Phylogeny, phylogeography and conservation genetics of the *brachyotis* group of rock-wallabies.

Petrogale brachyotis (short-eared rock-wallaby)

Submitted by

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THESIS SUMMARY

This thesis explores phylogenetic relationships amongst rock-wallabies, genus *Petrogale*, throughout Australia and in particular the phylogeographic relationships of three species (*brachyotis* taxonomic group) from north-west Australia. A combination of nuclear and mitochondrial DNA markers have been utilised to assess evolutionary history at several spatial scales. The first data chapter assesses broad scale relationships amongst rock-wallaby species with subsequent chapters examining the impacts of biogeographic processes on genetic diversification within the *brachyotis* group and conservation implications. These studies have increased our understanding of rock-wallaby evolution and provide valuable data to support the recognition of multiple species within *Petrogale brachyotis* (short-eared rock-wallaby). It has also established new hypotheses about the relationships of *P. burbidgei* (monjon) and *P. concinna* (nabarlek) to *P. brachyotis* within the *brachyotis* group. Although *P. brachyotis* is widespread, we have found this species is highly divergent across its range, with future management needing to ensure the survival of multiple highly diverse genetic lineages.

The phylogenetic analysis (chapter two) identified four distinct clades within *Petrogale*, with three comprising taxa with the ancestral karyotype (2n=22). The *brachyotis* group was the first to diverge and phylogenetic relationships within this lineage suggest the need for a focused phylogeographic study of this group and the likelihood of taxonomic revisions. There was support for *P. purpureicollis* being reinstated as a full species and *P. concinna* being classified within *Petrogale* rather than the monotypic genus *Peradorcas*. Ancestral habitat reconstructions suggested ancestral *Petrogale* were originally widespread across Australia and have undergone vicariance as a result of isolation caused by environmental/climatic changes during the Plio-Pleistocene.

The third chapter concentrated on north-west Australia, the main focus of this thesis, and its numerous proposed biogeographic barriers which have remained largely untested by phylogeographic studies. This thesis provides the first evidence of how these barriers have profoundly influenced the genetic differentiation of mammals within north-west Australia. Rock-wallabies, with their habitat specificity and naturally low gene flow are a good indicator

species for biogeographic barriers and provide good evidence of how past climatic cycles during the Plio-Pleistocene have influenced genetic differentiation across northern Australia.

The fourth chapter was more localised and focused on the genetic diversity of *P. brachyotis* within the Kimberley. This study provided evidence of greater connectivity of populations than previously recorded for any other rock-wallaby, highlighting how suitably connected habitat can allow dispersal of rock-wallabies across large distances. Large genetic differentiation was detected between the East and West Kimberley *P. brachyotis*, supporting the need for a reassessment of taxonomic classifications and conservation units within *P. brachyotis*.

The final data chapter examined the conservation status of the *brachyotis* group of rock-wallabies and outlined taxonomic reclassifications of *P. brachyotis*. This analysis provided evidence for division of *P. brachyotis* into two species. It is likely that additional taxonomic changes will be necessary in the future as further sampling and analyses are undertaken. Although this thesis has significantly advanced our understanding of the relationships within the *brachyotis* group, it also highlights the need for future work on this group of rock-wallabies.

STATEMENT OF AUTHORSHIP

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution to Sally Potter and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

This thesis represents an original and independent piece of research. All significant aspects of analysis and interpretation of results were done by myself. The thesis is presented as a series of papers. The nature of the collaborations indicated by the co-authorship of these papers takes two forms: 1) Authors D.A. Taggart, M.D.B. Eldridge and S.J.B. Cooper were included in recognition of the contribution they have made to my training as my supervisors. 2) Author C.J. Metcalfe was included as co-author in recognition of her providing *Cytb* sequence data. 3) Author J.Z. Paplinska was included as co-author in recognition of her training in genotyping microsatellites from rock-wallaby faecal samples and expert advice. I carried out all further analyses of this material. These contributions in no way diminish the originality or my overall contribution to the thesis.

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Sally Potter

CHAPTER 1: Introduction

Molecular genetics has played an integral role in increasing our understanding of biological diversity and its origins. The process of generating biodiversity starts with the development of population differentiation which can then lead to reproductive isolation and ultimately speciation (Beheregaray and Caccone 2007). Recent advances in molecular technology and statistical analysis (e.g., Hey and Nielsen 2004; Yang and Rannala 2006; Drummond and Rambaut 2007; Liu and Pearl 2007) from those outlined by Avise (1989), have improved the ability for genetic comparisons to be made across (phylogenetics) and within species (population genetics and phylogeography). The wealth of resulting data has not only proven useful in systematics (Templeton 1980; Avise 1994) but has increased our knowledge of the evolutionary processes that have generated biological diversity around the world (Avise and Walker 1998; Knowles and Maddison 2002).

As the rate of molecular evolution varies between different regions of the genome (e.g., nuclear and cytoplasmic), evolutionary divergence can be assessed at different hierarchical levels to elucidate different fundamental biological processes throughout the history of the organism (Riddle 1996). Across this broad framework, molecular markers have been used to examine a single population, quantifying genetic variation and estimating inbreeding and relatedness levels; to assess multiple populations, identifying structure and gene flow, sex-biased dispersal and mating systems and studying phylogeographic patterns within species using coalescent processes (Avise 2006). The discovery of the polymerase chain reaction (PCR) in the 1990s (Saiki *et al.* 1988; White *et al.* 1989; Mullis 1990; Erlich *et al.* 1991; Erlich and Arnheim 1992) greatly facilitated the direct sequencing of DNA which produced a revolution in our understanding of intra- and inter-specific differentiation (e.g., micro- and macro-evolution) (Avise 1994).

Micro- and macro-evolution are linked through the study of phylogeography, the geographical distribution of genealogical lineages, uniting phylogenetic relationships of populations within a species with the geographical distributions of those phylogenetic groupings (Avise *et al.* 1987; Avise 2000), and so elucidating historical demographic and evolutionary processes (Avise *et al.* 1987; Avise 1989, 2000; Riddle and Haffner 2006; Beheregaray 2008; Avise

2009; Hickerson *et al.* 2009; Joseph and Omland 2009). Phylogeography can be used to test biogeographic hypotheses, evolution of population differentiation, reproductive isolation and speciation. In addition, since the demographic history of a population influences its genealogy, inferences can also be made about temporal changes in the environment and its impact upon a population (Beheregaray 2008). It is these capabilities of phylogeography which have allowed it to contribute to our knowledge of speciation (Avise *et al.* 1998; Moritz *et al.* 2000; Hewitt 2001), historical biogeography (Avise 2000; Riddle and Haffner 2006), conservation biology (O'Brien 1994; Avise and Hamrick 1996; Smith and Wayne 1996; Moritz and Faith 1998; Fraser and Bernatchez 2001; Frankham *et al.* 2002) and taxonomy (Taberlet 1998; Beheregaray and Caccone 2007). Therefore, understanding the evolutionary history of species and the causes of their divergence can be assessed across a continuum, from population genetics and phylogeography, to phylogenetics and historical biogeography. The integration of phylogeography and phylogenetics using coalescent processes and species tree approaches will allow a greater interpretation of genetic patterns and diversity (Edwards 2009). Only together do these studies lead to a greater understanding of the evolutionary processes shaping biomes across the world and provide the necessary data to aid planning for biodiversity conservation under future environmental change (Moritz 1994; Hewitt 2004c; Byrne *et al.* 2008; Joseph and Omland 2009).

Macroevolution

Braby (2008) suggested a good understanding of taxonomy, systematic relationships and geographical distribution of biodiversity is required to elucidate biogeographical patterns and formulate hypotheses that can be tested using molecular approaches (phylogenetic and phylogeographic methods). The search to understand the processes that generate biological diversity and the differentiation of species requires a phylogenetic framework (Hillis and Bull 1991; Avise 1994). Interspecific variation (known as phylogenetics) assesses the genetic diversity between species to determine the evolutionary history of a group of reproductively isolated species or higher taxa (Avise 2009). Factors including mutation, selection, migration and genetic drift all impact the evolution of gene sequences and ultimately organisms (Avise 1994).

Phylogeographical studies have been used to address questions about the origins of lineages/taxa to delimit species boundaries, to test hypotheses about historical climatic changes on speciation, as well as to examine population dynamics at different spatial and temporal scales (Hickerson *et al.* 2009). Reconstructing the history of speciation has been improved through molecular dating methods, where ages at ancestral nodes in a phylogenetic tree can be estimated, clarifying how historical patterns and processes have influenced divergence and speciation of extant taxa (Avise 1998; Barraclough and Nee 2001). Divergence estimates based on fossil records or palaeo-distributions offer the opportunity to correlate environmental changes to the evolutionary processes leading to current genetic diversity and structural assemblages (Yang and Rannala 2006; Donoghue and Benton 2007; Swenson 2008; Moussalli *et al.* 2009). Where multiple co-distributed species are examined concordant vicariance patterns can be used to infer a general pattern of speciation, revealing the nature of cladogenesis and establishing common extrinsic causes (Cracraft 1982; Avise *et al.* 1987; Riddle 1996; Bermingham and Moritz 1998; Arbogast and Kenagy 2001; Ford and Blair 2005; Joseph and Omland 2009). Comparative phylogeographic studies are important in informing conservation management, through their identification of current biodiversity hotspots and the location of refugia (Bermingham and Moritz 1998; Moritz and Faith 1998; Avise 2000; Arbogast and Kenagy 2001; Kutcha and Meyer 2001; Moritz 2002; Beheregaray and Caccone 2007; Beheregaray 2008).

Concordant phylogeographic patterns among taxa can also be used to predict genetic variation of co-distributed organisms that are yet to be sampled (Avise 1998; Garrick *et al.* 2006) and can therefore be used as surrogate taxa for conservation planning (Schneider and Moritz 1999; Barraclough and Nee 2001; Hewitt 2004c; Kirchman and Franklin 2007). There are characteristics of some species which make them more useful as surrogates. For example, less mobile and more specialist taxa have been suggested as being more sensitive to the historical influence of climate change (Hewitt 2004a & b; Sunnucks *et al.* 2006). This is because limited dispersal facilitates geographic and genetic differentiation, with sex-biased dispersal and philopatry also helping to preserve genetic patterns created in the past (Cruzan and Templeton 2000). In addition, an assessment of cytoplasmic markers (chloroplast DNA and mitochondrial DNA) from organisms which exhibit such isolating characteristics can provide reconstructions of the historical processes leading to contemporary distributions (Cruzan and

Templeton 2000). As a result of phylogeographical studies, a number of scenarios have recognised glacial-interglacial cycles as mechanisms driving population differentiation and speciation through isolation of geographic populations (Vrba 1992; Riddle 1996; Schneider and Moritz 1999; Moritz *et al.* 2000; Hewitt 2001, 2004c). Numerous studies have found concordance in the contraction and survival of taxa in the same refugia and similar colonisation routes during subsequent range expansions (Avise 1992; Joseph *et al.* 1995; Schneider *et al.* 1998; Taberlet 1998; Hewitt 2001, 2004c).

As well as identifying processes driving population differentiation and speciation, molecular approaches have also enriched taxonomic classifications. In particular, the traditional biological species concept (BSC; species are characterised as "groups of actually or potentially interbreeding natural populations which are reproductively isolated from other such groups", Mayr 1963), has been enhanced by adding a phylogenetic/phylogeographic perspective to discussions of population relationships (Avise 1994). The BSC has been the most influential definition of species in population and evolutionary genetics, as well as in conservation biology (Frankham *et al.* 2004; Frankham 2010b) and remains the most widely applied. A particular complexity of other concepts such as the phylogenetic species concept, where species are "the smallest diagnosable cluster of individual organisms within which there is a parental pattern of ancestry and descent" (Cracraft 1983) concerns the type of evidence required to substantiate a monophyletic group warranting species recognition (Avise 1994). In contrast, the BSC allows a practical assessment based on genetic data by recognising the importance of genetic exchange, since species are defined as populations that exchange genetic material while those from different species do not (Frankham *et al.* 2004). Difficulties can still arise with the BSC, for example, defining allopatric species, but Frankham *et al.* (2004) suggests that calibration of genetic differentiation against other recognised species within the genus can in these circumstances be used for delineating species. However, it is clear that the most convincing species designations come from concordance of a wide array of information (e.g., morphology, chromosomes, nuclear and mitochondrial DNA) that demonstrates a lack of gene flow. Recent development of coalescent approaches (Yang and Rannala 2010) also provide future methods for species delimitation, with an integrative approach (e.g., phylogeographic, population genetic, morphological) the best method to resolve taxonomic uncertainties (Dayrat 2005; Yang and Rannala 2010).

Despite the controversy that exists over the appropriate „species concept" to use to delineate species, one commonality is clear: they are distinct entities which represent evolving lineages that we hope to conserve (Hey 2001).

Microevolution

Several processes influence intra-specific genetic variation, including; demographic processes, life-history traits, evolutionary forces, as well as historical biogeographic processes. Evolutionary forces include migration or gene flow, random genetic drift, mutation, recombination and natural selection (Avise 1994; Arbogast and Kenagy 2001). Demographic and life-history traits such as mating system, sex-biased dispersal and philopatry are also predicted to influence population differentiation (Avise 1994). The ability of phylogeographic approaches to examine intra-specific as well as inter-specific relationships has enabled this discipline to link population genetic analyses to phylogenetic analyses, extending comparative research to explore historical impacts on co-distributed species, as well as gene lineages within single species (Avise *et al.* 1987; Riddle 1996; Arbogast and Kenagy 2001). At a population genetic scale phylogeography has identified genetic structure and diversity of populations, gene flow, migration and dispersal patterns, concentrating on population and species responses to contemporary evolutionary processes and recent paleoclimatic events compared to the more historical processes of biological diversification (Bermingham and Moritz 1998; Kidd and Ritchie 2006).

Landscape characteristics also influence the distribution of organisms and their genetic diversity, ultimately affecting their long-term persistence (Fischer and Lindenmayer 2007). In converse, genetic structure can provide valuable insight into biological and ecological processes in a landscape (Holderegger and Wagner 2008). Factors impacting regional and local diversity include; dispersal ability, habitat specificity, population sizes and area requirements. These factors have been suggested to influence the long term response of taxa to past climatic fluctuations and environmental changes (e.g., local extinctions and recolonization) as well as future vulnerability (Braithwaite and Muller 1997; Hedrick 2000; Moussalli *et al.* 2009).

Advancement of molecular analyses (e.g., coalescent based methods) has allowed greater understanding of population genetics in respect to more recent historical events (e.g., patterns of migration and gene flow), increasing interpretation capabilities about intra-specific geographic variation (Hudson 1990; Crandall and Templeton 1993; Avise 1994; Cruzan and Templeton 2000; Hare 2001; Hey and Nielsen 2004; Knowles 2004; Emerson and Hewitt 2005). As a result of such sophisticated analytical methods (Edwards and Beerli 2000; Knowles and Maddison 2002), we now have the ability to estimate levels of gene flow or migration, population sizes and divergence times between populations using coalescent approaches (Hey and Nielsen 2004). This allows for evaluation of genetic connectivity but has the ability to be associated with demographic events linked to genealogical processes within a species, aiding our understanding of the processes involved in shaping speciation (Beheregaray and Caccone 2007).

Modern genetic markers (e.g., DNA sequences, microsatellites) have enhanced the ability of population genetics approaches to explore the evolutionary processes that have generated biological diversity (Sunnucks 2000; Hare 2001). Mitochondrial DNA (mtDNA) is the most commonly used molecular marker in animal phylogeographic studies as it shows little or no recombination, is present in high copy number in each cell, and has a rapid evolutionary rate which allows greater resolution of intra-specific patterns (Brown *et al.* 1979; Avise *et al.* 1987; Moore 1995; Avise 2000). By providing the first data widely used to reconstruct „gene genealogies" at the inter- and intra-specific level, mtDNA methods have enabled a link to be developed between micro- and macro-evolution (Avise 1989, 1994). As mtDNA is maternally inherited it has an effective population size one-fourth that of the nuclear genome and therefore takes less time for polymorphisms to become fixed in a population (Avise *et al.* 1987). Microsatellites have also been described as extremely informative DNA markers for inferring population genetic structure (Zhang and Hewitt 2003). Like mtDNA, the fast evolutionary rate allows these markers to differentiate between closely related species and individuals, shedding light on the recent history of taxa (Hebert *et al.* 2003).

Although very useful and widely used, mtDNA genealogies need to be interpreted with caution as they only present a single inference about the history of a population (and therefore only one estimate of the evolutionary scenario of a population). They can provide misleading

interpretations of the evolutionary history of an organism, especially when sex-biased dispersal or strong mating selection is present (Niegel and Avise 1986; Cronin 1993; Moritz 1994; Moore 1995; Hoelzer 1997; Bermingham and Moritz 1998; Hare 2001; Zhang and Hewitt 2003; Ballard and Whitlock 2004). In addition, saturation of nucleotide substitutions in mtDNA can inhibit the ability to distinguish accurate divergence estimates (Brown *et al.* 1979) and mitochondrial pseudogenes have been found in the genome of animals and sometimes have resulted in erroneous results and conclusions (Zhang and Hewitt 2003). Stochastic sorting of lineages, retention of ancestral haplotypes, hybridisation and introgression are all factors which may influence the gene genealogy of any given locus (Ferris *et al.* 1983; Tajima 1983; Niegel and Avise 1986; Pamilo and Nei 1988; Cronin 1993; Brower *et al.* 1996; Wilkins 2004; Kuo and Avise 2005; Joseph and Omland 2009), therefore testing hypotheses about the evolutionary history of a population requires multiple independent loci (population demography influences all neutral loci, whereas selection acts locally within a genome) (Fu and Li 1999; Avise 2000; Hare 2001; Luikart *et al.* 2003). It is well recognised that analysis of multiple unlinked loci is essential for characterising coalescent stochasticity and improving our knowledge of demographic history and divergences estimates (Edwards and Beerli 2000; Hare 2001; Knowles 2004; Beheregaray 2008). Analysis of both nuclear and cytoplasmic DNA is used in population genetic studies due to their differences in transmission and their evolution, which can reveal different aspects of population biology (Sunnucks 2000) and their ability to separate historical and contemporary processes that influence genetic diversity (Vandergast *et al.* 2007). Despite nuclear loci being expected to have less pronounced phylogeographic structure than cytoplasmic loci (due to their lower mutation rates and larger effective population sizes), they still provide valuable information about deeper coalescent processes and can help to elucidate processes that may lead to diversification and speciation (Wakeley and Hey 1997; Fu and Li 1999; Hare 2001; Nichols 2001).

Molecular genetics and conservation

Conserving biodiversity is greatly facilitated by an accurate understanding of taxonomy (Avise 2006). The correct identification and classification of species is often a vital and significant first step in conservation, since methods for species recovery usually require

adequate knowledge of species biology and factors that impact its survival (Mace 2004). Molecular systematics, phylogenetics and phylogeography have had an integral role in recent taxonomy, uncovering new species, resolving taxonomic uncertainties and identifying the most appropriate priorities and scale for conservation management (Avise 1989; Templeton 1994; Moritz 1995; Hedrick *et al.* 1996; Dubois 2003; Frankham *et al.* 2004; Frankham 2010a). The importance of taxonomy in conservation is emphasized further in the political arena, with government legislation using taxonomic distinctions as the basis for legal protection and management. For example, under the USA *Endangered Species Act 1973* (O'Brien 1994) and within Australia, the *Environmental Protection Biodiversity Conservation* (*EPBC*) *Act 1999* recognises species (including subspecies and distinct populations) for the purposes of the Act, along with State legislations (e.g., New South Wales *Threatened Species Conservation Act 1995*; Victoria *Flora and Fauna Guarantee Act 1988*; Western Australia *Wildlife Conservation Act 1950*; South Australia *National Parks and Wildlife Act 1972*).

For numerous species, a large proportion of the information required to conserve them is difficult to obtain directly, therefore genetic considerations play a central role in identifying risks to wild populations (Moritz and Faith 1998). Genetic diversity represents an essential component of biodiversity and as such, affects the ability of a populations to respond to environmental change (Frankham *et al.* 2002, 2004). The loss of diversity, effects of inbreeding, random genetic drift and accumulation of deleterious mutations are all associated with small populations and can cause declines and extinctions (Frankham *et al.* 2004). Therefore, understanding genetic differentiation within a species can be of importance to their conservation. Phylogeographical research has provided a valuable means to identify genetic differentiation within species and especially to detect genetically distinct and historically isolated populations (e.g., Evolutionarily Significant Units; ESUs) which can form a foundation for conservation planning (Avise 1994; Moritz 1994).

Alongside the continuing debate over „species concepts" lies a similar debate over the recognition and definition of ESUs within species (Crandall *et al.* 2000; Moritz 2002). These controversies reflect the continuum of processes between population differentiation and reproductive isolation as well as the time-scales over which this is achieved (Avise and Wollenberg 1997; deQueiroz 1998; Moritz 2002). Currently the most widely accepted and

applied definitions are those of Moritz (1994) who defined ESUs as "reciprocally monophyletic for mtDNA alleles and show significant divergence of allele frequencies at nuclear loci," avoiding the problem of determining how much divergence is sufficient. This definition infers that lineages (populations) that are historically isolated with minimal or no gene flow have independent evolutionary histories. Management units (MUs) were also defined by Moritz (1994) to represent populations that show evidence for restricted gene flow and demographic independence but may not represent an ESU. Management units are defined as "populations with significant divergence of allele frequencies at nuclear or mitochondrial loci, regardless of the phylogenetic distinctiveness of the alleles" (Moritz 1994). Management units are important for short term management addressing current population dynamics, whereas the ESUs are concerned more with long term management of historical population structure. These concepts were introduced to overcome the existing taxonomic conflict of defining species and the fact that some taxonomic labels (e.g., subspecies) may not accurately reflect the genetic diversity present within a species (Avise 1989; Moritz 1994; Fraser and Bernatchez 2001).

Biogeographical and evolutionary processes in northern Australia

A recent review by Beheregaray (2008) identified a wealth of phylogeographic data for organisms in the Northern Hemisphere (77% of empirical surveys). In comparison, the Southern Hemisphere is poorly explored and Australian mammals only account for 4% of the phylogeographic publications of mammals world-wide (Beheregaray 2008). Within Australia, the Wet Tropics of north-east Queensland is a good example of an intensely studied system that has incorporated phylogeographic analyses for a range of organisms in a comparative framework to reveal extensive insights into the regions historical processes (Joseph *et al.* 1995; Schneider *et al.* 1998; Hugall *et al.* 2002; Dolman and Moritz 2006; Moussalli *et al.* 2009). Utilising pollen records and fossil charcoal as evidence to estimate habitat changes, including contractions and expansions during fluctuating glacial periods (Kershaw 1983, 1993, 1994; Kershaw and Nix 1988; Hopkins *et al.* 1993; Hopkins *et al.* 1996), has uncovered connections between current species distributions and biogeographical processes. As a result, the impacts of pre-Pleistocene/Pleistocene and late-Quaternary climate fluctuations on spatial patterns of species and genetic diversity have emerged (Schneider *et al.* 1998; Schneider and

Moritz 1999). Evidence across a variety of taxa suggests that regional patterns of genetic diversity are largely determined by local extinctions and recolonization events driven by these fluctuating climatic changes, but differences in the degrees of diversity found across taxa highlights the importance of species-specific ecological characteristics (Moussalli *et al.* 2009)**.** More recently studies have begun to focus on the arid zone of Australia (discussed by Byrne *et al.* 2008) and some significant early patterns have begun to emerge. Complex patterns of contraction into localized refugia and subsequent expansions driven by dramatic fluctuations in aridity and temperature during the Plio-Pleistocene appear to have profoundly shaped the diversity of arid zone biota (Byrne *et al.* 2008). A rapid and recent expansion of arid zone endemics has been detected, with intra-specific diversification during the Pleistocene linked to hybridisation, parthenogenesis and polyploidy in some groups (Cooper *et al.* 2000; Byrne *et al.* 2001; Mummenhoff *et al.* 2004; McLeish *et al.* 2007; Byrne *et al.* 2008). Earlier divergence times (Pliocene or Late Miocene) have also been suggested for diplodactyline geckos (Oliver *et al.* 2007), amphipods (Cooper *et al.* 2007) and dasyurid marsupials (Blacket *et al.* 2001).

In comparison to numerous studies in the Australian arid zone and the Wet Tropics, the north-western region of Australia, from the Kimberley across to the Northern Territory, has received little attention. Broad biogeographic hypotheses across northern Australia were suggested by Cracraft (1982), with a vicariance hypothesis suggesting vicariance barriers between the Kimberley and Arnhem Land, and between these regions and Cape York (based on endemic bird taxa). These vicariant barriers have more recently been supported by Jennings and Edwards (2005) using nuclear gene phylogenies. A recent review by Bowman *et al.* (2010), however, identified limited biogeographical, phylogenetic and ecological studies of terrestrial plants and animals from this region. In light of numerous knowledge gaps for northern Australia, a focus is needed on utilising modern phylogenetic and phylogeographic methods to elucidate and test biogeographical patterns and hypotheses. The landscape of north-western Australia still remains relatively intact which allows a unique opportunity to assess natural processes of diversification in this area of high biodiversity (Braby 2008; Bowman *et al.* 2010). However, there are concerns emerging with widespread declines in small to medium-sized mammals recorded (Braithwaite and Griffiths 1994, 1996; Woinarski *et al.* 2001; Price *et al.* 2005) throughout the region. This has been associated with the

introduction of exotic herbivores (e.g., cattle), cats, land clearing and an increase in the frequency and intensity of fires, causing increased predation from a lack of cover and reduced resources (Burbidge and McKenzie 1989; Short and Smith 1994; Woinarski *et al.* 2001; McKenzie *et al.* 2007; Start *et al.* 2007; Legge *et al.* 2008).

Proterozoic sandstone dominates north-western Australia and forms a series of dissected escarpments amongst a range of habitats which are separated by areas of less complex topography. These flat lowland areas have been identified as potential biogeographic barriers to a wide variety of fauna, with the most obvious being Daly River and Bonaparte Gulf regions which separate the Kimberley and Arnhem Land (Northern Territory) Plateaus (Ford 1978; Bowman *et al.* 2010). A lack of fossils and palaeoecological information from this region (Bowman *et al.* 2010) makes it difficult to understand biogeographical patterns. However, phylogeographical studies offer an opportunity to assess geographical patterns and evolutionary history, and the ability to relate this to climatic and geological events by estimating divergence times using molecular clock methods. The Australian monsoon tropics have been suggested as the continent‟s last biological frontier (Braby 2008). We therefore need to concentrate resources to understand biogeographical patterns and processes in order to elucidate how current threats may impact biodiversity, and allow sustainable development of the region through effective conservation planning (Bowman *et al.* 2010).

Rock-wallabies: taxonomic history

The endemic Australian marsupial genus, *Petrogale* (rock-wallabies), provides an excellent example of how the use of genetic data can help resolve taxonomic uncertainties and define genetic units for management (Briscoe *et al.* 1982; Eldridge and Close 1993; Browning *et al.* 2001; Campeau-Péloquin *et al.* 2001; Eldridge *et al.* 2001c). Rock-wallabies have experienced a long history of taxonomic confusion and uncertainty with changes in the number of species recognised ongoing for decades. Frankham *et al.* (2004) described rock-wallabies as "populations „caught in the act‟ of speciating," with species and populations showing varying degrees of differentiation (e.g., morphology, chromosomes, allozymes and mtDNA). The complex taxonomic history of *Petrogale* taxa has largely been attributed to the group‟s recent and rapid radiation (0.5-3.7 million years ago) (Eldridge and Close 1993;

Campeau-Péloquin *et al.* 2001), which has resulted in many taxa being difficult to distinguish on traditional morphological criteria (e.g., pelage and skull morphology) (Eldridge and Close 1993). The application of molecular genetics to rock-wallaby systematics resulted in a significant shift towards stability in *Petrogale* taxonomy, with comprehensive genetic studies establishing a greater understanding of *Petrogale* inter-relationships and taxonomy (Briscoe *et al.* 1982; Calaby and Richardson 1988; Sharman *et al.* 1990; Eldridge and Close 1992, 1997; Bee and Close 1993; Eldridge *et al.* 2001c). Chromosomal analysis has been proposed as a reliable method for identifying *Petrogale* taxa (refer to Table 1.1), due to the group‟s exceptional karyotypic diversity and the inability to distinguish some taxa by traditional means (Sharman *et al.* 1990; Eldridge and Close 1992; Eldridge *et al.* 1992a & b; Eldridge 1997b). The remarkable chromosomal diversity of rock-wallabies has focused research on the inter-relationships of these taxa using a variety of techniques, including cytological analyses (C- and G-banding, chromosome painting), molecular analyses (allozyme and mitochondrial DNA restriction site electrophoresis) and studies of endo-parasites (Briscoe *et al.* 1982; Sharman *et al.* 1990; Bee and Close 1993; O'Neill *et al.* 2004). Cytogenetics, allozymes and mtDNA have also provided evidence to suggest speciation in this complex is ongoing with the introgression of genetic markers occurring between some species (Bee and Close 1993). The five north-east Queensland species (*P. assimilis*, *P. coenensis*, P. *godmani*, *P. mareeba*, *P. sharmani*) cannot be reliably distinguished by means other than their karyotypes, and although male hybrids are typically sterile and females sub-fertile they have similar or overlapping mtDNA and allozyme profiles (Eldridge and Close 1992; Bee and Close 1993), indicative of recent speciation or evidence for introgression among „species“. When speciation of an ancestor in two daughter species is recent, allelic differentiation between the descendant species may not be detectable (Avise 1994). Despite the considerable research effort and progress made in *Petrogale* systematics, relationships within this genus are still contentious with previous analyses unable to resolve some relationships, or not including important taxa (Eldridge and Close 1992; Campeau-Péloquin *et al.* 2001). Since 1837, 26 rock-wallaby taxa (species/subspecies) have been described (reviewed in Eldridge 1997b), with 16 species currently recognised (Van Dyck and Strahan 2008), divided into 21 chromosomal races (Eldridge and Close 1997).

Table 1.1: Summary of chromosome data (chromosomes 1-10) for *Petrogale* taken from Eldridge and Close (1997), excluding X chromosome illustrations (refer to Eldridge and Close 1997; idiogram of karyotypes). The ancestral karyotypes (*xanthopus* chromosomal group) are given in the top section, the *lateralis-penicillata* chromosomal group in the next section and the *brachyotis* chromosomal group in the bottom section. Chromosome number is given in the last column (2n). * = unchanged; e = addition of euchromatic short-arms; a = change to acrocentric; s and sm = change to subacrocentric; m = change to metacentric; i = inversion; - = fused to another chromosome.

Rock-wallabies are small-medium sized marsupials (1-12kg) and form one of the largest groups of extant macropodids. They are distributed across Australia, including some continental islands and inhabit complex rocky environments such as cliffs, gorges, outcrops and escarpments (Sharman and Maynes 1983; Burbidge and McKenzie 1989; Van Dyck and Strahan 2008). Suitable rocky areas can be set amongst a variety of habitats, ranging from rainforests and tropical woodlands, to dry open forest and deserts (Van Dyck and Strahan 2008). The patchy distribution of rock-wallabies, due to their habitat specificity, results in many populations experiencing periods of isolation. This isolation has been proposed to have facilitated the fixation of novel mutations and chromosomal rearrangements (Eldridge and Close 1993) thereby increasing their rate of speciation. An Australia-wide distribution, rapid and recent diversification, as well as exceptional chromosomal diversity, makes *Petrogale* an important model genus for studies of evolution, speciation and biogeographical processes within Australia.

Historically, *Petrogale* taxa have been divided into three chromosome groups, based on karyotypic similarity. The *brachyotis*, *lateralis-penicillata*, and *xanthopus* groups (refer to Table 1.1; Briscoe *et al.* 1982), remain in use today and only limited changes have been made to *Petrogale* taxonomy since (Calaby and Richardson 1988; Eldridge and Close 1992; Eldridge *et al.* 2001c). The *brachyotis* group consists of three species; *P. brachyotis, P. burbidgei* and *P. concinna*, distributed across north-western Australia (see Fig. 1.1). It is chromosomally the most divergent group of *Petrogale,* with all species sharing highly derived karyotypes that are distinct from all other rock-wallabies (Eldridge *et al.* 1992b; Eldridge and Close 1997; Campeau-Péloquin *et al.* 2001). The *brachyotis* group has received little attention in molecular systematic studies compared to other *Petrogale* species (Sharman *et al.* 1990; Eldridge *et al.* 1991, 2001; Eldridge and Close 1992; Bee and Close 1993; Pope *et al.* 1996; Eldridge 1997).

The *lateralis-penicillata* group consists of two recently differentiated species complexes, a western *lateralis* and an eastern *penicillata* complex, but relations of taxa within these complexes may be paraphyletic (Eldridge *et al.* 1991b). The *lateralis* complex consists of six chromosomally distinct taxa distributed throughout western and central Australia; *P. lateralis*

lateralis, P. lateralis West Kimberley race*, P. lateralis* MacDonnell Ranges race, *P. l. pearsoni, P. l. hackettii* and *P. purpureicollis* (Sharman *et al.* 1990; Eldridge *et al.* 2001c; see Fig. 1.1). The *penicillata* complex consists of eight chromosomally distinct species; *P. assimilis, P. coenensis, P. godmani, P. herberti, P. inornata, P. mareeba, P. penicillata, P. sharmani*, distributed parapatrically down the Great Dividing Range in eastern Australia (Eldridge and Close 1997; Fig. 1.1). The position of *P. purpureicollis* within the *lateralis-penicillata* group has been controversial. It has been proposed as the sister group to the *penicillata* complex based on DNA/DNA hybridisation (Campeau-Péloquin *et al.* 2001) and preliminary mtDNA control region sequence data (Eldridge *et al.* 2001c), although chromosome and allozyme data had placed it as a member of the *lateralis* complex (Sharman *et al.* 1990).

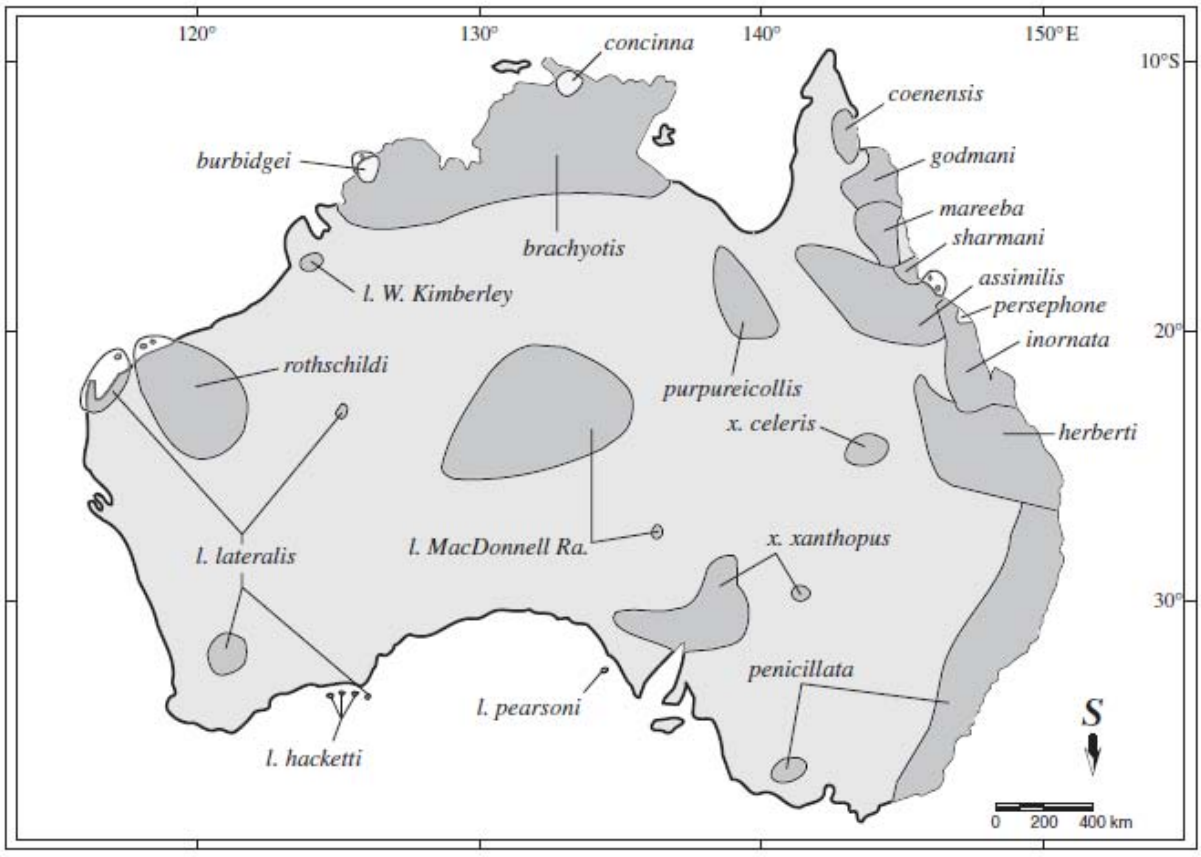

Figure 1.1: Map of *Petrogale* species distributions in Australia. Modified from Eldridge and Close (1993).

The *xanthopus* group consists of three large rock-wallaby species; *P. xanthopus, P. rothschildi* and *P. persephone* (see Fig. 1.1)*;* which all retain the ancestral (plesiomorphic) macropodine karyotype (2n=22) (Eldridge *et al.* 1992a; Eldridge and Close 1997). Campeau-Péloquin *et al.* (2001), using DNA/DNA hybridisation data, suggested that this was not a monophyletic group, with the affinities of *P. rothschildi* appearing to lie with the *lateralis-penicillata* group. However, the DNA/DNA hybridisation methodology was not completely effective for *P. rothschildi* (Campeau-Péloquin *et al.* 2001), and their conclusions were not definitive. The suggested close relationship of *P. persephone* and *P. xanthopus* (Maynes 1982) was also questioned in this DNA/DNA hybridisation study (Campeau-Péloquin *et al.* 2001), although the position of *P. persephone* and *P. xanthopus* in the phylogenetic trees was unstable and better resolution will be required to make any definitive conclusions of their relationship.

Numerous hypotheses have been proposed for the biogeographic history of *Petrogale*. Briscoe *et al.* (1982) suggested *Petrogale* originated in Western Australia with two invasions into the Great Dividing Range in eastern Australia. In contrast, Eldridge and Close (1997) suggested a *Thylogale*-like ancestor from eastern Australia, with movement west and a return to the east where chromosomally divergent lineages are now parapatric with the ancestral karyotype. *Petrogale* have frequently been associated with the relatively unspecialised macropodid genus *Thylogale* (pademelons), because they share a similar karyotype (Kirsch 1977; Briscoe *et al.* 1982; Sharman *et al.* 1990; Kirsch *et al.* 1995). However, *Dendrolagus* (tree-kangaroos) from New Guinea and north-east Queensland have also been suggested as the sister-group of *Petrogale* from a variety of different molecular studies (Ziegler 1977; Baverstock *et al.* 1989; Kirsch *et al.* 1997). It is now widely accepted that *Dendrolagus* is the sister taxon to *Petrogale* (Campeau-Péloquin *et al.* 2001; Westerman *et al.* 2002), with the most recent support coming from extensive marsupial nuclear DNA phylogenetic analyses (Meredith *et al.* 2008, 2009). However, the patterns of diversification within *Petrogale* remain unclear.

Avise (1994) highlighted that DNA/DNA hybridisation methods can fail to differentiate species and resolve inter-relationships, as shown within *Petrogale* (Campeau-Péloquin *et al.* 2001), where the phylogenetic positions of multiple taxa were unstable or the methodology proved ineffective. Enhanced resolution of species relationships with modern molecular data

is therefore required. In addition, the resolution of relationships amongst the basal *Petrogale* lineages (*P. persephone*, *P. xanthopus* and the *brachyotis* group) is needed to clarify the origins and biogeographic history of this genus. A greater understanding of the phylogenetic relationships within rock-wallabies will also provide useful information in a broader context by helping to elucidate the evolutionary and biogeographical processes that have been operating within Australia.

The *brachyotis* group of rock-wallabies

The *brachyotis* group of rock-wallabies consist of three species (*P. brachyotis*, *P. burbidgei* and *P. concinna*) restricted to north-western Australia (see Fig. 1.1). The distributions of both *P. burbidgei* and *P. concinna* are embedded within that of *P. brachyotis*. These are the only rock-wallaby species that occur sympatrically, making them of considerable evolutionary and ecological interest. Genetic data indicates that these three species form a monophyletic group (Briscoe *et al.* 1982; Sharman *et al.* 1990; Eldridge and Close 1997; Campeau-Péloquin *et al.* 2001), as their karyotypes are dramatically altered compared to all other *Petrogale*, sharing a suite of unique rearrangements including the addition of significant amounts of constitutive heterochromatin at the centromeres (Sharman *et al*. 1990; Eldridge *et al*. 1992b). Despite these similarities, *P. brachyotis, P. burbidgei* and *P. concinna* are each clearly differentiated as distinct species, being distinguished by a series of unique morphological and chromosomal features (Table 1.2). Their distributions also vary with *P. brachyotis* being the most widespread, extending across northern Australia from the Queensland/Northern Territory border to the west coast of the Kimberley, Western Australia; whereas *P. burbidgei* is confined to the coastal north-west Kimberley and *P. concinna* to higher rainfall regions of the Northern Territory and the Kimberley, Western Australia (Eldridge and Telfer 2008; Pearson *et al.* 2008; Sanson and Churchill 2008; see Fig. 1.1).

Table 1.2: Characteristics distinguishing members of the *brachyotis* group (*Petrogale brachyotis*, *P. burbidgei* and *P. concinna*). Chromosomal changes from the ancestral karyotype are taken from Eldridge and Close (1997), whilst the rest of the characteristics are summarised from *burbidgei* Kitchener and Sanson (1978), Eldridge and Telfer (2008), Pearson *et al.* (2008) and Sanson and Churchill (2008).

	P. brachyotis	***P. burbidgei***	***P. concinna***
Weight (kg)	3.7-4.4	<1.2	<1.4
Ears	Proportionally shorter compared to *P. burbidgei* and *P. concinna.* Usually less than half length of head.	Shorter than *P. concinna* (<35 mm).	More than 35mm.
Head stripe	Distinct head stripe (excluding Victoria River population).	Lack of a distinct head stripe.	Distinct head stripe.
Teeth	Maximum of four molars.	Maximum of four molars.	Supernumerary molars.
Chromosome 4	Submetacentric.	Ancestral.	Submetacentric.
Chromosome 5	Submetacentric.	Fused to chromosome 9.	Fused to chromosome 9.
Chromosome 8	Acrocentric, but with euchromatic short-arms.	Metacentric.	Metacentric.

Taxonomic boundaries and inter-relationships within the *brachyotis* group have long been controversial (Poole 1979; Briscoe *et al.* 1982; Calaby and Richardson 1988; Sharman *et al.* 1990; Sharman *et al.* 1995), with Eldridge (1997b) identifying the need for a detailed genetic (and morphological) study of this group as a priority. Recent genetic evidence suggests the taxonomy of the *brachyotis* group should be re-evaluated since exceptionally high levels of mitochondrial control region sequence divergence have been found within *P. brachyotis* (Telfer and Eldridge 2010). *P. brachyotis* is highly variable in morphology across its range (Sharman and Maynes 1983; Eldridge and Telfer 2008) and historically was described as five distinct taxa (4 separate species) (Sharman and Maynes 1983); *P. brachyotis* from Hanover Bay, Kimberley, WA (Gould 1841); *P. wilkinsi* (Roper River, NT) (Thomas 1926a); *P. longmani* (Groote Eylandt, off NT coast) (Thomas 1926a); *P. venustula* (Upper Daly River, NT) (Thomas 1926b)*;* and the subspecies *P. brachyotis signata* (South Alligator River, Mary River, Daly River region, NT) (Thomas 1926b). After much subsequent taxonomic confusion and uncertainty (see Eldridge 1997b), Sharman *et al.* (1990) synonymised all 4 taxa with *P. brachyotis* and recognised a single widespread species based on their shared, highly derived, unique 2n=18 karyotype. On morphological criteria it was subsequently suggested that *P. brachyotis* could be divided into three geographic races; a Kimberley race covering the Kimberley region, WA; a Victoria River race encompassing populations from the Victoria River district, NT; and an Arnhem Land race comprising populations formerly described as *P. longmani, P. venustula and P. wilkinsi* in the NT (Sharman and Maynes 1983), although Sharman *et al.* (1995) referred to these as populations rather than races.

Three subspecies of *P. concinna* have been described; *P. concinna concinna* (Victoria Rivers district, NT) (Gould 1842), *P. c. canescens* (Arnhem Land, NT) (Thomas 1909) and *P. c. monastria* (Kimberley region, WA) (Thomas 1926b). *P. c. concinna* is only known from the type specimen collected in 1839 and the other two subspecies have rarely been reported. *P. concinna* too has had a complicated taxonomic history, being removed from *Petrogale* to found the monotypic genus *Peradorcas* based on its presence of continuously erupting molars (Thomas 1904). However, more recent molecular evidence has not supported this arrangement, clearly placing *P. concinna* within *Petrogale* (Briscoe *et al.* 1982; Calaby and Richardson 1988; Eldridge 1997b), as a member of the *brachyotis* group. There is growing

concern about the conservation status of *P. concinna,* with evidence of ongoing declines (Briscoe *et al.* 1982; Churchill 1997) and its current status is unknown (Pearson 2009).

Petrogale burbidgei was only relatively recently discovered and described (Kitchener and Sanson 1978) and very little is known about its biology or population dynamics. Although populations of *P. burbidgei* and *P. brachyotis* currently appear stable, their future is under similar threats to those of other species in northern Australia (e.g., common brushtail possum, *Trichosurus vulpelcula* and northern quoll, *Dasyurus hallucatus*) (Kitchener 1978; Woinarski 1992; Braithwaite and Muller 1997; Woinarski *et al.* 2001) where it can no longer be assumed that any species is safe (Woinarski *et al.* 2001). At present northern Australia and especially the Kimberley is coming under increasing environmental pressure due to predation by feral cats, changed fire regimes and cattle grazing (Kerle 1998; Woinarski *et al.* 2001; Start *et al.* 2007). In addition, increased mining activity, cane toad invasion and climate change may exacerbate current environmental pressures. The conservation status of these north-west Australian rock-wallabies is either unknown or has not been reviewed in many years. It is therefore important to gain knowledge about ecological and genetic processes before they become threatened, like many of their congeners. Understanding their life-history strategies and evolutionary processes will assist in determining how threats may impact populations and in determining future management schemes to maintain genetic diversity and key populations. The ability to research *Petrogale* taxa before major declining impacts occurs, offers an important opportunity to understand the population biology of colonies free from the threatening impacts influencing many closely related taxa. As the threats against *Petrogale* species are increasing, it is important to maintain research efforts on individual species to ensure the survival of these endemic Australian species.

Since *P. brachyotis* inhabits rock outcrops across the Kimberley and northern Northern Territory, it provides an ideal candidate to examine the role of habitat discontinuities in promoting diversification in northern Australia. The habitat specificity of *Petrogale*, their small population sizes and limited dispersal (Hazlitt *et al.* 2004, 2006b; Piggott *et al.* 2006b) should make rock-wallabies sensitive indicators of long-term patterns of gene flow and so ideal candidates to elucidate historical biogeographic patterns and barriers. Therefore, *P. brachyotis* can provide useful information on the patterns of biodiversity in northern Australia

and when compared with co-distributed taxa should enable an increased understanding of the roles of biogeographic, demographic and evolutionary processes in generating current patterns of diversity.

