ215363
H38	GQ215437	GQ215364

Appendix 6.2 (contd)

H39	GQ215438	GQ215365
H40	GQ215439	GQ215366
H41	GQ215440	GQ215367
H42	GQ215441	GQ215368
H43	GQ215442	GQ215369
H44	GQ215443	GQ215370
H45	GQ215444	GQ215371
H46	GQ215445	GQ215372
H47	GQ215446	GQ215373
H48	GQ215447	GQ215374
H49	GQ215448	GQ215375
H50	GQ215449	GQ215376
H51	GQ215450	GQ215377
H52	GQ215451	GQ215378
H53	GQ215452	GQ215379
H54	GQ215453	GQ215380
H55	GQ215454	GQ215381
H56	GQ215455	GQ215382
H57	GQ215456	GQ215383
H58	GQ215457	GQ215384
H59	GQ215458	GQ215385
H60	GQ215459	GQ215386
H61	GQ215460	GQ215387
H62	GQ215461	GQ215388
H63	GQ215462	GQ215389
H64	GQ215463	GQ215390
H65	GQ215464	GQ215391
H66	GQ215465	GQ215392
H67	GQ215466	GQ215393
H68	GQ215467	GQ215394
H69	GQ215468	GQ215395
H70	GQ215469	GQ215396
H71	GQ215470	GQ215397
H72	GQ215471	GQ215398
H73	GQ215472	GQ215399
H74	GQ215473	
H75	GQ215474	
H76	GQ215475	
H77	GQ215476	
H78	GQ215477	
H79	GQ215478	
H80	GQ215479	
H81	GQ215480	
H82	GQ215481	
H83	GQ215482	
H84	GQ215483	
H85	GQ215484	
H86	GQ215485	
H87	GQ215486	
H88	GQ215487	
H89	GQ215488	

Appendix 6.3 Individuals of *Gomphiocephalus hodgsoni* and *Cryptopygus antarcticus antarcticus* for which both *cox1* and *cox2* were obtained. The haplotype code (H) subsequently assigned for each gene is given, as is the haplotype frequency (Hf) in order to show whether haplotypes that were common for *cox1* were also common for *cox2* in each species. Relationships among haplotypes across genes can be determined by referring to Figures 6.2 and 6.3. Population codes are as referred to in Table 6.1 and Figure 6.1.

Region	Population code	Individual	*cox1* H	Hf	*cox2* H	Hf
G. hodgsoni						
Granite Harbour	CR	1	H43	4	H35	1
		2	H43	4	H1	21
		3	H43	4	H1	21
		4	H43	4	H1	21
		5	H44	1	H1	21
	TR	6	H42	5	H1	21
		7	H42	5	H1	21
		8	H42	5	H1	21
		9	H42	5	H1	21
		10	H42	5	H2	1
		11	H45	3	H1	21
		12	H45	3	H1	21
		13	H45	3	H1	21
	DI	14	H14	16	H36	7
		15	H14	16	H36	7
		16	H14	16	H36	7
		17	H14	16	H36	7
		18	H14	16	H36	7
	FL	19	H32	27	H38	12
		20	H32	27	H38	12
		21	H32	27	H42	3
		22	H35	13	H1	21
		23	H35	13	H3	3
		24	H35	13	H3	3
		25	H35	13	H3	3
		26	H37	2	H7	1
	CG	27	H32	27	H42	3
		28	H35	13	H1	21
		29	H35	13	H36	7
		30	H38	17	H9	1
		31	H38	17	H32	2
		32	H38	17	H32	2
		33	H38	17	H33	6
		34	H38	17	H33	6
	BB	35	H32	27	H1	21
		36	H32	27	H38	12
		37	H32	27	H38	1
		38	H32	27	H38	12
		39	H32	27	H42	3
		40	H34	2	H39	2
		41	H35	13	H1	21
		42	H35	13	H1	21

Appendix 6.3 (contd)

		43	H35	13	H1	21
		44	H35	13	H1	21
		45	H35	13	H6	2
		46	H35	13	H6	2
		47	H36	1	H1	21
		48	H37	2	H8	1
		49	H38	17	H33	6
	ME	50	H32	27	H38	12
		51	H32	27	H38	12
		52	H32	27	H38	12
		53	H32	27	H38	12
		54	H32	27	H38	12
		55	H32	27	H38	12
		56	H32	27	H38	12
		57	H33	1	H41	1
		58	H34	2	H39	2
		59	H35	13	H1	21
	MS	60	H1	48	H14	2
		61	H1	48	H14	2
		62	H1	48	H17	3
		63	H1	48	H20	2
		64	H1	48	H20	2
		65	H3	2	H17	3
		66	H12	1	H18	1
		67	H13	1	H19	1
	SB	68	H1	48	H15	4
		69	H1	48	H15	4
		70	H1	48	H15	4
		71	H1	48	H16	1
		72	H1	48	H17	3
		73	H1	48	H21	1
		74	H11	1	H15	4
Dry Valleys	SJ	75	H14	16	H22	3
		76	H18	2	H22	3
		77	H18	2	H22	3
		78	H31	3	H23	1
		79	H39	6	H33	6
	CL	80	H38	17	H33	6
		81	H38	17	H34	1
		82	H39	6	H33	6
	HG	83	H1	48	H10	8
		84	H1	48	H10	8
		85	H1	48	H10	8
		86	H1	48	H10	8
		87	H1	48	H10	8
		88	H1	48	H30	1
		89	H6	32	H28	23
		90	H6	32	H28	23
		91	H6	32	H28	23
		92	H6	32	H28	23
		93	H6	32	H28	23
		94	H7	1	H28	23

Appendix 6.3 (contd)

		95	H14	16	H36	7
		96	H21	38	H56	11
		97	H21	38	H56	11
		98	H21	38	H56	11
	BG	99	H21	38	H12	1
		100	H21	38	H55	3
		101	H21	38	H55	3
		102	H21	38	H55	3
		103	H21	38	H56	11
		104	H21	38	H56	11
		105	H21	38	H56	11
		106	H21	38	H56	11
	LF	107	H1	48	H25	6
		108	H6	32	H28	23
		109	H6	32	H28	23
		110	H6	32	H28	23
		111	H6	32	H28	23
		112	H6	32	H28	23
		113	H6	32	H28	23
		114	H6	32	H28	23
		115	H6	32	H28	23
		116	H6	32	H28	23
		117	H6	32	H28	23
		118	H6	32	H28	23
		119	H6	32	H28	23
		120	H6	32	H28	23
		121	H6	32	H28	23
		122	H6	32	H28	23
		123	H6	32	H28	23
		124	H6	32	H29	1
		129	H8	1	H28	23
		130	H21	38	H11	1
		131	H21	38	H56	11
		132	H21	38	H56	11
		133	H21	38	H56	11
		134	H21	38	H57	1
		135	H22	1	H56	11
		136	H23	1	H58	1
Southern Dry Valleys	GV	137	H1	48	H10	8
		138	H1	48	H10	8
		139	H1	48	H25	6
		140	H1	48	H26	2
		141	H1	48	H26	2
		142	H1	48	H31	1
		143	H10	1	H13	1
		144	H24	36	H45	3
		145	H24	36	H45	3
		146	H24	36	H45	3
	MV	147	H1	48	H25	6
		148	H1	48	H25	6
		149	H1	48	H25	6
		150	H1	48	H27	2

Appendix 6.3 (contd)

Ross Island	CB	151	H24	36	H50	4
		152	H24	36	H50	4
		153	H24	36	H51	3
		154	H24	36	H51	3
		155	H24	36	H51	3
		156	H24	36	H52	1
		157	H26	1	H50	4
		158	H29	1	H54	1
	CC	159	H24	36	H43	11
		160	H24	36	H43	11
		161	H24	36	H43	11
		162	H24	36	H43	11
		163	H24	36	H43	11
		164	H24	36	H43	11
		165	H24	36	H43	11
		166	H24	46	H44	1
		167	H32	27	H43	11
	CY	168	H24	36	H50	4
		169	H24	36	H53	8
		170	H24	36	H53	8
		171	H30	1	H53	8
	BI	172	H14	16	H37	1
		173	H14	16	H47	2
		174	H14	16	H47	2
		175	H14	16	H48	4
		176	H14	16	H48	4
		177	H14	16	H48	4
		178	H14	16	H49	1
		179	H15	1	H5	1
		180	H16	1	H43	11
C. a. antarcticus						
Central	9	1	H46	1	H36	1
		2	H50	3	H44	3
		3	H51	1	H46	1
		4	H57	1	H45	1
		5	H58	2	H44	3
		6	H61	5	H47	1
		7	H63	1	H54	1
		8	H64	1	H59	1
		9	H68	1	H41	1
	11	10	H50	3	H60	4
		11	H52	1	H34	1
		12	H53	1	H48	1
		13	H54	1	H43	1
		14	H55	1	H40	1
		15	H58	2	H58	1
		16	H61	5	H60	4
		17	H61	5	H60	4
		18	H62	2	H52	1
		19	H62	2	H60	4
		20	H65	2	H61	1
	12	21	H12	1	H37	4

Appendix 6.3 (contd)

		22	H59	3	H56	1
		23	H59	3	H66	4
		24	H59	3	H66	4
		25	H60	1	H66	4
		26	H65	2	H66	4
		27	H67	1	H64	1
		28	H70	1	H37	4
	13	29	H37	1	H42	2
		30	H56	1	H62	1
		31	H81	3	H67	4
		32	H81	3	H67	4
		33	H81	3	H67	4

CHAPTER SEVEN:
BIOGEOGRAPHY OF SEVERAL CIRCUM-ANTARCTIC SPRINGTAILS

The work in this chapter is submitted for publication:

McGaughran, A., Stevens, M.I., Holland, B.R. Historical biogeography of the Circum-Antarctic springtail family Isotomidae. *Molecular Phylogenetics and Evolution*, submitted 7 September 2009.

7.1 ABSTRACT

Antarctic terrestrial ecosystems are structured by many of the same forces that influence evolution elsewhere; however the importance of isolation and its subsequent effects on population and species differentiation is perhaps easier to appreciate on the continent locked in ice. Here, we examine the effects of isolation over both ancient and contemporary timescales on evolutionary diversification and speciation patterns of springtail species in continental-, maritime- and sub-Antarctica, with special focus on members of the genus *Cryptopygus*. We employ phylogenetic analysis of mtDNA (*cox1*), and ribosomal DNA (rDNA; 18S and 28S) genes in the programmes MRBAYES and RAXML to estimate accurate regional phylogenetic relationships among these species. In addition, we use dating estimates and dispersal-vicariance analysis to establish the biogeographical pathway of *Cryptopygus* species in Antarctica and help elucidate connectivity among locations.

We show that contemporary distributions among members of the *Cryptopygus* group are largely a result of vicariant processes (with persistence in refugia) operating during the Miocene (23 – 5 Ma) on a widespread ancestral distribution. However, dispersal events over both ancient and contemporary time-scales have also been important for this group of species, and these appear to have chiefly followed oceanic and/or atmospheric currents in an easterly circum-Antarctic direction. Thus, the evolutionary history of these southern hemisphere springtails reflects a varied and diverse origin. The phylogenetic relationships estimated here call for a revision of current 'species' designations.

7.2 INTRODUCTION

Isolation across both ancient and contemporary scales is an important force influencing population structuring in Antarctic terrestrial environments. Previous glacial cycling and current dispersal barriers (e.g. glaciers), play a role in defining the limits of distribution of different species, but other less obvious forces are also important (Rogers 2007). For example, the non-uniformity of ecological properties such as availability of

liquid water and ice-free soil also influence population differentiation. Collectively, glaciological and ecological forces in terrestrial Antarctic ecosystems have provided a rich template of patchily distributed species across which the mechanisms of evolutionary processes such as speciation may be studied.

The perceived limited dispersal ability of species such as soil microarthropods means that their sub-populations are effectively isolated from one another on contemporary time-scales (e.g. Frati et al. 2001; McGaughran et al. 2008). In particular, intrinsic characteristics of springtails, including the absence of wings, limited desiccation tolerance and reduced body size, have marked effects on dispersal capabilities, such that evolution of genetic differentiation among populations in these species is likely (Frati et al. 2001). Antarctic springtails are therefore good candidates for studies of the processes that have led to contemporary species distributions.

Two processes in particular are often invoked to explain genetic and phylogenetic relationships among species: fragmentation of ancestral populations by *vicariant* events or by *dispersal* across a pre-existing barrier (Sanmartín & Ronquist 2004). Under a vicariant hypothesis, fragmentation of ancestral populations via an isolating (vicariant) event would be expected to lead to congruent phylogenetic trees among genera from geographically equivalent (i.e. formerly connected) locations (Zink et al. 2000). Alternatively, dispersal hypotheses consider range expansion common and use dispersal to explain close genetic links between geographically isolated species and incongruent tree topologies for different genera (Zink et al. 2000).

Palaeoclimatic changes (e.g. cooling and warming intervals; Clarke & Crame 1992) may account for vicariance of southern hemisphere fauna; however vicariant hypotheses usually explain relationships between organisms of the southern continents in terms of their shared Gondwanan ancestry. The break-up of Gondwana began in the late Jurassic (~157 Ma), following which progressive separations and the opening of the deep Drake Passage (~30 – 22 Ma) eventually led to the current positioning of the major southern landmasses (Lawver et al. 1992; Crame 1999; Convey et al. 2008), including Australia, New Zealand, Chile and Antarctica (Fig. 7.1). These are thus of sufficient age to have provided habitats in isolation to taxa over Gondwanan time-scales. In addition, sub-

Antarctic Îles Crozet (~8.7 My) and Îles Kerguelen (~100 My) (Fig. 7.1) could also have supported vicariant species distributions.

Elsewhere in the Southern Ocean, islands such as Heard, Macquarie and Marion Island (Fig. 7.1) have all become available for colonisation within the last million years and may be more likely targets for dispersal-derived species distributions. In the greater Antarctic realm ('continental' Antarctica; 'maritime' Antarctica – Antarctic Peninsula and surrounding islands; and 'sub'-Antarctica – defined here as islands between 40° and 60° latitude), currents in the Southern Ocean (Fig. 7.1) may provide dispersal routes for rafted specimens between locations over a variety of time-scales. As these currents (including the Antarctic Circumpolar Current (ACC), the West Wind Drift (WWD) and ultimately parts of the southern hemisphere subtropical gyres; Williams et al. 2003 and references therein) all flow from the west, dispersal in easterly directions may be important in determining colonisation patterns. Additionally a coastal current close to the continent flows in the opposite direction (Fahrbach et al. 1992). Other options for dispersal include wind- and bird-mediated (particularly via atmospheric currents in the case of wind), and contemporary introduction and/or spread by humans, particularly over recent time-scales (e.g. Stevens & Hogg 2002), is likely to be a strong dispersal vector, especially in the sub-Antarctic (e.g. Frenot et al. 2005; Hughes et al. 2006).

While it has been widely assumed that geographic location and the severity of the contemporary climate present insurmountable barriers to the colonisation of Antarctica today (e.g. Allegrucci et al. 2006), the evolutionary relationships among Antarctic taxa have been interpreted as indicating a mixture of dispersal and vicariant processes. For example, passive dispersal has been suggested for the introduction of springtails to 'eastern' Antarctica (Greenslade & Wise 1984) (see Fig. 7.1), and to explain the disjunct occurrence of populations of the springtail *Gressittacantha terranova* in northern Victoria Land (Fanciulli et al. 2001). In addition, a combination of recent range expansion and vicariance in the form of refugia has been suggested to explain distribution patterns of southern Victoria Land springtail *Gomphiocephalus hodgsoni* (e.g. McGaughran et al. 2008). More recently, a study comparing the phylogeographic structure of this latter species with the springtail *Cryptopygus antarcticus antarcticus* from the Antarctic Peninsula emphasised the importance of early colonisation events

from refugia in both springtail species (McGaughran et al. 2009). Indeed, the importance of long distance dispersal events in conjunction with vicariant patterns is becoming widely recognised in evolutionary studies (Sanmartín & Ronquist 2004 and references therein).

To date 25 springtail species have been described in Antarctica (Greenslade 1995), with most genera belonging to the Isotomidae, members of which are also widely distributed globally (Frati & Carapelli 1999). What is known of the evolutionary origins among these species draws back to the isolating nature of the Antarctic environs. Thus, within species, genetic differentiation over both small (< 1 km) and intermediate (tens to hundreds of kilometres) spatial scales is seen (e.g. Fanciulli et al. 2001; Frati et al. 2001; McGaughran et al. 2008, 2009; Clarke et al. 2009). Over larger spatial scales, the eastern and western parts of the Antarctic continent (see Fig. 7.1), are recognised as being very different, sharing no springtail species (Torricelli et al. submitted manuscript) across what has recently been referred to as Antarctica's "Gressitt Line" (Chown & Convey 2007) (Fig. 7.1).

In their separate geographical elements, the springtail fauna of Antarctica includes a high proportion of endemic genera. For example, of the 10 species in eastern Antarctica 60% of genera and all species are endemic (Wise 1967, 1971; Greenslade 1995; Stevens & Hogg 2006; Pugh & Convey 2008; Torricelli et al. submitted manuscript), six of which are from the family Isotomidae (Stevens et al. 2007). In Antarctica, the high occurrence of endemics is often taken as apparent evidence of divergence in isolation, suggesting survival of these groups through ancient times (Rogers 2007). In the context of Antarctic phylogeography, recent mtDNA work has confirmed that some species carry signals over multimillion year timescales (e.g. Stevens et al. 2006; Convey & Stevens 2007; Convey et al. 2008; McGaughran et al. 2009). Meanwhile, non-endemic species with wider cosmopolitan distributions are expected to represent more recent introductions. Therefore, contemporary species distributions likely reflect a varied origin for Antarctic springtails (Frati & Carapelli 1999) that includes both relic and more recent immigrant species (e.g. Wallwork 1973; Greenslade 1995; Marshall & Pugh 1996; Marshall & Coetzee 2000; Pugh & Convey 2000).

In addition to this heterogeneity, which still sees the origin of many Antarctic taxa as unclear (e.g. Frati et al. 2000), there is uncertainty pertaining to the phylogenetic relationships among Antarctic species. In Antarctica, springtail distribution and taxonomy received considerable attention in the 1960s and early 1970s (e.g. Gressitt et al. 1963; Wise & Gressitt 1965; Gressitt 1967; Strandmann 1967; Wise 1967, 1971; Wise & Spain 1967; Wise & Shoup 1971). However, classification and phylogenetic relationships among globally distributed springtails has been a subject of disagreement among various authors (e.g. D'Haese 2002, 2003; Xiong et al. 2008) and Antarctic species have received little attention in this context. In fact, only one paper (based on mtDNA *cox1* variation) studies phylogenetic relationships within Antarctic Isotomidae (Stevens et al. 2006).

Here, we significantly extend the work of Stevens et al. (2006), to achieve a more accurate reconstruction of the regional phylogenetic relationships among members of the springtail family Isotomidae from Antarctica and circum-Antarctic locations. We achieve this by adding several new individuals/locations to the existing *cox1* dataset, and combining analysis of this maternally-inherited mtDNA gene with bi-parentally inherited nuclear (18S and 28S rDNA) genes. We apply additional (phylogeographic) emphasis to the genus *Cryptopygus* by using dating estimates and dispersal-vicariance (DIVA) analysis to establish the evolutionary pathway of these species in Antarctica and to help elucidate connectivity among locations.

7.3 METHODS

7.3.1 Species and locations

The nominate species *Cryptopygus antarcticus* was described in 1901 (see Table 7.1). Subsequent work has shown there to be a variety of undescribed subspecies of this genus (e.g. Déharveng 1981; Potapov 2001; Rusek 2002; Déharveng et al. 2005; Stevens et al. 2006). Based on this, specimens of *Cryptopygus* (including several undescribed subspecies hereafter referred to as "*Cryptopygus a. 'complex'*") were extensively sampled from a variety of locations across their entire range throughout the continental, maritime and sub-Antarctic (Fig. 7.1). Twelve other related species

(including four geographically-relevant isotomid springtails selected as outgroup taxa), with more restricted geographic distributional ranges, were heterogeneously sampled based on specimen availability and successful sequence generation. In addition, several *cox1* sequences from Stevens et al. (2006) were downloaded from GenBank. All sampling information including location, species and relevant GenBank accession numbers are given in Table 7.1.

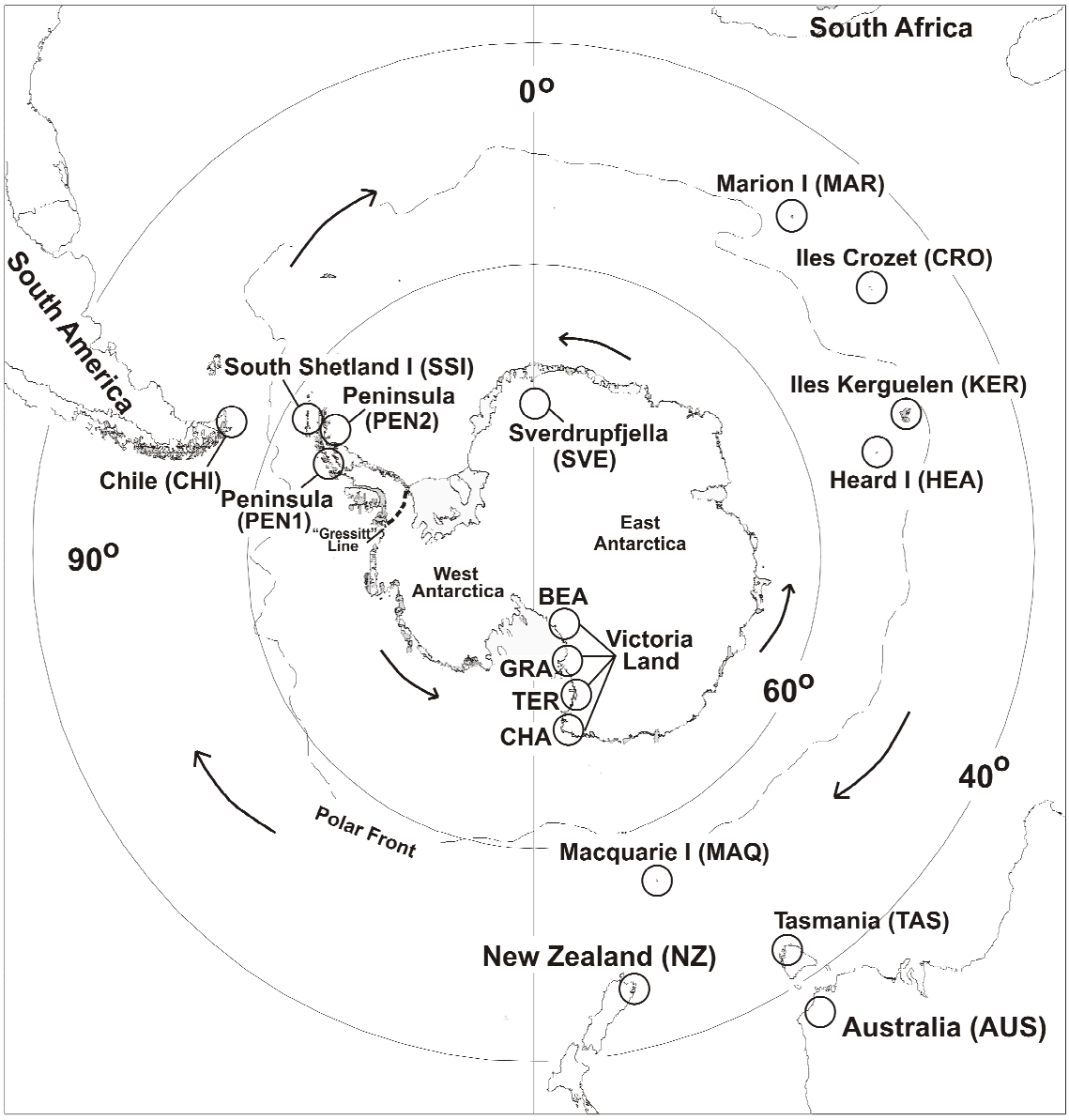

Figure 7.1 Distribution map of Antarctica and surrounding islands to show locations referred to in the text, and from which samples were collected.

7.3.2 Sequence generation

Total DNA was extracted from specimens following a 'salting out' protocol (Sunnucks & Hales 1996). Upon extraction, fragments of the mitochondrial cytochrome *c* oxidase subunit I (*cox1*), 18S rDNA and 28S rDNA genes were amplified using the universal primers TY-J-1460 or LCO1490 and HCO2198 (Folmer et al. 1994) for *cox1*; 18S1F, 18S3R, 18S3F, 18S5R, 18SA2.0, 18S9R (Giribet et al. 1996; Whiting et al. 1997) for 18S; and 28Srd1.2A, 28SB (Whiting 2002) or 28Sbout (Giribet et al. 2001; Prendini et al. 2003) for 28S (see Table 7.2).

Amplifications for each specimen used a 10 µl reaction volume containing 1 µl of extracted DNA (unquantified), 1x PCR buffer (Roche, Penzberg, Germany) 2.2 mM $MgCl_2$, 0.2 mM of each dNTP (Boehringer-Mannheim, Mannheim, Germany), 1.0 µM of each primer, and 0.5 U of Red Hot DNA polymerase (Thermo Scientific, United Kingdom). The thermal cycling conditions for *cox1* were: 94ºC for 1 min followed by five cycles of denaturation and polymerase amplification (94ºC for 1 min, 45ºC for 1.5 min, 1 min at 68ºC) followed by 35 cycles of 94ºC for 1 min, 51ºC for 1.5 min and 1 min at 68ºC, followed by 5 min at 72ºC; and for 18S were: initial denaturation at 94ºC for 3 min followed by 40 cycles of denaturation and polymerase amplification (94ºC for 30 s, 52ºC for 45 s and 1.30 min at 68ºC), followed by 4 min at 72ºC. Cycling conditions for 28S were the same as those used for 18S except for a 50ºC annealing temperature and a total cycle number of 35. All reaction products were purified using SAP/EXO (USB Corp., Cleveland, United States). Sequencing used forward and/or reverse primers and was performed directly on a capillary ABI3730 genetic analyser (Applied Biosystems Inc., California, United States) at the Allan Wilson Centre Genome Service, Massey University.

