Divergence of a mammal along a habitat gradient:

A study of the coppery brushtail possum, *Trichosurus vulpecula johnsonii*.

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STATEMENT ON THE CONTRIBUTION OF OTHERS

Financial Support

Financial support for this research was provided by James Cook University through IRA and Graduate Research School funding, and through a grant awarded by The Australia and Pacific Science Foundation. I was financially supported by an Australian Postgraduate Award stipend paid by the Australian Government.

Samples

Sam Price-Rees, with assistance from Katie Jones, captured 23 adult grey brushtail possums, two grey pouch-young, one coppery brushtail possum, and sampled three road kill possums for this study in 2005. The morphological measurements and genetic samples were collected for, and used in this study. Associate Professor John Winter contributed measurements and DNA samples from eight grey brushtail possums. One of these samples was not entered into this study as it was the pouch young of a female captured: juvenile measurements and the bias of including known offspring in a population genetics analysis meant that it could not be included in this study. Jane DeGabriel captured six coppery brushtail possums from the Atherton Tablelands for separate study, and contributed DNA and measurements from these animals to this study also.

Advice on methods

Associate Professor Steve Williams provided key recommendations on the field techniques and applications of distance sampling. He and Associate Professor John Winter also provided valuable advice regarding the distribution of brushtail possums along the eastern slopes of the Atherton Tablelands. Professor John Endler was instrumental in educating me on how to best perform colour analysis of fur photographs. Dr. Will Edwards provided advice on several statistical analyses and computer programs to utilize. Anna Pintor taught me how to run quantile regressions using my microsatellite relatedness/geographic distance data.

Supervisors

My four supervisors, Associate Professor Andrew Krockenberger, Associate Professor Brad Congdon, Professor Chris Johnson, and Professor Ross Crozier, together acquired the Australia and Pacific Science Foundation grant for this research, and provided their assistance and advice according to their different specialties in resolving the various difficulties and complexities that arose regarding the planning, implementation, analysis and reporting of this research.

Fieldwork

Many volunteers were instrumental in assisting the detection and capture of brushtail possums around the Atherton Tablelands: Steve Ryan, Anthony Mann, Westen Thomas, Luke, Peter Byrnes, Mel Commerford, Ilona and Pieter Moerman, Denise and Gareth, Sarah Schapel and William Hancock, Andrew Picone, Emma King, Louise Halritchie, Mandy Soymonoff, Martine Adriaansen, Madeline Ford, Tim Johns, Sarah Meyer, Natsumi Morita, Eri and Yuriko, Cherie Dugal. Russel Edwards, Brigitte and Georgina Humphries, Beau and Amy, Emma and Rick, Mel, Les and Mickey, each residents of Millaa Millaa, not only assisted my attempts to capture brushtail possums, but also very kindly allowed me to allow me to access their properties and to trap around their land in these endeavours.

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Michael Joyce and Pat Nagellega from QPWS were extremely helpful after cyclone Larry in helping me to access Herberton State Forest.

Johan Larson and Gabriel Porolak from James Cook University's Vertebrate Ecophysiology Laboratory volunteered their time in the field on numerous occasions, assisting in the capture of many brushtails.

DECLARATION ON ETHICS

The research presented and reported in this thesis was conducted within the guidelines for research ethics outlined in the *National Statement on Ethics Conduct in Research Involving Human (*1999*),* the *Joint NHMRC/AVCC Statement and Guidelines on Research Practice* (1997)*,* the *James Cook University Policy on Experimentation Ethics*. *Standard Practices and Guidelines* (2001), and the *James Cook University Statement and Guidelines on Research Practice* (2001). The proposed research methodology received clearance from the James Cook University Experimentation Ethics Review Committee (approval numbers A856, A1261 and A1262).

Signature: _____ Date:_____

Sarah Emily Kerr

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My rainforest adventure began three and a half years ago. It has had its ups and downs, and a steep learning curve, as I suspect most PhD projects do, but through the assistance and encouragement of the following amazing people, it has been an immensely rewarding experience, and I thank them all very much for all their help.

I have been fortunate to have had the guidance of four brilliant and encouraging supervisors, each with different, complementary specialties. Together they acquired a grant from the Australia and Pacific Science Foundation, an organization to which I extend considerable thanks for their financial support of this research. Associate Professor Andrew Krockenberger, my principle supervisor, was instrumental in providing advice on rainforest field methods; teaching me how to shoot and tranquilize possums, climb trees, and combining the two, demonstrating how to very quickly collect a possum from eight meters up a tree when it has fallen asleep hanging by just the very tip of its tail! I thank Andrew also for sharing his time and expertise throughout this project; in planning, fieldwork, data analysis, seminar preparations and thesis writing; he has been an amazing mentor throughout my time as a PhD student, and I feel very fortunate to have had his guidance. Also based in Cairns, Associate Professor Brad Congdon provided invaluable advice about genetics techniques and theory. Brad's generosity with his time in explaining many of the more advanced or technical genetic concepts is something for which I am immensely grateful, and was particularly important in the successful design of MC1R colour gene primers, in carefully exploring the genetic structure of Atherton Tablelands brushtail possums from many different perspectives, and assisted my ability to later explain all that work here. Professor Chris Johnson was instrumental in my undertaking this project, and I thank him very much for this opportunity, and for his time and advice throughout this study, particularly regarding evolutionary processes, brushtail behavior, statistics, and thesis write-up. Professor Ross Crozier guided me into the world of phylogenetic analysis, shared with me his passion for both the history and the potential of genetic research (conversations

that often led to us climbing his rather tall book cases looking for some ancient algorithm or vertebrate-specific text), he introduced me to Genbank and the delights of simple curiosity in this realm, and with his enthusiasm, debate, humour and patience, Ross helped me to develop a far greater set of skills both in the lab and away from it. I miss his guidance immensely. Specific to this project, Ross was instrumental in sharing his time and expertise to help me examine and interpret the genetic data obtained, proof-read thesis drafts, provided advice regarding phylogenetic analyses, and guided me through the maze of genetic analysis software and the pitfalls that can arise without checking the assumptions within the algorithms. I consider it a great privilege and myself incredibly fortunate to have had Ross as my supervisor, and it saddens me deeply to think that never again will we climb his bookcases searching for a key paper, because Ross's office is now empty, and that there will be no more conversations between us of possums, of genetics, insects, music, elegant algorithms, or historical events, because he is gone. Yet I am heartened by the idea that having been such an important mentor to not just myself but a very large array of students, colleagues, friends and family, Ross's legacy will be enduring.

I was very fortunate to receive guidance from Associate Professor Steve Williams about how to conduct distance sampling surveys. Steve and Associate Professor John Winter also gave me some extremely timely advice about the distribution of brushtail possums around the eastern edge of the Atherton Tablelands; this undoubtedly saved me many long nights of persistent spotlighting for possums that weren't there. Professor John Endler gave me some important advice about analyzing colour from fur photographs, and I thank him very much for sharing his time and expertise. Dr. Will Edwards provided assistance with several statistical analyses. In particular, with the help of Will, and of Anna Pintor, I was introduced to the wonderful possibilities of quantile regressions, and I thank them both for their help, time and advice.

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transect. I thank Sam very much for not only her work in the field trapping the grey brushtails, but in scouting the Tablelands for potential sites, and together with Katie Jones, for introducing me to the region when I began my PhD, sharing tips about life at James Cook Uni., rainforest fieldwork, possums, and finding North Queensland's best curry and kebabs in Atherton!

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Jane DeGabriel was conducting research at James Cook University's Townsville campus, studying the dietary tolerances of brushtail possums. She had six coppery brushtails from the Atherton Tablelands included in her captive study, and I thank Jane very much for providing me with DNA from these animals, and allowing me to come measure and photograph each of her coppery brushtails.

I discovered during the course of this research that there is a fourth colour morph of brushtail possum; the golden brushtail possum. With fur colour similar to that of a yellow labrador dog, these striking brushtails appear to have a recessive fur colour mutation. Though my attempts thus far to amplify the MC1R colour gene in these animals have been unsuccessful (ironically it seems this is due to some unlucky positioning of the very mutations I had hoped to find!), I would like to thank Wildworld nature park in Sydney for sending me DNA from one of their golden brushtails, Martin Fingland from Geckoes Wildlife, and Michael Pyne from Currumbin Sanctuary for their assistance tracking down golden brushtails and offering to provide brushtail DNA samples also.

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returned to find my three new friends looking somewhat uncertain about their situation, for which I do apologize, though I suspect it helped considerably that I did in the end return with the possum! Cherie Dugal interned with our lab and was a brilliant volunteer, assisting particularly with establishing a site near the very rainy town of Millaa Millaa.

Millaa Millaa was a fantastic place for fieldwork despite the continuous heavy rain, which unluckily, seemed to coincide with our arrival each trip! Russel Edwards, a town resident and QPWS ranger, was instrumental in establishing the Millaa Millaa site. I met Russel while tree-climbing in Ravenshoe for another of our lab's possum projects, and amusingly, it is at this same tree on numerous trips since that first meeting that we usually run into each other! Before my first trip to Millaa Millaa, Russel had talked to friends around town and gained cautious permission for me to trap around different properties. In the course of a single afternoon of introductions and setting up traps, the Millaa Millaa site was near-established. Thank you Russell for all your help, introductions and for sharing your enthusiasm about possums and tree-climbers. Thanks also to Beau and Amy, Emma and Rick, Mel, Les and Mickey who trusted me to wander around their properties at unusual hours without waking anyone up, and to trap possums without catching family pets!

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To my family and friends, who have put up with my constant talk of possums, Henry in particular, thank you so much for all your support, and for encouraging so much laughter and fun throughout my PhD project! I have been incredibly lucky to have made so many great friends at JCU in Cairns, such that it can often be difficult to feel like I'm going to work; a notion that to my mind suggests far less cheerful days. Instead, even through the longest and most tiring of days, these have been days of adventuring in the rainforest with friends, or working alongside them to solve my brushtail puzzle. And so I would like to thank Johan Larson and Gabriel Porolak, my funny, kind and enthusiastic teammates on so many rainforest adventures, and back in the lab, both of whom have scaled trees and enthusiastically traipsed through rainforests in the cold 3am darkness for my copperies! Thank you also to other lab members past and present: from Cairns, Katie Jones, for her help and encouragement as I learned to shoot, Gabriella Eiris, for her support and advice particularly in troubled PhD times, Megan Quenzer and Jeff Silverman, for so much fun in the field, and for introducing me further to the herpetological world, Toby Ross and Sue Tallarico for their encouragement and sharing amazing stories of PNG, Mel Commerford, for sharing in so much laughter and most importantly, for understanding how a few lines on a electrophoresis gel can mean celebratory dancing around the genetics lab! And in Townsville, thankyou to Stephen Kolomyjec, who taught me how to catch platypus and generously let me help weigh and measure one, Veronica Menz and Brooke Bateman, for their hospitality and warm welcome each time I came down south, sharing their mammal trapping tips, help acquiring genetics supplies, and occasional genetics commiserations.

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My amazing sister Takara has been an incredible strength, and I thank her for all her love and support, and for providing important perspective to my life. To this end I would like to thank also her husband, Shannon, and her five boys (three of whom came into the world during my time as a PhD student), Justin,

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ABSTRACT

I investigated the role of habitat in shaping mammalian evolution by studying the divergence of two parapatric subspecies, the common brushtail possum, *Trichosurus vulpecula vulpecula*, and the coppery brushtail possum, *T.v.johnsonii*, which are found in close proximity on the Atherton Tablelands in North Queensland, Australia. Their reputed distribution and colour differences, then unquantified, suggested that these subspecies may be candidates for evolution through parapatric speciation. This has never before been demonstrated in a mammal.

I discovered that along a habitat gradient from dry sclerophyll forest and mosaic ecotone to rainforest, brushtail possums differed significantly in fur colour. The two morphs were characterised by their fur colour saturation: its colour intensity. Possums with low colour saturation were grey and did not inhabit rainforest. Those with high fur colour saturation were a red-copper colour and did not occur in dry sclerophyll forest. There was a dichotomy in the shade of red expressed among coppery brushtails, which was either a red-orange or red-purple hue, with the different rainforest localities of brushtails significantly associated with this variation. Brushtails in ecotone were either coppery or grey. Fur colour did not vary with distance from the ecotone, nor did fur colour appear to change once established in early development. Ecotone habitats supported very few possums, which may suggest some potential for the ecotone to restrict gene flow along the habitat gradient. The population density in rainforest was 18.6 times that in ecotone, and dry sclerophyll forest supported 8.7 times more brushtails than ecotone habitat.

Coppery and grey brushtail possums were also morphologically distinct in body size. On average, coppery brushtails had ears that were 8mm shorter and 3.4mm thinner, legs that were 3.6mm shorter from knee to heel, and tails 34mm longer than grey brushtail possums of the Atherton Tablelands. There was no sexual dimorphism among brushtail possums along a habitat gradient for body size or colour, suggesting that sexual selection is unlikely to be acting upon these traits to promote divergence.

Comparing mitochondrial DNA control region sequences I found that the morphological distribution was not the result of secondary contact between reciprocally monophyletic populations: coppery and grey possums have evolved together in multiple, distantly related clades. Analyses of the morphology of these clades demonstrated that variation in body-shape morphology was not associated with genetic similarity but with fur colour. As such, possums with the same fur colour also shared body size morphology, whether they were from genetically distant clades or if they were genetically similar. These differences, together with the bimodal distribution of morphs along habitat gradients, indirectly suggested that selection is acting upon these morphological traits to produce the phenotypes and distribution observed.

Examination of the population structure of Atherton Tablelands brushtail possums using mitochondrial DNA control region sequences demonstrated that grouping populations by colour morphology did not explain genetic variation. Genetic differences between populations were not explained by the latitudinal, longitudinal, straight-line or elevation distances between them. However 30.1% of variation could be explained through the identification and separate grouping of the four populations that were closest to rainforest habitat. Along a habitat gradient, grey and coppery populations were distinct.

Investigation of population structure with microsatellite loci showed significant gene flow throughout the Tablelands. Along the habitat gradient, adjacent coppery and grey populations were genetically distinct despite this widespread gene flow. Indeed several geographically more distant populations were not distinct with these markers. This suggested that gene flow is restricted along the habitat gradient. However reproductive isolation was not complete: calculations revealed that gene are exchanged in both directions along the gradient.

Both grey and coppery brushtail possums reproduced synchronously, suggesting that there was no temporal discontinuity to gene flow. However this reproductive synchronicity may limit the potential for polygyny.

With selection acting on morphology and gene flow restricted along a habitat gradient, two possums separated by the same geographic distance should be less related if they are different morphs than if they are the same colour. However, while the pairwise relatedness between different brushtail colour morphs was significantly different to those among coppery brushtails, there was no difference to comparisons among greys. This may be a consequence of the higher population densities of coppery brushtails; relatives may possess smaller home ranges and be closer. However these genetic results did not match our theoretical expectations: possums of different colour morphs and habitats were not less related than possums of the same morph at the same distance of separation. My underlying assumption was that fur colour in the coppery brushtail possum, like so many other species, is a genetically determined trait. If true, then brushtail colour is inherited and determined via an unprecedented mechanism. While this can not be discounted, I questioned my underlying assumption.

Fur colour can also be phenotypically plastic. Climate and diet can determine fur colour, though usually only temporarily. Fur colour did not appear to change once established in early development. The presence and fine-scale distribution of coppery and grey brushtails in rainforest fragments suggested that climatic effects are highly unlikely to be determining fur colour. There has been one previous demonstration of diet permanently determining fur colour via maternal diet acting *in utero* in laboratory mice. I found that determination of brushtail fur colour by maternal diet and selection upon this trait better explains the observed morphological distribution and genetic structure. Further experimental research is needed to conclusively demonstrate this effect, to explore how widespread this capacity for coppery brushtail colour is, and to discover which foods are involved in fur colour expression. Such an congenital change would be highly significant in evolutionary research as it allows, without mutation, the widespread single generation adaptation of offspring to the environmental conditions experienced by the parent.

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CHAPTER ONE

An introduction to the case of the coppery brushtail possum

In this project I examined the role that habitat can play in shaping the evolution of mammals. There are many accounts of speciation in mammals, but none have described the action of habitat, independent of a geographic barrier, resulting in the evolution of two distinct species. Two subspecies of brushtail possum, *Trichosurus vulpecula johnsonii*; the coppery brushtail possum; and *Trichosurus vulpecula vulpecula*; the common brushtail possum, were candidates for having undergone genetic divergence without geographic isolation by occupying different, adjacent habitats, and thus were the focus of this study. It is my aim in this chapter to introduce the concepts of how new species are formed, how speciation can occur without a geographic barrier to gene flow, and to explain why the coppery brushtail possum is an exciting candidate for potentially having evolved in such a manner, when no other mammal has yet been shown to have done so.

1.1. Divergence and speciation

Evolution is a process of biological change. Central to evolutionary theory are concepts of how new species are formed (Darwin, 1859; Dobzhansky, 1970; Mayr, 2000). Speciation is the process by which a single species will diverge to form two or more new species (Mayr, 2000). This process is of great importance in understanding how extant species have come to exist, explaining why there are so many species despite the less diverse origins of life, and why extinction processes, particularly mass extinction events throughout Earth's history, have not permanently reduced the number of species (Benton, 1995); it is through speciation that species numbers can recover. The ability to build phylogenetic trees through the analysis of DNA has also allowed inferences to be made about divergence times of taxa from a 'common ancestor' (Webster *et al.,* 2003), this being the historical speciation point when one species has diverged into two or more species.

The point at which two divergent forms are considered separate species is determined by our species definition as the formation of reproductive isolation between two forms (Dobzhansky, 1970; Mayr, 2000). There are three key theoretical mechanisms of speciation: allopatric, parapatric and sympatric. Each is characterized by the type of barrier that prevents the gene flow between populations (Dobzhansky, 1970; Coyne and Orr, 2004). Allopatric speciation has been well demonstrated in many species, and occurs when there are physical geographic barriers to gene flow between two populations, such as rivers, mountains, oceans, and roadways (reviews by Rice and Hostert, 1993; Orr and Smith, 1998; Gavrilets, 2003). The direction of drift or the nature and intensity of selective pressures may differ in each geographic region, thus potentially altering the relative reproductive success of individuals in each population, the gene frequencies of that population over time, and ultimately the degree of reproductive isolation experienced by each form (Barton, 1989).

Non-allopatric speciation, whether sympatric or parapatric, occurs without a geographic barrier to gene flow (Mayr, 1942). This means that in the early stages of speciation, divergence occurs despite the homogenising effects of gene flow (Key, 1982). Historically this was believed to be impossible, with non-allopatric speciation thought either too improbable, given the need to produce divergence despite gene flow, or simply too rare and the conditions for such an event too specific to be of any great usefulness to evolutionary study (Mayr, 1942). Certainly there are far fewer examples of non-allopatric speciation than allopatric, but over time there has been a growing number of theoretical models demonstrating the myriad of ways in which this mode of speciation can occur (Maynard Smith, 1966; Dickinson and Antonovics, 1973; Udovic, 1980; Rice, 1987; Gavrilets *et al.,* 1998; Gavrilets *et al.,* 2000; Kirkpatrick and Ravigné, 2002), as well as increasing observations of non-allopatric divergence in the field among, for example, birds (Chesser and Zink, 1994; Smith *et al.*, 1997; Slabbekoorn and Smith, 2002), reptiles (Schneider *et al.,* 1999; Ogden and Thorpe, 2002), amphibians (Graham *et al*., 2004), fish (Barluenga *et al.,* 2006), and insects (Bush, 1969; Tauber and Tauber, 1989; Johannesson *et al.*, 1995). This growing evidence suggests that non-allopatric speciation may be far more

important and common than previously believed (Schluter, 1996; Schluter and Rambaut, 1996; Smith *et al.*, 1997; De Aguiar *et al.*, 2009; reviews by Orr and Smith, 1998; Schluter, 2001; Via, 2001; Coyne and Orr, 2004). While the smaller number of non-allopatric speciation events reported compared with the amount of allopatric speciation cases may reflect a true difference in the frequencies of each mode of speciation, it may also reflect the greater difficulty of finding and substantiating non-allopatric cases (Coyne and Orr, 2004). Allopatric speciation occurs when there is geographic division and reproductive isolation of two forms subject to differing selective pressures or drift over a significant period of time (Mayr, 1942). As such it may be possible to identify allopatric cases by looking for differences in the characteristics of two forms, or by investigating a geographic feature that is a candidate barrier to gene flow (Lessios *et al.,* 1998; Schneider *et al.*, 1998).

However in non-allopatric speciation the challenge is to identify divergent forms in the same geographic space. Sympatric speciation for example, refers to the divergence of two forms with overlapping distributions. There is no geographic divide and speciation usually begins with behavioural divergence, such as a change in feeding strategy among a proportion of the species which can lead to assortative mating and genetic divergence (Bush, 1969; Key, 1982; Dieckmann and Doebeli, 1999). Identification of taxa in the field is largely made from observations of appearance and distribution. It is unlikely that two morphs similar in these characteristics would be easily identified as being more than a single form, let alone thought to have become reproductively isolated. Aiding the detection of non-allopatric speciation events somewhat, behavioural differences between forms may also lead to morphological variation between them, for example if feeding strategy had effects on relative body size we may have a visual clue that in a single geographic area there is not one species, but two sister species. Indeed these morphological changes may play a very important role in promoting reproductive isolation (Schluter and Nagel, 1995; Dieckmann and Doebeli, 1999; Boughman, 2001; Barluenga *et al.,* 2006)

Parapatric speciation occurs with expansion, but not separation, of part of a population into adjacent new regions. There is no geographic barrier to gene flow, so like sympatric speciation, divergence occurs despite initial gene flow across the historic and new ranges (Endler, 1977; Futyama and Mayer, 1980; Barton *et al.,* 1988). Parapatric speciation is controversial however, because it requires that natural selection across different habitat types is not only able to drive significant morphological change, but that it might do so despite these homogenising effects of gene flow between the diverging forms (Key, 1982). Divergence among populations arises from adaptations to differing selective pressures among habitat types associated with factors such as predation pressure, resource types and availability, foraging requirements, and conspecific competition driving divergence between populations of the two localities (Key, 1982; Smith *et al.*, 1997).

Beyond the difficulties described above in detecting candidates of non-allopatric speciation, demonstrating cases of parapatric speciation is further complicated because allopatric speciation with later expansion and convergence of two forms along a hybrid zone can produce a similar distribution of morphs and genetic signature to that of parapatric speciation (Endler, 1982). Thus in order to demonstrate parapatric speciation, a hypothesis of allopatric speciation, thought to be the predominant mode of speciation, must be clearly falsified as having shaped the evolution of two divergent forms. Thus it is important to understand the current and historic geography of a region and its interaction with the distribution of the forms under investigation. Indeed to demonstrate parapatric speciation it may be vital for this habitat-associated speciation to occur in the same manner in multiple locations with a common ecological structure (Coyne and Orr, 2004). Divergence in the little greenbul, *Andropadus virens*, a bird that has altered its song to suit the acoustic qualities of the different forest types that greenbul populations inhabit, demonstrates well the difficulties of investigating parapatric speciation and the importance of genetic analysis in substantiating parapatric speciation events (Smith *et al.*, 1997; Slabbekoorn and Smith, 2002). Bird song can be vital in determining mate choice, and thus changes in song are often associated with assortative mating

between populations and potentially reproductive isolation between them to produce distinct species (Irwin, 2000; review by Slabbekoorn and Smith, 2002*b*). The greenbul is distributed continuously across a single geographic region of mosaic habitat, with the two bird forms differing in song, and by body size, though the physical differences between the two forms are at a scale of just a few millimetres; not traits that can be easily identified in the field (Smith *et al.*, 1997; Slabbekoorn and Smith, 2002). Genetic analysis of twelve greenbul populations demonstrated that with a given rate of gene flow between two populations, birds of the same habitat type were less morphologically divergent than those compared across different habitats (Smith *et al.*, 1997). However Smith *et al.,* (1997) did not demonstrate that reproductive isolation had occurred between the two forms. Based upon ecological, morphological and acoustic comparisons, there appears to have been parapatric divergence, possibly even speciation. But it is genetic analysis that plays a vital role in demonstrating significant reproductive isolation and establishing whether parapatric divergence has also led to parapatric speciation (Ogden and Thorpe; 2002), particularly as criticisms of parapatric speciation centre on the likelihood that parapatric divergence can progress to speciation despite initial gene flow between forms (Mayr, 1942; Coyne and Orr, 2004). These difficulties of detecting events of non-allopatric speciation and conclusively demonstrating that allopatric speciation is not responsible for the observations made, helps explain not only why there are few field examples of this mode of speciation, but why there has yet to be a demonstration of non-allopatric speciation in mammalian evolution, for which only allopatric speciation has thus far been demonstrated (Fitzpatrick and Turelli, 2006).