Study objectives

The overall aim of this thesis is to use molecular data to understand the biogeographical processes driving speciation in *Petrogale,* in particular within the *brachyotis* group and use this information to inform future conservation planning. Specifically, this study aims to:

1. Examine the phylogenetic relationships among *Petrogale* species using mtDNA and nDNA sequences to assess the biogeographical history of the genus and clarify phylogenetic relationships (Chapter two).
2. Use *P. brachyotis* and *brachyotis* group taxa to examine the biogeographic patterns that promote differentiation in the tropical monsoon fauna of north-western Australia (Chapter three).
3. Assess the fine-scale population structure of *P. brachyotis* across the Kimberley using microsatellite loci and mtDNA sequence data to determine whether the East and West Kimberley represent genetically differentiated populations (Chapter four).
4. Examine phylogeographic relationships within the *brachyotis* group to assess the evolutionary processes involved in the differentiation of populations and use these molecular data to reassess current taxonomic classifications (Chapter five).
5. Use the molecular data to make recommendations for the effective conservation management of *brachyotis* group taxa (Chapter five).

Statement of Authorship

Sally Potter (candidate)

Corresponding author: Prepared DNA extracts for PCR amplification, carried out DNA sequencing, analysed sequence data, wrote manuscript and produced all Figures.

Signed Date 12-1-2011

Steve J.B. Cooper

Supervised the direction of the study and provided advice on analyses and critically reviewed manuscript.

I give consent for Sally Potter (candidate) to include this manuscript for examination towards the degree of Doctor of Philosophy.

Signed Date 12/1/2011

Cushla J. Metcalfe

Provided DNA sequences of *Cytb* for analyses.

I give consent for Sally Potter (candidate) to include this manuscript for examination towards the degree of Doctor of Philosophy.

Signed Date 11 Jan 2011

David A. Taggart

Provided assistance with obtaining project funding and evaluated the manuscript.

I give consent for Sally Potter (candidate) to include this manuscript for examination towards the degree of Doctor of Philosophy.

Signed Date 11 Jan 2011

Mark. D.B. Eldridge

Provided samples for study, provided *Cytb* sequences, provided assistance with obtaining project funding, supervised the direction of the study, provided advice on analyses and critically reviewed manuscript.

I give consent for Sally Potter (candidate) to include this manuscript for examination towards the degree of Doctor of Philosophy.

Signed Date 10 Jan 2011

CHAPTER 2: Phylogenetic relationships of *Petrogale* and their biogeographic history within Australia

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Abstract

The rock-wallaby genus *Petrogale* is a group of habitat specialist macropodids endemic to Australia. Their restriction to rocky outcrops, with concomitant infrequent interpopulation dispersal, has been suggested as the cause for their recent and rapid diversification. Molecular phylogenetic relationships within and among species of *Petrogale* were analysed using mitochondrial (cytochrome oxidase c subunit 1, cytochrome b, NADH dehydrogenase subunit 2) and nuclear (omega-globin intron, breast and ovarian cancer susceptibility gene) sequence data with representatives that encompassed the morphological and chromosomal variation within the genus, including for the first time both *P. concinna* and *P. purpureicollis*. Four distinct clades were identified, 1) the *brachyotis* group, 2) *P. persephone*, 3) *P. xanthopus* and 4) the *lateralis-penicillata* group, with three clades including taxa with the ancestral karyotype (2n=22). Paraphyletic relationships within the *brachyotis* group indicate the need for a focused phylogeographic study of this group with subsequent taxonomic changes likely. There was also support for *P. purpureicollis* being reinstated as a full species and *P. concinna* being placed within *Petrogale* rather than in the monotypic genus *Peradorcas*. Bayesian analyses of divergence times suggest that episodes of diversification commenced in the late Miocene-Pliocene and then continued throughout the Pleistocene. Ancestral state reconstructions suggest that ancestral *Petrogale* were originally widespread across Australia and have undergone vicariance as a result of environmental/climatic changes. These vicariance patterns correlate with the timing of radiations in other arid zone vertebrate taxa across Australia.

Introduction

The endemic Australian rock-wallaby genus *Petrogale* currently includes 16 species (Van Dyck and Strahan 2008) divided into 21 chromosomal races (Eldridge and Close 1997). Rock-wallabies are small-medium sized marsupials (1-12kg) and form one of the largest groups of extant macropodids. They are distributed across Australia, including some continental offshore islands, and one species (*P. penicillata*) has been successfully introduced into Hawaii and New Zealand (Tomich 1969; Kramer 1971; Van Dyck and Strahan 2008). As their name implies, rock-wallabies inhabit rocky habitats, including cliffs, gorges, outcrops and escarpments, where complex rock formations provide protection from the environment and predators (Sharman and Maynes 1983; Burbidge and McKenzie 1989). Suitable rocky areas can be set amongst a variety of habitats, ranging from rainforests and tropical woodlands, to dry open forest and deserts (Van Dyck and Strahan 2008). The patchy distribution of rock-wallabies, due to their habitat specificity, results in many populations experiencing periods of isolation. This is thought to have increased their rate of speciation, facilitating the fixation of novel mutations and chromosomal rearrangements (Eldridge and Close 1993). Their broad distribution across Australia, rapid and recent diversification, as well as exceptional chromosomal diversity, makes rock-wallabies an important model genus for studies of evolution, speciation and biogeography.

The genus *Petrogale* has experienced a long history of taxonomic confusion and uncertainty with the number of species recognised being in a state of flux for decades (reviewed in Eldridge 1997b). This history of uncertainty has been largely attributed to the group‟s probable recent and rapid radiation (0.5-3.7 million years ago) (Eldridge and Close 1993; Campeau-Péloquin *et al.* 2001), which has resulted in many taxa being difficult to distinguish on traditional morphological criteria (e.g., pelage and skull morphology). Since J.E. Gray described the first rock-wallaby species in 1837, 26 different taxa (species/subspecies) have been described. Rock-wallabies exceptional chromosomal diversity led to a major focus on the inter-relationships of taxa in the 1980s and 1990s. Briscoe *et al.* (1982) overhauled *Petrogale* molecular systematics, using allozyme and cytological analysis, recognising 10 species and 20 chromosomal races, assembled into three chromosome groups; the *brachyotis, lateralis-penicillata,* and *xanthopus* groups. These groups remain in use today and only

limited changes have been made to *Petrogale* taxonomy since (Calaby and Richardson 1988; Eldridge 1997b; Eldridge *et al.* 2001c), including the description of three species in 1992 (Eldridge and Close 1992).

Although considerable progress has been made, relationships within this genus are still contentious with previous analyses either unable to resolve some relationships, or not including important taxa (Eldridge and Close 1992; Campeau-Péloquin *et al.* 2001). Although originally described as *Petrogale concinna* (Gould 1842), this species was subsequently transferred to found the monotypic genus *Peradorcas* (Thomas 1904). Numerous authors have indicated that *concinna* should be returned to *Petrogale* (Poole 1979; Briscoe *et al.* 1982; Calaby and Richardson 1988; Sharman *et al.* 1990; Eldridge and Close 1993), but there remains a lack of consensus (Strahan 1983, 1995; Meredith *et al.* 2008, 2009). Hence, the phylogenetic position of *P. concinna* remains uncertain and the most recent phylogenetic study of *Petrogale* did not include this taxon (Campeau-Péloquin *et al.* 2001). Uncertainty also surrounds the hypothesised close relationship of *P. persephone* and *P. xanthopus* (Maynes 1982) as chromosome analysis was unable to resolve their relationship due to the presence of symplesiomorphies (Eldridge and Close 1993, 1997) and DNA/DNA hybridisation produced variable relationships based on inclusion or exclusion of other taxa in analyses (Campeau-Péloquin *et al.* 2001). The relationships of *P. rothschildi* and *P. purpureicollis* are also contentious, with chromosomal studies placing *P. rothschildi* in the *xanthopus* group based on a shared ancestral karyotype (Sharman *et al.* 1990; Eldridge and Close 1993) and *P. purpureicollis* being, until recently, regarded as a subspecies of *P. lateralis* (Briscoe *et al.* 1982; Sharman *et al.* 1990; Campeau-Péloquin *et al.* 2001). More recently, it was proposed that *P. rothschildi* was more closely related to *P. lateralis*, based on DNA/DNA hybridisation analysis, however, the *P. rothschildi* sample could not be adequately labelled so relationships were only estimated and therefore lack strong support (Campeau-Péloquin *et al.* 2001). The affinity of *P. purpureicollis* to other *Petrogale* taxa is also unresolved. *P. purpureicollis* was re-established as a full species in 2001 (Eldridge *et al.* 2001c) and it is unclear whether it is most closely related to the eastern *penicillata* complex or to the western *lateralis* complex (Sharman *et al.* 1990; Eldridge *et al.* 1991a; Campeau-Péloquin *et al.* 2001). Clarifications of these phylogenetic relationships are important in order

to understand the biogeographical history associated with the pattern and timing of diversification of *Petrogale* within Australia.

The radiation of *Petrogale* is thought to have occurred after the Pliocene, during a period, or periods, of increasing aridity, and the change from closed forest to more open woodlands and grasslands (Maynes 1989). It has therefore been suggested that the ancestral *Petrogale* was associated with a closed forest environment on the east coast of Australia, similar to that which the „relict" *P. persephone* currently inhabits (Eldridge and Close 1997). The earliest records of *Petrogale* are from the late Pliocene (Flannery and Archer 1984), with the earliest fossils recorded ~4.0 million years ago (MYA) in the Bluff Downs Local Fauna in north-east Queensland (Archer 1976). Molecular dating estimated a split between *Dendrolagus* (tree-kangaroos) and *Petrogale* at 7.5 MYA during the mid-Miocene (Campeau-Péloquin *et al.* 2001), a time when other Australian-New Guinean marsupial clades were estimated to have separated (Kirsch and Springer 1993; Krajewski *et al.* 1993, 2000). Prideaux and Warburton (2010) suggest this estimate could be slightly late, based on the major morphological differences between these two genera and the fact that *Dendrolagus* and *Bohra* (an extinct tree-kangaroo genus) had already separated prior to the start of the Pliocene, making the divergence of *Petrogale* and *Dendrolagus* sometime before that. The radiation of extant *Petrogale* was estimated to have occurred between 3.7 MYA and 0.5 MYA, during the mid-to-late Pliocene and Pleistocene (Campeau-Péloquin *et al.* 2001). However, these dates based on DNA/DNA hybridisations are indirect estimates of the divergence of DNA and ignore the variation in rates of evolution that occur in the different genes by taking an average.

Contrasting biogeographic hypotheses have been proposed, with Eldridge and Close (1997) suggesting that ancestral *Petrogale* dispersed from eastern Australia across to the west, with a subsequent return to the east, where the more recently derived *penicillata* complex species (e.g., *P. assimilis*) occupy drier habitats. An earlier proposal, however, suggested that *Petrogale* originated in the Western Shield (comprising the Yilgarn and Pilbara Cratons in Western Australia), followed by an easterly dispersion across the mainland (Briscoe *et al.* 1982). A more recent hypothesis places the divergence of *Petrogale* from an eastern centre, with successive invasions to the west and no back-migration into the east (except for *P.*

persephone) (Campeau-Péloquin *et al.* 2001). This analysis places the *brachyotis* group as the ancestral lineage which conflicts with earlier studies that suggest *P. persephone* was the basal lineage (Sharman *et al.* 1990; Eldridge *et al.* 1992b; O'Neill *et al.* 1997). A drawback of this hypothesis, based on DNA/DNA hybridisation was that it excluded *P. rothschildi* due to unstable topologies. Exclusion of this taxon could greatly affect the biogeographic scenario due to its distribution on the west coast of Australia. Although chromosomal analysis is still recognised as the most reliable method for identifying most *Petrogale* taxa (Sharman *et al.* 1990; Eldridge and Close 1992; Eldridge *et al.* 1992a & b; Eldridge 1997b), the inability of these methods to resolve basal relationships within the genus makes it important to use alterative techniques to help understand the evolutionary history of *Petrogale*.

The current study, using molecular analyses of two nuclear (slow) and three mitochondrial (fast) evolving markers, aimed to clarify the phylogenetic relationships and biogeographic patterns within *Petrogale.* Specifically, the study aimed to (i) identify the major lineages within *Petrogale* using a rigorous character-based phylogenetic approach; (ii) establish relationships amongst the major lineages and resolve the continuing uncertainty surrounding the relationships of *P. rothschildi*, *P. purpureicollis* and *P. concinna*; and to, (iii) investigate the biogeographical history of the genus and estimate associated divergence times.

Materials and methods

Taxon Sampling

Samples were obtained from 18 individual *Petrogale* taxa representing 12 species, including three representatives of *P. brachyotis* from across its distribution (listed in Table 2.1; refer to Fig. 2.1 for species‟ distributions). Two genera have been suggested as the sister-genus to *Petrogale*. Eldridge and Close (1997) suggested a *Thylogale*-like ancestor based on their similar karyotypes, a suggestion also put forward by Sharman (1961), Briscoe *et al.* (1982) and Sharman *et al.* (1990). However, Campeau-Péloquin *et al.* (2001), based on DNA-DNA hybridisation analysis, suggested the genus *Dendrolagus* (tree-kangaroos) from New Guinea and northeast Queensland is the sister taxon to *Petrogale,* which is supported by Ziegler (1977), Baverstock *et al.* (1989), Kirsch *et al.* (1995) and Meredith *et al.* (2008), and is now

widely accepted. We therefore used three *Thylogale* (pademelons) and two *Dendrolagus* species as outgroups.

Frozen tissue (ear, muscle, liver and kidney) samples were obtained from museum tissue collections, and ethanol preserved ear biopsies were obtained from live trapped *P. brachyotis* individuals (Table 2.1). Sampled individuals represent all major *Petrogale* groups and 16 of the 23 currently recognised *Petrogale* taxa (species/subspecies/races). Samples from *P. c. concinna, P. c. monastria* were not available. *P. lateralis hacketti* was also not included and only one taxon (*P. assimilis*) was included to represent the five closely related north-east Queensland species (*P. coenensis, P. godmani, P. mareeba* and *P. sharmani, P. assimilis*), which are known to share an identical mitochondrial DNA haplotype and nuclear restriction fragment length polymorphisms (RFLPs) (Eldridge and Close 1992; Bee and Close 1993; Loupis and Eldridge 2001).

DNA extraction, PCR-amplification and sequencing

DNA was extracted from the 70% ethanol preserved tissue using the Gentra DNA Extraction Kit following the manufacturer‟s procedures (Gentra Systems). All frozen samples were extracted following a „salting out‟ method (Sunnucks and Hales 1996). Three mitochondrial gene segments and two nuclear regions were PCR amplified (see Table 2.2 for primers); cytochrome oxidase c subunit 1 (*COI*) and cytochrome b (*Cytb*) (two overlapping fragments), protein coding portions of the breast and ovarian cancer susceptibility gene (*BRCA1* – exon 11); NADH dehydrogenase subunit 2 (*ND2* gene); and the non-coding omega-globin intron 2 (*ω –globin*). These genes were chosen because of their proven utility in resolving interspecies relationships within marsupials and other vertebrates (Schneider *et al.* 1998; Osborne and Christidis 2001; Metcalfe 2002; Meredith *et al.* 2008; Malekian *et al.* 2010b). In addition, there are background data on their molecular evolution in marsupials (e.g., *ω –globin*; (Wheeler *et al.* 2001).

Table 2.1: Collection localities and sources of *Petrogale*, *Thylogale* and *Dendrolagus* taxa sampled in this study.

Common Name	Scientific Name	Location	GPS location or Lat/Long	Animal ID	Tissue ID	Museum Voucher
Allied rock-wallaby	*P. assimilis*[1]	Black Rock, Lyndhurst, Queensland (Qld)	-19.08 144.45	S920	EBU35568	none
Short-eared rock-wallaby	*P. brachyotis* Arnhem Land pop[1]	Umbrawarra Gorge, Northern Territory (NT)	-13.96 131.63	S1989	EBU35569	none
Short-eared rock-wallaby	*P. brachyotis* Victoria River pop[1]	Bradshaw Station, Victoria River District, NT	-15.50 130.10	S304	EBU35576	CM15306
Short-eared rock-wallaby	*P. brachyotis* Kimberley pop[2]	Doongan Station, Kimberley, Western Australia (WA)	-15.20 125.92	99375	ABTC99375	none
Monjon	*P. burbidgei*[1]	Mitchell Plateau, WA	-14.82 125.70	S982	EBU35577	AM22322
Nabarlek	*P. concinna*[1]	Mt Borradaile, NT	-12.05 132.90	S315	EBU35578	none
Brush-tailed rock-wallaby	*P. penicillata*[1]	Jenolan Caves, New South Wales (NSW)	-33.82 150.03	S766	EBU35579	CM16835
Unadorned rock-wallaby	*P. inornata*[1]	Apis Creek Station via Marlborough, Qld	-23.02 149.57	S163	EBU47373	CM15234
Black-footed rock-wallaby	*P. lateralis lateralis*[1]	Nangeen Hill, WA	-31.83 117.68	S972	EBU47374	CM16824
Black-footed rock-wallaby	*P. lateralis pearsoni*[1]	South Pearson Island, South Australia (SA)	-33.95 134.27	S1373	EBU47375	none
Black-footed rock-wallaby	*P. lateralis* West Kimberley race[1]	Erskine Range, West Kimberley, WA	-17.81 124.33	S1256	EBU47376	none
Black-footed rock-wallaby	*P. lateralis* MacDonnell Ranges race[1]	Heavitree Gap, Alice Springs, NT	-23.74 133.87	S969	EBU47377	AM29508
Herbert‟s rock-wallaby	*P. herberti*[1]	Dawson River, Sequens Station, Qld	-23.96 149.75	S455	EBU47378	CM12618
Proserpine rock-wallaby	*P. persephone*[1]	Gloucester Island, Qld	-20.04 148.45	S869	EBU47379	AM37280
Purple-necked rock-wallaby	*P. purpureicollis*[1]	Dajarra, Qld	-21.67 139.29	S888	EBU47380	CM16832
Rothschild‟s rock-wallaby	*P. rothschildi*[1]	Rosemary island, Dampier Archipelago, WA	-20.49 116.59	S204	EBU47381	CM15324
Yellow-footed rock-wallaby	*P. xanthopus xanthopus*[1]	Middle Gorge, Quorn, SA	-32.17 138.04	S359	EBU47382	none
Yellow-footed rock-wallaby	*P. xanthopus celeris*[1]	Captive, QPWS, Charleville, Qld		S1157	EBU47383	None
Goodfellow‟s tree-kangaroo	*D. goodfellowi*[1]	Captive, Taronga Zoo, Sydney, NSW		S1606	EBU47384	none
Lumholtz‟s tree-kangaroo	*D. lumholtzi*[1]	Hypipamee National Park, Qld	-17.43 145.49	S1611	EBU9231	AM32217
Red-necked pademelon	*T. thetis*[1]	Captive, Macquarie University, NSW		S1030	EBU47385	none
Red-legged pademelon	*T. stigmatica*[1]	Broken River Rd, Eungella National Park, Qld	-21.07 148.64	S1101	EBU63675	none
Brown‟s pademelon	*T. browni*[1]	Chimbu Province, Papua New Guinea	-06.67 145.00	S1150	ABTC44080	AM14825

[1] Australian Museum, [2] South Australian Museum

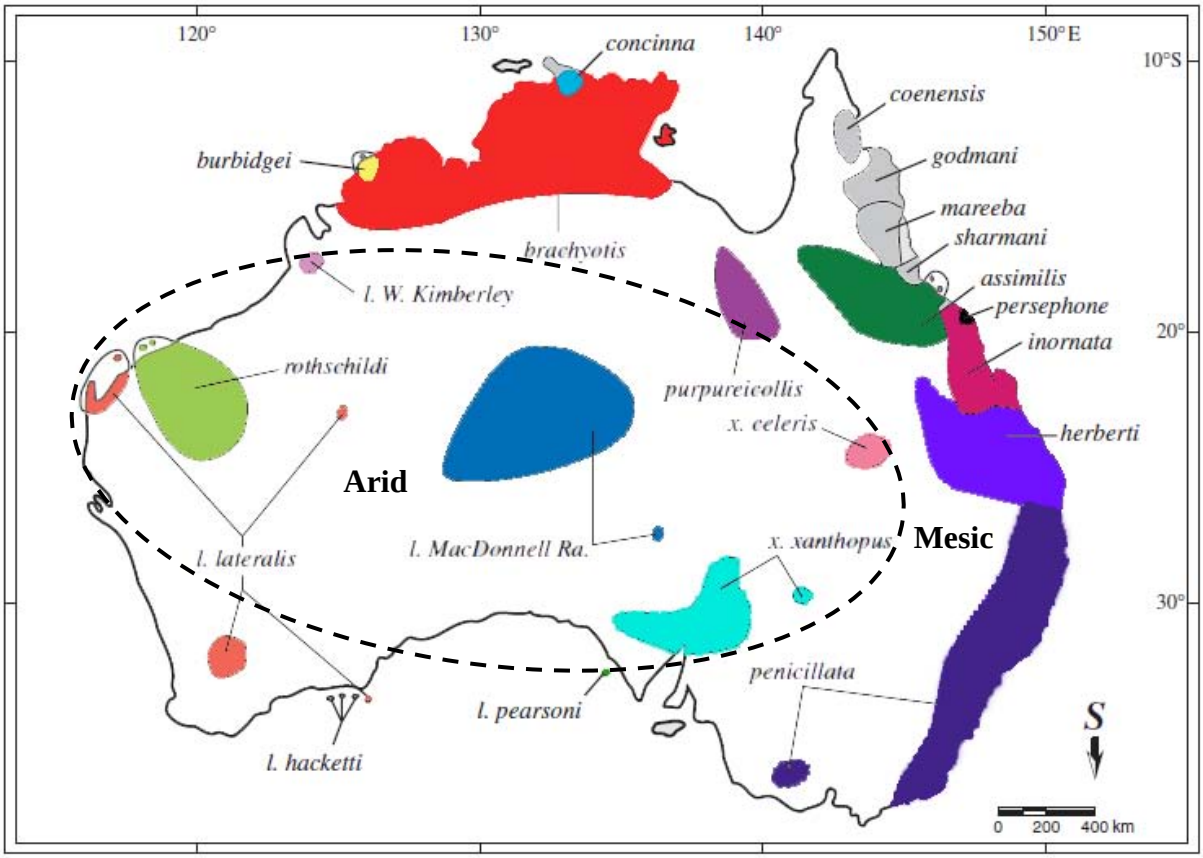

Figure 2.1: Distribution of *Petrogale* taxa in Australia. Ancestral area states (arid and mesic) are denoted by the dashed line. Taxa with distributions marked in grey are not included in this study. Map modified from Eldridge and Close (1993).

PCR-amplifications were carried out in 25μL reactions with approximately 100ng genomic DNA, 1 x PCR buffer (Applied Biosystems), 0.20mM dNTPs, 2.5mM $MgCl_2$, 2pmol primers (Table 2.2) and 0.1U Ampli*Taq* Gold^{TM} polymerase (Applied Biosystems). Thermocycling conditions were: initial denaturation at 94°C for 9 min; 34-40 cycles of 45 s at 94°C (denaturation), 45 s at 48°C (*ND2*), touchdown 55-50°C (*COI* & *Cytb*), 48-50°C (*BRCA1*) and 55°C (*ω-globin*) (annealing), and 1 min at 72°C (extension); and a final extension for 6 min at 72°C. PCR products were purified using Millipore MultiScreen PCR_{384}Filter Plates (Millipore) and directly sequenced at the Australian Genome Research Facility (AGRF).

Table 2.2: Primers used to amplify segments of *Cytb*, *ND2*, *COI*, *ω-globin* and *BRCA1* within *Petrogale* taxa.

Gene	Primer Name	Source	Sequence (5'-3')
COI	*M134	KN Armstrong, unpublished	GTATTTATCCTGCTTACTCTTAGTTAACAGC
	M73 (H)	(Schneider *et al.* 1998)	CCTATTGATAGGACGTAGTGGAAGTG
	M320 (Cox)	(Palumbi *et al.* 1991)	TGATTCTTTGGGCATCCTGAAG
	M31 (COA)		AGTATAAGCGTCTGGGTAGTC
Cytb	M5 (H15149)	(Kocher *et al.* 1989)	AAACTGCAGCCCCTCAGAATGATATTTGTCCTCA
	M495 (L14725)	(Pääbo 1990)	CGAAGCTTGATATGAAAAACCATCGTTG
	M496 (L15162)	(Taberlet *et al.* 1992)	GCAAGCTTCTACCATGAGGACAA
	M1282 (Mr2)	(Bulazel *et al.* 2007)	AGGGTGTTATACCTTCATTTTTGG
ND2	M635 (mmND2.1)	(Osborne and Christidis	GCACCATTCCACTTYTGAGT
	M636 (mrND2c)	2001)	GATTTGCGTTCGAATGTAGCAAG
BRCA1	G1798 (F-498MAC)	(Meredith *et al.* 2008)	CCAGAGGTAATCCTCAGAACTG
	G1799 (R11)		AGTTCTGAAAGTGGATTCTTT
	G1800 (F9)		CTGACCTRCAGCCTGAGGATTTCAT
	G1801 (R-1MAC9-20)		CTGCAGCTAGCTAACACTTGATC
ω-globin	G314	(Wheeler *et al.* 2001)	GGAATCATGGCAAGAAGGTG
	G424	(Blacket *et al.* 2006)	CCGGAGGTGTTYAGTGGTATTTTC

*Australian Centre for Ancient DNA housekeeping number

Sequence analysis

DNA sequences were edited using SEQED (version 1.0.3; Applied Biosystems), then aligned using CLUSTAL X (version 1.83; Thompson *et al.* 1997) and manually refined using SEAL (version 2.0a11; Rambaut 1996). Gaps were considered as indels and assigned „-" and where there were ambiguities at heterozygous sites, the base was considered uncertain „?". All sequences are available on CD (Appendix 1).

DNASP (version 5.0; Librado and Rozas, 2009) was used to determine parsimony informative sites, total number of polymorphic sites, nucleotide diversity and the number of haplotypes. This analysis was performed on individual genes, the mtDNA and nDNA datasets and the combined dataset (mtDNA + nDNA). Intra-specific sequence divergences were calculated, based on pairwise differences was estimated using PAUP* (version 4.0b10; Swofford 2002).

Phylogenetic analyses

Maximum likelihood (ML), maximum parsimony (MP) and Bayesian inference analyses were performed on each gene separately and on the concatenated mitochondrial alignment, nuclear and complete datasets, using RAXML (version 7.0.3; Stamatakis 2006; Stamatakis *et al.* 2008), PAUP* and MRBAYES (version 3.1.2; Ronquist and Huelsenbeck 2003; Huelsenbeck and Ronquist 2005) respectively. The Akaike Information Criterion (AIC) was implemented using MODELTEST 3.06 (Posada and Crandall 1998) in PAUP* (version 4.0b10) to determine the best-fit models for RAXML and MRBAYES. To determine optimal partitioning methods, preliminary Bayesian analyses of all possible partitions for each gene and combined dataset (partition by gene/codon/1st-2nd codon etc.) were run. The AIC for each individual partition was calculated and compared to the less complex partition to see if there was any significant difference between the fit of partition to the results. The mtDNA genes generated different models of evolution for each codon position from the MODELTEST 3.06 analyses, so these genes were partitioned by individual codon positions. The most complex partitions seemed to fit the data best for individual genes, but the nDNA dataset was partitioned by gene and the combined dataset was partitioned into mtDNA, coding nDNA and non-coding nDNA. The models of sequence evolution selected are in Table 2.3. In cases where exact models

suggested from MODELTEST 3.06 were not available in a program (RAXML and MRBAYES), a more general model (HKY, GTR) was selected that accounted for the most complex model rather than the least.

Table 2.3: Summary of nucleotide substitution models implemented in MRBAYES and RAXML, selected for data partitioning using the Akaike Information Criterion in MODELTEST 3.06.

Gene	Codon partition	DNA substitution model	Invariant sites	Substitution rates
COI	3	TrN (GTR)	Yes	Gamma distribution
ND2	3	TIM (GTR)	No	Gamma distribution
Cytb	3	TIM (GTR)	Yes	Gamma distribution
BRCA1	1+2	HKY	No	Equal
ω-globin	Intron	HKY	No	Equal
	Exon	TrNef (GTR)	Yes	Equal

Heuristic searches using 1000 randomised addition orders with tree-bisection-reconnection (TBR) branch swapping, and a stepwise addition starting tree were used for MP analyses and the DELTRAN option for character-state optimization was utilised as previously suggested (Cooper *et al.* 2007; Malekian *et al.* 2010a & b). The ML analyses were started from a complete random starting tree. The default rapid hill climbing algorithm was used together with its default settings on the partitioned dataset. Since mixed models could not be implemented in this program, the General time Reversible with rate variation among sites modelled with a discrete gamma distribution (GTR+G) model was applied to each partition to cover the more complex partition models (as GTR+G plus a proportion of invariant sites [I] model has errors, see RAxML manual for information). Bootstrap analyses were carried out for 1000 replicates, with multiple (100) searches per replicate and the best tree chosen from those runs was used in the calculation of bootstrap proportions. MRBAYES uses Metropolis-coupled Markov chain Monte Carlo (MCMC) sampling to calculate Bayesian posterior

probabilities. We used default settings for priors, random starting trees and four Markov chains (three hot, one cold), with sampling every 1000 generations. Analyses were terminated when the average standard deviation of split frequencies for the simultaneous analyses fell below 0.01 (≈2-10 million generations). TRACER (version 1.5; Rambaut and Drummond 2009) was used to check that the convergence of parameter estimates had occurred. Tracer was also utilised to identify when the log likelihood values of trees became stable, to define the appropriate cut off for initial instability of trees (usually 10% burn-in was suitable). Posterior probabilities were calculated after discarding the first 25% of the sampled trees as burn-in.

Partition homogeneity tests were not employed in this study to determine whether concatenating genes would significantly alter the phylogeny, as the incongruence length difference test and similar methods have been criticised for producing erroneous results and are unable to address phylogenetic accuracy questions (Barker and Lutzoni 2002; Ramirez 2006). There is also evidence that concatenating datasets improves the accuracy of phylogenies, despite no individual substitution model being used per partition (Gadagkar *et al.* 2005). As this topic is controversial, data partitions were assessed separately (individual genes) and analysed for mtDNA, nDNA and a combined dataset (mtDNA and nDNA). The data were explored, to assess the differences between individual gene trees and those from concatenated datasets. Also, a mixed model analysis was performed on the complete dataset using BAYESPHYLOGENIES (version 1.1; Pagel and Meade 2004, 2005) to assess the effects of data partitioning on the tree . This program uses a Bayesian approach together with a reversible-jump MCMC algorithm that fits more than one model of sequence evolution to determine the optimal number of models for an alignment (Pagel and Meade 2004, 2005). It implements a heterotachy model that calculates branch lengths on the tree based on a likelihood model, allowing for variation in rates of evolution in the tree (Pagel and Meade 2008). Different individual sites in the alignment are characterised without prior knowledge of the evolutionary process accommodating pattern heterogeneity (variation in evolution across sites) (Pagel and Meade 2004, 2005). The analysis was run for 3 million generations with default settings for priors, a random starting tree and one chain sampled every 10,000 generations. The GTR+G model of evolution with estimation of base frequencies was selected, so different rate matrices, rate parameters and base frequencies could be estimated.

Analyses were terminated when log likelihood values of the tree became stable and a 25% burn-in was removed. A consensus tree was viewed in BAYESTREES (version 1.0**;** http://www.evolution.reading.ac.uk/BayesTrees.html), which allows multiple branch lengths (heterotachy) to be viewed as posterior probabilities of more than one branch at that node.

We also analysed the nuclear and mitochondrial dataset using a species tree approach implemented in Bayesian Estimation of Species Trees (BEST version 2.2; Edwards *et al.* 2007b; Liu and Pearl 2007; Liu 2008; Liu *et al.* 2008). Model parameters were estimated from MODELTEST 3.06 (as described above) and included as unlinked partitions. The priors included an inverse gamma distribution ($\alpha = 3$; $\beta = 0.003$) for the population size and a uniform distribution (0.2, 1.8) for the gene mutation and were estimated as part of the analysis. The analysis was run for 50 million generations, sampling every 10,000 generations (5 million generations burnin). Parameters were assessed for convergence and mixing in TRACER (version 1.5; Rambaut and Drummond 2009). Runs were combined to produce a single consensus tree based on runs producing the same topology and similar posterior probabilities.

Molecular dating analyses

The program BEAST (version 1.5.3; Drummond *et al.* 2006; Drummond and Rambaut 2007) was used for molecular divergence estimation (Drummond *et al.* 2006; Drummond and Rambaut 2007). To explore the time-scale of diversification of *Petrogale*, the combined mtDNA and nDNA dataset of all species was analysed under a relaxed molecular clock using the Uncorrelated Lognormal prior, which estimates the rate of each lineage (tree branch) independently from a lognormal distribution. The data was partitioned into mtDNA and nDNA, with the following models of nucleotide substitution; GTR+G+I (mtDNA), HKY (nDNA) based on the model of sequence evolution suggested by MODELTEST 3.06 for these data partitions. The Yule prior was selected for the tree model and was fixed for all partitions.

Both „hard" and „soft" bound node constraints were employed to estimate divergence times. Whilst hard bound constraints are fixed, based on the fossil record, soft bound constraints are variable and are changed by the sequence data. Soft bounds are useful in studies where there

are fossil uncertainties, as they have a reliable assessment of estimation errors (Yang and Rannala 2006; Sanders and Lee 2007). Selection of fossil constraints was based on the methods of Meredith *et al.* (2008), where they utilised stratigraphic bounding to determine minimum and maximum constraints. The minimum ages are slightly varied from those reported by Meredith *et al.* (2008), based on more recent information (Prideaux pers. comm., Prideaux and Warburton 2010). Since the Australasian fossil record still has many gaps three prior distributions were implemented to account for the bias in the fossil record. The analyses were conservative as to the upper bounds (maximum), where the stratigraphic bounding was used from the fossil-bearing deposit one unit older than the next immediate oldest fossil-bearing layer not containing any fossils from the lineage of interest (Meredith *et al.* 2008). The three fossil constraints were selected based on the available fossil record. Monophyly was imposed for clades where fossil constraints were created to estimate most recent common ancestors (MRCAs) and this included a clade of all the data [*Thylogale* + (*Dendrolagus* + *Petrogale*)] and a clade excluding *Thylogale* individuals (*Dendrolagus* + *Petrogale*) and a clade including only *Petrogale* taxa. In the first analysis a lognormal prior distribution was implemented that estimates divergence times using a skewed model, with estimates more likely nearer the fossil record than the maximum bound.

Minimum divergence dates were 4.0 MYA for the node connecting all *Petrogale* taxa, 4.4 MYA for the node connecting *Dendrolagus* species to the *Petrogale* clade and 4.46 MYA for the node connecting *Thylogale* species to the rest of the taxa. The calibration point for the *Petrogale* clade is based on an undescribed species of *Petrogale* known from Bluff Down Local Fauna (Bartholomai 1978; Mackness *et al.* 2000; Prideaux 2004; Prideaux and Warburton 2010). The minimum calibration of 4.4 MYA for the node connecting *Dendrolagus* and *Petrogale* taxa is based on a *Dendrolagus* sp. known from the Hamilton deposits (Flannery *et al.* 1992; Prideaux and Warburton 2010), as this is the minimum possible age of this node based on all the available data. The oldest described species of *Thylogale*, *Thylogale ignis* (Hamilton Local Fauna; Flannery *et al.* 1992; Meredith *et al.* 2008) was found in capped basalt dated from the Pliocene and was used to calibrate the node connecting all taxa in the phylogeny. The maximum constraints on the nodes were all the same and set to 15.97 MYA, based on the absence of fossils in System C deposits at Riversleigh (Gradstein *et al.* 2004; Meredith *et al.* 2008).

Lognormal priors still bias against the possibility that the real dates for calibration nodes are much younger than the proposed fossil dates (owing to taxonomic error) (Sanders and Lee 2007), therefore the second analysis, implemented a normal distribution for the prior distribution model (as the Australian fossil record is patchy and the normal distribution models the non-directional uncertainty). This model used the same minimum and maximum bounds for the node constraints. Finally, a fixed model (set minimum and maximum bounds), using uniform distributions was implemented to estimate branch-specific substitution rates (Sanders and Lee 2007). Two independent MCMC chains were run for 50 million generations with sampling every 1000 generations and 10% burn-in of the posterior samples. Tracer (version 1.5; Rambaut and Drummond 2009) was used to examine if the chains had reached satisfactory convergence, based on effective sample sizes and posterior densities. To test that the data fitted the molecular clock model the coefficient of variation parameter was examined and the autocorrelation of rates in the phylogeny were assessed using the rate of covariance.

Biogeographic analyses

To test biogeographical hypotheses of *Petrogale* evolution, two different methods were implemented to assess ancestral area reconstructions. BAYESTRAITS (version 1.0; Pagel *et al.* 2004) was implemented, using a Bayesian MCMC method to derive posterior probabilities of the state of traits at three ancestral nodes in the *Petrogale* clade. Two states were analysed which correlate to area distributions found in Fujita *et al.* 2010 (as depicted in Fig. 2.1). A total of 8000 trees were used from Bayesian analysis of the combined mtDNA and nDNA data (excluding outgroups) to estimate trait rate parameters and ancestral states. The MRCA approach (Pagel *et al.* 2004) was used to infer the ancestral state for nodes connecting selected taxa in the tree, to assess broad-scale biogeographic patterns of *Petrogale*. The analysis used a reversible-jump hyperprior drawn from an exponential prior seeded from a uniform distribution (0.0-5.0). The analysis was run for 50 million generations, sampled every 1000 generations with a burnin of 2 million generations. Each run was repeated five times to ensure stability of the harmonic mean. Results were checked for acceptance rates of 20-40% and stationarity of the harmonic mean. The probability of each of the states at each node was averaged across all of the output, excluding the burnin.

The second program implemented to reconstruct ancestral distributions used dispersal-vicariance analysis (DIVA; Ronquist 1996) and was executed using the BEST tree reconstruction (excluding the outgroup taxa). The optimal reconstruction of ancestral area states is obtained using a three-dimensional cost matrix (Ronquist and Nylin 1990) and the ancestral state reconstruction is created based on the minimum optimal distributions for each node. The same two states used in BAYESTRAITS analysis were used for DIVA analysis. Analysis was set so that 1000 optimal distributions was the maximum saved for each ancestral node.

Results

Sequence analyses: mtDNA & nDNA

The mtDNA alignment comprised 3024 nucleotides (*COI* - 1315 bp, *Cytb* - 1140 bp, *ND2* - 569 bp). Overall a total of 1021 sites were variable among the 23 individuals, of which 751 sites were parsimony informative (*COI* – 324, *Cytb* – 273, *ND2* – 154). Nucleotide diversity of individual genes ranged from 9.1% (*COI*) – 10.8% (*ND2*). Sequences for three individuals (S1256 – *COI*, S1611 & S1606 – *Cytb*) included only partial data as a result of difficulties in sequencing. There were no ambiguities in the data to suggest that any of the mitochondrial sequences were nuclear copies (e.g., absence of stop codons or indels) and individual mtDNA markers generally produced concordant phylogenetic trees.

Among *Petrogale* taxa, the minimum mitochondrial sequence divergence was between *P. l. pearsoni* and *P. l. lateralis* (0.3%; 0.17%-0.46%) and the maximum was between *P. brachyotis* Kimberley population and *P. rothschildi* (10.9%; 10.5%-11.3%). The average sequence divergence amongst *Petrogale* taxa was 0.057 ± 0.053, while between *Petrogale* and *Dendrolagus/Thylogale* taxa it was 0.126 ± 0.013.

The nDNA alignment totalled 1787 nucleotides (*BRCA1* - 1048 bp, *ω-globin* - 739 bp). The *ω-globin* fragment consisted of 169 bp of coding sequence (Exon 2 and Exon 3) and 570 bp of non-coding sequence (Intron 2). There were 29 parsimony informative sites, together with

31 parsimony informative sites for *BRCA1* from an overall total of 129 polymorphic sites. One individual for *BRCA1* and five for *ω-globin* showed ambiguities that were coded as missing for further phylogenetic analyses.

The nDNA was less divergent than the mtDNA, with a total of 14 haplotypes for *ω-globin* and 20 haplotypes for *BRCA1*. Nucleotide diversity values were 0.012 between *Petrogale* taxa for both *ω-globin* and *BRCA1*. The maximum sequence divergence between different haplotypes in *ω-globin* was between *P. persephone* and *P. brachyotis* NT population (1.8%). For *BRCA1* maximum divergence was between *P. brachyotis* Kimberley population and *P. lateralis* West Kimberley race (1.3% sequence divergence). There were a total of seven indel events in the *ω-globin* intron region, four indels of 1 bp (three of which were just between *Petrogale* and outgroup taxa) and single indels of 2, 3 and 27 bps, which could be the result of one or more mutation events. Two indels were detected in *BRCA1* (one 3 bp and the other 15 bp), which code for six amino acids in *Thylogale* and *Dendrolagus* sequences that are deleted from the *BRCA1* gene of *Petrogale* taxa.

Phylogenetic analyses: mtDNA, nDNA & combined dataset

Within *Petrogale* four well resolved lineages were recovered from mtDNA and combined (mtDNA and nDNA) analyses, which were congruent with the BEST species tree (Figs. 2.2-2.4). These four lineages consisted of; 1) the *brachyotis* group, 2) *P. persephone*, 3) *P. xanthopus*, and 4) the *lateralis-penicillata* group (including *P. rothschildi* and *P. purpureicollis*). The congruence between the concatenated dataset and BEST reconstruction indicates that the overall topology is robust, so the BEST tree was therefore used to assess biogeographical hypotheses of *Petrogale* evolution. All *Petrogale* taxa formed a monophyletic group with high bootstrap and Bayesian posterior probability values (98-100% bs and pp; Figs. 2.2-2.3), except for the MP analysis of the combined nDNA data, which had two outgroup taxa (one *Dendrolagus* and one *Thylogale*) grouping within the *Petrogale* clade and low bootstrap support for a majority of the branches (58-100%, generally <89% bs; Fig. 2.4).

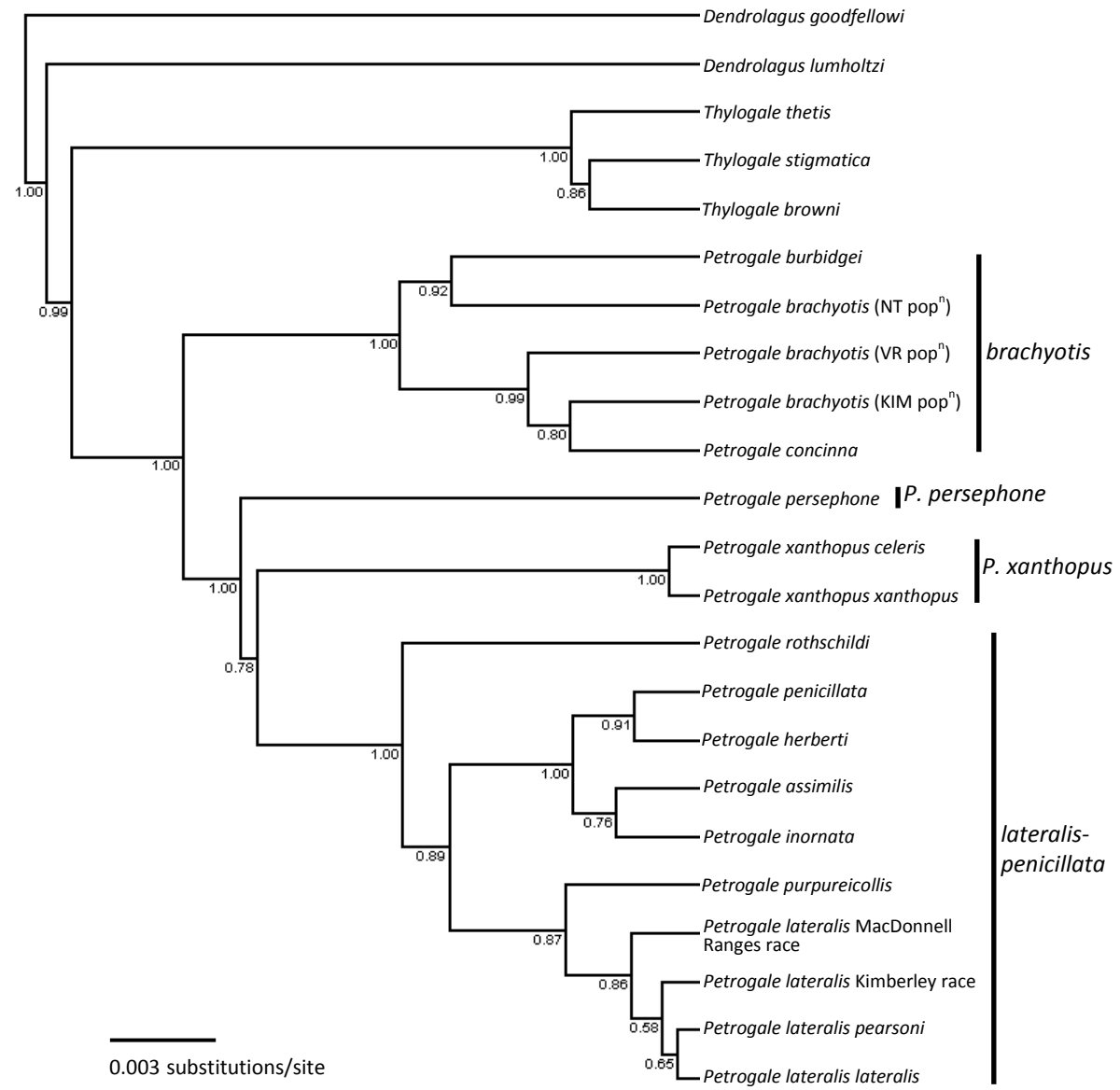

Figure 2.2: BEST species tree (phylogram) inferred using both nuclear and mitochondrial data, including posterior probabilities (adjacent to branches). Major clades are outlined by taxonomic group and taxon name. *P. brachyotis* is split into Northern Territory (NT), Victoria River (VR) and Kimberley (KIM) populations.