All sequences were checked for consistency with springtail DNA using the GenBank BlastN search. Nucleotide sequences from a total of 16 described species and several members of the undescribed *Cryptopygus a. 'complex'* (81 individuals in total) were edited in CONTIGEXPRESS (VECTOR NTI ADVANCE ver. 10.3.0, Invitrogen Corporation, United States) and aligned using ClustalW as implemented in MEGA ver. 4.1 (Tamura et al. 2007).

Table 7.1 List of all individuals used in this study (n), and relevant information, including species record, collection location, distribution assigned to dispersal-vicariance (DIVA) analyses, and relevant GenBank accession numbers (for *cox1* individuals only; see Stevens et al. 2006).

Species No.	Record	Location	DIVA distribution	n	Accession
Cryptopygus antarcticus antarcticus	Willem, 1901	Antarctic Peninsula – 1 (PEN1)	K	3	
		Antarctic Peninsula – 2 (PEN2)	K	4	
DQ285353-56					
		South Shetland Is. (SSI)	J	10	
DQ285359-66					
Cryptopygus antarcticus 'complex'		Australia (AUS)	E	1	DQ285377
		Chile (CHI)	B	5	
		Heard Is (HEA)	M	1	DQ285373
		Macquarie Island (MAQ)	D	7	
DQ285367-69,72					
		New Zealand (NZ)	H	1	DQ285370
		Tasmania (TAS)	E	2	
DQ285378-79					
Cryptopygus antarcticus maximus	Déharveng, 1981	Îles Kerguelen (KER)	I	8	
DQ285381-84					
		Macquarie Island (MAQ)	D	1	DQ285385
Cryptopygus antarcticus reagens	Déharveng, 1981	Îles Crozet (CRO)	G	10	
DQ285386-89					
Cryptopygus antarcticus travei	Déharveng, 1981	Marion Island (MAR)	A	1	
Cryptopygus caecus	Wahlgren, 1906	Marion Island (MAR)	A	1	
		South Shetland Is. (SSI)	J	2	
Cryptopygus cisantarcticus	Wise, 1967	Cape Hallett (CHA)	C	3	
Cryptopygus dubius	Déharveng, 1981	Marion Island (MAR)	A	8	
Cryptopygus sverdrupi	Lawrence, 1978	Sverdrupfjella (SVE)	O	1	
Cryptopygus tricuspis	Enderlein, 1909	Marion Island (MAR)	A	1	
Antarctophorus subpolaris	Salmon, 1962	Beardmore Glacier (BEA)	F	1	DQ285405

Table 7.1 (contd)

Gressittacantha terranova DQ285399-401	Wise, 1967	Terra Nova Bay (TER)	N	3	
Neocryptopygus nivicolus DQ285403-404	Salmon, 1965	Granite Harbour (GRA)	L	3	
Outgroup					
Desoria klovstadi	Carpenter, 1902	Cape Hallett (CHA)	n/a	1	DQ285296
Isotoma (Folsomotoma) marionensis	Déharveng, 1981	Marion Island (MAR)	n/a	1	
Isotoma (Parisotoma) notabilis	Schäffer, 1896	Marion Island (MAR)	n/a	1	
Isotomurus cf. palustris	Müller, 1876	Marion Island (MAR)	n/a	1	

Table 7.2 List of primers used in this study, and their sequences (5' to 3').

Primer	Sequence (5' to 3')
TY-J-1460	tacaatttatcgcctaaacttcagcc
LCO1490	gttcaacaaatcataaagatattgga
HCO2198	taaacttcagggtgaccaaaaaatca
18S1F	tacctggttgatcctgccagtag
18S5R	cttggcaaatgctttcgc
18S3F	gttcgattccggagaggga
18SBI	gagtctcgttcgttatcgga
18SA2.0	atggttgcaaagctgaaac
18S9R	gatccttccgcaggttcacctac
28SB	tcggaaggaaccagctac
28Srd1.2A	cccssgtaatttaagcatatta
28Sbout	cccacagcgccagttctgcttacc

7.3.3 Data exploration

Multiple datasets were generated: separate '*cox1*', '18S' and '28S' datasets, along with one dataset containing 27 individuals for which a sequence of each gene existed (i.e. with no missing data) ('allgenes dataset'; hereafter referred to as 'AG') and a complete dataset for all 81 individuals (i.e. with missing data) ('allindividuals'; or 'AI' dataset) (Table 7.3). The *cox1* dataset had no insertions or deletions, while any gaps generated in the alignment of 18S and 28S sequences were excluded from all analyses (as were nucleotides on either side of the gap until the next homologous site was encountered). A partition homogeneity test was performed in PAUP* ver. 4.0b10 (Swofford 2002) to see if the 18S and 28S datasets could be concatenated; this was shown to be an inappropriate option (P = 0.01), hence subsequent analyses treated all datasets separately, or with separate partitions.

Exploratory data analysis was performed on the individual gene datasets in the programme MRBAYES ver. 3.1.2 (Ronquist & Huelsenback 2003) using the GTR+I+Γ model as chosen under the Akaike Information Criterion (AIC) in MODELTEST ver. 3.7 (Posada & Crandall 1998). The Monte Carlo Markov Chain (MCMC) was run from 10-30 million iterations (to result in a final standard deviation of split frequencies of < 0.01) with a sampling frequency of 10,000. Three runs were performed for each dataset – one with all data and no constraints enforced (i.e. 'unconstrained'), one with all data

and the outgroup (corresponding to: *Desoria klovstadi*, *Isotomurus palustris*, *Isotoma (Folsomotoma) marionensis*, and *Isotoma (Parisotoma) notabilis*; Table 7.1) set as a constraint ('constrained'), and one with the outgroup excluded ('no outgroup'). These analyses were repeated using default settings (i.e. GTR+Γ) and estimating the proportion of invariable sites following a Maximum Likelihood (ML) approach in the programme RAxML ver. 7.0.4 on its web-server (Stamatakis et al. 2008).

Table 7.3 List of the DNA datasets (including outgroup taxa) used in this study, and information on sequence length (no. bp) and numbers of constant (C) and parsimony-informative (PI) sites.

Datasets	No. bp	C	PI
Individual			
cox1 (52 taxa)	367	210	146
18S (39 taxa)	427	377	24
28S (40 taxa)	502	410	53
Combined			
AG: *cox1*+18S+28S (27 taxa)	1296	997	199
AI: *cox1*+18S+28S (81 taxa)	1296	981	234

The 18S and 28S datasets behaved well during these analyses, with the outgroups consistently falling outside the ingroup in consensus networks generated in the programme SPLITSTREE ver. 4.10 (Huson & Bryant 2006) using a 10% burn-in (as determined via the programme TRACER ver. 1.4.1.; Rambaut & Drummond 2007) and a splits threshold of 0.1. However, this was not the case for analyses with the *cox1* dataset, hence, we re-coded third-codon positions as "RY" in this dataset and repeated the analyses in both MRBAYES and RAXML. Unfortunately, this did not correct the outgroup problem and (as expected) caused a loss in resolution.

7.3.4 Phylogenetic analysis

As a result of our exploratory data analysis (above), we concluded that the best approach to generating final tree hypotheses was to first determine the ingroup relationships using data with the outgroup excluded (RAXML/MRBAYES) and to then run RAXML/MRBAYES on the complete dataset (including the outgroup) with the ingroup relationships constrained to see where the outgroup was positioned in relation

to the (constrained) ingroup (see Holland et al. (2003), and Harrison et al. (2004), for discussion as to how an outgroup can disrupt a correct ingroup).

We followed this approach to generate trees for the AG (27 individuals with outgroup) and AI (81 individuals with outgroup) datasets. Specifically, we used the ingroup-only analyses to generate an ingroup hypothesis, with bootstrap values (RAxML) and posterior probabilities (MrBayes), and then ran each analysis with the ingroup constrained (MrBayes) or set as a backbone (RAxML) in order to get the best outgroup position. As the partition homogeneity test had indicated that it was not suitable to combine genes, we assigned partitions according to genes, such that each partition was able to evolve independently under the GTR+I+Γ model. All other parameter settings followed those employed during data exploration (see above). For MrBayes, this involved the settings: lset applyto=(1,2,3) nst=6 rates=invgamma; unlink statefreq=(all) revmat=(all) shape=(all) pinvar=(all); prset applyto=(all) ratepr=variable. This means that the branch lengths were linked across partitions; but that each partition was allowed its own GTR+I+G model parameters. Part of the RAxML output is a 'besttree' file which is the heuristically best tree based on un-bootstrapped data. For the AG and AI analyses, we used the RAxML besttree (no outgroup) as the final tree hypothesis, and rooted this according to the constrained analysis (above) following concordance checks of the besttree ingroup relationships against majority-rule consensus trees of the RAxML (bootstrap) and MrBayes results. In addition, these final AG and AI 'best' trees were checked against those generated for the individual-gene datasets.

7.3.5 Dating estimates

To obtain magnitude date estimates in order to help distinguish dispersal-vicariant events, we used the programme PAUP* to estimate GTR+I+Γ (as chosen under the AIC in ModelTest; see above) distances between sequences in the three individual-gene datasets, and the AG dataset. These percentage sequence divergence values were then averaged as appropriate to include only single representative individuals of each species and/or location (i.e. lineage). This resulted in a maximum of 26 individuals (with 3 lineages of *C. a. antarcticus*, 2 lineages of both *C. a. 'complex'* (Macquarie Island) and

C. a. maximus, 1 or 2 lineages of *C. caecus* depending on the dataset, and an additional 17 – 19 individual lineages depending on the dataset). The individuals that were common to all four datasets (i.e. *cox1*, 18S, 28S, AG) were then compared, and individuals from the *cox1* dataset were used to infer dating estimates among species and lineages. These were based on the molecular clock rate of 1.5 – 2.3% divergence per million years commonly applied to arthropod taxa (see Brower 1994; Juan et al. 1996; Quek et al. 2004). The divergence estimate for the lower bound (1.5%) is derived from uncorrected p-distances (see Quek et al. 2004), which do not correct for multiple changes at nucleotide sites, so will become increasingly biased towards underestimating dates as divergences become older. Thus, we perform our estimates using a divergence range (i.e. 1.5% – 2.3%), and caution that our branch lengths estimates will be longer and our older age estimates will likely be larger compared to dating estimates quoted in the literature for uncorrected p-distances.

7.3.6 Dispersal-vicariance analysis

To reconstruct the distribution history of this springtail group, we used the dispersal-vicariance approach implemented in the program DIVA ver. 1.1 (Ronquist 1996). In DIVA, a fully bifurcating phylogeny is used to parsimoniously optimise the distribution of ancestral species. The method is based on optimisation of a three-dimensional cost matrix derived from a simple biogeographic model. Distributions are described in terms of a set of unit areas, and speciation is assumed to divide ancestral distributions allopatrically into mutually exclusive sets of unit areas. DIVA finds the optimal distributions of ancestral species by minimising the number of dispersal and extinction events (Ronquist 1996).

Our DIVA analyses were based on the rooted besttree (see above) generated in RAXML for the AI dataset (no outgroup). We took this approach because the AI dataset contains all of the information in the AG dataset, but adds a wider taxon sample, thus is less likely to be affected by taxon sampling bias (e.g. Zwickl & Hillis 2002). We pruned this tree to obtain a final optimal tree hypothesis where each 'lineage' within each clade was represented. This resulted in a tree containing 32 individuals; defining 10 'lineages' of *C. a. antarcticus* (across two locations on the Antarctic Peninsula and

from the South Shetland Islands), 8 'lineages' of *C. a. 'complex'* (3 from Macquarie Island (from 2 clades), and 1 from each of Australia, Chile, Heard, New Zealand, and Tasmania), 3 lineages of *C. a. maximus* (1 from Marion Island and 2 from Îles Kerguelen), and 2 lineages of *C. caecus* (Marion Island and South Shetland Islands), as well as single lineages for the remaining species.

The distribution of each species was classified as present/absent in 15 different areas corresponding to those given in Table 7.1. The selection of these areas was based on the geographic distribution of species, and, where possible, matched springtail species endemism. In the optimisation, we also ran the analysis with the outgroup included, and both without constraint on the maximum number of ancestral areas allowed, and with 'maxareas' set to limit the maximum allowable number of geographical areas of ancestral species to 2, 3, 4, and 5.

7.4 RESULTS

7.4.1 Phylogenetic analysis

Information on the final datasets, including final sequence length, and number of constant and parsimony informative sites is given in Table 7.3. In Figures 7.2 and 7.3, we present the rooted 'besttree' results of the RAxML analyses for the AG and AI datasets (without outgroup; see Methods, section 7.3.4), respectively. Superimposed on these ML trees are the support values (bootstrap values from RAxML and the posterior probabilities generated in MrBayes) from the respective ingroup-only analyses. In Figure 7.2, the ingroup comprises two main groupings, one of which isolates *C. sverdrupi* (Sverdrupfjella) (1), *C. tricuspis* (Marion Island) (2), *C. caecus* (Marion Island) (3), *N. nivicolus* (Granite Harbour) (4), and *G. terranova* (Terra Nova Bay) (5). The other grouping ("6" in Fig. 7.2) consists of two sub-groups containing 6(a): *C. a. 'complex'* (Macquarie Island and Heard Island) + *C. a. 'complex'* (Macquarie Island), and *C. a. antarcticus* (Peninsula and South Shetland Islands) and *C. a. maximus* (Macquarie and Heard Islands); and 6(b): *C. a. 'complex'* (Tasmania) + *C. dubius* (Marion Island) + *C. cisantarcticus* (Cape Hallett) and *C. a. complex* (Chile) (Fig. 7.2).

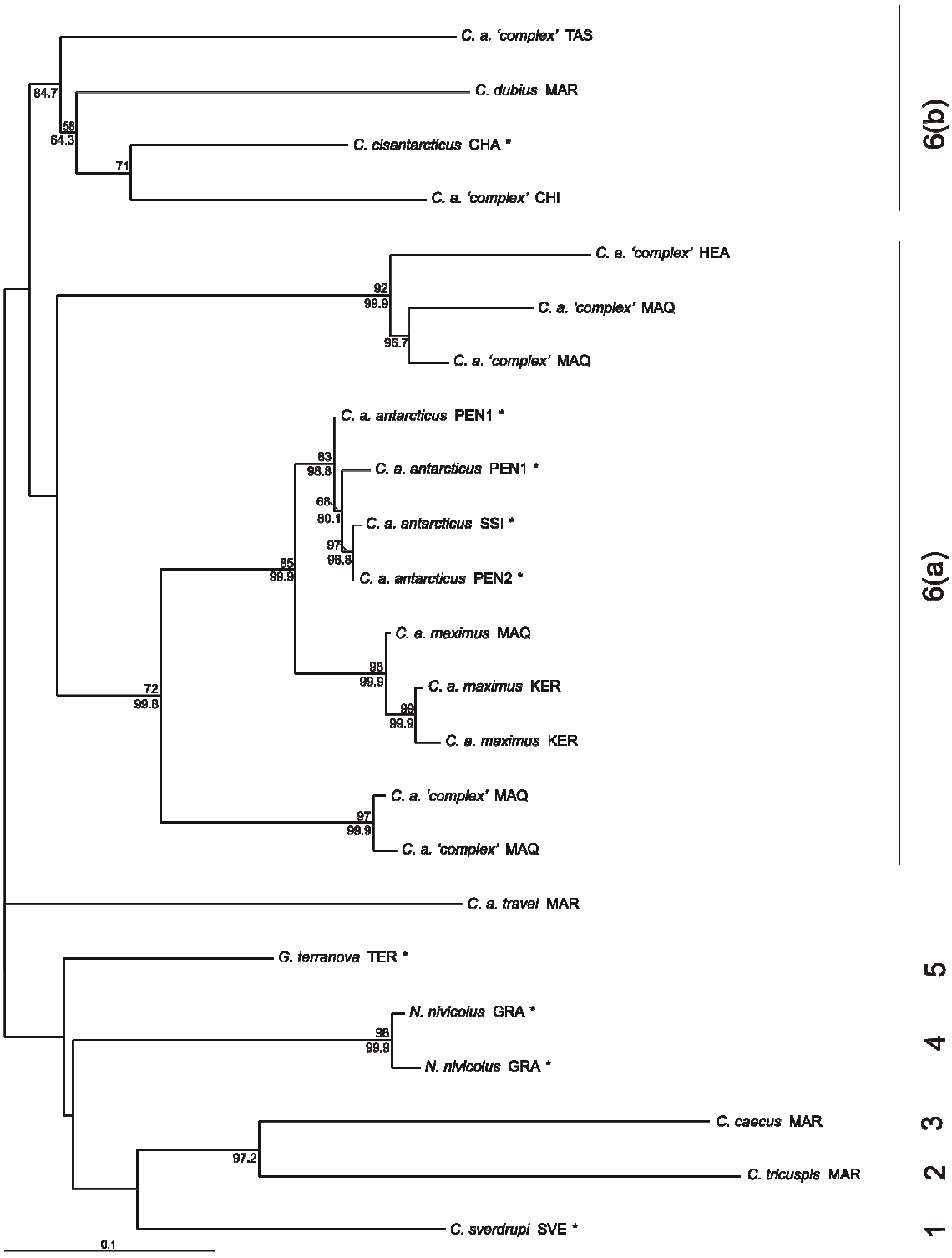

Figure 7.2 RAxML-generated 'besttree' for the allgenes ('AG') dataset of *Cryptopygus* and its close relatives. The outgroup (4 individuals) is not included here, although it was used to root this tree; see Methods, sections 7.3.3, 7.3.4), hence we show 23 of the 27 possible individuals of this dataset. ML bootstrap values (100 replicates) >50% are indicated above nodes with posterior probabilities from the MRBAYES Bayesian analyses (>50%) below nodes – these were obtained from analyses without the

(contd from p188) outgroup (see Methods, section 7.3.4). In each analysis, partitions according to the *cox1*, 18S and 28S genes were allowed to evolve independently under the GTR+I+Γ model (see Methods, section 7.3.4 for further information). Location codes correspond to: MAR: Marion Island, CHA: Cape Hallett, TER: Terra Nova Bay, SVE: Sverdrupfjella, GRA: Granite Harbour, TAS: Tasmania, CHI: Chile, HEA: Heard Island, MAQ: Macquarie Island, KER: Îles Kerguelen, PEN: Peninsula, SSI: South Shetland Islands. Antarctic locations are indicated with an asterisk. The numbers 1 – 6 on the right-hand side of the figure refer to the overall main groupings referred to in the text.

This analysis was unable to differentiate the position of *C. a. travei* (Marion Island) comparative to these two main groupings and we note that in several instances our bootstrap and posterior distribution values are low at deeper nodes of the tree (Fig. 7.2).

The addition of more individuals in the form of the AI dataset (with missing data) largely retains the overall groupings outlined above; however, the specific relationships between several groups have changed. For example, *C. sverdrupi* (group 1) and *A. subpolaris* (new to Fig. 7.3) come out as sister taxa, and these, along with *C. tricuspis* and *C. caecus* (groups 2 and 3) now fall as sister groups to subgroup 6(a) (Figs 7.2, 7.3). In addition, *C. a. travei* and *C. a reagens* (absent from Fig. 7.2) form a sister group within subgroup 6(b), while *N. nivicolus* (group 4) and *G. terranova* (group 5) now fall as sister groups to subgroup 6(b) (Figs 7.2, 7.3). Thus, the main rearrangement going from Figure 7.2 to 7.3 is that groups 1 – 5 from Figure 7.2 move closer to either of subgroups 6(a) or (b) in Figure 7.3. This change highlights the prevalence of genetic associations among geographically distinct locations, although we again note that our bootstrap and posterior distribution values are low at deeper nodes of the tree (Fig. 7.3).

Because employment of wider taxon sampling generally results in tree hypotheses that are less biased by systematic error (see Zwickl & Hillis 2002), we expect that the tree generated from the AI dataset is more accurate than that resulting from the AG analyses. However, the different analytical methods (i.e. RAxML/MrBayes) gave relatively concordant results across genes without outgroups (see Appendices 7.1, 7.2 and 7.3 for results from individual gene datasets). The six main groups (above) were supported without conflict in the individual *cox1* and 18S datasets, however there was some signal for groups 2, 3 and 4 falling in with 6(b), and group 5 falling in with 6(a) in the 28S dataset (Appendix 7.3).

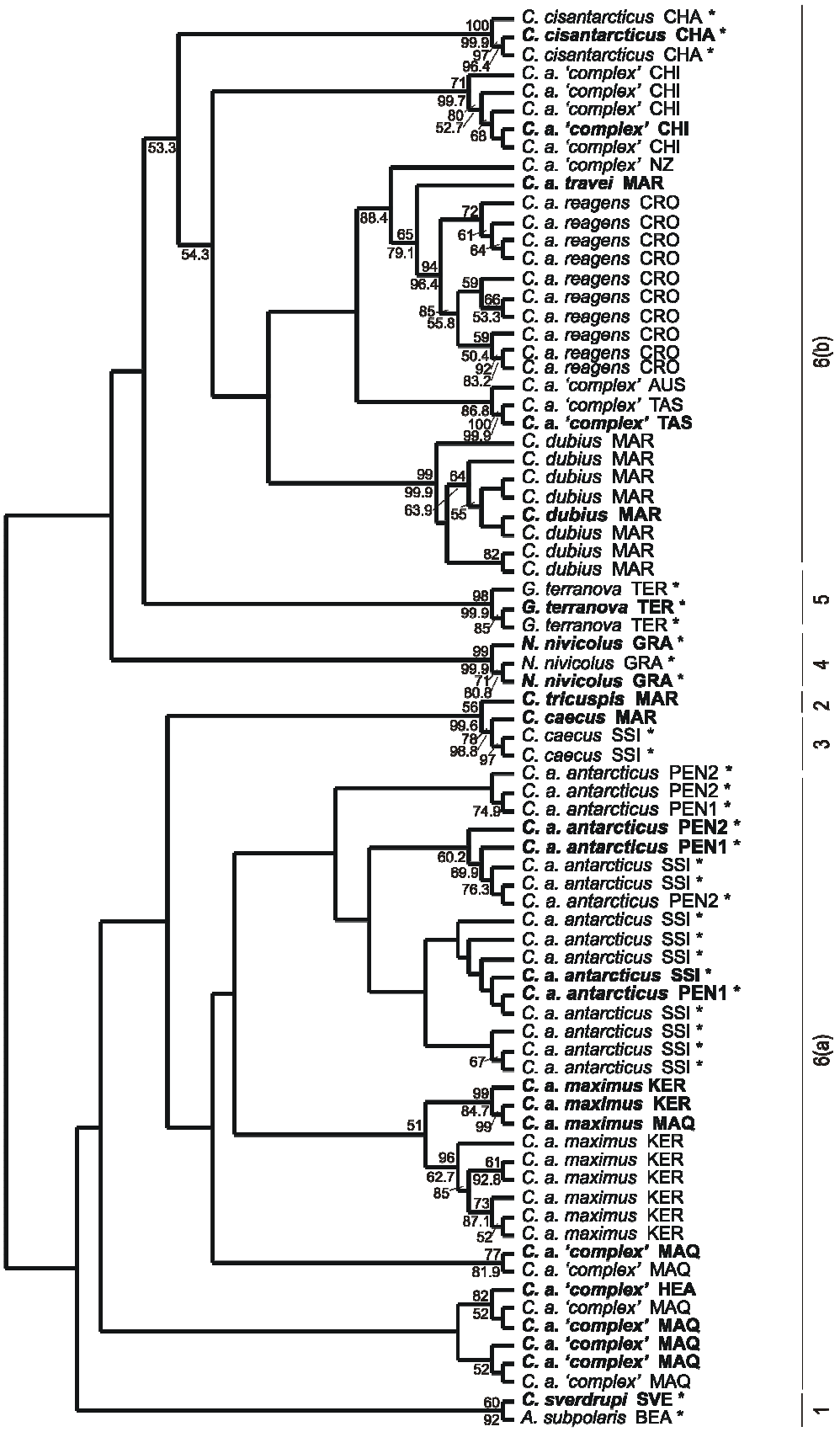
C. cisantarcticus CHA *
C. cisantarcticus CHA *
C. cisantarcticus CHA *
C. a. 'complex' CHI
C. a. 'complex' CHI
C. a. 'complex' CHI
C. a. 'complex' CHI
C. a. 'complex' CHI
C. a. 'complex' NZ
C. a. travei MAR
C. a. reagens CRO
C. a. reagens CRO
C. a. reagens CRO
C. a. reagens CRO
C. a. reagens CRO
C. a. reagens CRO
C. a. reagens CRO
C. a. reagens CRO
C. a. reagens CRO
C. a. 'complex' AUS
C. a. 'complex' TAS
C. a. 'complex' TAS
C. dubius MAR
C. dubius MAR
C. dubius MAR
C. dubius MAR
C. dubius MAR
C. dubius MAR
C. dubius MAR
G. terranova TER *
G. terranova TER *
G. terranova TER *
N. nivicolus GRA *
N. nivicolus GRA *
N. nivicolus GRA *
C. tricuspis MAR
C. caecus MAR
C. caecus SSI *
C. caecus SSI *
C. a. antarcticus PEN2 *
C. a. antarcticus PEN2 *
C. a. antarcticus PEN1 *
C. a. antarcticus PEN2 *
C. a. antarcticus PEN1 *
C. a. antarcticus SSI *
C. a. antarcticus SSI *
C. a. antarcticus PEN2 *
C. a. antarcticus SSI *
C. a. antarcticus SSI *
C. a. antarcticus SSI *
C. a. antarcticus SSI *
C. a. antarcticus PEN1 *
C. a. antarcticus SSI *
C. a. antarcticus SSI *
C. a. antarcticus SSI *
C. a. antarcticus SSI *
C. a. maximus KER
C. a. maximus KER
C. a. maximus MAQ
C. a. maximus KER
C. a. maximus KER
C. a. maximus KER
C. a. maximus KER
C. a. maximus KER
C. a. maximus KER
C. a. 'complex' MAQ
C. a. 'complex' MAQ
C. a. 'complex' HEA
C. a. 'complex' MAQ
C. a. 'complex' MAQ
C. a. 'complex' MAQ
C. a. 'complex' MAQ
C. a. 'complex' MAQ
C. sverdrupi SVE *
A. subpolaris BEA *
6(b)
5
4
2
3
6(a)
1

Figure 7.3 (p190) RAxML-generated 'besttree' for the allgenes ('AI') dataset of *Cryptopygus* and its close relatives. The outgroup (4 individuals) is not included here (although it was used to root this tree; see Methods, sections 7.3.3, 7.3.4), hence we show 77 of the possible 81 individuals for this dataset. ML bootstrap values (100 replicates) >50% are indicated above nodes, with posterior probabilities from the MRBAYES Bayesian analyses (>50%) below nodes – these were obtained from analyses without the outgroup (see Methods, section 7.3.4). In each analysis, partitions according to the *cox1*, 18S and 28S genes were allowed to evolve independently under the GTR+I+Γ model (see Methods, section 7.3.4 for further information). See Figure 7.2 for location codes – in addition, AUS: Australia, CRO: Îles Crozet, BEA: Beardmore Glacier. Individuals from Figure 7.2 are highlighted in bold; Antarctic locations are indicated with an asterisk. The numbers 1 – 6 on the right-hand side of the figure refer to the overall main groupings referred to in the text. Note that the branch lengths in this figure are not to scale. In addition, the branch lengths generated for this dataset were based on all three genes, however sister relationships within the ingroup depicted here were separated by 25% - 30% sequence divergence when relevant individuals from the *cox1* dataset were analysed, whereas divergence within species from different locations (i.e. *C. a. 'complex'* (Heard and Macquarie Islands), *C. a. antarcticus* and *C. a. maximus*) were separated by 2 – 6% *cox1* sequence divergence (see text for further details).