1.2. Detecting parapatric speciation with genetic analysis

The example of parapatric speciation in the little greenbul highlighted the importance of genetic analyses in detecting and validating this mode of evolution (Smith *et al.*, 1997). An example of the successful application of these genetic analyses lies in Ogden and Thorpe's (2002) research on *Anolis roquet* lizards. This was a notable study because it successfully examined speciation processes across both a geographic barrier that was a candidate for promoting

allopatric speciation, and across different habitat types to examine the role of habitat alone (Ogden and Thorpe, 2002). This critical role for genetic analyses in understanding and investigating candidate speciation events has arisen because different speciation mechanisms can leave distinct phylogenetic traces (Coyne and Orr, 2004). A distribution of two or more forms living in adjacent but not fully overlapping geographic ranges could be the product of parapatric speciation or it could be the result of secondary contact between historically divergent forms that had not necessarily evolved through parapatry (Endler, 1982; appendix 1.0). Different types of DNA can be examined and compared to determine a species evolutionary history from different perspectives. In order to falsify the hypothesis that allopatric speciation has contributed to the divergence of two forms, it is preferable to observe the following mitochondrial and microsatellite genetic signatures in multiple comparisons across similar ecological habitats (Coyne and Orr, 2004; appendix 1.0).

Mitochondrial DNA can be a particularly useful tool for investigating long-term change and is widely used to construct phylogenetic trees that show the path of divergence for a species or for populations, relative to other groups. Mitochondrial DNA is generally maternally inherited and mutates slowly relative to many other loci at a rate that depends on the species, location and function of the sequence; in mammals a rapid rate of control region mutation was documented at 0.32 mutations/base/million years (Sigurdardóttir *et al*., 2000). Thus even at relatively rapid mutation rates, mitochondrial DNA sequencing provides a tool with which to examine long term change. Yet care must be taken with data interpretation in light of evidence that mitochondrial DNA mutation rates can not be used to create reliable evolutionary 'clocks', and that selective pressures may act more strongly on mitochondrial DNA than previously thought; unlike nuclear DNA, mitochondrial genetic diversity does not correlate with species population sizes or ecology, suggesting that in general it is not neutral (Bazin *et al.*, 2006; Galtier *et al.,* 2009). In contrast to this general trend, within mammals the selective pressures acting upon mitochondrial DNA are near-neutral, and most likely a product of genetic drift, with genetic diversity highly variable between lineages and not correlated with population size, species

abundance or ecology (Nabholz *et al.*, 2008). Thus if two mammalian forms living in geographically adjacent, connected regions were examined using a portion of the mitochondrial DNA sequence, such as the non-coding control region, we would expect that if divergence has occurred through allopatric speciation with the current distribution formed by expansion and possibly convergence, then genetically distant lineages with significant morphological differences will be phylogenetically dichotomous and cluster by the morphological and behavioural differences they possess (Lande, 1980; appendix 1.0). Across multiple populations, morphological difference would probably be indicative of genetic distance also (Lande, 1980). Under parapatric speciation we might expect a similar distribution of haplotypes when examining one divergent population, but across many, with the same ecological differences producing similar resultant morphologies, we would expect that adjacent, morphologically dichotomous populations will be genetically more alike than groups sharing their morphological traits in distant populations (Coyne and Orr, 2004; appendix 1.0).

Microsatellite markers are genetic tools that are useful in examining more recent evolutionary change (Slatkin, 1995). Microsatellites are short sequences of DNA composed of many repeats of different two or three base pair groups. Although many microsatellite loci are neutral, non-coding and not subject to any apparent selective pressures, increasingly loci are being described that control the fine-scale phenotypic expression of genes and which are subject to selection either directly, or as a result of proximity to adjacent genes (Payseur and Nachman, 2000; Kashi and King, 2006). Microsatellites have a high mutation rate, and alleles are inherited from both parents which facilitates recombination with each generation, making them highly variable (Weber and Wong, 1993). This high variability allows greater detectability of the relatedness of individuals, and consequently of recent gene flow between groups (Slatkin, 1995; Marshall *et al*., 1998). In order to substantiate the occurrence of non-allopatric speciation, and rule out historic allopatric speciation, it must be demonstrated that reproductive isolation has occurred (Coyne and Orr, 2004). Morphs occupying adjacent, geographically connected regions, that mitochondrial DNA analyses indicate are not the product of secondary contact,

need to be examined using markers such as microsatellite loci to calculate the probable rates of gene flow across habitats between forms (Coyne and Orr, 2004). If parapatric divergence has progressed to speciation we would expect that there will be reproductive isolation and no gene flow between the morphs (Coyne and Orr, 2004). However if parapatric divergence has not yet led to full reproductive isolation of the two morphs, we would expect that gene flow between morphologically divergent forms will only be restricted (Coyne and Orr, 2004; appendix 1.0).

The likelihood that parapatric divergence will lead to speciation appears in theory to depend upon the strength of selection relative to the homogenizing effects of gene flow (Barton, 1989; Rice and Hostert, 1993; Nosil *et al*., 2003; Gavrilets, 2006). By examining multiple parapatric events across similar ecological boundaries, we can test whether selective pressures are playing a significant role in driving divergence, or if similar morphological distributions are due to chance drift (Slatkin, 1987; Smith *et al.,* 1997). In cases where forms differ in more than one trait, and when these morphologies are not linked, we would expect that under the effect of drift, either groups that are genetically similar will also be morphologically alike, or genetically distant groups with one morphological trait in common will differ with respect to their other morphological characteristics (Endler, 1977; Slatkin, 1987; appendix 1.0). Alternatively if selective pressures have shaped a particular morphological distribution across multiple parapatric divergence events, we would expect that the groups that are morphologically similar may not be those most closely related, but that genetically distant groups with one morphological trait in common may also share several unlinked morphological traits (Slatkin, 1987; appendix 1.0).

The conundrum of identifying parapatric speciation events is that there are a vast number of habitats to test and many species found across them. Thus to direct our search effort it is highly advantageous to search for forms displaying visible or audible differences across habitat types, not only because it is easier to detect in the field, but also because the evolution of these characteristic

differences may indicate that selection is sufficiently strong to shape morphology despite gene flow in early speciation. Examples of parapatric divergence thus far have included behavioural divergence by song (Smith *et al.*, 1997; Slabbekoorn and Smith, 2002), morphological differences by body size (Smith *et al.*, 1997) and variation in colour (Marchetti, 1993; Lodé, 2001), suggesting that these characteristics may be of particular value in detecting such evolution.

1.3. Colouration as an indicator of speciation

Colouration can determine how favourably animals are regarded by potential mates, as well as their ability to camouflage themselves, and thermoregulate (Nosil, 2004; Price, 2006; Mundy, 2007; Nadeau *et al.,* 2007). Thus, colour can be a trait under intense divergent selection in different habitats, and variation in colouration between populations can be indicative of genetic divergence (Boughman, 2001; Nosil, 2004; Endler *et al*., 2005).

The colour of animals is most commonly determined genetically (Price, 2006). The common genetic inheritance of colour has been utilized in animal husbandry for centuries, with its basic principles understood well before the concepts of genetics and evolution (Brewer, 1893; Lerner, 1957). Genetic determination of colour has more recently been demonstrated across a wide range of taxa, with one key gene, the Melanocortin-1-Receptor, in consort with four other genes **(**Miller *et al.,* 1997; Abdel-Malek *et al.,* 2001; Prasolova *et al.,* 2002; Kerns *et al.,* 2003; Gratten *et al.,* 2007), being responsible for colouration in birds (Theron *et al.,* 2001; Kerje *et al.,* 2003; Doucet *et al.,* 2004, Nadeau *et al.,* 2006; Baião *et al.,* 2007), reptiles (Rosenblum *et al.,*2004) and eutherian mammals (Miller *et al.,*1997; Kijas *et al.,* 1998; Wada *et al.,*1999; Everts *et al.,* 2000; Rouzaud *et al.,* 2000; Rieder *et al.,* 2001; Kerns *et al.,* 2003; Nachman *et al.,* 2003; Hoekstra *et al.,* 2004; Vage *et al.,* 2005; Fontanesi *et al.,* 2006; Hoekstra *et al.,* 2006; Mengel-Jørgensen *et al.,* 2006).

The Melanocortin-1-Receptor is an intron-less gene that controls the expression of eumelanin and phaeomelanin, and single base pair changes often control phenotypic colour expression (Majerus and Mundy, 2003; Mundy, 2005). The

potential research avenues using this gene are particularly exciting because although mutations do not always code for colour changes (Hosoda *et al.*, 2005), the pattern of mutations that do cause such variation have been identified in a wide range of species, allowing recent research that has involved the prediction of hair colour from a DNA sample alone, not just in extant species but those long extinct, such as the mammoth (Römpler *et al.*, 2006). Such prediction is less controversial in zoological, archeological or paleontological endeavours, but its application to forensics and medicine is legally and ethically complicated (Cho and Sankar, 2004; Branicki *et al.*, 2006; Lowrance and Collins, 2007).

In a few species however, colouration is not genetically determined. Colour can also be a phenotypically plastic trait, meaning that a single genotype can produce different phenotypes in response to specific environmental cues. Climate has been demonstrated to affect animal colour, with temperature and photoperiod causing seasonal fur colour changes in cryptic species such as the Arctic fox (Vage *et al.,* 2005). In Siamese cats a lower body temperature around the paws, tail, ears and face results in increased tyrosinase activity, thus increasing melanin production to cause darkening fur colour in these parts of the animal (Iljin and Iljin, 1930; Lyons *et al*., 2005). However determination of fur colour by climate is generally not permanent, with species changing colour seasonally, or in the case of the Siamese cat, new hair growth reflecting the temperature at which it developed (Iljin and Iljin, 1930).

Diet has also been demonstrated to temporarily affect colouration, perhaps the most dramatic example being that of the flamingo, which loses its bright pink colour unless its diet is high in carotenoids (Fox, 1962). This reaction is widespread though not as intense in other bird species, many of which will also acquire a pink hue while consuming diets high in carotenoids (Hill, 1993; Hays *et al.,* 2006). While these effects are temporary, some dietary effects are permanent. In laboratory mice specially bred for a specific genotype, the diet of the mother *in utero* affected the hue of her offspring; producing pups with varied shades of brown fur (Cropley *et al.,* 2006). However, out of the laboratory,

permanent determination of colour by diet has not been demonstrated in any species.

1.4. Mammalian speciation without geographic isolation

There has yet to be a demonstration of mammalian speciation without geographic isolation of the diverging forms. However it has been demonstrated that habitat-mediated mammalian genetic divergence can occur without geographic isolation (Lodé, 2001). Lodé's (2001) study of polecat populations is particularly significant to my research as the phenotypic difference between divergent forms was fur colour. The polecat colour morphs did not have strict habitat affinities, yet they were genetically divergent (Lodé, 2001). Following from this observation it would seem likely that a mammalian population with morphs that were parapatrically distributed, and which displayed strict habitat fidelity for differing habitat types would be even more divergent than the polecats, possibly to the point of speciation.

1.5. The coppery brushtail possum

The brushtail possum, *Trichosurus vulpecula* is a marsupial species native to Australia. An arboreal, nocturnal, usually herbivorous creature, the brushtail possum is widespread throughout eastern Australia (Kerle and How, 2008), and has become a serious pest in New Zealand (Cowan, 1992). Brushtail possums are territorial with male-biased juvenile dispersal in which males can move up to ten kilometers before establishing a territory (Clout and Efford, 1984; Johnson *et al.,* 2001). Females tend to settle adjacent to their mother's home range (Clout and Efford, 1984; Johnson *et al.,* 2001). Mostly grey in colour, a black morph can be found in New Zealand and Tasmania (Guiler and Banks, 1958; Kean, 1971) and a golden morph can be found in Tasmania (Fingland, 2005).

On the Atherton Tablelands of Far North Queensland there are two colour morphs of brushtail possum, which at present are classified as *Trichosurus vulpecula* subspecies (Kerle and How, 2008); the common, grey coloured

brushtail possum, *T.v.vulpecula*, and the coppery, red coloured, brushtail possum, *T.v.johnsonii*.

Common and coppery brushtail possums are found in close proximity in the Atherton Tablelands region and have distinct habitat distributions. The coppery form is found in rainforest, and the common grey morph is outside of this habitat type, in the drier, less dense, sclerophyll forest (Winter, 1984). These adjacent habitat types are commonly connected by less than a kilometre of mosaic ecotone. Coppery and grey brushtail possums are apparently distributed widely across the Atherton Tablelands within their respective habitats (Winter, 1984; Collins, 2003). Pleistocene glaciation is associated with the historic contraction of rainforest in the Wet Tropics of North Queensland, the creation of rainforest refugia and consequently the divergence of species across geographic features such as the Black mountain corridor (Winter, 1997; Schneider and Moritz, 1999). However there is no indication of such historic geographic division between these habitats on the Atherton Tablelands or the morphs that presently inhabit them. As the rainforest has expanded and contracted, and sclerophyll forest likely contracted and expanded concordantly, the two habitats would likely have shifted in their size and location but remained connected through the ecotone (Hopkins *et al.,* 1993; Kershaw, 1994). While an increased frequency of fire in dry periods may have created patches of cleared ground and resulted in the demise of some common brushtail populations living in dry sclerophyll forest (Hopkins *et al.,* 1990; 1993), the widespread distribution of both colour morphs suggests that allopatric speciation is unlikely to have occurred between these brushtail possums.

The coppery brushtail has been described without quantification, as a distinctive rainforest-dwelling possum that is heavier, and has a fur colour that is redder and darker grey, than the common brushtail possum of other Queensland populations (Winter, 1984). Kerle *et al.* (1991) also suggested that coppery brushtail possums were distinct in skull morphology and allozyme analysis, but did not provide any measure or indication that their specimens were in fact coppery brushtails; they are simply listed as being from unspecified localities across the vast Atherton Tablelands region. Because of this reputedly distinct

colour and body-shape morphology, Flannery (1994) suggested that these morphs be classified as distinct species, however genetic analysis of control region mitochondrial DNA by Collins (2003) suggested that the two brushtail morphs from the Atherton tablelands are reciprocally polyphyletic; multiple distinct mitochondrial lineages contain both morphs which are unlikely to be distinct at the species level. Such a phylogenetic structure may also suggest that the present distribution of morphs is the product of divergence, not convergence through secondary contact (Lande, 1980; Coyne and Orr, 2004). Yet, Collins' study (2003) was not detailed in its examination of the coppery brushtail possum. He examined coppery brushtail possum phylogeny at a large scale, with a small sample size, and no detailed measures of colour morphology, nor geographic and ecological distributions (Collins, 2003). Thus our sum understanding of Atherton Tablelands brushtail possum colour morphologies and distribution has until now been without any robust quantitative foundation. Looking more broadly, there have been two other key studies that examined brushtail possum fur colour, though both examined colour on a large scale, and neither quantitatively measured colouration, nor did either study examine the genetic distribution of different morphs (Guiler and Banks, 1958; Kean, 1971).

These studies, of the black and grey brushtail morphs in Tasmania (Guiler and Banks, 1958) and New Zealand (Kean, 1971), found that black brushtails tend to inhabit the more dense humid forests, and like the grey brushtails on the Atherton Tablelands, the common brushtails were distributed in the more open, less humid habitat. Kean (1971) hypothesized that brushtail colouration may be determined genetically, and if so, that the genes for climatic tolerance would be strongly linked to those for fur colour. Guiler and Banks (1958) demonstrated a correlation between coat colour frequencies and regional vegetation structure and rainfall. If brushtail distributions on the Atherton Tablelands do reflect a wider ecological trend, where greys inhabit the drier habitat and an alternative, melanistic morph occupies more humid regions, this may suggest a common environmental cause for such fur colour divergence. Perhaps, as Kean (1971) suggested, there is linkage between the genes for fur colour and climatic

tolerances. Alternatively there may be a more direct determination of fur colour by factors such as temperature and humidity, or as a result of dietary differences between the morphs. Whether brushtail fur colour is genetically or ecologically determined, the implications of either mechanism permanently determining colour in a mammalian species would be highly significant to our understanding of evolutionary processes. Ecological determination of fur colour introduces the capability for mammalian populations to make large phenotypic shifts in few generations without the need for widespread genetic mutation, bottleneck or allelic fixation. Genetic determination of fur colour in these subspecies that are reputedly found in adjacent, connected habitats may be indicative of either parapatric divergence and the early-stages of speciation; important processes about which little is known due to the difficulties inherent in detecting and monitoring parapatric speciation events (Tregenza, 2002; Via and West, 2008; Via, 2009; Nosil and Schluter, 2011) or it may indicate the complete speciation of forms, which would be novel in mammalian evolution and would suggest that new mammalian species can arise despite gene flow in response to the differing selective pressures of their environment (Key, 1982).

1.6. Aims and thesis structure

Thus the aim of this study has been to examine the morphology, distribution, phylogeography and genetic divergence of brushtail possums on the Atherton Tablelands, particularly along habitat gradients, where the coppery and common brushtail possums are reputedly found in close proximity. This situation presented an important opportunity to examine the divergence and potentially the speciation of mammalian subspecies not separated by a geographic barrier, should reproductive isolation have evolved also. Therefore, this project had three key questions at its core:

1. To what morphological and behavioural degree is the coppery brushtail possum, *Trichosurus vulpecula johnsonii*, distinct from the common form, *Trichosurus vulpecula vulpecula*?
2. Are these subspecific differences being maintained despite gene flow between coppery and common brushtail possums?

3. If so, are selective pressures playing a significant role in the divergence, or lack thereof, of these two brushtail possum subspecies?

Chapter Two answers the first of these questions by firstly testing the hypothesis that there are distinct morphological groups of brushtail possums, and then by evaluating the mutually exclusive hypotheses that the distribution of morphs has evolved as a result of either a) sympatric divergence, b) parapatric divergence, or c) allopatric divergence. Using data from the measurements of brushtail possums that were captured, and from surveys of their distribution using distance sampling techniques, the evidence concerning the hypothesis that sexual selection has shaped the divergence of forms, and observations pertinent to the hypothesis that fur colour is genetically determined are also discussed in chapter two. This chapter was the first step in defining the two subspecies and giving quantitative foundation to the concept of a coppery brushtail possum, which has until now been speculative. Having defined the differences in colour, body size, reproductive synchronicity, habitat preference and distribution of the common and coppery brushtail possums of the Atherton Tablelands, I then possessed a platform from which to investigate the final two questions to establish whether divergence and speciation were occurring between these two subspecies, and to better understand the mechanism of evolution shaping the morphologies and distribution of brushtails observed.

The final two questions are answered in Chapter Three, which presents the genetic analyses of Atherton Tablelands brushtail possums. In this third chapter I evaluated four evolutionary mechanisms, testing the mutually exclusive hypotheses of each (appendix 1.0):

1. whether the distribution of colour morphs is the product of a) parapatric divergence or b) allopatric divergence
2. whether full speciation has occurred between colour morphs, which would be supported by a) no gene flow across a habiat gradient and refuted by b) the detection of gene flow
3. whether the evolution of the morphologies observed have been more strongly shaped by a) genetic drift or b) natural selection

4. whether fur colour is genetically or environmentally determined in this case, specifically, examining the four mutually exclusive hypotheses that

a) fur colour is genetically determined in Atherton Tablelands brushtail possums

b) fur colour is a temporary plastic trait in these possums

c) fur colour is a plastic trait permanently determined by climate

d) that fur colour is a plastic trait permanently determined by diet.

I used three different types of genetic markers to test these hypotheses, and although each could be written separately to outline a single analysis and its results, the synthesis of data gathered using markers that act either on different evolutionary timescales or to examine a specific gene associated with colour, provides better comparison of these results and allows a comprehensive debate of the merits of the possible evolutionary mechanisms in action.

I first used a mitochondrial control region DNA sequence 467 base pairs long to examine the more historic genetic structure of these possums, testing whether secondary contact is responsible for the distributions observed, the cladistics of how and when coppery fur colour might have evolved, relationships between genetic structure and morphology, the role of selection in shaping morphology, and calculating probable rates of migration between colour morphs along a habitat gradient.

The second genetic tool set I utilised consisted of eight microsatellite loci, which allowed the exploration of more recent gene flow, comparing population structure calculated with microsatellite loci to those of mitochondrial DNA, determining rates and direction of gene flow along a habitat gradient, and examining how the type of pairwise comparison between two possums; whether among possums of the same colour, or between possums of different colours; affected the correlation between relatedness of possums and the geographic distance between them.

In Chapter Three I also report the results from the amplification of 642 base pair sequence of the Melanocortin-1-receptor gene in the brushtail possum. This

gene, discussed above, is highly conserved and determines fur, scale, skin and plumage colour across a wide range of taxa (Miller *et al.,*1997; Kijas *et al.,* 1998; Wada *et al.,*1999; Everts *et al.,* 2000; Rouzaud *et al.,* 2000; Rieder *et al.,* 2001; Theron *et al.,* 2001; Kerje *et al.,* 2003; Kerns *et al.,* 2003; Nachman *et al.,* 2003; Doucet *et al.,* 2004, Hoekstra *et al.,* 2004; Rosenblum *et al.,* 2004; Vage *et al.,* 2005; Fontanesi *et al.,* 2006; Hoekstra *et al.,* 2006; Mengel-Jørgensen *et al.,* 2006; Nadeau *et al.,* 2006; Baião *et al.,* 2007). This however is the first creation of primers and successful extraction of this colour-determining gene in a marsupial.

With the aid of these molecular tools I answer the project's final two core questions in Chapter Three, and further narrow the possible evolutionary mechanisms producing the morphologies and distribution described in Chapter Two to just two possible evolutionary mechanisms, both of which are novel to mammalian evolutionary study.

In Chapter Four I present my final conclusions; a synthesis of Chapters Two and Three, with recommendations for the management and conservation of the coppery brushtail possum, and for further investigation into this novel mechanism of mammalian evolution.

CHAPTER TWO

Morphology and distribution of brushtail possum subspecies, *Trichosurus vulpecula vulpecula* and *T.v.johnsonii* indicates a novel mode of mammalian divergence.

2.1. ABSTRACT

The morphologies and distributions of brushtail possum subspecies, *Trichosurus vulpecula vulpecula* and *T.v.johnsonii,* were examined in rainforest fragments and along habitat gradients spanning dry sclerophyll forest, ecotone and rainforest on the Atherton Tablelands in northeastern Australia's Wet Tropics. I found the two subspecies to be morphologically distinct, both in fur colour and body size. Unexpectedly, I found that within one subspecies, *T.v.johnsonii*, fur colour is complex: there were two red colour hues, with significant differences in this expression occurring between forests, although the mechanism for this is unclear. In general, each subspecies had strong and differing habitat affinities along habitat gradients that could not be explained by geographic barriers to movement: *T.v.vulpecula* occupied dry sclerophyll forest, and *T.v.johnsonii* the neighbouring rainforest. Both colour morphs were also, but less frequently found in mosaic ecotone habitat and in rainforest fragments, suggesting that their morphological differences, if phenotypically plastic, are unlikely to be determined by climatic variables. The low number of animals in the ecotone suggests that there is limited interation between the colour morphs and little dispersal through this habitat, thus restricting gene flow between habitat types. If brushtail fur colour is genetically determined, limited gene flow could enable parapatric divergence and result in the observed phenotypic differences. Therefore, I suggest that these two brushtail forms may represent a novel mode of mammalian divergence, occurring due to either the environmental determination of fur colour via maternal diet, or genetic determination of colour with parapatric genetic speciation.