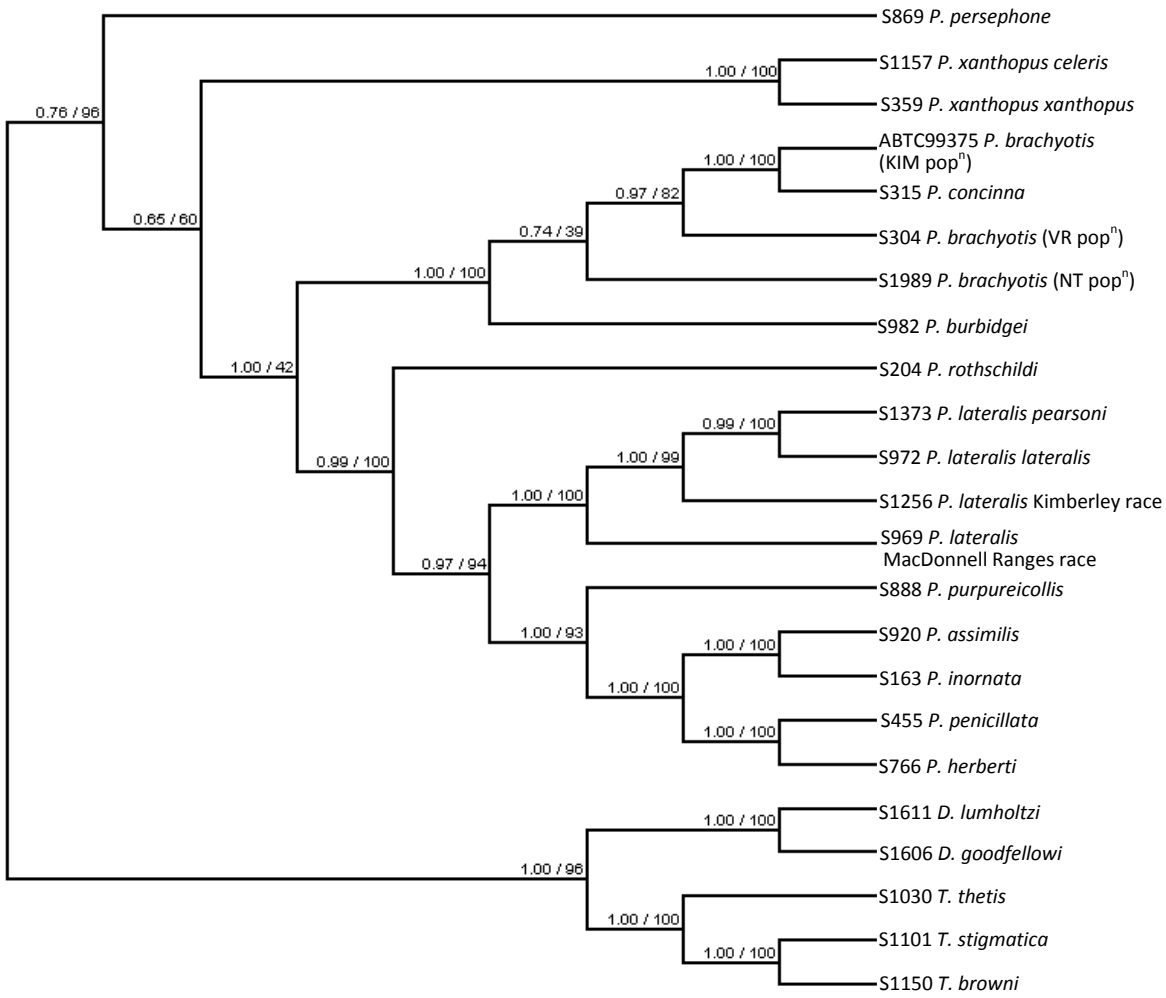

Figure 2.3: Mitochondrial tree (cladogram) of combined data (*COI, Cytb* and *ND2*) inferred using MrBayes version 1.3.2 using codon-partitions. The Bayesian posterior probabilities as decimals and ML bootstrap support as percentages. *P. brachyotis* is split into Northern Territory (NT), Victoria River (VR) and Kimberley (KIM) populations.

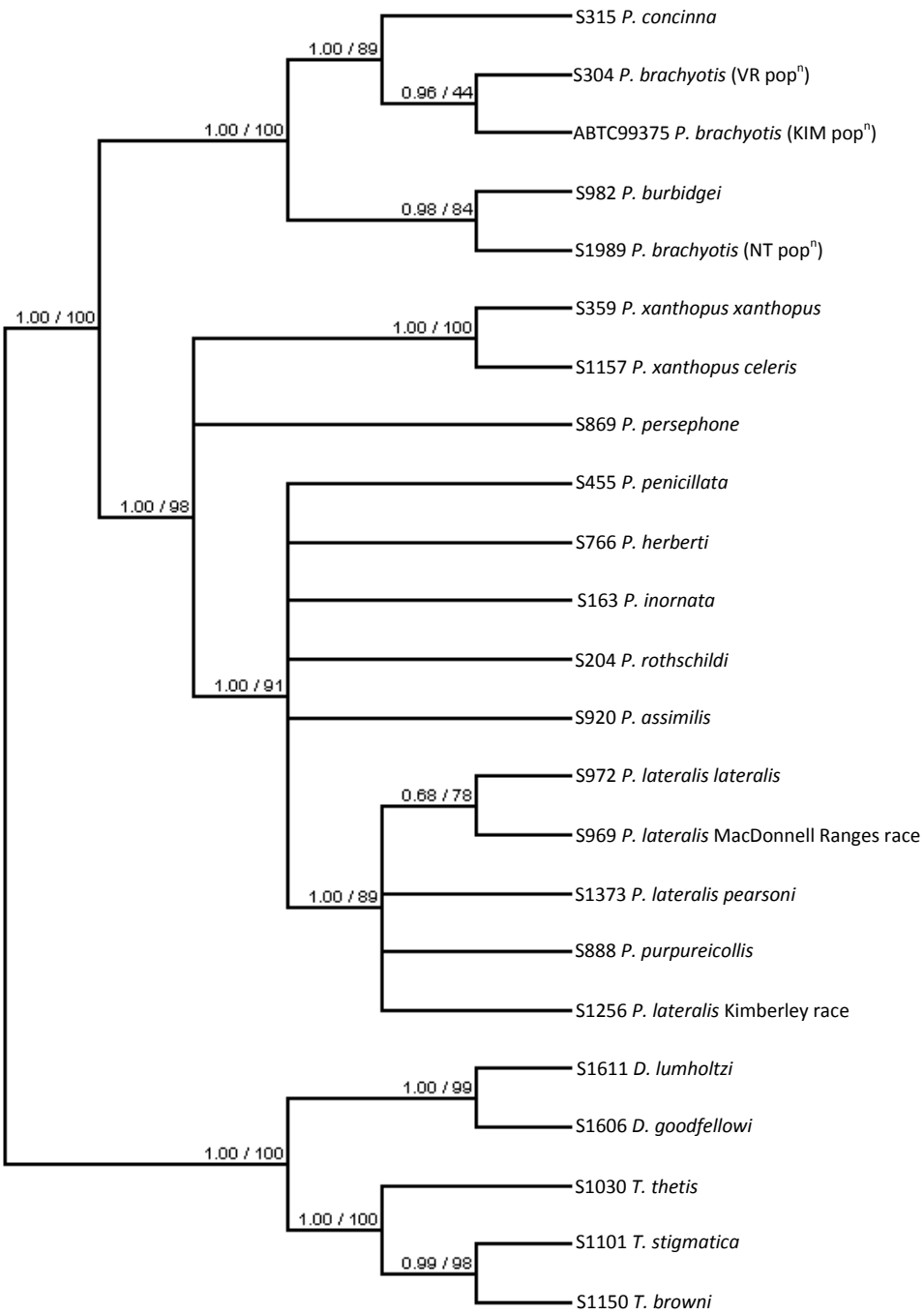

Figure 2.4: Concatenated nuclear tree (cladogram) based on a partitioned Bayesian inference inferred using MrBayes version 1.3.2. The Bayesian posterior probabilities are as decimals and ML bootstrap support as percentages. *P. brachyotis* is split into Northern Territory (NT), Victoria River (VR) and Kimberley (KIM) populations.

The different analyses and different datasets (mtDNA, nDNA and combined) showed some inconsistencies in the placement of the first three lineages (*brachyotis* group, *P. persephone*, *P. xanthopus*) and the relationship of *P. purpureicollis* within the *lateralis-penicillata* complex. The BEST analysis clearly separates the *brachyotis* group from the other taxa (pp = 1.0), but the relationships among *P. persephone*, *P. xanthopus* and the *lateralis-penicillata* complex remain unresolved. The relationship of *P. purpureicollis* to the *lateralis* and *penicillata* complexes appears also to be less well resolved. BEST and Bayesian analyses (combined dataset) showed *P. purpureicollis* as the sister lineage to the *lateralis* complex (87-99% support; Fig. 2.2), as did ML results but with very low support (30%), whereas mtDNA and MP (combined dataset) analyses resolved *P. purpureicollis* as a sister lineage to the *penicillata* complex (94-100% support; Fig. 2.3).

The mixed model phylogeny (results not shown) also highlighted uncertainty in the relationship of *P. purpureicollis* within the *lateralis-penicillata* group, grouping it with low support to the *lateralis* complex (54%). The first major clade was comprised of all members of the *brachyotis* group (*P. brachyotis*, *P. burbidgei*, *P. concinna*) and was well supported by ML, MP and Bayesian analyses, as well as the BEST analysis (100% bs and pp; Figs. 2.2-2.4). However, *P. brachyotis* was paraphyletic, with *P. burbidgei* forming the sister lineage to *P. brachyotis* from the Northern Territory, whilst *P. brachyotis* from the Kimberley and Victoria River clustered with *P. concinna*. Nuclear analysis had low support in resolving the relationship of *P. concinna* with Kimberley and Victoria River *P. brachyotis* (54-100%; Fig. 2.4), but mtDNA and combined data analyses showed *P. concinna* as the sister lineage to *P. brachyotis* from the Kimberley, in concordance with the BEST results (75-100% support; Figs. 2.2-2.3).

The second major clade consisted only of *P. persephone* which was not closely related to any other taxa and contained a long branch length indicating it diverged early in *Petrogale* evolution. The third major clade, which diverged around a similar time to *P. persephone*, comprised the two recently diverged subspecies of *P. xanthopus* which were not closely related to any other *Petrogale* taxa.

The fourth major clade (100% support; Figs. 2.2-2.3) was comprised of the most recently derived *Petrogale* taxa. This clade consists of all examined taxa from the *lateralis-penicillata* group (*lateralis*; *lateralis* subspecies and races; *penicillata; P. assimilis, P. herberti, P. inornata, P. penicillata*), as well as *P. purpureicollis* and *P. rothschildi.* The placement of *P. rothschildi* as the sister taxon to the *lateralis-penicillata* group is well supported in the combined, BEST and mtDNA results (100%; Figs. 2.2-2.3). However, on their own the nuclear data were unable to resolve the relationship of *P. rothschildi* with the eastern *penicillata* complex taxa (forming a polytomy with *P. penicillata, P. herberti, P. assimilis* and *P. inornata*), although it was distinguished from the western *lateralis* complex taxa.

Within the *lateralis-penicillata* clade, the four *P. lateralis* subspecies/races consistently formed a monophyletic group separate from the four eastern *penicillata* complex species (82-100%; Figs. 2.2-2.3). Within this monophyletic group *P. l. pearsoni* and *P. l. lateralis* were recovered as sister lineages, with *P. lateralis* West Kimberley race most closely related to these lineages, and *P. lateralis* MacDonnell Ranges race forming a sister lineage to these other three *P. lateralis* taxa. These finer scale relationships were well supported (95-100%; Fig. 2.3) in the mtDNA phylogeny, but to a lesser extent in the BEST reconstruction (58-86% pp; Fig. 2.2). Within the *penicillata* complex, *P. assimilis* and *P. inornata* formed sister lineages, as did *P. herberti* and *P. penicillata* in both the BEST reconstruction and the mtDNA based phylogeny (76-100% support; Figs. 2.2-2.3). The nDNA reconstruction was unable to resolve the relationships of these taxa (Fig. 2.4).

Molecular clock analyses

The relaxed lognormal clock analysis of the combined dataset produced the same topology of the phylogenetic tree as the Bayesian and ML analyses of the combined dataset (see Fig. 2.5 for BEAST phylogeny). The coefficient of variation averaged 0.12 (95% highest posterior density (HPD); lower: $4.14 \times 10^{-2} – 4.60 \times 10^{-2}$, upper: 0.205 - 0.210) for analyses of all three prior distributions (lognormal, normal and uniform). These values suggest a limited departure from the molecular clock, but that a lognormal relaxed clock is appropriate as the data are not strictly clock-like. The rate of covariance averaged between -0.046 to -0.047 (95% HPD; lower: -0.317 to -0.321, upper: 0.223 – 0.229) for the three analyses. These values span zero,

indicating that there is no significant evidence of covariance between parent and child branch rates and no strong evidence of autocorrelation of rates in the phylogeny (Drummond *et al.* 2006).

A fossil calibration with a lognormal prior distribution gave estimates of the most recent divergences between lineages, with the highest mean rate of evolution (7.2×10^{-3} substitutions per site per million years). A normal prior distribution for the fossil calibration gave estimates with the lowest mean rate of evolution (4.6×10^{-3} substitutions per site per million years). The uniform prior distribution analysis produced similar divergence estimates to those from the normal distribution for the basal divergences in the phylogenetic tree, but then became higher than the divergence estimates from the normal and lognormal priors for the most recent divergences, with a mean rate of evolution of 5.9×10^{-3} substitutions per site per million years.

Dating of the MRCA of *Petrogale* taxa was estimated between 5.94 – 11.32 MYA (95% HPD – minimum lower (*Ml* - from lognormal prior) 4.57 MYA to maximum upper (*Mu* – from normal prior) 12.91 MYA), when it shared an ancestor with *Dendrolagus* (refer to Appendix 2 for divergence estimates). The earlier split of *Thylogale* and *Dendrolagus* lineages from their MRCA was around the same time during the Miocene, estimated between 6.44 – 12.81 MYA (95% HPD – *Ml* 4.91 to *Mu* 14.31 MYA).

Divergence of the major clades within *Petrogale* occurred during the Miocene-Pliocene, with the *brachyotis* group diverging first between 4.33 – 8.70 MYA (95% HPD – *Ml* 4.00 to *Mu* 10.03 MYA) and *P. persephone* and *P. xanthopus* following closely (4.07 – 8.19 MYA, 3.55 – 7.55 MYA; 95% HPD – *Ml* 3.47 to *Mu* 9.34 MYA, *Ml* 3.07 to *Mu* 8.61 MYA, respectively). The majority of clade splits was estimated to have occurred during the Plio-Pleistocene, with divergences ranging as recent as 232,600 - 344,000 years ago (YA) and 141,100 – 506,000 YA (95% HPD – *Ml* 68,500 YA to *Mu* 378,600 YA; *Ml* 130,000 to *Mu* 590,600 YA; divergence of *P. l. lateralis* and *P. l. pearsoni*, and *P. xanthopus* subspecies respectively); to 1.32 – 2.65 MYA (95% HPD – *Ml* 908,100YA to *Mu* 2.75 MYA) between the taxa of the *penicillata* group and 2.65 – 5.05 MYA (95% HPD – Ml 1.95 to Mu 5.58 MYA) within the *brachyotis* group taxa.

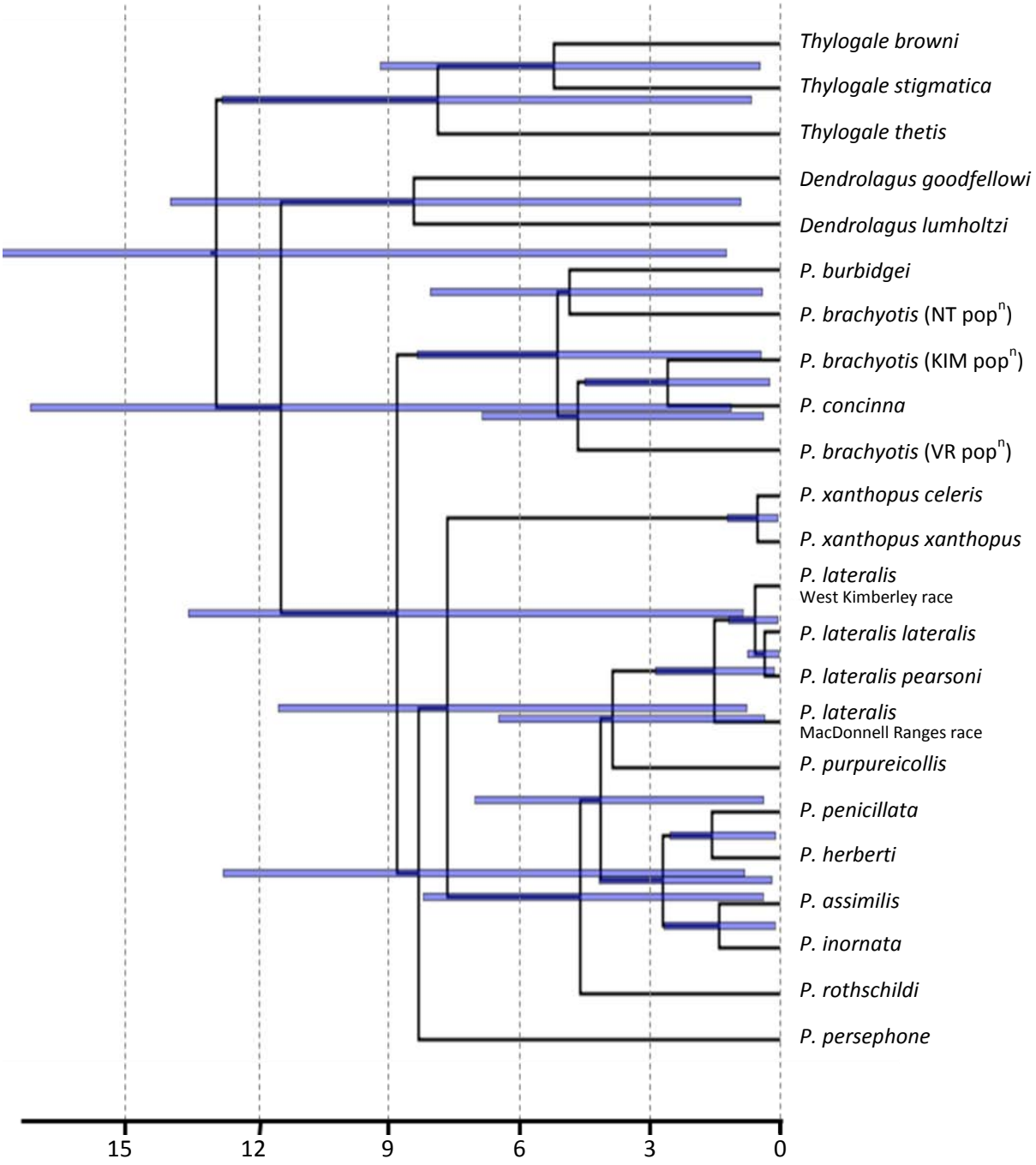

Figure 2.5: Chronogram inferred from a relaxed molecular clock in BEAST based on the normal prior distribution of fossil calibrations. Node bars are 95% confidence intervals and vertical bars infer the time-scale. *P. brachyotis* (NT – Northern Territory; VR – Victoria River; KIM – Kimberley populations). Scale bar is in millions of years ago (MYA), starting from the present (0 MYA) at the tips of the nodes.

Ancestral area reconstructions

BayesTraits analyses was used to assess the ancestral areas of the root node (1) for all *Petrogale* taxa, a second node (2) connecting the *brachyotis* group to *P. persephone* and a third node (3) connecting the *lateralis-penicillata* taxa, *P. rothschildi* and *P. purpureicollis*. The MRCA analysis which assesses the posterior probability of ancestral states at each of these nodes was unable to provide strong support for either ancestral state (arid or mesic; Fig. 2.1), with average probabilities of 50% for the three nodes (Fig. 2.6). The DIVA results, however, supported a mesic ancestral state at the root node for all *Petrogale* taxa. A mesic ancestral state was also recorded for the node connecting *P. persephone* to *P. xanthopus* and the *lateralis-penicillata* complex. Ancestral states at other major nodes connecting *P. rothschildi* with the *lateralis-penicillata* complex also had a mesic state, however the node connecting *P. xanthopus* to these taxa could not be resolved. The inability of BayesTraits to strongly support an ancestral state across the phylogeny, however, suggests that there is uncertainty in the inference of areas of origin of ancestral *Petrogale* taxa during its biogeographic history and the DIVA results must therefore be taken with caution.

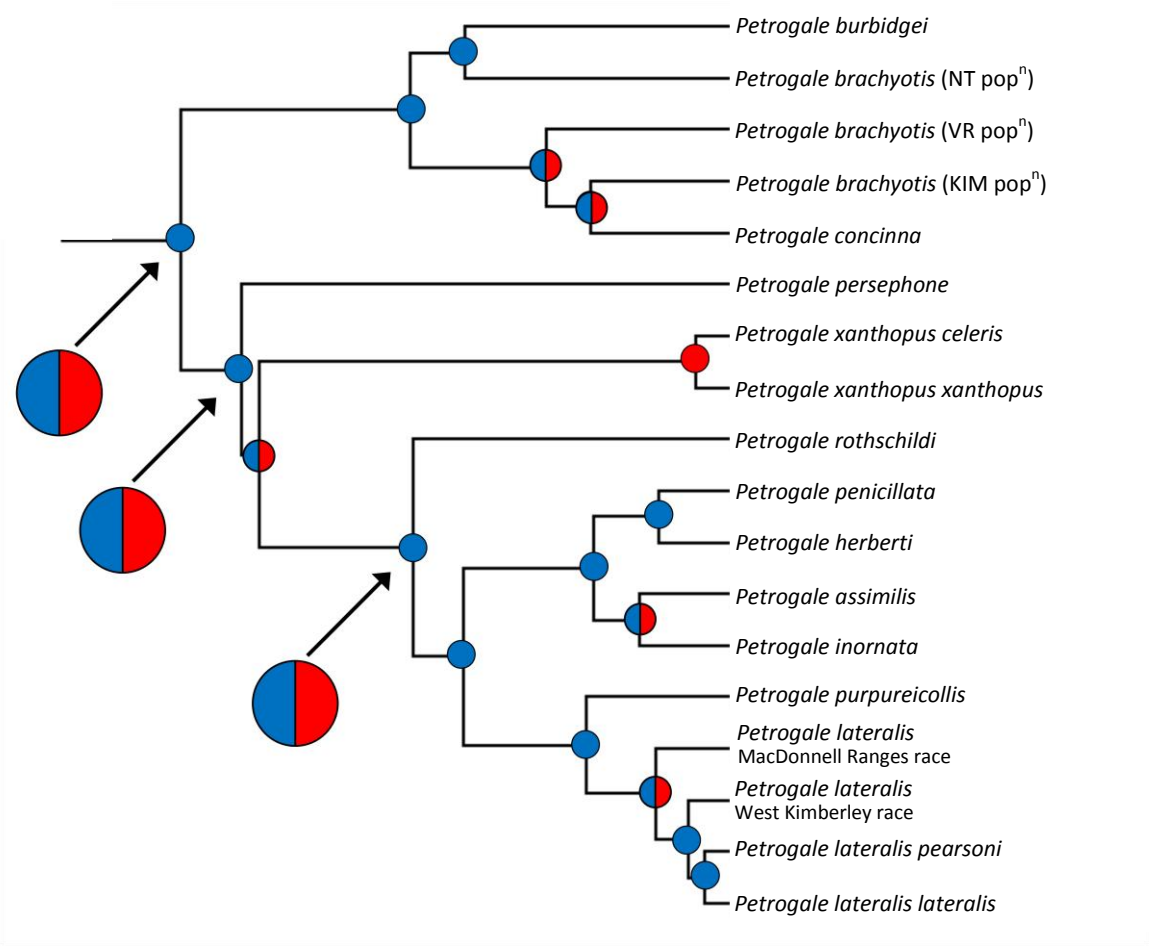

Fig. 6. Reconstruction of ancestral area states for *Petrogale* using DIVA and BayesTraits. DIVA reconstructions are displayed at each node and BayesTraits posterior probabilities are indicated for the three analysed nodes. (A) Red- north-eastern area, (B) Blue – northern area, (C) Yellow – southern area. *P. brachyotis* (NT – Northern Territory; VR – Victoria River; KIM – Kimberley populations).

Discussion

Molecular phylogenetic relationships

These phylogenetic analyses provide strong support for the presence of four major lineages within *Petrogale*, with the *brachyotis* group forming the sister lineage to all other *Petrogale* (Fig. 2.2). The four lineages consist of *P. persephone* and *P. xanthopus* forming two independent single-species lineages, with the *brachyotis* group and the *lateralis-penicillata* group (including *P. rothschildi* and *P. purpureicollis*) forming the other two. The identification of these lineages confirms that members of the *xanthopus* group (*P. persephone*, *P. rothschildi* and *P. xanthopus*) are not monophyletic. The results also highlight some unresolved relationships within *Petrogale* that will require further assessment. Although some individual tree topologies differed, we were able to identify sources of incongruence and compare these to the BEST phylogenetic tree which allowed us to explore the data critically for each node, each taxon and each taxonomic group. Overall the BEST tree, which was similar to nDNA and BEAST reconstructions (which recovered the same topology), was used as the basis for further biogeographic testing and phylogenetic hypotheses in this study.

The results indicate the *brachyotis* group is the sister lineage to all other *Petrogale*, with strong support from the independent nuclear markers and mixed model analysis of the combined dataset (results not shown), the combined nuclear data (Fig. 2.4), as well as the BEST and BEAST analyses (mtDNA and nDNA; Figs. 2.2 and 2.5). These results support the DNA/DNA hybridisation study that placed this group basal to other *Petrogale* (Campeau-Péloquin *et al.* 2001), but contradicts chromosomal studies that placed *P. persephone* and *P. xanthopus* as ancestral due to their retention of the 2n=22 ancestral macropodid karyotype (Eldridge and Close 1993, 1997). However, chromosome analyses do support the findings that the *brachyotis* group is monophyletic and highly divergent from other *Petrogale*, since these taxa share multiple complex chromosomal rearrangements and the presence of large amounts of centromeric constitutive heterochromatin not present in other *Petrogale* (Maynes 1989; Sharman *et al.* 1990; Eldridge *et al.* 1992b). Phylogenetic relationships within the *brachyotis* group are less clear. Three geographic representatives of *P. brachyotis* were included in the current analysis in the light of previous taxonomic uncertainties (Eldridge

1997b; Telfer and Eldridge 2010). The analyses indicated that *P. brachyotis* from the Northern Territory was more closely related to *P. burbidgei* (6.7% mtDNA SD) than to other *P. brachyotis* (7.1-7.4% mtDNA SD) and together they formed the sister group to *P. concinna* and *P. brachyotis* from the Kimberley and Victoria River. The paraphyly of *P. brachyotis* revealed in this analysis suggests that *P. brachyotis* as currently recognised consists of multiple species. This result highlights the need for a more detailed molecular and morphological study evaluating samples of *P. brachyotis* from across its distribution.

The placement of *P. concinna* with respect to *P. brachyotis* from the Kimberley and Victoria River was different in the nuclear and mitochondrial analyses (see Fig 2.3 and 2.4), potentially representing stochastic lineage sorting in the common ancestor or ancient introgression. The placement of *P. concinna* within *P. brachyotis* contradicts previous studies of chromosome morphology that placed *P. burbidgei* and *P. concinna* as sister taxa (Eldridge and Close 1993). Due to its rarity *P. concinna* is not often examined in phylogenetic analyses, with its inclusion only in the Eldridge *et al.* (1992) chromosomal study and recent broader marsupial and macropodoid phylogenetic papers of Meredith *et al.* (2008, 2009). The systematics of *P. concinna* has been controversial since its placement by Thomas (1904) in the monotypic genus *Peradorcas* on the basis of its unique (amongst marsupials) presence of continuously erupting molars. While some authors continue to support this arrangement (Sanson *et al.* 1985; Strahan 1983, 1995; Meredith *et al.* 2008, 2009) others argue that it should be returned to *Petrogale* (Thomas 1904; Poole 1979; Briscoe *et al.* 1982; Sanson *et al.* 1985; Calaby and Richardson 1988; Sharman *et al.* 1990; Eldridge and Close 1993; Meredith *et al.* 2008, 2009). It is evident from these data that *P. concinna* is not sufficiently distinct to warrant separate generic status and that it should be included within *Petrogale.*

Petrogale persephone forms a highly distinct (10% SD) single-species lineage within *Petrogale* and is the sister lineage to a monophyletic group comprising *P. xanthopus* and the *lateralis-penicillata* group. This arrangement contradicts previous suggestions of a close relationship between *P. persephone* and *P. xanthopus* (Maynes 1982) and chromosomal studies which placed *P. persephone, P. rothschildi* and *P. xanthopus* as members of the *xanthopus* group (Briscoe et al., 1982). There is now compelling evidence that the *xanthopus* group is polyphyletic and the similarity of their chromosomes is based on symplesiomorphies.

The highly allopatric distributions of *P. persephone* and *P. xanthopus* together with major differences in morphology and habitat preference (*P. xanthopus*: widespread semi-arid; *P. persephone*: restricted tropical coastal) also lend support to a distant relationship (Van Dyck and Strahan 2008). Although not supported by all analyses, the BEST tree indicates that the *P. persephone* lineage diverged prior to *P. xanthopus*, supporting previous proposals that *P. persephone* represents a relict species (Briscoe *et al.* 1982; Sharman *et al.* 1990). The position of *P. xanthopus* as the sister lineage to the *lateralis-penicillata* group (including *P. rothschildi*) is consistent with previous studies (Briscoe *et al.* 1982; Sharman *et al.* 1990; Eldridge and Close 1993; Campeau-Péloquin *et al.* 2001).

The relationship of *P. rothschildi* within *Petrogale* has also been contentious, with the species being placed in the *xanthopus* group based on its ancestral (2n=22) karyotype, but more recent molecular results have suggested a closer relationship to the *lateralis-penicillata* group (Briscoe *et al.* 1982; Sharman *et al.* 1990; Eldridge *et al.* 1992a; Campeau-Péloquin *et al.* 2001). The BEST analysis, together with reconstructions from combined datasets and mtDNA, provide strong support for a sister relationship of *P. rothschildi* with the *lateralis-penicillata* group. This finding supports previous allozyme studies (Briscoe *et al.* 1982; Eldridge and Close 1997) that, suggested a close relationship between *P. rothschildi* and the *lateralis* complex (Kirsch 1967; Hayman and Martin 1969; Close and Lowry 1990). DNA/DNA hybridisation analyses showed variation in the placement of *P. rothschildi* within the *lateralis* complex depending on the analysis performed, and ultimately it was excluded from analyses (Campeau-Péloquin *et al.* 2001). The unresolved polytomy for *P. rothschildi* and the *penicillata* group formed in the nDNA reconstruction is likely the result of incomplete lineage sorting and retention of ancestral polymorphisms.

Within *Petrogale*, the taxonomy and affinities of *P. purpureicollis* have been particularly unstable. It was originally described as a new species, based on its distinct morphology (Le Souef 1924), but was then reclassified as a subspecies of *P. inornata* (Troughton 1941). Subsequently, it was again reclassified as a subspecies of *P. penicillata* (Poole 1979) and then later, on the basis of chromosomal analyses, as a subspecies of *P. lateralis* (Briscoe *et al.* 1982). More recently, *P. purpureicollis* was reinstated as a full species based on mtDNA and additional chromosome data (Eldridge *et al.* 2001c). The results from the nuclear and

mitochondrial data presented here confirm that *P. purpureicollis* is a distinct species, but its relationships to the other *lateralis-penicillata* taxa were not well resolved. The BEST tree, as well as the combined and nuclear phylogenetic reconstructions (ML and Bayesian) suggest that *P. purpureicollis* is sister taxon to the *lateralis* complex, but low bootstrap support (as low as 33%) indicates uncertainty in this relationship. The mtDNA and parsimony reconstruction of the combined results place *P. purpureicollis* as a sister taxon to the *penicillata* complex, in support of previous DNA/DNA hybridisation analyses (Campeau-Péloquin *et al.* 2001). Discrepancies in these results are potentially caused by the short time period over which all of these lineages diverged, making it difficult to distinguish the position of *P. purpureicollis*. There is also the potential for retained ancestral characters through stochastic lineage sorting or introgression to influence tree reconstructions. Since retention of ancestral characters can produce the same patterns as introgression, these processes cannot be distinguished in this study (Niegel and Avise 1986; Pamilo and Nei, 1988; Doyle *et al.* 2003). Eldridge *et al.* (1991a) also could not resolve the position of *P. purpureicollis* using chromosome G-banding analyses, with evidence for either introgression between *P. purpureicollis* and the eastern *Petrogale* taxa or convergent chromosome evolution between *P. purpureicollis* and *P. lateralis* MacDonnell Ranges race. Although *P. purpureicollis* is currently allopatric with taxa from the *penicillata* group, past introgression at contact zones is possible, given the widespread evidence for introgression amongst eastern *Petrogale* species (Bee and Close 1993). Further data are required to fully resolve the relationship of *P. purpureicollis,* but based on the BEST results (87% pp) we suggest that *P. purpureicollis* is most closely allied to the *lateralis* complex. This proposal is consistent with chromosomal data linking *P. purpureicollis* to the *lateralis* complex, based on identical G-banding patterns with *P. lateralis* MacDonnell Ranges race (Sharman *et al.* 1990; Eldridge *et al.* 1991a) although it is the most divergent member of the *lateralis* complex (Eldridge *et al.* 2001b).

The relationships within the *penicillata* and *lateralis* complexes (excluding *P. rothschildi* and *P. purpureicollis*) were well resolved in these analyses, being consistent with previous studies based on morphological, allozyme, mtDNA and chromosomal analyses (Briscoe *et al.* 1982; Sharman and Maynes 1983; Sharman *et al.* 1990; Eldridge *et al.* 1991a). These results provide strong support for a close relationship between *P. l. lateralis and P. l. pearsoni*, with *P. l.* West Kimberley race forming the sister taxon to these two taxa and *P. l.* MacDonnell

Ranges race forming the most divergent *P. lateralis* taxon. Within the *penicillata* group there are two sister-lineage relationships (associated with parapatric distributions), one between *P. penicillata* and *P. herberti*, and the other between *P. assimilis* and *P. inornata*.

Divergence times and biogeography

Divergence dates estimated from fossil calibrations, such as those used in this study, should be taken with caution due to the patchy nature of marsupial fossils. However, they are used here as a general indication of divergences times within *Petrogale* to enable a general assessment of how geological and climatic events may have influenced diversification. The estimate of the split between tree-kangaroos (*Dendrolagus*) and *Petrogale* suggest this diversification occurred during the Miocene (6.0-11.3 MYA) during the commencement of aridification within Australia after a period of relatively stable wet and warm climatic conditions during the mid-Miocene (Martin 2006; Byrne *et al.* 2008). This transition in climate changed the landscape considerably and caused contraction of rainforests as open woodlands and chenopod habitat extended across central Australia (Martin 2006). This divergence estimate is consistent with those of Campeau-Péloquin *et al.* (2001) DNA/DNA hybridisation study (7.5 MYA), as well as those (5-10.4 MYA) based on the DNA sequence data of Meredith *et al.* (2008, 2009), coinciding with a time when many Australian-New Guinean marsupial clades separated (Kirsch and Springer 1993; Krajewski *et al.* 1993, 2000).

Within *Petrogale*, the divergence of three major lineages were also dated within the late Miocene-Pliocene, with the *brachyotis* group separating between 4.4-8.7 MYA, followed closely by the lineages leading to *P. persephone* (4.1-8.2 MYA) and *P. xanthopus* (3.6-7.6 MYA). These dates are slightly older than those found by Campeau-Péloquin *et al.* (2001), but are still within the early Pliocene and consistent with Meredith *et al.* (2008), who place the split of *P. concinna* and *P. xanthopus* at 4.3-6.1 MYA. That the first two of these three major lineages are distributed within the monsoon tropics (*brachyotis* group and *P. persephone*), suggests that the ancestor of these taxa inhabited mesic environments prior to their adaptation to more arid conditions. This is supported in the DIVA ancestral state reconstructions, where both the *brachyotis* group and *P. persephone* share a mesic ancestral

state, which then alters to an arid state for *P. xanthopus* taxa (Fig. 2.6). This hypothesis will need to be supported by further ancestral area reconstructions.

Further divergences within *Petrogale* were estimated to have occurred during the early Pliocene between 2.0-5.1 MYA, giving rise to the *P. rothschildi* lineage, the *lateralis* and *penicillata* complexes, the *P. purpureicollis* lineage, and multiple lineages within the *brachyotis* group. This period of divergence appears associated with a brief return of mesic conditions in Australia which may have allowed the expansion of *Petrogale* across the continent before the intense aridification in the mid-late Pliocene commenced (Martin 2006; Byrne *et al.* 2008; Fujita *et al.* 2010). During this period, it appears some *Petrogale* lineages (e.g., *P. rothschildi* and *P. lateralis*) began to inhabit and adapt to more arid environments in central and western Australia. The presence of divergent lineages with restricted distributions across Australia suggests *Petrogale* taxa have evolved in localised refugia where they could persist during periods of extreme aridity.

A more recent phase of divergences is estimated to have occurred throughout the Pleistocene when multiple taxa within the *lateralis* and *penicillata* complexes diverged (0.2-2.7 MYA), along with *P. concinna* and the Kimberley *P. brachyotis* lineages (1.1-2.5 MYA) and *P. xanthopus* subspecies (0.1-0.5 MYA). These divergence estimates coincide with more pronounced climatic cycles during the Pleistocene (Kershaw *et al.* 2003; Hocknull *et al.* 2007; Byrne *et al.* 2008; Fujita *et al.* 2010) which has been suggested to have modified species ranges across Australia (Fujita *et al.* 2010). Sharman *et al.* (1990) suggested derived chromosomes spread in eastern *Petrogale* through existing relatively continuous populations, with environmental and climatic factors rather than the chromosomal structural changes determining taxon distribution, although karyotypic changes may have contributed to the process of speciation through post-mating reproductive isolation at boundaries between parapatric taxa. However, the results support a hypothesis that environmental factors influenced speciation amongst eastern *Petrogale* through populations contracting during glacial maxima, isolating populations and facilitating genetic differentiation, including fixation of chromosomal changes in different geographic regions.

Attempts to reconstruct localities where ancestral *Petrogale* may have originated using ancestral state reconstruction revealed low support for ancestral states using BAYESTRAITS. This low posterior support for the two ancestral area states (arid and mesic) for the three nodes (in particular the root node and the node connecting the *lateralis-penicillata* taxa, *P. rothschildi* and *P. purpureicollis* taxa) highlights the widespread origins of ancestors to modern *Petrogale*. The habitat specificity of *Petrogale*, being restricted to rocky refugia, has likely enhanced the isolation experienced during these glacial-interglacial cycles of the Pleistocene. A hypothesis of widespread vicariance of *Petrogale* across Australia is concordant with a proposal by Sharman *et al.* (1990) who suggested Australia became occupied by a karyotypically homogeneous assemblage of *Petrogale*. The distribution of diverse *Petrogale* lineages across Australia and timing of diversification during the late Miocene-Pliocene with further speciation during the Pleistocene are concordant with a growing list of species (e.g., Bynoe‟s gecko (*Heteronotia binoei*; Fujita *et al.* 2010), king brown snake (*Pseudechis australis*; Kuch *et al.* 2005), *Ctenotus* and *Egernia* skinks (Chapple and Keogh 2004; Rabosky *et al.* 2007), agamid lizards (Hugall *et al.* 2008), elapid snakes (Kuch *et al.* 2005; Sanders *et al.* 2008), dasyurid marsupials (Blacket *et al.* 2001), old endemic rodents (Rowe *et al.* 2008) and suggests that the persistence of taxa in localised refugia has played an important role in diversification and speciation across Australia. These results support the numerous studies that illustrate the profound impact the mid to late Miocene-Pliocene and Pleistocene environmental changes have had on structure and diversity of different biomes (Byrne *et al.* 2008).

Conclusion

There are four major lineages within *Petrogale*; the *brachyotis* group, *P. persephone*, *P. xanthopus* and the *lateralis-penicillata* group (including *P. rothschildi*). Although *P. persephone, P. xanthopus* and *P. rothschildi* share similar 2n=22 karyotypes they are not monophyletic and represent three highly distinct lineages, with *P. rothschildi* being closely associated with the *lateralis-penicillata* group. *P. concinna* should be considered as part of the genus *Petrogale* and should no longer be referred to as *Peradorcas concinna*. The reinstatement of *P. purpureicollis* as a distinct species is supported and this species is likely to be the sister lineage to the *lateralis* complex, but further data from additional nuclear markers

are required to confirm this relationship. Future work needs to focus on resolving the relationships and taxonomy within the *brachyotis* group, with analyses suggesting that *P. brachyotis* as currently recognised consists of multiple species.

Ancestral *Petrogale* appear to have been widespread across a more mesic Australia, with climatic changes and glacial cycles of the late Miocene-Pliocene and Pleistocene associated with the diversification in this genus. It is likely that range contractions during glacial cycles resulted in isolated populations in localised refugia where divergence and speciation occurred. Increasing aridity resulted in the emergence of more arid-adapted taxa which have allowed *Petrogale* to persist throughout central and western Australia.

Statement of Authorship

Sally Potter (candidate)

Corresponding author: Prepared DNA extracts for PCR amplification, carried out DNA sequencing, analysed sequence data, wrote manuscript and produced all Figures.

Signed Date 12-1-2011

Mark D.B. Eldridge

Provided samples for study, provided assistance with obtaining project funding supervised the direction of the study, provided advice on analyses and critically reviewed manuscript.

I give consent for Sally Potter (candidate) to include this manuscript for examination towards the degree of Doctor of Philosophy.

Signed Date 10 Jan 2011

David A. Taggart

Provided assistance with obtaining project funding, sample collection and evaluated the manuscript.

I give consent for Sally Potter (candidate) to include this manuscript for examination towards the degree of Doctor of Philosophy.

Signed Date 11-1-2011

Steve J.B. Cooper

Supervised the direction of the study and provided advice on analyses and critically reviewed manuscript.

I give consent for Sally Potter (candidate) to include this manuscript for examination towards the degree of Doctor of Philosophy.

Signed Date 12/1/2011

CHAPTER 3: Phylogeography of the *brachyotis* group across northern Australia identifies multiple biogeographic barriers

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Abstract

Understanding of biogeographic patterns in the Southern Hemisphere is limited and the phylogeography of northern Australia is particularly poorly known. Northern Australia represents a relatively intact tropical monsoon landscape, with many endemic species. A major challenge is to understand the biogeographical processes that have shaped the distribution and diversity of taxa, without detailed knowledge of past climatic and environmental fluctuations. Although molecular data have great potential to address these biogeographical questions, only a few species, and no mammals, have been examined. Here, we use the widely distributed and abundant short-eared rock-wallaby (*Petrogale brachyotis*), together with the other *brachyotis* group taxa, the monjon (*P. burbidgei*) and nabarlek (*P. concinna*), to assess historical evolutionary and biogeographical processes in northern Australia. We sequenced ≈ 1000 bp of mitochondrial DNA (control region and *ND2*) and ≈ 3000 bp of nDNA (*BRCA1*, *ω-globin* and two anonymous loci) to investigate the phylogeographic structuring of populations within the *brachyotis* group and delineate the time-scale of diversification within the region. The results indicate deep phylogeographic structure, with a particularly large genetic discontinuity found between Arnhem Land and Kimberley regions. This major vicariant event, together with several other identified barriers has resulted in substantial diversification within the *brachyotis* group. Additional genetic structuring within *P. brachyotis* across the Kimberley, in the absence of any obvious barrier, suggests that additional barriers exist which could similarly influence genetic structure in other monsoonal biota.

Introduction

The impact of past climatic changes in driving species diversification, population genetic diversity and structural assemblages is becoming increasingly apparent through phylogeographic studies worldwide (Avise 2000; Yang and Rannala 2006; Donoghue and Benton 2007; Swenson 2008; Joseph and Omland 2009; Moussalli *et al.* 2009). The information gained from phylogeographic studies allows testing of alternative hypotheses about biogeography and speciation (Avise 2000; Moritz *et al.* 2000) and is also relevant for conservation (Moritz 1994; Hewitt 2004c; Byrne *et al.* 2008).

The historical biogeography of the Southern Hemisphere is poorly known in comparison to the wealth of data available for the Northern Hemisphere (Beheregaray 2008; Byrne *et al.* 2008). However, it appears to be emerging that aridification and climatic deterioration (cooling and drying) in the Southern Hemisphere has played an integral role in the speciation of flora and fauna throughout the Miocene and Pliocene (Richardson *et al.* 2001; Martin 2006; Ortiz-Jaureguizar and Cladera 2006; Byrne *et al.* 2008; Cowling *et al.* 2009; Fujita *et al.* 2010). Regular climatic fluctuations from glacial cycles during the Pleistocene have also caused periodic fragmentation, isolating taxa and creating further diversification (Richardson *et al.* 2001; Byrne *et al.* 2008; Bowman *et al.* 2010; Fujita *et al.* 2010). The Australian continent has become drier over the last 20 million years (MY) causing extreme aridity during glacial cycles (Williams 2000) and the contraction of rainforests and expansion of sclerophyllous vegetation (Martin 2006; Byrne *et al.* 2008). While recent research has focused on the Wet Tropics of coastal north-east Queensland (e.g., Joseph *et al.* 1995; Schneider *et al.* 1998; Moritz *et al.* 2000; Moritz *et al.* 2001; Stuart-Fox 2001; Hugall *et al.* 2002; Hugall *et al.* 2002; Dolman and Moritz 2006), southern and eastern Australia (e.g., James and Moritz 2000; Byrne *et al.* 2003; Wheeler and Byrne 2006; Edwards *et al.* 2007a; Garrick *et al.* 2007; Toon *et al.* 2007; Byrne 2008; Kearns *et al.* 2009; Edwards and Melville 2010; Malekian *et al.* 2010a) and the central Australian arid zone (e.g., Schodde 1982; Cooper *et al.* 2000; Strasburg and Kearney 2005; Ladiges *et al.* 2006; Cooper *et al.* 2007; Edwards 2007; Oliver *et al.* 2007), one of the major centres of endemism in northern Australia (the Top End and Kimberley) remains relatively unexplored. The Top End and Kimberley, located in the north-west of Western Australia and the northern Northern Territory have been identified as

containing the highest diversity of mammals, reptiles and birds within Australia (Woinarski 1992). However, for this monsoonal region there is a lack of knowledge of historical biogeography, evolutionary processes and the adaptive responses of organisms to ecological processes (Crisp *et al.* 2004; Braby 2008; Bowman *et al.* 2010). Biogeographical studies of terrestrial plants and animals from this region are limited (e.g., Ford 1978; Bowman 1992; Woinarski 1992) and even fewer phylogeographical studies have been published (Crisp *et al.* 2004; Bowman *et al.* 2010; Fujita *et al.* 2010), with three of the most comprehensive focusing on butterflies (Braby 2008) and birds (Jennings and Edwards 2005; Toon *et al.* 2010). With this region, only recently exposed to European impact, an ideal opportunity exists to assess the influence of natural biogeographic processes on diversification and speciation within a significant region of the Southern Hemisphere.