7.4.2 Dating estimates

Estimates of percentage sequence divergence among species (or lineages) were fairly consistent across the *cox1*, 18S, 28S and AG datasets, although as expected, these varied on an absolute scale depending on the gene. In particular, 18S and 28S sequence divergences were similarly low; values from individuals in the AG dataset were around 2-3-fold higher than these, and *cox1* values were 6-10-fold higher again (see Appendices 7.4, 7.5). Since a commonly employed molecular clock exists for the *cox1* dataset, we focus on these results for our dating estimates.

In the *cox1* dataset (containing 27 representative 'lineages'; see Methods, section 7.3.5, and Appendices 7.4, 7.5) sequence divergence ranged from 1 – 51% (mean: 30%) within the ingroup. The mean distance from the ingroup to the outgroup was 36%, and among 'subgroups' of the ingroup ranged from 25 – 35%. In the three cases where the same species existed across more than one location, comparatively small genetic distances separated these. This includes *C. a. antarcticus* from the two Peninsula and South Shetland Island locations (~2.7% divergent), *C. a. 'complex'* from Macquarie and Heard Islands (6%) and *C. a. maximus* from Îles Kerguelen and Macquarie Island (~2.6%). Finally, a mean distance of 51% separated the two Macquarie Island lineages in this dataset and divergence among *C. a. 'complex'* individuals averaged 30%.

Using the generalised invertebrate molecular clock rate of 1.5 – 2.3% divergence per My suggests that the deepest splits within *Cryptopygus* occurred 22.2 – 34.0 Ma (i.e. 51% sequence divergence), while the shallowest splits have occurred in the last

million years and up to ~2.6 – 4 Ma (for *C. a. 'complex'* from Macquarie and Heard Islands).

7.4.3 Dispersal-vicariance analysis

The best dispersal-vicariance analysis constructions (i.e. with the least number of dispersals) in all cases were obtained using no outgroup and no 'maxareas' restrictions (see Methods, section 7.3.6). In addition, the ancestral distributions towards the root were the same both with and without outgroups included, most likely as a result of the limited geographic distributions of the outgroup taxa. Increasing the 'maxareas' settings, as well as dramatically increasing the number of inferred dispersal events (to ~20), increased the number of alternative putative ancestral areas proposed.

The ancient distribution of *Cryptopygus* was defined as: "ABCDEFGHIKLNO(JM)", thus always encompassing 13 areas and possibly including areas J and M (Fig. 7.4, see also Table 7.1); as is the usual tendency in DIVA, this root node distribution is large and includes many (or all) of the areas occupied by the terminal species. In particular, the ancestral distribution at the nodes encompassing subgroup 6(a): "DFIKO(AJM)" and 6(b): "BCEGHLN(A)" (Fig. 7.4), were difficult to restrict and thus may have been widespread. Interestingly, there is little overlap in distribution between this main 6(a)/6(b) split, which largely involves maritime- and sub-Antarctic groups (i.e. DIK(JM)) separating from continental southern hemisphere groups (i.e. BCEHLN) (Fig. 7.5), and suggests that a vicariant event separated these lineages early in the evolutionary history of the group.

The optimisation without outgroup and with no restriction on 'maxareas' inferred a biogeographic scenario requiring nine dispersal events (Fig. 7.4). Exact dispersal events depend on selection of specific ancestral areas when > 1 is considered parsimonious, however four dispersal events are present in all scenarios (indicated by '*' on tree branches in Fig. 7.4) and these are between: Macquarie Island and Îles Kerguelen, and the Antarctic Peninsula and South Shetland Islands.

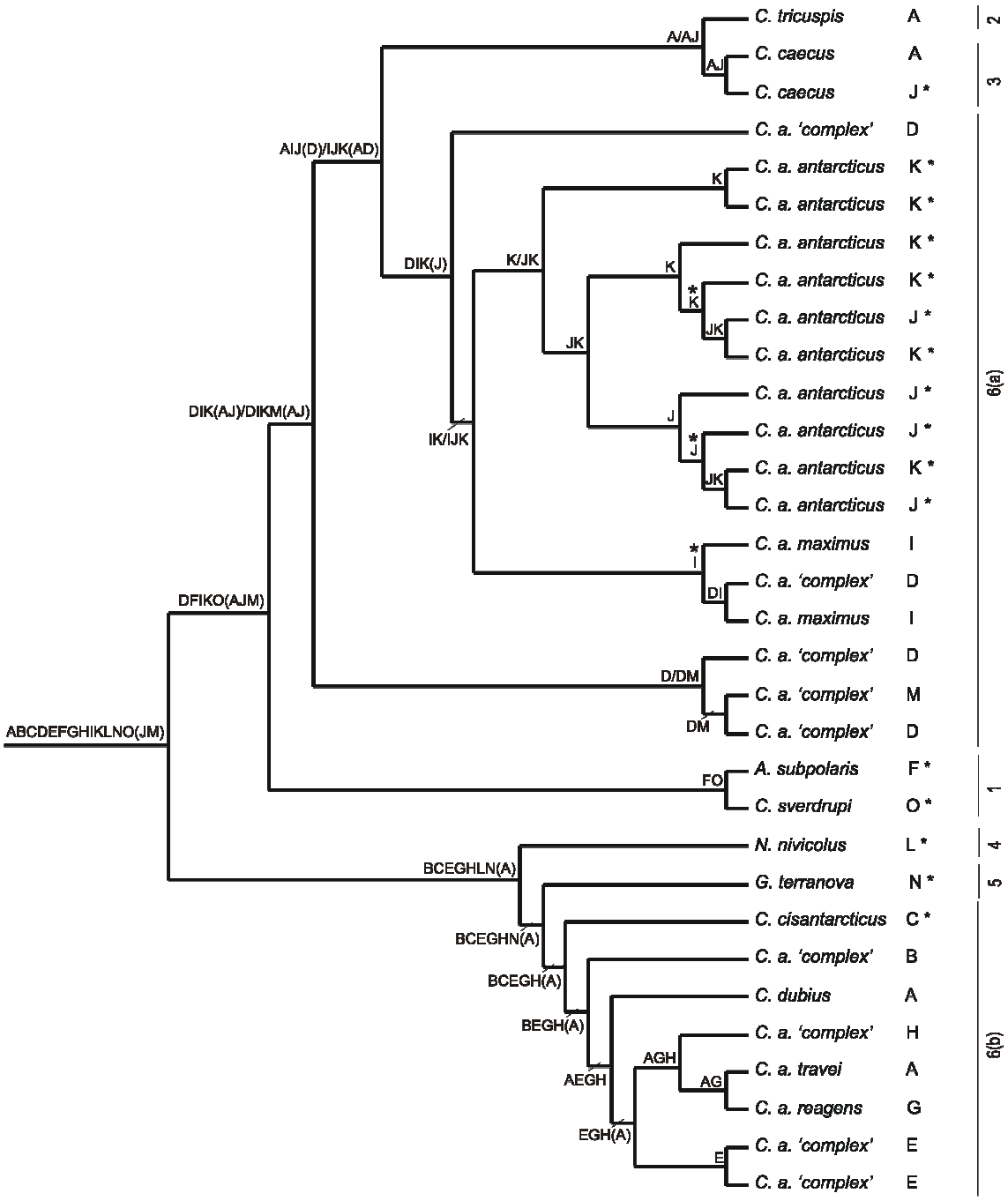

Figure 7.4 The tree hypothesis used for dispersal-vicariance (DIVA) analyses, based on a pruned version of the RAxML-generated besttree for the AI dataset shown in Fig. 7.3. Distributions correspond to: A: Marion Island, B: Chile, C: Cape Hallett, D: Macquarie Island, E: Australia, Tasmania, F: Beardmore Glacier, G: Îles Crozet, H: New Zealand, I: Îles Kerguelen, J: South Shetland Islands, K: Peninsula, L: Granite Harbour, M: Heard Island, N: Terra Nova Bay, O: Sverdrupfjella. Distributions above nodes correspond to the ancestral distributions predicted by DIVA, while those beside species labels indicate the contemporary distribution of the relevant species. Asterisks beside species names indicate individuals from Antarctic locations, while those on branches indicate cases where a dispersal event was indicated under all DIVA optimisations. The numbers 1 – 6 on the right-hand side of the figure refer to the overall main groupings referred to in the text.

With a total of 32 individuals, there are up to 31 possible events. Since nine of these are dispersal events and extinction events are considered a cost in DIVA (Ronquist 1996), many vicariant and/or speciation episodes are also predicted (i.e. an upper limit of 22). Thus the biogeographic scenario inferred by DIVA suggests that the current distribution is a result of a combination of vicariance and dispersal events from a disjunct ancestral distribution (see Fig. 7.5).

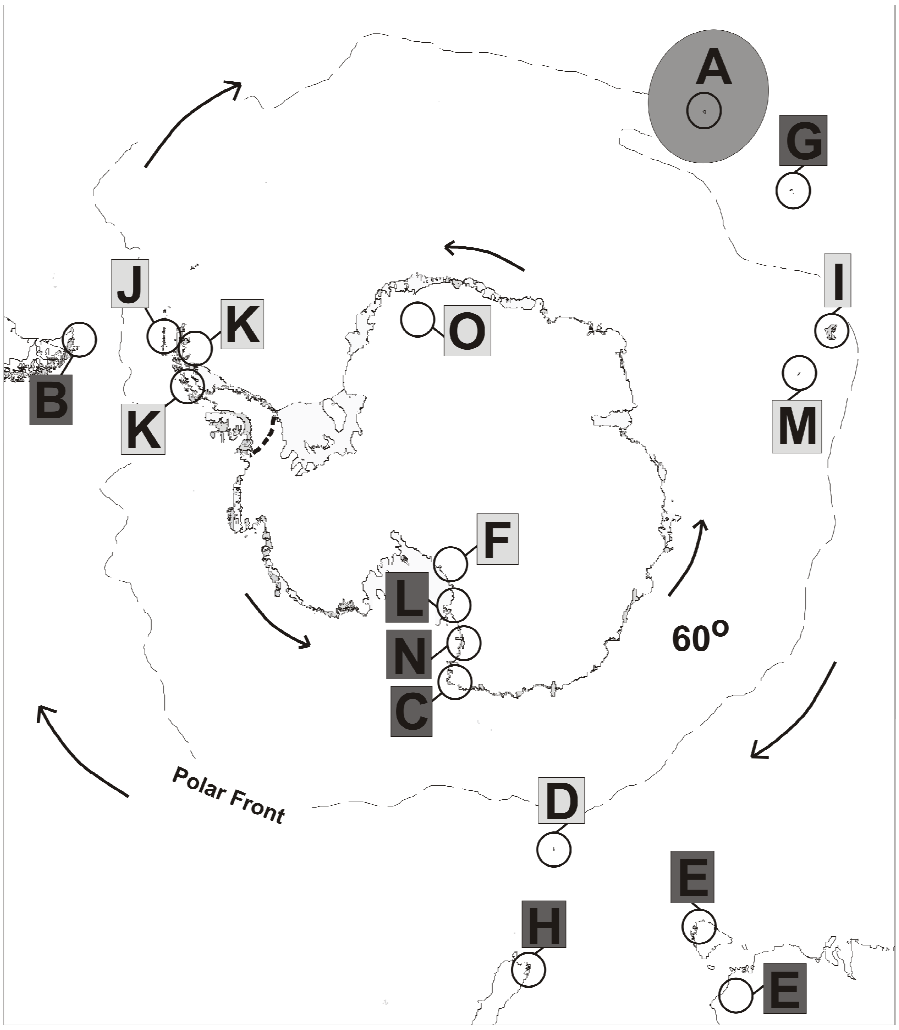

Figure 7.5 Re-worked version of Fig. 7.1 to show species distributions according to the results of the DIVA analysis for the ancient split of lineages at the nodes encompassing subgroups 6(a) (inferred distribution: DFIKO(AJM); dark grey shading) and 6(b) (inferred distribution: BCEGHLN(A)"; light grey shading) from Fig. 7.4; location 'A' in the figure is coloured with medium grey shading to highlight that it is the only location inferred in these distributions which bridges this ancient split. Distributions correspond to those used in Table 7.1 and Fig. 7.4. See text and associated figures for further details.

7.5 DISCUSSION

The phylogenetic results presented here are largely consistent with those of Stevens et al. (2006), in particular confirming the placement of our groups 4, 5, and 6(a and b) (Fig. 7.3). The addition of new species and genes in this study sees us place *A. subpolaris* differently – as sister to subgroup 6(a) (this species grouped with *C. dubius* – our group 6(b) – in Stevens et al. 2006 and here, groups with *C. sverdrupi* – absent in Stevens et al. 2006) (Figs 7.2, 7.3). In addition, we find lineages of *C. a. 'complex'* from Macquarie and Heard Islands grouping with 6(a) – these grouped with our 6(b) in Stevens et al. (2006). Finally, we introduce two additional Marion Island species (*C. tricuspis* and *C. caecus*) to further the reconstruction presented here and these both fall within group 6(a) (Fig. 7.3).

In all of our phylogenetic analyses, the bootstrap and posterior distribution support values were quite low at the deeper nodes of our trees. In addition, we had problems with inconsistent outgroup separation in our *cox1* dataset. Given the uncertainty in taxonomy of this group, it may be possible that the disruption of ingroup sequences in *cox1* is not an artefact but rather a description of reality. However, the 18S, 28S, AG and AI datasets do not support this idea – all resolved the outgroup correctly in all analyses. Nevertheless, our results do not support monophyly for species within Antarctic *Cryptopygus*. For example, several paraphyletic relationships involved *Cryptopygus antarcticus* subspecies (*C. a. antarcticus*, *C. a. 'complex'*, and *C. a. maximus*). In addition, our analyses including all individuals found sister relationships between various *Cryptopygus* species (e.g. *C. a. travei*, *C. caecus*, *C. sverdrupi*, *C. tricuspis*) as well as several non-*Cryptopygus* species (*G. terranova*, *N. nivicolus*, and *A. subpolaris*), indicating non-monophyly for Antarctic *Cryptopygus* as a whole. Thus, it is clear that more work needs to be done to further resolve relationships for both Antarctic *Cryptopygus* and Antarctic Isotomidae in general.

In the meantime, the subspecies recognised so far by taxonomists (see Table 7.1) are confirmed in this molecular approach. The levels of divergence identified among the composite of undescribed *'C. a. complex'* species as well as the recognized sub-species (i.e. *Cryptopygus antarcticus maximus*, *reagens*, *travei*) calls for a taxonomic

revision of this genus, suggesting that the subspecies in particular should be elevated to species status (see also Stevens et al. 2006).

The high level of genetic divergence between species of this group also suggests that they have persisted in Antarctica over multi-million year time-scales. For example, the *cox1* dating estimates suggest that the deepest evolutionary splits among lineages occurred 22.2 – 34.0 Ma (during the Miocene). This corresponds to a largely vicariant scenario explaining the evolutionary history of these Antarctic *Cryptopygus*. In this scenario, ice sheet oscillations through time drive repeated shifts in the distribution of land species, leading to fragmentation and isolation (allopatric speciation) in refugia during glacial maxima (Convey & Stevens 2007; Rogers 2007; Convey et al. 2008). Several recent studies have invoked such vicariant explanations for contemporary distributions of Antarctic taxa (e.g. Marshall & Coetzee 2000; Allegruci et al. 2006; Stevens et al. 2006). In the current study, the ages of sub-Antarctic island Îles Crozet (~8.7 My) and Îles Kerguelen (~100 My), as well as the more ancient southern landmasses (Australia, Tasmania, New Zealand, Chile, and the Antarctic Peninsula and continent) fit well with our suggested Miocene divergence dates, underscoring the significance of vicariant events in shaping contemporary distributions of the *Cryptopygus* group.

However, relationships among the shallow paraphyletic lineages in this study suggest that dispersal over small geographic scales has also been significant in the evolutionary history of these species. This includes recent divergence among *C. a. antarcticus* from the Peninsula and South Shetland Islands (< 1 Ma) and among *C. a. maximus* lineages from Îles Kerguelen and Macquarie Island (~1 – 2 Ma), each of which were predicted in the DIVA analysis. In addition, phylogenetic (sister) relationships between Macquarie and Heard Island lineages of *C. a. 'complex'* as well as subspecies including *C. a. travei* (Marion Island), *C. a. 'complex'* (New Zealand) and *C. a. reagens* (Îles Crozet), and *C. tricuspis* (Marion Island) and *C. caecus* (Marion Island and South Shetland Islands), suggest some form of dispersal over more ancient timescales (i.e. ~2.6 – > 20 Ma). Taken together, these results indicate that oceanic currents between latitudes 40º and 60º may have aided dispersal among sub-Antarctic islands, most likely in an easterly direction. Certainly, the WWD has favoured such

dispersal in circum-Antarctica, where the islands are proposed to act as 'stepping stones' to aid this process (McDowall 1970; Fleming 1979; Winkworth et al. 2002). The age of habitat availability for several of the sub-Antarctic islands, namely Heard (~1 My), Marion (~0.3 My) and Macquarie (~0.7 My) Islands (Stevens et al. 2006), fit well with several of these dispersal scenarios (although in the case of volcanic Marion Island, close faunal ties between this and nearby (~19 km) Prince Edward Island (~8 – 18 My) allow vicariant scenarios to also play a role; analysis of samples from the latter locality would strengthen this suggestion of a deep divergence for the Marion/Prince Edward Island fauna). However, the large genetic distances separating the two Macquarie Island lineages (~ 51%) must reflect separate colonisations of two distinct evolutionary lineages to this recently available habitat.

An implicit assumption in Antarctic biogeography which suggests that obliteration of habitats following successive glacial events restricts many species to being recent colonists is gradually lessening its hold on Antarctic biogeography (see Convey & Stevens 2007; Convey et al. 2008). Indeed, a more accurate biogeographic scenario for Southern Hemisphere terrestrial taxa places both ancient and recent elements in the mix (e.g. Stevens et al. 2006, 2007; Mortimer & Jansen van Vuuren 2007; McGaughran et al. 2008). Our findings, as outlined above, support this shift in paradigm.

In addition, the dispersal-vicariance analyses support this conclusion, assigning 9 dispersal events and an upper bound of 22 vicariant events from a disjunct ancestral distribution to optimally explain current distribution patterns. We feel the tendency of DIVA to suggest a biased large root node distribution in some cases (Ronquist 1996) allows us to say little about the true nature of the ancestral distribution here and look forward to advances in the field of biogeographic inference (e.g. Nylander et al. 2008). However, faunal similarities have been used in the past to suggest that a large former insular area of evolution may be a feature of several sub-Antarctic islands (Gressitt 1970), thus supporting the idea of widely dispersed ancestral taxa in some circum-Antarctic species. In particular, the DIVA analysis highlighted a separation between largely (younger) maritime- and sub-Antarctic groups (i.e. DIK(JM)) and (older) continental southern hemisphere groups (i.e. BCEHLN) (see Fig. 7.5). The only exceptions to this proposed vicariant split were F (Beardmore Glacier) and O

(Sverdrupfjella), which both fell in with the sub-Antarctic group, and G (Îles Crozet) which grouped with the continental locations. In the former case, the genetic distances separating the species found at F and O (*A. subpolaris* and *C. sverdrupi*; respectively) are large enough to suggest missing (possibly continental Antarctic) lineages, while the age of Îles Crozet (~8.7 My) may explain its grouping with older landmasses. Interestingly, A (Marion Island) is the only location to overlap this DIVA-split and again, the proximity of the older Prince Edward Island landmass may be important here.

In summary, the evolutionary history of this group of Antarctic springtails reflects an origin of predominant (vicariant) Miocene isolation interspersed with occasional episodes of subsequent contact (dispersal). We suggest that the former must imply the continuous presence of refugia with appropriate environmental conditions, and that the latter has been aided in some part by the WWD and other oceanic currents. Collectively, this sees the contemporary distribution of the studied southern hemisphere springtails as deriving from multiple independent evolutionary lineages from which largely paraphyletic species relationships have derived. The high level of genetic divergence associated with this diversification calls for a revision of current 'species' designations and suggests the need for extreme care to prevent human-mediated dispersal among these clearly unique sub-Antarctic and Antarctic springtail populations. Future investigation of this issue should focus on those sub-Antarctic and southern temperate species of *Cryptopygus* that were unable to be included in this study, such that more general conclusions about wider Antarctic biogeography may be drawn. In particular, it will be interesting to see if additional sub-Antarctic species are more closely related to South-American, South-African, Australian or New Zealand species than to their Antarctic counter-parts.