2.2. INTRODUCTION

There have been demonstrations of non-allopatric divergence and speciation in birds (Chesser and Zink, 1994; Slabbekoorn and Smith, 2002), reptiles (Schneider *et al.,* 1999), amphibians (Graham *et al.*, 2004), fish (Barluenga *et al.,* 2006), and insects (Bush, 1969; Tauber and Tauber, 1989). In mammals non-allopatric divergence has been demonstrated, however these cases do not show a progression to the speciation of forms (Lodé, 2001; Fitzpatrick and Turelli, 2006). Of the possible modes of speciation only allopatric speciation has thus far been demonstrated in mammals (Fitzpatrick and Turelli, 2006).

Identifying cases of non-allopatric speciation can be difficult because parapatric and sympatric forms occupy a continuous range, with differences in behaviour typically resulting in reproductive isolation (Futyama and Mayer, 1980; Dieckmann and Doebeli, 1999). These behavioural differences may drive the evolution of more visible differences between forms, which can indicate that divergence is occurring (Barluenga *et al.*, 2006). For example, visible differences between sexes can be indicative of sexual selective pressures that can act to create reproductive isolation (Wilson *et al.*, 2000; Knight and Turner, 2004). Because colouration is most commonly determined genetically (Price, 2006) and variation in colour morphology can thus be indicative of genetic divergence (Boughman, 2001; Lodé, 2001; Nosil, 2004; Endler *et al*., 2005), the presence of two distinct brushtail possum colour morphs on the Atherton Tablelands of Far North Queensland has been considered indicative of genetic divergence among brushtail possums in this region (Flannery, 1994). The reputed distribution of these morphs, occupying distinct but adjacent habitats, is also characteristic of parapatric divergence (Key, 1982). Yet we lack knowledge of whether there is any sexual dimorphism or breeding seasonality in these populations; characteristics that can be indicative of potential reproductive isolation through sexual selection or reduced polygyny (Trivers, 1972; Wilson et al., 2000; Isaac and Johnson, 2003; Knight and Turner, 2004). If the degree of divergence is great enough that the two colour morphs have evolved into distinct species, this would be highly significant as it would be the first

mammalian demonstration of non-allopatric speciation, thus altering our understanding of the speciation mechanisms acting on mammals.

At present the two colour morphs of brushtail possum are classified as subspecies of *Trichosurus vulpecula* (Kerle and How, 2008): the common, grey coloured brushtail possum, *T.v.vulpecula*, and the coppery, red coloured, brushtail possum, *T.v.johnsonii*. Common and coppery brushtail possums are reportedly found in close proximity in the Atherton Tableland region with the coppery form found in rainforest, and the common grey morph in the adjacent drier, less dense, sclerophyll forest (Winter, 1984). However there has been no study quantifying the colour morphology and distribution of brushtail possums on the Atherton Tablelands. Our understanding of these subspecies and their evolution is currently derived from samples lacking critical measurements and from unquantified field observations.

Thus the aim of this study was to examine the morphology and distribution of brushtail possums on the Atherton Tablelands, particularly along habitat gradients from dry sclerophyll forest to rainforest; where the coppery and common brushtail possums are reputedly found in close proximity. Understanding how these two morphs differ and interact is a vital first step in discovering how the coppery brushtail possum has evolved.

2.3. METHOD

2.3.1. Distribution of morphs of the brushtail possum

Distance sampling surveys of two rainforest fragments and two ecotonal habitat gradients (Fig. 2.1) were conducted between April and October in 2007 on the Atherton Tablelands, North Queensland, Australia. Distance sampling is a non-capture survey technique used to examine the population size and distribution of a particular animal group by searching along a transect or from a single point, and measuring how far located animals are from the transect/point (Buckland *et al.*, 2004). From these distance measurements the relationship between the

probability of animal detection and distance from the transect can be used to calculate the effective area surveyed in each habitat type (Buckland *et al.*, 2004). This method is preferable to trapping, which can be far more labour intensive; indeed trapping the coppery brushtail possum was generally ineffective as they tend not to come down out of the canopy, being able to cross from tree to tree directly or using vines. Distance sampling is also advantageous because unlike quadrat or fixed-width transects, all animals seen can be recorded, and we avoid the frustration of omitting records, for example, of animals sitting just a few meters beyond a fixed transect boundary.

The rainforest fragments surveyed were reputed to contain coppery brushtail possums, and their relatively isolated locations allowed a comparison of coppery brushtail possum distribution with, and in the absence of an ecotone. Although it is possible that there is a very small amount of migration of brushtail possums between each rainforest fragment and nearby rainforest, these patches are isolated from dry sclerophyll forest. The first rainforest fragment (F1 in Fig 2.1), 26 hectares of the endangered type 5B complex notophyll vine forest (Tracey, 1982), is located on the edge of the town of Atherton (55K 338340, UTM 8093580), and is surrounded by a matrix of farmland and urban properties. The nearest forest tract to this fragment lies approximately 2500m to the west and is type 14 dry tall open forest (Tracey, 1982). The second rainforest fragment (F2 in Fig 2.1), 19 hectares of type 5B rainforest (Tracey, 1982; Freeman *et al.,* 2008) is located along a road opposite the Curtain Fig National Park, near Yungaburra (55K 347490, UTM 8090830). Primarily surrounded by cleared fields, this fragment is approximately 600m from the national park, which conserves 260 hectares of 5B rainforest (Tracey, 1982; Freeman *et al.,* 2008).

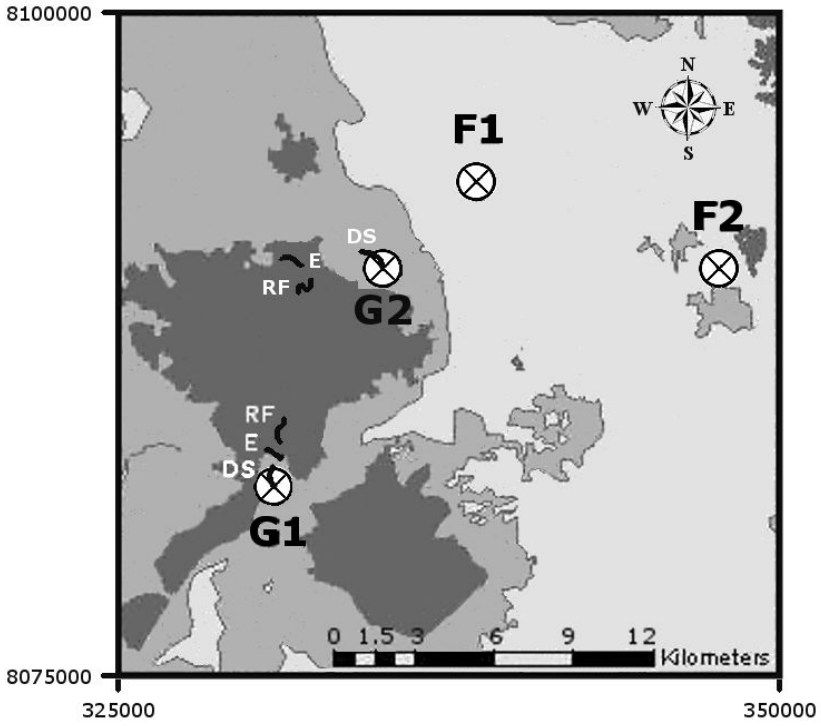


Fig. 2.1. The sites used for distance sampling surveys in the region of Far North Queensland, Australia. The white circles with crosshairs are the locations of the four sites. 'G1' and 'G2' indicate the location of the two habitat gradient transects in Herberton State Forest, with transects in dry sclerophyll forest (DS), mosaic ecotone (E) and rainforest (RF) also shown for each. 'G1'is also the gradient along which grey and coppery brushtails were captured and measured. 'F1' is the rainforest fragment north of the town of Atherton, and 'F2' is the rainforest fragment near the Curtain Fig National Park. The distribution of rainforest (shaded in the darkest grey), forest (the middle shade of grey) and unforested areas (light grey) is also shown. This simple grading of forest density suggests that the G2 ecotone is within rainforest, however this is inaccurate (Tracey, 1982). The map shows the region within the coordinates 55K: 325000-350000, UTM: 8075000-8100000 in AGD55 UTM grid, and was created using ArcGIS v.9.2 (ESRI Inc., 2006), incorporating maps from James Cook University (School of Tropical Environment Studies) and the Commonwealth of Australia (Geoscience Australia, 2001).

The two habitat gradients (G1 and G2 in Fig. 2.1) sampled were located in Herberton State Forest (55K 332000, UTM 8083000) on opposite sides of Mt Baldy, and were separated by a straight-line distance of 4.4 kilometers. At lower altitudes the forest in each case is type 14 tall open forest (Tracey, 1982). With increasing altitude there is a transition from dry sclerophyll forest to an ecotone approximately one kilometer wide, which is type 13 vine forest with eucalyptus (Tracey, 1982) This ecotone then gives way to rainforest consisting of type 9 simple microphyll vine-fern forest (Tracey, 1982). These two sites were chosen because they had gradients in vegetation, were inhabited by brushtail possums, had tracks that facilitated access to each habitat type, but which were closed to the public without a permit, which from a safety and public relations perspective is advantageous when using firearms to tranquilise mammals.

Three transects were established along each of the habitat gradient sites: one in each habitat type; dry sclerophyll forest (labeled 'DS' in Fig. 2.1), ecotone ('E' in Fig. 2.1) and rainforest ('RF' in Fig.2.1). These habitat types were identified using established criteria that describe the species composition and density of these vegetation groups (Tracey, 1982). Three replicate surveys were conducted for these six transects, giving a total of eighteen surveys. In addition, each rainforest fragment was surveyed twice. I attempted to establish 1000m transects in all habitat types, however there were two exceptions to this. One rainforest fragment was only 900m long, making it impossible to establish a longer transect, and the narrow ecotone along one of the habitat gradients allowed only a 600m transect to be established; unfortunately there was not another nearby, wider area of ecotone forest. In one replicate survey of a habitat gradient rainforest transect, visibility became too poor due to cloud to continue beyond 650m; the other two surveys of this transect sampled the full 1000m. Surveys were conducted at night, using hand-held spotlights to detect the eyeshine of mammals along the transects. Where possible surveys were conducted at a similar time in the lunar month and were not conducted in rain. An average walking pace of 25meters/min (1.5 Km/h) was maintained, with the time required to take distance measurements and notes excluded from the survey time. The number of surveyors was also kept as consistent as possible,

with just two people searching in most surveys, to standardize survey effort and levels of acoustic and visual disturbance. For each habitat gradient survey replicate, the dry, ecotone and rainforest transects were surveyed in a single night, but with varying order and direction along each transect. Straight line distance to the animal from the spotting location, perpendicular distance from the transect line, height of the animal's location, canopy height and GPS location of the observer when standing on the transect, perpendicular to the animal (i.e. distance along the transect), were recorded for each animal seen, and possums were classed as either coppery, grey or 'colour not visible', which could occur when the possum was simply too far away or too well hidden by foliage. All arboreal mammal species were recorded.

I tested the probability that the distribution of grey and coppery brushtail possums along a habitat gradient was being produced by chance. Having observed a particular number of brushtail possums in each habitat type, I randomized the observations from the full habitat gradient transect for 5000 replicates, sub-sampling the observed number of possums for each habitat (using a macro in Microsoft Excel (Microsoft Corporation, 2003; appendix 2.0)), and calculated the probability that the observed numbers of each colour morph in each habitat could have been produced by random assortment across the habitat gradient for comparison with the frequency distribution generated. Brushtail possum density was calculated using the method of Buckland *et al.* (2004; calculations in appendix 2.1) with all animals sighted used to calculate f(x), g(x), w, μ, before calculating the brushtail possum population density and its standard error. Other statistical comparisons for each colour morph of mean distance and height values for each survey were calculated using Minitab statistical software (Minitab Inc., 1998).

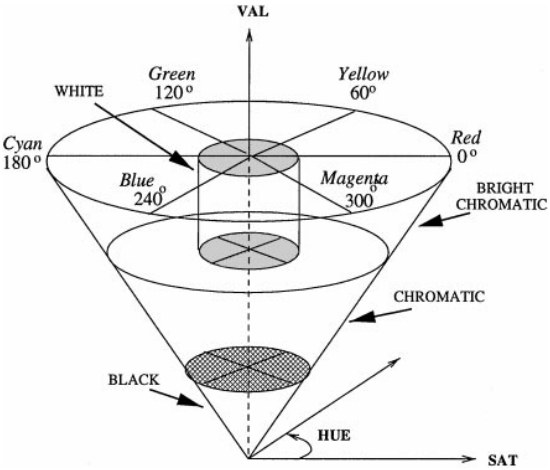
2.3.2. Morphology

A total of 69 live and road-kill brushtail possums were collected in multiple locations across the Atherton tablelands (Appendix 2.2). Of these, seven sets of capture measurements and DNA samples were generously donated by Associate Professor John Winter after trapping possums in another study. For the remaining possums collected, live possums were captured either with baited

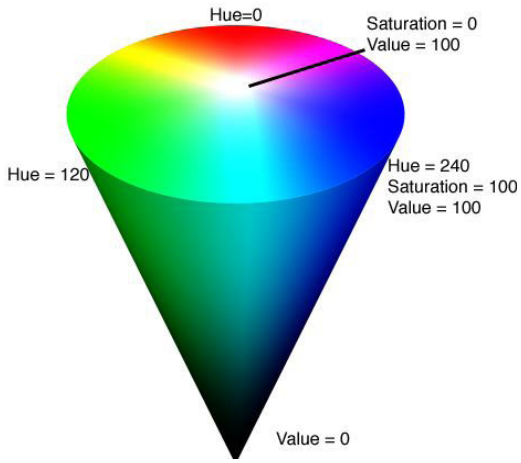
cage traps (1326 trap nights) placed at tree bases in forests, and on roof-tops and window-sills in Milla Milla, or using darts fired from a gas-powered air rifle (Black Wolf, Tranquil Arms Company, Seymour, Vic., Australia), which were loaded with 30mg Zoletil, (Virbac Pty Ltd) in 0.15 ml, in accordance with permits WISP03171005, ATH05/021, ATH06/019, ATH07/024, and ethics approvals A856, A1261 and A1262. Possums captured in cage traps were not sedated as they are relatively easy to handle once in a cotton bag

Measurements of body mass, head length and width, ear length and width, tail length, hind limb length from the top of the knee to the heel, foot length, testes width and length, and pouch condition were obtained where possible for each possum. ANOVA, PCA and T-tests using these measures were conducted using SPSS statistical software (SPSS Inc., 2007).

Photographs were taken of the fur at six points on the body: belly, chest, shoulders, flank, thigh and rump. In order to standardize the photographs taken, the Canon 5.0MP Powershot G5 camera with a ring flash attached, was fitted to a cylindrical tube and the same settings were used for each photograph, as follows: shutter speed: 2000, aperture: 8.0, ISO: 50. To take a photo, the camera was fitted into the tube, and the end of the tube pressed onto the possum's fur in such a way that no external light contributed to the photograph. At the end of the cylindrical tube was a circular piece of grey card, cut to allow a square 7cm by 7cm area of fur to be photographed. Analysis of the RGB (red green blue) colour composition, measuring the frequency with which each colour occurred in the selected region of the digital photograph, was conducted using MVH Image freeware (Pickle and Kirtley, 2004). Approximately 100,000 pixels were sampled in each assessment of fur colour, and 10,000 for the control grey card. These percentage RGB values were converted to a raw score out of 255, as is the range for RGB values, and then into HSV (hue saturation value) colour space (Fig. 2.2; Androutsos *et al.*, 1999). These HSV fur colour values were then corrected for slight variations in the flash angle and battery life using the grey card at the bottom of the photography tube as a standard. The HSV values of the card in an individual photograph were subtracted from the



A. (From Androutsos *et al.*, 1999)



B. (From Caradini, 2001)

Fig. 2.2. These diagrams depict HSV colour space a) diagrammatically, and b) graphically. Hue is measured in degrees; a colour wheel, shown on the top surface of the cone. Value is a measure of black shading and is shown as the vertical axis running through the center of the cone. A low percentage value reflects a black colour, and a high percentage value demonstrates a white colouration. Saturation is a measure of brightness and colour intensity. In HSV colour space it depicted as the distance from the center of the cone. High percentage saturation will produce a bright, more intense colour.

mean HSV values calculated from all the card measurements to determine the variation of any specific photograph from standard conditions. This difference was then added to the HSV values of the fur colour. Hue is measured in degrees and is more easily understood using a colour wheel (Fig. 2.2). Filling the 360° range of the colour wheel are a plethora of hues, with the primary hues of red, yellow and blue located at 0°, 60°, and 240° respectively. With the degree of hue for fur colour ranging between 260.7º (-99.3º) and 149.17º, measurements between 260º and 360º were for the purpose of statistical analyses converted to a negative number, lest the calculation of means mistakenly imply that the brushtails have a green hue (Fig. 2.2). Mean values calculated for comparison of colour by habitat did not include chest measurements, as variation in the number of males captured in each habitat would probably cause bias due to the greater secretions of sternal scent glands in males, which produce a slick, darkly pigmented stripe in the surrounding chest fur. This dark patch is generally larger and more prominent in males.

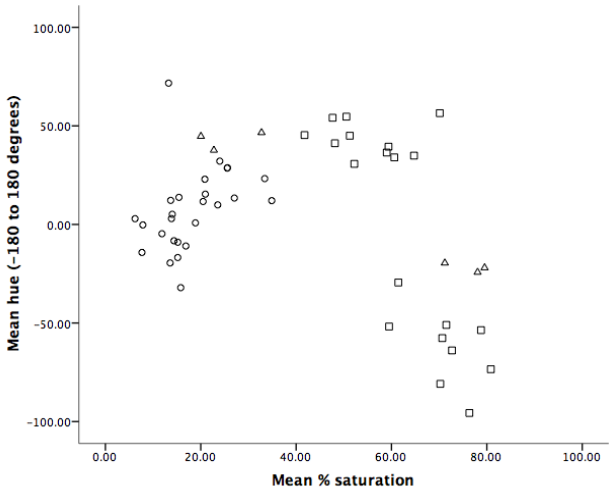
2.3.3. Breeding synchrony

Calculations of birth dates of pouch young were made with either head or body length, using the equations of Tyndale-Biscoe (1954) and Gemmell and Hendrikz (1993) which describe brushtail possum (*T.v.vulpecula)* developmental growth rates. For the purpose of statistical comparison of the reproductive timing of coppery brushtail possums with that of grey brushtail possums with a t-test, the number of days until birth from the first of June each year was used to define the time of breeding. This date was chosen as it marked the start of the month in which six of the nine young were born. It should be noted that because collection of data on pouch young was incidental to the main study, sample sizes are small, with only the pouch young of 4 female grey brushtail possums and 5 female coppery brushtail possums available for comparison.

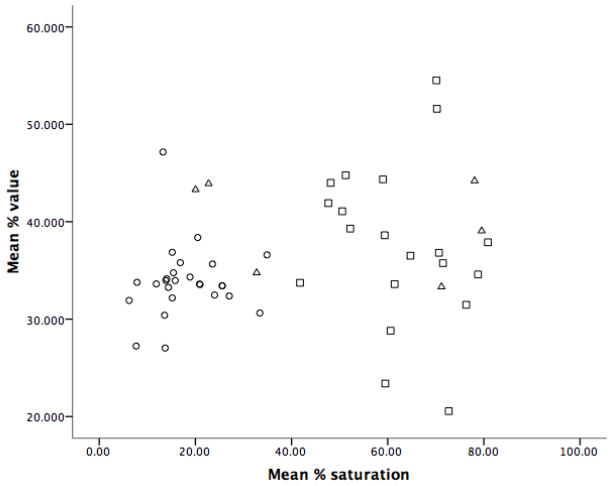
2.4. RESULTS

2.4.1. Morphology of captured brushtail possums

The results demonstrated that there are two main colour morphs of brushtail possum on the Atherton Tablelands; coppery and grey. Although there was a small amount of inter-site variation in these two fur colours, there were no intermediate colour morphs. Fur colour differed significantly between possums in different habitat types (Figs. 2.3, 2.4). Differences in fur colour saturation were most definitive, with significant saturation differences occurring between possums from different habitats at every photograph site on the possum's fur (belly: $F_{2,44}$=69.012, P<0.001; chest: $F_{2,34}$=31.996, P<0.001; flank: $F_{2,43}$ =82.953, P<0.001; rump: $F_{2,44}$=101.373, P<0.001; shoulder: $F_{2,44}$=42.974, P<0.001; and thigh: $F_{2,43}$=48.23, P<0.001), as well as in mean individual fur colour ($F_{2,46}$=112.371, P<0.001). Possums from the dry sclerophyll forest were less saturated than possums from rainforest. The percentage Value for mean individual fur colour did not differ significantly between habitats ($F_{2, 46}$=2.318, P=0.11), though on fur of the rump ($F_{2, 44}$=9.368, P<0.001), and thigh ($F_{2, 43}$ =9.888, P<0.001) there was significant variation, with dry sclerophyll possums having a fur colour that was a darker shade of grey than possums from ecotones and rainforest. Likewise there was no significant variation in mean hue across all fur photograph sites ($F_{2, 46}$=0.348, P=0.708), but possums from different habitats did have significantly different shoulder hues ($F_{2, 44}$=53.06, P<0.001), with dry sclerophyll possums displaying a red-orange hue while ecotone and rainforest possums displaying a red-purple hue on their shoulders. Brushtails from the ecotone displayed colouration akin to either 'rainforest' or 'dry sclerophyll' fur types; they did not form a separate colour morph or an intermediate one (for example, an intermediate morph may have saturation values of thirty to fifty percent; Figs. 2.3 and 2.4). Rainforest brushtail possums appear to express one of two reddish hues; either a slightly orange-red or a slightly purple-red hue (Fig. 2.4, photographs B and C). Expression of these different hues was not determined by sex (t=1.09, df=17, P=0.29), whether the animal was an adult or juvenile (t=2.42, df=4, P=0.073), nor by rainforest type



A



B

Fig. 2.3. The mean composition of brushtail fur colour in HSV colour space for animals living in dry sclerophyll forest (circles) ecotone habitat (triangles), or rainforest (squares). Hue, the colour of the fur, is measured in degrees and is scaled between -180º and 180º, to avoid the calculation of means resulting in brushtails being assigned a green hue (Fig. 2.2). Value is the HSV colour component that indicates the amount of black and white in the colour, with increased percentage Value associated with a colour that is less black. Saturation is a measure of how intense the colour is, with a high saturation value associated with bright colours. Graph A shows the colour morphologies for possums of each habitat using the saturation and hue. Graph B shows how colour morphology differs across the three habitat types in measures of saturation and value.

A

B

C

Fig. 2.4. Photographs of shoulder fur representative of the brushtail possum colour morphs observed in dry sclerophyll and in rainforest, which are distinct in the amount of fur colour saturation. In photograph A the dry sclerophyll 'grey brushtail' form is shown, and in photographs B and C the two morphs of rainforest 'coppery brushtail' colour are pictured. In photograph B the red-orange coppery morph with hue greater than 0º is shown. In photograph C the red-purple coppery morph with a hue less than 0º is shown. Brushtail possums from the ecotone were either grey or coppery brushtails; they did not display a distinct colour variety.

(t=-1.96, df=18, P=0.066). However the categorization of rainforest possums by capture site did demonstrate significant differences in hue of possums at different rainforest sites geographically separated by as much as thirty-four kilometers (appendix 2.2; $F_{3, 18}$=13.20, P<0.001).Regression of fur colour on the distance of a possum from the ecotone, demonstrated that neither hue (R^2=0.003, $F_{1, 31}$=0.108, P=0.744), saturation (R^2=0.081, $F_{1, 31}$=2.735, P=0.108), nor value (R^2=0.004, $F_{1, 31}$=0.117, P=0.735) correlated significantly with distance from the ecotone.