The tropical monsoon region of Northern Australia represents a unique ecosystem, comprising a range of habitats and natural landscapes that are relatively intact and unaffected by human-induced fragmentation compared to eastern and southern Australia. The monsoonal climate is characterised by dry winters and wet summers which drives the vegetation type and associated distribution of taxa (Braby 2008; Bowman *et al.* 2010). Tropical habitats contracted north during aridification in the Miocene and late Pliocene and the monsoonal conditions commenced following the rise of the Tibetan Plateau (3.4-7.2 MY ago; MYA) and the closing of the Isthmus of Panama which created the ocean and wind currents controlling the climatic conditions (Haug and Tiedemann 1998; An 2000; Fujita *et al.* 2010). It wasn‟t until the Pleistocene that the monsoonal tropics experienced fluctuating environmental changes associated with global glacial cycles which developed the more contemporary arid-adapted vegetation (reviewed in Fujita *et al.* 2010). Amongst the savannah of north-west Australia lie disjunct sandstone outcrops occupied by a suite of habitat specialists which may exhibit congruent biogeographical patterns. Phylogeographic analyses are lending support for recurrent vicariant speciation events in response to two major barriers in northern Australia, the Carpentarian Gap and Ord Arid Intrusion (Keast 1961; Ford 1978; Cogger and Heatwole 1981; Hnatiuk and Pedley 1985; Cracraft 1986; Woinarski 1992; Ladiges *et al.* 2003; de Bruyn *et al.* 2004; Jennings and Edwards 2005; Ozeki *et al.* 2007; Lee and Edwards 2008). However, a major obstacle to greater understanding of the region‟s biogeography is inadequate knowledge of species distributions, systematic relationships and taxonomy

(Schneider *et al.* 1998; Braby 2008). Surrogate taxa have been used to provide patterns of biodiversity for conservation planning where limited sampling of taxa is available from across their distribution and using this information to improve our understanding of the roles of environmental change, vicariance and dispersal on biodiversity patterns (especially when able to assess in a comparative framework with co-distributed taxa) (Schneider and Moritz 1999; Barraclough and Nee 2001; Hewitt 2004c; Moritz *et al.* 2005; Kirchman and Franklin 2007). Collectively, the phylogeographic data gained from various taxa will provide an understanding of the ecology and evolution of the monsoon tropics and determine whether common historical/demographic processes or different species‟ biology influence spatio-temporal biogeographic patterns.

The *brachyotis* group of rock-wallabies, the short-eared rock-wallaby (*Petrogale brachyotis*), monjon (*P. burbidgei*), and nabarlek (*P. concinna*) are ideal candidate taxa to examine the role of habitat disjunction (or discontinuities) in promoting diversification in northern Australia. The geographic distribution of the most widespread species (*P. brachyotis*) ranges from the Northern Territory/Queensland border to the north-western Western Australian coastline and southwards to the 1000mm rainfall isohyets (Sharman and Maynes 1983; Eldridge and Telfer 2008). *P. burbidgei* and *P. concinna* are sympatric with *P. brachyotis* across their range, with *P. burbidgei* confined to the far north-west Kimberley (Western Australia), as well as Bigge Island (18,000 ha) and several smaller islands off the Kimberley coast (Pearson *et al.* 2008), and *P. concinna* distributed in disjunct populations in the Northern Territory and Kimberley (Sanson and Churchill 2008). Due to paraphyletic relationships amongst the *brachyotis* group taxa (Chapter 2), all taxa are included here to explore the biogeographic history of northern Australia and to gain a full understanding of their evolutionary history. These species inhabit rocky outcrops throughout northern Australia, which is dominated by fire-prone savannah woodland (Eldridge and Telfer 2008). However, the rocky areas favoured by rock-wallabies act as a buffer against fire (Braithwaite and Muller 1997), which has enabled these species to remain relatively unaffected from the recently changed fire regimes which are seriously impacting the fauna of the region (Woinarski *et al.* 2001; Yibarbuk *et al.* 2001; Legge *et al.* 2008), therefore providing a valuable model organism to assess biogeographic processes across northern Western Australia and the Northern Territory.

Petrogale brachyotis is a medium-sized rock-wallaby (2.2-5.6 kg) that is morphologically variable across its range. Historically it was described as five distinct taxa (4 separate species) (Gould 1841; Thomas 1926a, 1926b), but since all share a highly derived autosomal karyotype (2n=18) only a single species is currently recognized (Sharman *et al.* 1990). However, exceptionally high levels of mitochondrial DNA (mtDNA) sequence divergence within and between *P. brachyotis* populations in the Northern Territory have recently been reported (Telfer and Eldridge 2010). This suggests that the distribution of genetic diversity within *P. brachyotis* is not currently well understood and a more comprehensive study is required to determine the inter-relationships of populations and the impact of historical biogeographic processes on this taxon**.**

Petrogale concinna (2n=16) is a similar but much smaller rock-wallaby (<1.4 kg), with a disjunct distribution across north-west Australia. Three subspecies have been described; *P. concinna concinna* (Victoria River district, NT) (Gould 1842), *P. c. canescens* (Arnhem Land, NT) (Thomas 1909) and *P. c. monastria* (Kimberley region, WA) (Thomas 1926b). *P. c. concinna* is only known from the type specimen collected in 1839 and the other two subspecies have rarely been reported. Only samples from *P. c. canescens* have been examined using genetic techniques and the distinction between the described subspecies (based on minor morphological differences) remains uncertain (Eldridge 1997b).

Petrogale burbidgei (2n=16) is the smallest species of rock-wallaby (<1.2 kg) and was only relatively recently discovered and described (Kitchener and Sanson 1978). It has a highly restricted distribution, being confined to rugged areas of King Leopold sandstone in the north-west Kimberley. *P. burbidgei'*s remote and largely inaccessible habitat has led to little research on this species, and nothing is known about its general biology or population dynamics. The habitat specificity of *Petrogale*, their small population sizes and limited dispersal (Hazlitt *et al.* 2004, 2006b; Piggott *et al.* 2006b) should make them sensitive indicators of long-term patterns of gene flow and so ideal candidates to elucidate historical biogeographic patterns and barriers.

Here we use phylogeographic analyses of the *brachyotis* group to examine the factors promoting diversification of the tropical monsoon fauna of northern Australia. Specifically, we use mitochondrial and nuclear DNA sequence data to (i) determine phylogeographic patterns of population differentiation within *P. brachyotis* and the broader *brachyotis* group (ii) infer the biogeographic barriers associated with these patterns (iii) estimate the timing of these divergences, and (iv) compare these findings with other studies to assess whether known barriers influence population differentiation within the *brachyotis* group and establish whether additional biogeographic barriers exist in northern Australia.

Materials and methods

Sampling and DNA extraction

Samples were obtained from 101 *P. brachyotis* from 28 populations throughout the species‟ distribution; eleven *P. burbidgei* from Mitchell Plateau (*n*=7), Prince Regent River (*n*=2), Enid Falls (1) and Bigge Island (*n*=1), Western Australia; and a single *P. concinna canescens* from Mt Borradaile in the Northern Territory (see Fig. 3.1 and Table 3.1). Due to limited sampling of *P. concinna* (*n*=1), its evolutionary history and relationship to other *brachyotis* group taxa cannot be reliably assessed and will not be discussed in detail. The number of samples per locality varied from 1-22 (average 3.5), and included eight representative *P. brachyotis* used in a previous study (Telfer and Eldridge 2010). All individuals were sequenced for the mtDNA fragments and a subsample of individuals (40-70), including representatives of various mtDNA haplotypes and geographical locations, was sequenced for the nuclear DNA (nDNA) loci. One *P. penicillata* (brush-tailed rock-wallaby), was used as an outgroup based on analysis of *Petrogale* phylogenetic relationships (Chapter 2).

Samples comprised 5mm ear biopsies collected in the field from live trapped animals or frozen and ethanol preserved ear, muscle, liver or kidney from the tissue collections of the South Australian, Western Australian and Australian Museum. DNA was extracted from the ethanol preserved ear biopsies using the Gentra DNA Isolation Kit (Gentra Systems), and from all other tissues following the „salting out‟ methods of Sunnucks and Hales (1996).

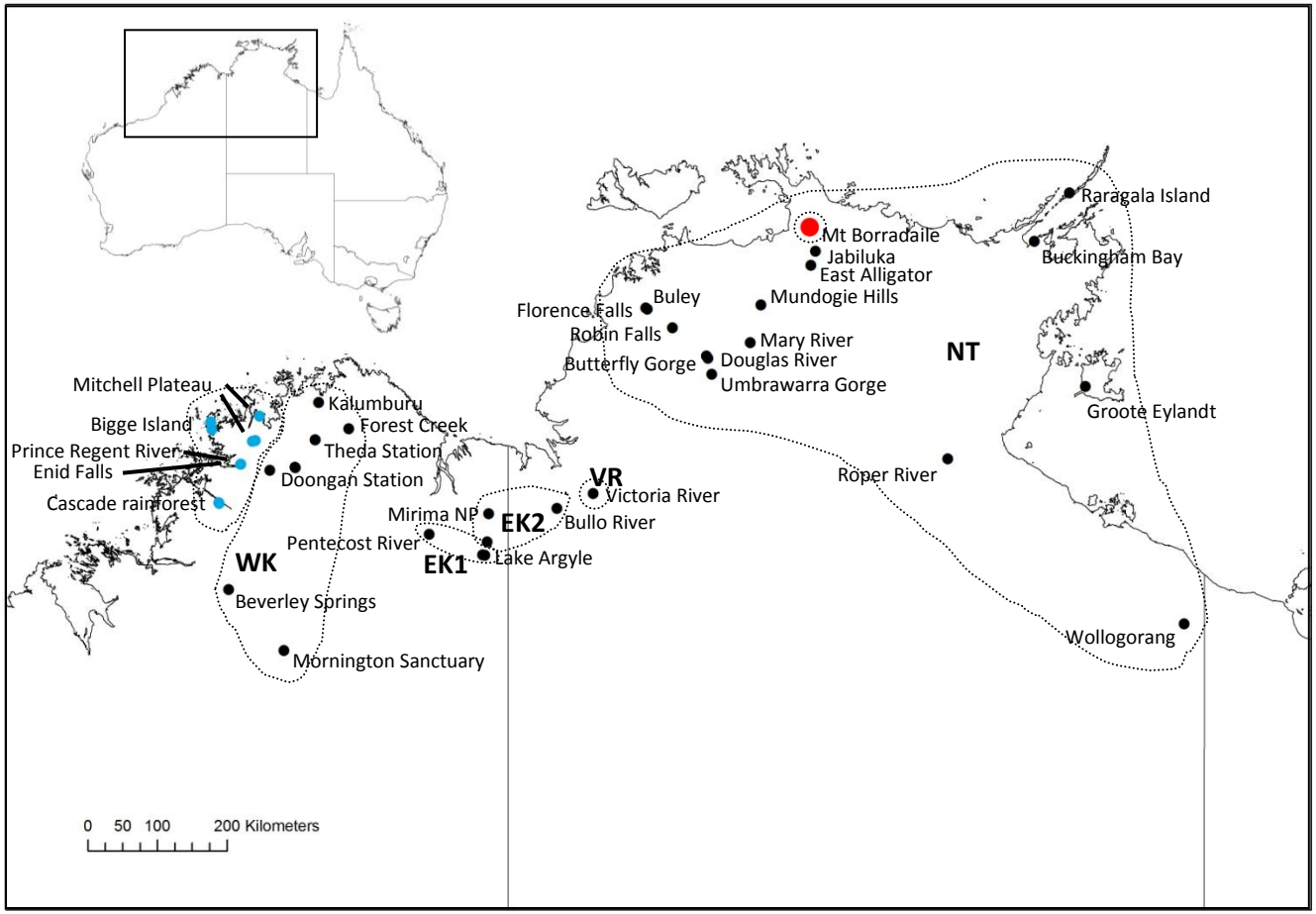


Figure 3.1: Map of northern Australia showing sampling localities and clade distributions of *Petrogale brachyotis* (black), *P. burbidgei* (blue) and *P. concinna* (red). Clades for *P. brachyotis* are labelled according to geographic location and are enclosed within the dashed lines. WK = West Kimberley, EK1 = East Kimberley 1, EK2 = East Kimberley 2, VR = Victoria River, NT = Northern Territory.

Table 3.1: Collection localities of *Petrogale brachyotis*, *P. burbidgei* and *P. concinna* used in this study.

Species	Location	State/ Territory	Sample ID	Latitude/Longitude	
P. brachyotis	Butterfly Gorge	NT	S1976, S1981	-13.72	131.56
	Umbrawarra Gorge	NT	S1982, S1989	-13.96	131.63
	East Alligator, Kakadu NP	NT	S1990, S2000	-12.36	132.98
	Robin Falls	NT	S2031	-13.36	131.13
	Florence Falls, Litchfield NP	NT	S2038	-13.10	130.78
	Buley, Litchfield NP	NT	S2052, S2056	-13.12	130.80
	Raragala Island	NT	ABTC29289	-11.61	136.26
	Wollogorang	NT	S1178	-17.19	137.74
	Mt Borradaile	NT	S318	-12.05	132.90
	Jabiluka, Kakadu NP	NT	S341	-12.55	132.92
	Groote Eylandt	NT	S92	-14.12	136.47
	Buckingham Bay	NT	S1368	-12.24	135.80
	Mundogie Hills	NT	S2082	-13.06	132.27
	Roper River (Ngukurr)	NT	S258, S259	-15.05	134.68
	Mary River	NT	S262, S263	-13.55	132.13
	Butterfly gorge, Douglas River	NT	S264, S265, S266, S267	-13.75	131.58
	Bradshaw Station, Victoria River	NT	S304	-15.50	130.10
	Bradshaw Station, Victoria River	NT	S305, S306	-15.20	129.80
	Big Knob waterhole, Bullo River	NT	S268, S269, S270, S271	-15.69	129.63
	Cyprus Valley, Theda Station, Kimberley	WA	ABTC100692, ABTC100693, ABTC100694, ABTC100695, ABTC100696, ABTC100697, ABTC100698, ABTC100699, ABTC100700, ABTC99396, ABTC103330, ABTC103331, ABTC103332	-14.81	126.51
	Mornington Sanctuary	WA	ABTC100701	-17.53	126.10
	Forest Creek	WA	ABTC101504	-14.66	126.94
	Mirima NP	WA	ABTC103494	-15.76	128.75

Table 3.1: Collection localities of *Petrogale brachyotis*, *P. burbidgei* and *P. concinna* used in this study.

Species	Location	State/ Territory	Sample ID	Latitude/Longitude	
P. brachyotis	Monsmont Island, Lake Argyle	WA	ABTC103495, ABTC103496, ABTC103497, ABTC103498, ABTC103499, ABTC103500, ABTC103501, ABTC103502, ABTC103503, ABTC103504, ABTC103505	-16.30	128.70
	Bullanyin Island, Lake Argyle	WA	ABTC103506, ABTC103507, ABTC103508, ABTC103509, ABTC103510, ABTC103511, ABTC103512	-16.29	128.67
	Lake Argyle (dam wall)	WA	S960, S962	-16.13	128.73
	Couchman, Doongan Station	WA	ABTC103715, ABTC99376, ABTC99377, ABTC99378, ABTC99379, ABTC99380, ABTC99381, ABTC99382, ABTC99383, ABTC99384, ABTC99385, ABTC99386, ABTC99387, ABTC99388, ABTC99389, ABTC99390, ABTC99391, ABTC99392, ABTC99393, ABTC99394, ABTC99395	-15.17	126.25
	Mitchell River, Doongan Station	WA	ABTC99375	-15.20	125.92
	El Questro, Pentecost River	WA	S272, S273, S274, S275, S277	-16.03	127.98
	Kalumburu	WA	S291, S292, S293	-14.32	126.55
	Beverley Springs	WA	S294	-16.74	125.39
	Beverley Springs	WA	S295	-16.51	125.33
P. burbidgei	Bigge Island	WA	ABTC101500	-14.57	125.16
	Surveyor's Pool, Mitchell Plateau	WA	ABTC101502	-14.67	125.17
	Enid Falls, Prince Regent River	WA	ABTC101503, ABTC101597	-15.12	125.54
	Prince Regent River	WA	ABTC101599	-15.63	125.26
	Crystal Creek, Mitchell Plateau	WA	S278, S279, S280, S281	-14.50	125.79
	Mertens Creek Mitchell Plateau		S982, S983	-14.82	125.73
P. concinna	Mt Borradaile	NT	S315	-12.05	132.90
P. penicillata	Ingles Rd Watagans	NSW	S1776	-32.76	151.11

DNA amplification, cloning and sequencing

Polymerase chain reaction (PCR) was used to amplify the following gene segments; mitochondrial control region (*CR*) and NADH dehydrogenase subunit 2 (*ND2*); non-coding nuclear *ω -globin* (intron 2; omega-globin gene), protein coding portions of exon11 of the breast and ovarian cancer susceptibility gene (*BRCA1*) and two anonymous nuclear markers (coded A1 & A2) (see Table 3.2 for primers). These markers were chosen because they have shown value in resolving marsupial interspecies relationships (Fumagalli *et al.* 1997; Osborne and Christidis 2001; Meredith *et al.* 2008; Malekian *et al.* 2010b).

The anonymous loci were amplified using primers G1732/ G1733; and G1738/ G1739 (see Table 3.2 for primer sequences). To develop these primers, genomic DNA from a gel extraction (selecting for ≈1 kb fragments) was digested with BamHI and BfuCI (New England BioLabs Inc.) and then cloned using the pGEM-T Easy Vector System (Promega). Cloned inserts were PCR-amplified using M13 forward (5‟-GTAAAACGACGGCCA-3‟) and reverse (5‟-CAGGAAACAGCTATGAC-3‟) plasmid vector primers and were sequenced for one *P. brachyotis* and one *P. burbidgei* (monjon). Primers (G1732/ G1733; and G1738/ G1739) were then designed for fragments that revealed variation between these two taxa.

PCR-amplifications were carried out in 25µL reactions with approximately 100ng genomic DNA, 1 x PCR buffer (Applied Biosystems), 0.20mM dNTPs, 2.5mM $MgCl_2$, 2 pmol corresponding primers and 0.1 U Ampli*Taq* Gold^{TM} polymerase (Applied Biosystems). Thermocycling was performed on a Corbett palmcycler under the following conditions: initial denaturation at 94°C for 9 min; 34-40 cycles of 45 s at 94°C (denaturation), 45 s at 48°C (*ND2*), 48-50°C (*BRCA1*), 52°C (A1) and 55°C (*CR*, *ω-globin*, A2) (annealing), and 1 min at 72°C (extension); and a final extension for 6 min at 72°C. PCR products were cleaned using Millipore MultiScreen PCR_{384}Filter Plates (Millipore) and then sequenced in both directions with the BigDye Terminator V3.1 Cycle Sequencing Kit (Applied Biosystems) at the Australian Genome Research Facility (AGRF) using an automated AB 3730xl capillary sequencer (Applied Biosystems).

Table 3.2: Primers used to amplify segments of *CR*, *ND2*, *BRCA1*, *ω-globin*, A1 and A2.

Gene	Primer Name	Source	Sequence (5'-3')
CR	L15999M	(Fumagalli *et al.* 1997)	ACCATCAACACCCAAAGCTGA
	H16498M		CCTGAAGTAGCAACCAGTAG
ND2	M635 (mmND2.1)	(Osborne and Christidis 2001)	GCACCATTCCACTTYTGAGT
	M636 (mrND2c)		GATTTGCGTTCGAATGTAGCAAG
BRCA1	G1798 (F-498MAC)	(Meredith *et al.* 2008)	CCAGAGGTAATCCTCAGAACTG
	G1799 (R11)		AGTTCTGAAAGTGGATTCTTT
	G1800 (F9)		CTGACCTRCAGCCTGAGGATTTCAT
	G1801 (R-1MAC9-20)		CTGCAGCTAGCTAACACTTGATC
ω-globin	G314	(Wheeler *et al.* 2001)	GGAATCATGGCAAGAAGGTG
	G424	(Blacket *et al.* 2006)	CCGGAGGTGTTYAGTGGTATTTTC
A1	G1732		ATAAACCCAAAGACCAAAAG
	G1733		GAAAAACGATGAGAAAGAAG
A2	G1738		GCTTCTATGACTTCCCCT
	G1739		CCGTATTGTGTTACTGTG

We tested for the presence of numts (nuclear pseudogenes of mtDNA) via a comparative serial dilution of potentially ambiguous mtDNA fragments against dilutions of a known amplifiable nDNA region, the X linked glucose-6-phosphate dehydrogenase (*G6PD* ; Loebel and Johnston 1997). Once the nDNA fragment failed to amplify, the mtDNA fragment amplified from the same template dilution was sequenced to compare with the original sequence. Results provided evidence that all mtDNA sequences obtained were from the mitochondrial genome.

Sequence analyses

DNA sequences were edited using SEQED (version 1.0.3; Applied Biosystems), then aligned using CLUSTAL X (version 1.83; Thompson *et al.* 1997) and manually refined using SEAL (version 2.0a11; Rambaut 1996). Gaps were considered as indels and assigned „-" and sequence ambiguities at heterozygous sites in nuclear regions were resolved using PHASE (version 2.1; Stephens *et al.* 2001; Stephens and Donnelly 2003). PHASE applies a Bayesian

statistical method to reconstruct haplotypes from population genotypic data, analysing single nucleotide polymorphisms (SNPs) from all taxa to infer probabilities of bases at ambiguous heterozygous sites. Sequences that could not produce accurate probabilities were removed (≈ 0.09) from further analyses as these unresolved ambiguities could produce erroneous results.

DnaSP (version 5.10; Librado and Rozas 2009) was implemented to estimate the number of haplotypes (*H*), polymorphic sites, nucleotide diversity (π) and haplotype diversity (*h*) (Rozas *et al.* 2003). Intra-specific sequence divergence among mtDNA haplotypes was estimated using the Tamura-Nei (Tamura and Nei 1993) model selected by Modeltest 3.06 (Posada and Crandall 1998) for the combined mtDNA dataset using PAUP* (version 4.0b10; Swofford 2002).

To test for evidence of recombination in *BRCA1*, *ω-globin*, A1 and A2 fragments, estimates of the minimum number of recombination events (Rm; Hudson and Kaplan 1985) and the "four gamete" test were calculated using DnaSP (version 5.10; Librado and Rozas 2009). Loci that revealed recombination were divided into segments of sequence free of recombination for neutrality and demographic analyses outlined below.

Phylogenetic analyses

Maximum likelihood (ML), maximum parsimony (MP) and Bayesian inference analyses were performed on the concatenated mitochondrial alignment, using RAxML (version 7.0.3; Stamatakis 2006; Stamatakis *et al.* 2008), PAUP* (version 4.0b10; Swofford 2002) and MrBayes (version 3.1.2; Ronquist and Huelsenbeck 2003; Huelsenbeck and Ronquist 2005). Analyses were run on a subset of samples that represented all haplotypes, to reduce run time and maximise the resolution of phylogenies under the different analysis criteria. The Akaike Information Criterion was implemented using Modeltest 3.06 (Posada and Crandall 1998) in PAUP* (version 4.0b10) to determine the best-fit models for RAxML and MrBayes. As the exact model suggested from Modeltest 3.06 was not available in these programs, the more complex General time Reversible (GTR) model was applied, with rate variation among sites modelled with a discrete gamma distribution (G) and proportion of invariant sites (I) for MrBayes. The GTR+G model was applied for RAxML, as the GTR+G+I model was

considered unreliable by the author (see RAxML manual for information). Heuristic searches using 1000 randomised addition orders with tree-bisection–reconnection (TBR) branch swapping, and a stepwise addition starting tree were used for MP analyses. The DELTRAN option for character-state optimization was utilised for MP trees, as there is a bug in PAUP* (Mac version) in the default ACCTRAN option which can result in erroneous branch lengths in output trees. ML analyses were started from a complete random starting tree and bootstrap analyses were carried out using 1000 pseudo-replicates, with multiple (100) searches per replicate, using the rapid Bootstrap analysis. MrBayes uses Metropolis-coupled Markov chain Monte Carlo (MCMC) sampling to calculate Bayesian posterior probabilities. We used default settings for priors, random starting trees and four Markov chains (three hot, one cold), with sampling every 1000 generations, for each of two independent analyses run simultaneously. Analyses were terminated when the average standard deviation of split frequencies for the simultaneous analyses fell below 0.01 (≈2-10 million generations). Tracer (version 1.5; Rambaut and Drummond 2009) was used to check that the convergence of parameter estimates had occurred. Tracer was also utilised to identify when the log likelihood values of trees became stable, to define the appropriate cut off for initial instability of trees (usually 10% burn-in was suitable). Posterior probabilities were calculated after discarding the first 25% of the sampled trees as burn-in.

Intraspecific evolutionary relationships are often better visualised using phylogenetic networks, as they allow assessment of persistence of ancestral haplotypes, recombination and multifurcations (Posada and Crandall 2001; Cassens *et al.* 2005) and low genetic variation within nuclear sequence data sets can be problematic for estimation of a phylogenetic tree. For each nuclear locus, therefore, an unrooted phylogram was constructed using the neighbour joining (NJ) distance approach in PAUP* (version 4.0b; Swofford 2002). As outlined by Dolman and Moritz (2006) these networks may not represent true genealogies, but allow for visual assessment of how alleles cluster with respect to mtDNA defined lineages. A parsimony haplotype network was also constructed for each locus using TCS (version 1.21; Clement *et al.* 2000), revealing similar configurations, but with multiple potential connections that possibly reflect recombination (Dolman and Moritz 2006) between nuclear haplotypes (results not shown).

Population genetic and historical demographic analyses

Analysis of molecular variance (AMOVA) was used to examine phylogeographic structure amongst geographically localized populations of *P. brachyotis*, *P. burbidgei* and *P. concinna*. AMOVA analyses were performed in Arlequin (version 3.11; Excoffier *et al.* 2005) and the populations identified in the mitochondrial phylogenetic analysis; *P. burbidgei*, *P. concinna*, *P. brachyotis* (Northern Territory; NT), *P. brachyotis* (Victoria River; VR), *P. brachyotis* (West Kimberley; WK) and *P. brachyotis* (East Kimberley; EK1 and EK2). Various groups of populations were tested to identify the most significant population structure, with significance of Φ_{ST} evaluated by 10,000 permutations.

The Tajima‟s test of neutrality (Tajima‟s *D*; Tajima 1989) was implemented to detect deviations from neutrality which could represent influences of selection. Demographic expansion and selection were assessed using Fu‟s Fs (Fu 1997) and R_2 tests (Ramos-Onsins and Rozas 2002) run in DnaSP (version 5.10; Librado and Rozas 2009), with negative Fs and low R_2 values suggesting demographic expansion or selection. These two tests are the most powerful tests for determining population growth (Ramos-Onsins and Rozas 2002). R_2 tests for population growth by comparing the difference between the number of singleton mutations and the average number of nucleotide differences between sequences and is powerful when dealing with limited sample sizes and appears to show little influence from recombination (Ramos-Onsins and Rozas 2002). Tests were conducted for regional groups supported by the AMOVA and phylogenetic clades inferred in the mtDNA Bayesian tree, with significance assessed with 1000 coalescent simulations under a constant population size model. These tests were run on recombination free data, as well as sequences with recombination to make sure results were congruent and that recombination did not influence the results.

The isolation with migration program (IMa; Hey and Nielsen 2004) was used to determine estimates of migration rates between two populations and distinguish models of gene flow. We employed this program for the divergence events between regional groups supported by AMOVA and mtDNA phylogenetic analyses, comparing geographically adjacent populations (*P. concinna* could only be included in other population comparisons, due to only a single

individual being sampled, that was insufficient to form an independent population for analysis). For each divergence event the following parameters were estimated, scaled by the neutral mutation rate (μ, geometric mean across loci of mutation rate per year per locus); θ_1, θ_2, effective population diversity for the two populations since divergence ($\theta_1 = 4\ N_1\mu$; where N_1 is the effective size of population 1); θ_A, effective population diversity for the ancestral population, m_1 and m_2, directional migration rates between the descendant populations (number of migrants per mutation $m_1 = m_1/\mu$ converted to population migration rate $M = 2\ N_1m_1$); and t, time since divergence. Since $4\ N_1\mu \times m_1/\mu/2 = 2\ N_1m_1$ (population migration rate), you can estimate the population migration rate (effective rate at which genes come into a population per generation) by $\theta_1 \times m_1/2$.

All loci were analysed together, with the infinite site (IS) model applied to all nuclear loci and the HKY model applied to the mtDNA data. IMa assumes no recombination in loci and selective neutrality (infinite sites model), so it was only used to analyse segments of genes free from recombination and evidence of selection. For the loci that did not pass the four-gamete test, the locus was divided into regions free of recombination. From these, a random sequence segment for each locus was then selected for IMa analyses. Several short preliminary metropolis coupled Monte Carlo Markov chains (MCMCMC) simulations were run to optimise prior boundaries for the parameters, with wide initial runs to ensure prior distributions for the model parameters were appropriate. The final simulation for each divergence was carried out with random seeds, 12 chains with geometric heating and run for 280 hours with a burnin period of 28 hours. Results from these runs confirmed convergence upon parameter distributions and additional repeat analyses ensured adequate MCMCMC mixing for all parameters.

Since an accurate molecular rate of evolution is needed to estimate dates of divergence in IMa and this was not available, divergence times were estimated using the program BEAST (version 1.5.3; Drummond *et al.* 2006; Drummond and Rambaut 2007), utilising multiple fossil constraints (refer to Chapter 2 for methods). Divergence estimates were based on *ND2*, *BRCA1* and *ω-globin* sequence data, under a relaxed molecular clock using the Uncorrelated Normal prior, which estimates the rate of each lineage (tree branch) independently from a normal distribution. The data were partitioned into mtDNA and nDNA, with the following

models of nucleotide substitution; GTR+G (*ND2*), HKY (nDNA) based on the model of sequence evolution suggested by Modeltest 3.06 for these data partitions. Two independent MCMC chains were run for 100 million generations with sampling every 10,000 generations and 25% burn-in of the posterior samples. Tracer (version 1.5; Rambaut and Drummond 2009) was used to examine if the chains had reached satisfactory convergence, based on effective sample sizes and posterior densities. To test that the data fitted the molecular clock model the coefficient of variation parameter was examined and the autocorrelation of rates in the phylogeny were assessed using the rate of covariance.

Results

Sequence variability

Control region (*CR*) and *ND2* fragments were analysed together; 1185 bp (599 bp *CR*; 586 bp *ND2*) were obtained from 113 individuals (101 *P. brachyotis*; 11 *P. burbidgei* and one *P. concinna*). A total of 378 sites were variable and 303 sites were parsimony informative within the *brachyotis* group, while 349 sites were variable and 269 sites were parsimony informative within *P. brachyotis* alone. All sequences are available on CD (Appendix 1). A total of 57 haplotypes were detected (Table 3.3), 49 in *P. brachyotis*, seven in *P. burbidgei* and one in *P. concinna***.** Reduced numbers of individuals were sequenced for the nuclear loci due to technical constraints, therefore a total of 40 individuals were sequenced for *BRCA1*, 68 for *ω-globin*, 57 for A1 and 66 for A2 (Table 3.3). In the nuclear analyses two haplotypes were analysed for each individual for each locus, to account for ambiguities at heterozygous sites. Within the 1044 bp fragment of *BRCA1*, 21 sites (2.0%) were variable and 14 were parsimony informative. Of the 537 bp sequenced for *ω-globin*, 22 (4.1%) variable sites were identified with 20 being parsimony informative. A 571 bp and a 680 bp fragment was sequenced for the anonymous markers A1 and A2, and these markers had 33 (5.8%) and 28 (4.1%) variable sites, respectively (Table 3.3), of which 30 and 24 sites were parsimony informative (respectively). Sequences are available on CD (Appendix 1). There were 32 indel (insertion or deletion) events detected within the *CR* and 2 indel events detected for A1, A2 and *ω-globin*. There was no recombination detected for *BRCA1* but 3-5 minimum recombination events were inferred for the other three loci, with 10-21 pairs of sites showing the four gametic types.

Table 3.3: Descriptive statistics for the five loci including non-recombined fragments used in this study. Sequence length (*L*) includes alignment gaps, number of samples (*n*) includes number of alleles for nuclear loci, *H* is the number of haplotypes, *S* is the number of segregating sites, *h* is the haplotype diversity and π is the nucleotide diversity. Loci in *italics** indicate fragments free of recombination for loci that showed evidence of recombination.

Marker	*L*	*n*	*H*	*S*	*h*	π
mtDNA	1159	113	57	378	0.96 ± 0.10	0.0758
BRCA1	1044	80	17	21	0.85 ± 0.02	0.0023
ω-globin	537	136	28	22	0.88± 0.02	0.0072
*ω-globin**	171	136	6	5	0.57 ± 0.04	0.0054
A1	571	114	31	33	0.93 ± 0.01	0.0099
*A1**	223	114	14	14	0.78 ± 0.03	0.0095
A2	680	132	25	28	0.86 ± 0.02	0.0040
*A2**	173	132	6	5	0.59 ± 0.03	0.0041

Mitochondrial phylogeography

Eight geographically constrained monophyletic groups of haplotypes (clades) were recovered from the combined mtDNA parsimony, likelihood and Bayesian analyses (73-100% bs and 0.96-1.00 pp; refer to Fig. 3.2 for phylogenetic tree). *P. brachyotis* was paraphyletic, with individuals from the Northern Territory forming a sister lineage to a clade comprised of *P. brachyotis* from the Victoria River region and Kimberley (comprising three independent lineages; two East Kimberley and one West Kimberley lineage), as well as *P. burbidgei* and *P. concinna* (Fig. 3.2). The five concordant, well supported, geographically structured *P. brachyotis* lineages represented *P. brachyotis* from the following regions: Northern Territory (NT), from near the Queensland border west to the Mary River, including some Arnhem Land islands; Victoria River (VR), in the western Northern Territory; West Kimberley (WK), from Beverley Springs north-eastwards to Kalumburu in the north Kimberley and south at Mornington Sanctuary; and two East Kimberley clades (EK1 and EK2), EK1 comprising individuals from Monsmont Island in Lake Argyle to Pentecost River in WA (≈82 km west)

and EK2 consisting of individuals from Bullanyin Island in Lake Argyle eastwards to Bullo River in the far west of NT (≈123 km east) (see Fig. 3.1 for localities). *P. burbidgei* formed a separate clade with the exception of one individual (ABTC101599) which was highly divergent from other *P. burbidgei* taxa (9.6-10.6% sequence divergence; SD) and formed a sister lineage to *P. concinna* instead of the *P. burbidgei* clade. The *P. burbidgei* clade formed a sister lineage to *P. brachyotis* (VR, EK1, EK2 and WK) and so was embedded within *P. brachyotis*, separating populations from the NT from those elsewhere in its distribution (Fig. 3.2). The single exemplar of *P. concinna* also formed an independent lineage, divergent from all others (4.9-12.5% SD), but was a sister lineage to *P. brachyotis* (EK1, EK2 and WK; Fig. 3.2). There was 16.8-18.8% SD between *P. penicillata* (outgroup) and *P. brachyotis* (NT, VR, EK1, EK2 and WK), 17.7% SD compared to *P. concinna* and up to 20% SD compared to *P. burbidgei*.

The *P. brachyotis* (NT) clade was the most divergent lineage with 9.6-15% SD between it and the other *P. brachyotis* lineages (9.1-14% SD compared to *P. burbidgei*). Within this clade, samples from Kakadu National Park, Litchfield National Park, Douglas River and Mary River were clustered both geographically and genetically, and showed sequence divergence of 0.3-3.2% between haplotypes (see Fig. 3.2). Samples from further to the east including Buckingham Bay in Arnhem Land, Raragala Island of the NT coast, as well as Roper River were more divergent (1.8-5.7% SD). A *P. brachyotis* specimen (S1178) from Wollogorang Station near the NT/Queensland border was especially divergent from all other haplotypes both within the NT clade (9-12.1% SD) and from the VR and Kimberley (KIM) lineages (10.5-13.0**%** SD; Fig. 3.2). There was strong support for an early divergence (0.99 pp and 100% bs) of this lineage at a similar time to the clades comprising VR and NT haplotypes (Fig. 3.2). The Groote Eylandt *P. brachyotis* individual (S92) also showed a high divergence (7.3-12.1% SD) from other haplotypes within the NT lineage. The branching pattern for S92 varied, with ML and MP analyses strongly supporting (100% bs) it as the sister lineage to all other NT samples (excluding the more divergent S1178; see Fig. 3.2). The Bayesian analyses, however, placed it as sister lineage to the Kakadu group described above, but support for this arrangement was low (50% pp) suggesting a lack of resolution.

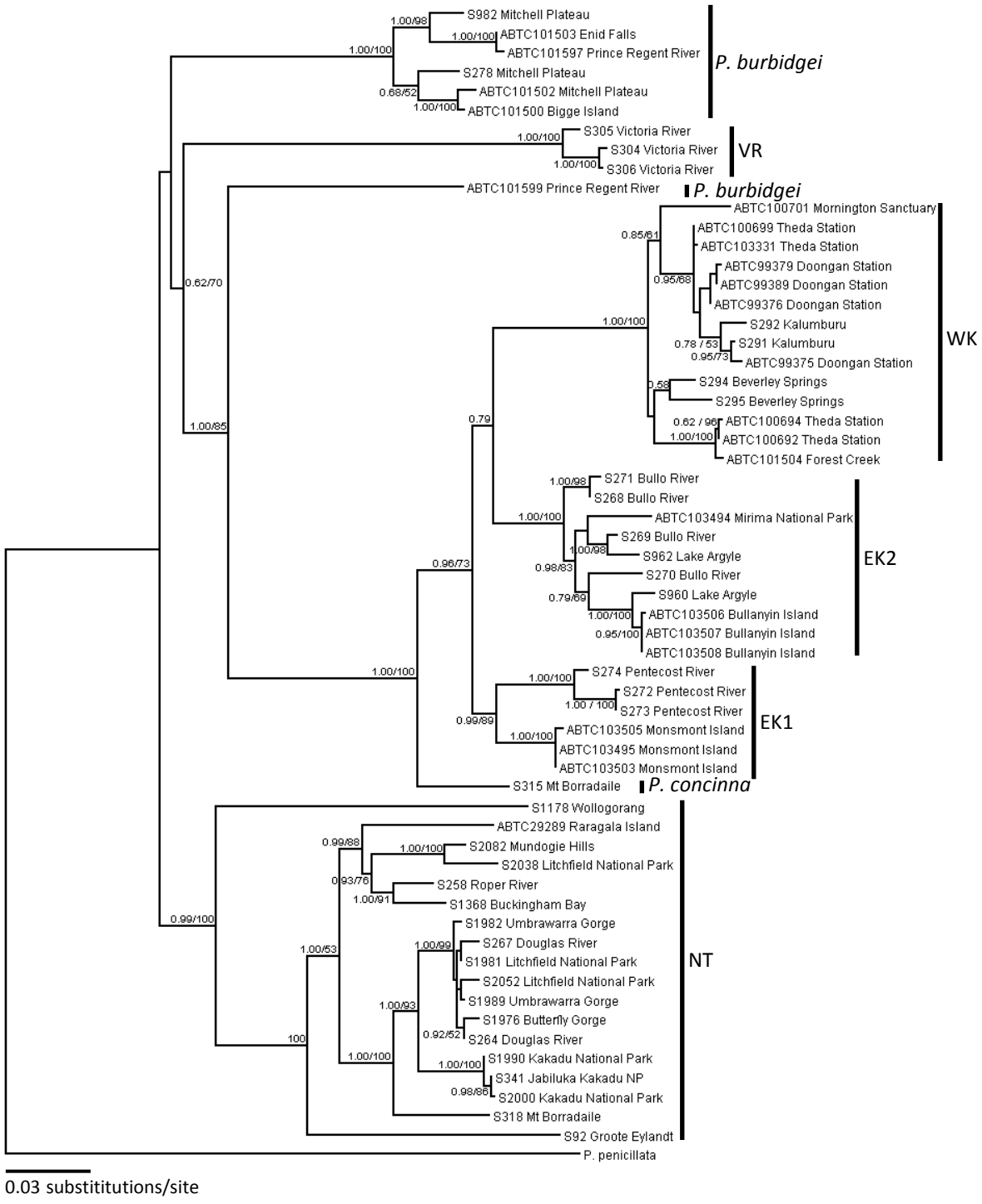


Figure 3.2: Maximum likelihood mtDNA phylogenetic tree of *P. brachyotis* (NT, VR, EK1, EK2 and WK), *P. burbidgei* and *P. concinna* with *P. penicillata* used as an outgroup to root the tree. The Bayesian posterior probabilities are represented as decimal values and the ML bootstrap values are as percentages (only includes support > 0.5 or 50%). Haplotypes from the different geographic populations are outlined to the right of the tree.

Although the *P. burbidgei* clade was well supported (100% bs and pp), the node connecting it to *P. brachyotis* (NT) was not well resolved with low bootstrap support from ML analysis (<50% bs; refer to Fig. 3.2) and in the Bayesian reconstruction it formed a polytomy with the *P. brachyotis* (NT) lineage and the remaining taxa. The relationship of the *P. brachyotis* (VR) lineage was also not well resolved, with the parsimony reconstruction showing a polytomy for it and all other taxa. In addition, low bootstrap and posterior probability support (70% and 62%, respectively, Fig. 3.2) suggests the node connecting *P. brachyotis* (VR) to *P. burbidgei* was not well resolved. *Petrogale burbidgei* individuals (excluding ABTC101599) were also highly divergent, with 9.6-12.8% SD compared to *P. brachyotis* (VR and KIM), *P. concinna* and individual ABTC101599. Within the *P. burbidgei* clade there was up to 4.3% SD between haplotypes. The three *P. brachyotis* (VR) haplotypes were all closely related (0.2-1.4% SD), whilst this lineage was highly divergent compared to *P. concinna* and *P. brachyotis* (KIM) haplotypes (10.6-12.5% SD).

Within the *P. brachyotis* Kimberley lineage (KIM; EK1, EK2 and WK) there was up to 6% SD between EK1 and EK2 lineages and 4.9-7.8% SD between all *P. brachyotis* (KIM) lineages (EK1, EK2, WK). The nodes connecting EK1, EK2 and WK lineages had reduced bootstrap support from ML analysis (<50% and 73%) indicating that they may not be well resolved (Fig. 3.2).

Lineage relationships inferred from nuclear regions

As expected, the nuclear markers showed less sequence variation (maximum SD 0.8% - 2.3%; *BRCA1* and *ω-globin*, respectively) and resolution than the mtDNA; therefore only unrooted phylograms of nDNA loci are presented (Fig. 3.3). For the A2 marker, the majority of haplotypes from the *P. brachyotis* (NT) population formed a distinct cluster that was not found in any other population (with one exception, S269 from EK2). For *BRCA1*, all *P. burbidgei* individuals shared a single haplotype (or allele) and *P. brachyotis* (VR and KIM) shared haplotypes that were not present in *P. brachyotis* (NT) (Fig. 3.3). Most of the *P. brachyotis* (NT) haplotypes formed a monophyletic group, independent from *P. brachyotis* (VR and KIM), *P. burbidgei* and *P. concinna*, with the exception of highly divergent (mtDNA) individuals from Groote Eylandt (S92) and Wollogorang (S1178) which formed

unique haplotypes. *P. concinna* formed independent haplotypes for *BRCA1* and A1. There were many shared alleles between the mtDNA groups across the four loci; nine between *P. brachyotis* (NT) and *P. brachyotis* (KIM), five between *P. brachyotis* (VR) and *P. brachyotis* (KIM), four between *P. burbidgei* and *P. brachyotis* (KIM), two between *P. concinna* and *P. brachyotis* (KIM) and one between *P. burbidgei* and *P. brachyotis* (NT; refer to Fig. 3.3). No alleles were shared uniquely between *P. brachyotis* (NT) and *P. brachyotis* (VR), *P. brachyotis* (NT) and *P. concinna*, *P. burbidgei* and *P. concinna*, or *P. brachyotis* (VR) and *P. burbidgei* or *P. concinna*.

Population structure

AMOVA analyses of all loci (nuclear and mtDNA) found *P. brachyotis* (NT) to be significantly differentiated ($P<0.05$) from the other two *P. brachyotis* mtDNA groups (VR and KIM), *P. burbidgei* and *P. concinna* (Table 3.4). The most significant proportion of variation between groups for mtDNA was obtained when samples were distributed among five groups (*P. brachyotis* NT, VR, KIM; *P. burbidgei* and *P. concinna*). For *ω-globin* however, the most significant proportion of the genetic variation was among seven groups (five listed above, but with *P. brachyotis* KIM split into EK1, EK2 and WK groups). For A1, the variation among groups was greatest with a similar group structure to *ω-globin*, but with the two EK populations pooled into a single group. The percentage of total variation distributed among the selected groups for each locus ranged from 26.5% in A1 (*P. brachyotis* in four geographic groups; NT, VR, EK and WK) to 52.4% in A2 (*P. brachyotis* in two geographic groups; NT, VR/KIM; refer to Table 3.4). ABTC101599 was removed from AMOVA analysis due to lack of confidence in its taxonomic classification based on such high mtDNA sequence divergence compared to other *P. burbidgei* individuals (see Chapter 5 for discussion on its classification).

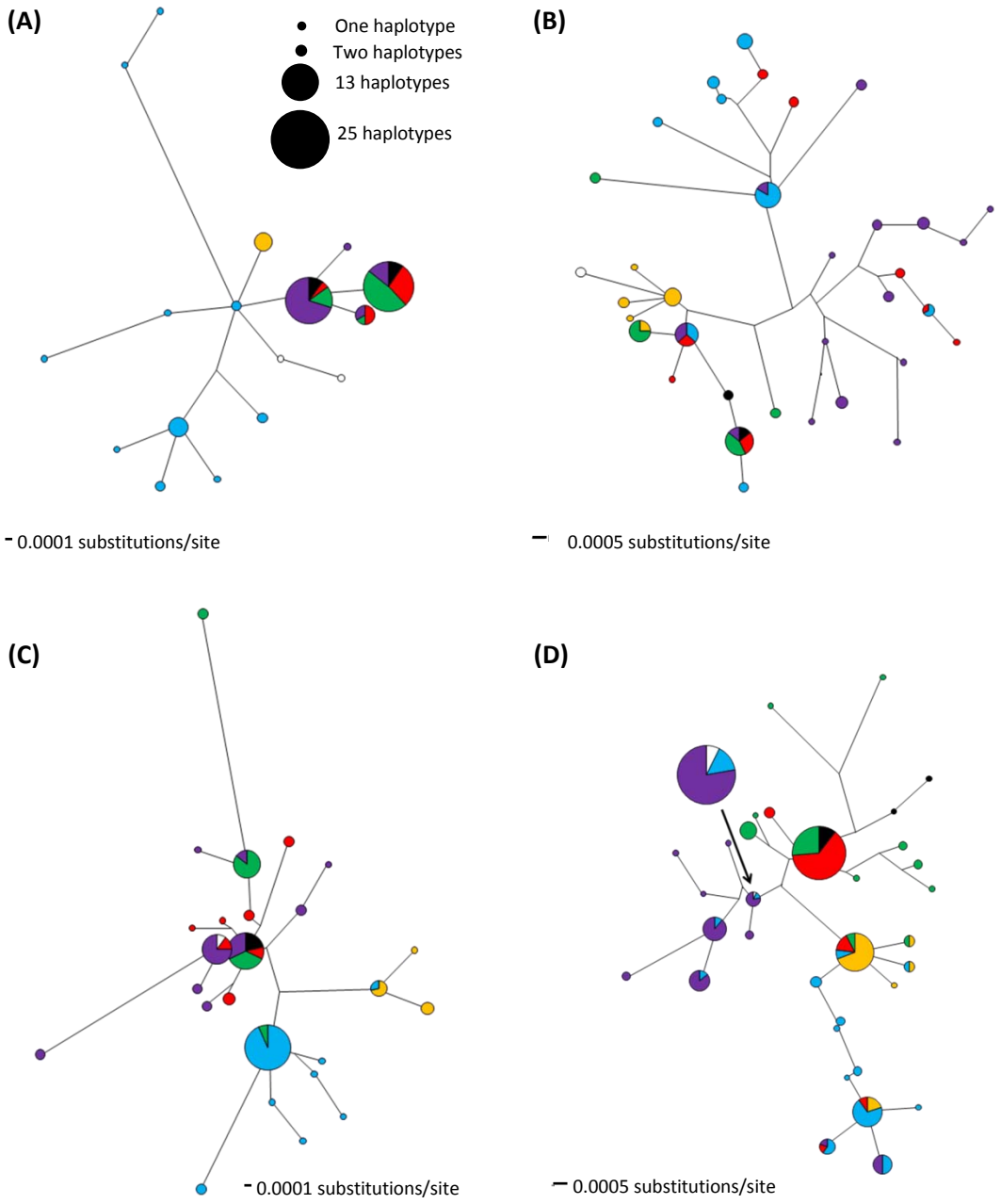


Figure 3.3: Nuclear haplotype networks (unrooted phylograms using the NJ distance approach with Tamura-Nei model); (A) *BRCA1*; (B) A1; (C) A2; (D) *ω-globin*. Circle size is scaled to allele frequency and branches scaled to the number of mutational steps; *P. brachyotis* (NT) - blue, *P. brachyotis* (VR) - black, *P. brachyotis* (EK1) – red, *P. brachyotis* (EK2) – green, *P. brachyotis* (WK) -purple, *P. burbidgei* - yellow and *P. concinna* - white.