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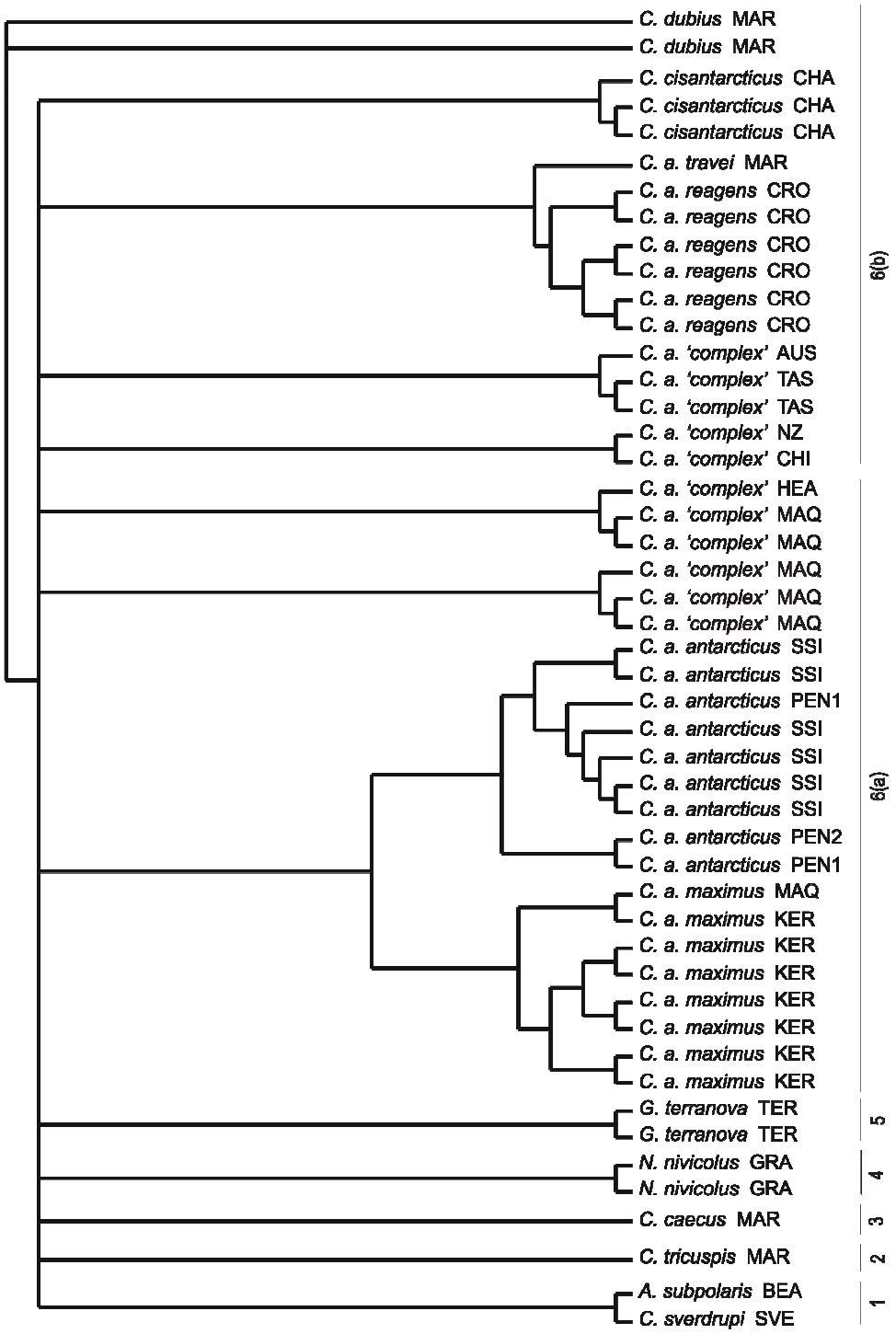
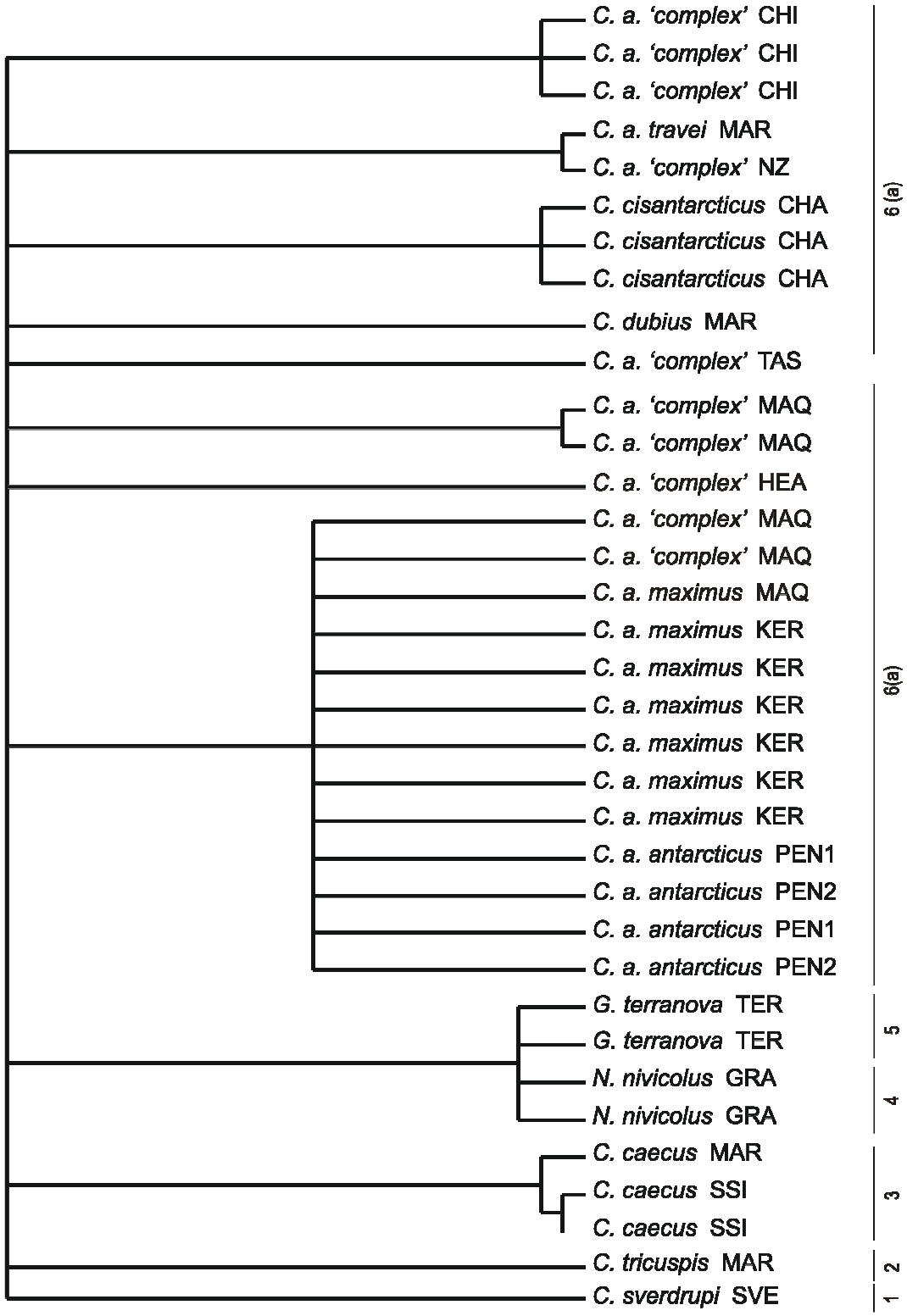
7.8 APPENDICES

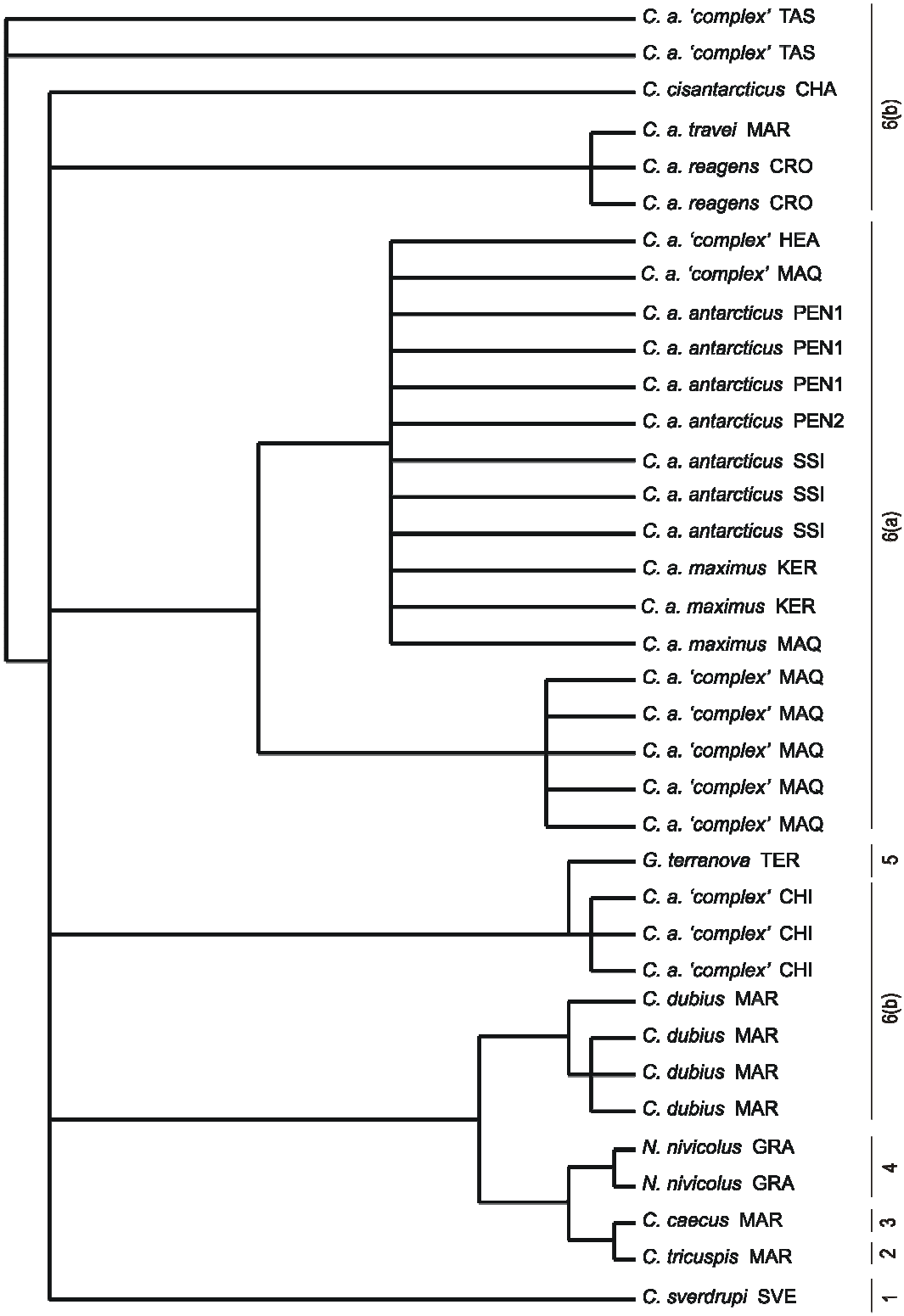
Appendix 7.1 50% majority-rule consensus tree of the 100 bootstrap trees generated by RAxML under GTR + I + Γ model for the *cox1* dataset of 48 taxa (no outgroup) of *Cryptopygus* and its close relatives. Species and location codes correspond to Fig. 7.1, Table 7.1. The numbers 1 – 6 on the right-hand side of the figure refer to the overall main groupings referred to in the text.



Appendix 7.2 50% majority-rule consensus tree of the 100 bootstrap trees generated by RAxML under GTR + I + Γ model for the 18S dataset of 35 taxa (no outgroup) of *Cryptopygus* and its close relatives. Species and location codes correspond to Fig. 7.1, Table 7.1. The numbers 1 – 6 on the right-hand side of the figure refer to the overall main groupings referred to in the text.



Appendix 7.3 50% majority-rule consensus tree of the 100 bootstrap trees generated by RAxML under GTR + I + Γ model for the 28S dataset of 36 taxa (no outgroup) of *Cryptopygus* and its close relatives. Species and location codes correspond to Fig. 7.1, Table 7.1. The numbers 1 – 6 on the right-hand side of the figure refer to the overall main groupings referred to in the text.



Appendix 7.4 Matrix of pairwise (GTR+I+Γ) sequence divergence values separating individuals representative of species and/or locations. Lower diagonal presents means for the relevant 20 individuals from the allgene ('AG') dataset; upper triangle presents means for the relevant 26 individuals from the *cox1* dataset.

	Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27
1	*C. a. antarcticus* PEN - L1		0.017	0.02	0.282	0.265	0.343	0.203	0.343	0.267	0.357	0.114	0.098	0.277	0.273	0.33	0.254	0.26	0.287	0.313	0.342	0.263	0.297	0.414	0.262	0.415	0.414	0.38
2	*C. a. antarcticus* PEN - L2			0.033	0.297	0.299	0.36	0.222	0.361	0.285	0.359	0.134	0.123	0.3	0.26	0.345	0.285	0.301	0.313	0.328	0.337	0.268	0.298	0.46	0.244	0.45	0.453	0.4
3	*C. a. antarcticus* SSI - L3	0.01			0.286	0.292	0.377	0.216	0.378	0.266	0.376	0.127	0.129	0.276	0.258	0.329	0.288	0.268	0.308	0.327	0.344	0.298	0.325	0.411	0.27	0.43	0.48	0.4
4	*C. a. 'complex'* AUS					0.272	0.388	0.265	0.388	0.204	0.228	0.312	0.3	0.254	0.305	0.351	0.303	0.291	0.397	0.363	0.336	0.274	0.32	0.382	0.265	0.409	0.517	0.39
5	*C. a. 'complex'* CHI	0.08		0.08			0.315	0.198	0.316	0.179	0.278	0.282	0.332	0.272	0.354	0.303	0.19	0.272	0.215	0.308	0.308	0.239	0.31	0.302	0.239	0.315	0.434	0.32
6	*C. a. 'complex'* HEA	0.06		0.07		0.09		0.509	0.006	0.263	0.391	0.468	0.391	0.299	0.346	0.327	0.368	0.301	0.346	0.32	0.399	0.405	0.318	0.383	0.286	0.426	0.419	0.38
7	*C. a. 'complex'* MAQ - L1	0.05		0.05		0.06	0.08		0.509	0.278	0.338	0.25	0.253	0.287	0.301	0.322	0.251	0.25	0.343	0.241	0.373	0.248	0.34	0.32	0.286	0.435	0.285	0.33
8	*C. a. 'complex'* MAQ - L2	0.08		0.09		0.09	0.02	0.10		0.263	0.391	0.47	0.392	0.299	0.346	0.327	0.369	0.313	0.346	0.339	0.4	0.405	0.319	0.384	0.286	0.427	0.452	0.39
9	*C. a. 'complex'* NZ										0.301	0.265	0.28	0.296	0.328	0.272	0.246	0.259	0.231	0.322	0.323	0.243	0.245	0.278	0.165	0.398	0.399	0.31
10	*C. a. 'complex'* TAS	0.08		0.08		0.08	0.09	0.08	0.09			0.358	0.341	0.298	0.351	0.334	0.482	0.317	0.35	0.406	0.387	0.293	0.352	0.388	0.33	0.386	0.362	0.37
11	*C. a. maximus* KER - L1	0.03		0.03		0.08	0.08	0.05	0.10		0.08		0.026	0.259	0.31	0.341	0.283	0.344	0.31	0.404	0.385	0.309	0.309	0.42	0.285	0.442	0.518	0.42
12	*C. a. maximus* MAQ - L2	0.02		0.03		0.08	0.07	0.05	0.09		0.08	0.01		0.264	0.289	0.343	0.272	0.32	0.293	0.376	0.373	0.307	0.301	0.449	0.251	0.389	0.51	0.4
13	*C. a. reagens* CRO														0.206	0.3	0.292	0.287	0.342	0.364	0.337	0.285	0.306	0.4	0.291	0.416	0.487	0.4
14	*C. a. trawei* MAR	0.07		0.07		0.09	0.08	0.07	0.10		0.08	0.08	0.07			0.336	0.345	0.323	0.371	0.422	0.315	0.388	0.335	0.476	0.336	0.503	0.34	0.41
15	*C. caecus* MAR - L1	0.09		0.09		0.09	0.09	0.09	0.10		0.09	0.09	0.09		0.10		0.311	0.339	0.276	0.356	0.35	0.296	0.313	0.338	0.258	0.537	0.37	0.38
16	*C. cisantarcticus* CHA	0.07		0.07		0.06	0.08	0.06	0.09		0.09	0.07	0.07		0.08	0.09		0.275	0.324	0.31	0.287	0.299	0.288	0.409	0.263	0.461	0.526	0.42
17	*C. dubius* MAR	0.08		0.08		0.09	0.09	0.08	0.09		0.09	0.09	0.09		0.09	0.10	0.07		0.367	0.338	0.362	0.258	0.31	0.305	0.265	0.361	0.337	0.32
18	*C. sverdrupi* SVE	0.07		0.08		0.07	0.08	0.08	0.10		0.09	0.08	0.07		0.09	0.09	0.07	0.10		0.28	0.266	0.277	0.31	0.332	0.246	0.348	0.379	0.33
19	*C. tricuspis* MAR	0.09		0.09		0.10	0.09	0.08	0.11		0.10	0.10	0.10		0.11	0.09	0.09	0.11	0.09		0.363	0.301	0.392	0.339	0.291	0.43	0.355	0.35
20	*A. subpolaris* BEA																					0.297	0.345	0.445	0.291	0.432	0.403	0.39
21	*G. terranova* TER	0.07		0.07		0.07	0.09	0.06	0.10		0.07	0.07	0.07		0.08	0.09	0.07	0.08	0.07	0.09			0.188	0.334	0.17	0.395	0.294	0.3
22	*N. nivicolus* GRA	0.07		0.07		0.09	0.08	0.08	0.09		0.08	0.07	0.07		0.08	0.08	0.07	0.09	0.08	0.10		0.06		0.36	0.223	0.341	0.363	0.32
23	*D. klovstadi* CHA	0.12		0.12		0.12	0.12	0.11	0.12		0.12	0.12	0.13		0.14	0.11	0.12	0.12	0.12	0.10		0.12	0.12		0.277	0.377	0.253	0.302
24	*I. marionensis* MAR	0.08		0.08		0.09	0.08	0.08	0.09		0.09	0.08	0.08		0.10	0.08	0.08	0.09	0.08	0.07		0.07	0.07	0.09		0.349	0.354	0.326
25	*I. notabilis* MAR	0.10		0.10		0.10	0.11	0.11	0.12		0.10	0.10	0.10		0.12	0.12	0.11	0.11	0.10	0.11		0.10	0.09	0.11	0.09		0.422	0.382
26	*I. palustris* MAR	0.14		0.14		0.14	0.13	0.12	0.14		0.12	0.15	0.15		0.13	0.13	0.15	0.13	0.14	0.12		0.12	0.13	0.11	0.11	0.13		0.343
27	Outgroup (mean)	0.11		0.11		0.11	0.11	0.10	0.12		0.11	0.11	0.11		0.12	0.11	0.11	0.11	0.11	0.10		0.10	0.10	0.1	0.09	0.11	0.11	

Appendix 7.5 Matrix of pairwise (GTR+I+Γ) sequence divergence values separating individuals representative of species and/or locations. Lower diagonal presents means for the relevant 24 individuals from the 18S dataset; upper triangle presents means for the relevant 26 individuals from the 28S dataset.

	Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27
1	*C. a. antarcticus* PEN - L1		0.00	0.00	0.00	0.04	0.00	0.00	0.00		0.02		0.00	0.02	0.02	0.03		0.01	0.03	0.01	0.04	0.02	0.03	0.07	0.04	0.04	0.10	0.06
2	*C. a. antarcticus* PEN - L2	0.00		0.00	0.00	0.04	0.00	0.00	0.00		0.02		0.00	0.02	0.02	0.03		0.01	0.03	0.01	0.04	0.02	0.03	0.07	0.04	0.04	0.10	0.06
3	*C. a. antarcticus* SSI - L3	0.00	0.00		0.00	0.04	0.00	0.00	0.00		0.02		0.00	0.02	0.02	0.03		0.01	0.03	0.01	0.04	0.02	0.03	0.07	0.04	0.04	0.10	0.06
4	*C. a. 'complex'* AUS					0.04	0.00	0.00	0.00		0.02		0.00	0.02	0.02	0.03		0.01	0.03	0.01	0.04	0.02	0.03	0.07	0.04	0.04	0.10	0.06
5	*C. a. 'complex'* CHI	0.02	0.02	0.02			0.04	0.04	0.04		0.05		0.04	0.05	0.05	0.06		0.03	0.05	0.03	0.08	0.04	0.05	0.11	0.07	0.07	0.12	0.09
6	*C. a. 'complex'* HEA	0.01	0.01	0.01		0.02		0.00	0.00		0.02		0.00	0.02	0.02	0.03		0.01	0.03	0.01	0.04	0.02	0.03	0.07	0.04	0.04	0.10	0.06
7	*C. a. 'complex'* MAQ - L1	0.01	0.01	0.01		0.01	0.01		0.00		0.02		0.00	0.02	0.02	0.03		0.01	0.03	0.01	0.05	0.02	0.03	0.07	0.04	0.05	0.11	0.07
8	*C. a. 'complex'* MAQ - L2	0.06	0.06	0.06		0.04	0.05	0.05			0.02		0.00	0.02	0.02	0.03		0.01	0.03	0.01	0.05	0.02	0.03	0.07	0.04	0.05	0.11	0.07
9	*C. a. 'complex'* NZ	0.03	0.03	0.03		0.02	0.03	0.02	0.07																			
10	*C. a. 'complex'* TAS	0.01	0.01	0.01		0.00	0.02	0.01	0.04	0.02			0.02	0.02	0.02	0.04		0.03	0.04	0.02	0.05	0.03	0.03	0.08	0.04	0.05	0.10	0.07
11	*C. a. maximus* KER - L1	0.00	0.00	0.00		0.02	0.01	0.01	0.06	0.03	0.01																	
12	*C. a. maximus* MAQ - L2	0.00	0.00	0.00		0.02	0.01	0.01	0.06	0.03	0.01	0.00		0.02	0.02	0.03		0.01	0.03	0.01	0.05	0.02	0.03	0.07	0.04	0.05	0.11	0.07
13	*C. a. reagens* CRO	0.03	0.03	0.03		0.02	0.03	0.02	0.07	0.02	0.02	0.03	0.03		0.00	0.03		0.02	0.03	0.02	0.05	0.02	0.03	0.08	0.04	0.05	0.11	0.07
14	*C. a. travei* MAR	0.02	0.02	0.02		0.02	0.03	0.01	0.06	0.02	0.02	0.02	0.02	0.00		0.03		0.02	0.03	0.02	0.05	0.02	0.03	0.08	0.04	0.05	0.11	0.07
15	*C. caecus* MAR - L1	0.03	0.03	0.03		0.02	0.03	0.03	0.06	0.04	0.02	0.03	0.03	0.04	0.04			0.03	0.05	0.03	0.03	0.04	0.03	0.06	0.03	0.05	0.10	0.06
16	*C. caecus* SSI - L2	0.01	0.01	0.01		0.01	0.01	0.02	0.05	0.03	0.01	0.01	0.01	0.03	0.03	0.01												
17	*C. cisantarcticus* CHA	0.02	0.02	0.02		0.01	0.03	0.01	0.05	0.03	0.01	0.02	0.02	0.02	0.02	0.03	0.02		0.02	0.01	0.05	0.01	0.03	0.08	0.04	0.05	0.11	0.07
18	*C. dubius* MAR	0.04	0.04	0.04		0.02	0.04	0.04	0.05	0.04	0.02	0.04	0.04	0.04	0.04	0.04	0.03	0.03		0.03	0.06	0.03	0.05	0.09	0.05	0.06	0.12	0.08
19	*C. sverdrupi* SVE	0.03	0.03	0.03		0.04	0.04	0.03	0.08	0.04	0.04	0.03	0.03	0.04	0.04	0.05	0.04	0.03	0.05		0.04	0.02	0.02	0.07	0.03	0.04	0.10	0.06
20	*C. tricuspis* MAR	0.02	0.02	0.02		0.01	0.01	0.02	0.05	0.04	0.01	0.02	0.02	0.03	0.03	0.02	0.01	0.01	0.03	0.04		0.05	0.04	0.06	0.02	0.05	0.09	0.06
21	*G. terranova* TER	0.01	0.01	0.01		0.01	0.01	0.01	0.05	0.02	0.01	0.01	0.01	0.01	0.01	0.03	0.02	0.01	0.03	0.02	0.02		0.03	0.08	0.04	0.05	0.11	0.07
22	*N. nivicolus* GRA	0.01	0.01	0.01		0.01	0.01	0.01	0.05	0.02	0.01	0.01	0.01	0.01	0.01	0.03	0.02	0.01	0.03	0.02	0.02	0.00		0.08	0.03	0.05	0.10	0.06
23	*D. klovstadi* CHA	0.04	0.04	0.04		0.03	0.04	0.04	0.05	0.05	0.03	0.04	0.04	0.05	0.05	0.04	0.03	0.04	0.05	0.06	0.03	0.04	0.04		0.04	0.07	0.09	0.07
24	*I. marionensis* MAR	0.02	0.02	0.02		0.02	0.02	0.03	0.04	0.04	0.02	0.02	0.02	0.04	0.04	0.03	0.02	0.03	0.03	0.05	0.01	0.02	0.02	0.03		0.04	0.08	0.05
25	*I. notabilis* MAR	0.03	0.03	0.03		0.01	0.02	0.03	0.05	0.03	0.01	0.03	0.03	0.03	0.03	0.03	0.01	0.02	0.03	0.04	0.02	0.02	0.02	0.02	0.02		0.09	0.06
26	*I. palustris* MAR	0.04	0.04	0.04		0.03	0.03	0.04	0.05	0.05	0.03	0.04	0.04	0.04	0.04	0.04	0.03	0.03	0.04	0.06	0.03	0.04	0.04	0.03	0.02	0.03		0.09
27	Outgroup (mean)	0.03	0.03	0.03		0.02	0.03	0.03	0.05	0.05	0.02	0.03	0.03	0.04	0.04	0.03	0.02	0.03	0.04	0.05	0.02	0.03	0.03	0.03	0.06	0.05	0.03	

CHAPTER EIGHT:

THESIS CONCLUSION

8.1 FUTURE RESEARCH

At the onset of this work, knowledge surrounding both the ecophysiology and evolutionary history of springtails of the Antarctic was scarce, in reality largely being limited for the former to a single maritime Antarctic species (Chown & Convey 2007) and for the latter to a specific region – Victoria Land (Frati et al. 2001; Fanciulli et al. 2001; Stevens et al. 2007; McGaughran et al. 2008).

The studies described in this thesis have gone some way toward addressing this; in particular, adding molecular genetic data on springtails from the Antarctic Peninsula (Chapter Six), considerably developing understanding of evolution of a springtail in southern Victoria Land (Chapters Two and Three), and greatly expanding knowledge of the evolutionary history of the genus *Cryptopygus* throughout Antarctica and its surrounding islands (Chapter Seven). In addition, knowledge of springtail evolution in the sub-Antarctic across both molecular genetic and physiological scales has been enhanced (Chapters Four and Five). Nevertheless, it is already clear that these studies are merely a catalyst and much work remains necessary to complement and enhance what is presented here.

From a molecular genetic perspective, the practical logistics associated with working on Marion Island highlighted the need for an informative guide to the springtails at this location. In particular, the ongoing research into invertebrates of this region (e.g. Chown & Crafford 1992; Chown 1993; Chown & Klok 2003; Klok & Chown 2003, 2005; McGeogh et al. 2006; Mortimer & Jansen van Vuuren 2007) would greatly benefit from this. Thus, during the 2007 season, representative collections of all springtail species on the island were made, and these have since been analysed genetically for mtDNA *cox1* and rDNA 28S genes. Analysis of this work will employ a combination of taxonomic and phylogenetic approaches such that users of the guide will be able to identify species based on both morphological and molecular genetic characteristics. It is hoped that this work will provide the impetus for further whole-island studies such that a catalogue of springtail species throughout the peri-Antarctic region may be assembled.

In addition to the future molecular genetic analyses planned above, the next bold move forward for this field should entail further use of molecular approaches (e.g. Rogers 2007) such as the new high throughput sequence technology becoming available. Focus should extend to generation of complete mitochondrial genomes for Antarctic species (e.g. as achieved recently for *C. a. antarcticus*; Carapelli et al. 2008, and *Friesea grisea*; Torricelli et al. submitted manuscript) to enable reconstruction of phylogenetic relationships at a variety of taxonomic levels and also examination of evolutionary rate variation across genes and species. This would help to further tease out the intricacies of the evolutionary process, from origins to subsequent adaptation, interaction and evolution of Antarctic species.

If there is a demonstrable need for holistic molecular genetic guides to the Antarctic springtails, then such a need also exists for comprehensive contemporary physiological guides to these taxa. For example, Chapter Two provides the first information about metabolism in a continental Antarctic springtail, and the first such data on any Antarctic springtail since the 1970s (Sinclair et al. 2006). Further, the approaches to studying temporal and spatial metabolic variation applied in Chapters Two and Three are relatively novel, and such in-depth analyses of physiological profiles in springtails are rare (e.g. Kauri et al. 1975; Testerink 1983; van der Woude & Joosse 1988).