In addition, during four months of observing of a grey back young with a coppery mother living along the ecotone of a habitat gradient, neither the young nor his mother appeared to change colour. This lack of colour change consistent with observations of coppery brushtail possums kept in a captive feeding study by DeGabriel *et al.* (2009), (including possums analysed here) which were kept for approximately five months on a basal diet of cereal and common vegetables. For 28 days during this period plant secondary metabolites were added to their diet. After five months these possums had retained their distinctive colour, maintaining the same high saturation fur colour as that of non-captive coppery brushtails (t=-1.77, df=20, P=0.092)

2.4.2. Body dimensions by morph

Comparison of brushtails by colour morph demonstrated that coppery brushtails have significantly longer tails than greys (t=5.02, df=64, P<0.001), being on average 34 ± 6.8 mm longer. Coppery ear length (t=-4.41, df=60, P<0.001) and width (t=-3.20, df=50, P=0.002) were significantly less than that of greys, on average 8 ± 1.8mm shorter and 3.3 ± 1.1mm narrower (appendix 2.3). Coppery brushtails also had significantly shorter hind legs (t=-2.38, df=60, P=0.021), greys had an average hind limb heel-knee length 3.6 ± 1.5 mm longer (appendix 2.3). Coppery and grey possums did not differ significantly in body mass (t= 0.178, df=52, P=0.86), head length (t=-0.182, df=57, P=0.857), head width (t=0.183, df=46, P=0.856), or foot length (t=-0.219, df=55, P=0.827; appendix 2.3).

2.4.3. Sexual Dimorphism

No sexual dimorphism was evident for body size among possums with coppery colouration. This was the case when all individuals were compared, and for the Herberton State Forest transect specifically. Grey brushtails from Herberton State Forest displayed no significant sexual dimorphism, however among all grey brushtails sampled, significant sexual dimorphism was detected in foot length ($t=-2.589$, $df=34$, $P=0.014$), with male foot length on average 1.7 ± 0.9mm longer and in the mean hue of fur colour ($t=-2.287$, $df=25$, $P=0.031$), with males having a hue that was 19.0º ± 8.3º more orange than females, though with a low saturation (Fig 2.3) there is little expression of this colour.

2.4.4. Distribution of brushtail morphs

A total of eight grey brushtails and thirteen coppery brushtails were sighted in the two rainforest fragments surveyed. An additional five brushtails were either too far into the fragment or too obscured by leaves to assess their colour. Along the two habitat gradients a sum of nine grey and no coppery brushtails were recorded when distance sampling the dry sclerophyll forests. Randomization tests indicate that this is not due to chance ($z=4.12$, $P<0.001$). Furthermore, of the twenty-one brushtails trapped (across 853 trap nights) in dry sclerophyll forest, all were grey. One grey brushtail was observed in the ecotone but no coppery brushtails, however three coppery brushtails were found in this habitat when capturing possums for detailed morphological measurements. Along the rainforest transects a total of twelve coppery brushtails were sighted with distance sampling surveys, but no grey brushtails. This assortment was also unlikely to be due to chance ($z=4.48$, $P<0.001$). In addition, while spotlighting for possums to capture in the rainforest (including 26 nights of clear weather, which gave high enough visibility to often note the colour of possums seen but not captured) I saw no grey brushtails. As the effort to capture each coppery was usually quite large, I would typically see between five and ten other rainforest brushtails before finding one able to be safely darted, and visiting the site over several years, I became familiar with many individuals and their preferred trees; identifying them from scars and patterns of ear tears they collect from fighting; leading me to believe that I was seeing most, if not all, of

the brushtails along the rainforest section in which I was darting. None were grey. Seven coppery brushtails were recorded as carrying coppery back young, three grey brushtails were carrying grey back young, and three coppery females with grey back-young were observed: two in fragments, and one in the ecotone of Herberton state forest.

2.4.5. Height and depth characteristics of brushtail possums spotted

Within rainforest fragments there were no significant differences between the colour morphs with regards to their mean height from the ground (t=0.42, df=4, P=0.69), relative height (animal height as a proportion of canopy height; t=-0.06, df=4, P=0.95) or the observed distances off the track (t=1.98, df=2, P=0.19) recorded with each survey. Along habitat gradients there was no difference between the two colour morphs with respect to their observed distance from the track (t=-1.62, df=4, P=0.18), height (t=0.14, df=3, P=0.89), nor their relative height (t=1.3, df=5, P=0.25).

2.4.6. Brushtail population density along habitat gradients

The density of brushtail possums found along habitat gradients differed significantly by forest type ($F_{2, 15}$=10.52, P=0.001). Along habitat gradients the density of brushtail possums in dry sclerophyll forest was 1.588 ± 0.686 brushtails per hectare. In the ecotone there were 0.182±0.132 brushtails per hectare. In the rainforest there were 3.392 ± 0.559 possums per hectare.

2.4.7. Reproductive synchronicity

There was no significant difference between coppery and grey brushtail possums along a habitat gradient in their timing of reproduction, though it should be noted that sample sizes were small (t=1.01, df=7, P=0.35; appendix 2.4).

2.5. DISCUSSION

2.5.1. Identification of brushtail morphs

Along a habitat gradient of dry sclerophyll forest, ecotone and rainforest, brushtail possums exhibited two distinct colour morphologies (Figs. 2.2 and 2.3). Dry sclerophyll and rainforest brushtail possums differed significantly by their fur colour saturation level across all parts of their body, creating two distinct morphs: coppery and grey. Brushtail possums from the ecotone were either coppery or grey, but not an intermediate or separate colour. In addition, among coppery brushtails, defined as having fur colour that has high percentage saturation, there were two types of red hue expressed: a red-orange hue or a red-purple hue (Fig. 2.4, photographs B and C). The expression of different coppery brushtail hues differed significantly between but not within capture sites. This may be indicative of differing environmental effects between forest sites, or genetic divergence between their possums, however the discovery of this previously unreported variation in hue may with further investigation provide an important avenue of investigation into coppery brushtail evolution.

Apart from fur colouration, the coppery and grey colour morphs displayed several significant differences in body size. Coppery brushtails had significantly longer tails, shorter hind legs, and ears that were shorter and narrower than grey brushtails'. No sexual dimorphism was detected in either colour morph along the habitat gradient, though in grey brushtails from all across the Atherton Tablelands some sexual dimorphism was apparent in mean hue colour and in foot length. However neither of these characteristics is useful in defining the body-shape morphology of the two brushtail colour morphs. Past descriptions of coppery brushtail possums, which were largely based on qualitative assessments, did not suggest that coppery and grey brushtail possums from the Atherton Tablelands differed across such an array of morphological characteristics (Winter, 1984; Kerle *et al.*, 1991; Flannery, 1994; Collins, 2003). My findings contradict the qualitative observations by Winter (1984) that coppery brushtails are heavier and larger in overall dimensions than grey brushtails. The ability, described here, to explicitly identify a coppery brushtail

possum, not only by fur colour but by body-shape morphology establishes a significant platform from which to then evaluate their distribution.

Distance sampling data of the wider distribution of brushtail colour morphs demonstrated that along habitat gradients grey brushtails are not found in rainforest and coppery brushtails do not occur in dry sclerophyll forest. This, together with the characteristics observed above, confirmed the brushtail possum distributions described by Winter (1984), and substantiated the parapatric distribution of the two forms.

Fur colour in brushtails does not appear to change once established. In a five month-long captive study (that included possums analysed here), coppery brushtail possums fed on a basal diet of cereal and common vegetables for all but 28 days during this period had not changed colour. Recurrent field observations in the current study of a grey back young with a coppery mother living along the ecotone also demonstrated that over the four months that this young was observed developing, following his mother and closely matching his behavior to hers, neither the colour of the mother nor her offspring changed. Thus any determination of colour, whether genetic or environmental, is likely to occur in early pouch life, when brushtail hair structure is developed (Gibbs, 1938) and appears to be permanent.

Quantifying the morphology and distribution of the coppery and common grey brushtail possum on a local scale across the Atherton Tablelands was a crucial first step in understanding the evolution of the coppery brushtail possum. With this information we can now narrow the range of possible mechanisms by which this unique colour morph has evolved to occupy its present distribution.

2.5.2. Genetic determination of colour

The genetic determination of fur colour in the brushtail possum would seem a likely mechanism, given that colouration is genetically determined by highly conserved genes across a wide range of species: birds (Theron *et al.,* 2001; Kerje *et al.,* 2003; Doucet *et al.,* 2004, Nadeau *et al.,* 2006; Baião *et al.,* 2007),

reptiles (Rosenblum *et al.,*2004) and eutherian mammals (Miller *et al.,*1997; Kijas *et al.,* 1998; Wada *et al.,*1999; Everts *et al.,* 2000; Rouzaud *et al.,* 2000; Rieder *et al.,* 2001; Kerns *et al.,* 2003; Nachman *et al.,* 2003; Hoekstra *et al.,* 2004; Vage *et al.,* 2005; Fontanesi *et al.,* 2006; Hoekstra *et al.,* 2006; Mengel-Jørgensen *et al.,* 2006). Indeed Kean (1971) believed that genetic determination of colour explained colour distributions in New Zealand and Tasmanian brushtail possums. The disjunct distribution of Tablelands brushtail possums, with the strong habitat affinities of each colour morph along habitat gradients is indicative of parapatric divergence, and possibly speciation. Although this dichotomous distribution may indicate the very first detection of non-allopatric speciation in a mammal, divergence and speciation without a geographic barrier to gene flow has been demonstrated in other taxa (Bush, 1969; Tauber and Tauber, 1989; Chesser and Zink, 1994; Schneider *et al.,* 1999; Slabbekoorn and Smith, 2002; Graham *et al.*, 2004; Barluenga *et al.,* 2006). Thus research into the evolutionary mechanisms and history of the coppery and grey brushtail possums on the Atherton Tablelands may not only provide novel information about wider mammalian evolutionary mechanisms, but also demonstrate the commonality of evolutionary processes across a diverse range of taxa.

If fur colour in the coppery and common brushtail possum is genetically determined, then the morphological distribution observed may be the product of either secondary contact or parapatric divergence. The results of Collins (2003), though lacking small-scale detail, suggested that tablelands brushtail possums are reciprocally polyphyletic, with both colour morphs found in multiple clades. Thus it is unlikely that the observed distribution is the result of secondary contact between previously isolated, reciprocally monophyletic populations. The presence of two adjacent but distinct morphs is indicative of parapatric speciation, however in order to overcome the homogenizing effects of gene flow and diverge to the extent observed, there would need to be a behavioural barrier to gene flow along the habitat gradient (Key, 1982).

Breeding seasonality is one reproductive behavioural mechanism that can restrict gene flow, if males are limited in the time they have to find a mate (Trivers, 1972). Brushtail possums along this habitat gradient appeared to be synchronous breeders, with no significant difference between the two colour morphs in their seasonality. As male brushtail possums do not typically increase their testes volume to increase their reproductive competitiveness, it is sexual dimorphism with a bias towards larger males that is generally indicative of the increased sexual competition that arises from asynchronous breeding (Gilmore, 1969; Isaac and Johnson, 2003). The lack of sexual dimorphism in body size displayed along a habitat gradient suggests that the breeding season of Tablelands brushtail possums is synchronous and that there is less potential for polygyny (Isaac and Johnson, 2003). When reproductively active females are common for a limited, shared time period there is less benefit for males that invest in greater body size compared with an asynchronous system where the defense and competition for one of a few reproducing females is advantageous to gain paternity (Isaac and Johnson, 2003). However, while there appears to be no temporal barrier to gene flow between the two colour morphs, the mating behaviour of this species could inhibit gene flow in a synchronously breeding population. Male brushtail possums typically court females by following them for up to a month (Winter, 1976). In the case of Atherton Tablelands possums, if a possum in its non-preferred habitat is subject to higher rates of predation, due hypothetically to a greater conspicuousness associated with an individual's fur colour, the subjection to such selective pressures over the month needed to court a female may limit the ability of grey males to successfully court females in the rainforest or coppery males to court females in dry sclerophyll forest.

Another potential behavioural restriction on gene flow may arise from differing habitat preferences (Lodé, 2001; Edelaar *et al*., 2008). The low population density of brushtails recorded in the ecotone may be indicative of it acting as an ecological barrier along the habitat gradient by reducing the number if interactions between individuals of different colour morphs and affecting rates of migration between rainforest and dry sclerophyll forest. With just 0.182 possums per hectare in the ecotone, the rainforest supported 18.6 times more

brushtails per hectare, and the dry sclerophyll forest was inhabited by 8.7 times more brushtails. Dispersal studies have demonstrated that other mammal species will avoid migrating into habitats different to their natal environment (Haughland and Larson, 2004; Mabry and Stamps, 2008). If this is also the case for the dispersal of brushtail possums, habitat preference could act to restrict gene flow between ecologically distinct forms by affecting both the interactions between adults of different colour morphs that hold territories closest to the mosaic ecotone, and by discouraging the male biased dispersal of juveniles (Clout and Efford, 1984) into habitat not typical for their morphology (Rice, 1984; Edelaar, 2008).

Brushtail possums from the ecotone did not display a colour morph distinct from the dry sclerophyll and rainforest possums, suggesting that if genetically determined, colour is not a trait encoded by alleles or combinations of genes that might allow the development of a colour with intermediate saturation levels; one of these two colours would be the dominant trait. In surveying populations from both habitat gradients and rainforest fragments, three coppery females were observed with grey back young. No grey females were observed with coppery back young. However these were incidental sightings during surveys and not the results from a dedicated survey of back young colour. Nonetheless these few observations are consistent with coppery colour as a recessive characteristic and grey the dominant trait. Indeed grey fur colour is the phenotype most common in brushtail possum populations in both Australia and New Zealand (Kean, 1971). At least one brushtail colour morph, the rare golden brushtail fur colour, appears from preliminary observations of inheritance patterns and distribution (Fingland, 2005) to be a genetically determined trait recessive to grey colour. If grey coat colour is also dominant to coppery pelage, then even with limited gene flow, and even if multiple genes are involved in coppery fur expression, we would expect along a habitat gradient for there to be a low frequency of grey coloured brushtails in the rainforest as dominant alleles are exchanged (Wright, 1931). This was not observed; rainforest brushtails can be a dark red/black colour but no grey possums were detected in this habitat type. Thus any theory of genetic determination of coat colour in brushtail

possums also requires an explanation for the absence along habitat gradients of a small number of grey brushtail possums in the rainforest, which we would otherwise expect to observe with gene flow or with occasional cross-habitat migration events between the two adjacent forest types.

There are two candidate explanations for the absence of grey brushtails in rainforest along habitat gradients. The first is that the ecotone deters brushtails, acting as a barrier to both gene flow and dispersal despite its small size relative to brushtail possum home ranges (Clout and Efford, 1984), thus preventing any dispersal of greys into rainforest, and stopping gene flow across the gradient. The morphological divergence observed could have evolved via a drift, selection or a combination of the two (Dobzhansky, 1970). The second candidate explanation is that strong natural selective pressures such as predation reinforce the genetic divergence begun by differing ecological preferences (Key, 1982). The narrow ecotone may not prevent dispersal or gene flow along a habitat gradient. Indeed the observation of brushtails living in this mosaic habitat indicate the potential for mating between both forms in this intermediate zone; however selection would act against possums in habitat not occupied by their colour morph, such that grey possums might share genes with coppery brushtails in the ecotone, but if a grey possum dispersed into rainforest, its fitness would decline dramatically.

Predation and nutritional tolerances are two key pressures that might affect fitness in this manner (Mundy, 2007; DeGabriel *et al.,* 2009). The colour of an animal can be crucial in camouflage, and is thus a key characteristic upon which natural selection can strongly act (review by Mundy, 2007). In the forests of the Atherton Tablelands, brushtail possums, which are nocturnal, are preyed upon by snakes and by birds such as owls, both of which may be able to distinguish colour with moonlight, or at least detect the higher degree of visual contrast associated with the increased conspicuousness of a possum in their non-preferred habitat (Hecht and Pirenne, 1940; Kanowski, 1998; Shine, 1998; Hayes *et al*., 2006). Nutritional tolerance appears to differ between brushtail populations and colour morphs, and problems accessing a suitable diet may

cause a reduced fitness in brushtails that move into the habitat of the opposite colour morph (DeGabriel *et al.*, 2009). If the fur colour of these brushtails is determined genetically, these two hypotheses accounting for the distributions observed can be tested by examining the populations for the distinctive genetic signatures characteristic of restricted gene flow and selection. A demonstration that parapatric speciation occurs in mammalian evolution would destabilize our present hypotheses that geographic separation is paramount in the evolution of new mammalian species and significantly alter our perspectives of environmental variation and its importance in generating biodiversity. However a genetic determination of fur colour is not the only candidate hypothesis to explain the observed morphological distribution of brushtail possums across the Atherton Tablelands.

2.5.3. Environmental determination of a phenotypically plastic trait

Climate and dietary effects are the two key ways in which colour can be environmentally determined in other species (Iljin and Iljin, 1930; Fox, 1962; Hill, 1993; Vage *et al.,* 2005; Cropley *et al.,* 2006; Hays *et al.,* 2006). However, the detection of both coppery and grey brushtail forms in both the ecotone habitat and in rainforest fragments would seem to make a climatic influence unlikely, particularly if colour is determined during early development while the young is *in utero* or in the pouch (Gibbs, 1938). This is because in these disturbed rainforest fragments and in more open habitat types, young brushtails of different morphs would very probably be exposed to the same temperature, humidity, and photoperiod prior to birth, when developing in their mother's pouch, and once emerged from the pouch (Gemmell *et al.*, 1997). Distance survey measurements indicated that coppery and common brushtails in rainforest fragments were observed at similar heights, relative heights and distances from the track. Given that for nocturnal arboreal mammals the most important determinate of microclimate temperature and humidity is the proximity to the forest edge (Chen *et al.*, 1993; Pohlman *et al.*, 2009) this suggests that the two morphs are exposed to similar microclimates within rainforest fragments. Thus I considered phenotypic plasticity mediated by climate to be unlikely as the cause of the different fur colours of grey and coppery brushtail possums. In the case of the coppery and grey brushtail possums of the Atherton Tablelands this

also makes it unlikely that genes effecting humidity tolerance are linked with the genes determining fur colour, as suggested to be the mechanism linking colour and habitat in New Zealand and Tasmanian brushtail populations (Kean, 1971).

The permanent determination of colour by diet has been demonstrated in laboratory mice (Cropley *et al.,* 2006), but not in any wild mammalian species. In this laboratory example, the varied shades of brown fur in different litters were the product of an congenital trait determined *in utero* by the diet of their mother (Cropley *et al.,* 2006). Methyl donors in the diet of the mother can inhibit the transcription of certain alleles on the *agouti* gene of her offspring, such that with increased consumption of these chemicals her litter would develop fur that was a darker shade of brown (Waterland and Jirtle, 2003; Cropley *et al*., 2006). Unlike these mice, brushtail possums from the Atherton Tablelands displayed fur colours that were dichotomous by saturation levels, with no intermediate shades between coppery and grey. This may suggest that there is a simpler developmental pathway controlling fur colour saturation that is either activated or inactivated by the presence of a key chemical in the diet. However the variation observed in the hue of coppery brushtail possums from different rainforest sites may be indicative of some correlation between the quantity of key nutrients and the shade of colour produced, as was the case in mice (Cropley *et al.,* 2006). Indeed if possum fur colour is determined via a similar pathway as that observed in mice, this may explain not only why different rainforest sites produced coppery brushtail possums with differing hues, but may also reflect a wider effect of diet on brushtail fur colour if one considers that in both New Zealand and Tasmanian brushtail possum populations, the more melanistic forms were consistently found in complex, dense, wet habitats, and grey brushtails had an affinity with drier, less complex, more open woodland (Guiler and Banks, 1958; Kean, 1971). However if dietary intake does determine fur colour hue on a fine scale, we might expect to see intermediate shades of red in animals closer to the mosaic ecotone, where maternal diet may be more mixed, and where the trees providing nutrients important in determining colour would likely be less abundant. This was not the case. Neither hue, saturation, nor value varied with distance from the ecotone.

Furthermore, in rainforest fragments with limited access to non-rainforest vegetation we would expect more uniformity in colour and no grey brushtails. In contrast, eight grey brushtails were sighted during the four surveys performed.

The presence of both colour morphs in rainforest fragments complicates the argument that diet determines offspring fur colour. Even if the phenotype produced can only be grey or coppery and not a variety of intermediate shades as has been demonstrated with mice (Cropley *et al.,* 2006), we would expect that the common rainforest habitat shared by grey and coppery brushtails in rainforest fragments would result in a population that would all be a similar colour, not the distinct coppery and grey forms observed. Yet not only are there grey coloured adults, but some coppery females in fragments had grey coloured back young. Perhaps there is fine scale resource partitioning within these fragments which results in dietary variation between female brushtails, such that if some nutritional threshold is not reached, the biochemical pathways needed to produce coppery fur colour will not be activated, and a female will have grey offspring. A biochemical pathway controlling melanin production is one such candidate for controlling a switch between coppery and grey colour. In other species, melanin biochemical pathways control the amount of phaeomelanin (red/yellow colour) and eumelanin (brown/black colour) in each hair to produce different fur colours (Fontanesi *et al.*, 2006). Given that coppery and grey possums express similar hues, it may be that they also have similar ratios of phaeomelanin to eumelanin in their fur, with their observed differences in saturation produced by coppery brushtail fur containing larger quantities of these melanins.

The ratio of eumelanin to phaeomelanin expression has been associated with differences in bone density in barn owl populations, suggesting that colour expression may have a cost (Roulin *et al.*, 2006). If fur colour is determined by maternal diet, then early nutrition may also determine the other body-shape morphological differences associated with the two colours expressed: tail and leg length, and ear size. If for example, phaeomelanin expression confers a greater cost than eumelanin expression, or if the diet associated with this

expression results in some nutritional deficiency, we might expect coppery brushtails to demonstrate a general morphological decrease in characteristics such as body size or mass compared to grey brushtail possums. This was not the case, suggesting that any trade-off with colour or nutritional effect is a complex one, affecting only a few characteristics. If there is an effect of nutrition only on ear size, leg length, and tail length; the morphological measures that differ between the two forms; we might expect one morph to have smaller measures for all these traits. Coppery brushtails have legs and ears that are typically smaller than grey brushtail possums, yet they have longer tails: there is no clear demonstration of a simple nutritional effect on these other body-shape morphological characteristics. Thus, if diet is determining fur colour, the differences between coppery and grey brushtails in tail, leg and ear length are probably produced through complex pathways in which tail length is affected differently by diet than the other traits (Scheiner *et al.*, 1991; Thompson, 1992).

Selective pressures may also be an important factor in maintaining the morphological divergence observed if fur colour and other morphological traits are determined by diet. It is possible that a female will have a young of a different fur colour to her own if consuming a diet atypical for her colour morph, and does not reach a critical threshold to activate key melanin pathways. Juvenile brushtails undergo a male-biased dispersal, with individuals migrating up to ten kilometers (Clout and Efford, 1984; Johnson *et al.,* 2001). The ecotone is less than a kilometer wide, and even if brushtails were to disperse like other mammal species, with a preference for remaining in habitat similar to their natal environment (Haughland and Larson, 2004; Mabry and Stamps, 2008), it is likely that some individuals would migrate into unfamiliar forest atypical for their colour morphology. In consideration of their longevity (typically up to eight years in the wild; Winter, 1980; Isaac, 2005), together with the dispersal and migration of individuals in other circumstances such as loss of territory, with bushfires, cyclones, and other events promoting territorial instability (Inions *et al*., 1989), we would expect along habitat gradients to find adult brushtails in habitats atypical for their colour morph. However I found no brushtail morphs in atypical habitats, suggesting that there is some maintenance of the morphological

divergence of forms and their affinity with different forest types. As discussed earlier, predation is a key candidate for this selective process, and there are a number of predators in the region with the potential to distinguish either colour or visual contrast (Hecht and Pirenne, 1940; Kanowski, 1998; Shine, 1998; Hayes *et al*., 2006). If selective pressures differ across habitat types, with advantage conferred preferentially to one colour morph over the other in each environment, this may explain the morphological distributions observed, and the strong association between habitat type and colour morphology despite the dispersal and migration capabilities of brushtail possums.

Searching for a biochemical pathway determining offspring fur colour, examining the effects on body-shape morphological characteristics, then testing for the presence and sensitivity of a threshold would be extremely difficult, requiring several years with captive studies (Kerle *et* al., 1991; Baker and Gemmell, 1999). However if dietary determination of fur colour is responsible for the phenotypic divergence observed, such a mechanism would alter our understanding of the potential for animals, especially mammals, to rapidly change with their environment and highlight the importance and dramatic effects of congenital traits in evolutionary processes.