Table 3.4: Results of analyses of molecular variance for mtDNA, *BRCA1*, *ω-globin*, A1 and A2 for the following groups: *P. brachyotis* (Northern Territory), *P. burbidgei*, *P. brachyotis* (Victoria River), *P. concinna* and three *P. brachyotis* (Kimberley) groups; EK1, EK2 and WK. Number of groups for each marker is defined by (d.f. + 1) and represents the number of groups within *P. brachyotis* (e.g., mtDNA; 4 d.f., 5 groups total, 3 *P. brachyotis* groups; NT, VR and KIM).

	Among groups				Among sites – within groups				Among individuals – within sites			
Markers	**d.f.**	**Variance**	Φ_{ST}	**% Var**	**d.f.**	**Variance**	Φ_{ST}	**% Var**	**d.f.**	**Variance**	Φ_{ST}	**% Var**
mtDNA	4	40.96	0.469*	46.9	4	31.63	0.831*	36.2	103	14.75	0.682*	16.9
BRCA1	3	0.840	0.510*	51.0	5	0.425	0.768*	25.8	71	0.382	0.526*	23.2
ω-globin	6	0.877	0.368*	36.8	2	0.201	0.452*	8.4	131	1.31	0.133	54.8
A1	5	0.968	0.265*	26.5	2	0.201	0.320*	5.5	106	2.49	0.075	68.0
A2	3	0.991	0.524*	52.4	5	0.230	0.646*	12.2	123	0.670	0.255*	35.4

*Significant difference (P-value < 0.05) between fixation indices (F_{ST} /F_{SC} /F_{CT}) and variance components.

Historical demographic analyses

Tests of neutrality suggested little evidence for non-neutral processes of evolution (e.g., selection or expansion) with non-significant Tajima‟s *D*, R_2 and F_S values for all groups, with the exception of the *P. brachyotis* (NT) group (results; Table 3.5). For *P. brachyotis* (NT), significant R_2 values were detected for *BRCA1* and A2, whilst significant F_S values were found for A2 and *ω–globin*. There were also significant R_2 values detected in *P. burbidgei* for two markers (*ω–globin* and A1), suggesting the possibility of demographic expansion in this species.

There was substantial recombination in the genealogical history of *ω-globin*, A1 and A2 according to the four gamete test and minimum number of recombination events. This reduced the nuclear fragments from 537 bp, 571 bp and 680 bp to 171 bp, 223 bp and 173 bp for *ω-globin*, A1 and A2, respectively (which reduced the number of segregating sites to 5, 14 and 5 for recombinant-free segments used in IMa analyses; refer to Table 3.3). Tests of neutrality performed on all recombination-free segments used in IMa analyses were not significant for either Tajima‟s *D* or Fu‟s Fs ($P<0.05$), indicating that there is no evidence of selection acting on the four nuclear loci.

Relative parameter estimates were used to assess and contrast *P. brachyotis* (NT), *P. burbidgei*, *P. brachyotis* (VR), *P. concinna* and *P. brachyotis* (EK1, EK2 and WK) groups (assuming the neutral mutation rate to be consistent) using a nested design, to assess whether groups were isolated (refer to Fig. 3.2 for groups; *P. concinna* was only included in groups with other *brachyotis* taxa). Marginal posterior density distributions of migration (m_1, m_2) and population diversity (θ_1, θ_2, θ_A) between the eight mtDNA clades (listed above) were derived (e.g., *P. brachyotis* NT compared to *P. burbidgei*, *P. brachyotis* VR, EK1, EK2, WK and *P. concinna*; *P. burbidgei* compared to *P. brachyotis* VR, EK1, EK2, WK and *P. concinna*) and are shown in Fig. 3.4. Peak posterior estimates for m_1, m_2, θ_1, θ_2, θ_A and *t* and respective credibility intervals (90% highest posterior densities) are presented in Table 3.6. Substituting the estimates of these parameters from the peak posterior heights, we estimated the population migration rates (estimated by θ_1 x $m_1/2$; Table 3.7). While we were able to obtain reliable estimates of θ_1, θ_2, m_1

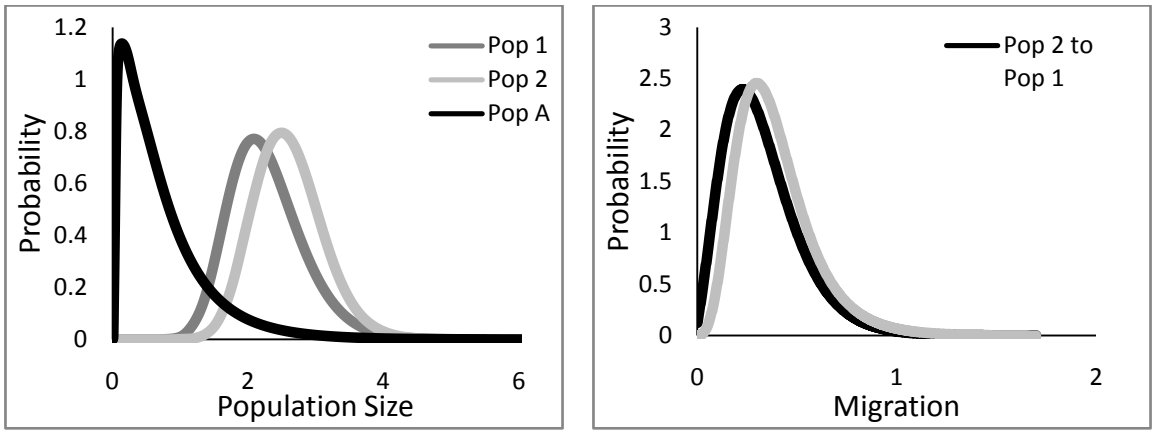
and m_2 for all pairs of populations this was not always attainable for θ_A and t. These parameters have little to no affect on estimates of migration rate though (Nielsen and Wakeley 2001), therefore it does not alter the confidence in these migration rate estimates.

Population migration rates between outlined mtDNA clades (displayed in Table 3.7) were all below one (<1; a critical value below which population divergence is expected to occur; Wright 1931). The upper 90% HPD was just above one (1.31 and 1.39) for comparisons between *P. brachyotis* (NT) and *P. burbidgei* (including *P. brachyotis* VR, EK1, EK2, WK and *P. concinna*) and between *P. burbidgei* and *P. brachyotis* (VR and EK1, EK2, WK) including *P. concinna*.

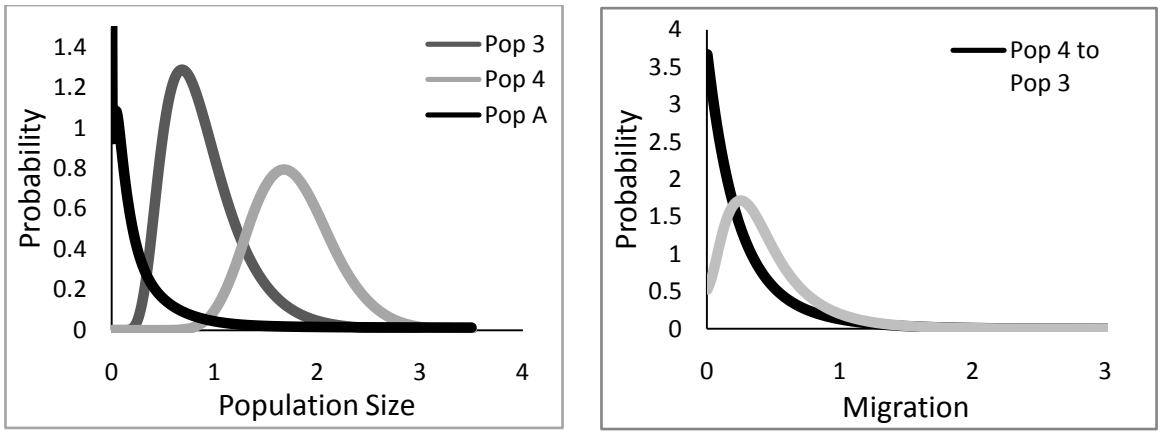
Since the marginal posterior density distributions of migration estimates peaked close to zero we can have confidence that they indicate a lack of gene flow in either direction between the populations in these comparisons (refer to Fig. 3.4). Population migration rates between *P. brachyotis* EK2 and WK also had fairly low estimates of upper 90% HPD (1.25, 1.91, respectively; Table 3.7) suggesting a lack of gene flow between these regions. Despite the peak posterior migration rate between *P. brachyotis* EK1 and EK2/WK close to zero for these comparisons, the upper bounds of the 90% HPD ranged up to 7.29 migrants per population (refer to Table 3.7), a level of effective gene flow well above the value <1 indicated to prevent differentiation through genetic drift among populations within a single species. This indicates a low confidence surrounding the peak posterior values between EK1 and EK2/WK based on the current data. The effective sample size (EES) for the *P. brachyotis* (VR) comparison to *P. brachyotis* (EK1, EK2, WK) individuals and *P. concinna* indicated that there was not enough data to identify the model and therefore results from this comparison are not reliable.

Table 3.5: Demographic results of each independent locus for all geographic subdivisions of *P. brachyotis* (NT – Northern Territory, VR – Victoria River, WK – West Kimberley and two East Kimberley – EK1 and EK2) including results for *P. burbidgei*. Significant *P*-values, highlighted in **bold** (**0.05***, **0.01****), indicate population expansion (R_2 and Fs) and non-neutral selection of loci (Tajima‟s *D* values). All recombination-free segments used in IMa analyses were not significant for Tajima‟s *D*.

Gene	Geographic Subdivision	R_2	Fs	Tajima's *D*
mtDNA	NT	0.124	0.009	-0.106
	VR	0.316	-	-
	EK1	0.137	0.042	-0.105
	EK2	0.144	0.169	-0.162
	WK	0.109	-0.418	-0.150
	P. burbidgei	0.161	0.607	-0.014
BRCA1	NT	**0.158***	0.021	-0.008
	VR	0.364	0.312	-0.004
	EK1	0.207	0.302	-0.019
	EK2	0.195	0.247	-0.096
	WK	0.165	0.281	-0.049
	P. burbidgei	-	-	-
A1	NT	0.118	-0.251	-0.068
	VR	0.360	0.325	-0.006
	EK1	0.142	0.111	-0.687
	EK2	0.142	0.014	-0.074
	WK	0.119	-0.008	-0.099
	P. burbidgei	**0.175***	0.218	-0.050
A2	NT	**0.130****	**0.075****	**-0.098****
	VR	-	-	-
	EK1	0.157	0.019	-0.026
	EK2	0.141	0.087	0.041
	WK	0.118	0.104	-0.071
	P. burbidgei	0.220	0.290	-0.011
ω-globin	NT	0.119	0.075	-0.082
	VR	0.321	0.512	-0.080
	EK1	0.142	0.143	-0.030
	EK2	0.138	**0.063****	-0.104
	WK	0.109	-0.004	-0.045
	P. burbidgei	**0.171****	0.196	-0.094

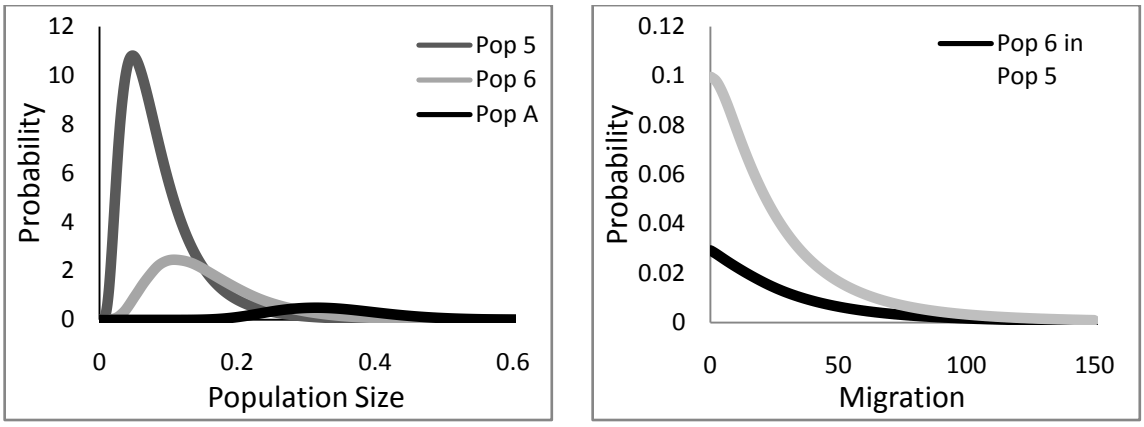


(A)

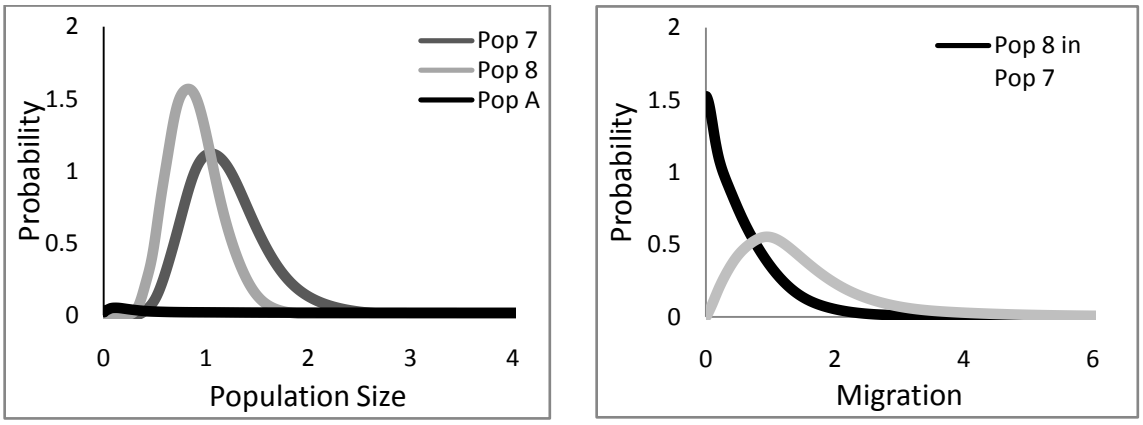


(B)

Figure 3.4: Marginal posterior density distributions of migration (scaled by neutral mutation rate) and population size (for two daughter populations and ancestral population – Pop A) derived from fitting the isolation with migration model to four nuclear loci and mtDNA for four divergence events, (A) *Petrogale brachyotis* (NT) – Pop 1 and *P. burbidgei* + *P. brachyotis* (VR + KIM) + *P. concinna* – Pop 2; (B) *P. burbidgei* – Pop 3 and *P. brachyotis* (VR + KIM) + *P. concinna* – Pop 4.



(C)



(D)

Figure 3.4: Marginal posterior density distributions of migration (scaled by neutral mutation rate) and population size (for two daughter populations and ancestral population – Pop A) derived from fitting the isolation with migration model to four nuclear loci and mtDNA for four divergence events, (C) *P. brachyotis* (EK1) – Pop 5 and *P. brachyotis* (EK2 + WK) – Pop 6; and (D) *P. brachyotis* (EK2) – Pop 7 and *P. brachyotis* (WK) – Pop 8.

Table 3.6: Results of IMa analysis of mtDNA subdivisions within the *brachyotis* group. All estimates shown here are scaled by the neutral mutation rate (μ): θ_1 and θ_2 represent the population size for the two populations; θ_A is the ancestral population size of the two populations; m_1 and m_2 represent the migration rate at which genes come into the two populations (m_1 genes come into population 1 and m_2 are the migration rate at which genes come into population two); and t indicates the divergence time between the two populations. HPD90Hi represents the upper bound of the estimated 90% highest posterior density interval and HPD90Lo is the lower bound of this density interval. Population one is the first named population$_1$ and population two is the second population$_2$ labelled.

Comparison	θ_1	θ_2	θ_A	m_1	m_2	t
P. brachyotis NT$_1$/*P. burbidgei*$_2$	2.09	2.49	0.14	0.23	0.30	1.33
	(1.39-3.14)	(1.76-3.40)	(0.02-1.56)	(0.04-0.60)	(0.10-0.67)	(0.88-1.81)
P. burbidgei$_1$/*P. brachyotis* VR$_2$	0.68	2.15	0.01	0.00	0.26	1.44
	(0.34-1.44)	(1.43-3.08)	(0.01-3.69)	(0.00-0.74)	(0.00-0.85)	(0.47-3.89)**?**
P. brachyotis EK1$_1$/*P. brachyotis* EK2+WK$_2$	0.05	0.27	3.14	0.08	0.03	0.01
	(0.01-0.16)	(0.10-0.68)	(2.04-4.65)	(0.08-77.63)	(0.03-21.43)	(0.00-0.02)
P. brachyotis EK2$_1$/P. brachyotis WK$_2$	1.05	0.83	0.58	0.01	0.95	2.73
	(0.59-1.81)	(0.47-1.32)	(0.03-44.43)	(0.01-1.38)	(0.10-2.90)	(0.08-35.13)?

*NT – Northern Territory population, VR – Victoria River population, EK$_1$ – East Kimberley population including Monsmont Island and Pentecost River, EK$_2$ – East Kimberley population including Bullanyin Island and Bullo River, WK – West Kimberley population (outlined from mtDNA phylogroups).

Table 3.7: Population migration rates (m_1, m_2), which represent the migration rate at which genes come into the two populations (m1 genes come into population 1 and m2 are the migration rate at which genes come into population two) estimated from IMa results Table 6 (θ_1 x m_1/2). HPD90Hi represents the upper bound of the estimated 90% highest posterior density interval and HPD90Lo is the lower bound of this density interval given in brackets (HPD90Lo-HPD90Hi). Population one is the first named population$_1$ and population two is the second population$_2$ labelled.

Population Divergences	m_1	m_2
P. brachyotis NT$_1$/*P. burbidgei*$_2$	0.24 (0.03-0.94)	0.37 (0.09-1.39)
P. burbidgei$_1$/*P. brachyotis* VR$_2$	0.00 (0.00-0.53)	0.28 (0.00-1.31)
P. brachyotis EK1$_1$/*P. brachyotis* EK2+WK$_2$	0.00 (0.00-6.21)	0.00 (0.00-7.29)
P. brachyotis EK1$_1$/P. brachyotis WK$_2$	0.01 (0.00-1.25)	0.39 (0.02-1.91)

Divergence estimates from BEAST analysis of fossil constraints using a normal distribution place the divergence of *P. brachyotis* (NT) from *P. burbidgei* during the early Pleistocene-Pliocene (2.24 million years ago, MYA; 95% HPD – minimum lower, *Ml* – 2.55 MYA to maximum upper, *Mu* –6.23 MYA; refer to Fig. 3.5 for phylogeny with divergence estimates). The divergence of both *P. brachyotis* (NT) and *P. burbidgei* from *P. concinna* and *P. brachyotis* (VR and KIM) was estimated at 2.70 MYA (*Ml* – 2.55 MYA; *Mu* – 6.23 MYA). *P. brachyotis* (VR) was estimated to have diverged at a similar time from *P. concinna* and *P. brachyotis* (KIM), 2.18 MYA (*Ml* – 2.05 MYA; *Mu* – 5.36 MYA), whilst divergences of *P. concinna* from *P. brachyotis* (KIM) and between *P. brachyotis* lineages within the Kimberley were estimated during the Pleistocene (0.13-1.64 MYA; Mu – 0.04 MYA; Ml – 3.36 MYA).

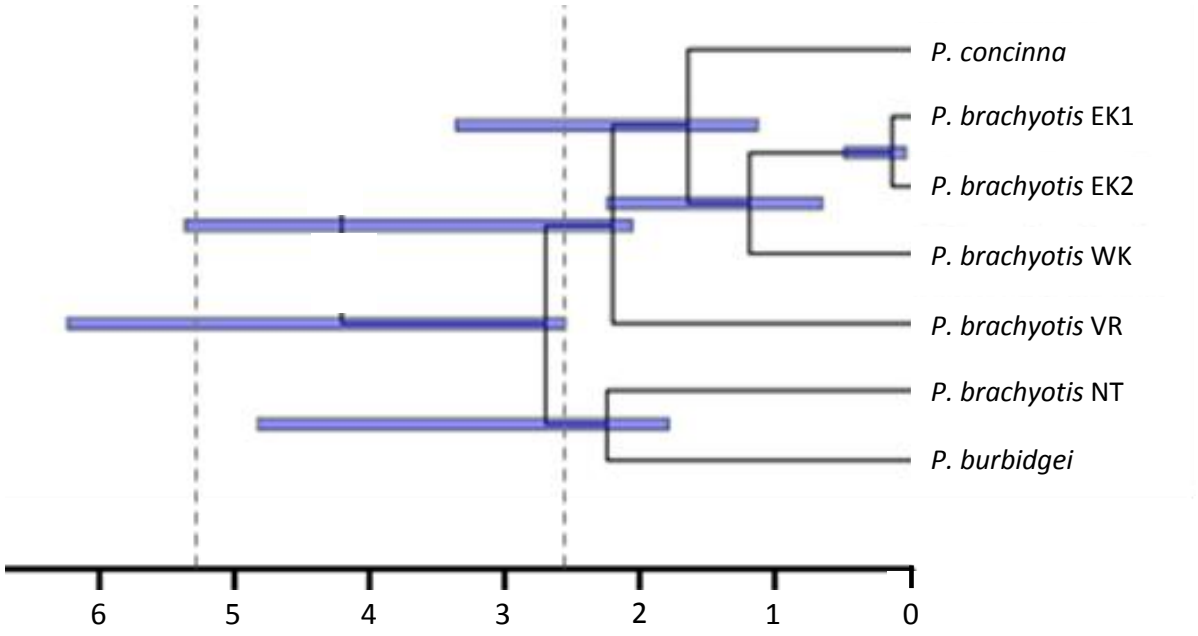


Figure 3.5: Chronogram inferred from a relaxed molecular clock in BEAST based on the normal prior distribution of fossil calibrations. Scale bar is in millions of years ago (MYA), starting from the present (0 MYA) at the tips of the nodes. Node bars are 95% confidence intervals and vertical bars infer the Pliocene (5.332 MYA) and Pleistocene (2.588 MYA) time-scale.

Discussion

Phylogeographic patterns and biogeographical processes

Analyses of mtDNA and four independent nuclear loci identified eight genetic lineages within the *brachyotis* group, distributed over discreet geographic regions in north-western Australia. The first lineage comprised haplotypes of *P. brachyotis* distributed throughout the Top End and Gulf of Carpentaria region of the Northern Territory (hereafter referred to as the Top End lineage). The other lineages included; samples of *P. burbidgei*, *P. brachyotis* individuals from Victoria River, *P. brachyotis* individuals from the Kimberley together with *P. brachyotis* individuals from north-western Northern Territory (Bullo River) that separated into three geographic regions (a West Kimberley and two East Kimberley lineages), a lineage consisting of the *P. concinna* individual and another for individual ABTC101599 (*P. burbidgei*). The boundary between the Top End *P. brachyotis* lineage and the other taxa (excluding *P. concinna*) lies around the Daly River, NT. This lowland region associated with a discontinuity in sandstone ranges and a band of arid country that extends northwards from central Australia to the Joseph Bonaparte Gulf, has been referred to in the biogeographic literature as the Victoria River Drainage barrier (Schodde and Mason 1999), the Bonaparte Gap (Ford 1982) or the Ord Arid Intrusion (Bowman *et al.* 2010). In this study we will refer to the Daly River barrier, as the location of the major separation within the *brachyotis* group.

In the mtDNA analysis *P. brachyotis* was paraphyletic, a phenomenon not uncommon in phylogeography (Funk 2003; Joseph and Omland 2009) and often attributed to incomplete lineage sorting, introgression (Joseph and Omland 2009) or incorrect taxonomy (e.g., two species being mistakenly treated as one) (Funk 2003). The finding that *P. burbidgei* formed a sister lineage to *P. brachyotis* from Victoria River and the Kimberley (VR and KIM) and not *P. brachyotis* from the Northern Territory (NT) is likely the result of incorrect taxonomy. The presence of such large sequence divergences between *P. burbidgei* and *P. brachyotis* (NT, VR and KIM; 9.1-14% SD) make it unlikely that these highly divergent lineages resulted from incomplete lineage sorting or recent hybridisation. In addition to the genetic divergence, both

taxa have different chromosome numbers (2n=16, *P. burbidgei* and *P. concinna*; 2n=18, *P. brachyotis*) and maintain different body sizes in sympatry (Eldridge and Telfer 2008; Pearson *et al.* 2008), so there is strong evidence that they are separate gene pools and reproductively isolated. Ancient hybridisation, however, cannot be ruled out, with evidence of hybridisation amongst parapatric species of *Petrogale* on the Australian east coast indicating this is possible in rock-wallabies (Sharman *et al.* 1990; Bee and Close 1993). However, the consistent pattern in the data, both nuclear and mitochondrial (AMOVA, mtDNA sequence divergence, with estimated divergence times during the Pliocene) seems to point to vicariance as the cause for the observed significant divergence between *P. brachyotis* (NT) and *P. burbidgei*, *P. brachyotis* (VR and KIM) with the Daly River Barrier separating the Top End lineage from all other lineages (excluding *P. concinna*).

The support from nuclear markers for significant divergence between these two regions indicates the mtDNA divergence is not just an outcome of sex-biased dispersal, but results from the long term lack of connectivity and gene flow leading to substantial differentiation within the *brachyotis* group. The most significant evidence comes from coalescent results, which incorporate the stochasticity of mutation and genetic drift (Joseph and Omland 2009) and indicated no effective gene flow between these two lineages (0.24, 0.37; migration into Top End and Kimberley lineages, respectively), reinforcing the Daly River region as a major historical barrier to gene flow for the *brachyotis* group.

The deep division of the *brachyotis* group is consistent with previous biogeographical analyses which similarly identified the Daly River region as a major vicariance barrier between Top End and Kimberley taxa/populations including breaks in the distribution of ~12 pairs of bird species (Keast 1961; Ford 1978; Schodde and Mason 1999; Jennings and Edwards 2005; Joseph and Omland 2009), as well as at least one rodent (Kitchener 1989), a carnivorous marsupial (Kitchener 1988) and butterflies of the genus *Nesolycaena* (Braby 2008). Since all of these species are associated with rugged sandstone habitat, their shared pattern of geographical structure implies a shared biogeographical history, which might be expected for habitat

specialists (see Bowman *et al.* 2010). Currently the only estimated divergence time for this major vicariant barrier comes from grass finches (*Poephila* sp.), where Jennings and Edwards (2005) estimated a split between taxa of 0.34 MYA, during periods of Pleistocene aridity. Pleistocene aridity was suggested earlier by Keast (1961) as being a cause for vicariance in numerous bird species across this region but without specific dates. Results, however, indicate that the *brachyotis* group first started to diverge earlier, during the Plio-Pleistocene (2.70 MYA). The Pliocene saw intense aridification of Australia but with a brief return to warm mesic conditions early on (3-5 MYA) (Byrne *et al.* 2008). The Pliocene climatic changes were suggested to have influenced the Australian biota by causing taxa adapted to mesic conditions to become isolated in refugia, whilst population expansion was expected for arid-zone lineages with increased aridification (Byrne *et al.* 2008), much the same as the glaciers influenced Northern Hemisphere biota. The presence of population expansions in the monsoonal biome similar to the patterns in the arid-zone lineages indicate that this region was similarly affected by aridification during the Pliocene and Pleistocene (Byrne *et al.* 2008; Fujita *et al.* 2010). Fujita *et al.* (2010) reinforced the notion that the monsoonal tropics included multiple refugial areas, especially in regions with relatively complex topography (e.g., Kimberley and Top End). The presence of multiple genetically divergent lineages across northern Australia within the *brachyotis* group supports the presence of multiple refugial areas. The presence of different estimated divergence times between *Peophila* and the *brachyotis* taxa suggests that vicariance events have occurred several times during the evolutionary history of this region in the Plio-Pleistocene.

Within *Petrogale* generally, and *P. brachyotis* specifically, localised refugia have been suggested to provide areas during glacial cycling where taxa differentiated and persisted during periods of extreme aridity (Telfer and Eldridge 2010; Chapter 2). Ancestral *Petrogale* are suggested to have inhabited mesic environments prior to their adaptation to more arid conditions during the Pliocene (Chapter 2). The effect climatic cycles played on the differentiation of taxa is a result of differing species‟ biology (e.g., vagility) and consequently different divergence times emerge between organisms. The location of refugia, however, may not have changed during the Pliocene and Pleistocene, therefore giving concordant biogeographical patterns for many taxa. The earlier

onset of divergence during the Pliocene for the Top End and Kimberley lineages is concordant with the divergence of many arid-zone taxa, including: dasyurid marsupials (Krajewski *et al.* 2000; Blacket *et al.* 2001), agamid lizards (Melville *et al.* 2004; Hugall *et al.* 2008), old endemic rodents (Rowe *et al.* 2008), invertebrates (Leys *et al.* 2003; Cooper *et al.* 2007; Cooper *et al.* 2008), elapids (Sanders *et al.* 2008) and geckos (Fujita *et al.* 2010), whose divergences began during the late Miocene/Pliocene, with subsequent intra-lineage structuring continuing throughout the Pleistocene for some taxa.

At a similar time during the Plio-Pleistocene (2.24-2.70 MYA; early divergence from *P. brachyotis* NT), *P. burbidgei* was estimated to have diverged from *P. brachyotis* (VR and KIM) and *P. concinna*. There is no obvious barrier for this deep divergence, with the distribution of *P. burbidgei* embedded within that of *P. brachyotis* (VR and KIM). One hypothesis previously proposed by Maynes (1989) was that the smaller sized rock-wallabies (*P. burbidgei* and *P. concinna*) originated through dwarfism as a result of isolation on islands. Under this scenario, an island population became reproductively isolated as a result of allopatric divergence and subsequently recolonised the mainland during favourable climatic cycles, allowing secondary contact between *P. burbidgei* and *P. brachyotis* (KIM). This resulted in the current sympatric distribution of these species. *P. burbidgei* from Bigge Island formed a close relationship to individuals from the mainland (up to 4.3% SD), indicating recent divergence (e.g., Pleistocene divergence in line with SD found between *P. brachyotis* KIM individuals), supporting a view of common ancestry in more recent times than the original divergence from *P. brachyotis* (NT). Further sampling of *P. burbidgei* from other islands off the Western Australia coast and mainland, as well as individuals of *P. concinna* throughout its range are required to support/reject this hypothesis and elucidate whether the evolution of *P. burbidgei* and *P. concinna* occurred at the same time or as a result of multiple evolutionary processes throughout the Plio-Pleistocene. Based on the limited data it appears that the speciation of *P. burbidgei* and *P. concinna* was not related (i.e., they are not sister taxa), but the result of independent evolutionary processes.

Although the region between the Daly River and the Joseph Bonaparte Gulf has been proposed as the major biogeographic barrier dividing the monsoonal biota of north-western Australia, these analyses suggest this region actually consists of multiple independent biogeographic barriers that have each caused significant differentiation, particularly within *P. brachyotis*. The most prominent of these occurs within the Kimberley lineage (excluding *P. burbidgei*), separating mtDNA lineages of *P. brachyotis* from the Victoria River region from those found further west. The 10.4-12.5% SD between haplotypes from these geographic regions is equivalent to that found between *P. brachyotis* from Victoria River and the Northern Territory (10.5-13%). In addition, the Kimberley and Victoria River *P. brachyotis* mtDNA lineages are paraphyletic, with *P. concinna* from the Northern Territory being placed as the sister lineage to *P. brachyotis* (KIM) rather than *P. brachyotis* (VR). This seemingly anomalous relationship needs to be assessed further, to understand whether incomplete lineage sorting, ancient hybridisation or inadequate current taxonomic classifications are the cause. Data from only one individual of *P. concinna* makes this assessment difficult and further sampling of *P. concinna* throughout its range is required. The lack of monophyly between *P. brachyotis* from the Victoria River and Kimberley regions in the nuclear haplotype networks could potentially be the result of slower timing to reach coalescence (four times more slowly than mtDNA under a neutral model of evolution) and a lower rate of mutations between taxa (Hare 2001). There was not enough data for the *P. brachyotis* (VR) and *P. brachyotis* (KIM) comparison to fit a model to determine population migration rates, therefore further sampling will be necessary to assess if there is gene flow between the Kimberley and Victoria River *P. brachyotis*. However, the AMOVA results for *ω-globin* and A1 found significant genetic variation amongst the *P. brachyotis* lineages when they were separated into Northern Territory, Victoria River as well as East and West Kimberley groups. The identification of Victoria River *P. brachyotis* as a genetically distinct entity strengthens the suggestions of Sharman and Maynes (1983) that *P. brachyotis* comprised three morphologically distinct races located in Arnhem Land, Victoria River region and the Kimberley. Based on these results it appears there is potentially reduced gene flow between the Kimberley and Victoria River *P. brachyotis*, with significant mtDNA divergences (larger than the 8.8% SD

between *P. penicillata* and *P. herberti*; Browning *et al.* 2001) and significant levels of genetic differentiation (based on AMOVA results) among these regions for *ω-globin* and the A1 locus.

The *P. brachyotis* samples we examined from the Victoria River region were collected north-east of the Victoria River, but west of the Daly River. The arid country around the Victoria River was outlined by Ford (1978) as a putative biogeographic barrier and equates to the Victoria River Drainage (VRD) barrier of Schodde and Mason (1999). This region also has less continuous sandstone habitat which is likely to have contributed to the geographical separation and genetic differentiation within the *brachyotis* group. Estimates of the divergence time suggest that this barrier was also creating differentiation between *P. brachyotis* during the early Pleistocene/late Pliocene (2.19 MYA), with aridification (as described earlier), causing lineages to contract to available refugia (associated with the sandstone escarpment between Victoria and Daly Rivers). More extensive sampling of *P. brachyotis* in the region between the Victoria and Daly Rivers is required to identify the full distribution of this divergent lineage. Studies of other species across north-western Australia have tended to only sample individuals from Arnhem Land and the Kimberley with limited sampling in the broader region between. Therefore, the existence of these barriers may have previously been undetected and their impact combined, in the absence of fine scale sampling across the region. Further sampling of species right across this geographic range is needed to elucidate whether these patterns of multiple phylogenetic breaks within the Victoria/Daly River region are common across taxa or whether they are specific to *brachyotis* group rock-wallabies and their unique life history traits.

River systems, presumably because they restrict dispersal (Avise 2009), appear to play a key role in separating rock-wallaby taxa. In eastern Australia the Fitzroy River separates *P. herberti* (Herbert‟s rock-wallaby) and *P. inornata* (unadorned rock-wallaby); the Burdekin and Bowen River separates *P. inornata* from *P. assimilis* (allied rock-wallaby) and; *P. mareeba* (Mareeba rock-wallaby) and *P. godmani* (Godman‟s rock-wallaby) are separated by the Mitchell River (Bee and Close 1993). It is therefore not surprising that the Daly and Victoria Rivers appeared associated with significant genetic structuring in the *brachyotis* group.

The mitochondrial data also indicate that there is significant divergence (6-7%) within the Kimberley region (for *P. brachyotis*), with three mitochondrial lineages associated with geographically localised regions in the eastern and western Kimberley. The 5.5-7.6% SD between these lineages is greater than differences between subspecies and evolutionarily significant units (ESUs) of other rock-wallaby species (Pope *et al.* 1996; Browning *et al.* 2001; Eldridge *et al.* 2001b)**.** Population structure (AMOVA) analyses of two nuclear markers (*ω-globin* and A1) also identified significant genetic variation amongst the East and West Kimberley regions, but only one marker (*ω-globin*) supported a West and two East Kimberley mitochondrial lineages (EK1, EK2 and WK). As support for these more minor phylogeographic patterns is based only on the mtDNA phylogeny and AMOVA results from one or two nuclear markers they must be viewed with caution since similar structure can result from stochastic lineage sorting (Niegel and Avise 1993; Barraclough and Nee 2001; Irwin 2002; Moussalli *et al.* 2009) and isolation by distance. There is a lack of confidence in the isolation with migration results which, although suggesting no effective gene flow between the East and West Kimberley (population migration rate <1), show an upper 90% HPD indicating the potential for high levels of gene flow maintaining connectivity between these regions. Therefore population migration values also need to be taken with caution and connectivity between these regions needs to be further assessed using multiple sources of data (e.g., fine-scale nuclear markers - microsatellites). Interestingly, the sympatric white-quilled rock-pigeon displays concordant geographical structure, with recognition of western Kimberley *Petrophassa albipennis alisteri* and eastern Kimberley *P. a. albipennis* subspecies on morphological criteria (Storr 1977; Ford 1978).

There is a lack of an obvious barrier (e.g., a major river) between the East and West Kimberley, however, there is the presence of a basalt range extending north to south in the central Kimberley (Griffin *et al.* 1993). Although this geological expanse was present in the Kimberley well before the estimated divergence of East and West Kimberley *P. brachyotis* (0.13-1.18 MYA), there is the potential that the sandstone previously deposited on top of this basalt provided suitable connectivity between these regions, but then the erosion of this sandstone during the Pleistocene, exposed the basalt (which lacks the complex rock structure necessary to sustain *Petrogale*

populations) and may have created a barrier between these two regions. Glacial and interglacial cycles causing range contractions (Russell-Smith *et al.* 1993) on either side of this potential barrier may also have contributed to the differentiation of populations. Range contractions of taxa into localised refugia are concordant with many studies of taxa in the Wet Tropics of north Queensland (Schneider *et al.* 1998; Moritz *et al.* 2000; Stuart-Fox 2001; Hugall *et al.* 2002; Dolman and Moritz 2006). The two East Kimberley mtDNA lineages are located either side of the Ord River, which runs from the Cambridge Gulf 650km inland and is one of Western Australia"s largest river systems and may therefore represent a significant barrier for dispersal. The Ord River region was proposed by Ford (1978) as a biogeographical barrier due to its greater aridity.

Further sampling is also required in the far east of *P. brachyotis'* distribution, where two geographically isolated samples (S1178, Wollogorang; S92, Groote Eylandt) were found to be highly divergent (up to 13% and 14.4% mtDNA SD respectively) indicating the potential presence of additional biogeographic barriers and phylogeographic structure. The high level of divergence between the Wollogorang individual (S1178) from near the Queensland border and all other Northern Territory haplotypes is potentially significant as the lowland area south and east of the Roper River has been proposed as a vicariant barrier (between Arnhem Land and the McArthur-Nicholson region) for other taxa (Ford 1978) including *Collurincincla woodwardi* (sandstone strike-thrush) and two endemic species of rock-rats *Zyzomys maini* (Arnhem Land rock rat) and *Z. palatalis* (Carpentarian rock rat) (Kitchener 1989).

Conclusion

The results from this study suggest that aridification during the Pliocene created separation of *brachyotis* populations from Arnhem Land and the Kimberley as more mesic habitats contracted into areas close to the major sandstone ranges. Subsequently, during periods of glacial-inter-glacial cycles, further periodic contraction and isolation of *P. brachyotis* populations occurred

around the Joseph Bonaparte Gap, Ord and Victoria Rivers resulting in additional genetic differentiation and lineage structuring.

Kuo and Avise (2005) assert that spatially concordant phylogeographic breaks across independent neutral loci normally emerge only in the presence of longstanding historical barriers to gene flow. Emergence of long-term historical barriers to gene flow across northern Australia is becoming evident as a result of comparisons between co-distributed taxa. The consistency in the location of the identified barriers across different organisms suggests that they are likely to have influenced a wide range of flora and fauna in north-western Australia. The Top End and Kimberley are continuing to emerge as regions of past refugia for organisms and high endemism. This biogeographic pattern is not then the result of a single event, but rather ongoing demographic processes associated with the changing environment of northern Australia.

A notable feature of this *brachyotis* group dataset is the high levels of mtDNA sequence divergence amongst populations, both within and between the major lineages. Since the substantial mtDNA sequence divergence observed amongst *P. brachyotis* lineages (5.5-14.4% SD) is equivalent or exceeds the levels reported between other rock-wallaby species, it appears we are dealing with multiple species and a formal taxonomic revision is in preparation (Chapter 5).

Statement of Authorship

Sally Potter (candidate)

Corresponding author: Prepared DNA extracts for PCR amplification, carried out DNA sequencing and microsatellite genotyping, analysed sequence data and genotypes, wrote manuscript and produced all Figures.

Signed Date 12-1-2011

Mark. D.B. Eldridge

Provided assistance with obtaining project funding supervised the direction of the study, provided advice on analyses and critically reviewed manuscript.

I give consent for Sally Potter (candidate) to include this manuscript for examination towards the degree of Doctor of Philosophy.

Signed Date 10 Jan 2011

Steve J.B. Cooper

Supervised the direction of the study and provided advice on analyses and critically reviewed manuscript.

I give consent for Sally Potter (candidate) to include this manuscript for examination towards the degree of Doctor of Philosophy.

Signed Date 12/1/2011

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Provided assistance in non-invasive DNA extractions and microsatellite genotyping, provided advice on techniques and analyses of DNA from faecal samples and reviewed the manuscript.

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Signed Date 10-1-2011

David A. Taggart

Provided assistance with obtaining project funding, sample collection and evaluated the manuscript.

I give consent for Sally Potter (candidate) to include this manuscript for examination towards the degree of Doctor of Philosophy.

Signed Date 11-1-2011

CHAPTER 4: Habitat connectivity among populations of the Short-eared Rock-wallaby (*Petrogale brachyotis*) in the Kimberley, Australia

Sally Potter, Mark D.B. Eldridge, Steve J.B. Cooper, Justyna Z. Paplinska, David A. Taggart

Abstract

Habitat fragmentation can cause significant genetic differentiation among populations. With species‟ biology potentially enhancing the effects of habitat fragmentation, it is often difficult to assess the relative role each aspect plays in genetic differentiation, unless a species can also be studied in a continuous habitat. Considerable genetic differentiation among populations has been reported in rock-wallaby (*Petrogale*) species across Australia, but most previous studies occurred in fragmented habitats and few have focused on widespread and abundant species in more continuous habitat. *Petrogale brachyotis* provides an opportunity to assess natural population structure and levels of gene flow in a relatively intact and continuous environment across the monsoonal tropics in north-western Australia. This study used 12 microsatellite loci from 84 individuals from six localities together with mitochondrial DNA (mtDNA) haplotypes from 51 tissue samples (four locations), to examine patterns of population structure and dispersal within and among populations of *P. brachyotis* in the Kimberley, Western Australia. Population genetic analyses of mtDNA and microsatellite data showed relatively low genetic differentiation among populations separated by up to 67 km. The inferred genetic connectivity of these populations suggests that in suitable habitat *P. brachyotis* can potentially disperse far greater distances than previously reported for rock-wallabies in more fragmented habitat. High levels of genetic relatedness within populations were found although there was no evidence of inbreeding, which suggests mechanisms such as male-biased dispersal could be in place to avoid inbreeding. Populations from the East and West Kimberley were highly differentiated, with no contemporary or long-term gene flow apparent; indicating that past biogeographic processes may have led to isolation of these regions.

Introduction

Habitat fragmentation is a major threat to global biodiversity, through its negative impacts, direct and indirect, on population dynamics (Harris 1984; Gilpin and Hanski 1991; Frankham *et al.* 2002). Fragmentation prevents or reduces dispersal between populations, restricting gene flow and resulting in increased genetic drift and genetic differentiation among populations (Lacy 1987; Frankham *et al.* 2002). The isolation associated with fragmentation can also accelerate the loss of genetic diversity and increase inbreeding, resulting in populations that are less able to adapt to environmental changes and so have an increased risk of extinction (Frankham *et al.* 2002, 2004).

Landscape characteristics alone, however, do not establish population structure, with individual species‟ biology contributing significantly to the genetic differentiation of populations (Kraaijeveld-Smit *et al.* 2007). Life-history traits including dispersal ability and sex-biased dispersal, mating system, habitat specialisation and generation time, all impact population dynamics and the distribution of genetic diversity (Slatkin 1994; Hedrick 2000). Even in continuous habitat these factors alone can lead to genetic differentiation (Ehrich and Stenseth 2001). It can therefore be difficult to separate the effects of life-history traits from those of habitat fragmentation when assessing population structure. Investigating the factors that influence population differentiation in contiguous habitat provides insight into genetic structure prior to the impacts of fragmentation (Macqueen *et al.* 2008). This can be extremely informative for management strategies, as comparison of genetic connectivity between populations within homogeneous and fragmented landscapes often reveal large differences attributed to anthropogenic processes (Stow *et al.* 2001). Fragmented populations may not solely result from habitat fragmentation, since other factors including resource availability, climatic conditions, presence of competitors and predators can also limit the spatial distribution of species (Fischer *et al.* 2005). Genetic studies can be valuable in identifying the impact of anthropogenic processes and so enable better informed conservation management decisions (Lindenmayer and Fischer 2006).

Genetic methods are now widely used to explore population structure of organisms, with microsatellite markers, in particular, being widely employed to investigate dispersal, genetic

variation and genetic connectivity of populations, as well as uncovering aspects of species‟ biology and evolution (Sunnucks *et al.* 2000; Balloux and Lugon-Moulin 2002). Non-invasive DNA sampling methods have also proven increasingly useful for understanding elusive species, enabling data to be gained on dispersal, relatedness and population structure (Creel *et al.* 2003; Piggott *et al.* 2006b; Ruibal *et al.* 2009; Smith *et al.* 2009). Many population genetic studies have aimed to identify how genetic diversity is distributed across the landscape for the purpose of designing effective conservation management (Moritz 1995; Cracraft *et al.* 1998; Lemes *et al.* 2003; Miller *et al.* 2006), to preserve future genetic viability and evolvability within species (Moritz 1994; Piggott *et al.* 2006b).