During the work summarised in Chapter Three, several additional experiments were performed at Cape Bird on *G. hodgsoni* in order to further examine physiological tolerance of this species. One noteworthy finding of these experiments is that, contrary to the commonly held belief that springtails cannot survive air dispersal due to the threat of desiccation, *G. hodgsoni* can disperse at least short distances via wind, as has been shown recently for the maritime Antarctic springtail *C. a. antarcticus* (Hawes et al. 2007). Upon further analysis, this work will provide a physiological summary of *G. hodsgoni*, covering rafting ability, desiccation tolerance and air dispersal to complement the existing published metabolic summaries (e.g. McGaughran et al. 2009a, submitted manuscript (i.e. Chapter Three)). In addition to perhaps providing the impetus for publication of similar work on Antarctic species, this work will add to our knowledge of dispersal abilities and colonisation processes of Antarctic taxa.

In Chapter Three, samples from Garwood valley were analysed to examine spatial variability of metabolic rates of *G. hodgsoni* in more climatically extreme locations. Completing thesis-based ideas for future research, this preliminary work was enhanced during the period 24 December 2008 – 19 January 2009 in a final Antarctic field season at Garwood Valley to provide further information on how energy is partitioned at a location with a shorter active season and colder temperatures. Specifically, the work on temporal variation in metabolic rates at Cape Bird (Chapter Two) was replicated at Garwood Valley to further examine spatial effects on temporal variability.

Data from this visit are still being collated and statistically analysed. However, early results show that, across 'measurement periods' metabolic rates at Garwood Valley were very low in the first measurement period, but got progressively higher, reaching a peak during the third measurement period before dropping off again at the end of the season. Rates at Garwood Valley were lower than those measured at Cape Bird in the early part of the season, but became more comparable as the season progressed. This result is particularly encouraging, in that it confirms the findings in Chapters Two and Three of very low early-season metabolic rates, thus strengthening support for systematic underlying seasonal variation in metabolic rate for this species, as has been widely found in the Antarctic marine environment (Clarke 1991a,b, 1993, 1998; Peck et al. 2006). The next step in this project will involve examination of the microclimate data at Garwood Valley to determine the role of environmental cues (or a lack thereof) in defining the patterns identified to date.

In addition to the future work outlined above, the next step for this project should involve the gathering of additional spatial information. In particular, investigation of metabolic rates of *G. hodgsoni* from more northern Dry Valleys and/or Granite Harbour would be beneficial, as these regions are postulated to have served as refugia in historical times (e.g. McGaughran et al. 2009a,b) and so may harbour a greater degree of physiological diversity than the regions studied to date.

8.2 THESIS SUMMARY

Studies examining between-population variation are important for advancing understanding of how differences in various attributes evolve and may be affected by extrinsic factors (Lardies et al. 2004). In the Antarctic terrestrial environment, seasonal environmental variability (unpredictability) over various time-scales is likely to have played an important role in influencing the evolution of characteristic life history strategies employed by resident species (Convey 1996; Vernon et al. 1998). This thesis has examined the adaptive strategies of Antarctic terrestrial taxa by investigating the effects of both the immediate contemporary environment and the environmental conditions that have factored over historical time-scales, on the physiological responses and genetic structuring of individuals/populations.

Variation in physiological traits is often a result of extrinsic forces; however the ways in which this variation is partitioned are often poorly understood (Chown et al. 1999). Chapter Two of this thesis addressed this by examining metabolic responses of the springtail *Gomphiocephalus hodgsoni* to the unpredictable Antarctic environment. The key finding of this work was the ability of *G. hodgsoni* to vary metabolic rate systematically with progression through the austral summer. Intra-seasonal and temperature-independent variation in mass-specific metabolic rates clearly linked metabolic rate elevation with biological function in this species. In addition response to temperature at relatively short time-scales was shown to be an important part of the life history strategy of *G. hodgsoni*, which appears capable of holding up a physiological and behavioural 'mirror' to its surroundings and reflecting appropriate responses to the local unpredictable Antarctic habitat.

Chapter Three extended the work of Chapter Two by incorporating an annual element to the investigation of temporal variation in individual metabolism of *G. hodgsoni*, in conjunction with a spatial element to detect physiological variation as it may relate to geographically-influenced environmental variation. This study found significant differences between metabolic rates across two years of measurement at Cape Bird, which were potentially attributed to a different microhabitat temperature profile between seasons in addition to an underlying systematic pattern of seasonal

variation in rate. Spatial analysis revealed higher metabolic rates in *G. hodgsoni* populations from sites further inland (where environmental conditions are more 'extreme'). Potential explanations for this latter finding include (but are not limited to) body mass and molecular genetic differences among locations, and local acclimation of *G. hodgsoni* populations in the form of metabolic cold adaptation (MCA).

Through the investigations in Chapters Two and Three, it is clear that the life history strategy of Antarctic terrestrial arthropods such as *G. hodgsoni* is governed by a matrix of factors (both intrinsic and extrinsic). Over temporal scales, both seasonal variation and local microhabitat temperatures and relative humidity clearly have strong influences on metabolic rate variation, including setting restrictive limits on activity early in the season. From a spatial perspective, metabolic rate variation may be caused ultimately by differences among populations in energy assimilation and expenditure (i.e. MCA) in response to climatic and habitat differences and/or a combination of these and other factors including genetic variability and its relationship to fitness. Functional consequences of the temperature-independent component of metabolic rate variation identified in Chapters Two and Three may be particularly important given the nature of current climate change predictions (e.g. warming of up to 5°C in the maritime Antarctic and Peninsula regions; King & Harangozo 1998). For example, it is interesting to speculate on how contemporary springtail populations may respond to warming predictions – including whether the degree of temperature independence highlighted here is strong enough to buffer populations from warming in general, and how populations may be affected by an absence or mis-timing of early season temperature cues.

The inextricably linked nature of physiological and molecular genetic parameters of individuals and populations (e.g. Martin & Palumbi 1993; Gillooly et al. 2001), that was identified in Chapters Two and Three was investigated explicitly in Chapters Four and Five. Indeed, an underlying hypothesis of these chapters was that, if genetic differentiation could be demonstrated between populations of the same species, physiological differentiation would also be predicted. In Chapter Four, measurement of metabolic rates of the springtail *Cryptopygus antarcticus travei* from six geographically distinct populations on sub-Antarctic Marion Island were supplemented with mtDNA

(*cox1*) haplotype analysis to examine both physiological and genetic population divergence. Through this novel approach, Chapter Four showed that physiological distinctions among populations may be phylogeographically correlated in addition to possibly paralleling local differences in environmental selection pressures. Thus, as physiological and genetic parameters are linked, so too are historical and contemporary factors importantly entwined in defining current patterns among populations.

In Chapter Five, the relationship between metabolic rate and mtDNA (*cox1*) mutation rate in *C. a. travei* demonstrated in Chapter Four was investigated in greater detail. The 'metabolic rate hypothesis' of nucleotide mutation was tested in a novel way using data on metabolic rates and root-to-tip distances on an mtDNA tree. This correlation analysis found evidence for an indirect relationship between these two variables, in particular showing that metabolic rates in *C. a. travei* populations are related to the underlying phylogeny (mtDNA tree) of this species. Thus, the study provided some support for a relationship between metabolic rate and the rate of (mtDNA) mutation. It is hoped that further investigation of this question based on the unique intra-specific approach and novel application of methodology demonstrated in Chapter Five, using larger datasets with greater statistical power and genetic variability, will further tease out the intricacies of this issue.

Chapters Six and Seven focused on molecular genetic variation. Patterns of variation among populations are brought about by historical and contemporary processes and study of these patterns provides important insights into the roles of geographic and ecological processes in species divergence and subsequent biogeographic structure (Bermingham & Moritz 1998, Turner et al. 2000). Chapter Six used molecular genetic data to examine phylogeographic hypotheses in the springtails *G. hodgsoni* and *C. a. antarcticus* from continental Antarctica and the Antarctic Peninsula, respectively. In both species, the phylogeographic patterns found were consistent with limited dispersal pathways, intra-regional (re-)colonisation and glacial cycling during the Pleistocene. In particular, both species showed strong genetic architecture, and refugia during glacial episodes have played an important role in population persistence within their respective regions over time. Expansion from these refugia during inter-glacial periods has led to comparable patterns of demographic

growth following colonisation to peripheral populations in each species. However, the analytical approaches employed revealed that colonisation in the Peninsula species occurred earlier in the Pleistocene and subsequent divergence in isolation has left only faint ancestral traces in these populations. The ancestral genetic heritage remains more obvious in the continental species which may have a more measured life pace.

Phylogenetic data was employed in Chapter Seven to examine relationships among the springtail genus *Cryptopygus* and taxonomically related species across their distribution in the continental, maritime and sub-Antarctic. Examination of multiple genetic loci indicated that both dispersal and refugial-based vicariant processes have been important in shaping the contemporary distribution of this particular suite of species. In particular, the origins of Antarctic *Cryptopygus* are proposed to have involved a disjunct (distribution) ancestral species and, through time, colonisation events with dispersal often following circum-polar currents (e.g. the West Wind Drift; Williams et al. 2003) have been important. Thus, the evolutionary history of these Antarctic springtails reflects a varied and diverse origin, and contemporary phylogenetic relationships identified here call for a revision of current 'species' designations.

Collectively, the results of this thesis have highlighted the distinctiveness (both physiological and genetic) of individuals and populations of springtail species over several spatial scales in Antarctica. Given that individual elements within Antarctic ecosystems are so clearly unique, it is important that steps are taken now in response to both climate warming predictions (see above) and the ever-present possibility of human-mediated dispersal events if we are to maintain these exclusive components of Antarctica. Chapter Six concluded with a recommendation for additional assessment of the ways in which differences between regions may have influenced distinct evolutionary histories in local taxa. At the heart of this recommendation is a recognition of the need to understand variation among populations if we are to understand how differences among individuals have evolved; indeed, if we are to understand *evolution*. As stated at the outset (Section 1.1) of this thesis, our understanding of diversity relates to the history of life upon Earth, expressed through change of form (i.e. variation) in time and space. In staying loyal to the concept that variation in genetic and physiological features over temporal and spatial scales forms

the substance of evolution, this thesis has come full circle. In doing so, the multi-faceted methodologies applied throughout this work have advanced understanding of the current picture of evolution in polar environments as exemplified by Antarctic springtails.

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THESIS APPENDICES

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Patterns of population genetic structure for springtails and mites in southern Victoria Land, Antarctica

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Abstract

We sequenced the mitochondrial (mt) DNA cytochrome *c* oxidase subunit I gene to examine comparative phylogeographic patterns for the springtail *Gomphiocephalus hodgsoni* and the mite *Stereotydeus mollis* throughout their ranges in southern Victoria Land, Antarctica. Our aim was to extend previous genetic work to encompass a large ice-free area in the Dry Valleys. In particular, we sought to determine if this new region harboured high levels of genetic diversity and if patterns of genetic structure were congruent across taxa. Phylogenetic and nested clade analyses for *G. hodgsoni* and *S. mollis* showed similar patterns of population sub-structuring among locations and highlighted several potential refugia that may have existed during glacial maxima. We identified greater levels of genetic divergence in *S. mollis* and suggest that there is a nucleotide substitution (mutation) rate difference between *S. mollis* and *G. hodgsoni*, and/or that *S. mollis* has had a longer association with the Antarctic landscape.

Keywords: Glacial refugia; Mitochondrial DNA; *Stereotydeus mollis*; *Gomphiocephalus hodgsoni*; Phylogeography

1. Introduction

There are several processes that may influence contemporary patterns of population genetic structure (e.g. gene flow, genetic drift, fragmentation). In particular, repeated glacial cycles are likely to have had a marked influence on contemporary species' ranges and population sizes through their effect on habitat and/or refugial availability (e.g. Knowles, 2001; Rowe et al., 2004). Such effects are likely to be particularly strong in Antarctica, where the local fauna has been exposed to a climate of increasing severity (including more than ten glacial cycles over the last million years (Hays et al., 1976)) since its isolation in the southern ocean following the break-up of Gondwana. The continent has been covered in a permanent ice sheet for ~34 My, and the variable extent of this sheet over time has likely resulted in repeated shifts of species distributions in both the land and marine realms of the Antarctic (Rogers, 2006).

It is well documented that several Antarctic locations have remained ice-free throughout the Last Glacial Maximum (e.g. coastal areas, Burgess et al., 1994; Gore et al., 2001; Hodgson et al., 2001; lakes, Cromer et al., 2006). In addition, continental regions including Dronning Maud Land (Marshall and Pugh, 1996), the Prince Charles Mountains (Fink et al., 2000), the Antarctic Peninsula (Pugh and Convey, 2000), southern Victoria Land (Stevens and Hogg, 2003, 2006) and the Ellsworth Mountains (Convey and McInnes, 2005) have had ice-free areas for millions of years which are likely to have enabled long-term survival of terrestrial taxa in refugia (Cromer et al., 2006; Convey

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and Stevens, 2007). Consequently, refugia are likely to have served as centres for preservation of biodiversity in fluctuating environments (Garrick et al., 2007).

Populations that become restricted to refugia may undergo divergence in isolation (e.g. through changes in population size, founder events, genetic drift), especially when numbers of individuals within sub-populations are small. Population differentiation and potentially speciation can result (Vandergast et al., 2007), and founder events may also increase genetic differences between populations (Rogers, 2006). In the Antarctic, high levels of genetic structure have been found for several terrestrial taxa on relatively short spatial scales (e.g. Marshall and Coetzee, 2000; Fanciulli et al., 2001; Frati et al., 2001; Stevens and Hogg, 2003, 2006; Nolan et al., 2006). In particular, Stevens and Hogg (2003) found 14 mtDNA (COI) haplotypes from 45 springtail (*Gomphiocephalus hodgsoni*) individuals from throughout southern Victoria Land in a pattern consistent with divergence of allopatric populations located in refugia. Across the same region, 18 haplotypes were identified from 32 mite (*Stereotydeus mollis*) individuals (Stevens and Hogg, 2006). In northern Victoria Land, Stevens et al. (2007), found 26 mtDNA (COII) haplotypes (of which only a single haplotype was found in more than one population) from 69 individuals for the springtail *Desoria klovstadi*, while Fanciulli et al. (2001) also showed a pattern of high genetic differentiation for the springtail *Gressittacantha terranova*. Notably, the genetic analysis of *G. terranova* showed three genetically distinct groups, with very little gene flow between them, and isolation thought to be caused by major glacial systems (Fanciulli et al., 2001).

Such genetic structuring is common for terrestrial invertebrates in the sub-Antarctic (e.g. Grobler et al., 2006; Mortimer and Jansen van Vuuren, 2007; Stevens et al., 2006; Myburgh et al., 2007), and elsewhere (e.g. Paulay, 1985; Gillespie and Roderick, 2002; Gillespie, 2004; Garrick et al., 2007), since many species have limited dispersal ability, which affects gene flow (Peterson and Denno, 1998). In addition, these organisms often have specific habitat requirements, such that small patches of suitable habitat are often separated by large areas that are uninhabitable. Under these circumstances, the effects of glaciations are likely to be important (see Rogers, 2006, and references therein). However, unlike many non-Antarctic regions where post-glacial population expansion from refugia has been a rapid and extensive process (Rogers, 2006), the Antarctic environment continues to limit habitat availability and the signature of glacial impacts on population structure of Antarctic terrestrial biota is still legible. Therefore, the fragmented Antarctic terrestrial landscape provides an ideal opportunity to examine patterns of species distribution and genetic population structure, and to test hypotheses related to the evolutionary history of taxa relative to environmental change brought about by glacial cycling.

Arthropod diversity is limited in southern Victoria Land and consists of three species of springtail and four species of mite (Strandtmann, 1967; Wise, 1971; see also Adams

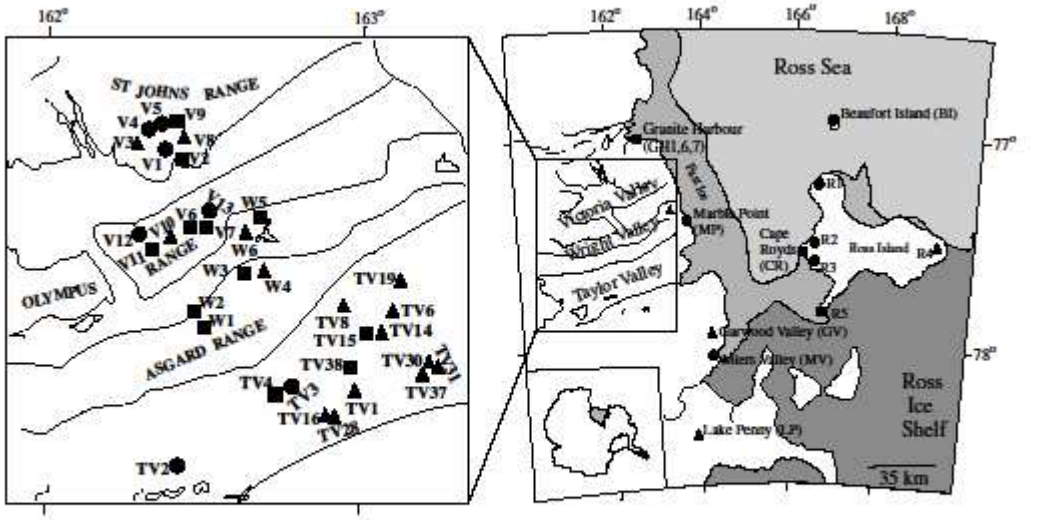

Fig. 1. Sampling locations for *Gomphiocephalus hodgsoni* and *Stereotydeus mollis* in southern Victoria Land, Antarctica. Inset: distribution of sampling sites for *G. hodgsoni* and *S. mollis* within Victoria, Wright and Taylor Valleys. Codes correspond to those given in Table 1. Solid squares correspond to locations where only *S. mollis* was found; solid triangles to locations where only *G. hodgsoni* was found; solid circles to locations where both species were found.

et al., 2006; Sinclair and Stevens, 2006). We selected *Gomphiocephalus hodgsoni* Carpenter, 1908 (Collembola: Hypogastruridae) and *Stereotydeus mollis* Womersley and Strandtmann, 1963 (Acari: Penthalodidae) for a comparative study because they are the most numerically dominant and widespread of the seven species, are endemic, and are often found together across their distributional ranges. Such comparative approaches are particularly useful for phylogeographic studies as they may help to identify common factors that have influenced population genetic structure (e.g. Bohonak, 1999).

This study extends earlier work on *G. hodgsoni* and *S. mollis* from southern Victoria Land (Stevens and Hogg, 2003, 2006; Nolan et al., 2006), the sampling for which omitted a large ice-free area in the McMurdo Dry Valleys. This unstudied area may be important from a genetic perspective since it lies close to Taylor Valley, which has been suggested as an important refugial location for extant populations in southern Victoria Land (Stevens and Hogg, 2003, 2006). Here we targeted this extensive ice-free area to incorporate individuals from Wright and Victoria Valleys (Fig. 1) into the existing dataset. Phylogenetic and phylogeographic analyses were used to identify patterns of haplotype diversity and distribution, and to test the hypothesis that these valleys would harbour high levels of genetic diversity and thus form part of a greater Dry Valley refugium during glacial maxima. We predicted that genetic linkages (represented by haplotype sharing) would exist between Dry Valley and geographically distant populations, with additional unique haplotypes at distinct locations resulting from divergence in isolation since colonisation. Furthermore, we tested the hypothesis that both taxa would display similar patterns of genetic structuring owing to a shared phylogeographic history.

2. Materials and methods

2.1. Study area and collection of taxa

The McMurdo Dry Valleys consist of some 4000 km^2 of high relief ice-free mountain polar desert topography and, with a mean annual temperature of −20 °C and a precipitation level of ~80 mm water equivalent, the area is described as a hyper-arid cold polar desert (Marchant and Denton, 1996). Taylor, Wright and Victoria Valleys make up the largest ice-free area in the region and these are separated by the Asgard and Olympus Ranges (Fig. 1). The mountains are higher further inland (>2000 m), and become progressively lower towards coastal regions (Marchant and Denton, 1996).

Gomphiocephalus hodgsoni and *S. mollis* are generally restricted to areas of high soil moisture and/or access to water (e.g. lake edges, snow patch edges, moist stream beds) and were collected from the underside of stones using an aspirator. Sampling targeted more than 40 sites across ten geographic locations. During January 2004, Wright and Victoria Valleys were sampled, and 16 sites contained one or both species (4 for *G. hodgsoni*; 8 for *S. mollis*, 4 for both species) (Fig. 1 and Table 1). Further samples from 28 sites (16 for *G. hodgsoni*; 4 for *S. mollis*; 8 for both species) and eight additional locations were included from previous studies, thus extending the sample size to encompass the distributional range for both species (Fig. 1). Upon collection, *G. hodgsoni* and *S. mollis* specimens were stored in 95% ethanol and identified using the original and/or relevant identification keys (e.g. Strandtmann, 1967; Wise, 1971).

2.2. DNA extraction, amplification and sequencing

Total DNA was extracted from one to three specimens from each site (Table 1), following the DNeasy tissue extraction kit (Qiagen, Hilden, Germany). Upon extraction, a fragment of the mitochondrial cytochrome *c* oxidase (COI) gene was amplified using the universal primers LCO1490 (5′-ggt caa caa atc ata aag ata ttg ga-3′) and HCO2198 (5′-taa act tca ggg tga cca aaa aat ca-3′) (Folmer et al., 1994) for *G. hodgsoni*, and COI-2R (5′-ggr tar tcw gar taw cgt ncg wgg tat-3′) and COI-2F (5′-tty gay cci dyi ggr gga gga gat cc-3′) for *S. mollis* (Otto and Wilson, 2001). Amplifications for each *G. hodgsoni* and *S. mollis* specimen used a 25 µl reaction volume containing 3 µl of the extracted DNA (unquantified), 1× PCR buffer (Roche, Penzberg, Germany) 2.2 mM $MgCl_2$, 0.2 mM of each dNTP (Boehringer-Mannheim, Mannheim, Germany), 1.0 µM of each primer, and 0.5 U of *Taq* DNA polymerase (Roche). The thermal cycling conditions for *G. hodgsoni* were: 94 °C for 1 min followed by five cycles of denaturation and polymerase amplification (94 °C for 1 min, 45 °C for 1.5 min, 1 min at 72 °C followed by 35 cycles of 94 °C for 1 min, 51 °C for 1.5 min and 1 min at 72 °C, followed by 5 min at 72 °C; and for *S. mollis* were: initial denaturation at 94 °C for 1.5 min followed by 40 cycles of denaturation and polymerase amplification (94 °C for 20 s, 55 °C for 30 s and then 1.5 min at 72 °C), followed by 5 min at 68 °C.

All reaction products were purified using the QIAquick PCR Purification Kit (Qiagen) or using SAP/EXO (USB Corp., Cleveland, OH, USA). Sequencing used forward and reverse primers, and was performed directly either on a MegaBACE DNA Analysis System (Amersham Biosciences, Buckinghamshire, England) at the University of Waikato DNA sequencing facility, or on a capillary ABI3730 genetic analyser (Applied Biosystems Inc., Foster City, CA) at the Allan Wilson Centre Genome Service, Massey University.

2.3. Phylogenetic analyses

Unique mtDNA sequences were lodged with GenBank (Accession Nos.: DQ305356–DQ305360 for *G. hodgsoni* and DQ305361–DQ305367; DQ305369–DQ305384 for *S. mollis*). Previous samples already lodged with GenBank included AY294564–AY294566; AY294571–AY294573;

Table 1
Sampling locations and site codes for *Gomphiocephalus hodgsoni* and *Stereotydeus mollis* throughout southern Victoria Land, Antarctica

Location	Lat (S)	Long (E)	Species	Site
Wright Valley	77°30.52′	162°10.13′	*S. mollis*	W1
	77°31.09′	162°11.26′	*S. mollis*	W2
	77°27.50′	162°33.38′	*S. mollis*	W3
	77°26.44′	162°42.52′	*G. hodgsoni*	W4
	77°26.50′	162°35.44′	*S. mollis*	W5
	77°27.44′	162°36.40′	*G. hodgsoni*	W6
Victoria Valley	77°19.31′	161°53.54′	Both species	V1
	77°21.43′	162°06.06′	*S. mollis*	V2
	77°19.31′	161°52.17′	*G. hodgsoni*	V3
	77°25.15′	161°48.33′	Both species	V4
	77°33.15′	161°48.20′	*S. mollis*	V6
	77°42.05′	161°57.23′	*S. mollis*	V7
	77°31.03′	161°50.28′	*G. hodgsoni*	V8
	77°31.05′	161°50.46′	*S. mollis*	V9
	77°41.55′	161°57.20′	Both species	V10
	77°34.05′	162°04.50′	Both species	V13
Taylor Valley	77°39.44′	163°05.50′	*G. hodgsoni*	TV1
	77°45.50′	162°02.14′	Both species	TV2
	77°38.34′	162°46.30′	Both species	TV3
	77°38.32′	162°46.28′	*S. mollis*	TV4
	77°36.45′	163°24.08′	*G. hodgsoni*	TV6
	77°34.08′	163°09.21′	*G. hodgsoni*	TV8
	77°38.29′	163°17.41′	*G. hodgsoni*	TV14
	77°38.30′	163°17.50′	*S. mollis*	TV15
	77°41.12′	162°52.20′	*G. hodgsoni*	TV16
	77°33.05′	163°20.28′	*G. hodgsoni*	TV19
	77°41.13′	162°57.00′	*G. hodgsoni*	TV28
	77°36.26′	163°30.03′	*G. hodgsoni*	TV30
	77°36.46′	163°31.37′	*G. hodgsoni*	TV31
	77°37.16′	163°29.21′	*G. hodgsoni*	TV37
	77°37.26′	163°06.34′	*G. hodgsoni*	TV38
Garwood Valley	78°01.11′	164°03.24′	*G. hodgsoni*	GV
Miers Valley	78°05.46′	163°45.32′	Both species	MV
Granite Harbour	77°00.52′	162°36.05′	*G. hodgsoni*	GH1
	77°01.52′	162°30.05′	*G. hodgsoni*	GH6
	77°02.18′	162°28.11′	*S. mollis*	GH7
Marble Point	77°26.07′	163°49.34′	Both species	MP
Lake Penny	78°18.36′	163°24.28′	*G. hodgsoni*	LP
Ross Island	77°13.16′	166°26.49′	Both species	R1
	77°32.46′	166°09.47′	Both species	R2
	77°38.02′	166°26.33′	Both species	R3
	77°27.48′	169°11.49′	*G. hodgsoni*	R4
	77°51.10′	166°40.48′	*S. mollis*	R5
Beaufort Island	76°55.54′	166°54.49′	Both species	BI

AY294575; AY294582–AY294585; AY294591; AY294603–AY294604 for *G. hodgsoni* (Stevens and Hogg, 2003) and DQ305368; DQ305385–DQ305398 for *S. mollis* (Stevens and Hogg, 2006). Individual sequences were verified as being derived from the relevant taxa using the GenBank BLAST algorithm, checked for open reading frames and then aligned using SEQUENCHER ver. 4.7 (Gene Codes Corp., Michigan) sequence editor. Data were then analysed using PAUP* ver. 4.0b10 (Swofford, 2002) and MrBayes ver. 3 (Ronquist and Huelsenbeck, 2003). Preliminary phylogenetic analyses utilized several prostigmatic mite sequences (GenBank Accession Nos.: AF142132–AF142143; Otto and Wilson, 2001) and hypogastrurid springtail sequences as outgroups (see also Stevens and Hogg, 2006).