2.5.4. Conclusions

In summary, this study has demonstrated that there are two distinct colour morphs of brushtail possum on the Atherton Tablelands, which can also be identified by the body dimensions that correlate strongly with each group. These two colour morphs are distributed parapatrically along a habitat gradient, with strong, differing habitat affinities; coppery brushtail possums in the highest population densities in the rainforest and grey brushtails in the dry sclerophyll forest, and both colour morphs inhabiting the narrow ecotone that separates the two, though in very low population densities. There was no sexual dimorphism, suggesting that sexual selection is not a significant influence on the evolution of these two morphs, and reproduction appears to be synchronous, potentially reducing both the capacity for polygyny and for gene flow across habitat types. Both colour morphs occur in habitat fragments, suggesting that fur colour is not

a plastic trait determined by climate. Observations of pouch young and captive brushtail possums suggest that once fur colour is determined, it is permanent for the lifetime of the animal. Two main candidate mechanisms for determining fur colour in Atherton Tablelands brushtail possums seem the most likely: parapatric speciation or phenotypic plasticity.

Further research is needed to conclusively establish which of these two mechanisms are producing this unusual morphological distribution. A captive study controlling maternal diet as offspring develop would determine whether colour is phenotypically plastic in this species. Unfortunately, without a nutrient candidate for this mechanism such a captive study would be difficult to design with any great confidence in producing the observed wild phenotypes. However an experimental common-garden approach might be successful in producing a common phenotype and thus demonstrating the potential for a congenital mechanism in this instance. Alternatively, genetic investigation into rates of gene flow, phylogeographic structure, looking for selection effects, and examining common base pair differences in genes associated with colouration, will provide information on the genetic determination of brushtail colour. Such molecular tools can also be used to conclusively falsify the possibility that the observed distribution of morphs is due to secondary contact between two divergent taxa, a hypothesis already weakened by Collins (2003). A conclusive answer to how the coppery and grey brushtail possums have come to occupy their present distribution would provide important knowledge about mechanisms of speciation that can be difficult to detect, are generally considered rare across many taxa and which have not yet been observed or applied in studies of mammalian evolution. It would allow greater insight regarding the capacity for habitat to promote divergence, and uniquely, for the environment to directly determine phenotypic expression. This is of particular importance not only for mammalian research, but also for a wide range of taxa and their surrounding environments, as it may lead to significantly greater understanding of the mechanisms that result in ecological networks such as complex rainforest ecosystems, and thus their management and conservation.

CHAPTER THREE

Phylogenetic relationships, parapatric divergence and fur colour in Atherton Tablelands brushtail possums, *Trichosurus vulpecula*.

3.1. ABSTRACT

I utilized a 467 base pair sequence of control region mitochondrial DNA, seven microsatellite loci and a 642 base pair sequence of the melanocortin-1-receptor to compare brushtail possums from populations in different geographic locations, examining both coppery and grey morphs within each. I found that the the distribution of colour morphs was not the result of secondary contact between previously isolated monophyletic lineages. Parapatric speciation had not occurred, as there was gene flow at a rate of approximately two brushtails per generation in each direction along the sclerophyll-rainforest gradient. In general, at a given distance of geographic separation along the rainforest-dry sclerophyll habitat gradient, genetic similarity was not an accurate indicator of morphology. If brushtail fur colour is genetically determined, then it is inherited in a unique manner not previously documented. The hypothesis that fur colour is phenotypically plastic and determined by maternal diet, or some other habitat specific factor, better explained the observed phylogeny: the fact that grey brushtail clades can evolve from coppery ones, and why possums of the same colour morph were just as related to those of the opposite morph at the same distance of separation. Thus, I propose that all Atherton Tablelands brushtail possums, both grey and coppery, share a mutation that has made fur colour a congenital trait, with offspring coat colour determined in early development, possibly by maternal diet. This mechanism is a novel evolutionary trait in wild mammalian species, and would mean that with environmental change there can be without mutation, a dramatic, widespread phenotypic change of the population in response to altered conditions experienced by adult females. Due to the significance of such an evolutionary mechanism, I recommend

experimental testing of this trait to better describe the mode and thresholds of colour determination in these brushtail possums.

3.2. INTRODUCTION

The distribution of coppery and grey forms of the brushtail possum on the Atherton Tablelands and their associated morphologies (both colour and body-size) could be the result of one of six evolutionary scenarios in which morphology is either genetically or environmentally determined. Each should affect the genetic identities of Atherton Tablelands brushtail possums in different ways:

3.2.1. Evolutionary hypotheses in which brushtail possum morphology is genetically determined.

Hypothesis 1a: The observed morphological distribution is the product of two genetically divergent brushtail possum populations making contact some time in the past, and then persisting on the Atherton Tableland as coppery and grey brushtail populations into the present. Their current distribution may reflect strong habitat preferences, competition between them over resources, or selection against either morph migrating into different ecological niches (Coyne and Orr, 2004). If the current distribution of coppery and grey brushtails is the product of secondary contact, we would predict that across multiple populations of the Atherton Tablelands, coppery and grey morphs will be genetically divergent, with dichotomous reciprocally monophyletic ancestral mitochondrial DNA lineages (Lande, 1980; appendix 1.0).

Hypothesis 1b: that the distribution of brushtail possums is not a product of secondary contact in this region, but has instead arisen through parapatric divergence. We would predict that populations across the Atherton Tablelands may be genetically more similar to adjacent populations with a different morphology than geographically distant populations that share the same morphology (Coyne and Orr, 2004; appendix 1.0). Collins (2003) suggested that coppery brushtails are polyphyletic in origin and that secondary contact is an

unlikely scenario. However, because Collins' (2003) study had a small number of samples from the very large area of the Atherton Tablelands, lacking notations of the specific locations, a measure of fur colour, or the habitat type of each possum, this evolutionary scenario requires further examination.

Hypothesis 2: along habitat gradients there is no gene flow between coppery and grey brushtail populations. We would predict that that brushtails of similar colour morphology along the rainforest-dry sclerophyll gradient will be genetically more similar to each other than to possums of a different morphology when separated by the same geographic distance, and that the calculated rate of migration will be very low (Wright, 1931; Wright, 1943; Slatkin, 1987; Smith *et al.*, 1997; appendix 1.0). This absence of gene flow could occur if the ecotone deters brushtails and acts as a barrier to both gene flow and dispersal despite its small size relative to brushtail possum home ranges. Without gene flow any morphological and genetic divergence could have evolved genetic drift, selection or a combination of the two (Dobzhansky, 1970).

Hypothesis 3a: That selection acts to reinforce the habitat preferences of each morph. We would predict that at a given geographic distance between two brushtails across the habitat gradient, possums of the same colour morphology and habitat will be genetically more similar than possums of different colours and habitats (Wright, 1943; Slatkin, 1987; Smith *et al.*, 1997), Across the many coppery and grey populations of the Atherton Tablelands, we would expect that geographic proximity between populations rather than their morphological similarity would be the best indicator of genetic similarity (Coyne and Orr, 2004), and that genetically distant brushtails of the same colour morph will share other morphological traits as a result of selection (Wright, 1931; Lande, 1976).

Hypothesis 3b: That morphological divergence is that product of genetic drift. We would predict that across many populations of brushtail possum on the Atherton Tablelands, genetically different populations will all so differ in multiple morphological traits (Dobzhansky, 1970; appendix 1.0).

3.2.2. Hypotheses in which brushtail possum morphology is environmentally determined

Hypothesis 4: A historical mutation shared by both coppery and grey brushtails on the Atherton Tablelands enables offspring to develop into either morph depending on the environmental conditions they experience as pouch young or *in utero*. Colour morphology is a congenital trait permanently determined by the environmental conditions of early development, most likely by maternal diet, which may provide chemical cues that affect fur colour differently beyond a threshold level (chapter two, 2.5.3.). Because brushtails are philopatric, with females tending to remain near their natal range (Clout and Efford, 1984; Johnson *et al.,* 2001), all offspring will likely acquire the same morphology as their mother if her surrounding flora has altered little since she was a pouch young. Unusual maternal behaviour affecting the nutrition (and thus the morphology) of her pouch young, dispersal, or migration, could all result in possums with atypical morphologies inhabiting each forest type. The absence of these atypical brushtails suggests that a selective advantage is conferred to one morph over the other in each habitat to maintain the morphological divergence observed (Smith *et al*., 1997; Nosil, 2004). While this may produce a reduction in gene flow along the habitat gradient, and allow some divergence by drift, on a larger scale we would not expect morphological differences to necessarily be correlated with genetic distances. Thus, under a model of maternal dietary determination of offspring fur colour, we would not necessarily anticipate that possums of the same colouration and habitat will be genetically more similar than possums of different colours and habitats when any isolation-by-distance effect is accounted for (Wright, 1943).

3.2.3. Genetic Toolbox

Three genetic markers were used in this study to determine which of the above evolutionary scenarios have produced the observed distribution of coppery and grey brushtails on the Atherton Tablelands. Using several genetic markers allows us to examine the processes over different time-scales and from different evolutionary perspectives, due their different modes of inheritance and functionality. Control region mitochondrial DNA is selectively near-neutral in

mammals (Nabholz *et al.*, 2008), inherited from only one parent, usually the mother (on average there is atypical paternal inheritance only once in every 1000-10000 offspring; Zouros *et al.*, 1994), and changes very little in comparison with other neutral markers such as the short tandem repeats of DNA targeted by microsatellite markers (Hewitt, 2001). Comparisons of mitochondrial control region sequences allow us to examine matriarchal lineages and deduce the history of interactions between populations over millions of years (Sigurdardóttir *et al.*, 2000). Though some regions of mitochondria are more informative than others, with regions such as cytochrome b not variable enough in brushtail possum lineages (Taylor and Foulkes, 2004), while control region mitochondrial DNA has been used successfully to map out phylogenetic relationships in this taxon (Collins, 2003). Microsatellite markers allow the examination of the length of short, non-coding, highly repetitive pieces of DNA (Payseur and Nachman, 2000). Microsatellites are inherited from both parents and recombinate frequently during meiosis, making them useful for examining recent genetic movements such as detecting gene flow or determining relatedness between individuals (Weber and Wong, 1993; Slatkin, 1995; Marshall *et al.,* 1998).

Genetic studies can also examine genes that are involved in the expression of a particular trait. This can be extremely difficult for several reasons. First, the genetic information of an organism can be composed of tens of thousands of protein-coding loci , for example, in the opossum 18,648 protein coding genes were initially identified with whole-genome sequencing (Mikkelsen *et al.,* 2007), and simply narrowing down these options to target a particular gene, let alone designing molecular primers to isolate this sequence presents great difficulties. Secondly the expression and regulation of a trait may be complex, influenced by the environment, regulatory changes, or encoded by multiple genes (Glazier *et al*., 2002; Cropley *et al*., 2006).

In the case of fur colour, the Melanocortin-1-Receptor, in consort with four other genes; agouti signaling protein (ASIP), TYRP1, dopachrome tautomerase (DCP), micropthalmia transcription factor (MITF); (Miller *et al.,* 1997; Abdel-

Malek *et al.,* 2001; Prasolova *et al.,* 2002; Kerns *et al.,* 2003; Gratten *et al.,* 2007), is responsible for colouration in birds (Theron *et al.,* 2001; Kerje *et al.,* 2003; Doucet *et al.,* 2004, Nadeau *et al.,* 2006; Baião *et al.,* 2007), reptiles (Rosenblum *et al.,*2004) and eutherian mammals (Miller *et al.,*1997; Kijas *et al.,* 1998; Wada *et al.,*1999; Everts *et al.,* 2000; Rouzaud *et al.,* 2000; Rieder *et al.,* 2001; Kerns *et al.,* 2003; Nachman *et al.,* 2003; Hoekstra *et al.,* 2004; Vage *et al.,* 2005; Fontanesi *et al.,* 2006; Hoekstra *et al.,* 2006; Mengel-Jørgensen *et al.,* 2006). The Melanocortin-1-Receptor is an intron-less gene that controls the expression of eumelanin and phaeomelanin (Fontanesi *et al.*, 2006). It appears to be the more influential of the five colour genes, with mutations in this gene able to override those in other genes associated with colour determination (Miller *et al.,* 1997; Abdel-Malek *et al.,* 2001; Gratten *et al.,* 2007). Single base pair changes in the Melanocortin-1-Receptor gene typically control phenotypic colour expression (Majerus and Mundy, 2003; Mundy, 2005). Mutations do not always code for colour changes (Hosoda *et al.*, 2005), but by examining a species and comparing the sequences of individuals with and without a particular fur colour, we can find mutations exclusive to those with the colour being investigated (Glazier *et al.*, 2002).

In this study I examined the genetics of coppery and grey brushtail possums along a habitat gradient and across the Atherton Tablelands using a 467 base pair sequence of control region mitochondrial DNA, 8 microsatellite loci, and a 642 base pair sequence of the Melanocortin-1-receptor. Using these genetic markers to examine the distribution of brushtail possums on the Atherton Tablelands from different inheritance and temporal perspectives, I sought to discover whether the morphological differences observed are the product of secondary contact, or alternatively if they are the product of divergence *in situ*, whether these forms are being maintained despite gene flow between coppery and grey brushtail possums, and if so, whether selective pressures are playing a significant role in maintaining this divergence. By answering these three questions I hoped to evaluate which of the five candidate evolutionary mechanisms is most likely to be responsible for the distribution of morphs observed. All but the theory of secondary contact, which was previously thrown

into doubt by Collins (2003), are unique in the study of mammalian evolution and highly significant in our wider understanding of evolutionary mechanisms. Learning how the coppery brushtail has evolved to occupy its present distribution could greatly alter our perception of the role of the environment in evolution and of the broad applicability that some more controversial evolutionary mechanisms may have to a very diverse range of taxa.

3.3. METHOD

3.3.1. Collection of samples

A total of 69 live and road-kill brushtail possums were collected in multiple locations across the Atherton tablelands (Appendix 2.2). Of these, seven sets of brushtail possum body-size measurements and DNA were generously donated by Associate Professor John Winter after being collected in another study. For the remaining possums collected, live possums were captured to obtain DNA, body size and morphological measurements, either with baited cage traps (1326 trap nights) placed at tree bases in forests, and on roof-tops and window-sills in Milla Milla, or using darts fired from a gas-powered air rifle (Black Wolf, Tranquil Arms Company, Seymour, Vic., Australia), which were loaded with 30mg Zoletil, (Virbac Pty Ltd) in 0.15 ml, in accordance with permits WISP03171005, ATH05/021, ATH06/019, ATH07/024, and ethics approvals A856, A1261 and A1262. Possums captured in cage traps were not sedated as they are relatively easy to handle once in a cotton bag. A small ear clipping was taken from each possum and preserved in 70% ethanol, then stored at 4º Celsius. After morphological measurements were taken as per the method described in chapter two (2.3.2) and each possum had recovered from the tranquilizers administered, they were released at the site of capture.

3.3.2. Extraction

DNA was extracted from tissue biopsies using the 'salting out' method of Sunnucks and Hales (1996), with modification for improved volumetric and temporal specification of the tissue being used as follows. Each single tissue sample was blotted dry to remove any excess ethanol, sliced into smaller

pieces, and added to a 1.5 ml tube containing 500 µl TNES (50mM Tris, pH 7.5, 400mM NaCl, 20mM EDTA, 0.5% SDS) and 2.5µl Proteinase K (10 mg/ml). The tissue was ground using a microfuge tube pestle to increase the surface area to volume ratio of the sample, before being placed in a rotating incubator at 37°C for five hours. After incubation, DNA was precipitated by adding 142µl 5M NaCl and manual shaking for 15 seconds. The protein-salt precipitate was pelleted by centrifuging at 11,500 xg for five minutes. A total of 400 µl of the supernatant was precipitated with 400 µl 100% ethanol, pelleted by centrifuging for 7 minutes at 11,500 xg then the pellet was washed with 600 µl of 70% ethanol, pelleted again and then air dried. The final DNA pellet was resuspended in 20 µl of TE (Tris 10 mM, EDTA (Na_2) 1 mM pH 8.0) and stored at 4°C for periods of up to one week, or at –20°C for preservation over longer periods. In one case both mother and offspring were sequenced at the target MtDNA locus, and in four cases both were genotyped. Although both mother and offspring MtDNA sequences, and all eight microsatellite genotypes were used to test for sequence amplification quality and marker heritability, only one of each pair was included in further statistical analyses.

3.3.3. Mitochondrial DNA analysis

The extracted DNA was quantified using lambda standards of 1 ng/µl, 2 ng/µl, 4 ng/µl and 10 ng/µl on a 1.5% agarose gel then diluted to make a 10 ng/µl DNA stock. Two primers, Mt15996L and DiMt15866H (Crowther *et al.,* 2003) were used to amplify a 467bp sequence from the mitochondrial DNA control region. Samples and control wells were cycled in 20µL reactions, with 0.1mM dNTPs, 3.5mM $MgCl_2$, 0.5u Taq polymerase (New England Biolabs), 1x Thermopol buffer (20 mM Tris-HCl,10 mM $(NH4)_2SO_4$,10 mM KCl, 2 mM $MgSO_4$, 0.1% Triton X-100, pH 8.8 at 25°C), 0.1µM of each primer, and 50ng DNA. PCR protocol: 92°C for 1 minute; 30 cycles of 92°C for 10 seconds, 60°C for 30 seconds, 75°C for 1 minute; 75°C for 5 minutes (Crowther *et al*., 2003). PCR products were purified through 200 µl of 6% Sephadex G-50 rehydrated in milli-Q water loaded onto a Whatman 350 neutral filter plate, then centrifuged for 2 minutes at 2000 xg to form a column. The column efficiency was checked by centrifuging the plate as above with 10 µl of milli-Q water loaded onto the

column. PCR products were then loaded onto the columns and centrifuged as above. The final flow through product from the column was collected into a sterile plate. This product was then visualised on a 1.5% agarose gel to check that a single clean band of the expected size had amplified, and the remaining PCR product was prepared for sequencing in both directions by creating two subsamples of DNA for each possum tested, adding Mt15996L to one subsample and DiMt15866H to the other as per the specifications of the Australian Genome Research Facility (AGRF), which performed the final sequencing reaction.

Raw and summarised outputs from the AGRF's sequencing were visually checked using sequence scanner v1.0 (Applied Biosystems, 2005). Only sequences of high quality with high quantity peaks were loaded into BioEdit (Hall, 1999) for clustal x alignment using the preset values (Thompson *et al.*, 1997). A sum 35 different haplotypes were recorded from 63 individuals, though this excluded known offspring, which were only used to check heritability and quality of the sequence. To check that the desired mitochondrial DNA region had been sequenced, haplotypes were compared with those of Collins (2003) and with two archived Genbank records (Phillips *et al.*, 2001; Eymann *et al.*, 2007). Haplotypic data were exported using DNAcollapser (Villesen, 2007), to Arlequin (Schneider *et al*., 2000) for calculations of population structure both past and present. The historical influences of demographic change and selective pressures on population structure were examined by testing for population expansion and neutrality. A population that has undergone unimodal expansion displays a mismatch distribution approximated by a Poisson curve (Rogers and Harpending, 1992), while stable populations have a mismatch distribution that is more 'ragged' due to the random extinction of some lineages over time (Harpending, 1994). Harpending's raggedness index measures the shape of the mismatch distribution, providing an indication of population stability (Harpending, 1994). If the raggedness value is statistically significant, this means that there is deviation from a 'ragged' mismatch distribution of haplotypes, which is characteristic of the gradual loss of haplotypes in a stable population. With significant deviation from this neutral situation, which we would

expect when examining mammalian mitochondrial DNA (Nabholz *et al.*, 2008), the null hypothesis of no historical expansion is rejected (Harpending, 1994). Arlequin (Schneider *et al*., 2000) was utilised to calculate raggedness with the command to run 1000 replicate simulations. Because Harpending's raggedness index is thought by some to be too conservative an evaluation of population expansion with an assumption of neutral selective pressures (Ramos-Onsins and Rozas, 2002), Tajima's (1989) D-test was also calculated using Arlequin (Schneider *et al*., 2000), with final statistics produced from 1000 simulations. This test calculates the probability of historical population expansion by using the number of segregating sites and the average number of pairwise differences between sequences, and tests this against a model of neutral mutation (Tajima, 1989).

Arlequin (Schneider *et al*., 2000) was also used for AMOVA to test for population structuring. individuals were grouped into putative populations based upon either their colour morphology, locality (Fig. 3.1), or both. The coppery and grey populations, which are differentiated by the habitats they occupy (chapter two, 2.4.4.), were initially treated as separate populations in data input. Where no significant genetic difference existed between the two colour morphs, they were treated as a single population. The pairwise F_{st} values from these comparisons between populations (Arlequin: Schneider *et al*., 2000) and the average F_{st} for each population (the mean value of all pairwise F_{st} scores from comparisons between the selected population and the other seven tablelands populations) were examined together with geographic measurements made using ArcGIS v.9.2 (ESRI Inc., 2006), incorporating maps from James Cook University (School of Tropical Environment Studies) and the Commonwealth of Australia (Geoscience Australia, 2001) to find whether there were any relationships between population structure and geography.

Paup v4.0 (Swofford, 2002) was used to calculate neighbour joining (Fig.3.2.) and maximum parsimony (Fig. 3.3.) trees. The calculation and inclusion of two types of phylogenetic trees does not reflect a lack of confidence in their results,


325000
350000
8100000
8075000
7
2
3
1C
1G
4
5
6
N
W
E
S
0 2 4 8 12 16
Kilometers

Fig. 3.1. The region of Far North Queensland, Australia in which the coppery and grey brushtail possum subspecies were studied. The mean locations of the brushtail possum populations used in analyses are each labelled with a white circle with crosshairs and annotated with a number from one to seven. Population one (from an area that overlaps in part with the G1 gradient site marked in Fig.2.1.) is separated by the two morph types into '1C' (coppery brushtail possums) and '1G' (grey brushtail possums). The brushtail possums of population two are from Atherton, population three is from 'Nassers Farm', a rainforest fragment to the East of Atherton, population four is from 'Drovers' an area South of Herberton, population five is from West of Ravenshoe (collected by John Winter), population six is from Millaa Millaa, population seven were found in Mareeba. The distribution of rainforest (dark grey), woodland/open forest (light grey), unforested areas (unshaded), and water bodies (stippled shading) is also shown. The grid of geographic coordinates is given in AGD55 UTM measure. This map was created using ArcGIS v.9.2 (ESRI Inc., 2006), incorporating maps from James Cook University (School of Tropical Environment Studies) and the Commonwealth of Australia (Geoscience Australia, 2001).

but instead reflects the established practice in phylogenetic research of being conservative with probabilistic data by examining the same phylogeny from different perspectives and with different underlying assumptions (Ronquist, 2004; Raffiudin and Crozier, 2007). Neighbour-joining analysis builds trees in a stepwise manner using a distance matrix, which in this case was calculated using the Jukes-Cantor formula (Jukes and Cantor, 1969; Posada and Crandall, 2001), and takes individuals separated by the least genetic distance to build outward branch by branch (Penny *et al.,* 2007). Five thousand replicates were used in calculations of bootstrap values to test the reliability of this tree. Maximum parsimony analysis finds the minimal number of mutations to create a network and in building hundreds of trees, looks for the most parsimonious model over the whole tree (Penny *et al.*, 2007). These two search methods allow us to look for common consensus or find areas of an organism's evolution that are uncertain. Default settings were generally used, though the maximum parsimony tree was created using a heuristic search, with the 500 highest scoring trees saved in calculation of the consensus values. A median joining network was also created using Network (version 4.6.0.0; available at fluxus-engineering.com; Bandelt *et al.,* 1999), to help visualise the base pair differences and haplotype sharing between individuals of the Atherton Tablelands. The default settings, with epsilon=0, were used in this network, which contained no outgroups, showing only the relationships between Tablelands brushtails.

Analysis of morphological similarities across different genetic clade groups was conducted using body size measurements detailed in the previous chapter, which were reduced to two variables using Principal Component Analysis and subject to MANOVA using SPSS statistical software (SPSS Inc., 2007). The morphology of a single reciprocally monophyletic genetic clade (Fig. 3.3, clade one) was compared to the pooled morphology for three less sampled ancestral clades of the Atherton Tablelands (Fig. 3.3, group two).