The impacts of fragmentation versus life-history processes have rarely been investigated in mammals yet are fundamental to understanding population declines and future management (Peacock and Smith 1997; Wolff *et al.* 1997; Amos and Balmford 2001; Walker *et al.* 2008)**.** To date, population genetic studies of rock-wallabies (*Petrogale* spp.) have focused on threatened and highly fragmented populations in southern Australia, including the yellow-footed (*P. xanthopus*), brush-tailed (*P. penicillata*) and black-footed (*P. lateralis*) rock-wallabies (Pope *et al.* 1996; Eldridge *et al.* 1999, 2001b; Browning *et al.* 2001; Hazlitt *et al.* 2006a; Piggott *et al.* 2006b). These rock-wallabies are now mostly present in small, isolated colonies with limited dispersal and gene flow between them (Jarman and Bayne 1997; Hazlitt *et al.* 2004, 2006a). Rock-wallabies appear to have lower genetic diversity (allelic diversity and heterozygosity within populations and higher genetic divergences between populations) compared to other macropodoids which has been linked to their patchy distribution as a result of their habitat specificity (complex rocky outcrops and escarpments) (Hazlitt *et al.* 2006a; Piggott *et al.* 2006b; Eldridge *et al.* 2010). However, this assumption may be flawed (Eldridge *et al.* 2010), with studies being biased towards highly fragmented rock-wallaby species that have declined as a result of habitat clearance and the impact of introduced predators (e.g., red fox, *Vulpes vulpes*) and hunting (Maxwell *et al.* 1996). Additional analyses of rock-wallabies in more continuous habitat would elucidate whether this apparent low diversity is a function of species‟ biology or a result of anthropogenic processes.

A recent study by Telfer and Eldridge (2010) on the widespread and common short-eared rock-wallaby (*P. brachyotis*) from the Northern Territory, northern Australia, aimed to assess

population structure in more continuous habitat, but nevertheless revealed highly structured populations. This suggests that rock-wallabies are predisposed to being highly structured as a result of their strong association with rocky habitat (Piggott *et al.* 2006a). Even though the Northern Territory retains largely intact vegetation between populations of *P. brachyotis*, the sandstone gorges and outcrops, which represent core rock-wallaby habitat (sampled by Telfer and Eldridge 2010), were quite disjunct and this seems likely to have led to the highly structured genetic diversity found in this species. Since Telfer and Eldridge (2010), it has been suggested that *P. brachyotis* actually represents multiple species (Chapters 2 & 3); with an eastern species from the Northern Territory being separated from *P. brachyotis* whose distribution is now centred on the Kimberley region of north-west Western Australia (refer to Chapter 5). The Kimberley differs from the areas sampled by Telfer and Eldridge (2010) in that it contains not only more extensive dissected sandstone plateaus and ranges but also greater connectivity of rocky habitat, with less separation of these broader sandstone habitats across the landscape. Therefore this landscape provides an opportunity to assess the population dynamics of a rock-wallaby within intact and more continuous rocky habitat, unlike previous studies. Sequence divergence between mtDNA haplotypes from within the Northern Territory (Chapters 3) is greater than within the Kimberley indicating populations in the Kimberley have less genetic differentiation and potentially represent populations where there is contemporary gene flow. Therefore *P. brachyotis* in this region could provide a suitable reference for the genetic diversity and population structure of a pre-decline or „healthy" population of rock-wallaby, which could be used to help set appropriate goals for the management of declining and threatened populations (Eldridge *et al.* 2004a).

Northern Australia comprises a unique environment, which is relatively intact and unaffected by many of the threatening processes which have impacted eastern and southern parts of Australia. This tropical monsoon biome encompasses a vast range of natural landscapes and habitats from tropical rainforests, savanna and open woodland areas, to dissected sandstone plateaus and escarpments. The Kimberley occupies the entire northern area of Western Australia and contains large expanses of Proterozoic sandstone plateaus which provide residence to a large number of organisms (Bowman *et al.* 2010). This sandstone expanse contains some of the highest mammal species richness across the Australian monsoonal tropics and is associated with endemic species of reptiles, birds and mammals (Woinarski

1992). An understanding of the evolution and biogeography of organisms, as well as the species‟ biology from this area of high biodiversity is lacking (Bowman *et al.* 2010). The mammals of the Kimberley have had relatively few threats, but human impacts, associated with increased fire frequency and intensity, introduction of invasive species (e.g., cane toads), mining, grazing and predation by feral animals, threaten to cause faunal declines and extinctions (Woinarski 1992; Braithwaite and Muller 1997; Eliot *et al.* 1999; Woinarski *et al.* 2001; McKenzie and Burbidge 2002). Currently there is no information about the population biology of *P. brachyotis* from the Kimberley and it is not known whether they represent metapopulations or discrete isolated colonies across the region. The phylogeographic and fine-scale population structure of *P. brachyotis* within the Kimberley is also unclear, with mtDNA data (Chapters 3) suggesting some differentiation between the East and West Kimberley populations. Since mtDNA only represents maternal gene flow patterns it is important to assess population structure using other techniques.

In this study we used microsatellite loci and mtDNA sequence data to investigate the genetic diversity and fine-scale population structure of *P. brachyotis* across the Kimberley. The aims were to: (i) assess genetic diversity and population differentiation of *P. brachyotis* in a relatively connected environment, (ii) assess phylogeographic patterns of *P. brachyotis* and determine whether the East and West Kimberley populations represent genetically differentiated populations, and (iii) investigate dispersal patterns and whether sex-biased dispersal is present within *P. brachyotis*.

Materials and methods

Sampling and DNA extraction

Tissue samples were obtained from 51 live trapped *P. brachyotis* from four sites in the Kimberley, WA (Fig. 4.1), collected over three years of trapping. For the purposes of the population analyses in this study all samples were pooled over years, with major changes in allele frequencies over seasons unlikely due to the long lived nature of rock-wallabies (e.g., up to 12 years; Bee and Close 1993).

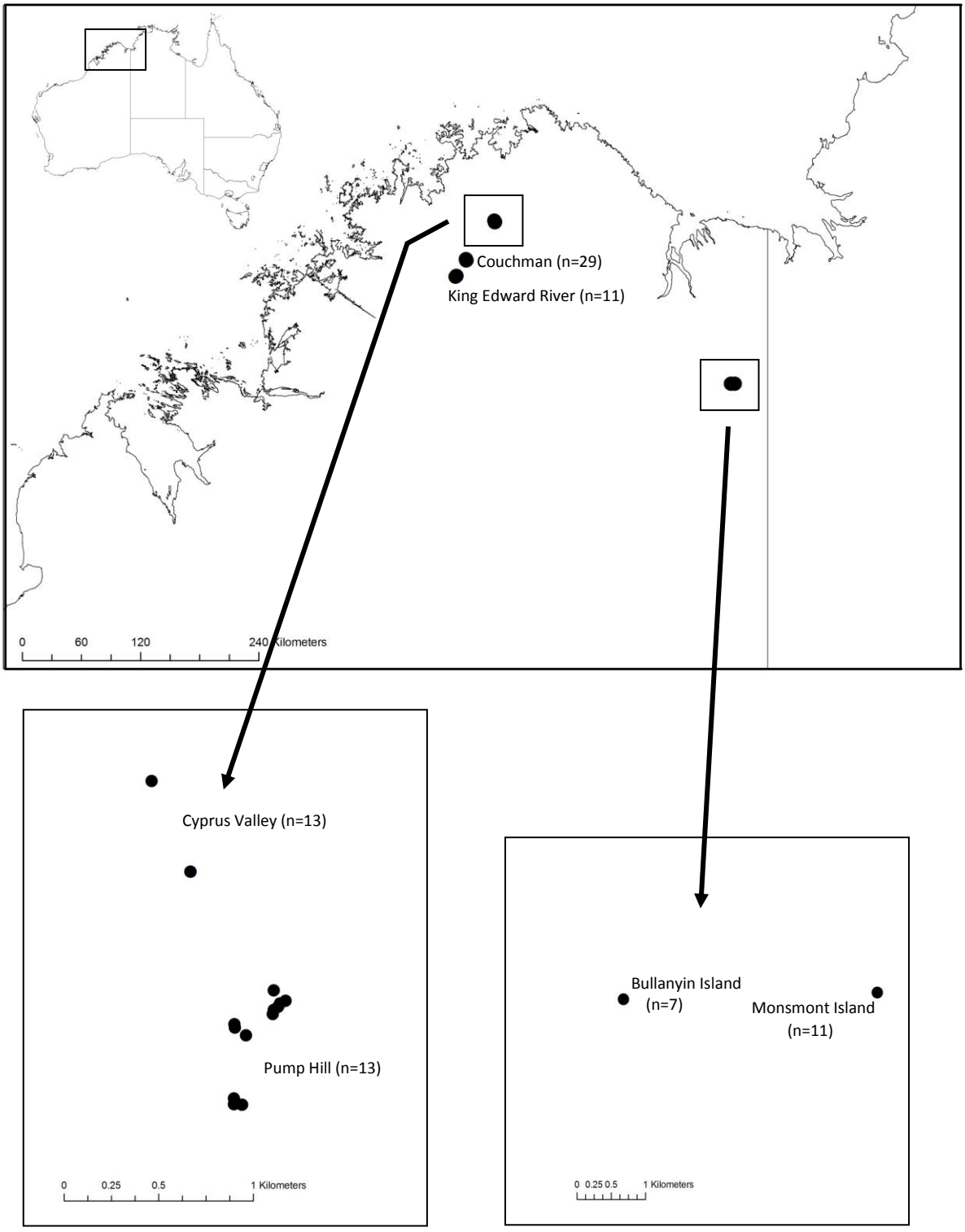


Figure 4.1: The distribution and sampling localities of *Petrogale brachyotis* along with sample sizes at each site in the Kimberley, Western Australia.

Rock-wallabies inhabit rugged terrain (Van Dyck and Strahan 2008) and in the Kimberley this can be in areas where vehicle access is limited, therefore we utilised non-invasive sampling, with a further 33 faecal samples obtained from three sites (six site localities overall). Distances between sites ranged from ≈1 km between Cyprus Valley and Pump Hill in the West Kimberley (up to 67 km between King Edward River and Cyprus Valley sites) and ≈3.5 km between two islands in Lake Argyle in the East Kimberley to ≈290 km between East and West Kimberley sites (Fig. 4.1). These sampled site localities do not encompass all discrete rocky habitats, so will represent a broad assessment of relationships amongst sites across the Kimberley and will be referred to as populations in this study. The sample sizes for each population do not necessarily correlate to population density, but were based on sample availability and PCR success. Only tissue samples were used in the mtDNA analyses, due to technical difficulties in getting mtDNA sequences from faecal samples, but all samples were used in microsatellite analyses.

Tissue samples comprised 5mm ear biopsies collected in the field via trapping and preserved in 70% ethanol. Fresh faeces were individually stored in brown paper envelopes and frozen (-20°C) for preservation, from the day of collection, during transportation and in the laboratory. DNA was extracted from the tissue samples using the Gentra DNA Isolation Kit (Gentra Systems) and from faecal samples using the QIAamp DNA Stool Kit (QIAGEN) according to the manufacturer"s instructions (pg 26) with small modifications from Piggott and Taylor (2003) („Method A"). Instead of following steps 1-4, we pipetted 5mL of Buffer ASL into a 50mL falcon tube and added scats into the tube. Tubes were placed on a rotor for 30 mins to wash the epithelial cells off the scat pellets. Falcon tubes were then spun down in a centrifuge for 1 min at full speed and then the protocol from step 5 was followed (two 2mL tubes were used per falcon tube for the following steps and then combined in the spin column step). Modifications to the remaining protocol included using two InhibitEX tablets (one for each tube, two per reaction); centrifugation for 5 mins instead of 3 mins for steps 7 and 8; addition of 800µL Buffer AW1 and AW2 in steps 17 and 18 instead of 500µL; and addition of 50µL Buffer AE in step 20 instead of 200µL. Negative controls were included in all faecal extractions to test for contamination and extractions were carried out in a laboratory isolated from routine DNA preparations.

DNA amplification, microsatellite genotyping and mtDNA sequencing

Polymerase chain reaction (PCR) was used to amplify two mitochondrial gene segments; *CR* (control region), L15999M (5‟-ACCATCAACACCCAAAGCTGA-3‟) and H16498M (5‟-CCTGAAGTAGCAACCAGTAG) (Fumagalli *et al.* 1997); *ND2* (NADH dehydrogenase subunit 2), mmND2.1 (5‟-GCACCATTCCACTTYTGAGT-3‟) and mrND2c (5‟-GATTTGCGTTCGAATGTAGCAAG-3‟) (Osborne and Christidis 2001).

A total of 12 polymorphic microsatellite loci were screened for all individuals: Pa297, Pa593, Pa597 from the allied rock-wallaby (*P. assimilis*; Spencer *et al.* 1995); Me2, Me14, Me15, Me16, Me17 from the tammar wallaby (*Macropus eugenii*; Taylor and Cooper 1998); Y76, Y148, Y170 from the yellow-footed rock-wallaby (*P. xanthopus*; Pope *et al.* 1996); G26-4 from the eastern-grey kangaroo (*M. giganteus*; Zenger and Cooper 2001). In addition, a SRY male-specific marker from the brush-tailed rock-wallaby (*P. penicillata*; O'Neill *et al.* 1997) was used to sex the faecal samples. Primer mixes (PM) consisted of 1µL of each fluorescently labelled primer (100µM) made up to 25µL with H_2O in the following combinations: PM1 – Y170, Pa297, Pa593; PM2 – Y151, Y148; PM3 – Y76, Me15; PM4 – Pa385, SRY; PM5 – Me17, Me2; and PM6 – Pa597, Me14, Me16, G26-4. Pa385 and Y151 were subsequently excluded from analyses due to overlapping genotypes.

PCR-amplifications of tissue samples were carried out in 10µL reaction volumes following the methods of Miller *et al.* (2010) using a Multiplex PCR kit (Qiagen); with approximately 30ng genomic DNA, 5µL Multiplex MasterMix, 0.1mM BSA, 0.2µM of each primer, 3µL H_2O. Thermocycling was performed on a Corbett palmcycler using an initial HotStarTaq activation step at 95°C for 15 min; 34-50 cycles of 30 s at 94°C (denaturation), 90 s at 58°C (Primer Mix 1, PM 2, PM4), 56°C (PM3) and touchdown 60-50°C (60°C, 57°C, 55°C, 52°C and 50°C for 10 cycles each; PM 5) (annealing), and 90 s at 72°C (extension); and a final extension for 10 min at 72°C. PCRs were pooled: Pool 1 included 4µL PM 1, 3µL PM 2, 4µL PM 3, and 4µL PM 4; and Pool2 included 4µL PM5, 4µL PM6. Pooled PCR products were cleaned using Millipore MultiScreen PCR_{384}Filter Plates (Millipore) following the manufacturer‟s protocols. Serial dilutions of 1:40 for Pool 1 and 1:20 for Pool 2 were then

genotyped at the Australian Genome Research Facility (AGRF) with an automated ABI 3730xl capillary sequencer (Applied Biosystems).

Multiplex PCR-amplifications of faecal samples were carried out in 25µL reactions using the Qiagen Multiplex kit (Qiagen); with 15µL Multiplex MasterMix, 2.5µL Q solution, 1.5-3.5µL dH_2O, 0.2µM of each primer and 2µL of DNA extract. The following conditions were used for amplification: initial denaturation 94°C for 5 min; 50 cycles of 30 s at 94°C (denaturation), 30 s at 56°C (PM 1 and 3), 54°C (PM2) and touchdown 60-54°C (60°C, 58°C, 56°C for 10 cycles each, 54°C for 20 cycles ; PM 4-6) (annealing), and 30 s at 72°C (extension); and a final extension for 30 min at 72°C. Primer mixes PM1 and PM3 were pooled together, as were primer mixes PM4, PM5 and PM6 into two separate MultiMix PCR reactions unlike the individual PCRs used for the tissue samples. PCR samples were pooled as above and then 1µL of pooled product was added to 0.3µL Liz500 and 8.7µL Hi-Di for genotyping and analysed on a 3730 DNA Analyser (Applied Biosystems).

DNA from non-invasive techniques can produce yields with lower quality and quantity, we therefore replicated all PCRs three times according to Piggott *et al.* (2006a) to control for allelic dropout (stochastic non-amplification of one allele) and ensure reduction in type 1 errors. Following Piggott *et al.* (2006a) we accepted a heterozygous genotype if each allele was present in two out of three replicates and only accepted a homozygous allele if only one allele was identified in all three replicates. When running the sex marker an internal positive control was used to distinguish females from samples that failed to amplify (by having it in a multiplex reaction with Pa385 – Pa385 results not included). A *post hoc* method was implemented, whereby samples that matched at all but four alleles were assigned the same identity (one individual removed from further analyses). This approach aims at eliminating overestimation biases of population abundance because the probability of individuals differing at two or more loci was high (Ruibal *et al.* 2009). Allelic dropout and false alleles were estimated for each microsatellite locus and sample using GIMLET (v3.2; Valière 2002), with total number of positive amplifications and genotyping errors per locus and sample computed. Following the methods of Ruibal *et al.* (2009), the frequencies of allelic dropout were then used to estimate a locus-specific rate using the equations ADO_1 from Broquet and Petit (2004). The microsatellite DNA fragments were scored using GENEMAPPER v4.0

(Applied Biosystems) for allele size and quantification. Samples were double-checked for genotyping errors in GENEMAPPER when mismatches at a locus were identified amongst the three repeats. To ensure duplicate samples of faecal DNA were not included in the analysis, only unique multilocus genotypes were used.

Population genetic and phylogenetic analyses

Mitochondrial DNA sequences were edited using SEQED (version 1.0.3; Applied Biosystems), then aligned using CLUSTAL X (version 1.83; Thompson *et al.* 1997) and manually refined using SeAl (version 2.0a11; Rambaut 1996). Sequences are available on CD (Appendix 1). Gaps were considered as indels and assigned „-". DnaSP (v5.10; Librado and Rozas 2009) was implemented to estimate the number of haplotypes (*H*), polymorphic sites, nucleotide diversity (π) and haplotype diversity (*h*) (Rozas *et al.* 2003). Intra-specific sequence divergence among mtDNA haplotypes was estimated using the Kimura 3-parameter (Kimura 1981) model selected by Modeltest 3.06 (Posada and Crandall 1998) for the combined mtDNA dataset using PAUP* (v4.0b10; Swofford 2002).

Conformance to Hardy-Weinberg equilibrium and linkage disequilibrium for each microsatellite locus within each population was assessed using GENEPOP (v3.2; Raymond and Rousset 1995) employing the Markov chain method with 1000 iterations and *P*-values adjusted using the sequential Bonferroni procedure (Rice 1989). Allelic diversity (*AD*; average number of alleles per locus), observed and expected heterozygosity (H_O, H_E respectively) were calculated for each population in ARLEQUIN (v3.11; Excoffier *et al.* 2005), and allelic richness (*AR*; allelic diversity corrected for sample size) was estimated using FSTAT (v2.9.3; Goudet 1995). Differences in H_E, *AD* and *AR* between the populations were tested using a Wilcoxon signed rank test with loci as the pairing factor using PASW Statistics, Release Version 17.0.2 (SPSS, Inc.). The number of rare alleles (frequency $\leq$ 5%) and the number of unique alleles (A_U) were calculated in GenAlEx 6 (Peakall and Smouse 2006).

Multilocus F_{IS} was also calculated at each locus for each population using FSTAT (version 2.9.3; Goudet 1995), and then tested by 1000 permutations using Weir and Cockerham"s

estimator (Weir and Cockerham 1984). F_{IS} was also calculated for males and females individually for each population.

Genetic structure and phylogeography

To assess whether individuals sampled at specific localities represent discreet genetic populations, a Bayesian clustering method in STRUCTURE 2.1 (Pritchard *et al.* 2000) was used to infer population structure. This method assumes no *a priori* geographical information and uses genotype data to infer population groups that minimise Hardy-Weinberg and linkage disequilibrium. Analyses were performed on the entire microsatellite dataset and then on separated East and West Kimberley samples. We estimated the most likely number of genetic clusters/groups (K) from between one to eight for the entire dataset, one to six for the West Kimberley samples and one to four for the East Kimberley samples. The parameters used were the admixture ancestry model, correlated allele frequencies model, a burnin length of 10,000 iterations and a run length of 100,000 Markov chain Monte Carlo repetitions. Each run of K was repeated 6 times and two methods of identifying K were employed, as it can be difficult to determine the optimal value of K (Evanno *et al.* 2005). The first approach was to assess ΔK values (a measure of the second order rate of change in the likelihood of K; Evanno *et al.* 2005), with the highest ΔK value representing the most likely K. The second method assessed the Q (fractional membership of each individual in each cluster) plot and log likelihood values to assess where individuals were assigned and with what probability the data fit the hypothesis of K clusters. STRUCTURE was not used as an assignment method to identify putative dispersers as it assumes all populations have been sampled which is an unrealistic assumption in this study.

Analysis of molecular variance (AMOVA) was used to examine the extent of phylogeographical structuring with the program Arlequin (v3.11; Excoffier *et al.* 2005), assessing mtDNA differentiation (pairwise Φ_{ST}) amongst the four populations from which tissue samples were collected (based on 10,000 permutations) and microsatellite differentiation (pairwise F_{ST}) among all six populations (based on 10,000 permutations). To visualise the genetic similarity among the six populations, a neighbour-joining (NJ) tree was constructed using the program MEGA (v5; Tamura *et al.* 2007), based on a pairwise matrix of

Nei‟s *D* (Nei 1978) of microsatellite data generated in GenAlEx 6 (Peakall and Smouse 2006).

Maximum likelihood (ML), maximum parsimony (MP) and Bayesian inference analyses were performed on the concatenated mitochondrial alignment of *CR* and *ND2* using RAxML (v7.0.3; Stamatakis 2006; Stamatakis *et al.* 2008), PAUP* (v4.0b10; Swofford 2002) and MrBayes (v3.1.2; Ronquist and Huelsenbeck 2003; Huelsenbeck and Ronquist 2005) respectively. The Akaike Information Criterion was implemented using Modeltest 3.06 (Posada and Crandall 1998) in PAUP* (v4.0b10) to determine the best-fit models for RAxML and MrBayes. As the exact model suggested from Modeltest 3.06 was not available in these programs the more complex General time Reversible (GTR) model was applied, with rate variation among sites modelled with a discrete gamma distribution (G). Heuristic searches using 1000 randomised addition orders with tree-bisection–reconnection (TBR) branch swapping, and a stepwise addition starting tree were used for MP analyses. The DELTRAN option for character-state optimization was utilised for MP trees, as there is a bug in PAUP* (Mac version) in the default ACCTRAN option which can result in erroneous branch lengths in output trees. ML analyses were started from a random starting tree and bootstrap analyses were carried out for 1000 replicates, with multiple (100) searches per replicate, using the rapid Bootstrap analysis. MrBayes uses Metropolis-coupled Markov chain Monte Carlo (MCMC) sampling to calculate Bayesian posterior probabilities. We used default settings for priors, random starting trees and four Markov chains (three hot, one cold), with sampling every 1000 generations. Analyses were terminated when the average standard deviation of split frequencies for the simultaneous analyses fell below 0.01 (≈2-10 million generations). Tracer (v1.5; Rambaut and Drummond 2009) was used to check that the convergence of parameter estimates had occurred. Tracer was also utilised to identify when the log likelihood values of trees became stable, to define the appropriate cut off for initial instability of trees (usually 10% burn-in was suitable). Posterior probabilities were calculated after discarding the first 25% of the sampled trees as burn-in.

Estimation of dispersal patterns using assignment tests

Putative first generation migrants and their population of origin were identified using the program GeneClass 2.0 (Piry *et al.* 2004). Using the Bayesian method of Rannala and Mountain (1997) the likelihood of each genotype in each population was estimated, following the Paetkau *et al.* (2004) Monte Carlo resampling method. This re-sampling method estimates a probability of excluding the individual from each population with a critical P value of 0.99 to reduce inaccurate assignment of immigrants and is relatively insensitive to missing alleles (Paetkau *et al.* 2004). We used the comparison of the likelihood of each individual in its „home" population to the most likely of the other populations, but took into account that all possible populations were not sampled (L=L_home). Individuals significantly different from their home population were only assigned to another population as the „home" if the significance was above 0.99. Immigrants that did not meet the P value of 0.99 were considered immigrants from unsampled colonies. Methods followed Piggott *et al.* (2006b) and despite the small sample sizes for some populations, using the Bayesian method, with more than 10 loci, and FST values close to 0.1 or greater between populations, the procedure should be quite accurate (Cornuet *et al.* 1999; Paetkau *et al.* 2004).

Relatedness within populations

Relatedness between individuals in each population was examined for overall pairwise relatedness and sex-specific relatedness using the program GenAlEx 6 (Peakall and Smouse 2006). Pairwise relatedness (r) estimates were calculated using the method of Queller and Goodnight (1989). An average of pairwise relatedness within populations was estimated using GenAlex using the Pops Mean option with 9999 permutations and 9999 bootstraps to estimate the 95% confidence interval around r. This was used to test the assumption that the average pairwise relatedness values did not differ significantly from random assortments of relatedness values (unrelated individuals).

We tested for correlation between genetic (microsatellite data) distance (Nei"s D_A; Nei *et al.* 1983) and geographical distance using Mantel tests implemented in AIS (Alleles in Space;

Miller 2005) for all locations, for males and females individually and for the West Kimberley individuals alone, based on 1000 permutations.

Results

Genetic diversity

A total of 1182 base pairs (bp) of mtDNA was analysed (596 bp *CR*; 586 bp *ND2*) from 52 individuals (30 females, 21 males; and one *P. penicillata* was included as an outgroup). Within the mtDNA fragment 120 sites were variable, of which 116 sites were parsimony informative. Seven different indels were detected amongst the *P. brachyotis* individuals within the *CR* fragment. Thirteen mtDNA haplotypes were identified (see Table 4.1), with haplotype and nucleotide diversity highest in the Cypress Valley population (h=52.6%, π=0.77%; Table 4.2).

A total of 40 females (10 identified from SRY sex specific marker) and 44 males (23 identified from faecal samples using the SRY marker) were genotyped for the six populations (refer to Appendix 1 for genotypes). All microsatellite loci in each of the six populations conformed to Hardy-Weinberg equilibrium after sequential Bonferroni corrections, except for the Pump Hill population which revealed a significant heterozygote deficit at one locus (Pa597). There was significant linkage disequilibrium after sequential Bonferroni corrections only within the King Edward River population between the following sets of loci: Me14 and Y148, Me15 and Y148, Me14 and Me15, Me15 and Pa593, Pa297 and Y76, Pa593 and Y148. This could be the result of recent admixture. All other populations examined revealed linkage equilibrium, so analyses were carried out on the full dataset. No false alleles were detected but 23 allelic differences were identified of the 1188 faecal amplifications, giving a genotyping error rate of 1.9%. The amplification success rate was 84% for both loci and samples, with allelic dropout rates of 2.8% detected across samples and 3.5% across loci. G26-4 had the highest allelic dropout rate (19.3%) compared to all other loci (1.4-4.4%).

The average number of alleles detected per locus was 6.13 ± 2.24 ranging from 2 to 15 alleles within populations. East Kimberley populations (Monsmont and Bullanyin Islands) had

significantly lower *AD* values compared to West Kimberley populations (Couchman, Cyprus Valley and Pump Hill; *P*=0.002-0.005). When *AD* was corrected for sample size (*AR*), Monsmont Island was again significantly lower compared to the three West Kimberley populations (*P*=0.002), whereas Bullanyin Island was only significantly lower when compared to the Pump Hill population (for both H_E and *AR*; 0.002, 0.003 p-values respectively). The West Kimberley H_E, *AD* and *AR* values were not significantly different between populations except for *AD* between the Couchman and King Edward River (*P*=0.005). No significant inbreeding (F_{IS}) was detected for any population at any locus (P>0.05), or for males or females within the populations. No rare alleles (frequency ≤ 5%) were detected in any population but unique alleles were detected in all populations ranging from 1% in King Edward River and Pump Hill populations to 7% (Bullanyin Island population). A total of 71 alleles (48%) were only found in the West Kimberley populations and 25 alleles (17%) were unique to the East Kimberley populations.

Table 4.1: Location of sampled *Petrogale brachyotis* populations and the number of identified mtDNA haplotypes (*H*).

Population	Latitude/Longitude	Sample Size	*H*
Couchman	204755 8321547	29	A (17)
			B (1)
			C (2)
Cypress Valley	231724 8361617	13	D (1)
			E (1)
			F(9)
			G(2)
Monsmont Island	467949 8198163	11	H (1)
			I (9)
			J (1)
Bullanyin Island	464389 8198066	7	K (1)
			L (5)
			M (1)
King Edward River*	194873 8305693	11	
Pump Hill*	232154 8360873	13	

*No mtDNA sequencing was done from the King Edward River and Pump Hill populations due to technical difficulties in getting mtDNA sequences from faecal samples.

Table 4.2: Summary of genetic diversity indices for sampled populations of *Petrogale brachyotis*. *h* haplotype diversity, π nucleotide diversity, *AD* allelic diversity, *AR* allelic richness (allelic diversity corrected for sample size; n=7), H_E mean expected heterozygosity, A_U unique alleles, F_{IS} inbreeding levels. All F_{IS} values were not significantly different from zero.

Sample Location	***h* (± SD)**	**π**	***AD* (± SD)**	***AR* (± SD)**	**H_E (± SD)**	**A_U**	***FIS***
Couchman	0.279 ± 0.123	0.00063	8.3 (± 3.1)	5.3 (± 1.5)	0.745 (± 0.16)	9	-0.003
Cyprus Valley	0.526 ± 0.153	0.00775	6.9 (± 2.4)	5.6 (± 1.7)	0.755 (± 0.20)	7	0.041
Monsmont Is	0.182 ± 0.144	0.00032	4.1 (± 1.5)	3.6 (± 1.3)	0.573 (± 0.19)	6	-0.035
Bullanyin Is	0.286 ± 0.196	0.00025	4.4 (± 1.5)	4.4 (± 1.5)	0.661 (± 0.17)	10	0.030
King Edward River			6.3 (± 2.8)	5.2 (± 2.1)	0.763 (± 0.15)	2	-0.096
Pump Hill			6.8 (± 2.1)	5.4 (± 1.5)	0.771 (± 0.15)	2	0.042

Genetic structure

Initial STRUCTURE results for all the microsatellite data indicated a geographically distinct East and West Kimberley grouping (K=2), with all individuals assigned to each cluster at probabilities >0.9. However, when the East and West Kimberley genetic clusters were analysed separately, the ΔK value was highest for two in each East and West analysis, indicating both regions are best represented by two additional genetic clusters (each K=2; see Fig. 4.2), making, in total, K=4 the most likely number of genetic clusters. Within the West Kimberley analysis, not all individuals were assigned to a cluster (probabilities >0.8), with fifteen individuals having a mixed assignment (probabilities <0.8) and three individuals (ABTC99393, KER09028, JA09019) clustering with a group different to those corresponding to their sampling region. Generally, one cluster consisted of individuals from the Couchman and King Edward River populations, and the second cluster of individuals from Cyprus Valley and Pump Hill (see Fig. 4.2 for assignment of individuals to clusters). The East Kimberley assignments corresponded to the sampled localities of the two islands in Lake Argyle, with only one individual not assigned to a cluster (ABTC103504). For the purposes of looking at fine-scale genetic structure within the West Kimberley, we kept all four populations from this region for analyses.

Population differentiation (Φ_{ST}) in mtDNA was highly significant between the four West Kimberley populations (Cyprus Valley, Monsmont Island, Bullanyin Island and Couchman) with values ranging from 0.856 to 0.995 (see Table 4.3). All microsatellite pairwise population comparisons (F_{ST}) were significant after sequential Bonferroni corrections aside from one, Cyprus Valley versus Pump Hill (0.022-0.059; see Table 4.3). The range of F_{ST} values between the East and West Kimberley populations and F_{ST} between the two East Kimberley populations were orders of magnitude higher (0.184-0.274; 0.166 respectively). The high F_{ST} value between the East Kimberley populations is likely to have resulted from each of them being from separate islands. The existence of an East and West Kimberley structure was also clearly illustrated in the NJ distance tree, based on the microsatellite data, where long branch lengths separated lineages from the East and West Kimberley as a result of large genetic distances between these two regions (Fig. 4.3).

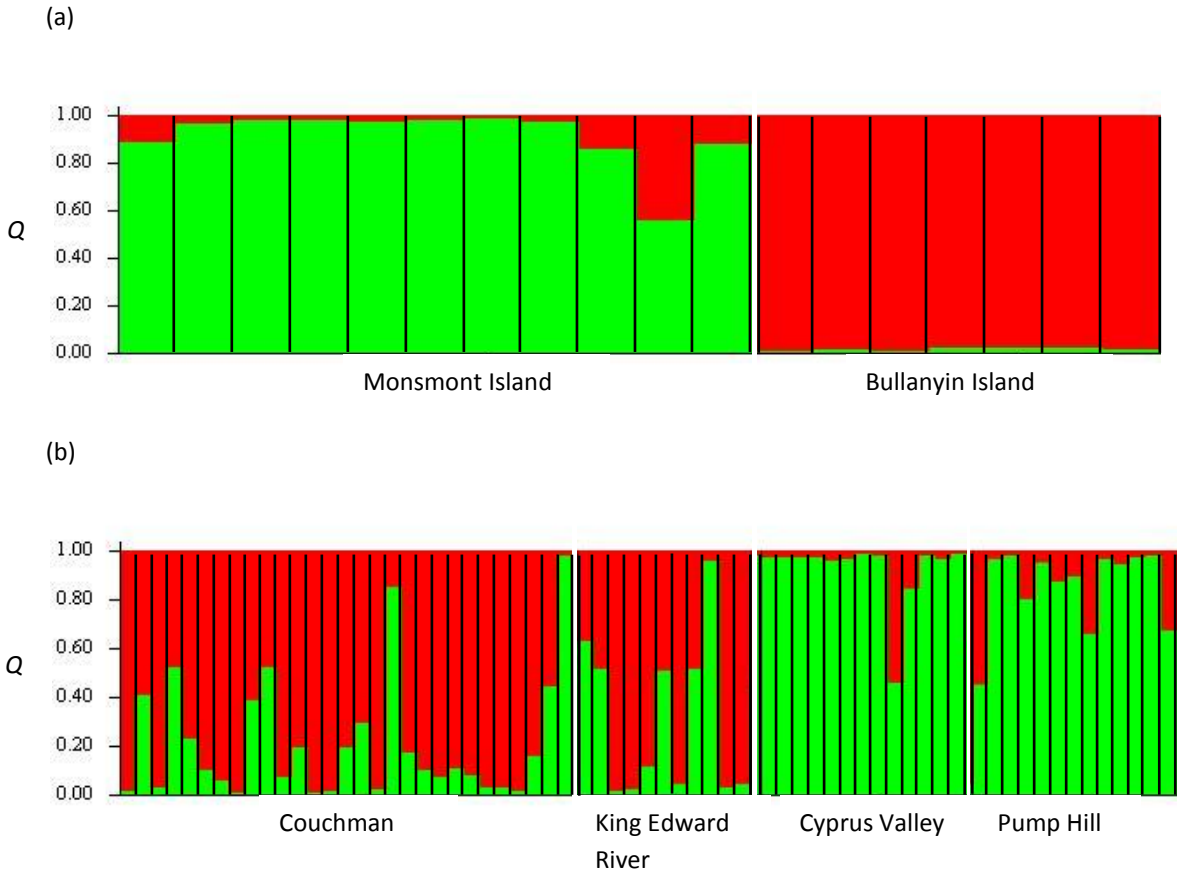


Figure 4.2: (a) Proportional membership (*Q*) of each *P. brachyotis* individual in the East Kimberley to the two clusters identified by STRUCTURE; (b) Proportional membership (*Q*) of each *P. brachyotis* individual in the West Kimberley to the two clusters identified by STRUCTURE. Each individual is represented by a single vertical bar.

Table 4.3: Genetic differentiation between sampled *Petrogale brachyotis* populations (Couchman – C; Cyprus Valley – CV; Monsmont Island – M Is; Bullanyin Island – B Is; King Edward River – KER; and Pump Hill – PH). Pairwise Φ_{ST} (mtDNA) above and F_{ST} (microsatellites) below the diagonal. *significant (P < 0.05) after Bonferroni correction.

	C	CV	M Is	B Is	KER
C		0.856*	0.993*	0.993*	
CV	0.043*		0.935*	0.929*	
M Is	0.256*	0.274*		0.995*	
B Is	0.202*	0.209*	0.166*		
KER	0.027*	0.058*	0.248*	0.184*	
PH	0.051*	0.022	0.265*	0.201*	0.059*

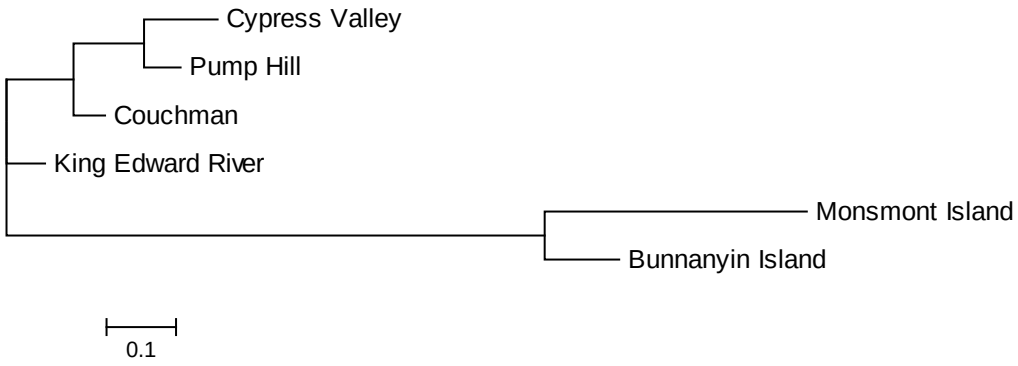


Figure 4.3: Unrooted neighbour-joining tree displaying Nei‟s genetic distance (Nei‟s *D* from microsatellite genotypes) between sampled populations.

MtDNA phylogeography

There were no shared haplotypes between any of the populations (Couchman, Cyprus Valley, Bullanyin Island and Monsmont Island). Three distinct groupings of haplotypes were apparent from the phylogenetic analyses (Fig. 4.4). One clade comprised the haplotypes from Monsmont Island (H, I, J), with the second clade consisting of haplotypes from Bullanyin Island (K, L, M). The remaining haplotypes (A–G) from the Couchman and Cyprus Valley populations (West Kimberley) formed the third clade, with 0.2-2.9% sequence divergence (SD) between haplotypes (0.2-0.3% SD amongst haplotypes from Couchman and 0.4-2.9% SD amongst Cypress Valley haplotypes). Divergence between the two West Kimberley populations was low in comparison to that between the East and West Kimberley populations (6.4-7.1% SD), as well as between Monsmont and Bullanyin Islands in the East Kimberley (5.6-5.9% SD). All three methods of tree reconstruction produced the same phylogeny (ML tree shown in Fig. 4.4, with ML bootstrap supports and Bayesian posterior probabilities).

Estimation of dispersal patterns using assignment tests

Four putative immigrants were detected amongst the six populations using GeneClass 2.0, including individuals from Couchman (JA09019), Monsmont Island (ABTC103504), King Edward River (KER09028) and Pump Hill (PH09017). No first generation female immigrants were detected, with all four of these potential dispersers being males. Two individuals (JA09019 and KER09028) had the highest likelihoods of coming from the Pump Hill population and PH09017 had the highest likelihood of coming from the King Edward River population, but these individuals were not assigned to any population with a probability of 0.99. ABTC103504, however, was excluded from all populations with no other sampled population having a higher likelihood of being „home" despite the individual not having a high enough probability (0.99) of belonging to its „home" population. Since all four individuals could not be assigned to a population with high probability (0.99) it is likely they have originated from populations not sampled in this study. These results are consistent with STRUCTURE outputs, where KER09028 and JA09019 group with a different cluster to the rest of their population and ABTC103504 has a mixed genotype and could not be assigned to a cluster.

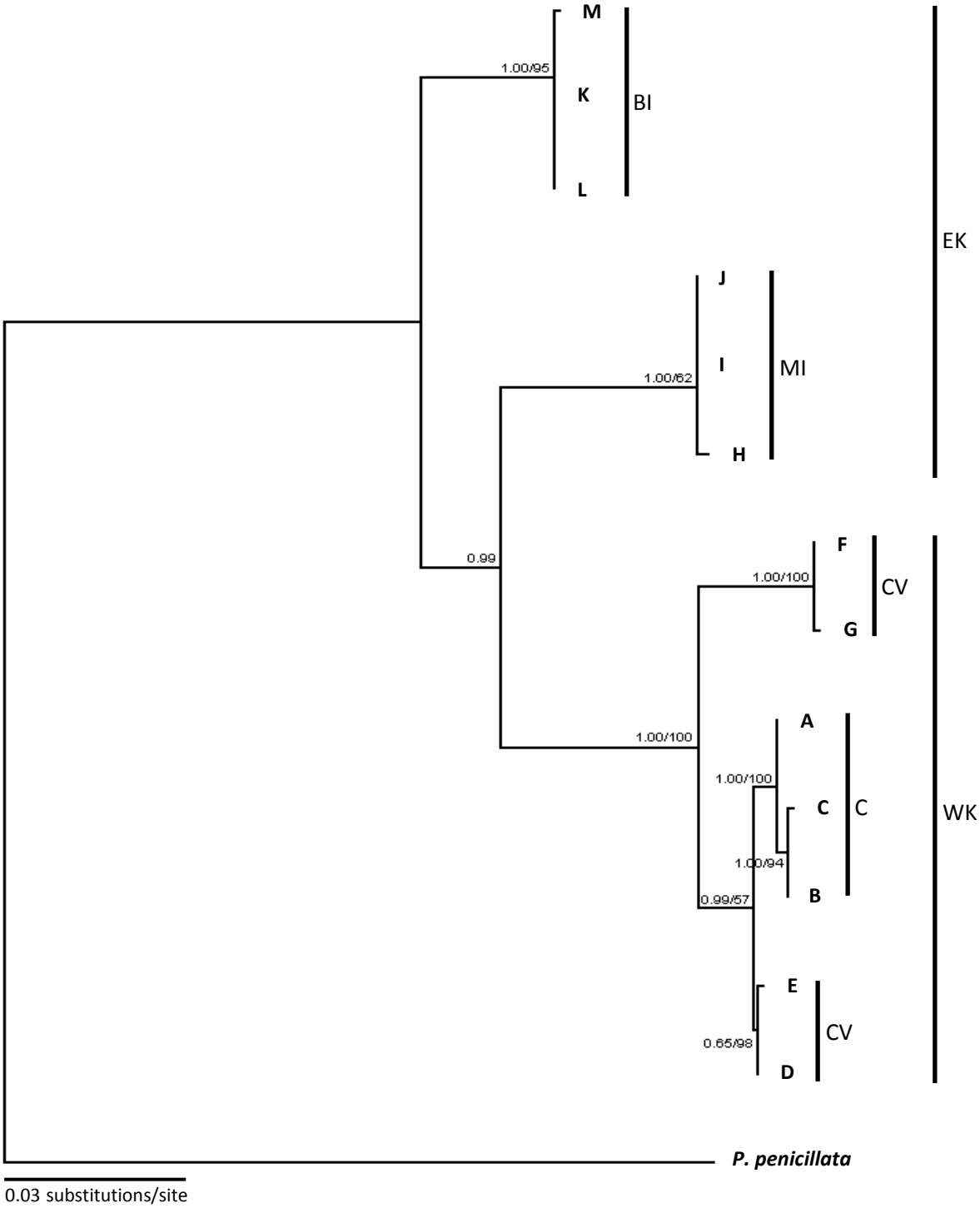


Figure 4.4: Maximum likelihood reconstruction of *P. brachyotis* haplotypes with *P. penicillata* as outgroup. Bootstrap values are represented as percentages and Bayesian posterior probabilities are represented as decimal values. Haplotypes are grouped into geographic regions (BI – Bullanyin Island, MI – Monsmont Island, C – Couchman, CV – Cyprus Valley; EK – East Kimberley, WK – West Kimberley).

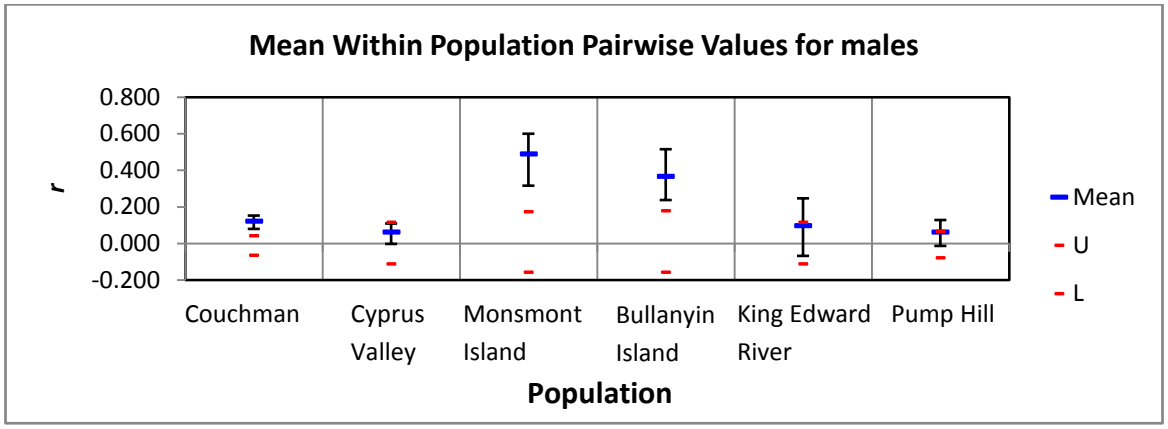
Relatedness within populations

All pairwise intra-population means were highly significant ($P< 0.007$) from the null hypothesis of individuals (male and female) being unrelated. All *r* means fell outside the 95% confidence interval (CI) permuted under the null hypothesis, but populations from Cyprus Valley, King Edward River and Pump Hill had 95% CI about the mean which overlapped with the upper 95% CI permuted from the null hypothesis. These populations were less related but still showed greater relatedness than expected by chance.

The results from female and male pairwise relatedness were different. Females were significantly related (significant *r* means for intra-population analyses) compared to the null hypothesis (unrelated) for all populations except Pump Hill, whereas males only revealed significant results for Monsmont Island, Bullanyin Island and the Couchman populations (refer to Fig. 4.5). For Cyprus Valley females the 95% CI about the mean value overlapped with the upper 95% CI permuted from the null hypothesis.

Mantel tests indicated significant correlations of both male and female genetic divergence and geographic distances ($P<0.001$) across the Kimberley, with females having a slightly higher correlation of genetic similarity to geographic distance (0.73 versus 0.68 for males). Results for the West Kimberley alone revealed significant, yet far lower, correlations between genetic and geographic distances (0.30, $P<0.001$), with slightly greater genetic similarity to geographic distance for females (0.36, $P<0.001$) than for males (0.25, $P<0.001$).

(a)



(b)

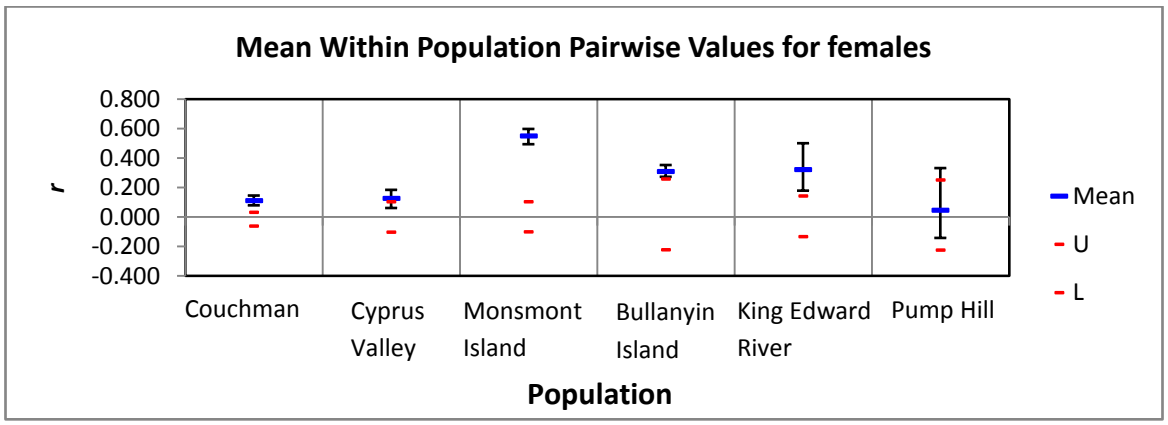


Figure 4.5: (a) Mean pairwise relatedness within populations for males with error bars around the mean. (b) Mean pairwise relatedness within populations for females. U and L are upper and lower 95% confidence bounds of the null hypothesis of no difference across populations (from 10,000 permutations).