χ^2 tests as implemented in PAUP* were used to determine whether the assumption of equal base frequencies among sequences was violated in three subsets of the data: all sites, parsimony-informative sites, and third codon positions only. Modeltest ver. 3.7 (Posada and Crandall, 1998) was used to determine the appropriate substitution model for maximum likelihood (ML) heuristic searches (using all unique sequences) with 500 bootstrap replicates. The model selected for *G. hodgsoni* was TrN+I and for *S. mollis* was TrN+I+Γ (see Supplementary Information online for details); all other options in PAUP* remained as default. Maximum parsimony trees were also generated using the default settings in PAUP*. Bayesian phylogenetic analyses (MrBayes ver. 3.0b4) were used to perform a partitioned-likelihood Bayesian search (substitution model obtained using MrModeltest ver. 2.2 (Nylander, 2004)). Four incrementally heated Metropolis-coupled Markov chain Monte Carlo (MCMCMC) were each run for 10,000,000 generations, sampling trees and parameters every 100 generations, with the first 100,000 generations discarded as burn-in determined from plotting log-likelihood values against generation time in TRACER 1.3 (Rambaut and Drummond, 2007). The consensus (majority-rule) tree was obtained from 180,000 trees sampled after the initial burn-in period, obtained with the sumt command using MrBayes, and visualised using TreeView ver. 1.6.6 (Page, 1996). The Bayesian and MP trees displayed the same tree topologies as the ML, thus ML trees (with Bayesian posterior probabilities) are presented. Distance matrices of pairwise nucleotide sequence divergence were calculated (uncorrected and corrected distances) using all unique sequences (see Appendices; Supplementary Information online). Comparisons of log-likelihood scores (using χ^2 tests) for ML trees with and without a molecular clock enforced (performed for springtail and mite alignments using PAUP*) indicated that these sequences were evolving in a clocklike manner ($P > 0.05$, in both cases). Subsequently, we estimated age among lineages based on an arthropod strict molecular clock conservative calibration of 1.5–2.3% divergence per million years derived from comparisons between geological and molecular data (Brower, 1994; Juan et al., 1996; Quek et al., 2004).

2.4. Population structure analyses

ARLEQUIN ver. 3.01 (Excoffier et al., 2005) was used to explore genetic characteristics and partitioning of nucleotide diversity (presence of population structure) for *G. hodgsoni* and *S. mollis*. We computed: haplotype (h) and nucleotide (π) diversity indices (Nei, 1987) separately for each location; hierarchical analysis of molecular variance (AMOVA; Excoffier et al., 1992) to compare geographic locations, with statistical significance of variance components tested with 16,000 permutations; pairwise differences

(φ-$_{st}$ values) between haplotypes using simple distances; and Tajima's D (Tajima, 1989) and Fu's F_s (Fu, 1997) to test for selective neutrality.

2.5. Nested clade analyses

For both *G. hodgsoni* and *S. mollis*, we estimated a haplotype network using the algorithm of Templeton et al. (1992) in TCS ver. 1.21 (Clement et al., 2000). We used a connection limit of 95% for both species. For *S. mollis* the analysis was unable to connect all groups when this connection limit was used, hence we also performed an analysis where we allowed a maximum number of mutational steps of 60, and used this information only to suggest possible points where the distinct groups may connect. Populations were specified by their GPS coordinates and sample sizes, and then defined into a nested structure including outgroup probabilities (Castelloe and Templeton, 1994), following the nesting rules described in Crandall (1996). Finally, nested geographical distance analyses were performed using GEODIS ver. 2.4 (Posada et al., 2000, 2002) to obtain a measure of how any one particular clade was distributed geographically compared to its closest evolutionary sister clades. Clades with statistically significant values of Dc, Dn or I–T were assessed using Templeton's 2005 inference key (http://darwin.uvigo.es/software/geodis.html), allowing us to deduce which factor(s) (e.g. restricted gene flow, past fragmentation, range expansion), may have caused significant spatial association among haplotypes.

3. Results

3.1. mtDNA sequence variation

We used 471-bp (157 codons) of unambiguous alignment (no insertions or deletions) from a total of 96 *G. hodgsoni* individuals and 504-bp (168 codons) of unambiguous alignment from a total of 61 *S. mollis* individuals for all analyses. Nucleotide composition averaged over all sequences showed an A–T bias of 64.8% (A = 26.9%, T = 37.9%, C = 19.2%, G = 16.0%) for *G. hodgsoni*, and 69.2% (A = 36.0%, T = 33.2%, C = 16.0%, G = 14.8%) for *S. mollis*. Base frequencies were not significantly heterogeneous among sequences for all sites, parsimony-informative sites and third codon sites. There were 20 variable (15 parsimony-informative) nucleotide substitutions for *G. hodgsoni*, and 103 variable (93 parsimony-informative) nucleotide substitutions for *S. mollis* (Appendix 2, Supplementary Information online). The nucleotide substitutions among haplotypes revealed low sequence divergence among individuals of *G. hodgsoni* (up to 2.1%, uncorrected *p*-distance), while divergence among *S. mollis* individuals was considerably higher (up to 14.5%, uncorrected *p*-distance) (Appendix 1, Supplementary Information online).

3.2. Phylogenetic analyses

For *G. hodgsoni* there were 20 unique haplotypes ranging in sequence divergence from 0.2% to 2.1%. The ML tree for *G. hodgsoni* showed three main groups: (1) a disjunct mixture of haplotypes from individuals throughout southern Victoria Land; (2) one individual with haplotype G11 from the Dry Valleys—1.9% divergent; (3) 21 individuals with haplotype G9 from the Dry Valleys—2.1% divergent from all other haplotypes. Within the former group, an additional three sub-groups can be identified: (a) haplotypes from individuals from all locations except Beaufort Island; (b) a Beaufort Island group (G19 and G20); and (c) G8 from the Dry Valleys (Fig. 2a).

Unique haplotypes were present at several locations: Victoria Valley (4), Taylor Valley (4), Beaufort Island (2), Ross Island (3), Lake Penny (1) and Garwood Valley (1) (Fig. 2a). Haplotype sharing was common, with 20 haplotypes shared among 96 *G. hodgsoni* individuals and six (or 30%) of the total haplotypes present in individuals from more than one location. For example, haplotype G7 was present in Victoria Valley (V8), Granite Harbour (GH1, GH6) and Ross Island (R1), and haplotype G3 was present in Victoria Valley (V1, V3, V8, V10), Miers Valley (MV), Taylor Valley (TV1), and Lake Penny (LP) (Fig. 2a).

For *S. mollis* there were 36 unique haplotypes ranging in sequence divergence from 0.2% to 14.5%. As for *G. hodgsoni*, the ML tree showed three main groups: (1) a mixture of haplotypes from individuals throughout southern Victoria Land, but excluding Beaufort Island; (2) a group containing haplotypes present in individuals from Victoria and Wright Valleys and Beaufort Island—12.9% divergent from all other haplotypes; and (3) a Victoria Valley haplotype (S14)—11.1% divergent (Fig. 2b). However, unlike the disjunct mixture of haplotypes within group (1) for *G. hodgsoni*, group (1) for *S. mollis* shows strong genetic structure, with two main groups ((a) all locations except Beaufort Island and Granite Harbour; and (b) multiple Victoria Valley individuals and one Granite Harbour individual) separated by ~9% sequence divergence (Fig. 2b).

Unique haplotypes were found at several locations: Wright Valley (6), Victoria Valley (16), Taylor Valley (3), Miers Valley (2), Ross Island (3), Marble Point (1), Beaufort Island (1), and Granite Harbour (1) (Fig. 2b). Of a total of 36 haplotypes from 61 individuals, only three (or 8.3%) of the total haplotypes were found in more than one location. For example, individuals with haplotype S8 were present in Victoria Valley (V1), Taylor Valley (TV2, TV3, TV15), and Ross Island (R1), while those with haplotype S30 were found in Taylor Valley (TV15) and Ross Island (R1, R2, R3) (Fig. 3a). Therefore the level of haplotype sharing among *S. mollis* individuals was lower than that found for *G. hodgsoni* (8.3% vs. 30%); although the sample size was lower for *S. mollis*.

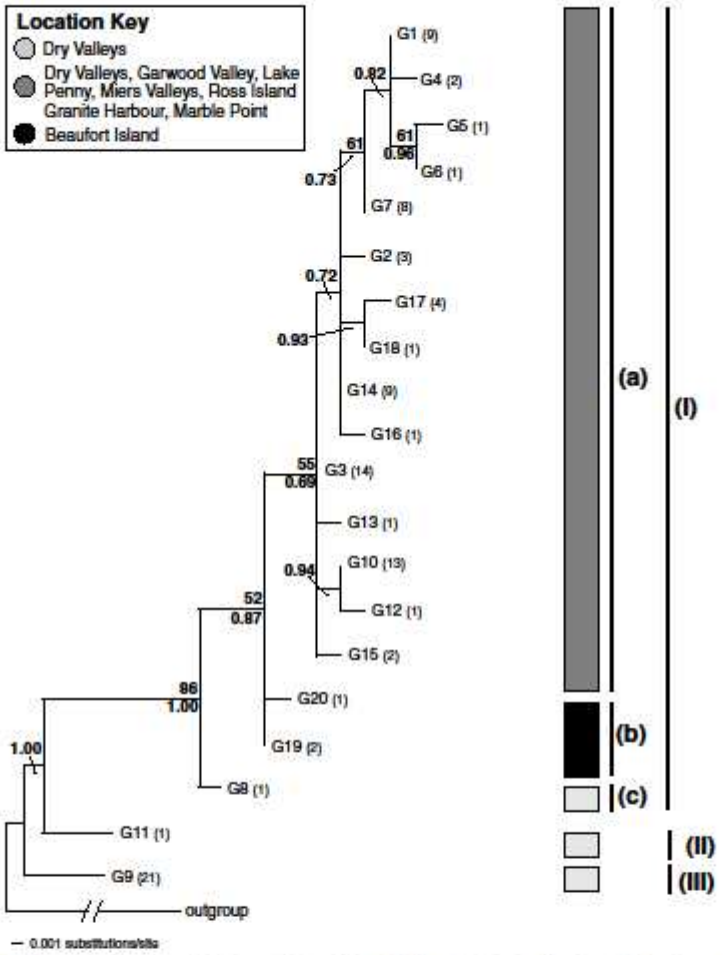

Fig. 2a. Maximum likelihood tree for *Gomphiocephalus hodgsoni* derived from Modeltest (see Section 2) using a 471-bp fragment of the mtDNA (COI) gene and only unique sequences. Bootstrap confidence limits (500 replicates) greater than 50% are shown in bold type above nodes and posterior probabilities greater than 0.55 from the Bayesian analysis are shown below nodes. The three main groupings and sub-groupings referred to in the text are indicted on the right, where coloured bars also indicate geographic locations (see accompanying key); the numbers of identical haplotypes present at any site is given in parentheses.

3.3. Population structure analyses

Haplotype diversity (h) for *G. hodgsoni* ranged from 0.29 to 0.80 (excluding locations where $N < 4$) and was highest in Victoria Valley and Ross Island ($h = 0.80$ and 0.73 for Victoria Valley and Ross Island, respectively). The location with lowest haplotype diversity (for $N > 4$) was Wright Valley, however despite intensive searching, we were only able to find springtails at the lower end of this valley; this lower spatial sampling may account for the finding of lower diversity. Measures of nucleotide diversity (π) were highest in Taylor and Victoria Valleys, as were estimates of effective population size $\theta(S)$ (Table 2). These findings are likely to be affected by sampling bias since locations with higher sample sizes portray higher haplotype and nucleotide diversity statistics. AMOVA analysis revealed a high level of genetic structure, with 45% ($P < 0.001$) of variation apportioned among geographic locations (Table 3). Most φ_{-st} values were large and significant, indicating long-term isolation and low gene flow between locations for *G. hodg-*

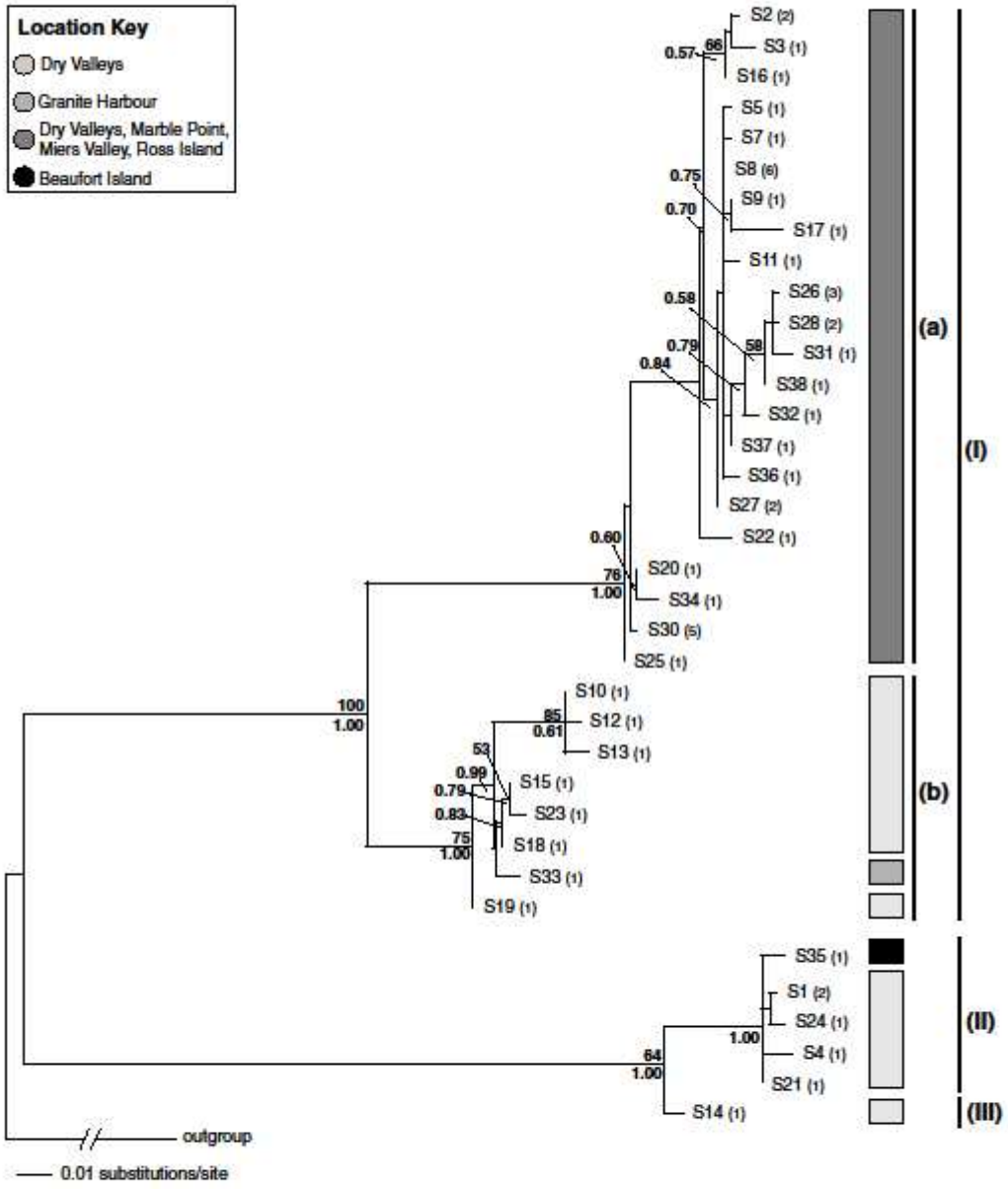

Fig. 2b. Maximum likelihood tree for *Stereotydeus mollis* derived from Modeltest (see Section 2) using a 504-bp fragment of the mitochondrial DNA (COI) gene and only unique sequences. Bootstrap confidence limits (500 replicates) greater than 50% are shown in bold type above nodes and posterior probabilities greater than 0.55 from the Bayesian analysis are shown below nodes. The three main groupings and two sub-groups referred to in the text are indicated on the right, where coloured bars also indicate geographic locations (see accompanying key); the numbers of identical haplotypes present at a site is given in parentheses.

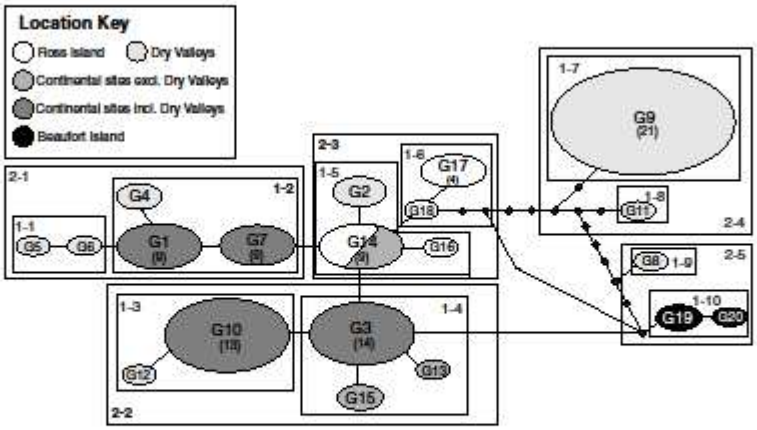

Fig. 3a. Nested clade design for the 20 unique mtDNA (COI) haplotypes from 96 individuals of *Gomphiocephalus hodgsoni*. Geographic locations are indicated by haplotype colour (see accompanying key). Number of individuals with each haplotype is identified by the size of the haplotype when $n < 3$ and by size and in parentheses when $n > 3$. Missing haplotypes/mutational steps are indicated by an '●'. The clades are identified using a two number system, where the first number refers to the nesting hierarchy and the second is an arbitrary, individual clade identifier. Thin-lined polygons enclose 1-step clades; broken lined polygons enclose 2-step clades. Significant clades are indicated by their bold type clade numbers (see Table 4).

Table 2
Population statistics and genetic characteristics of sampled locations for *Gomphiocephalus hodgsoni* and *Stereotydeus mollis*: *h*, haplotype diversity; π, nucleotide diversity, θ (*S*), effective population size (Nei, 1987); all statistics calculated in Arlequin, ver. 3.01 (Excoffier et al., 2005)

Species	Genetic population	No. of individuals	No. of haplotypes	No. of polymorphic sites	*h* (SD)	π (SD)	θ (SD)	Distribution of haplotypes within populations
G. hodgsoni	Victoria Valley	18	8	10	0.797 (0.089)	0.005 (0.003)	2.907 (1.321)	G01–G08
	Wright Valley	7	2	3	0.289 (0.196)	0.002 (0.002)	1.224 (0.836)	G01–G02
	Taylor Valley	37	5	12	0.568 (0.056)	0.009 (0.005)	2.875 (1.154)	G03, G09–12
	Garwood Valley	1	1	0	1.000 (0.000)	0.000 (0.000)	0.000 (0.000)	G13
	Miers Valley	4	1	0	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	G03
	Granite Harbour	6	3	2	0.600 (0.215)	0.001 (0.001)	0.876 (0.684)	G01, G07, G14
	Marble Point	3	1	0	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	G10
	Lake Penny	4	2	1	0.667 (0.204)	0.001 (0.002)	0.545 (0.545)	G03, G15
	Ross Island	17	5	4	0.728 (0.083)	0.003 (0.002)	1.183 (0.691)	G07, G14, G16–18
	Beaufort Island	3	2	1	0.667 (0.314)	0.001 (0.002)	0.667 (0.667)	G19–20
S. mollis	Victoria Valley	22	17	93	0.965 (0.028)	0.065 (0.033)	25.512 (8.725)	S08–24
	Wright Valley	8	6	76	0.929 (0.084)	0.078 (0.043)	29.311 (12.921)	S01–5, S07
	Taylor Valley	11	6	18	0.873 (0.071)	0.013 (0.008)	6.146 (2.749)	S08, S25–28, S30
	Miers Valley	2	2	9	1.000 (0.500)	0.018 (0.019)	9.000 (6.708)	S31–32
	Granite Harbour	1	1	0	1.000 (0.000)	0.000 (0.000)	0.000 (0.000)	S33
	Marble Point	2	2	16	1.000 (0.500)	0.032 (0.033)	16.000 (11.662)	S27, S34
	Ross Island	8	5	15	0.786 (0.151)	0.015 (0.009)	5.785 (2.822)	S08, S30, S36–38
	Beaufort Island	1	1	0	1.000 (0.000)	0.000 (0.000)	0.000 (0.000)	S35

soni. Finally, no departures from neutrality were detected (results for Tajima's *D* and Fu's F_s tests were non-significant and are not shown).

Haplotype diversity (*h*) ranged from 0.79 to 0.97 for *S. mollis* from locations where $N > 4$, thus was higher and less variable for this species compared to *G. hodgsoni*. However, the pattern of high diversity was comparable, with Victoria, Wright and Taylor Valleys and Ross Island showing similarly high estimates of (*h*) (Table 2). Measures of *S. mollis* nucleotide diversity (π) were an order of magnitude higher than that found for *G. hodgsoni* but had the same overall pattern of higher values in selected locations. This is likely to be a sampling artefact, since, for both species the locations with greater sample sizes show higher haplotype and nucleotide diversity values. Estimates of effective population size θ(*S*) also conformed to this pattern (Table 2). Tajima's *D* and Fu's F_s tests detected no departures from neutrality (non-significant) and are not shown. AMOVA analysis revealed some genetic structure, with ~20% ($P = 0.002$) of variation apportioned among loca-

tions (Table 3). A much greater proportion of variation was partitioned within *S. mollis* locations (~80%) than for *G. hodsgoni* (~55%, Table 3), and this is consistent with the low level of haplotype sharing found for this species. Fewer φ_{-st} values were large and significant for *S. mollis* than for *G. hodgsoni*.

3.4. Nested clade analyses

The nested cladogram for the 20 unique *G. hodgsoni* haplotypes (from 96 individuals) consisted of ten 1-step clades and five 2-step clades, and the maximum number of mutational steps between haplotypes was seven (Fig. 3a). Analysis of suitable clades with Templeton's inference key inferred "allopatric fragmentation" for clades 1-2 and 2-2 while "long distance colonisation possibly coupled with subsequent fragmentation or past fragmentation followed by range expansion" was the most likely explanation for the patterns observed at the total cladogram level (see Table 4).

The nested cladogram for the 36 unique *S. mollis* haplotypes (from 61 individuals) consisted of eighteen 1-step clades and six 2-step clades. The number of mutational steps between groups was high, thus we separated the cladogram into three distinct groups and proposed linkages between groups based on a maximum number of 60 mutational steps between groups (Fig. 3b). Insight into processes shaping local population differentiation was gained at the total cladogram level, where "restricted gene flow with isolation by distance" was inferred (Table 4).

Nested cladograms for both taxa show a small number of cases of unresolved relationships (e.g. for *G. hodgsoni*, between clades 2-2, 2-3 and 2-5; for *S. mollis* within clade 1-16) (Figs. 2b and 3b). This can, in some cases, confound attempts to reconstruct accurate evolutionary trees and infer phylogenetic history (Xu, 2000; Posada et al., 2002). Consequently, any inferences made regarding these clades need to be interpreted with caution.

4. Discussion

The mtDNA (COI) gene was relatively homogeneous among *G. hodgsoni* individuals, with only 20 variable nucleotide sites across 471-bp. The most genetically distinct *G. hodgsoni* lineages were closely related (up to 2.1% divergence). In contrast, 103 nucleotide sites were variable and levels of divergence were much higher for *S. mollis* (up to 14.5% for some individuals). Sequence divergence values for *G. hodgsoni* were consistent with intraspecific divergences previously found for springtails (e.g. Soto-Adames, 2002; Stevens and Hogg, 2003; Hogg and Hebert, 2004; Myburgh et al., 2007). However, sequence divergences for mites are variable in the literature. Several authors have found similarly high levels of purportedly intraspecific divergence (e.g. Anderson and Trueman, 2000; Salomone et al., 2002; Schaefer et al., 2005; Stevens and Hogg, 2006). In particular, genetic divergence in the mite *Steganacarus carlosi* was up to 7.8% for specimens from the same geographic location, with values ranging from 16.8% to 23.4% for populations from different islands of the Canary

Table 3
Analysis of molecular variance (AMOVA) (Excoffier et al., 1992) results for *Gomphiocephalus hodgsoni* and *Stereotydeus mollis*, as implemented in Arlequin ver. 3.01 (Excoffier et al., 2005)

Species	Source of variation	df	Sum of squares	Variance components	Percentage of variation	P
G. hodgsoni	Among locations	9	0.191	0.00213 Va	45.01	
	Within locations	90	0.234	0.00260 Vb	54.99	<0.001
	Total	99	0.426	0.00473		
	Fixation Index F_{ST}: 0.450					
S. mollis	Among locations	7	0.399	0.00567 Va	19.53	
	Within locations	47	1.097	0.02335 Vb	80.47	0.002
	Total	54	1.496	0.02901		
	Fixation Index F_{ST}: 0.195					

Statistical significance of variance components in AMOVA tested with 16,000 permutations.