Lamarc 2.0 (Kuhner, 2006) was utilised to calculate the maximum likelihood migration rates between populations along the habitat gradient, with four

replicate calculations made. Making migration calculations with a single marker can sometimes produce errant results, thus I also used microsatellite markers (see below) to corroborate these results and more accurately estimate rates of migration.

3.3.4. Microsatellite analysis

Microsatellite analysis was performed by the Australian Genome Research Facility (AGRF), with extracted, quantified DNA sent as per their protocols. Genotypes for 66 individuals were generated using eight polymorphic microsatellite loci; Tv12, Tv16, Tv19, Tv27, Tv53, Tv54, Tv58, Tv64; from Taylor and Cooper (1998). Raw and scored genotype results received from AGRF were visually checked for signal strength and stutter effects. Amplification was generally very strong, with size and shape of alleles distinctive, and we only confirmed the scoring of an allele if it was of sufficient quantity in the electropheronograms and not part of a stutter pattern. Repeat allele scores were attained for 8 individuals that were run twice across 46 loci, and checked for scoring error. The microsatellite scores were then analysed using Arlequin (Schneider *et al*., 2000) to test for linkage disequilibrium between loci, null alleles, and conformity to Hardy-Weinberg equilibrium. Both tests used the default settings, such that 1000 permutations were used in testing linkage disequilibrium, and Markov chains 1 000 000 units in length were used to run an exact test of Hardy-Weinberg assumptions. Arlequin (Schneider *et al*., 2000) was also used to calculate associated AMOVAs, with individuals grouped into putative populations based upon either their morphology, locality (Fig. 3.1), or both.

GenAlEx (Peakall and Smouse, 2006) was used to calculate pairwise relatedness (Queller and Goodnight, 1989) and geographic distance between individuals. The correlation between these two factors was tested in three types of pairwise comparisons; among coppery brushtails, among grey brushtails, and between grey and coppery possums. This was done using two analyses; quantile regression analysis, which was conducted using R version 2.9.1 (R Development Core Team, 2009), with the subroutine ‘quantreg’ (Koenker, 2009) and ANCOVA, which was calculated using SPlus 8.0 (Insightful Corp., 2007).

While the genotype and geographic position of each individual was used in two comparisons, each pairwise comparison was independent. To further support the proposition that any relationships observed are not the product of confounding influences, I used macros (Appendix 3.0) in Microsoft Excel (Microsoft Corporation, 2003) to generate 5000 random combinations of these relatedness-geographic distance values (rbrhodes, 2007), calculated the slope of the linear relationship describing each combination, then used this distribution of 5000 slopes to test the probability that the slope observed was a due to chance (Congdon *et al*., 1997; Congdon *et al*., 2000). Migrate (Beerli, 2008) was used to calculate maximum likelihood migration rates along the habitat gradient transect, with four replicates of this probability analysis conducted. Apart from specifying that the data type was microsatellite loci, defult settings were used. As discussed previously for calculations of migration rates with mitochondrial DNA, the use of a single marker or locus to calculate migration vectors can give erroneous results, thus I have used multiple markers, and treat these data conservatively.

3.3.5. Isolation of Melanocortin-1-receptor gene

Total genomic DNA was quantified on 0.8% agarose gel then diluted to make a 10 ng/µl DNA stock. Brushtail possum specific primers for the MC1R gene were designed by comparing known sequences from the chicken (Genbank accession number: AY220303), jaguar (Genbank accession number: AY237396), jaguarundi (Genbank accession number:AY237399), pocket mouse (Genbank accession number: AY258937), arctic fox (Genbank accession number: AJ786717) and pig (Genbank accession number: AY365251) with the opossum genome (Genbank accession number: LOC100027632). Two primers were designed to amplify a 642bp sequence: Tv-MC1R-1 (5'-TTG ACC CTG GGG CTG GTA AGC-3') and Tv-MC1R-2 (5'-TTC CGA AGC TCT TGA CTG CGG A-3'). This captured sequence aligns with the 177-821bp region of the MC1R gene in *Mus musculus* (AB306322.1), the locality associated with most of the known colour variation in this gene (Majerus and Mundy, 2003; Mundy, 2005). Amplification was achieved in a 19.2µl reaction, with 0.16 µM Tv-MC1R-1, 0.l6 µM Tv-MC1R-2, 2.6 µM $MgCl_2$, 0.31 µM dNTP's, 1x Thermopol buffer

(20 mM Tris-HCl,10 mM $(NH4)_2SO_4$,10 mM KCl, 2 mM $MgSO_4$, 0.1% Triton X-100, pH 8.8 at 25°C), 1u Taq polymerase (New England Biolabs), and 10ng DNA. Control reactions were run at the same time as brushtail possum samples were being processed, and later examined on an electrophoresis gel to check for cross-contamination of DNA. The PCR protocol was designed to amplify with high specificity to begin with using lower annealing temperatures, and then by increasing the annealing temperature to optimise reaction rates I hoped to maximise the replication of these targeted sequence regions in the following cycles: 94°C for 3 minutes; 5 cycles of 94°C for 30 seconds, 50°C for 30 seconds, 72°C for 1 minute; 5 cycles of 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 1 minute; 10 cycles of 94°C for 30 seconds, 60°C for 30 seconds, 72°C for 1 minute; 10 cycles of 94°C for 30 seconds, 65°C for 30 seconds, 72°C for 1 minute; 72°C for 5 minutes. PCR products were purified through 200 µl of 6% Sephadex G-50 rehydrated in milli-Q water loaded onto a Whatman 350 neutral filter plate, then centrifuged for 2 minutes at 2000 *x*g to form a column. The column efficiency was checked by centrifuging the plate as above with 10 µl of milli-Q water loaded onto the column. PCR products were then loaded onto the columns and centrifuged as above. The final flow through product from the column was collected into a sterile plate. This product was then checked for the amplification of a single clean band of the expected size on a 1.5% gel, and the remaining PCR product was prepared for sequencing in both directions by creating two subsamples of DNA for each possum tested, adding Tv-MC1R-1 to one subsample and Tv-MC1R-2 to the other as per the specifications of the Australian Genome Research Facility (AGRF), which performed this final sequencing reaction. Output from the AGRF's sequencing was checked using sequence scanner v1.0 (Applied Biosystems, 2005) and loaded into BioEdit (Hall, 1999) for clustal x alignment (Thompson, 1997). The default alignment settings were used.

3.4. RESULTS

3.4.1. Mitochondrial control region DNA sequence

3.4.1.1. Phylogeny of Tablelands brushtail possums

Thirty five different haplotypes were recorded from 63 individual brushtails on the Atherton Tablelands (Figs. 3.2 and 3.3; appendix 3.1). Figures 3.2 and 3.3 illustrate the genetic distance between individuals of the Atherton tablelands using neighbour joining and maximum parsimony methods respectively. The haplotypes of two possums, one from New Zealand (Genbank accession number AF357238; Phillips *et al.,* 2001) and another from New South Wales (Genbank accession number EF166067, Eymann *et al.,* 2007) were used as outgroups in both analyses. There appeared to be at least four brushtail possum clades present on the Atherton Tablelands (Fig. 3.3) Only the top group, clade one, is a monophyletic group of haplotypes. In general, Atherton Tablelands brushtail possums are polyphyletic. In many cases individuals of different colour morphs share haplotypes and belong to the same clade (Fig. 3.3, Figure 3.4). Supposing that grey fur colour is the ancestral state, since it is the predominant colour Australia-wide, the phylogeny demonstrates that there has been at least one reversion to grey fur colour from a coppery clade; clade 3, with only grey brushtails, and the branch of mostly grey brushtail possums within clade one both seem to have evolved from coppery brushtail clades (Fig. 3.3).

The mismatch distribution of pairwise base pair differences between individuals obtained using a 467bp sequence of mitochondrial DNA control region did not conform to a model of historical sudden population expansion (Harpending's raggedness index=0.013, P=0.625; SSD=0.021, P=0.514; Fig. 3.5). The spread and clustering of haplotypes from which this mismatch distribution derives does not appear to have an uneven or biased distribution of colour morphs (Fig. 3.4) and individuals from a single location can have diverse haplotypes (Fig. 3.3). Tajima's test of neutrality also demonstrated no significant selection on this section of the mitochondrial DNA control region, and no population expansion (D=-0.748, P=0.25).

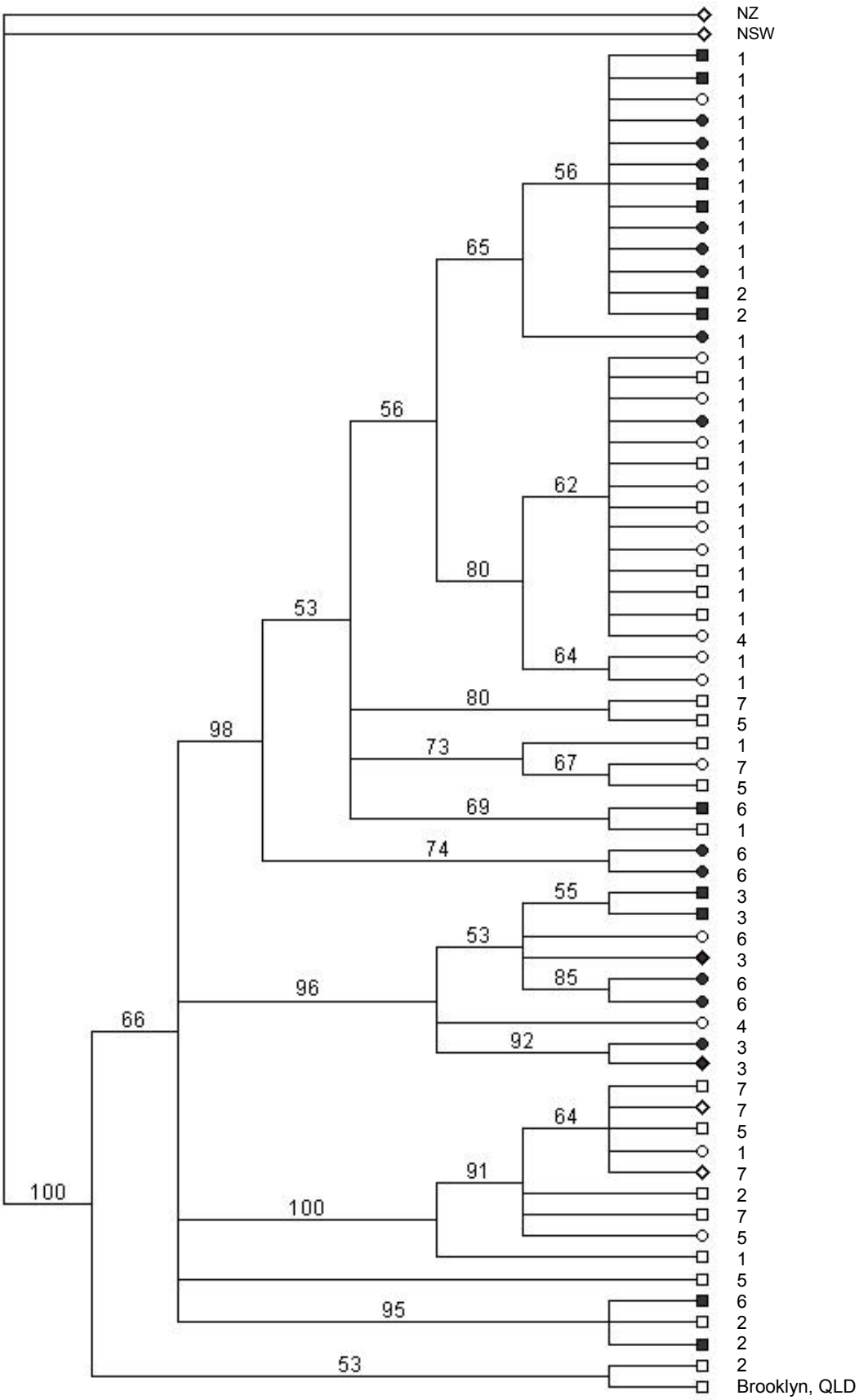

NZ
NSW
1
1
1
1
1
1
56
1
1
1
65
1
1
2
2
1
1
1
1
56
1
1
1
1
62
1
1
1
1
80
1
1
1
4
64
1
1
80
7
5
98
53
73
1
67
7
5
69
6
1
74
6
6
55
3
3
53
6
3
85
6
96
6
66
4
92
3
3
7
7
64
5
1
7
91
100
2
100
7
5
1
5
6
95
2
2
53
2
Brooklyn, QLD

Fig. 3.2. Neighbour joining tree of Atherton Tablelands brushtail possum mitochondrial DNA haplotypes with bootstrap values shown. Created using Paup v4.0 (Swofford, 2002), and calculated using a Jukes-Cantor distance matrix, two mitochondrial sequences from Genbank were used as outgroups; 'NZ': a New Zealand possum (Genbank accession number AF357238, Phillips *et al.,* 2001) and 'NSW': a possum captured in New South Wales (Genbank accession number EF166067, Eymann *et al.,* 2007). The gender of each brushtail possum is indicated by either a square: male, a circle: female, or a diamond: gender unknown. Possums with coppery fur colour have this shape are shaded black, and grey brushtail possums are shaded white. The two outgroup possums at the top of the tree have an unknown colour morphology. The column of numbers to the right of these symbols indicates the capture site for each possum, which are also illustrated on the map in Fig.3.1.

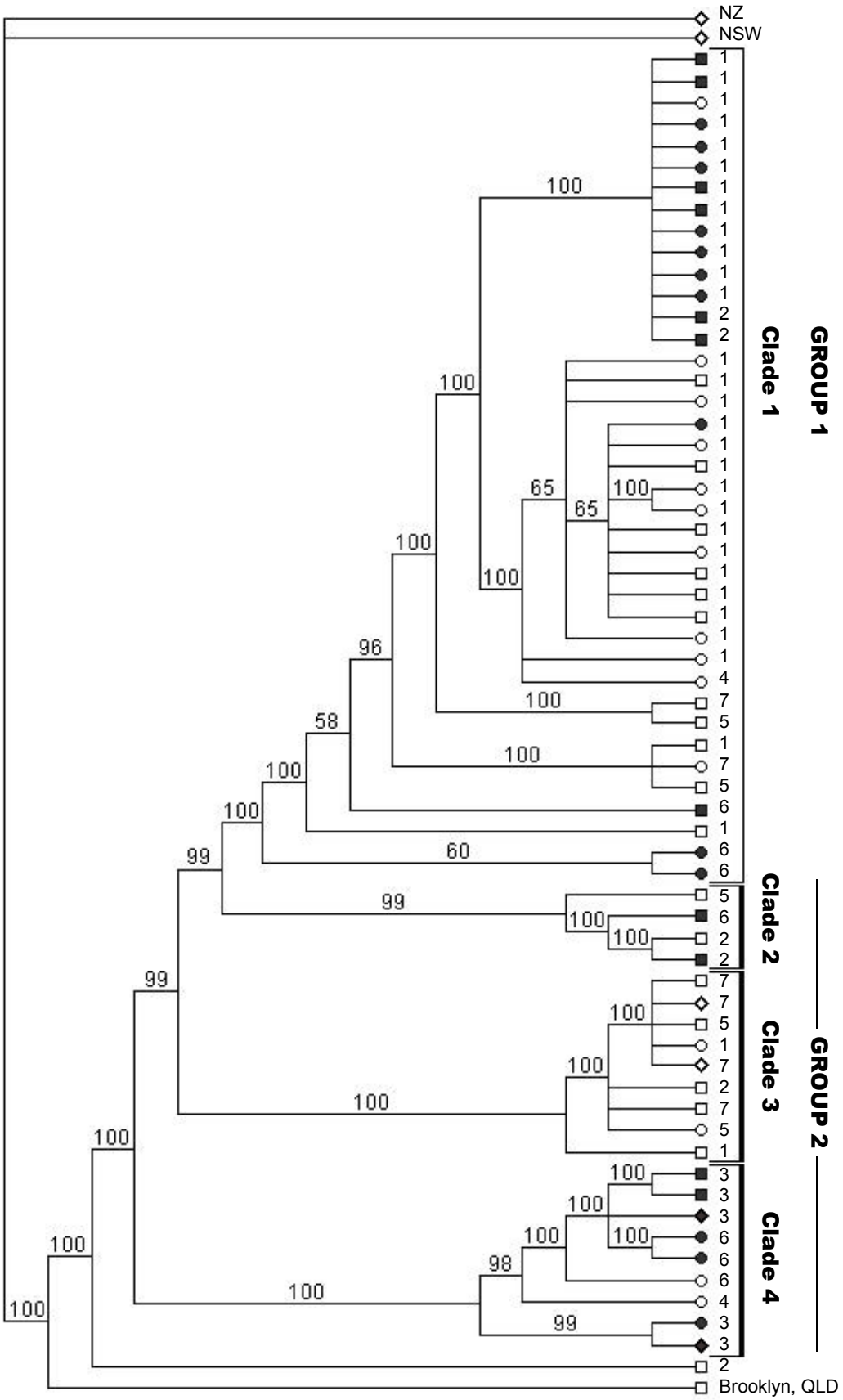

NZ
NSW
Brooklyn, QLD
GROUP 1
GROUP 2
Clade 1
Clade 2
Clade 3
Clade 4

Fig. 3.3. Maximum Parsimony tree of Atherton Tablelands brushtail possum mitochondrial DNA control region haplotypes with consensus values shown. Created using Paup v4.0 (Swofford, 2002), two mitochondrial sequences from Genbank were used as outgroups; 'NZ': a New Zealand possum (Genbank accession number AF357238, Phillips *et al.,* 2001) and 'NSW': a possum captured in New South Wales (Genbank accession number EF166067, Eymann *et al.,* 2007). The gender of each brushtail possum is indicated by either a square: male, a circle: female, or a diamond: gender unknown. Possums with coppery fur colour are shaded black, and grey brushtail possums are shaded white. The two outgroup possums at the top of the tree have an unknown morphology. The column of numbers to the right of these symbols indicates the capture site for each possum, which are also illustrated on the map in Fig.3.1. Four apparent clades are shown by brackets on the far right hand side of the figure. The top group, clade one, is monophyletic. The bottom three clades are apparently ancestral to the first, and due to smaller sample sizes, were grouped for the purpose of morphological Principal Component Analysis and AMOVA this grouping is indicated by a thicker bracket.

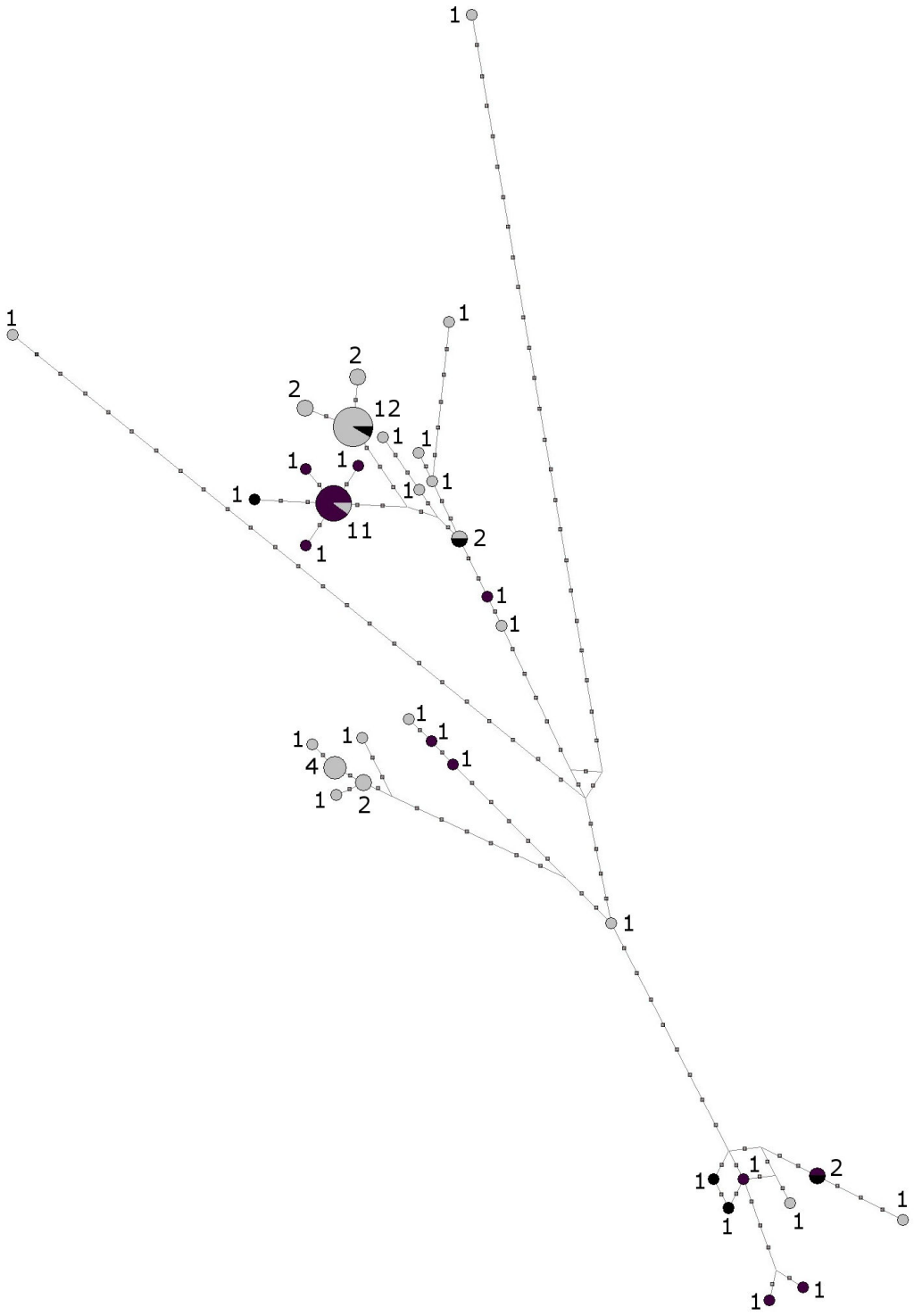

1
1
2
2
12
1
1
1
1
1
1
1
11
1
2
1
1
1
1
1
1
1
4
1
2
1
1
1
1
1
2
1
1
1
1
1
1

Fig. 3.4. Median joining network of mitochondrial DNA haplotypes shows the number of base pair distances (epsilon=0, other default settings used) between possums of the Atherton Tablelands. The grey and black circles (or fractions thereof) represent grey and coppery coloured individuals respectively. Circle size reflects the number of individuals sharing a particular haplotype, with this number also shown beside each circle. The number of base pair differences between two haplotypes is indicated by the number of square markers along the branches. Network (version 4.6.0.0; available at fluxus-engineering.com; Bandelt et al., 1999) was used to construct this tree, which was annotated using Gimp (version 2.6, available at www.gimp.org).

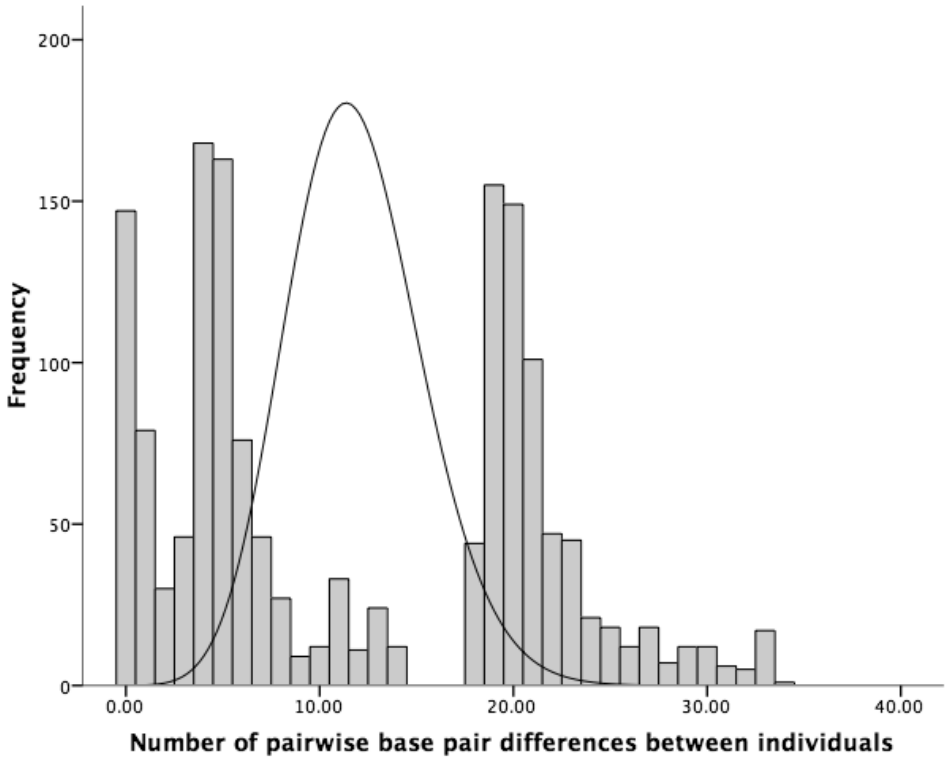


Fig. 3.5 Mismatch analysis of a 467bp sequence of mitochondrial DNA control region for Atherton Tablelands brushtail possums. The line shows the expected distribution under a model of unimodal population expansion (Slatkin and Hudson, 1991).