Discussion

This study provides the first comparison of rock-wallaby populations in a landscape which contains large areas of interconnected habitat. This greater habitat connectivity is consistent with this data showing lower genetic differentiation across the West Kimberley compared to the high levels previously recorded for *Petrogale* taxa elsewhere (see below). *P. brachyotis* like other *Petrogale* species is a habitat specialist, but these results suggest connectivity between suitable habitat patches plays a critical role in maintaining gene flow and reducing genetic differentiation across the landscape. It appears that both habitat fragmentation and species‟ biology may shape population dynamics, but these results provide insight into how the effects of fragmentation may have impacted threatened species of rock-wallabies. The lower levels of genetic differentiation found in the West Kimberley are not shared between the two East Kimberley populations or the East and West Kimberley regions, which is likely the result of biogeographic processes, but could also be a consequence of isolation by distance.

Genetic diversity of Kimberley rock-wallabies

Within the West Kimberley there were no significant differences in the genetic diversity (*AD*, *AR*, H_E) between populations, except for *AD* between the Couchman and all other West Kimberley populations. This result is most likely due to the larger sampled Couchman population, as *AR* (accounts for population size) is not significant between these populations. Microsatellite diversity (*AD* and H_E) from the West Kimberley was generally higher than other rock-wallaby species (*P. penicillata*, *P. lateralis*, *P. brachyotis*, *P. xanthopus*), but lower than *P. assimilis* (allied rock-wallaby; see Table 4.4). This general pattern of greater genetic diversity compared to other rock-wallabies is potentially a result of larger population sizes and greater gene flow, creating larger metapopulations.

Table 4.4: Reported allelic diversity (*AD*) and average heterozygosity (H_E) at polymorphic microsatellite loci in 25 populations of *Petrogale* which were assessed using an assortment of the markers used in this study.

Taxon	No. of individuals (and site)	No. of loci	*AD*	H_E	Reference
Petrogale assimilis	128 (Black Rock)	5	11.6	0.86	(Spencer and Marsh 1997)
	15 (Little Black Rock)	5	8.6	0.85	(Spencer *et al.* 1995)
Petrogale brachyotis	32 (East Alligator)	10	7.8	0.78	(Telfer and Eldridge 2010)
	14 (Florence Falls)	10	3.4	0.55	(Telfer and Eldridge 2010)
	30 (Buley)	10	4.6	0.62	(Telfer and Eldridge 2010)
Petrogale penicillata	28 (Ingles Rd)[T]	11	5.2	0.70	(Eldridge *et al.* 2004c)
	15 (Crocodile Rock)[T]	7	5.1	0.65	(Piggott *et al.* 2006b)
	17 (Rocky Creek)[T]	7	5.1	0.65	(Piggott *et al.* 2006b)
	20 (Bowmans Rd)[T]	11	5.1	0.69	(Eldridge *et al.* 2004c)
	44 (Wolgan, Main)[T]	7	4.9	0.65	(Piggott *et al.* 2006b)
	54 (Hurdle Creek)[T]	12	4.5	0.67	(Hazlitt *et al.* 2006a)
	17 (Broke)[T]	11	4.0	0.64	(Eldridge *et al.* 2004c)
	15 (Perseverance Dam)[T]	12	3.4	0.57	(Hazlitt *et al.* 2006a)
	20 (Jenolan 1985, captive)[T]	11	3.2	0.50	(Eldridge *et al.* 2004c)
	18 (Kawau Is, New Zealand)[T]	11	2.6	0.44	(Eldridge *et al.* 2001a)
	10 (Jenolan 1996, captive)[T]	11	2.5	0.45	(Eldridge *et al.* 2004c)
	19 (Kangaroo Valley)[T]	12	2.4	0.37	(Paplinska 2006)
	8 (Rocky Plains Creek)[T]	11	1.9	0.34	(Browning *et al.* 2001)
Petrogale lateralis lateralis	19 (Tutakin)[T]	11	4.0	0.62	(Eldridge *et al.* 2004a)
	31 (Sales Rock)[T]	11	3.5	0.55	(Eldridge *et al.* 2004c)
	15 (Exmouth)[T]	10	3.4	0.62	(Eldridge *et al.* 1999)
	32 (Mt Caroline East)[T]	11	3.3	0.51	(Eldridge *et al.* 2004a)
	19 (Mt Caroline West)[T]	11	3.1	0.49	(Eldridge *et al.* 2004a)
	19 (Nangeen Hill)[T]	11	2.5	0.41	(Eldridge *et al.* 2004a)
	43 (Querekin)[T]	11	2.2	0.27	(Eldridge *et al.* 2004a)
	28 (Barrow Island)[T]	10	1.2	0.05	(Eldridge *et al.* 1999)
Petrogale xanthopus celeris	50 (Hill of Knowledge)	4	7.5	0.69	(Pope *et al.* 1996)
Petrogale x. xanthopus	69 (Flinders Ranges)*[T]	7	7.8	0.75	(Zenger *et al.* 2003)

*Samples pooled from multiple localities.

T: Threatened taxon (Maxwell *et al.* 1996).

There were significant differences in microsatellite diversity between populations from the East and West Kimberley, however, with *AD*, *AR* and H_E generally lower on Monsmont and Bullanyin Islands compared to the West Kimberley populations. This reduced diversity is likely due to these populations being located on small (50-100 ha) islands within Lake Argyle, which were formed in 1972, when the Ord River Irrigation Scheme developed Lake Argyle as a major storage reservoir**.** Eldridge *et al.* (2010) reported high levels of microsatellite diversity associated with macropodoid populations from relatively abundant or widespread species, whilst low levels were typically associated with populations showing a restricted distribution including those found on islands. The theory behind the reduced diversity expected on islands is due to the loss of variation as a result of finite population size and the associated problems of small populations (e.g., inbreeding, genetic drift; Frankham 1997; Frankham *et al.* 2002, 2004). Other populations of rock-wallabies on islands have shown reduced genetic diversity, with the black-footed rock-wallaby revealing an extreme example of this phenomenon (Eldridge *et al.* 1999). There was, however, no evidence for inbreeding due to non-random mating in Monsmont and Bullanyin Island populations for either males or females despite high levels of relatedness (Queller and Goodnight 1989; mean estimator) within these populations (0.251-0.797 Monsmont Island; 0.118-0.693 Bullanyin Island) and low genetic diversity. Mating system preferences such as kin avoidance and sex-biased dispersal have been suggested to facilitate inbreeding avoidance (Hazlitt *et al.* 2006b), with other studies of *P. penicillata* recording high levels of genetic diversity with no evidence of inbreeding within isolated colonies (Browning *et al.* 2001; Eldridge *et al.* 2004c; Hazlitt *et al.* 2004; Piggott *et al.* 2006b). These processes could be occurring within these island populations of *P. brachyotis* and have been suggested to help maintain genetic diversity within *Petrogale* populations (Spencer *et al.* 1997).

Population differentiation across the Kimberley

The West Kimberley revealed low to moderate F_{ST} values from the microsatellites (0.022-0.059) compared to other species of rock-wallabies using similar combinations of microsatellite loci (Pope *et al.* 1996; Eldridge *et al.* 2001b; Hazlitt *et al.* 2006a; Piggott *et al.* 2006b). Cyprus Valley and Pump Hill populations (≈1.2 km apart) were the only populations which were not significantly differentiated. However, the relatively low levels of genetic

differentiation (F_{ST} values) found at distances of up to 67 km are in contrast to those reported for rock-wallaby populations in closer proximity, but within more heavily modified intervenory landscapes; including *P. penicillata* (0.072, <10km; Hazlitt *et al.* 2006a), *P. xanthopus* (0.107, 10-70 km; Pope *et al.* 1996) and *P. lateralis* (0.238, <10 km; Eldridge *et al.* 2001b). They are also far lower than that found in *P. brachyotis* from the Northern Territory which revealed limited contemporary and long-term gene flow between populations 1.2 km apart (F_{ST}: 0.085; Telfer and Eldridge 2010). This lower level of genetic differentiation indicates that populations in the West Kimberley are more genetically connected, likely the result of the relatively pristine environment and greater continuity of sandstone escarpments.

STRUCTURE results identified two genetic groups in the West Kimberley which were related to geographic locality, indicating genetic connectivity at distances up to 18 km (Couchman and King Edward River populations). A large proportion of individuals had admixed ancestry (22.7%) and could not be placed into a single genetic cluster, with individuals from three out of the four populations revealing mixed genotypes (Cyprus Valley excluded). Some individuals from the Couchman and King Edward River populations even had a higher probability of clustering with the Cyprus Valley and Pump Hill group (up to 67 km away) in comparison to the rest of their populations. Additionally, three first generation migrants (KER09028, JA09019 and PH09017) were detected indicating gene flow is occurring across this landscape, creating low genetic differentiation between populations. These immigrants are likely to have come from unsampled populations which suggest that gene flow among *P. brachyotis* in the West Kimberley occurs via interspersed populations not sampled in this study.

Phylogenetic results of mtDNA provide no evidence for long term isolation of females between populations in the West Kimberley, with Cyprus Valley and Couchman populations forming a clade comprising haplotypes with only 0.2-2.9% SD. The mtDNA haplotypes from these two populations do not form reciprocally monophyletic clades, with haplotypes from Cyprus Valley being more closely related to those from the Couchman (0.2% SD) than to other haplotypes from Cyprus Valley. This similarity could be interpreted as retained ancestral polymorphisms in isolated populations; but given the F_{ST} and STRUCTURE results

and reduced isolation by distance correlations compared to those in the East Kimberley (0.30 vs. 0.67), greater connectivity in this region seems more likely.

F_{ST} and STRUCTURE analyses revealed no evidence for population differentiation between Cyprus Valley and Pump Hill populations, suggesting contemporary gene flow between these two sites located on either side of the Morgan River. This permanent waterway does not appear to represent a significant barrier to gene flow despite rivers generally being shown to impact *P. brachyotis* in the east of its range and other rock-wallaby species and populations (Bee and Close 1993; Piggott *et al.* 2006b; Chapters 2 & 3). Dispersal across the Morgan River is most likely occurring during the dry season, when river levels are lower, exposing potential paths for individuals to traverse the kilometre between outcrops. It is likely that these populations represent a large panmictic population, as genetic clustering methods grouped these two sampled sites together. Further assessment of population and home range sizes may identify at what level fine-scale population structure exists at this location.

Evidence of gene flow within the East Kimberley was limited by the sampling regime. However, the F_{ST} values between the two geographically close East Kimberley populations (0.166) were comparable to those found between the East and West Kimberley (0.184-0.274). This high level of differentiation within the East Kimberley was supported by the mtDNA data, not only with significant Φ_{ST} values between Monsmont and Bullanyin Islands, but also from haplotypes from each island forming two distinct clusters showing up to 5.9% sequence divergence. The genetic clustering method of Pritchard *et al.* (2000) also identified two genetic clusters, corresponding to the two sampled island populations, within this region.

Some level of genetic differentiation within the East Kimberley is anticipated since it is unlikely that individuals are traversing the 3.5 km of water to cross between islands. However, divergence/differentiation seems larger than expected (discussed below in phylogeography section). One intriguing outcome was the detection of a first generation migrant on Monsmont Island, suggesting that there is more than one population or family group of *P. brachyotis* on this island (given the high level of relatedness); that people are shifting individuals onto the islands; or that rock-wallabies are able to swim. Given that rock-wallabies appear to be poor dispersers over water (Robinson *et al.* 1996), the most likely

scenario is that more demes exist on this 50 ha island than were sampled. This is consistent with pairwise relatedness results which indicate that this individual (ABTC103504), while being similar, has lower values (average r=0.34) compared to all other individuals in the population (average r=0.55). Since these islands populations are not broadly typical of the East Kimberley, further analysis of other less isolated populations will be necessary to examine the processes influencing the population dynamics and gene flow of *P. brachyotis* in this region.

Sex-biased dispersal

Amongst the six populations sampled in this study, four first generation migrants were detected all of which were male. This does not discount the possibility of female dispersal (Eldridge *et al.* 2001b has shown it occurs), but the strong female philopatry indicated by the mtDNA results and low differentiation for microsatellite markers suggests these rock-wallabies display sex-biased dispersal. Limited female-mediated gene flow is consistent with other rock-wallabies (Hazlitt *et al.* 2004, 2010; Piggott *et al.* 2006b) and supports suggestions that dispersal for macropodoids is generally male-biased (Eldridge *et al.* 2010). The isolation by distance results also indicate greater female genetic similarity correlations to geographic distance compared to males, in both the overall and the West Kimberley analyses (0.73 vs. 0.68; 0.36 vs. 0.25, respectively). The relatedness results also indicate that females are generally more related than males within populations, with significant relatedness differences between sexes detected in the King Edward River and Monsmont Island populations. These results are consistent with other studies where male-biased dispersal has been detected in brush-tailed rock-wallabies (Hazlitt *et al.* 2004, 2006a).

Population structure of Kimberley P. brachyotis

Populations in the East and West Kimberley were clearly differentiated by each of the different methods used in this study. Both phylogenetic analysis of mtDNA (ML reconstruction) and microsatellites (NJ tree) indicate a clustering of populations from the East Kimberley separate from an assemblage of West Kimberley populations, with larger sequence divergences between the East and West providing strong evidence of genetic isolation

between these regions. We also detected significant F_{ST} and Φ_{ST} values between all populations from these two regions. Initial STRUCTURE analysis also recognised two discrete genetic clusters, separating individuals from the East Kimberley from those from the West. Additionally, the East and West Kimberley possess a large percentage of unique alleles (17-48%). This is consistent with the high levels of mtDNA sequence divergence identified between haplotypes from the West Kimberley and the East Kimberley (up to 7.1 % SD), which are similar to levels of divergence found between subspecies and ESUs in other rock-wallabies (e.g., 5.3% *P. xanthopus*, Pope *et al.* 1996; Eldridge *et al.* 2001b; 5% *P. lateralis,* Eldridge *et al.* 2001b; 7.6% *P. penicillata*, Browning *et al.* 2001), but over much greater distances (400-1000 km) apart. The sequence divergence we have detected between *P. brachyotis* from the East and West Kimberley at distances up to 290 km, together with high F_{ST} values (microsatellite analyses), suggest that these two regions represent highly differentiated populations showing long-term genetic isolation. Although these conclusions are based on a relatively small sample size from the East Kimberley, there is also evidence from mitochondrial and nuclear sequencing of individuals from other sites in the East Kimberley which supports a lack of contemporary or long term gene flow between the East and West Kimberley (Chapters 2 & 3).

Phylogeography

The cause of the divergence between East and West Kimberley *P. brachyotis* populations is not clear, as there are few obvious physical barriers. There is a large basalt range running north to south in the central Kimberley that formed as a result of Carson Volcanics depositing tholeiitic basalt between 1790-1835 million years ago (MYA) (Griffin *et al.* 1993). Although basalt rarely weathers to produce suitable rock-wallaby habitat the formation of the Carson Volcanics long predates the estimated divergence of East and West Kimberley *P. brachyotis.* However, the sandstone subsequently deposited over this region may only initially have provided suitable rock-wallaby habitat and therefore connectivity between the East and West Kimberley, but once it eroded away, the basalt zone was exposed and created a barrier for *P. brachyotis* dispersal as a zone lacking the complex rock structure necessary to sustain *Petrogale* populations. The divergence between the East and West Kimberley may also have been influenced by glacial and interglacial cycles during the Pleistocene, where large-scale

environmental changes caused range contractions and expansions of many species (Russell-Smith *et al.* 1993). This was suggested to have influenced the population structure of *P. brachyotis* in the Northern Territory (Telfer and Eldridge 2010) and between the Kimberley and the Northern Territory (Chapter 2). In this scenario *P. brachyotis* populations may have contracted into eastern and western mesic rocky refugia during arid phases and over time differentiated from each other. During these cycles, the ongoing processes of sandstone weathering may have led to the loss of habitat connecting the East and West Kimberley resulting in the continued separation of these populations.

Isolation by distance may also have influenced the genetic structure of *P. brachyotis* across the Kimberley, with the genetic differentiation between the East and West Kimberley resulting from a low level of gene flow (particularly female) across the landscape (≈290 km). The level of genetic differentiation between Monsmont and Bullanyin Island populations appears to be greater than would be expected just from a lack of gene flow as a result of Lake Argyle being created 40 years ago. Since *Petrogale* longevities of up to 12 years have been suggested in the wild (Bee and Close 1993), it is unlikely sufficient generations have passed to cause the genetic differences detected. We suggest this extensive differentiation represents longer term separation, as a consequence of these two populations originally being located on either side of the Ord River (pers. comm. David Pearson) which is likely to have restricted gene flow between these two populations long before the creation of Lake Argyle. Based on previous studies this split is estimated to have occurred around 0.13 MYA (Chapter 3) and could be related to the impacts of glacial and interglacial cycles mentioned above.

Conclusions and future implications

Low microsatellite divergence together with weak genetic structure of mtDNA within the West Kimberley provides evidence of genetic connectivity across this landscape, with lower genetic differentiation over larger distances than previously reported for any other rock-wallaby species. Given the limited sampling of populations relative to the range of *P. brachyotis*, the reduced genetic differentiation recorded illustrates how relatively intact and continuous habitat can facilitate gene flow across this region and enhance the diversity and persistence of rock-wallaby populations. These findings are in contrast to studies of *P.*

brachyotis in the Northern Territory where populations were genetically differentiated at distances of 1.2 km, but where rocky habitat is patchier. This study suggests that habitat connectivity plays a critical role in enabling dispersal within this species, which exhibits high philopatry and strong habitat specificity. Despite evidence of male-biased dispersal and female philopatry, these results suggest that species‟ biology does not necessarily lead to genetic differentiation of rock-wallabies, with low genetic differentiation observed across suitable habitat.

We found clear evidence that *P. brachyotis* from the East and West Kimberley are highly differentiated, based on long term isolation of gene flow between these regions potentially impacted from the effects of Pleistocene climatic cycling. In the West Kimberley, sex-biased dispersal appears to play a role in maintaining gene flow between populations and this together with possible kin avoidance mechanisms could potentially influence mating systems to facilitate inbreeding avoidance in *P. brachyotis*. Further sampling of individuals in the East Kimberley is required to assess the genetic connectivity of populations within this region, but it is evident some long term processes have separated populations in this region.
Non-invasive techniques were used effectively in this study and we anticipate that this approach now offers the opportunity to assess population dynamics of other cryptic and uncommon species in this rugged and inaccessible environment. Sampling of other species from across the Kimberley is needed to add insight into the biogeography of this region and determine the relative impacts of climatic change and individual species‟ biology.

Statement of Authorship

Sally Potter (candidate)

Corresponding author: Prepared DNA extracts for PCR amplification, carried out DNA sequencing, analysed sequence data, wrote manuscript and produced most Figures.

Signed Date 12-1-2011

David A. Taggart

Provided assistance with obtaining project funding, sample collection and evaluated the manuscript.

I give consent for Sally Potter (candidate) to include this manuscript for examination towards the degree of Doctor of Philosophy.

Signed Date 11-1-2011

Steve J.B. Cooper

Supervised the direction of the study and provided advice on analyses and critically reviewed manuscript.

I give consent for Sally Potter (candidate) to include this manuscript for examination towards the degree of Doctor of Philosophy.

Signed Date 12/1/2011

Mark. D.B. Eldridge

Provided samples for study, provided the morphological characteristics table and photographs, provided assistance with obtaining project funding supervised the direction of the study, provided advice on analyses and critically reviewed manuscript.

I give consent for Sally Potter (candidate) to include this manuscript for examination towards the degree of Doctor of Philosophy.

Signed Date 10 Jan 2011

Preamble:

This chapter (Chapter 5) is based on the same analyses to that provided in Chapter 3, but has a focus on conservation and taxonomic issues rather than phylogeography. This Chapter was set out as a separate manuscript to allow in depth explanations for taxonomic changes within the *brachyotis* group. In addition, morphological data is included in this chapter, setting it apart from Chapter 3.

CHAPTER 5: Genetic differentiation within the *brachyotis* group outlines new taxonomic classifications and conservation units

Sally Potter, David A. Taggart, Steve J.B. Cooper and Mark D.B. Eldridge

Abstract

Molecular techniques can provide essential data to resolve taxonomic uncertainties and identify conservation units within species. The *brachyotis* group of rock-wallabies currently consist of three well differentiated species (*Petrogale brachyotis*, *P. burbidgei* and *P. concinna*) whose inter-relationships remain uncertain. All three species are endemic to north-western Australia and are sympatric over parts of their distribution. DNA sequence analyses of two mitochondrial (mtDNA) regions (*CR*, *ND2*) and four nuclear loci (*BRCA1*, *ω-globin,* two anonymous loci) have found substantial divergence within and between these species. Phylogenetic analyses indicate that *P. brachyotis* (*sensu lato*) is paraphyletic and represents at least two separate species: *P. brachyotis* (*sensu stricto*) from the Kimberley and western Northern Territory (NT), and *P. wilkinsi* from the northern and eastern NT. In addition, *P. brachyotis* (*sensu stricto*) has been separated into four evolutionarily significant units (ESUs) that show reciprocal monophyly for mtDNA and significant divergence of allele frequencies at nuclear loci. Substantial genetic divergence remains within both *P. brachyotis* **(***sensu stricto***)** and *P. wilkinsi* and additional research may result in additional species and/or ESUs being recognised. Although data are limited, both *P. burbidgei* and *P. concinna* represent distinct lineages with *P. burbidgei* being the sister species to *P. brachyotis* (Victoria River) and *P. concinna* the sister taxon to *P. brachyotis* (Kimberley). Additional studies of the diminutive *P. concinna* are urgently needed as it appears to be declining and knowledge of population structure across its range is lacking. This study has identified considerably more diversity than previously recognised within the *brachyotis* group, with important implications for the effective conservation management of these rock-wallabies.

Introduction

Molecular data are now frequently used to resolve taxonomic uncertainties and identify conservation units within species, as well as understand patterns and processes of evolution and speciation (Avise 1989; Templeton 1994; Dubois 2003; Frankham *et al.* 2004; Frankham 2010a). Accurate taxonomy is crucial to conservation efforts, which aim to maintain unique evolutionary lineages, as erroneous lumping or splitting of taxa can profoundly influence management decisions (Frankham *et al.* 2004). Taxonomic uncertainties are best resolved using an integrative approach such as combining data from phylogeography, morphology, population genetics, ecology and behaviour (Dayrat 2005). Controversy continues over the appropriate concept to use in delineating species, but covering the array of species concepts and the ensuing debate is not the aim of this paper (see Hey 2000). The biological species concept (Mayr 1993) has been the most influential definition of species in population and evolutionary genetics, as well as in conservation biology (Frankham *et al.* 2004; Frankham 2010b). This concept allows practical application using genetic data by recognising the importance of genetic exchange and defines species as populations that exchange genetic material while those from different species do not (Frankham *et al.* 2004). Defining allopatric populations using genetic markers can be difficult, but Frankham *et al.* (2004) suggests calibration of genetic differentiation against other recognised species within the genus. However, the most convincing evidence of specific status comes from the concordance of a wide array of information (e.g., morphology, chromosomes, nuclear and mitochondrial DNA). One commonality amongst the controversy surrounding species definitions is clear; they are distinct entities which represent evolving lineages that we hope to conserve (Hey 2001).

In addition to delineating species, molecular analyses have been increasingly utilised to define units within species which show significant genetic differentiation and that require separate management. The definition of these units is another contentious topic (Bowen 1999; Dimmick *et al.* 1999; Paetkau 1999; Crandall *et al.* 2000; Goldstein *et al.* 2000; Fraser and Bernatchez 2001). Moritz (1994, 2002) described criteria to identify evolutionarily significant units (ESUs), which apply to historically isolated populations (Frankham *et al.* 2004). Moritz (1994) defines ESUs as populations "reciprocally monophyletic for mtDNA alleles and show

significant divergence of allele frequencies at nuclear loci". This definition continues to be broadly applied in conservation biology and will be the criteria implemented in this study.

The endemic Australian marsupial genus, *Petrogale* (rock-wallabies), provide good examples where using genetic data has resolved taxonomic uncertainties and defined ESUs (Briscoe *et al.* 1982; Sharman *et al.* 1990; Eldridge and Close 1993; Browning *et al.* 2001; Campeau-Péloquin *et al.* 2001; Eldridge *et al.* 2001c). These iconic macropodids demonstrate significant genetic differentiation across Australia, as a result of recent and rapid speciation (Chapter 1). Rock-wallabies are an example of "populations „caught in the act" of speciating," (Frankham *et al.* 2004) with species and populations showing varying degrees of differentiation (e.g., morphology, chromosomes, allozymes and mtDNA), with many exhibiting only partial reproductive isolation. Most *Petrogale* species from the east coast of Australia can only be definitively identified by genetic analysis, with cryptic morphology impeding previous taxonomic classification (Eldridge *et al.* 1991b; Eldridge and Close 1992, 1993, 1997; Bee and Close 1993). Northern rock-wallaby species, the short-eared rock-wallaby (*P. brachyotis*), monjon (*P. burbidgei*) and nabarlek (*P. concinna*), which comprise the monophyletic *brachyotis* group, have so far received little attention in molecular systematic studies compared to other *Petrogale* (Sharman *et al.* 1990; Eldridge *et al.* 1991b, 2001c; Eldridge and Close 1992; Bee and Close 1993; Pope *et al.* 1996; Eldridge 1997a).

Taxonomic boundaries and inter-relationships within the *brachyotis* group have long been controversial (Poole 1979; Sharman *et al.* 1990; Sharman *et al.* 1995), with Eldridge (1997b) identifying the need for a detailed genetic (and morphological) study of this group as a priority. Recent molecular studies have identified exceptionally high levels of mitochondrial DNA (mtDNA) sequence divergence within and between *P. brachyotis* populations (Telfer and Eldridge 2010; Chapters 2 and 3) that are similar to, or exceed, divergences recorded between other rock-wallaby species (Browning *et al.* 2001; Chapter 2). These high levels of divergence suggest *P. brachyotis* may represent multiple species (Chapter 2) and reinforces the need for a comprehensive molecular analysis. Additionally, phylogenetic data (Chapter 2) indicate *P. burbidgei* and *P. concinna* are not sister taxa as previously suggested by chromosomal analysis (Sharman *et al.* 1990; Eldridge *et al.* 1992b; Eldridge and Close 1997), but represent sister lineages to different *P. brachyotis* populations (Chapter 2), indicating that

any review of the taxonomic status of *P. brachyotis* populations needs to include all species from the *brachyotis* group.

Although forming a monophyletic group within *Petrogale* (Briscoe *et al.* 1982; Sharman *et al.* 1990; Eldridge and Close 1997; Campeau-Péloquin *et al.* 2001; Chapter 2)*, P. brachyotis, P. burbidgei* and *P. concinna* are considered distinct species on the basis of their different chromosome numbers/structure (Sharman *et al.* 1990; Eldridge *et al.* 1992b), body size (see below), morphology (e.g., continually erupting molars, *P. concinna*; proportionally shorter ears, *P. brachyotis*; lack of distinct head-stripe, *P. burbidgei*) (Thomas 1926a; Kitchener and Sanson 1978) and their existence in sympatry without evidence of hybridisation. Their distributions also vary with *P. brachyotis* being the most widespread extending from the Queensland/Northern Territory (NT) border to the west coast of Western Australia (WA); whereas *P. burbidgei* is confined to the coastal Kimberley region of north-western WA and *P. concinna* to higher rainfall regions of the NT and the Kimberley, WA (Eldridge and Telfer 2008; Pearson *et al.* 2008; Sanson and Churchill 2008). These three species are the only *Petrogale* species to occur in sympatry, making their evolutionary history of considerable interest.

The widespread *P. brachyotis* is highly variable in size (2.2-5.6 kg) and morphology across its range (Sharman and Maynes 1983; Eldridge and Telfer 2008) and historically was described as five distinct taxa (4 separate species)(Sharman and Maynes 1983); *P. brachyotis* from Hanover Bay, western Kimberley, WA (Gould 1841); *P. wilkinsi* (Roper River, NT) (Thomas 1926a); *P. longmani* (Groote Eylandt, off Arnhem Land coast, NT) (Thomas 1926a); *P. venustula* (Upper Daly River, NT) (Thomas 1926b)*;* and the subspecies *P. brachyotis signata* (South Alligator River, Mary River, Daly River region, NT) (Thomas 1926b). After much taxonomic confusion and uncertainty (see Eldridge 1997b), Sharman *et al.* (1990) synonymised all 4 taxa with *P. brachyotis* and recognised a single widespread species based on their shared, highly derived, unique 2n=18 karyotype. Based on morphological criteria it was subsequently suggested that *P. brachyotis* could be divided into three geographic races; a Kimberley race covering the Kimberley region, WA; a Victoria River race encompassing populations from the Victoria River District, NT; and an Arnhem Land race comprising populations formerly described as *P. longmani, P. venustula and P. wilkinsi* in the NT

(Sharman and Maynes 1983), although Sharman *et al.* (1995) referred to these as populations rather than races.

Petrogale concinna (2n=16) is a much smaller rock-wallaby (≈1.4 kg), with a disjunct distribution across north-west Australia and three subspecies have been described; *P. concinna concinna* (Victoria Rivers District, NT) (Gould 1842), *P. c. canescens* (Arnhem Land, NT) (Thomas 1909) and *P. c. monastria* (Kimberley region, WA) (Thomas 1926b). *P. c. concinna* is only known from the type specimen collected in 1839 and the other two subspecies have rarely been reported. *P. concinna* too has had a complicated taxonomic history, being removed from *Petrogale* to a monotypic genus *Peradorcas* based on the presence of continuously erupting molars (Thomas 1904). However, molecular evidence has not supported this arrangement, clearly placing *P. concinna* within *Petrogale* (Briscoe *et al.* 1982; Calaby and Richardson 1988; Eldridge 1997b; Chapter 2), as a member of the *brachyotis* group. There is a growing concern about the conservation status of *P. concinna*, with evidence of ongoing declines (Briscoe *et al.* 1982; Churchill 1997), but their current status is unknown and of concern (Pearson 2009). The latest International Union for Conservation of Nature (IUCN) red list referred to them as „data deficient" with an unknown population trend (IUCN 2010). Few individuals have been seen in the field since 1991 when numerous NT populations were observed, but could not be captured (Churchill 1997). A survey in 2007 (Pearson and Churchill, unpublished) revisited many locations that Churchill (1997) had sighted *P. concinna* between August 1989 and September 1991, with no sightings except a single individual at Mt Borradaile, NT. It seems likely then that populations have declined dramatically over the past two decades. The behaviour of *P. concinna* is generally cryptic and it can be difficult to distinguish them from *P. burbidgei* and young *P. brachyotis* in the wild. These attributes, together with its remote habitat and aversion to enter traps has made this species problematic to study (Churchill 1997). Only samples from *P. c. canescens* have been examined using genetic techniques and the distinction between the described subspecies (based on minor morphological differences) remains uncertain (Eldridge 1997b).

Petrogale burbidgei (also 2n=16 – but different chromosome morphology to *P. concinna*) is the smallest species of rock-wallaby (≈1.2 kg) and was only relatively recently discovered and described (Kitchener and Sanson 1978). It has a highly restricted distribution being confined

to rugged areas of King Leopold sandstone in the wetter far north-west Kimberley, as well as Bigge Island (18,000 ha) and several smaller Islands off the Kimberley coast. *P. burbidgei's* remote and largely inaccessible habitat has led to little research being conducted on this species, and very little is known about its general biology or population dynamics.

In this study sequence data from mitochondrial and nuclear DNA (mtDNA, nDNA) were used to, (i) assess the level of genetic differentiation amongst populations of *P. brachyotis* throughout its range; (ii) compare these data with morphological variation to test the appropriateness of current taxonomic classifications; and (iii) assess the presence of conservation units (e.g., ESUs) within *P. brachyotis*.

Materials and methods

Samples and sequence analyses

Sequence data: one hundred and one *P. brachyotis*, eleven *P. burbidgei* and a single *P. concinna canescens* were sampled as described in Chapter 3 (see Fig. 5.1 for map of locations) and a single sample of *P. penicillata* (brush-tailed rock-wallaby) was included as an outgroup. Mitochondrial and nuclear sequence data (mtDNA – *CR*, *ND2*; nDNA – *BRCA1*, *ω-globin*, and anonymous markers A1 and A2) outlined in Chapter 3 was used in the current study.

Phylogenetic analyses (maximum likelihood - ML; maximum parsimony – MP; and Bayesian) of mtDNA were performed following the methods of Chapter 3 and pairwise genetic distances were calculated in PAUP* (version 4.0b Swofford 2002). An unrooted phylogram for each nuclear locus was also constructed, in PAUP* (version 4.0b Swofford 2002) using the neighbour joining (NJ) distance approach, as outlined in Chapter 3.

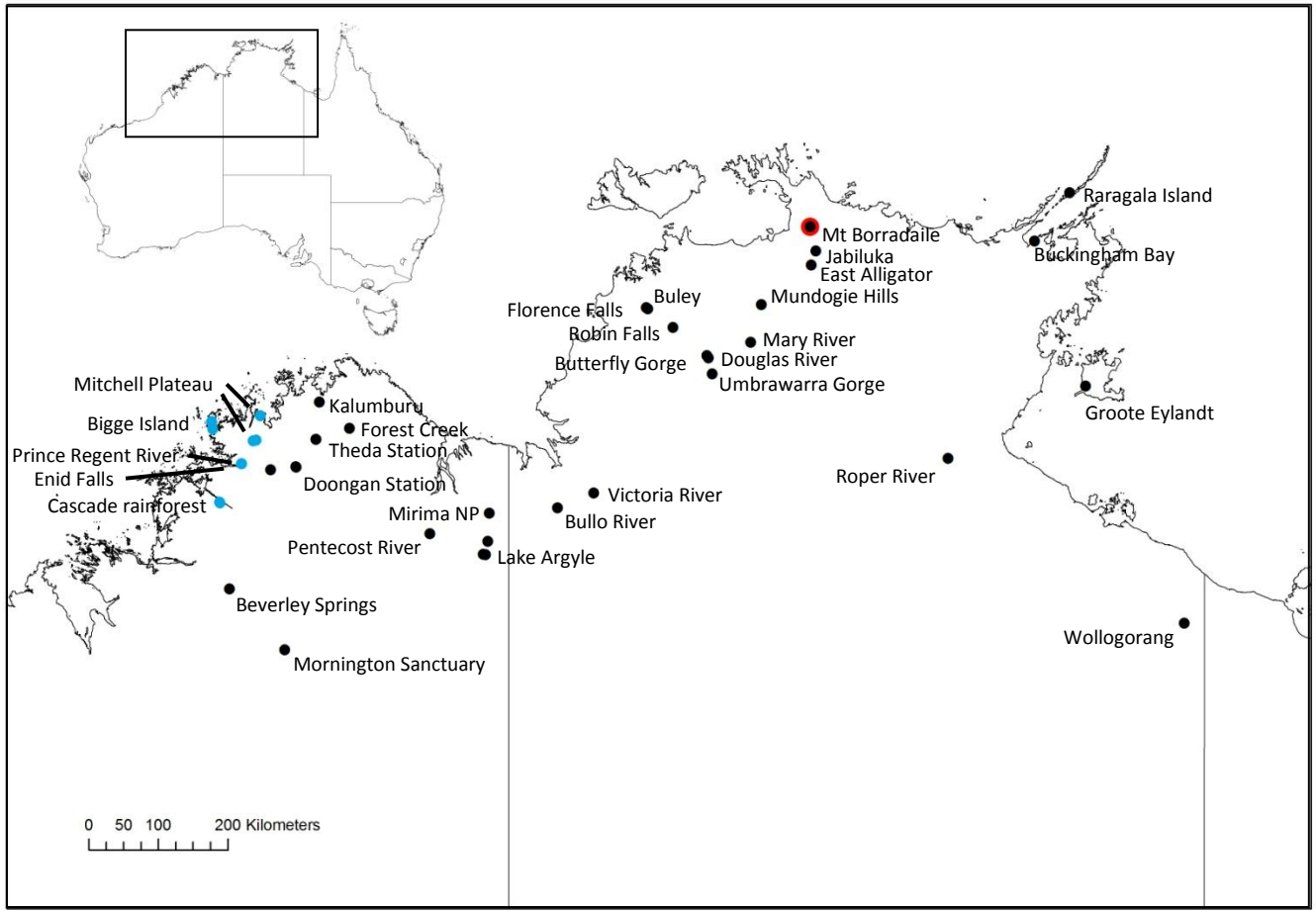


Figure 5.1: Sampling localities and locality names of *Petrogale brachyotis* (black), *P. burbidgei* (blue) and *P. concinna* (red) in northern Australia.

Analysis of molecular variance (AMOVA) was used to examine genetic differentiation amongst geographically localized populations of *P. brachyotis*, *P. burbidgei* and *P. concinna* using ARLEQUIN (v3.11; Excoffier *et al.* 2005) following methods of Chapter 3 to determine if significant genetic variation was present amongst populations. Parameters described in Chapter 3 (scaled by the neutral mutation rate, μ) were used to estimate migration rates between populations (effective gene exchange) and distinguish models of gene flow using a coalescence-based approach using the isolation with migration program (IMa; Hey and Nielsen 2004).

Morphological assessment

Skins of *P. brachyotis* specimens from the Australian Commonwealth Scientific and Research Organization (CSIRO) Australian National Wildlife Collection (ANWC) (n=67) and the Western Australian Museum (n=38) were examined. Details of pelage markings (e.g., location and intensity) and colouration were compared between specimens from across the distribution of *P. brachyotis*, including individuals from all of the major regions sampled in this study.

Results

Sequence analyses

Previous analyses reported in Chapter 3 provided evidence that *P. brachyotis* from the Northern Territory is the sister lineage to all other *brachyotis* group taxa (clade comprised of *P. brachyotis* from the Kimberley and Victoria River region, as well as *P. burbidgei* and *P. concinna*; refer to Fig. 5.2 for mtDNA phylogenetic tree). Three highly divergent monophyletic clades (10.4-15% sequence divergence, SD; see Table 5.1 for SD between lineages) were recovered for *P. brachyotis* from the following regions; Northern Territory (NT), Victoria River (VR) and Kimberley (KIM; refer to Fig. 5.2). *P. burbidgei* formed a monophyletic clade with the exception of one individual (ABTC101599) which was highly divergent from other *P. burbidgei* taxa (9.6-10.6% SD; see Table 5.1) and formed a sister lineage to *P. concinna* instead of the *P. burbidgei* clade.

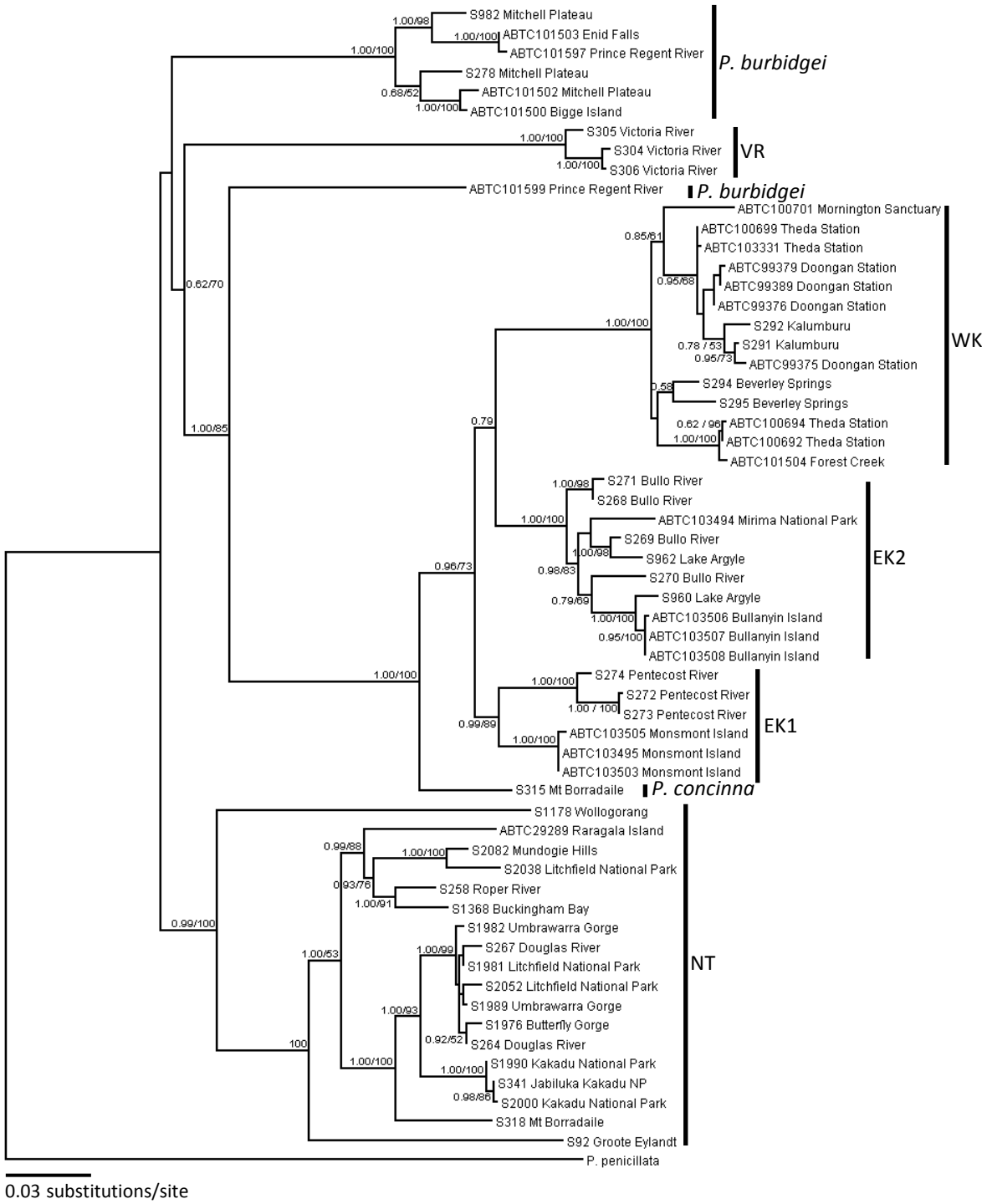


Figure 5.2: Phylogenetic tree of mtDNA of *P. brachyotis* (NT, VR, EK1, EK2 and WK), *P. burbidgei* and *P. concinna* taxa with *P. penicillata* as the outgroup used to root the tree. The Bayesian posterior probabilities are in decimals and the ML bootstrap values are as percentages. Haplotypes from the different geographic populations are labelled with different colours.

Table 5.1: Pairwise genetic sequence divergence between mtDNA lineages of the *brachyotis* group using a Tamura-Nei distance model. *P. brachyotis* lineages are split into a NT – Northern Territory, VR – Victoria River, WK – West Kimberley and two East Kimberley lineages (EK1 and EK2). ABTC101599 represents the independent lineage of *P. burbidgei*.

	NT	***P. burbidgei***	**VR**	**ABTC101599**	***P. concinna***	**EK1**	**EK2**	**WK**
NT	0.3-12.1							
P. burbidgei	9.1-14	0.2-4.3						
VR	10.7-12.9	10.4-12.3	0.2-1.4					
ABTC101599	9.6-11.3	9.6-10.5	11.8-12.6	-				
P. concinna	10.7-13.2	9.8-10.6	10.3-10.8	9.1	-			
EK1	10.4-14.2	10.3-11.8	11.2-12.5	9.4-11.2	4.9-5.7	0-4.8		
EK2	10.4-14.3	10.2-12.2	10.6-12.2	10.1-11.1	5.3-6.3	4.4-6.4	0-3.3	
WK	11.3-15	11.1-12.8	10.7-12.5	9.7-10.6	5.9-7.2	5.7-7.2	5.9-7.8	0.1-3.8

The *P. burbidgei* clade formed a sister lineage to *P. brachyotis* (VR and KIM) and so was embedded within *P. brachyotis*, separating populations from the NT from those elsewhere in its distribution (VR, KIM; Fig. 5.2). The single exemplar of *P. concinna* was also an independent lineage forming the sister lineage to *P. brachyotis* (KIM), thus separating *P. brachyotis* individuals from the Victoria River and Kimberley (Fig. 5.2).

Within *P. brachyotis* (NT) high levels of mtDNA divergence were detected, with two individuals (S1178, near the QLD border; S92, Groote Eylandt) showing 7.3-12% SD from other NT individuals (Table 5.1). There was also considerable divergence revealed within *P. brachyotis* (KIM), with individuals clustering into three (well supported; 89-100% bs and pp, see Fig. 5.2) distinct geographical lineages (4.9-7.8% SD between lineages); a clade containing individuals from the West Kimberley (WK), Beverley Springs to Kalumburu in the northern Kimberley; a clade comprising individuals from Monsmont Island in Lake Argyle north-west to Pentecost River in the East Kimberley (EK1); and a clade consisting of individuals from Bullanyin Island in Lake Argyle (East Kimberley) eastwards to Bullo River in the far west of NT (EK2).

Unrooted nuclear phylograms (neighbour-joining trees) as reconstructed in Chapter 3 (Fig. 3.3) are depicted in Fig. 5.3. *P. burbidgei* individuals shared a single haplotype for *BRCA1* and there were no shared haplotypes between *P. brachyotis* (NT) and *P. brachyotis* (VR and KIM). Two of these *P. brachyotis* (NT) individuals from Groote Eylandt (S92) and Wollogorang (S1178) contained divergent haplotypes compared to the other *P. brachyotis* (NT) haplotypes. A similar pattern for *P. brachyotis* (NT) for the A2 marker was found, with most individuals containing a single haplotype with the inclusion of one individual from *P. brachyotis* (EK2). No other haplotypes were shared amongst *P. brachyotis* (NT) and *P. brachyotis* (VR and KIM). There were many shared alleles between the mtDNA groups as outlined in Chapter 3. No alleles were shared uniquely between *P. brachyotis* (NT) and *P. brachyotis* (VR), *P. brachyotis* (NT) and *P. concinna*, *P. burbidgei* and *P. concinna*, or *P. brachyotis* (VR) and *P. burbidgei* or *P. concinna* (see Fig. 5.3).