Table 4
Clades with significant geographical structure ($P < 0.05$) and their biological interpretation according to Templeton's inference key (2005) for mtDNA (COI) data from *Gomphiocephalus hodgsoni* and *Stereotydeus mollis*

Species	Clade	χ^2-statistic	P	Chain of inference	Inference
G. hodgsoni	1-2	33.514	<0.001	1-2-3-5-15-16-NO	Allopatric fragmentation
	2-2	22.059	0.035	1-2-3-4-9-10-YES	Allopatric fragmentation
	2-3	12.019	0.042	1-2-INCONCLUSIVE	No tip/interior status
	Total cladogram	0.000	<0.001	1-2-3-5-6-13-YES	Long distance colonisation possibly coupled with subsequent fragmentation OR past fragmentation followed by range expansion
S. mollis	Total cladogram	139.193	0.014	1-2-3-4-NO	Restricted gene flow with isolation by distance

Clades not showing genetic or geographic variation are excluded (no test is possible within such nested categories).

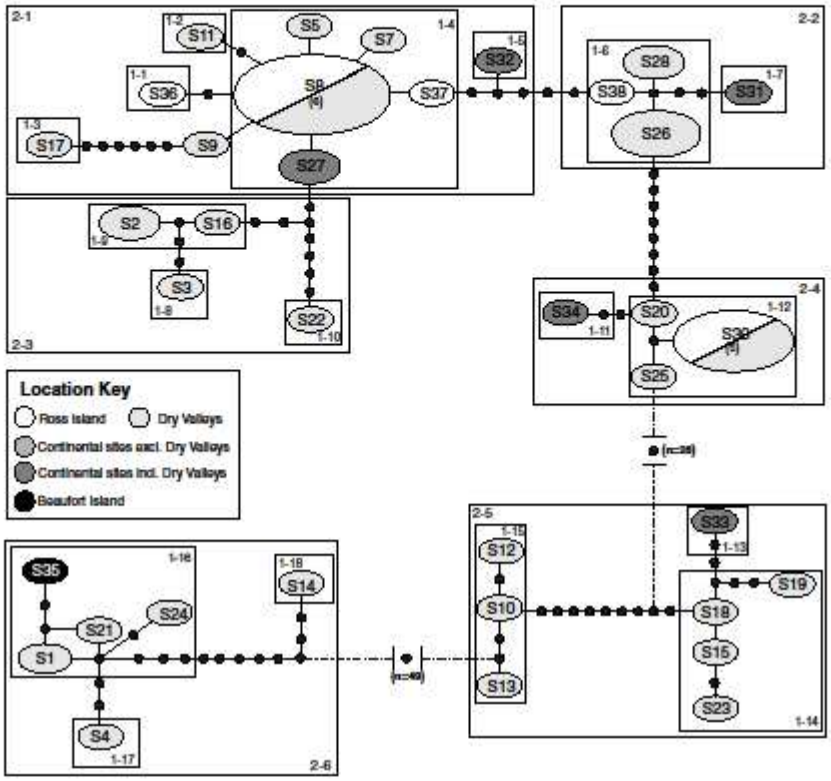

Fig. 3b. Nested clade design for the 36 unique mtDNA (COI) haplotypes from 55 individuals of *Stereopydeus mollis*. Geographic locations are indicated by haplotype colour (see accompanying key). Number of individuals with each haplotype is identified by the size of the haplotype when $n < 3$ and by size and in parentheses when $n > 3$. Missing haplotypes/mutational steps are indicated by an '●'. In cases where distinct groups occur, the proposed link between distinct groups (see Section 2) is indicated by a '–––| ●|–––', with the number of mutational steps between these groups given with this symbol. The clades are identified using a two number system, where the first number refers to the nesting hierarchy and the second is an arbitrary, individual clade identifier. Thin-lined polygons enclose 1-step clades; broken lined polygons enclose 2-step clades.

group (Salomone et al., 2002). In contrast, Mortimer and Jansen van Vuuren (2007) found just 2.2% sequence divergence for the mite *Eupodes minutus* in the sub-Antarctic. However, this species is likely to have a relatively recent origin (<1 Ma) on Marion Island.

The molecular clock calibration of 1.5–2.3% divergence per million years commonly applied to arthropods (see Brower, 1994; Juan et al., 1996; Quek et al., 2004) suggests that *G. hodgsoni* populations in this study have diverged within the last million years (i.e. during the Pleistocene/Holocene), with an earlier (up to 9.6 Ma) Pliocene/Miocene divergence for *S. mollis*. Previous work (Stevens and Hogg, 2006), suggested that these taxa have both experienced a similar glaciological history predominantly driven by Mio-Pliocene glacial cycling, and that the discrepancy in mtDNA divergences may be the result of differences in nucleotide substitution rates between these two species. A faster rate has been suggested for some mite taxa (e.g. Murrell et al., 2005) while a slower rate for *G. hodgsoni* was suggested by Stevens and Hogg (2006). Variation in nucleotide substitution rate can arise due to disparity between species in generation times, activity and other life history factors (see Martin and Palumbi, 1993). Thus, physiological differences between mites and springtails may be responsible for any rate differences, through their effect on mutation (evolutionary) rates. If there is a rate discrepancy between these species, then the proposed 1.5–2.3% molecular clock rate is subject to a high level of uncertainty, particularly for the mites.

The haplotype distribution patterns for *S. mollis* and *G. hodgsoni* are broadly similar in that there are three main genetic groups for both species, which may suggest a common phylogeographic history for these species (Stevens and Hogg, 2006). However, the patterns are also different

enough to indicate that other explanations may be equally appropriate. For example, *S. mollis* shows greater genetic structure at the sub-group level than that found in *G. hodgsoni*. Consistent with this, is the pattern of greater haplotype sharing among locations in *G. hodgsoni* than in *S. mollis* (50% and 22% and 30% and 8.3%, for *G. hodgsoni* and *S. mollis* for Stevens and Hogg (2006), and the present study, respectively). While Stevens and Hogg (2006), found similar numbers of haplotypes for these species (14 for *G. hodgsoni*, 18 for *S. mollis*), here we found a much higher number for *S. mollis* (36), than for *G. hodsoni* (20), despite a smaller sample size for *S. mollis*. Finally, the nested clade and AMOVA analyses performed here provide additional support for greater genetic structure in *S. mollis*. Combined, these factors suggest that *S. mollis* populations may have had a longer period of isolation (e.g. in refugia) and are more genetically differentiated as a result.

If *S. mollis* has been associated with the Antarctic landscape for a longer period, then *G. hodgsoni* could be either a more recent arrival or the victim of a more recent and severe bottleneck. Certainly, the greater desiccation resistance and better dispersal ability of *S. mollis* (Sinclair and Sjursen, 2001; Sjursen and Sinclair, 2002) may mean that this species has a greater capacity to respond to environmental disturbance and recover more rapidly from a bottleneck event. Greater dispersal capacity and earlier access to habitats would presumably enable longer periods in isolation (e.g. during glacial maxima), and potentially allow speciation to occur. A long isolated evolutionary history is thought to indicate an ancient origin for the mite *S. carlosi*, which also exhibits surprisingly high divergences between populations (Salomone et al., 2002) and Cromer et al. (2006) suggested that a series of temporally overlapping refugia best explained the continued presence (perhaps pre-dating the Gondwanan break-up) of the freshwater invertebrate *Daphniopsis studeri* on the Antarctic continent. While we were unable to detect any morphological differences in *S. mollis*, it is also possible that, with such high 'intraspecific' sequence divergences, cryptic species are present. With the isolating Antarctic conditions, genetic divergence among isolated populations may extend to speciation without reproductive isolation, leading to difficulties in the definition of species.

Within the McMurdo Dry Valleys, we found: (1) more haplotypes at inland sites at the western ends of the valleys; and (2) that inland sites (e.g. V8 and V10) of higher altitude often contained several different haplotypes. The former may suggest potential expansion of extant populations from western refugia following an eastern colonisation route towards the Ross Sea. The latter may indicate that populations persisted in elevated locations during glacial advances across valley floors, as postulated by Nolan et al. (2006) for Taylor Valley during the last glacial period. The Dry Valley region harboured many of the haplotypes found elsewhere in southern Victoria Land suggesting that this area may have served as a refuge during glacial periods. In particular, genetic links between the Dry Valleys and Ross Island for both species suggest an initial colonisation of Ross Island from a Dry Valleys individual, with subsequent divergence in isolation at this location accounting for those haplotypes now unique to Ross Island (e.g. G16–G18 and S36–S38). Further links between continental Antarctic locations (e.g. Marble Point) and the Dry Valleys (e.g. G10, S27) may also have arisen via dispersal from the Dry Valley region. Thus, initial dispersal from a Dry Valley refugium may have been followed by the formation of multiple refugia at newly colonised locations (e.g. Ross Island, Beaufort Island) (see also Stevens and Hogg, 2003, 2006).

In summary, Mio-Pliocene glacial cycles, through their effect on spatial and temporal variation in habitat structure and quality, appear to have played an active role in driving population structure for *G. hodgsoni* and *S. mollis*. While these Antarctic taxa display broadly similar patterns of phylogeographic structure, the presence of greater genetic structure in *S. mollis* suggests that: (1) there is a nucleotide substitution rate difference between these species; and/or (2) *S. mollis* has persisted in southern Victoria Land through a greater number of glacial cycles than *G. hodgsoni*. Future work focusing on investigation of physiological parameters capable of affecting evolutionary rates may help to resolve this issue further.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2007.10.003.

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Phylogeographic structure suggests multiple glacial refugia in northern Victoria Land for the endemic Antarctic springtail *Desoria klovstadi* (Collembola, Isotomidae)

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We carried out a phylogeographic study using mtDNA (COII) for the endemic springtail *Desoria klovstadi* (formerly *Isotoma klovstadi*) from northern Victoria Land, Antarctica. Low levels of sequence divergence (≤ 1.6%) across 26 unique haplotypes (from 69 individuals) were distributed according to geographic location. Cape Hallett and Daniell Peninsula contained the highest nucleotide (both > 0.004) and haplotype (both > 0.9) diversity with 10 (of 16) and 8 (of 12) unique haplotypes, respectively. All other populations (Football Saddle, Crater Cirque, Cape Jones) had lower diversity with 2–4 unique haplotypes. Across the 69 individuals from five populations there was only a single haplotype shared between two populations (Daniell Peninsula and Football Saddle). Furthermore, nested clade analyses revealed that some of the Daniell Peninsula haplotypes were more closely related to Football Saddle haplotypes than to any other population. Such discrete haplotype groupings suggest historical (rare) dispersal across the Pleistocene (1.8 mya–11 kya) and Holocene (11 kya–present), coupled with repeated extinction, range contraction and expansion events, and/or incomplete sampling across the species range. The nested clade analyses reveal that a common pattern of climatic and geological history over long-term glacial habitat fragmentation has determined the geographic and haplotype distributions found for *D. klovstadi*.

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Introduction

Antarctica has been isolated from other continents by a wide oceanic belt (ca., 40°S–66°S) for at least 28 million years (Schultz 1995; Lawver & Gahagan 1998; McLoughlin 2001). Since that time the terrestrial landscape has been increasingly dominated by long-term habitat fragmentation, with little more than 0.3% of the 14 million km^2 of the continent ice-free today (British Antarctic Survey 2004; Peat *et al.* 2007), and more than 10 major glacial cycles over the last 1 million years (Hays *et al.* 1976). It is now well established that the Ross Sea sector (e.g., Victoria Land) is one of the few continental regions whose terrestrial fauna are unlikely to be recent colonisers from subantarctic or temperate regions (Wise 1967; Brundin 1970; Stevens *et al.* 2006a). Consequently, terrestrial life in this region has evolved *in situ* as a unique collection of endemic fauna and flora (Stevens *et al.* 2006a; see also Adams *et al.* 2006 for review).

Prolonged low temperatures and increased glacial activity have meant that many locations in Victoria Land became further isolated and the survival of taxa, particularly the terrestrial invertebrates, could only be possible in ice-free refugia, such as nunataks (Wise 1967; Frati *et al.* 1997; Stevens & Hogg 2003). Evidence to support the availability of terrestrial habitats throughout the Trans-Antarctic Mountains (including Victoria Land) is increasing (e.g., Denton *et al.* 1993; Prentice *et al.* 1993; Marchant & Denton 1996; Denton & Hughes 2000), and it is likely that the biogeography in this region at least reflects, in part, the historical persistence of biota since Antarctica became glaciated (see also Stevens *et al.* 2006a, and references within). Nunataks would have provided

possible refuges during the last glaciation in this region, but many terrestrial habitats have only become available for (re-)colonisation from these refuges within the current interglacial (< 17 000 years). Habitat fragmentation also influences species distribution on a smaller scale, with populations being effectively isolated across short geographic distances, particularly where glaciers or other landscape features may represent barriers to effective dispersal. The effects of habitat fragmentation may be enhanced in those organisms, such as arthropods, with limited mobility and reduced likelihood of passive dispersal (Marshall & Cotzee 2000). Therefore, habitat fragmentation generally impedes demographic processes (e.g., mobility and resulting gene flow), particularly in the context of range contraction/expansion among glacial populations (Hewitt 2000).

To address questions concerning the sources of biodiversity as well as pathways of dispersal and the formation of communities in the fragmented Antarctic landscape, phylogeographic analyses are ideal, as they can illuminate processes of speciation and population history of organisms (e.g., Garrick *et al.* 2004). Unfortunately, there are few phylogeographic studies that have examined the importance of Pleistocene glacial events in shaping the distribution of genetic diversity for the terrestrial fauna of continental Antarctica (Courtright *et al.* 2000; Fanciulli *et al.* 2001; Frati *et al.* 2001; Stevens & Hogg 2003; see Stevens & Hogg 2006a for review).

Victoria Land, in the Ross Sea sector, represents an ideal system to examine hypotheses related to Pleistocene speciation and the evolutionary persistence of Antarctic taxa relative to long-term perturbations. The endemic springtail *Desoria klovstadi* (Carpenter 1902) (previously *Isotoma klovstadi*, see Stevens *et al.* 2006b) from northern Victoria Land is among the most mobile arthropods in Antarctica with a larger body size and a well functioning furca ('jumping organ'). Here we examined the spatial distribution of mitochondrial DNA haplotypes for *D. klovstadi* in a phylogeographic context to determine the relative contributions of past fragmentation, range expansion and recurrent, restricted gene flow.

Materials and methods

Collections, DNA extraction and sequencing

Individuals of *D. klovstadi* were collected from five populations in northern Victoria Land, Antarctica: Cape Jones (73°17′S, 169°22′E), Crater Cirque (72°37′S, 169°22′E), Daniell Peninsula (72°42′S, 169°36′E), and two sites on Hallett Peninsula–Football Saddle (72°30′S, 169°42′E) and Cape Hallett (72°25′S, 169°20′E) (Fig. 1). All individuals at each site (see Table 1) were collected with an aspirator in an area of about 100 m^2 (see Frati *et al.* 2001; Stevens & Hogg 2002 for details), and stored in liquid nitrogen or 95% ethanol in the field, and later stored at –80 °C or in absolute ethanol until needed for DNA extraction.

In addition to 40 individuals sequenced previously (Frati *et al.* 2001) total genomic DNA was extracted from a further 29 entire individuals using one of three protocols: (i) a quick protocol in which single specimens were homogenised in 50 µL of a solution containing PCR buffer, 1% Tween 20 and 0.1 mg/mL proteinase k. The homogenate was then incubated for 15 min at –80 °C, 2 h at 65 °C and 15 min at 100 °C; (ii) a CTAB method (Boyce *et al.* 1990, as modified in Shahjahan *et al.* 1995) followed by 'salting out' (Sunnucks & Hales 1996) or phenol extraction and ethanol precipitation; or (iii) using the 'salting out' technique only.

Polymerase chain reaction (PCR) amplification (Saiki *et al.* 1998) was carried out using a 10 or 25 µL reaction volume consisting of 1–3 µL of template DNA (not quantified), 1× PCR buffer (Roche), 2.2 mM $MgCl_2$, 0.2 mM of each dNTP (Bochringer Mannheim), 1.0 µM of each primer and 0.06–0.2 units of *Taq* DNA polymerase (Roche) on a Biometra T1 thermocycler (Whatman Biometra) or a Perkin Elmer 2400 Thermal Cycler. The thermal cycling conditions were: 30 cycles with 45 s of denaturation at 95 °C, 1 min of annealing at 47 °C (the first 5 cycles) and 50 °C (the remaining 25 cycles) and 1 min 10 s of extension at 72 °C. Two primers which flank the COII gene in the mitochondrial genome were used: COIIa (5′-AATATGGCAGATTAGTGCA-3′) and COIIb (5′-GTTTAAGAGACCAGTACTT-3′) (Frati *et al.* 2001). All reaction products (~790 bp) were purified by using SAPEXO (USB Corp.), or band-excised using the Perfectprep gel cleanup kit (Eppendorf) or the Concert Rapid Gel Extraction System (Life Technologies). Purified PCR products were sequenced (using COIIa and in some cases COIIb to resolve ambiguities; and/or two internal primers; see Frati *et al.* (2001) for complete details) directly using BigDye™ Terminator chemistry (Perkin-Elmer Applied Biosystems). Sequencing was performed on a Perkin Elmer 373A automated sequencer at the Core Facility of Italian National Agency for New Technologies, Energy and Environment (ENEA) in Rome, or on a capillary ABI3730 genetic analyser (Applied Biosystems Inc.) at the Allan Wilson Centre Genome Service, Massey University, New Zealand.

Population structure analyses

Sequences were checked for open reading frames (using MacClade ver. 4; D.R. Maddison & W.P. Maddison 2000) to check for the presence of nuclear copies or other unintended sequence types (Sunnucks & Hales 1996) and were confirmed as being consistent with springtail DNA using the GenBank BLASTn search. All 69 sequences (EMBL accessions: AJ312973–AJ313012, Frati *et al.* 2001; new accessions: EF119745–EF119773) were aligned using SEQUENCHER (Gene Codes ver. 4.6) sequence editor. This alignment (with trace files) identified some assignment errors in the original sequences from Frati *et al.* (2001), which have been corrected here and in the accessions, namely that haplotypes B and C are identical

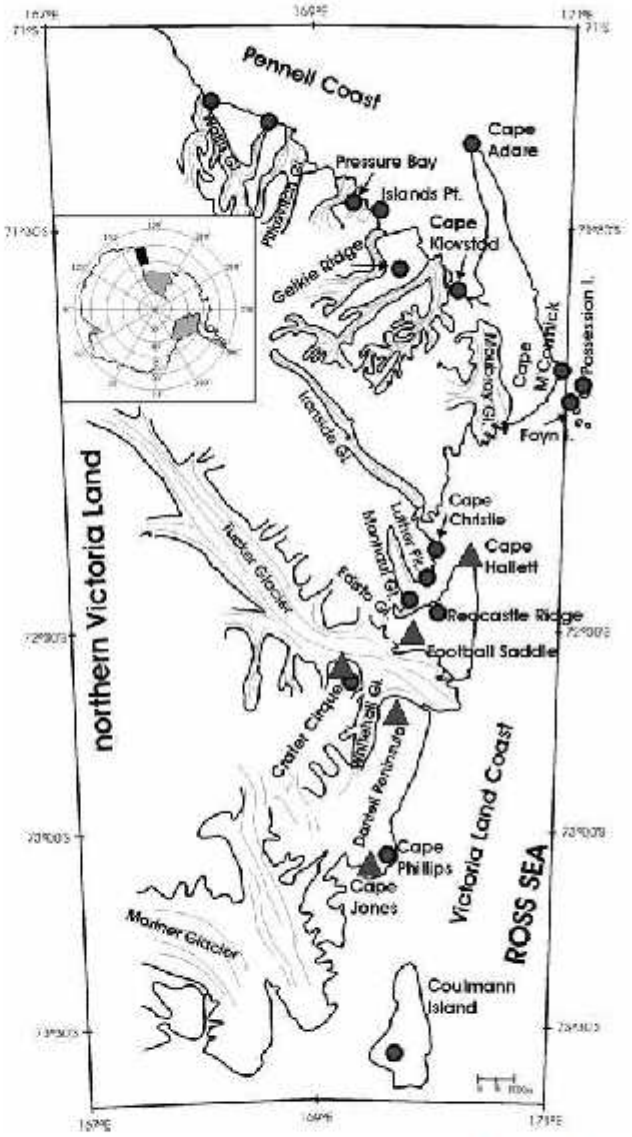


Fig. 1 The known distribution for *Desoria klovstadi* in northern Victoria Land, Antarctica (Ross Sea Region) (indicated by an '●'), and the location of the five populations used in this study (indicated by an '▲'). Inset indicates the location of northern Victoria Land in relation to Antarctica.

to haplotype A; haplotype R is identical to haplotype O; haplotype N is identical to haplotype I; and three incorrect substitutions change the sequence of haplotype M. Given these corrections, here we retain population and individual codes from Frati *et al.* (2001) (e.g., CJ1, FS2, etc.) but have applied new haplotype codes (i.e., A–Z).

PAUP* (ver. 4.0b10) (Swofford 2002) was used to examine assumptions of homogenous base frequencies, variable sites

Table 1 The 29 variable nucleotide sites from the 678-bp mtDNA (COII) fragment among the 26 unique haplotypes for *Desoria klovstadi*.

	Nucleotide position																											Amino acid				
	2	4	4	1	1	1	2	2	2	2	2	2	2	3	3	3	4	4	4	4	5	5	5	5	6	6	6	6				2	2
	2	5	6	1	1	1	1	1	2	3	4	5	8	9	1	2	6	4	5	6	2	5	1	4	2	5	3	4	3	5	1	1	1
Population	4	5	6	0	1	4	0	6	5	1	9	2	2	5	5	7	9	7	0	2	5	1	4	2	5	3	4	3	6	6	7	9	9
A CH1	T	T	A	T	C	T	A	T	C	T	G	A	T	A	T	A	C	G	G	C	G	T	C	G	A	A	T	C	A	Ile	Ile	Thr	Ile	Phe
B CH2, 3	.	G	.	.	.	.	.	.	T	.	C	.	.	.	.	.	T	A	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	Tyr
C CH4, 5	.	G	.	.	.	.	.	.	T	.	C	.	.	.	.	.	T	A	A	.	.	.	.	.	.	.	.	.	T	.	.	.	.	Tyr
D CH6, 7	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	Tyr
E CH8, 9, 12, 13	.	G	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	Tyr
F CH10	.	G	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	G	T	.	.	.	Met	Tyr
G CH11	.	G	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	T	.	.	.	.	Tyr
H CH14	.	G	.	.	.	.	.	.	T	.	C	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	Ala	.	Tyr
I CH15	.	G	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	T	.	.	.	.	Tyr
J CH16	.	.	.	.	.	.	.	.	T	.	C	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	Tyr
K FS1-4, 6, DP3	.	G	.	.	.	.	G	.	T	.	C	G	.	.	.	.	.	A	.	.	.	.	.	.	.	.	C	.	T	.	.	.	.	Tyr
L FS5, 7, 10	.	G	.	C	G	G	G	.	T	.	C	G	.	.	.	.	.	A	.	.	.	.	.	.	.	.	C	.	T	.	Thr	.	.	Tyr
M FS8	.	G	.	C	G	.	G	.	T	.	C	G	.	.	.	.	.	A	.	.	.	.	.	.	.	.	C	.	T	.	Thr	.	.	Tyr
N FS9	.	G	.	.	G	G	G	.	T	.	C	G	.	.	.	.	.	A	.	.	.	C	.	.	.	.	C	.	T	.	Met	.	.	Tyr
O CC1-14	.	G	G	.	.	.	G	C	T	.	C	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	T	Val	.	.	.	Tyr
P CC15	.	G	G	.	.	.	G	C	T	.	C	.	.	.	.	.	.	A	.	.	A	.	.	.	.	.	.	.	T	Val	.	.	.	Tyr
Q DP1, 4, 12	.	G	.	.	.	.	G	C	T	.	C	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	Tyr
R DP2, 5	.	G	.	.	.	.	G	.	T	C	C	.	.	.	C	.	.	A	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	Tyr
S DP6	.	G	.	.	.	.	G	.	T	.	C	G	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	Tyr
T DP7	.	G	.	C	G	G	G	C	T	.	C	.	G	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	T	.	Thr	.	.	Tyr
U DP8, 10	.	G	.	C	G	G	G	.	T	C	C	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	T	.	Thr	.	.	Tyr
V DP9	.	G	.	C	G	G	G	.	T	.	C	G	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	T	.	Thr	.	.	Tyr
W DP11	C	G	.	.	.	.	G	.	T	.	C	G	.	.	.	.	.	A	.	.	.	.	.	.	.	G	.	.	T	.	.	.	.	Tyr
X CI1, 5, 7, 16	.	G	.	.	.	.	G	.	T	.	C	.	.	G	.	G	.	A	.	.	.	.	A	A	G	.	.	.	T	.	.	Ala	.	Tyr
Y CI2, 4, 6, 9-14	.	G	.	.	.	.	G	.	T	.	C	.	.	G	.	G	.	A	.	.	.	.	A	A	.	.	.	.	T	.	.	Ala	.	Tyr
Z CI3, 8, 15	.	G	.	.	.	.	G	.	T	.	C	.	.	G	.	G	.	A	.	.	.	.	A	.	.	.	.	.	T	.	.	Ala	.	Tyr

Haplotype A from Cape Hallett (CH) was used as the reference sequence; the number following the population codes (Football Saddle (FS), Daniell Peninsula (DP), Cape Jones (CJ) and Crater Cirque (CC)), indicates the individual sequenced. All substitutions occur at third codon positions, except for those at 46 and 295 (first codon position) and at 110 and 656 (second codon position). Nucleotide position associated to amino acid changes shown in 'bold' type, and the amino acid residues where these changes occur (relative to the reference haplotype) are shown.