3.4.1.2. Morphology by cladistics group

Principal Component Analysis (PCA) of brushtail body-shape morphology on the Atherton Tablelands condensed the measurements taken into two composite factors. When differentiating individuals by cladistic group (Fig. 3.3; note that clades two, three and four are pooled) and colour morph in MANOVA analyses, only one PCA factor varied significantly (Factor 1: $F_{3,27}$=2.43, P=0.087; Factor 2: $F_{3,27}$=25.48, P<0.001). This composite morphological measure, PCA Factor two, primarily describes the variation observed in ear length and width and tail length; body-shape morphological factors that distinguish coppery and grey brushtail possums (chapter two: 2.4.2.). Colour form did have a significant association with the variation of PCA factor two ($F_{2,26}$=24.21, P<0.001; Fig. 3.6), however the mitochondrial DNA clade groups were not significantly associated with the variation observed in this measure of body-shape morphology ($F_{2,26}$=0.137, P=0.188; Fig. 3.5). An outlier in the coppery brushtail group of clade one had little influence on the significance of observed relationships (with the outlier excluded from analyses: variation in PCA factor 1: $F_{3,26}$=2.59, P=0.074; Factor 2: $F_{3,26}$=25.45, P<0.001; association of cladistic group with variation in PCA factor 2: $F_{2,25}$=1.773, P=0.190; association of colour form with variation in PCA factor 2: $F_{2,25}$=24.92, P<0.001), though it is unclear why this individual might appear to possess a distinct body-shape morphology.

The expression of coppery coat colour with either a red-purple or red-orange hue correlates with the rainforest locality of each animal (chapter two, 2.4.1). Further testing of coppery fur colour expression and its relationship to the three mitochondrial DNA clades containing coppery brushtail possums, demonstrated that the genetic lineage of a possum was not correlated with which coppery hue it expressed ($F_{2,18}$=1.61, P=0.227; clades bracketed in Fig. 3.3).

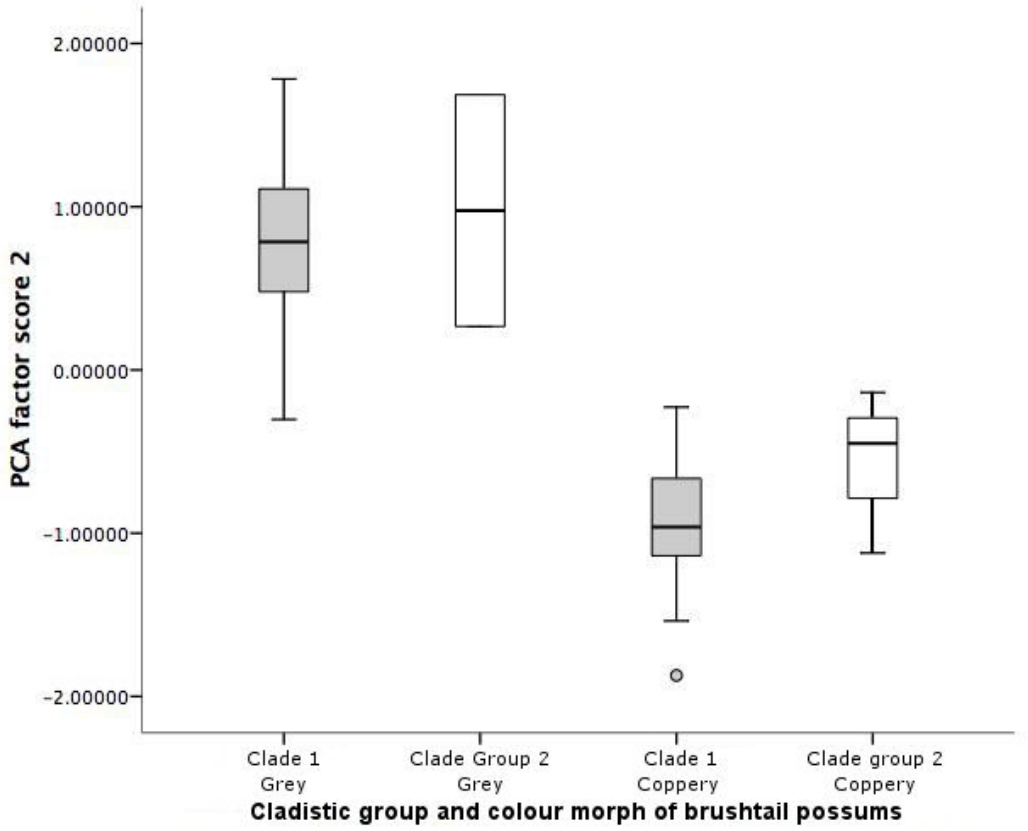


Fig. 3.6. Distribution of Principal Component Analysis (PCA) factor 2, which was the only factor from the PCA to vary significantly by cladistic group and colour form (PCA Factor 1: $F_{3,27}$=2.43, P=0.087; PCA Factor 2: $F_{3,27}$=25.48, P<0.001). The boxplots are differentiated by clade group and colour form. Clade one is shaded and clade group two unshaded. The first two boxplots show PCA factor score 2 for grey brushtail possums in each of these clade groups, and the third and fourth boxplots show the range of values observed in coppery brushtail possums.

3.4.1.3. Population structure in Tablelands brushtail possums obtained using control region mitochondrial DNA

There was no significant haplotypic population structure when possums were grouped by colour morph (AMOVA: Variation between groups=-0.662, df=1, P=0.70772 ± 0.00895; Variation between populations within groups=3.447, df=10, P<0.001; Variation within populations=4.044, df=49, P<0.001).

There was a significant relationship between the pairwise aerial distances and pairwise F_{st} of the populations ($F_{1,26}$=4.53, P=0.042). There was also a significant negative linear relationship between the average F_{st} for each brushtail possum population (appendix 3.2) on the Atherton Tablelands and the base ten logarithm of the distance from rainforest ($F_{1,6}$=11.13 P=0.016). Populations closest to rainforest were genetically more different but in moving away from rainforest the brushtail populations are less genetically distinct from all others on the Atherton Tablelands and have lower average F_{st}.

The most parsimonious population grouping, which maximised the variation between groups and minimised variation within them, explained 31.4% of genetic variation. This population structuring separated the four populations found in closest proximity to rainforest; Herberton State Forest coppery brushtail possums (population '1C' in Fig.3.1), Herberton State Forest grey brushtail possums (population '1G' in Fig.3.1), Millaa Millaa possums(population '6' in Fig.3.1) and Nassers cattle farm(population '3' in Fig.3.1), differentiating them from each other and from the other tablelands populations (Variation between groups =2.19, df=4, P=0.015±0.004; Variation between populations within groups =0.64, df=3, P=0.617±0.014; Variation within populations =4.15, df=52, P<0.001).

Pairwise F_{st} values between populations were not significantly correlated with other geographic characteristics (Fig. 3.1) such as pairwise latitudinal distance

($F_{1,26}$=1.37, P=0.253), longitudinal distance ($F_{1,26}$=2.836, P=0.104), or elevation difference ($F_{1,26}$=0.138, P=0.713).

3.4.1.4. Gene flow along the habitat gradient

Using mitochondrial DNA, the rate of gene flow ($4N_em$) between coppery brushtail possums and grey brushtail possums was estimated to be 1.01±0.051 (S.E.) individuals per generation out of the rainforest, and 0.93±0.052 (S.E.) individuals per generation out of dry sclerophyll forest.

3.4.2. Microsatellite Markers

3.4.2.1. Linkage disequilibrium and Hardy-Weinberg equilibrium

No significant linkage disequilibrium was detected across the eight loci. Three loci were found to significantly deviate from Hardy-Weinberg equilibrium when the genotypes of all Atherton Tablelands brushtail possums were considered together (Tv19: N=64, P=0.001; Tv27: N=64, P=0.015; Tv54: N=65, P<0.001; appendix 3.3). Only Tv54 showed consistent disequilibrium across each population, probably as a result of heterozygote deficiency (F_{is}=0.63) or possibly sex-linkage at this locus. As a result it was removed from further analyses.

3.4.2.2. Population structure in Tablelands brushtail possums

No significant population structure was detected across the Atherton Tablelands brushtail possum populations using microsatellite markers. The most informative population grouping was when populations two and three (Fig. 3.1) were grouped together. However this population structuring explained only 2.7% of total variation, with 97.2% of variation occurring within populations (variation between groups = 0.080, df=6, P=0.063±0.007; variation between populations within groups = 0.004, df=1, P=0.318±0.014; variation within populations = 2.887, df=120, P<0.001). Along the habitat gradient the coppery and grey brushtail possum populations were significantly different (F_{st} =0.038, P<0.001; appendix 3.4).

3.4.2.3. Interaction between the relatedness of individuals and geographic separation

There was a significant negative linear relationship between the base ten logarithm of pairwise geographic separation of brushtail possums along the habitat gradient and their relatedness ($F_{1,526}$ =4.183, P=0.041; Fig. 3.7). This relationship, with a coefficient of -0.036 was unlikely to have been produced by chance, as a randomized slopes test demonstrated (t=98.127, DF=4999, P<0.0001).

There were no linear relationships between relatedness and geographic distance (linear or base ten logarithm) among coppery brushtails possums (linear geographic distance: $F_{1,64}$=0.086, P=0.770; $\log_{10}$ geographic distance: $F_{1,64}$=0.240, P=0.626; Fig. 3.7), for brushtail possums that were different colour morphs (linear geographic distance: $F_{1,250}$=0.817, P=0.367; $\log_{10}$ geographic distance: $F_{1,250}$=0.326, P=0.33 Fig. 3.6), or among grey brushtail possums along this habitat gradient (linear geographic distance: $F_{1,208}$=.287, P=0.59; $\log_{10}$ geographic distance: $F_{1,208}$=.586, P=0.445; Fig. 3.7).

The correlation between relatedness and geographic distance was significantly different between the three types of pairwise comparisons (ANCOVA; linear geographic distance: $F_{3,523}$=3.62, P=0.013; $\log_{10}$ geographic distance: $F_{3,523}$ =5.99, P<0.001): among coppery brushtails, among grey brushtails, and between one coppery and one grey brushtail possum. The correlation of relatedness and geographic distance between different colour morphs was significantly different to that observed in comparisons among coppery brushtails (linear geographic distance: $F_{2,315}$=5.57, P=0.004; $\log_{10}$ geographic distance: $F_{2,315}$=9.16, P<0.001), but not to those among grey brushtail possums (linear geographic distance: $F_{2,459}$=1.27, P=0.282; $\log_{10}$ geographic distance: $F_{2,459}$=2.53, P=0.081).

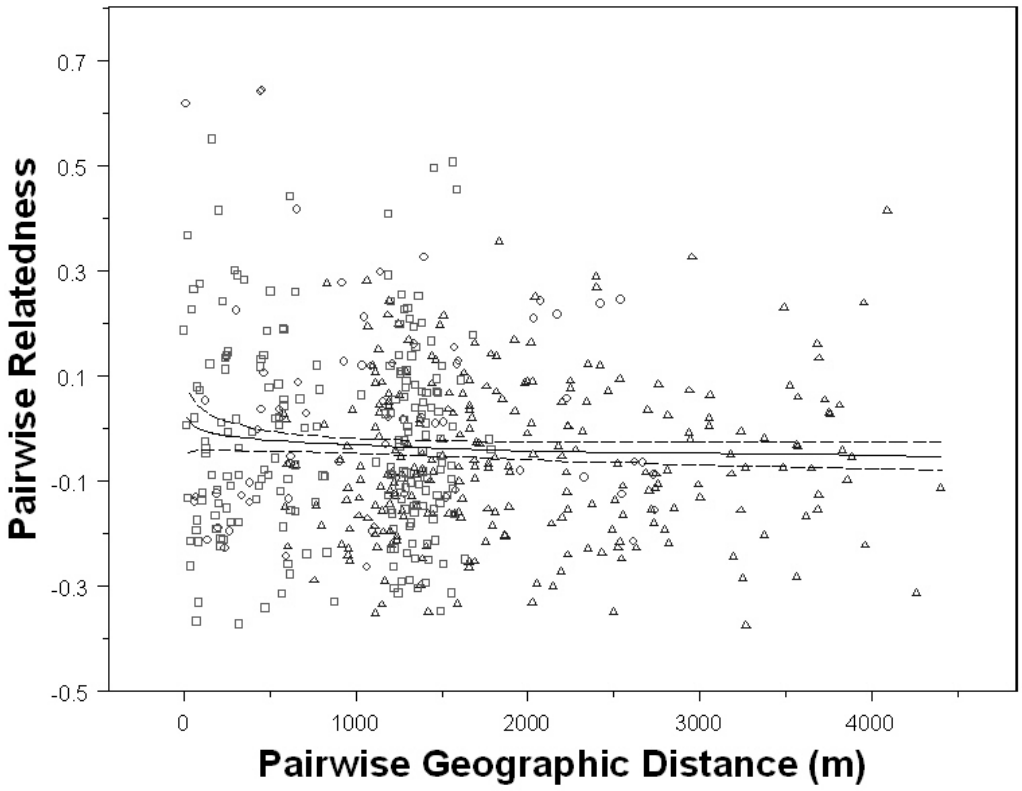


Fig. 3.7. The pairwise relatedness (Queller and Goodnight, 1989) and geographic distance between pairs of brushtail possums along a habitat gradient. The comparisons between two coppery possums are shown with circular markers, comparisons between grey brushtails are illustrated with square symbols, and comparisons with one possum of each colour morph are each shown by a triangle. The pairwise relatedness and $\log_{10}$geographic distance between all brushtail possums, which had a statistically significant relationship ($F_{1,526}$=4.183, P=0.041), is shown here by the solid black line, and flanked by grey dashed lines marking the 95% confidence interval.

3.4.2.4. Gene flow along the habitat gradient

Using microsatellite markers, the rate of gene flow ($4N_em$) between coppery brushtail possums and grey brushtails was calculated to be 2.721 ± 0.110 (S.E.) individuals per generation out of the rainforest, and 1.932 ± 0.132 (S.E.) individuals per generation out of dry sclerophyll forest. These estimates are slightly higher but statistically consistent with the earlier mitochondrial DNA estimates of gene flow (T=1.122, DF=14, P=0.281) and suggest that on average 1.648 ± 0.194 brushtail possums are moving in each direction between habitat types along the habitat gradient.

3.4.3. Melanocortin-1-receptor (MC1R) sequence

A 642 base pair MC1R sequence was successfully amplified in these *Trichosurus vulpecula* subspecies (Appendix 3.5). This sequence aligns with the 177-821bp region of the MC1R gene in *Mus musculus* (Genbank accession number: AB306322.1), the locality associated with of most of the known intra-specific colour variation caused by this gene (Majerus and Mundy, 2003; Mundy, 2005). *Trichosurus vulpecula* and *Mus musculus* share 70.2% of base pairs along this sequence. The MC1R sequences of eight possums from four different populations were examined, with the DNA of four coppery and four grey brushtail possums compared. However there were no base pair differences observed between the two colour morphs: the sequences were identical.

3.5. DISCUSSION

3.5.1. Phylogeny of Atherton Tablelands brushtail possums

My results indicated that brushtail possums from the Atherton Tablelands have polyphyletic origins, with both coppery and grey morphs occuring in multiple genetically distant clades (Figs. 3.2 and 3.3). This supports the results of Collins (2003), and interestingly, some of the same haplotypes were detected in this

study. This phylogenetic structure is not what would be expected after the secondary contact of previously isolated monophyletic populations (Lande, 1980), therefore the different colour morphs observed have arisen via the divergence of one form into many.

Previously, I had narrowed the number of potential explanations for the distribution of brushtail morphs to six putative mechanisms:

1a. That the distribution of coppery and grey brushtail possums on the Atherton Tablelands is the direct result of two genetically divergent populations, one coppery and one grey, making secondary contact.

1b. That the distribution of coppery and grey brushtail possums on the Atherton Tablelands has arisen through parapatric divergence.

2. that brushtail morphology (colour and body-shape) is genetically determined, and that along habitat gradients the mosaic ecotone forest acts as a barrier to prevent gene flow, allowing genetic and morphological divergence through either drift or selection.

3a. that natural selection reinforces the habitat preferences of each morph. Along a habitat gradient, brushtails of the same morphology should be genetically more similar. However on a wider scale across multiple distant populations geographic proximity rather than morphological similarity would be indicative of genetic likeness, with possums that share a common colour morphology also having a similar body shape (Wright, 1931; Lande, 1976; appendix 1.0).

3b. that genetic drift has produced the morphological variation observed. Across multiple distant populations we would expect that either genetic similarity correlates with morphological similarity, or that genetically distant brushtails of the same colour morph will vary in body shape (Lande, 1976; appendix 1.0).

4. that morphology is not genetically determined. It is a congenital trait that allows newborn possums the ability to be either coppery or grey depending on the environment they experience in early development. Morphological divergence is the product of environmental conditions, and the correlation between morphology and habitat is reinforced by selective

pressures. Gene flow may occur across the ecotone, and across the larger geographic scale of the Atherton Tablelands genetic similarity would not be indicative of morphology.

In light of the phylogenetic distribution observed, my first hypothesis of secondary contact being principally responsible for shaping the morphological distribution of coppery and grey brushtail possums should be considered very unlikely. Closer inspection of the phylogeny of Atherton Tablelands brushtail possums (Fig. 3.5) demonstrated that the distribution did not conform to a model of sudden population expansion, further weakening arguments for secondary contact after allopatric divergence (Rogers and Harpending, 1992). This suggests that the population is in an equilibrium state (Rogers and Harpending, 1992). The current distribution of coppery and grey brushtail possums reflects a long-term, stable arrangement.

Both colour morphs were found in genetically distant clades (Fig. 3.3). This suggested that genetic ancestry is a poorer indicator of morphology than habitat type, which was previously found to correlate strongly with fur colour (chapter two: 2.5.1.). This does not mean that brushtail fur colour can not be genetically determined, but it does suggest that any mutation(s) responsible for determining fur colour in the Atherton Tablelands brushtail possums have evolved more recently than the divergence of the population into these clades. To produce the phylogeny observed these same mutations must have occurred repeatedly across many divergent lineages. However, despite this reoccurrence, these mutations for coppery fur colour do not seem to have evolved in any other populations in Australia or New Zealand (Collins, 2003).

Furthermore, if fur colour is genetically determined in these brushtails, then on at least one occasion a clade with a majority of possums expressing grey fur colour phenotype has evolved from a clade which expresses mainly the coppery phenotype (Fig. 3.3). This is significant because the common phenotype of brushtail possums is grey fur colour (Collins, 2003), though there is limited expression of black, coppery or blond fur also. Golden fur colour appears to be

a trait recessive to grey colour (Fingland, 2005), and some coppery females were observed to have grey offspring, but not *vice versa*, suggesting that grey fur colour is likely the dominant fur colour phenotype. If so, a mostly coppery clade would have in the population a high proportion of key recessive allele(s) for one or several genes, making the evolution of a grey clade from such a source population unlikely without selection (Wright, 1931; Coyne *et al*., 1997). Alternatively, if fur colour in the Atherton Tablelands brushtail possums were a congenital trait, these populations may share a mutation that evolved in a common ancestor before their divergence into clades. As detailed previously (chapter two: 2.5.3.), the expression of such a congenital trait to affect colour determination in brushtail possums would most likely be influenced by the environmental conditions experienced in early development, maternal diet in particular (Cropley *et al.*, 2006). If this were the case, then habitat type should better predict morphology than ancestry. These two mechanisms, in which the morphological divergence observed is either genetically or environmentally determined, create a dichotomy in the interpretation of subsequent results. Thus I will present here separate critiques of each, with the aim of falsifying the three remaining evolutionary hypotheses; as discussed above, my hypothesis of secondary contact was rejected after examining the phylogenetic distribution of Tablelands brushtail possums.

3.5.2. An interpretation of the evidence assuming that morphology in Atherton Tablelands brushtail possums is genetically determined.

3.5.2.1. Selection versus drift

If there is linkage between the genes encoding for the expression of colour and non-colour morphological traits, then genetic drift can result in possums of the same colour also being similar in body-shape. However drift alone could not explain the assortment of the two morphs into differing habitats, especially in a system of male-biased dispersal, as occurs in brushtail possums (Clout and Efford, 1984, Johnson *et al*., 2001). The genetic drift of linked colour and body-shape morphological traits and the action of selection to produce the observed morphological distribution (chapter two) would also be associated with genetic

divergence between possums of different colours (Dobzhansky, 1970). As this has not occurred (Fig 3.6), the action of drift on linked colour and non-colour phenotypes does not adequately explain the observed distribution. Alternatively, if selection was acting upon unlinked morphological traits to shape the genotypes observed, we would expect either that genetically similar possums would also be more alike in colour and body-shape morphology, or that possums of the same colour morph from genetically distant clades would display variation in body-shape morphologies (Lande, 1976). However this was not observed. Genetically similar possums could have very different non-colour morphologies, and genetically distant possums of the same colour share the same body dimensions (Fig. 3.6). This suggests that drift alone could not have driven the evolution of these two forms, and that selective pressures are acting to produce consistent body-shape characteristics in possums of the same colour, despite them belonging to different genetic lineages (Wright, 1931; Lande, 1976). Variation in coppery hue did not correlate with mitochondrial DNA structure, but with forest locality (chapter two: 2.4.1.), suggesting that there may be some fine scale variation in selective pressures across the different rainforests of the Wet Tropics.

Colouration can be very important in processes of sexual selection, in natural selection by providing camouflage from predators, and in thermoregulation (Price, 2006; review by Mundy, 2007; Nadeau *et al.*, 2007). In the nocturnal, endothermic brushtail possum, variation in colour is unlikely to affect thermoregulation. In other species that have evolved sexually selected visible traits, it is common for there to be visible dimorphism between the sexes (Lande, 1980b). This was not observed among coppery or grey brushtail possums, suggesting that colour is not likely to be under sexual selection in these brushtail possums. Further to this, in other brushtail populations sexual selection typically occurs via male competition for territory and access to resources such as food and females (Clinchy *et al*., 2004; Isaac and Johnson, 2003). A key indicator of sexual selection in this species is male-biased size dimorphism (Clinchy *et al*., 2004; Isaac and Johnson, 2003). The lack of sexual dimorphism displayed for body size in either coppery or grey brushtail possums

on the Atherton Tablelands also suggests that sexual selection is not a significant evolutionary force in these possums (chapter two: 2.5.1; Clinchy *et al*., 2004; Isaac and Johnson, 2003). Thus it seems likely that any selection of fur colour occurs through differential crypsis and consequent predation. Indeed grey brushtail possums appear to camouflage well against the grey-barked eucalypts of the dry sclerophyll forest, and the dark red colour of coppery brushtails is difficult to spot against the mosaic of dark greens and reds and purples in the rainforest. Though variation in coppery hue between rainforest sites may reflect some adaptation under selection to slight differences in background environmental colour (Marchetti, 1993; Endler *et al*., 2005), it is fur colour saturation levels that distinguish the two forms, thus it also seems likely that it is not the contrast in hue, but colour saturation that is most important in a brushtail possum's ability to camouflage with its environment. Candidate predators of brushtail possums on the Atherton Tablelands principally include snakes and owls (Kanowski, 1998; Hayes *et al*., 2006). Although these predatory groups may not typically have a wide range of colour vision, most are able to detect with moonlight the degree of visual contrast between prey animals and their surroundings, which may increase for possum occupying their non-preferred habitat (Hecht and Pirenne, 1940; Kanowski, 1998; Shine, 1998).