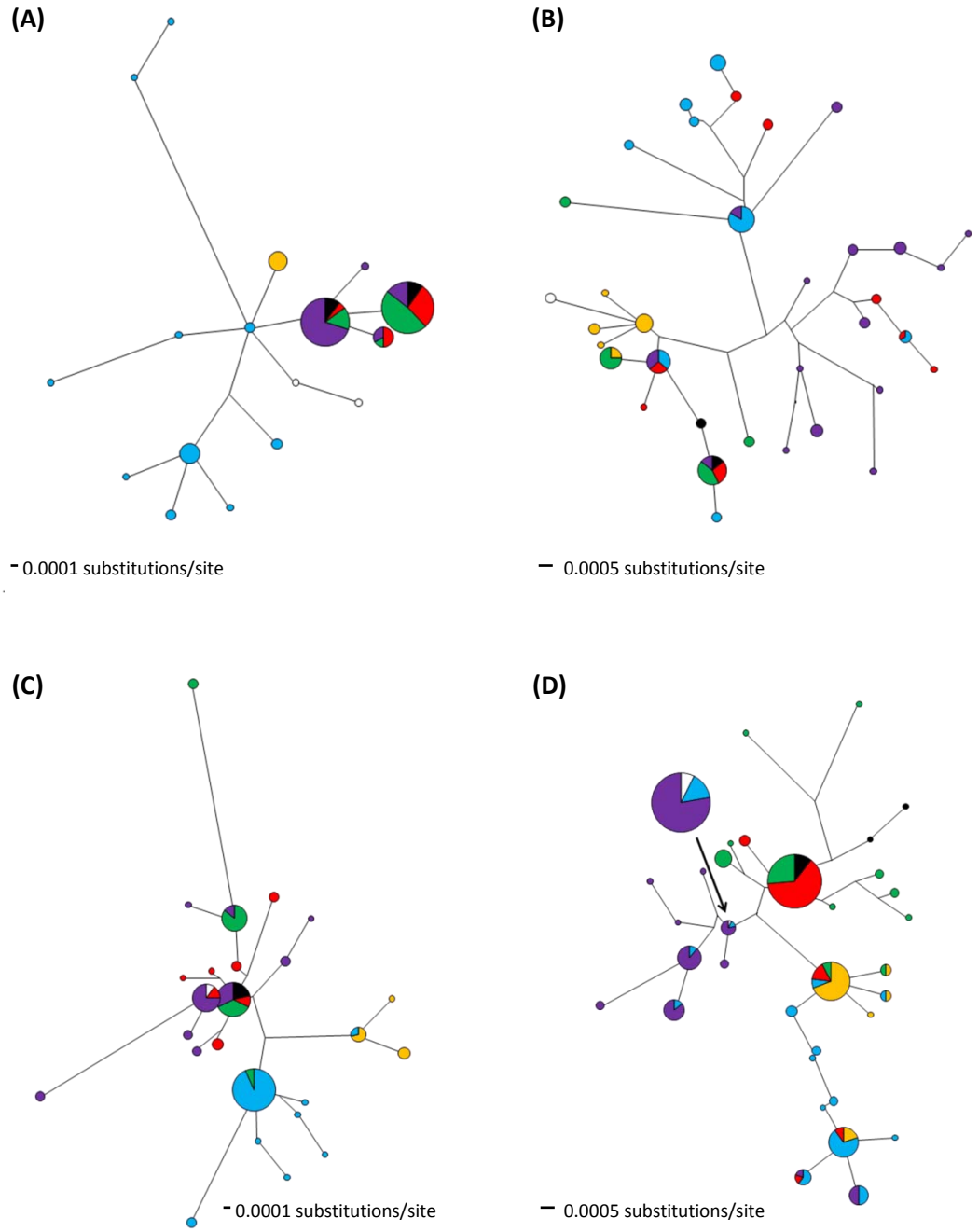


Figure 5.3: Nuclear haplotype networks (unrooted phylograms using a NJ distance approach with Tamura-Nei model); (A) *BRCA1*; (B) A1; (C) A2; (D) *ω-globin*. Circle size is scaled to allele frequency and branches scaled to the number of mutational steps; *P. brachyotis* Northern Territory (NT) - blue, *P. brachyotis* Victoria River (VR) - black, *P. brachyotis* East Kimberley (EK1) – red, *P. brachyotis* (EK2) – green, *P. brachyotis* West Kimberley (WK) - purple, *P. burbidgei* - yellow and *P. concinna* - white.

AMOVA analyses of all loci (nuclear and mtDNA) found *P. brachyotis* (NT) to be significantly differentiated ($P<0.05$) from the other two *P. brachyotis* mtDNA groups (VR and KIM), *P. burbidgei* and *P. concinna* (Table 5.2). The most significant proportion of variation between groups for mtDNA was recorded when *P. brachyotis* was separated into three groups (NT, VR and KIM). For *ω-globin* and A1, variation between groups was at a maximum when Kimberley *P. brachyotis* was split into a West Kimberley (WK) and East Kimberley (EK) group (two EK groups for *ω-globin*; EK1 and EK2; see results in Table 5.2).

Estimates of population migration rates (estimated by $\theta_1 \times m_1/2$; Chapter 3) between the five mtDNA clades (*P. brachyotis* NT, VR, KIM, *P. burbidgei*, *P. concinna*) and between the three geographical lineages within Kimberley *P. brachyotis* (EK1, EK2, WK) are presented in Table 5.3. Population migration rates between all populations were all estimated to be below one (<1; a critical value below which population divergence is expected to occur; Wright 1931). The upper 90% highest probability density (HPD) was only slightly higher than one (1.31 and 1.39) for comparisons between *P. brachyotis* (NT) and *P. burbidgei* (including *P. brachyotis* VR, KIM and *P. concinna*) and between *P. burbidgei* and *P. brachyotis* (VR and KIM) including *P. concinna.* Since these estimates peaked close to zero and based on these low upper 90% HPD values we can have confidence that these estimates indicate a lack of gene flow in either direction between these taxa in these comparisons. The comparison between *P. brachyotis* (EK2) and *P. brachyotis* (WK) also had fairly low estimates of upper 90% HPD (1.25, 1.91, respectively) suggesting limited gene flow between these regions, however the upper 90% HPD estimates between *P. brachyotis* (EK2) and *P. brachyotis* (EK2/WK) lacks confidence surrounding the peak posterior estimate, with values up to 7.29 migrants per population. This upper 90% HPD estimate for migration between these two populations (6.21 and 7.29) would allow genetic connectivity between populations to be maintained. As outlined in Chapter 3, *P. brachyotis* (VR) could not be compared to *P. brachyotis* (KIM) due to insufficient data for this population.

Table 5.2: Results of analyses of molecular variance for mtDNA, *BRCA1*, *ω-globin*, A1 and A2 for the following groups: *P. brachyotis* (Northern Territory), *P. burbidgei*, *P. brachyotis* (Victoria River), *P. concinna* and three *P. brachyotis* (Kimberley) groups; EK1, EK2 and WK. Number of groups for each marker is defined by (d.f. + 1) and represents the number of groups within *P. brachyotis.*

	Among groups				Among sites – within groups				Among individuals – within sites			
Markers	**d.f.**	**Variance**	Φ_{ST}	**% Var**	**d.f.**	**Variance**	Φ_{ST}	**% Var**	**d.f.**	**Variance**	Φ_{ST}	**% Var**
mtDNA	4	40.960	0.469*	46.9	4	31.630	0.831*	36.2	103	14.750	0.682*	16.9
BRCA1	3	0.840	0.510*	51.0	5	0.425	0.768*	25.8	71	0.382	0.526*	23.2
ω-globin	6	0.877	0.368*	36.8	2	0.201	0.452*	8.4	131	1.310	0.133	54.8
A1	5	0.968	0.265*	26.5	2	0.201	0.320*	5.5	106	2.490	0.075	68.0
A2	3	0.991	0.524*	52.4	5	0.230	0.646*	12.2	123	0.670	0.255*	35.4

ABTC101599 was removed from the analysis due to lack of confidence in its taxonomic classification. *Significant difference (*P*-value < 0.05) between fixation indices (F_{ST} /F_{SC} /F_{CT}) and variance components.

Table 5.3: Population migration rates (m_1, m_2), which represent the migration rate at which genes come into the two populations (m_1 genes come into population 1 and m_2 are the migration rate at which genes come into population two) estimated from IMa results Table 3.5 (θ_1 x $m_1/2$) in Chapter 3. HPD90Hi represents the upper bound of the estimated 90% highest posterior density interval and HPD90Lo is the lower bound of this density interval given in brackets (HPD90Lo-HPD90Hi). Population one is the first named population$_1$ and population two is the second population$_2$ labelled.

Population Divergences	m_1	m_2
P. brachyotis NT$_1$/*P. burbidgei*$_2$	0.24 (0.03-0.94)	0.37 (0.09-1.39)
P. burbidgei$_1$/*P. brachyotis* VR$_2$	0.00 (0.00-0.53)	0.28 (0.00-1.31)
P. brachyotis EK1$_1$/*P. brachyotis* EK2+WK$_2$	0.00 (0.00-6.21)	0.00 (0.00-7.29)
P. brachyotis EK1$_1$/P. brachyotis WK$_2$	0.01 (0.00-1.25)	0.39 (0.02-1.91)

Morphological assessment

Although there was considerable variation in pelage characteristics amongst *P. brachyotis* populations (see Fig. 5.4), three major patterns of colouration and markings were evident (Table 5.4), with each being restricted to a specific geographic region. A fourth pelage type was also identified in a limited number of specimens from the southern Gulf of Carpentaria.

Figure 5.4: Specimens representing the variation in morphology within *P. brachyotis.* (a) Front to back: CM7975 Nourlangie Rock, NT; CM15306 (RW288) Lobby Creek, 'Bradshaw', NT; and CM15305 (RW273) Kalumburu, WA. (b) Left to right: CM7975 Nourlangie Rock, NT; CM15306 (RW288) Lobby Creek, 'Bradshaw', NT; and CM 15305 Kalumburu, WA. These individuals represent *P. brachyotis* from NT, VR and KIM respectively.

(a)

(b)

Table 5.4: Pelage characteristics of *P. brachyotis* populations from four geographic regions (after Sharman and Maynes 1983). Sample sizes (*n*) are given for each geographic region, along with a description of the distribution of the region.

Character	Kimberley Region	Victoria River region	Top End region	Gulf of Carpentaria region
Location	From the Kimberley east to Victoria River.	Between Victoria and Fitzmaurice Rivers.	From the Daly River east to Roper River, including Groote Eylandt.	North Island and Wollogorang.
Number of samples	*n*=55	*n*=4	*n*=43	*n*=3
Coat colour and markings	Light grey – grey/brown above, some more reddish.	Pale grey above, some yellowish.	Dark grey – grey/brown above. Some more yellowish brown.	Light brown – light grey-brown above.
	White to greyish-white below.	Slightly paler below.	Paler to white below	Paler below
	Less distinctly marked than Top End. Extent varies among populations. Napier Downs most distinctly marked and quite grey.	Markings almost absent.	Distinctly marked. Southern populations' paler but still more distinctly marked than Kimberley or Victoria River.	Less distinctly marked than Top End.
Dorsal stripe	Indistinct dark brown dorsal stripe, from forehead extending to the shoulders in some specimens.	Hint of darker dorsal marking, mainly between ears.	Prominent dark brown/black dorsal stripe from forehead to beyond shoulders.	Faint dorsal stripe more prominent on crown. Darker and extending to the shoulders in Wollogorang.
Auxillary patch	Light grey shoulder stripe behind dark auxillary patch. Extent and intensity of both quite variable.	Barely discernable small pale marking behind faint and indistinct dark auxillary patch.	Distinct white (occasionally yellowish) shoulder stripe behind dark well developed auxillary patch.	Pale shoulder stripe behind dark auxillary patch.
Side stripe	No side stripe.	No side stripe.	Pale side stripe.	Faint side stripe.
Limb colour	Faint hip stripe rarely.	No hip stripe.	Pale hip stripe commonly.	Pale hip stripe.
	Limbs same colour as body or lighter.	Limbs same colour as body or more yellowish.	Limbs distinctly and often brightly coloured; from yellow through orange to almost reddish brown.	Limbs same colour as body or lighter.
Face colour	Face similarly coloured to neck /shoulders or darker.	Face similarly coloured to neck /shoulders or darker.	Face light brown to orangey-brown.	Face darker than body and more reddish.
Tail colour	Tail generally same colour as body on dorsal surface, sides lighter.	Tail generally same colour as body or lighter.	Tail generally lighter than body on dorsal surface, sides more yellowish.	Tail darker than body.
	Terminal 1/3 of tail darker; brown to black.	Terminal 1/3 – 1/5 of tail darker; brown.	Terminal 1/3 -1/5 tail darker; brown to almost black.	Terminal 1/3 -1/5 tail darker; reddish brown to almost black.
	Tail darker on dorsal surface especially towards tip.		Tail darker on dorsal surface especially towards tip.	In Wollogorang, tail darker on dorsal surface especially towards tip.
			Roper River, overall more yellowish including yellow shoulder stripe.	
			Groote Eylandt, overall more dark silvery-grey, including grey shoulder stripe. Auxillary patch black.	

Discussion

Six major mtDNA lineages were identified within the *brachyotis* group; *P. brachyotis* formed three geographically separate lineages (NT, VR, KIM), *P. burbidgei* two lineages (ABTC101599 forming an independent lineage) and single *P. concinna* lineage. *P. brachyotis* was found to be paraphyletic, with *P. burbidgei and P. concinna* embedded within it, suggesting that *P. brachyotis* represents multiple taxa. Alternative explanations, including incomplete lineage sorting or recent introgression are considered unlikely given the large divergences and long divergence times involved (Chapter 3). The possibility of introgression occurring in the distant past (e.g., in the ancestor of *P. concinna* and *P. brachyotis* from the Kimberley) cannot be ruled out, however, the multiple divergent lineages within the *brachyotis* group occurring in sympatry reflects long-term isolation. The mitochondrial sequence divergence found amongst the three identified *P. brachyotis* lineages (10.6-15%) is similar or greater than the divergences found between recognised species of *Petrogale* (e.g., *P. penicillata* and *P. herberti* 8.8% SD; average of 12.2% SD between *P. penicillata, P. assimilis, P. lateralis* and *P. purpureicollis*; Browning *et al.* 2001; Eldridge *et al.* 2001c; and 9.1-14% SD between *P. burbidgei* and *P. brachyotis*/*P. concinna*). Therefore on mtDNA criteria alone, there is some justification for recognising each of the three major *P. brachyotis* lineages as a distinct species. Such a decision would also be consistent with the morphological data since there is largely concordance between the geographical distribution of the genetic lineages and the distribution of the three major patterns of pelage characteristics. However morphological differences can be maintained by selection even in the presence of gene flow (Moritz *et al.* 2000; Dolman and Moritz 2006). In addition, we consider it unwise to elevate populations to full specific status based largely on divergence at a single genetic locus, since the most convincing evidence of species status under the BSC comes from evidence of lack of gene flow (i.e., reproductive isolation) across both nuclear and mitochondrial loci (Moore 1995; Fu and Li 1999; Avise 2000; Edwards and Beerli 2000; Hare 2001; Knowles 2004; Beheregaray 2008; Avise 2009). Significant divergence at a single locus can result as a consequence of stochastic coalescent processes (e.g., lineage sorting, ancestral polymorphisms, introgression) (Barraclough and Nee 2001; Kuo and Avise 2005; Riddle *et al.* 2008) or sex-biased dispersal in the case of a mtDNA phylogeny (Niegel and Avise 1993; Avise 2000; Irwin 2002; Moussalli *et al.* 2009), therefore concordance across

multiple independent neutral loci needed to provide compelling evidence of a break in gene flow.

In this current study, an AMOVA analyses of nDNA sequence data from four loci detected significant genetic differentiation within *P. brachyotis,* and the existence of two genetically isolated populations, one comprising *P. brachyotis* from the Northern Territory (NT) and the other *P. brachyotis* from the Victoria River and Kimberley (VR and KIM). In addition, numerous private alleles were present in these two groups further supporting genetic differentiation between them. The geographic location of this major divergence within *P. brachyotis* nDNA corresponds to a well known biogeographic barrier (Daly River barrier see Chapter 3) and there is evidence for divergent mtDNA lineages on either side of the barrier. The presence of spatially concordant biogeographic breaks across independent neutral loci normally emerges only in the presence of longstanding historical barriers to gene flow (Kuo and Avise 2005). Isolation with migration results also supported a lack of gene flow between *P. brachyotis* (NT) and *P. burbidgei*, *P. brachyotis* (VR and KIM) and *P. concinna*, but data were insufficient to provide reliable estimates of migration levels between *P. brachyotis* VR and KIM. There were also numerous shared nuclear haplotypes between individuals from VR and KIM giving us additional grounds for not separating *P. brachyotis* (VR) and *P. brachyotis* (KIM) as distinct species, as might be suggested from the mitochondrial data alone.

The phylogenetic position of the much smaller *P. burbidgei* within *P. brachyotis* (*sensu lato*) further reinforces the discontinuous relationship between *P. brachyotis* populations from the NT and VR/KIM first noted in Chapter 2. This lack of mtDNA monophyly, together with significant differentiation in all nuclear AMOVA comparisons, no evidence of current gene flow and morphological divergence between *P. brachyotis* from these regions highlights a major historical and contemporary lack of genetic exchange. From these data we propose that *P. brachyotis* (*sensu lato*) represents two highly divergent species; one distributed from the Kimberley and north-west Northern Territory and another from the northern and eastern Northern Territory. Since the type locality of *P. brachyotis* is from the western Kimberley (Gould 1841), the western species becomes *P. brachyotis* (*sensu stricto*), while the eastern species should be known as *P. wilkinsi* based on the earliest description of a specimen from this region (Roper River, southwest Arnhem Land; Thomas 1926a).

Further evidence of genetic differentiation within *P. brachyotis* was detected from the phylogenetic analysis of nuclear sequence data, but this was not consistent across loci. AMOVA results for *ω-globin* and A1 found the most significant genetic differentiation between populations when *P. brachyotis* was separated into a Victoria River, as well as East and West Kimberley populations (two East Kimberley populations for *ω-globin*), whereas this level of differentiation was not found for *BRCA1* or A2. This variation in genetic differentiation is not surprising given the slower rates of evolution in nDNA, therefore a lack of divergence may represent a lack of resolution rather than evidence of gene flow between these regions. Additional nuclear markers should help resolve this differentiation. Compared to other rock-wallaby species (e.g., *P. penicillata*, Eldridge *et al.* 2001a; Browning *et al.* 2001), substantial genetic divergence is present within both *P. brachyotis* (*sensu stricto*) and *P. wilkinsi*, mostly associated with known biogeographic barriers (Chapter 3). Despite *P. brachyotis* (VR) revealing high levels of mitochondrial sequence divergence (up to 12.5% SD) from *P. brachyotis* (KIM) and forming an independent monophyletic clade, there is currently not sufficient evidence from nuclear markers to support further separation of these lineages into distinct species. Unrooted nuclear phylograms displayed shared alleles between *P. brachyotis* (VR and KIM) for all markers and AMOVA results only supported significant variation between these geographic regions for two nuclear markers (*ω-globin* and A1). These results suggest *P. brachyotis* (VR) is historically isolated from *P. brachyotis* (KIM), with reciprocally monophyletic mtDNA alleles and significant divergence in nuclear loci. Based on the Moritz (1994) criteria for defining ESUs, *P. brachyotis* VR and KIM clearly form separate ESUs and should be managed as separate evolutionary lineages in the future for the purposes of maximising genetic diversity within the species. Since the VR specimens were all from a single locality, greater sampling around the Victoria River region and an examination of additional nuclear loci including microsatellites is recommended to better clarify the nature and extent of gene flow across this region.

Within the Kimberley three reciprocally monophyletic clades were recovered from mtDNA analysis (EK1, EK2, WK), highlighting a lack of female gene flow across the Kimberley. These three Kimberley lineages showed similar levels of mtDNA sequence divergence (4.9-7.8%) as reported between subspecies and ESUs in other rock-wallabies (e.g., 5.3% *P. xanthopus*, Pope *et al.* 1996; 5% *P. lateralis*, Eldridge *et al.* 2001c; 7.6% *P. penicillata*,

Browning *et al.* 2001). The nDNA sequence analyses provided limited evidence of isolation across the Kimberley, with one marker (*ω-globin*) revealing significant variation between these three geographic regions. Results for other loci were inconsistent, with AMOVA analysis of A1 revealing geographic structure where the two East Kimberley lineages (EK1 and EK2) were grouped together, separate from the west Kimberley lineage, whereas mtDNA reconstructions indicated the EK1 lineage formed a sister clade to the WK lineage rather than the geographically adjacent EK2 lineage. However, population migration rates between the East and West Kimberley suggested reduced gene flow between these regions, as did the Bayesian clustering of microsatellite genotypes (Chapter 4). Overall, in light of the reciprocal monophyly in mtDNA and significant differentiation at nuclear loci three ESUs are provisionally recognised within the Kimberley, one in West Kimberley (WK) and two in the east (EK1, EK2). Since the sampling in the East Kimberley is limited, additional sampling and microsatellite analyses are recommended to further test for levels of connectivity across this region.

Within *P. wilkinsi* there was also high levels of mtDNA sequence divergence, similar or greater to that found between taxa (up to 12% SD), but little evidence of geographic structuring. The greatest divergences were between individuals S92 from Groote Eylandt and S1178 from Wollogorang which differed by 7.3-12% compared to all other sampled *P. wilkinsi*. S1178 also had unique alleles for nDNA loci and unique pelage (Table 5.4). Since both these localities, from the far east of *P. wilkinsi*"s distribution, are only represented by single individuals it is difficult to interpret their significance. Further sampling from these localities and between Roper River and the Queensland/NT border is required to elucidate whether populations from these regions represent additional ESUs or distinct taxa. The population on Groote Eylandt was previously described as a separate species (*P. longmani*; Thomas 1926a) and its pelage is much more of a dark silvery-grey (with a grey shoulder stripe) although still strongly marked as is typical of *P. wilkinsi* (Table 5.4).

The mtDNA analysis also showed that *P. burbidgei* was paraphyletic, with one individual (ABTC101599 from Prince Regent River, Western Australia) forming a separate lineage from all the other sampled *P. burbidgei*, which were monophyletic and closely related (up to 4.3% SD). ABTC101599 showed significant sequence divergence (9.6-10.6%) from the other *P.*

burbidgei and formed a sister lineage to *P. concinna* and *P. brachyotis* (KIM). There are several possible explanations for this result. Firstly, populations of *P. burbidgei* may be more highly structured for mtDNA than previously thought. Secondly, this individual may have been misidentified in the field and actually represents a specimen of *P. c. monastria*, which also occurs in the Prince Regent River region of the Kimberley and is morphologically very similar to *P. burbidgei* (Kitchener and Sanson 1978). This would explain its significant divergence from *P. burbidgei* and the sister relationship of this lineage to *P. c. canescens* (Arnhem Land subspecies). However, if correct, the high levels of divergence found between these two *P. concinna* subspecies suggest the possibility that each is likely to represent a distinct species. Thirdly, it is also possible that ABTC101599 represents a currently unknown new *Petrogale* taxon. Further sampling of *P. burbidgei* throughout its range and also *P. concinna* especially from the Kimberley is now required to resolve this anomaly and clarify the relationships and taxonomic classification of the three described *P. concinna* subspecies.

Telfer and Eldridge (2010) suggested Litchfield National Park was a secondary contact zone between divergent populations of *P. brachyotis* (now *P. wilkinsi*) based on the exceptionally high levels of divergence between mtDNA haplotypes found within this population. This study indicates that these high levels of divergence are common across the distribution of *P. brachyotis* and *P. wilkinsi* and are unlikely to be the result of secondary contact. Instead, the strong geographical structuring of mitochondrial lineages reflects a pattern of long term persistence of populations and limited female gene flow across northern Australia, indicating isolation by distance patterns of divergence, correlating to patterns seen in other *Petrogale* taxa (e.g., *P. penicillata*, *P. lateralis*, *P. brachyotis* NT) (Eldridge *et al.* 2001b; Hazlitt *et al.* 2006a; Piggott *et al.* 2006b; Hazlitt *et al.* 2010; Telfer and Eldridge 2010). Rather than regularly recolonising habitat patches across the landscape, it appears they have remained confined to particular habitat patches and regions across northern Australia (as outlined in Chapter 3), leading to a high level of divergence between populations. This study demonstrates that widespread species such as *P. brachyotis* can be genetically highly structured and the preservation of this diversity requires management and protection at a finer scale.

Conclusion

This study has brought greater clarity to the distribution of diversity within the *brachyotis* group and the scale over which it should be managed. MtDNA, nDNA and morphological analyses indicate that the current taxonomy does not accurately reflect the true nature of inter-relationships and divergences within the *brachyotis* group. *P. brachyotis* (*sensu lato*) was identified as being highly divergent and paraphyletic for mtDNA with *P. burbidgei* and *P. concinna* embedded within it. Based on these data we suggest that *P. brachyotis* (*sensu lato*) be split into two species; populations from the NT becoming *P. wilkinsi*, whilst populations from the Victoria River region westwards through the Kimberley remaining as *P. brachyotis* (*sensu stricto*). Both species are characterised by high levels of intra-specific divergence with this study recommending that at least four ESUs be recognised within *P. brachyotis*, and that additional sampling and analysis assess the potential for the far eastern populations of *P. wilkinsi* to represent additional ESUs or species.

Future research also needs to focus on *P. concinna*, which appears to be rare or absent over most of its previously recorded range (which likely reflects the lack of knowledge surrounding this elusive species) and may be declining, similarly to other co-distributed small mammals. Urgency is required in regards to clarifying the conservation status of *P. concinna* as well as taxonomic classification of subspecies, which have not been assessed by current molecular techniques. Such a study will need to include a combined effort from biological surveys, additional genetic analyses, plus basic ecology to gather as much information about these taxa as possible, so that Australia doesn‟t lose another mammal to extinction.

CHAPTER SIX: General Discussion

Synthesis

Rock-wallabies are an intriguing genus, due to their recent and rapid diversification, exhibiting exceptional chromosomal diversity yet often similar morphology and mitochondrial genomes. My research has built on previous studies to elucidate the biogeographic processes impacting on *Petrogale* and has highlighted the numerous processes occurring throughout *Petrogale* evolution that have lead to diversification and speciation. Four major lineages were identified within *Petrogale*; the *brachyotis* group, *P. persephone*, *P. xanthopus* and *lateralis-penicillata* group (including *P. rothschildi*), which phylogenetic relationships are only partially concordant with those based on chromosome morphology (Briscoe *et al.* 1982; Sharman *et al.* 1990; Eldridge and Close 1997). My study has also clarified some long-standing uncertainties, confirming the placement of *P. concinna* within the genus *Petrogale* rather than in *Peradorcas* and supporting the reinstatement of *P. purpureicollis* as a distinct species. Estimated divergences of three major lineages (excluding the *lateralis-penicillata* group) place divergences within the late Miocene-Pliocene (3.6-8.7 MYA). The distribution of the majority of these taxa within the monsoon tropics (*brachyotis* group and *P. persephone*), suggests that the ancestor of these taxa most likely inhabited mesic environments prior to their adaptation to more arid conditions. Divergence estimates placed further speciation events throughout the Pliocene and Pleistocene, when it appears some *Petrogale* lineages (e.g., *P. rothschildi* and *P. lateralis*) began to inhabit and adapt to more arid environments in central and western Australia.

My study has been the first in-depth examination of the *brachyotis* group of rock-wallabies, contributing to our knowledge of population and phylogeographic structure, taxonomy and management of these taxa. *P. brachyotis* was shown to be paraphyletic, with extensive genetic differentiation amongst lineages indicative of multiple separate species and conservation units. *P. burbidgei* formed a monophyletic clade with the exception of one individual (ABTC101599; whose taxonomic classification has been questioned) and *P. concinna* from the Northern Territory formed the sister lineage to *P. brachyotis* from the Kimberley. Based on the limited data in my study it appears that the speciation of *P. burbidgei* and *P. concinna*

was not a related process (i.e., they are not sister taxa), but the result of independent evolutionary processes.

A particular focus of the current study was to increase our understanding of the biogeographic history of northern Australia, from Arnhem Land in the Northern Territory (NT), across to the Kimberley in Western Australia (WA). Multiple significant barriers to gene flow emerged, some of which had previously been suggested (e.g., Ord Arid Intrusion also referred to as Victoria River Drainage barrier or the Bonaparte Gap). However, evidence from my study suggest the region around the Daly and Victoria Rivers and the Joseph Bonaparte Gulf, which were previously proposed as a single major biogeographic barrier dividing the monsoonal biota of north-western Australia, may actually represent multiple independent biogeographic barriers that have each caused significant differentiation, particularly within *P. brachyotis*. In addition, a new barrier was detected between the East and West Kimberley, best explained by the presence of a basalt range extending north to south in the central Kimberley (Griffin *et al.* 1993). I propose that this range previously provided habitat connectivity between these regions, but erosion of the sandstone covering this area during the Pleistocene, exposed the basalt, creating a barrier for *P. brachyotis*. These data suggest that if co-distributed species have been similarly impacted by these biogeographical processes then considerable, currently undetected, and unmanaged diversity is likely to exist across north-western Australia.

A significant outcome from my research is the suggested reclassification of *P. brachyotis* (*sensu lato*) into two separate species; *P. brachyotis* (*sensu stricto*) from the Kimberley and western NT, and *P. wilkinsi* from the northern and eastern NT. This arrangement better reflects the deep genetic divergence found between *P. brachyotis* (*sensu lato*) from the NT and Kimberley and is concordant with morphological characteristics (Sharman *et al.* 1995). In addition, the multiple ESUs defined within *P. brachyotis* (*sensu stricto*) reflect major historic and contemporary barriers to gene flow and indicates that *P. brachyotis* can no longer be considered a single entity in future conservation and management.

Future Research Directions for northern Australia

The examination of multiple co-distributed species across northern Australia is now required to elucidate whether the patterns and timing of divergences reported here are common amongst organisms from the region. While current evidence indicates that the region between the Daly and Ord Rivers represents an important vicariant barrier (or more likely series of barriers) in northern Australia, phylogeographic data remain limited and incongruent in estimated divergence times. Further comparisons are now required to determine whether there has been simultaneous divergences within unrelated organisms or whether the region is characterised by repeated patterns of range expansion and contraction during climatic glacial cycles (as in the Queensland Wet Tropics). In the Wet Tropics, repeated range contractions and expansions have resulted in variable divergences between taxa as a consequence of different species‟ ecologies (Schneider *et al.* 1998; Schneider and Moritz 1999; Moritz *et al.* 2000; Ford and Blair 2005). Despite distinct bioregions emerging across the monsoon tropics (e.g., Top End and Kimberley) that reflect the environmental history of the region (Bowman *et al.* 2010), the differing sensitivities of species‟ biology to these changes and therefore their impact remains unclear.

The high levels of genetic differentiation and multiple novel biogeographic barriers identified in this study highlights the need for ongoing research to more accurately identify areas of high diversity (often associated with historical refugia) as priorities for conservation efforts (Mesibov 1994; Faith *et al.* 2003; Garrick *et al.* 2006). The Kimberley and Top End regions of northern Australia are known to have high levels of biodiversity endemism (Woinarski 1992). However, this diversity may be significantly underestimated and further exploration is needed to define refugia and associated habitat in order to ensure protection of historical genetic diversity, as well as adaptive potential across a broad spectrum of taxa. As genetic diversity (both historical and adaptive) is important to conserve (both within and across regions), and the strategies to maintain each are different (Moritz *et al.* 2000), evaluation of co-distributed species will be required to generate predictions for taxa that have yet to be sampled (Avise 1998).

Surrogate taxa have been highlighted as significant for assessing overall patterns of biodiversity and aiding establishment of conservation priorities (Prendergast *et al.* 1999; Margules and Pressey 2000; Moritz *et al.* 2001). Invertebrates have been suggested as better biodiversity surrogates than vertebrate taxa, because of their finer-scale distribution and limited mobility which improves their ability to capture spatial patterns of biodiversity (Ferrier *et al.* 1999; Moritz *et al.* 2001; Yeates *et al.* 2002; Garrick *et al.* 2006; Sunnucks *et al.* 2006). Based on this assumption, evaluation of invertebrates across the Kimberley and Northern Territory should be a priority and may provide further evidence of biogeographic barriers and their impact on patterns of biodiversity. The evaluation of invertebrates across northern Australia would also provide additional information to aid the creation of additional protected areas to preserve and maintain biodiversity, as well as informing the management of existing protected areas. However, such studies would require a vastly improved understanding of species taxonomy and current distributions, together with greater survey effort especially across the Kimberley and in the Joseph Bonaparte Gulf where multiple barriers may be present. Better communication between survey teams will be required to ensure adequate sampling of taxa from across the region and the collection and lodgement of genetic samples with museums to ensure the assessment of phylogeographic patterns in both an inter-specific analysis and a comparative framework.

Given the poor state of basic knowledge of the northern Australian invertebrate fauna and a lack of taxonomic expertise it is unlikely that invertebrates alone will be able to rapidly provide the data necessary for conservation planning. Therefore it is likely that a broad collection of species will be required (Zink 1996) in a comparative study to understand the ecological, genetic and taxonomic diversity of north-western Australia. An ideal mammalian group on which to focus would be the rock-rats (*Zyzomys* spp.) which have a similar distribution to the *brachyotis* group of rock-wallabies. In addition to three endemic species isolated to the rocky refugia of the Kimberley, Arnhem Land and Gulf of Carpentaria escarpment (*Z. woodwardi*, *Z. maini*, *Z. palatalis*) is a sympatric common widespread species (*Z. argurus*). An evaluation of how these two very different distributions originated is likely to shed light on the timing and nature of historical biogeographic processes and their interaction with life-history strategies. Currently there is research in progress on a variety of other vertebrate taxa from north-western Australia including, *Planigale* spp., (Aplin and

Blackett pers. comm.), antilopine wallaroos (Eldridge, Ritchie and Johnson, pers. comm.) reptiles and amphibians; *Uperoleia* spp. (Cautulo and Keogh, pers. comm.) and *Diporiphora* and *Lophognathus* (Melville, pers. comm.). These studies of a suite of co-distributed taxa promise to reveal much about the phylogeographic patterns of Northern Australia and will greatly increase our understanding of the past climatic and biogeographic processes that have shaped the biota of this biodiverse region.

Future research within the *brachyotis* group of rock-wallabies

My population genetic study found that most *P. brachyotis* populations in the Kimberley despite appearing isolated in discreet patches of suitable habitat, did not reveal any of the problems frequently associated with small populations (e.g., inbreeding and low diversity), as had previously been reported for *P. brachyotis* populations in the Northern Territory (Telfer and Eldridge 2010). Exceptions were the Lake Arygle island populations where I found greater relatedness and lower allelic diversity, allelic richness and expected heterozygosity compared to populations from the West Kimberley, confirming the importance of habitat connectivity to maintain gene flow. For the non-island populations, there was strong evidence to indicate the maintenance of genetic variation and connectivity throughout the West Kimberley, with some direct evidence of dispersal between populations. Under the assumption that genetic variation based on neutral markers (e.g., microsatellites, anonymous DNA sequences) equates to variation in genes involved in adaptive traits, it is predicted that *P. brachyotis* populations in the West Kimberley are genetically healthy. They may still remain vulnerable to ecological threats and human-induced impacts (e.g., land clearing, fire, mining etc.). Sampling for my microsatellite study was limited, particularly in the East Kimberley. Therefore supplementary research would benefit from more widespread sampling to assess the distribution and abundance of genetic diversity, patterns of gene flow and potential biogeographic barriers (e.g., Ord River) throughout the range of *P. brachyotis*.

Additional sampling and analysis of populations, from both *P. brachyotis* and *P. wilkinsi*, not included in this study are also required to assess the distribution and validity of the ESUs and potential ESUs identified in this study. This sampling should particularly focus on the East Kimberley, the region between the Victoria and Daly Rivers, as well as the Gulf of

Carpentaria escarpment. It is likely that further sampling and additional analyses will result in further refinement to the number of species and/or ESUs being recognised. For example the highly divergent Victoria River ESU and Wollogorang specimen (S1178) may prove to actually represent distinct species, while the two identified East Kimberley ESUs, could potentially be merged if additional sampling indicates greater connectivity across this region. In addition, I recommend that a comprehensive study of skull morphometrics across the range of *P. brachyotis* and *P. wilkinsi* be carried out to look for concordant patterns with the genetic data and facilitate the identification of specimens collected in the field or currently held in museum collections.

The limited samples of *P. burbidgei* and, in particular, *P. concinna* available in this study means that future research needs to focus on these taxa. Unfortunately our study has added little clarity to the conservation concerns for *P. concinna* and has elucidated little about its origins, population structure or the taxonomic uncertainty surrounding its three described subspecies. Despite increasing concern about its conservation status and the urgent need for additional information, research on *P. concinna* has proved particularly intractable because they are difficult to observe, identify and trap in the field. The successful utilisation of DNA from *P. brachyotis* scats demonstrated in this study however provides a potential way forward. In conjunction with genetic sampling of museum specimens (e.g., ancient DNA), it should now be possible to assess the phylogeographic structure of *P. concinna* and test the validity of the currently recognised subspecies, their current distribution and conservation status. With additional genetic data and samples, collected across a greater area of their distribution, a more meaningful understanding of the phylogeographic relationships within *P. concinna* will be forthcoming which will also help determine the evolutionary and biogeographic history of the rest of the *brachyotis* taxa.

In particular, the intriguing origins of these two dwarf rock-wallabies (*P. burbidgei* and *P. concinna*) will require additional sampling and analysis to unravel. There appears to be little genetic differentiation between *P. burbidgei* individuals from Bigge Island compared to those from the mainland suggesting a more recent vicariance, and *P. concinna* from the Northern Territory forms a more recently derived lineage from a common ancestor with *P. brachyotis* (Kimberley) compared to *P. burbidgei* suggesting *P. burbidgei* and *P. concinna* did not share

a most recent common ancestor, so the dwarf characteristics of these rock-wallabies has evolved multiple independent times. More extensive sampling of other island populations of both species is required to test any hypotheses (e.g., the evolution of dwarfism, their evolutionary origins, biogeographic history and conservation status).

Research priorities for *Petrogale*

The *Petrogale* phylogeny constructed in this study was not fully resolved and in the past this has been explained by introgression and hybrid speciation (Eldridge and Close 1993; Campeau-Péloquin *et al.* 2001; Kirsch *et al.* 2010). Additional sequence data will be required to obtain better resolution of several nodes, for example, those connecting the first three lineages - the *brachyotis* group, *P. persephone* and *P. xanthopus*; and the relationship of *P. purpureicollis* within the *lateralis-penicillata* group. Additional fossil data will also allow better and more robust estimates of divergence dates between lineages of *Petrogale*.

Since this phylogenetic study did not include all currently extant taxa, *P. lateralis hacketti* (Hackett"s rock-wallaby) will need to be included along with the remaining northeast Queensland species (*P. coenensis*, *P. inornata*, *P. mareeba* and *P. sharmani*) in any further study. In this current study *P. assimilis* was included as a representative of this later group of closely related northeast Queensland species, since these five taxa share a single mtDNA restriction fragment length polymorphism (RFLP) haplotype. Relationships within this group are likely to be complex but interesting. The karyotype of each species are definitive and different (Sharman *et al.* 1990; Eldridge *et al.* 1991b), but all other markers trialled thus far (e.g., mtDNA, nDNA RFLPs) show that polymorphisms are shared across taxa (Bee and Close 1993; Kirsch *et al.* 2010). The similar genomic profiles of these five species may have resulted from introgression, supporting previous suggestions (Bee and Close 1993; Eldridge and Close 1993; Campeau-Péloquin *et al.* 2001), or could be the result of a recent origin and retention of ancestral polymorphisms. Therefore there is the need for a large study, sampling throughout the range of each of the eight parapatric species of the *penicillata* complex. A detailed fine-scale analysis using microsatellites, as well as nuclear and mtDNA sequences is likely to be necessary to assess relationships and evidence of introgression. This research

would provide a useful model to shed additional light on the evolutionary processes which currently limit our understanding of the complex biogeographic history of eastern *Petrogale*.

Introgressive hybridisation has been detected amongst chromosomal races of the Australian morabine grasshopper (genus *Vandiemenella*, *viatica* species group), with population structure based on nuclear data similar to that based on chromosome variation, but mtDNA forming different phylogeographic groupings (Kawakami *et al.* 2007). As a result, a decrease in nuclear gene flow was suggested to be associated with chromosomal variation, whereas mitochondrial gene flow was suggested to be autonomous from such variation (Kawakami *et al.* 2007, 2009). The differences of nuclear and mtDNA may also be influenced by the small effective population size of mtDNA, which may allow fixation of foreign mtDNA (introgressed from parapatric taxa) from founder effects or drift following hybridisation with little or no gene flow at nuclear loci (Avise *et al.* 1984). Within morabine grasshoppers (Kawakami *et al.* 2007), the evidence suggests range contractions and expansions which supports this founder hypothesis, and is also consistent with studies of the *Mus musculus*/*domesticus* species complex (Boursot *et al.* 1993) and another grasshopper species complex (*Caledia captiva*) (Arnold *et al.* 1999). Within *Petrogale*, allopatric speciation has previously been proposed (within the *penicillata* complex), whereby chromosomal rearrangements replaced themselves along the coast creating reproductively isolated populations (Sharman *et al.* 1990). My phylogenetic study also suggests range contractions and expansions within *Petrogale.*. In addition, Barker and Close (1990) suggested *P. penicillata* and *P. herberti* have undergone a succession of contacts and divisions, based on electrophoresis of lice, supporting this theory. As outlined for the morabine grasshoppers (Kawakami *et al.* 2007), assessment of nuclear sequence data from individuals at contact zones will allow the most beneficial investigation of the mechanisms of reproductive isolation.

My results have allowed a more thorough interpretation of the biogeographic history of *Petrogale*, however, they also highlight the difficulties in determining ancestral states when using a small dataset. It would therefore be useful to assess the biogeographic history of *Petrogale* in comparison with closely related taxa (e.g., *Dendrolagus* and *Thylogale* taxa). This would require thorough sampling of species across both genera, and the inclusion of

outgroups to reconstruct ancestral origins of taxa. Meredith *et al.* (2008) has established phylogenetic relationships of macropod genera using single exemplar species for each genus. A comprehensive analysis of the biogeographic history of *Petrogale* (and closely related taxa) will require more extensive sampling. However, as samples of many Papua New Guinean *Dendrolagus* and *Thylogale* species are limited and difficult to obtain, non-invasive techniques may be a useful approach. With the development of techniques (e.g., 454 multiplex sequencing) there is potential that such samples will be valuable, however current procedures will need to be refined before results can be accepted without concern (especially for nDNA sequences due to the lower copy of nDNA compared to mtDNA), although these techniques are becoming more cost effective and viable (Davison *et al.* 2002; Piggott 2004).

Conclusion

This study links evolutionary processes from the micro- to the macro- level and reveals how these can be used to understand the biogeographic history of organisms and inform their future conservation. Biogeographic processes occurring at the intra-specific level often aid our understanding of processes at the inter-specific level, with the *brachyotis* group experiencing a similar pattern of diversification to other rock-wallabies across Australia. This pattern of diversification is concordant with that found for many other taxa in both the arid zone of Australia and in the Wet Tropics of north-east Queensland, highlighting the broad impact that climatic cycles and aridification during the Pliocene and Pleistocene has had on organisms throughout Australia.

Appendix 1

Sequence data and microsatellite genotypes:

Chapter 2 alignments: sequences are labelled by taxon name and identification number.

Chapter 3 alignments: sequences are labelled by identification number and can be cross-referenced with Table 1 from Chapter 3 for species names and locations.

Chapter 4 alignments: sequences are labelled by identification number and population reference (C – Couchman, CV – Cyprus Valley, MI – Monsmont Island and BI – Bullanyin Island). Microsatellite genotypes are labelled by identification number in order of population (1 – Couchman, 2 – Cyprus Valley, 3 - Monsmont Island, 4 – Bullanyin Island, 5 – King Edward River, 6 – Pump Hill) and microsatellite loci are identified.

Appendix 2

Divergence estimates of *Petrogale* based on fossil calibrations from Meredith *et al.* (2008) using the program BEAST. Analyses used a relaxed molecular clock using a uniform, lognormal and normal prior distribution. Divergence times were estimated based on a combined dataset including; *COI*, *Cytb*, *ND2*, *BRCA1* and *ω–globin*.

Clade	Lognormal	Normal	Uniform
Thylogale + *Dendrolagus* + *Petrogale*	6.45 (5.28 – 7.76)	12.81 (6.49 – 14.31)	11.06 (5.24 – 13.86)
Dendrolagus + *Petrogale*	5.95 (4.87 – 6.91)	11.32 (5.84 – 12.91)	8.57 (4.80 – 12.43)
brachyotis group + *P. persephone* + *P. xanthopus* + *lateralis-penicillata* group	4.39 (4.04 – 5.33)	8.70 (4.45 – 10.03)	6.87 (4.00 – 9.58)
P. persephone + *P. xanthopus* + *lateralis-penicillata* group	4.07 (3.63 – 5.05)	8.18 (4.17 – 9.40)	6.35 (3.52 – 8.98)
P. xanthopus + *lateralis-penicillata* group	3.56 (3.23 – 4.64)	7.55 (3.79 – 8.62)	5.80 (3.18 – 8.26)
P. burbidgei + *P. brachyotis* (NT) + *P brachyotis* (VR) + *P. concinna* + *P. brachyotis* (KIM)	2.65 (1.95 – 3.02)	5.05 (2.38 – 5.58)	3.78 (1.93 – 5.30)
P. rothschildi + *penicillata* group + *lateralis* group	2.51 (2.00 – 3.03)	4.53 (2.36 – 5.53)	4.18 (1.93 – 5.27)
P. burbidgei + *P. brachyotis* (NT)	2.47 (1.71 – 2.76)	4.79 (2.10 – 5.04)	3.57 (1.72 – 4.82)
P. brachyotis (VR) + *P. concinna* + *P. brachyotis* (KIM)	2.08 (1.69 – 2.71)	4.57 (2.09 – 4.96)	3.37 (1.66 – 4.69)
penicillata group + *lateralis* group	2.18 (1.75 – 2.70)	4.05 (2.13 – 4.93)	3.49 (1.73 – 4.69)
P. purpureicollis + *P. lateralis*	1.98 (1.54 – 2.44)	3.78 (1.88 – 4.45)	3.11 (1.54 – 4.25)
P. penicillata + *P. herberti* + *P. assimilis* + *P. inornata*	1.32 (0.91 – 1.52)	2.65 (1.12 – 2.75)	1.99 (0.90 – 2.59)
P. brachyotis (KIM) + *P. concinna*	1.12 (0.83 – 1.52)	2.54 (1.05 – 2.71)	1.92 (0.83 – 2.56)
P. lateralis MacDonnell Ranges race + *P. lateralis* West Kimberley race + *P. lateralis lateralis* + *P. lateralis pearsoni*	0.74 (0.53 – 1.01)	1.48 (0.67 – 1.79)	1.41 (0.53 – 1.68)
P. assimilis + *P. inornata*	0.78 (0.46 – 0.89)	1.37 (0.58 – 1.58)	1.22 (0.44 – 1.46)
P. penicillata + *P. herberti*	0.61 (0.38 – 0.77)	1.54 (0.48 – 1.36)	1.04 (0.38 – 1.27)
P. xanthopus celeris + *P. xanthopus xanthopus*	0.14 (0.13 – 0.34)	0.51 (0.17 – 0.59)	0.42 (0.13 – 0.54)
P. lateralis West Kimberley race + *P. lateralis lateralis* + *P. lateralis pearsoni*	0.31 (0.16 – 0.36)	0.55 (0.21 – 0.63)	0.45 (0.16 – 0.58)
P. lateralis lateralis + *P. lateralis pearsoni*	0.23 (0.07 -0.22)	0.34 (0.09 – 0.38)	0.29 (0.74 – 0.34)

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