(variable amino acids using MacClade) and sequence divergence among all sequences. We used Arlequin ver. 3.01 (Excoffier *et al.* 2005) to explore genetic characteristics and partitioning of nucleotide diversity (presence of population structure); we calculated haplotype (h) and nucleotide (π) diversity indices (Nei 1987) for the species as a whole, and separately for each population; analysis of molecular variance (AMOVA; Excoffier *et al.* 1992) with statistical significance evaluated for 16 000 permutations; pairwise differences (Φ_{st} values) between haplotypes using simple distances; and Tajima's D (Tajima 1989) and Fu's F_s (Fu 1997) to test for selective neutrality. However, Tajima's D and Fu's F_s tests did not detect departures from neutrality, and were non-significant ($P < 0.05$, in all cases), and are not shown.

Haplotype network and nested clade analyses

A haplotype network was estimated for *D. klovstadi* using the programme TCS ver. 1.21 (Clement *et al.* 2000) and was defined into a nested structure following the nesting rules of Crandall (1996). An input file with user-defined distances (kilometres between sampling sites calculated using: (i) 'as the crow flies' and (ii) along open/free water via common glacial melt-water streams and near-shore coastal regions), and outgroup weights specified were then created and run in the programme GEODIS ver. 2.4 (Posada *et al.* 2000) in order to perform nested geographical distance analyses on the *D. klovstadi* haplotypes. This programme assesses the geographical association(s) between clades by measuring the geographical range of the clades (Dc, the clade distance); the distance between haplotypes across tip and interior clades (Dn, the nested clade distance); and the average interior clade and tip clade distances (I-T, the interior vs. tip distance (Templeton *et al.* 1995; Templeton 1998)). Clades with statistically significant values of Dc, Dn or I-T in the GEODIS output were assessed with the updated Inference Key for the Nested Haplotype Tree Analysis of Geographical Distances of Templeton (http://darwin.uvigo.es/software/geodis.html) to separate population structure from population history

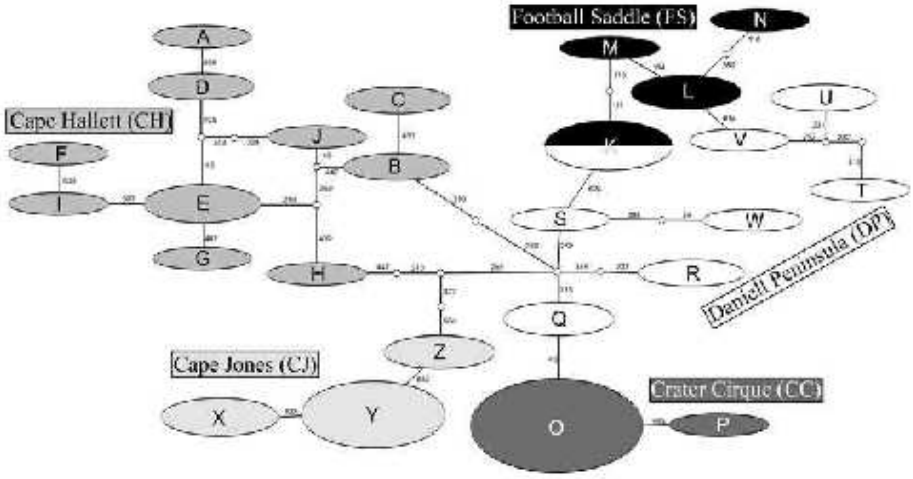


Fig. 2 Haplotype network analysis (statistical parsimony) depicting relationships among the *Desoria klovstadi* haplotypes from the five populations (26 unique haplotypes from 69 individuals) using the mtDNA (COII) gene. Relative size of ellipses indicate frequency of each haplotype (see Table 3), and numbers at each branch correspond to the location for that mutational step shown in Table 1.

by deducing which factor(s) (e.g., restricted gene flow, past fragmentation, range expansion) caused significant spatial association among haplotypes.

Results

mtDNA haplotype diversity and population structure

A 678-bp fragment was used in all analyses. No insertions, deletions or stop codons were detected. The nucleotide composition of all sequences was biased towards A and T (A = 31%, T = 33%, C = 21%, G = 14%). Base frequencies were homogenous across sequences as tested for all sites (χ^2_{204} = 1.89, P = 1.00), for the 29 variable sites only (χ^2_{204} = 39.19, P = 1.00), and for the 226 third codon sites only (χ^2_{204} = 9.19, P = 1.00). Among the 69 *D. klovstadi* sequences there were 29 variable (22 parsimony-informative) nucleotide substitutions that resulted in 26 unique haplotypes (Table 1). At the first and second codon positions there were only four variable sites (positions 46 and 295, 110 and 656, respectively), with most variability occuring at the third codon position (86%) (see Table 1). The nucleotide substitutions resulted in six amino acid changes (at five positions): two at the first codon position (site 46 in all Crater Cirque (CC) individuals, and site 295 in all Cape Jones (CJ) individuals and a single Cape Hallett individual (CH14)); two at the second codon position (site 110 in Daniell Peninsula (DP) and Football Saddle (FS), and site 656 in CH1); and two at the third codon position (site 111 in DP and FS, and site 633 in CH10) (Table 1).

Percentage of sequence divergence between haplotypes ranged from 0.1% (1 substitution) to 1.6% (11 substitutions) (Table 2). These values were generally highest when comparing Cape Hallett (range 0.4%–1.6%) or Cape Jones (0.6%–1.6%) with all other populations; while comparisons among these other populations were lower (0.1%–1.2%) (Table 2).

Haplotype diversity (h) ranged from 0.133 to 0.925 for the five populations (Table 3). CH showed the highest haplotype diversity (h = 0.925) with 10 haplotypes (A–J) identified from 16 individuals; similar levels of diversity were present at DP (h = 0.924) with 8 haplotypes (K, Q–W) from 12 individuals (Table 3). Measures of nucleotide diversity (π) were highest in these populations also, as were estimates of effective population size θ(S) (Table 3). The FS population contained 4 haplotypes (K–N) among 10 individuals, CJ had 3 haplotypes (X–Z) from 16 individuals, and at CC only 2 haplotypes (O, P) were identified from 15 individuals (Table 3). Shared haplotypes between populations occurred in only one instance — five individuals at FS were identified with haplotype K, and this haplotype was also identified for a single individual at DP (Table 3; Fig. 2).

Table 2 Uncorrected distances between all 26 unique haplotypes from 69 *Desoria klovstadi* individuals for the 678-bp mtDNA (COII) fragment.

		A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X	Y
Cape Hallett	A																									
	B	0.009																								
	C	0.010	0.001																							
	D	0.001	0.007	0.009																						
	E	0.004	0.004	0.006	0.003																					
	F	0.007	0.007	0.009	0.006	0.003																				
	G	0.006	0.006	0.007	0.004	0.001	0.004																			
	H	0.007	0.004	0.006	0.006	0.003	0.006	0.004																		
	I	0.006	0.006	0.007	0.004	0.001	0.001	0.003	0.004																	
	J	0.006	0.003	0.004	0.004	0.004	0.007	0.006	0.004	0.006																
Football Saddle	K*	0.012	0.006	0.007	0.010	0.007	0.010	0.009	0.007	0.009	0.009															
	L	0.016	0.010	0.012	0.015	0.012	0.015	0.013	0.012	0.013	0.013	0.004														
	M	0.015	0.009	0.010	0.013	0.010	0.013	0.012	0.010	0.012	0.012	0.003	0.001													
	N	0.016	0.010	0.012	0.015	0.012	0.015	0.013	0.012	0.013	0.013	0.004	0.003	0.004												
Crater Cirque	O	0.012	0.006	0.007	0.010	0.007	0.010	0.009	0.007	0.009	0.009	0.006	0.010	0.009	0.010											
	P	0.013	0.007	0.009	0.012	0.009	0.012	0.010	0.009	0.010	0.010	0.007	0.012	0.010	0.012	0.001										
Daniell Peninsula	Q	0.010	0.004	0.006	0.009	0.006	0.009	0.007	0.006	0.007	0.007	0.004	0.009	0.007	0.009	0.001	0.003									
	R	0.012	0.006	0.007	0.010	0.007	0.010	0.009	0.007	0.009	0.009	0.006	0.010	0.009	0.010	0.006	0.007	0.004								
	S	0.010	0.004	0.006	0.009	0.006	0.009	0.007	0.006	0.007	0.007	0.001	0.006	0.004	0.006	0.004	0.006	0.003	0.004							
	T	0.016	0.010	0.012	0.015	0.012	0.015	0.013	0.012	0.013	0.013	0.010	0.006	0.007	0.009	0.007	0.009	0.006	0.010	0.009						
	U	0.015	0.009	0.010	0.013	0.010	0.013	0.012	0.010	0.012	0.012	0.009	0.004	0.006	0.007	0.009	0.010	0.007	0.006	0.007	0.004					
	V	0.015	0.009	0.010	0.013	0.010	0.013	0.012	0.010	0.012	0.012	0.006	0.001	0.003	0.004	0.009	0.010	0.007	0.009	0.004	0.004	0.003				
	W	0.013	0.007	0.009	0.012	0.009	0.009	0.010	0.009	0.007	0.010	0.004	0.009	0.007	0.009	0.007	0.009	0.006	0.007	0.003	0.012	0.010	0.007			
Cape Jones	X	0.016	0.010	0.012	0.015	0.012	0.015	0.013	0.009	0.013	0.013	0.010	0.015	0.013	0.015	0.010	0.012	0.009	0.010	0.009	0.015	0.013	0.013	0.012		
	Y	0.015	0.009	0.010	0.013	0.010	0.013	0.012	0.007	0.012	0.012	0.009	0.013	0.012	0.013	0.009	0.010	0.007	0.009	0.007	0.013	0.012	0.012	0.010	0.001	
	Z	0.013	0.007	0.009	0.012	0.009	0.012	0.010	0.006	0.010	0.010	0.007	0.012	0.010	0.012	0.007	0.009	0.006	0.007	0.006	0.012	0.010	0.010	0.009	0.003	0.001

Haplotypes are indicated by their codes (A–Z).

*Note: Haplotype K at Football saddle was also identified from a single individual at Daniell Peninsula.

Table 3 Sampling statistics and genetic characteristics for the five populations for *Desoria klovstadi*.

Population	No. of individuals	No. of haplotypes	No. of polymorphic sites	h (SD)	π (SD)	θ (SD)	Distribution of haplotypes within populations
Cape Hallett	16	10	11	0.925 (0.047)	0.0045 (0.0028)	3.315 (1.501)	A, B, C, D, E, F, G, H, I, J
Football Saddle	10	4	4	0.711 (0.118)	0.0027 (0.0019)	1.414 (0.861)	K, L, M, N
Crater Cirque	15	2	1	0.133 (0.112)	0.0002 (0.0002)	0.308 (0.308)	O, P
Daniell Peninsula	12	8	11	0.924 (0.058)	0.0057 (0.0035)	3.643 (1.722)	K, Q, R, S, T, U, V, W
Cape Jones	16	3	2	0.625 (0.093)	0.0011 (0.0009)	0.603 (0.452)	X, Y, Z

h, haplotype diversity; π, nucleotide diversity; θ, theta (S) (Nei 1987). All statistics calculated in Arlequin ver. 3.01 (Excoffier *et al.* 2005). Haplotype K is the only shared mtDNA haplotype (shown in bold).

Table 4 (a) Analysis of molecular variance (AMOVA) (Excoffier *et al.* 1992) results for *Desoria klovstadi*, as implemented in Arlequin ver. 3.01 (Excoffier *et al.* 2005). (b) Pairwise population φ_{-st} values for populations within *D. klovstadi*.

(a)

Source of variation	df	Sum of squares	Variance components	Percentage of variation	P
Among populations	4	0.179	0.00318 Va	71.47	< 0.001
Within populations	64	0.081	0.00127 Vb	28.53	—
Total	68	0.260	0.00444	—	—

(b)

Region					
Cape Hallett (CH)	—	—	—	—	—
Football Saddle (FS)	0.65*	—	—	—	—
Crater Cirque (CC)	0.72*	0.87*	—	—	—
Daniell Peninsula (DP)	0.45*	0.33*	0.53*	—	—
Cape Jones (CJ)	0.77*	0.87*	0.95*	0.70*	—

Statistical significance of variance components in AMOVA tested with 16 000 permutations; '*' indicates $P < 0.05$.

The haplotype network (Fig. 2) shows that haplotypes from CH are highly related, as are those from CJ. The haplotypes found at DP and FS are linked together by no more than two substitutions and individuals from both populations appear nested within each other (Fig. 2), yet are separated geographically by the Tucker Glacier (Fig. 1). The 2 haplotypes found at CC are closely related and linked to haplotype Q from DP (Fig. 2), and separated by the Whitehall Glacier (Fig. 1). AMOVA analysis revealed a high level of genetic structure, with ~71% of variation apportioned among populations ($P < 0.001$) (Table 4a). All φ_{-st} values were large and significant (Table 4b), indicating long-term historical isolation and low gene flow between populations for *D. klovstadi*.

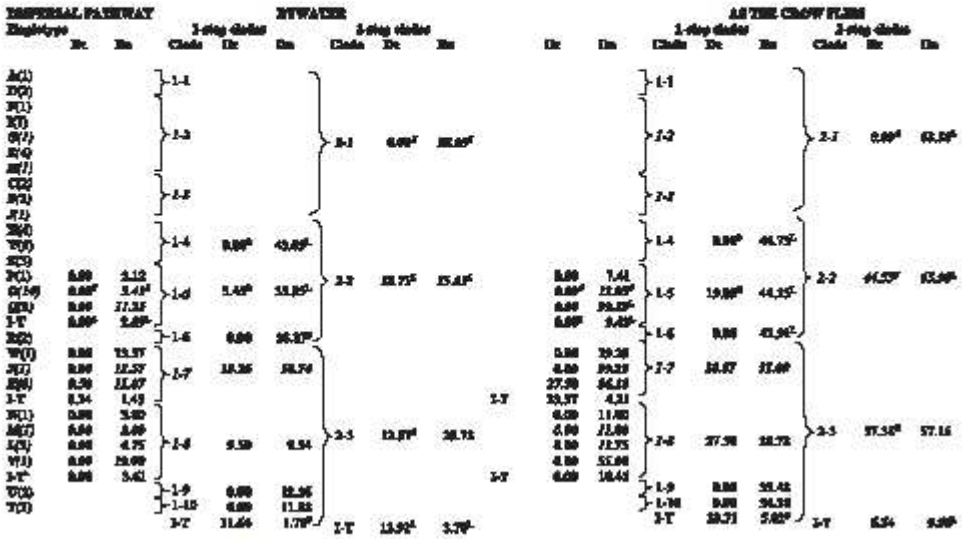
Nested clade analyses

The nested cladogram for *D. klovstadi* contained 26 unique haplotypes from 69 individuals and provided eleven 1-step clades (i.e., clades where one mutational step separates two adjacent haplotypes) and three 2-step clades (two mutational steps separate adjacent haplotypes) (Table 5; Fig. 3). The maximum number of mutational steps between haplotypes was five. Nested contingency analysis revealed significant association of clades and geographical distance for clades 1-6, 2-2, 2-3 and the total cladogram, which were subsequently analysed using Templeton's inference key (Table 6). Clade 2-2 gave an inconclusive outcome because it consisted of only tip clades, hence no tip/interior status could be determined (Table 6). However, the most likely explanation for the patterns observed for clade 1-6 and the total cladogram the inference was 'restricted gene flow/dispersal with some long distance dispersal over intermediate areas not occupied by the species OR past gene flow followed by extinction of intermediate populations' (results were identical whether data utilised distances between populations as 'km by water' or 'km as the crow flies', except for the total cladogram for which 'long distance colonisation/and or past fragmentation' was inferred when the user-defined distance was 'km as the crow flies') (see Table 6).

Discussion

The mtDNA (COII) gene revealed a heterogeneous haplotype organisation among the five *D. klovstadi* populations, with 26 haplotypes (from 69 individuals) structured according to geographic location. Only 1 haplotype was shared among populations–haplotype K was present in Football Saddle (FS) and Daniell Peninsula (DP). This may appear surprising given the limited geographic distances between these populations. However, previous studies have shown that terrestrial invertebrate taxa in Antarctica are often characterised by high levels of sub-structuring and local mitochondrial diversity even on fine geographical scales (e.g., Frati *et al.* 2001; Stevens & Hogg 2003; Nolan *et al.* 2006). While these studies identified a relatively high number of haplotypes for the number of individuals examined, levels of divergence among haplotypes were low and haplotype sharing among populations was very limited. The present study is consistent with this earlier work, and our nested clade analyses support long-term historical

Table 5 Nested clade distance analysis of mtDNA (COII) haplotypes observed in *Desoria klovstadi* under the assumption of dispersal 'by water' (geographic distance calculated along putative free-water routes) or 'as the crow flies'.



Brackets reflect the nesting structure (see Fig. 3). Dc and Dn are clade and nested clade distances, respectively (for details see Templeton *et al.* 1995). Interior vs. tip contrasts for Dc and Dn are indicated with 'I-T' in the corresponding clade, with interior clades given in italic type. Superscript 'S' and superscript 'L' indicate that distance measures are significantly smaller and larger, respectively, than expected under random distribution of haplotypes.

fragmentation and isolation with low gene flow between populations for *D. klovstadi*.

It has been proposed that this common pattern of geographical and genetic subdivision/structuring among species and locations in Antarctica is due to a common biogeographic legacy (e.g., Brundin 1970; Stevens & Hogg 2006a). In particular, Pleistocene and Holocene glacial cycles, through their effect on spatial and temporal variation in habitat structure and quality (Thompson *et al.* 1971), appear to have played an active role in driving allopatric fragmentation of terrestrial invertebrate populations in Antarctica. Indeed, the genetic distances between *D. klovstadi* individuals (up to 1.6% divergence) and a strict molecular clock calibration of 1.5%–2.3% divergence per million years (see Stevens *et al.* 2006b) suggests that populations in this study most likely diverged around 0.94 to 1.4 million years ago. This time frame supports the impact of Pleistocene and Holocene glaciations on present day phylogeographic patterns for this taxon, and compares remarkably closely to the divergences (up to 2.0% across the species range) reported for the southern Victoria Land springtail *Gomphiocephalus hodgsoni* (Stevens & Hogg 2003, 2006b).

Several studies have investigated the impact of Pleistocene climate changes on phylogeographic patterns, and these have found greater genetic diversity at localised ice-free regions (i.e., refugia) relative to glaciated sites (e.g., Weider & Hobæk 2003; Rowe *et al.* 2004). The presence of glacial refugia can explain the persistence of populations through historical glacial cycles. In the present study, the high levels of genetic diversity in the Daniell Peninsula (DP) and Cape Hallett (CH) populations may represent either a large population size or post-glacial remnants sourced from multiple refugia that persisted throughout glacial cycles. In contrast, the low levels of genetic diversity found for Football Saddle (FS), Cape Jones (CJ) and Crater Cirque (CC) populations are potentially a result of post-glacial expansion (founder events) of a few individuals or a relatively small (bottlenecked) population. Alternatively, each of the five populations may represent its own historical refugium, and indeed, multiple refugia are not uncommon (e.g., Stevens & Hogg 2003; Weider & Hobæk 2003; Rowe *et al.* 2004).

Geological and glaciological evidence for persistent terrestrial refugia is presently lacking for northern Victoria Land. However, in East Antarctica the polar cap was thought to

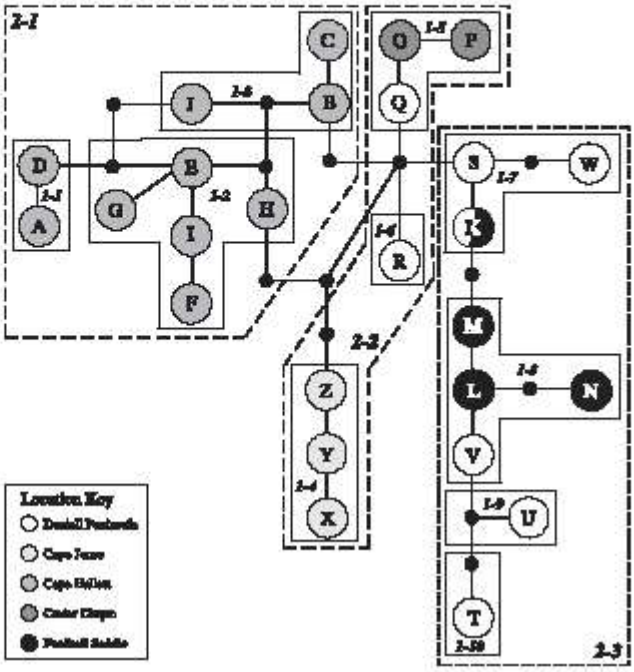


Fig. 3 Nested clade design for the mtDNA (COII) 26 unique haplotypes from 69 individuals of *Desoria klovstadi*. Haplotypes are indicated by their codes (A–Z). Missing haplotypes/mutational steps are indicated by an '●'. The clades are identified using a two number system, where the first number refers to the nesting hierarchy and the second is an arbitrary, individual clade identifier. Thin-lined polygons enclose 1-step clades (i.e., clades where one mutational step separates two adjacent haplotypes); broken-lined polygons enclose 2-step clades (two mutational steps separate adjacent haplotypes).

Table 6 Clades with significant geographical structure ($P < 0.05$) and their biological interpretation for mtDNA (COII) *Desoria klovstadi*.

Clade	χ^2-statistic	*P*	Chain of Inference	Inference
1-6	18.000	< 0.001	1-2-3-5-6-7-8-YES	Restricted gene flow/dispersal with some long distance dispersal over intermediate areas not occupied by the species OR past gene flow followed by extinction of intermediate populations
2-2	48.000	< 0.001	1-2-INCONCLUSIVE	No tip/interior status
Total Cladogram	120.572	< 0.001	1-2-3-5-6-7-8-YES	Restricted gene flow/dispersal with some long distance dispersal over intermediate areas not occupied by the species OR past gene flow followed by extinction of intermediate populations*

Clades not showing genetic or geographic variation are excluded (no test is possible within such nested categories).
*Note: results were identical whether data utilized distances between populations as 'km by water' or 'km as the crow flies', except in this case where 'long distance colonization and or past fragmentation' was inferred for the total cladogram for distance in 'km as the crow flies'.

have established as early as the Miocene (ca., 14 mya). Extensive debate (reviewed in Barrett 1996) exists over whether this ice cap has persisted almost unchanged to modern times due to temperature stability (Haq *et al.* 1987), or whether it has undergone large fluctuations in the last 3–5 my, with extensive changes in the ice cover (Webb *et al.* 1984; Webb & Harwood 1991). In any event, the glacial cycles occurring during the Quaternary will have certainly influenced the turnover of deglaciated land available for the survival of soil invertebrates, but that refugia existed in multiple regions along the Trans-Antarctic mountains (Thompson *et al.* 1971; see also Nolan *et al.* 2006, Stevens *et al.* 2006a).

Interestingly, the entire distribution for *D. klovstadi* is contained on the northern region of northern Victoria Land (Fig. 1). This region coincides precisely with the Robertson Bay and Bowers Arc terranes that form a 'wedge' between the Mariner Glacier along the Victoria Land coast (see Fig. 1) to Bower Mountains (near Rennick Glacier, 70°S, 162°E)

located west from Cape Adare along the Pennell Coast (see Fig. 1 in Roland *et al.* 2004). These terranes are thought to have 'docked' with the Wilson terrane sometime in the Mesozoic to Cenozoic (Roland *et al.* 2004), suggesting that this species may have 'arrived' in Antarctica through tectonic means or that these terranes have provided refugia specific for *D. klovstadi* not found elsewhere.

The populations we analysed are distributed in the southern half of the total known distribution for *D. klovstadi* (see Stevens *et al.* 2006b) and thus might represent peripheral populations. Given the potential metapopulation scenario, peripheral populations would have a high probability of going through local extinction/re-colonisation cycles. Such fluctuating populations would lead to haplotypes being representative of the most recent colonisers (which are potentially not represented in the populations here), rather than showing any genealogical signature associated with a history of gene flow. Accordingly, further extensive sampling across the known range for *D. klovstadi* is required (although currently logistically impractical) in order to more completely illustrate the historical processes that have shaped population and haplotype distribution.

In contrast to an overall pattern of isolation and fragmentation, we found a close association between Football Saddle (FS) and Daniell Peninsula (DP) populations (e.g., Figs 2, 3; and also supported by our nested clade analyses). While the main finding was 'restricted gene flow/dispersal with some long distance dispersal over intermediate areas not occupied by the species or past gene flow followed by extinction of intermediate populations', the inference for clade 2-3 (which contains DP and FS populations) was 'contiguous range expansion'. A feasible explanation for the association between these populations is their likely proximity to Tucker Glacier melt-water streams (see Fig. 1). The Tucker Glacier itself is likely to have posed a barrier to other *D. klovstadi* populations, thus contributing to the overall patterns of fragmentation and isolation by distance associated with glaciers seen here and elsewhere (Fanciulli *et al.* 2001; Stevens & Hogg 2006a). However, in the case of the DP and FS populations, with wind-mediated dispersal being highly unlikely (Marshall & Cotzee 2000), the melt-water streams may have provided a possible means of transport between populations, enabling gene flow and potentially accounting for the genetic links we find. Indeed, the one instance of haplotype sharing in this study is between these two populations, and melt-water streams have been suggested as the primary dispersal mechanism for springtails in Victoria Land (Nolan *et al.* 2006).

In summary, we interpret the phylogeographic structuring, haplotypic diversity and limited inter-population mixing seen for *D. klovstadi* to result from long-term historical fragmentation and isolation coupled with limited gene flow for this endemic Antarctic springtail. In addition, multiple refugia are likely to have allowed persistence of *D. klovstadi* populations in northern Victoria Land since the initial glaciation of Antarctica and throughout subsequent glacial cycles.

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