3.5.2.2. Population structure in Tablelands brushtail possums

In a scenario where selection has shaped the observed morphological distribution, it is expected that phenotypic differences would indicate an underlying genetic divergence between coppery and grey brushtail possums (Lande, 1976). The population structure observed in Atherton Tablelands brushtail possums using control region mitochondrial DNA demonstrated that there was no significant population structuring by colour morph, and possums of different colours shared haplotypes (Figs. 3.2 and 3.3). Rather, genetic variation was best described by the proximity of a population to rainforest, whether grey or coppery. Given that the phylogenetic distribution indicates no sudden expansions and a general long-term stability of the Atherton Tablelands brushtail populations, the propensity for populations near rainforest to be the more different likely reflects their regular isolation from each other by some

common divisive influence, perhaps fire (Kershaw *et al.*, 1993), rather than a bottleneck, then expansion event that might be attributed to global glaciation periods (Schneider and Moritz, 1999). Fire would tend to burn dry sclerophyll forest more frequently than rainforest (Hopkins *et al.*, 1993). While a fire may only burn a portion of the dry sclerophyll forest connecting two less fire-affected rainforest populations, this would be significant in producing periodic migration, even deaths within clades inhabiting this drier habitat type, would reduce the short-term availability of food, and the longer term access to tree hollows in which to shelter (Inions *et al*., 1989). The repeated impact of fires on phylogeography through the possible elimination of some genetic lineages and the disruption to brushtail movement may have acted in the long term to slow gene flow between rainforest populations, particularly when those rainforests are connected by dry sclerophyll forest. Therefore, populations closer to rainforest would probably be more distinct from other Atherton Tablelands populations due to their greater stability and isolation in this habitat type, while populations further from rainforest would be less distinct, due to the repeated mosaic recolonisation of burned forests over time (Hopkins *et al.*, 1993).

Mitochondrial DNA, which was used to investigate the phylogenic distribution discussed above, is slower to recombinate than nuclear DNA, is generally capable of showing only the maternal lineage, and in this case, its haplotypic distribution is limited over multiple generations by the dispersal patterns of female brushtail possums (Moritz *et al.*, 1987; Hewitt, 2001; Isaac and Johnson, 2003; Clinchy *et al.*, 2004). As such it is highly useful in examining long term evolutionary change, but is not the best genetic marker to detect more recent gene flow and the relatedness of individuals of different brushtail morphs (Moritz *et al*., 1987). Microsatellite markers allow us to examine the length of highly variable DNA sequences that are full of base pair repeats. The higher rate of mutation compared with control region mitochondrial DNA (Brown *et al.,* 1993; Weber and Wong, 1993) and increased genetic variation via the inheritance of an allele from both parents gives a greater resolution of more recent migration and divergence among populations (Hewitt, 2001).

The use of microsatellite loci demonstrated that on a more recent evolutionary timescale, the populations of Atherton Tablelands brushtail possums have not all experienced the same levels of gene flow. The lack of significant population structure suggests that considerable gene flow has been occurring across the Tablelands (Loveless and Hamrick, 1984). However along the habitat gradient, microsatellite analysis demonstrated that the coppery and grey populations of Herberton State forest were significantly different from each other. With their preferred habitat types separated by less than a kilometer, we might expect that populations more distantly separated would also be genetically distinct (Wright, 1943). Yet there were no significant differences between the four neighbouring populations (population two, from a rainforest fragment, population three, from around the town of Atherton, and populations four and five, from dry sclerophyll forest) which were separated from each other by 2.5 to 23.5 kilometers (Fig. 3.1). This suggests that there is some factor that has acted to restrict gene flow between coppery and grey populations along habitat gradients, a factor that therefore distinguishes habitat gradients as being distinct from other environments inhabited by Tablelands brushtail possums.

3.5.2.3. Gene flow restriction along a habitat gradient

At a smaller spatial scale, over habitat gradients spanning approximately five kilometers, coppery brushtail possums were found in the rainforest, grey brushtails occupied the dry sclerophyll forest and a small number of both colour morphs were seen in the ecotone (chapter two: 2.4.4.). It is this ecotone that is unique to habitat gradients and not found in comparisons between these other populations. Mitochondrial DNA and microsatellite population structuring demonstrated that coppery and grey populations along the habitat gradient were distinct. Calculations of migration rates between these two parapatric colour morphs differed slightly with mitochondrial DNA results providing an estimate of approximately one possum per generation from each population successfully crossing into the other habitat type, while the results calculated from microsatellite loci suggested that this may be closer to two. Given the differences in how these markers are inherited, with mitochondrial DNA typically conserved through matrirachial lineages (Zouros *et al.*, 1994), and microsatellites being inherited from both parents, this slight variation may

suggest that these populations experience male-biased dispersal of juveniles, as has been documented in other brushtail possum populations (Clout and Efford, 1984; Johnson *et al.*, 2001). The upper estimate of microsatellite gene flow of 4NeM= 2.72, is not a high rate, and suggests that gene flow was restricted, but that reproductive isolation has not occurred (Wright, 1931). This falsifies the second hypothesis that divergence is in this case a product of the ecotone preventing all gene flow: if morphology is genetically determined, then it is occurring despite some gene flow along the habitat gradient.

In order to investigate this restriction on gene flow, I tested whether at a given distance of separation, the relatedness of two possums was affected by whether they shared colour morphology and habitat. I would expect that under selection, the phenotypic divergence I observed would also indicate genetic divergence (Wright, 1931; Lande, 1976). Furthermore, with a restriction in gene flow along the habitat gradient, I would expect that at the same distances of geographic separation, relatedness between opposite coloured brushtails will be lower than between brushtails of the same morphology (Wright, 1943; Slatkin, 1987; Smith *et al.*, 1997). This is not what I observed.

In general there was a negative linear relationship between pairwise relatedness and the base ten logarithm of geographic distance. Quantile regression demonstrated that this correlation was limited to the 80% quantile i.e. there is a significant relatedness-distance effect only within the 20% most closely related individuals. There were no statistically significant correlations between relatedness and geographic distance for each individual type of pairwise comparison; whether between different coloured brushtail possums, among grey brushtail possums or among coppery brushtails; for each comparison an individual was likely to be just as closely related to possums nearby as to those far away. It may be that the distances examined were too short, with the detectability of any relationship between relatedness and geographic separation being a product of the scale of home range and dispersal distances relative to the length of the transect. Higher population density, smaller home ranges, and shorter dispersal distances of males from their natal

range would promote a higher number of nearby, close relatives, and would increase the detectability of an isolation-by-distance effect. There is a higher brushtail population density in rainforest compared with dry sclerophyll forest (chapter two: 2.4.6.), which may produce smaller home ranges in this habitat type (Trewhella *et al*., 1988; Glessner and Britt, 2005; Dahle *et al*., 2006). Female brushtail possums typically settle near their mother's range, while male brushtails disperse as juveniles (Clout and Efford, 1984; Johnson *et al.*, 2001). If home range size is small, the dispersal distance of males need not be as great either, as the distance to get to non-relatives is reduced (Johnson and Gaines, 1990; Bowman *et al*., 2002). Thus the higher population densities of coppery brushtail possums compared with grey brushtails may be indicative of smaller home ranges and dispersal distances, and an increased ability to detect a correlation between relatedness and geographic distance when one exists (Trewhella *et al*., 1988; Bowman *et al.*, 2002; Glessner and Britt, 2005; Dahle *et al*., 2006).

Although a coppery brushtail possum was likely to be just as closely related to a nearby coppery as to a distant one, at a given geographic distance, coppery brushtail possums were likely to be to be more closely related to another coppery brushtail than to a grey, and a grey brushtail possum is just as likely to be related to another grey as to a coppery brushtail. Importantly, this means that after accounting for isolation by distance effects, along a habitat gradient the pairwise relatedness of two individuals of different colour morphology may be just as closely related to two possums of the same colour. In the situation where phenotypic traits under selection have diverged, and gene flow between adjacent populations of these different morphs is restricted, it is unprecedented that there would not be a correlation between the type of morphological comparison made and relatedness over distance (Wright, 1943; Slatkin, 1987; Smith *et al.*, 1997; Moritz *et al.,* 2000), making this result difficult to rationalize. An alternative, more likely explanation for the observed pattern in the distribution of brushtail morphs, is the possibility that colour is not directly genetically determined, but rather it is determined by habitat in some form of phenotypic plasticity.

3.5.3. An interpretation of the evidence assuming that fur colour in Atherton Tablelands brushtail possums is phenotypically plastic.

3.5.3.1. The evolution of a phenotypically plastic trait

The presence of both colour morphs in multiple clades, and the absence of coppery brushtails in other parts of Australia (Collins, 2003) suggests that the capacity to have a coppery coat colour is unique to Atherton Tablelands brushtail possums. In Chapter two I proposed that fur colour may be a phenotypically plastic trait, and based upon observations of both colour morphs in similar climates, and that young brushtail possums to keep the same colour once they have hair, I suggested that fur colour may be determined by maternal diet during early development. In Cropley *et al.*'s (2006) study of the effects of maternal diet on laboratory mice, fur colour was a congenital trait, with an underlying genetic mutation allowing phenotypic plasticity in fur colour. Variation in pelage was activated by the presence of key chemicals obtained *in utero,* which permanently altered the biochemical pathways followed within the cells controlling hair development, in turn affecting the ratios of melanin and eumelanin expressed in each hair on the mouse throughout its lifetime (Abdel-Malek *et al.*, 2001; Cropley *et al*., 2006). In Tablelands brushtails it is possible that a congential mutation allowing either coppery or grey fur colour evolved in an ancestral clade (Fig. 3.3), and has been conserved in extant clades due to its utility as a mechanism to provide camouflage across the fine-grained spatial scale of variation in habitat types on the Atherton Tablelands. Variation in the hue of coppery brushtail fur colour may indicate this adaptability across different rainforest localities, with colour correlated to forest site, not mitochondrial DNA lineage. Intraspecific plant chemistry can vary significantly between forest patches of a similar habitat type as a result of differing geological, climatic, and ecological conditions (Chapman *et al*., 2003). Therefore if two brushtail possums from different rainforest sites were to feed on the same quantities and species in their own forest patch, the chemical composition of the diets may differ. If fur colour of coppery brushtail possums is determined by maternal diet, it seems most likely that the interaction between coppery brushtail dietary

chemistry and the expression of hue occurs in both coppery morphs via the same biochemical pathways, but perhaps similar to Cropley *et al*.'s (2006) mice, the quantity or quality of key chemicals determines colour as different biochemical thresholds are met.

3.5.3.2. Selection and non-colour, body-shape morphologies

Selection has apparently acted upon brushtail possums to produce highly similar morphologies with respect to both fur colour and body-shape across the different clades (Fig. 3.6). For example, Grey brushtail possums have larger ears and shorter tails than coppery brushtail possums (chapter two: 2.4.2). The acoustic qualities of rainforest are very different to dry sclerophyll forest (Slabbekoorn and Smith, 2002). Variation in ear size between grey and coppery brushtail possums may reflect some adaptation to this, as larger ears can be associated with increased hearing ability (Coles *et al.*, 1989). Variation in the structural characteristics of different rainforest localities, and in particular, differences in the visual characteristics of each forest, may also explain why fine-scale variation in the hue of coppery fur colour correlates with rainforest locality, but not mitochondrial DNA structure. If the visual background of a particular rainforest site affects the intensity and direction of selection in each region, the evolution and conservation of a mutation allowing direct, fine-scale adaption to the surrounding environment would seem highly advantageous in providing camouflage to a dispersing species over many generations in a complex environment (Marchetti, 1993; Endler *et al*., 2005).

If we assume that fur colour is phenotypically plastic and determined by habitat (possibly through maternal diet), these other highly correlated traits of ear size, leg and tail length must also be determined by diet in some way (Scheiner *et al.*, 1991). In some owl species there is a trade-off between eumelanin colour expression and skeletal calcium density (Roulin *et al*., 2006). Among Atherton Tablelands brushtail possums the expression of red colour is most strongly associated with smaller ear size and hind leg length, while grey colour is associated with shorter tail length (chapter two, 2.4.2.). If the expression of each fur colour promotes a different nutritional and physiological tradeoff either as a

consequence of a cost associated with colour expression in brushtails, or as a consequence of a difference in diet that also leads to colour change, this would explain the observed morphological dichotomy between coppery and grey brushtail possums. However it seems unlikely that fur colour expression causes general nutritional deficiencies, as I observed no consistent trend in the morphological responses of each brushtail form; each colour morph had some skeletal characteristics that were smaller, some the same size, and some larger than the opposite morph. It is more likely that the developmental pathway controlling coat colour is linked with physiological controls for ear size, leg and tail length, such that the same selective pressures acting on fur colour have directed these other traits also (Scheiner *et al.*, 1991; Thompson, 1992).

3.5.3.3. Genetic structure and fur colour as a phenotypically plastic trait

The hypothesis that fur colour is determined by maternal diet provides a more likely explanation for the phylogenetic distribution observed, by providing a mechanism in which grey clades might easily evolve from coppery ones. Coppery brushtail females living in mosaic ecotone habitat had grey young, thus if coppery brushtail possums underwent a period of gradual expansion, we would expect to see an increase in population densities near and possibly in the ecotone, which should result in higher birth rates of grey brushtail possums in this mosaic habitat type. The dispersal of possums from this habitat into low population density dry sclerophyll forest might in time establish a new clade, especially if there is biased dispersal away from unfamiliar habitat types (Rice, 1984; Haughland and Larson, 2004; Edelaar *et* al., 2008; Mabry and Stamps, 2008), or resource paucity in regions between the colour forms following natural disasters (Hopkins *et al.,* 1990; Hopkins *et al.,* 1993). Alternatively if the coppery brushtails maintained their territories but the rainforest contracted, this would leave them without their preferred habitat and make the next generation more likely to have grey fur colour, providing they could reproduce before the mosaic ecotone transitioned to dry sclerophyll forest and selective pressures eliminated the population. Paleoecological data indicates that there has been widespread net rainforest contraction across the Atherton Tablelands over approximately 140,000 years (Kershaw, 1994). DeGabriel *et al.* (2009) found

that coppery brushtail possums have a high tolerance to plant secondary metabolites, defensive plant compounds which can greatly limit the quantities of vegetation consumed and nutrient intake of brushtails. This tolerance may have assisted survival when the forest became drier and transitioned into dry sclerophyll forest, as the eucalyptus species that largely comprise such forest would present coppery brushtail possums with new plant secondary metabolites and other compounds in their diet (DeGabriel *et al.*, 2009).

This plastic mechanism by which grey clades could evolve from coppery ones also explains the observations made using microsatellite data that at a given distance, possums of different colour morphology can be just as closely related to one another as brushtails of a shared colour (Fig. 3.6). An assumption of colour determination by maternal diet suggests that phenotypic divergence can occur without genotypic divergence. If the fur colour of these brushtail possums is environmentally determined by maternal diet, it is unlikely that there would be a selective bias on the alleles shared through gene flow based on colour morphology; proximity to the ecotone would be key to producing young with a fur colour not shared by their mother and allowing successful movement of these offspring and their alleles into a different habitat type. Differences in population density and the home range establishment of females is likely to result in more relatives at close proximity in high population density forest, so that within a given short-range transect distance, coppery brushtails are likely to have more relatives than grey brushtails (Johnson and Gaines, 1990; Knight *et al.*, 1999; Johnson *et al.*, 2001; Bowman *et al.*, 2002). Therefore, in a situation of fur colour being determined by maternal diet, and the observation of phenotypic divergence with reduced gene flow between morphs, but without directional selective bias on the genes shared between the two habitats, we would expect that at small distances of separation the relatedness among coppery brushtail possums will be higher than between a coppery and a grey brushtail possum or among grey brushtails (Knight *et al.*, 1999; Bowman *et al.*, 2002). Over larger geographic distances there is no expectation that possums separated by the same distance should be any differently related to others of the same colour morph than to those of a different morph (Wright, 1943). This is

what I observed (Figs. 3.3 and 3.6), suggesting that the determination of fur colour by maternal diet, although a mechanism not observed before in wild mammalian populations, but which has one precedent in Cropley *et* al.'s laboratory experiment with mice (2006), may be the best explanation for the distribution of morphs observed and their genetic characteristics.

Although coppery brushtail possums are only found on the Atherton Tablelands, this mode of colour determination may be extended to account for the distribution of black brushtail colour morphs in Tasmania and New Zealand. In these regions, as on the Atherton Tablelands, the more melanistic brushtail morph inhabits the wetter, denser, more diverse forest, and despite small distances between habitat types the grey form is found in the drier, sparser less diverse forest (Guiler and Banks, 1958; Kean, 1971). If diet can determine brushtail fur colour, I would hypothesize that the diets of black New Zealand and Tasmanian possums may be similar to coppery brushtail possums in that some key component that activates melanin pigmentation pathways to produce their distinctive coat colour.

The identical sequences between coppery and grey Atherton Tablelands brushtails along 642 base pairs of the Melanocortin-1-receptor (MC1R) gene may be indicative of the location of a shared mutation allowing fur colour to be a congenital trait. MC1R is a highly conserved functional gene that in concert with four other genes **(**Miller *et al.,* 1997; Abdel-Malek *et al.,* 2001; Prasolova *et al.,* 2002; Kerns *et al.,* 2003; Gratten *et al.,* 2007), is responsible for colouration in many diverse taxa: birds (Theron *et al.,* 2001; Kerje *et al.,* 2003; Doucet *et al.,* 2004, Nadeau *et al.,* 2006; Baião *et al.,* 2007), reptiles (Rosenblum *et al.,*2004) and eutherian mammals (Miller *et al.,*1997; Kijas *et al.,* 1998; Wada *et al.,*1999; Everts *et al.,* 2000; Rouzaud *et al.,* 2000; Rieder *et al.,* 2001; Kerns *et al.,* 2003; Nachman *et al.,* 2003; Hoekstra *et al.,* 2004; Vage *et al.,* 2005; Fontanesi *et al.,* 2006; Hoekstra *et al.,* 2006; Mengel-Jørgensen *et al.,* 2006). MC1R controls the production of phaeomelanin; responsible for red or yellow colour; and eumelanin, which produces black or brown colouration (Fontanesi *et al.*, 2006). I expected that because of the strong conservation of MC1R across a wide

range of taxa, its frequent role in colour determination, and the red colouration of the coppery brushtail possum (possibly due to phaeomelanin production) that there should be a mutational difference between the two colour morphs in MC1R. The identical sequences between the two morphs alone falsify neither theory of colour determination in Atherton Tablelands brushtails: whether developmental phenotypic plasticity, or genetic determination of colour via a novel mechanism and parapatric divergence. Though as I have already come to question the assumption that fur colour is genetically determined, and considering that my hypothesis that fur colour is an environmentally determined congenital trait is better supported by my results, it is possible that mutation shared by coppery and grey brushtail possums on the Atherton Tablelands is located on MC1R and in response to differing environmental conditions in early development, perhaps through methylation (Cropley *et al*., 2006), fur colour expression in each animal can be either grey or coppery. If such a mutation is limited to brushtail possums of the Atherton Tablelands, then comparisons of their sequences with those from grey brushtail possums less likely to have this mutation, such as individuals geographically distant from this Tablelands lineage, would assist in locating the gene's position.

3.5.4. Conclusions

I suggest that the best explanation for the morphological (Chapter Two) and genetic distributions observed is that Atherton Tablelands brushtail possums share a mutation that has allowed fur colour to evolve into a congenital, environmentally determined trait, with offspring coat colour determined in early development, most likely by maternal diet. It is possible that fur colour is genetically determined by some novel mechanism of genetic trait determination, with processes of parapatric divergence producing the distribution observed, however at this time such a hypothesis is not well supported by the evidence at hand; genetic similarity is not predictive of morphology, and fur colour does not seem to be an inherited trait.

A definitive demonstration of the effects of maternal diet on offspring phenotype is needed and could be undertaken using an experiment in captivity, breeding

brushtails to examine conformity to Mendel's inheritance laws and testing the offspring colour frequencies against the colour ratios produced when controlling the diet of females during pouch young development to produce young of different colours. Despite the challenges of such an intensive experiment, there are considerable advances to be made from investigating and conclusively demonstrating the processes behind coppery brushtail possum evolution. The evolution in a wild mammalian population of a visible congenital trait that is subject to strong selective pressures, but with its expression determined by maternal diet would be highly significant in our understanding of evolution. It presents mammalian evolutionary research with a novel mode of change that allows for widespread single generation adaptation by offspring to new conditions in response to the environmental conditions experienced by the parental generation. This is significant because it means that we must question our historic assumption that the likelihood and frequency of genetic mutation limits the capacity for change in the prevailing phenotype of a mammalian population (Lande, 1980). This mode of adaptation allows a species to change between forms relatively quickly in an evolutionary sense, without significant skew to allelic diversity or even a significant genetic bottleneck, and if necessary to return to their original form again with little if any genetic trace of such phenotypic shifts.

CHAPTER FOUR

Thesis Synthesis

The aim of this study was to investigate the mechanisms responsible for the morphological and genetic distribution of coppery and grey brushtail possum forms on the Atherton Tablelands in Australia's Wet Tropics. The reputed parapatry of these two colour morphs presented an important opportunity to examine the role that habitat may play in mammalian diversification independent of geographic isolation. Using brushtail possums as a model for habitat-mediated mammalian evolution, I have sought to answer three key questions:

1. To what morphological and behavioural (habitat preferences, reproductive synchronicity) degree is the coppery brushtail possum, *Trichosurus vulpecula johnsonii*, distinct from the common form, *Trichosurus vulpecula vulpecula*?
2. Are these subspecific differences being maintained despite gene flow between coppery and common brushtail possums?
3. If so, are selective pressures playing a significant role in the divergence, or lack thereof, of these two brushtail possum subspecies?

To answer these questions I examined five important evolutionary influences and indicators, testing the mutually exclusive hypotheses of each:

1. that distinct morphological groups of brushtail possums have a) evolved on the Atherton Tablelands, or b) are not detectable
2. that the distribution of morphs is the result of a) sympatric divergence, b) parapatric divergence, or c) allopatric divergence
3. that between populations along a habitat gradient there is a) gene flow, or b) reproductive isolation
4. that the evolution of the morphologies observed is the product of a) genetic drift, or b) natural selection
5. that fur colour in Atherton Tablelands brushtail possums is either a) genetically determined, b) a temporary environmentally determined,

plastic trait, c) is a plastic trait permanently determined by climate, or d) a plastic trait permanently determined by diet.

In this chapter I summarize the main findings of my thesis (illustrated summary in appendix 4.0), outline their implications for wider research and conservation, and provide recommendations for further study of this unusual evolutionary trait.

4.1. Morphological and behavioural comparisons

Coppery and common grey brushtail possums can be distinguished by their habitat affinities, colour and body-shape morphologies. Visually, these two morphs can be characterised by differing amounts of fur colour saturation; the intensity of colour expressed. Along habitat gradients from dry sclerophyll forest, through mosaic ecotone, to rainforest, these two forms have very strong and different habitat affinities; possums with low levels of colour saturation expressed a grey colour and did not inhabit rainforest, while brushtails with high levels of fur colour saturation expressed a red coloured coppery phenotype, and did not occur in the dry sclerophyll forest. Brushtails in the ecotone expressed either a coppery or grey colour, they did not display a distinct colour morphology. This distribution confirmed the suggestions of Winter (1984) that each colour morph was habitat specific, however the distribution of coppery fur colour was more complex than expected. Coppery brushtail possum fur colour was characterised by high saturation levels but there was a dichotomy in the shade of red expressed, which was either a red-orange or a red-purple hue. These differences in hue correlated with the rainforest locality of each brushtail, and were not explained by genetic divergence, suggesting they may be due to environmental influences. However, it is important to note that fur colour did not appear to change once established in early development, thus if environment does in some way determine colour, its influence is made early in a possum's life, and is permanent.

The distribution of brushtail possums in the mosaic ecotone that divides the two habitat types by less than a kilometre was significant not only because it was inhabited by both colour morphs, but also because they occurred there at very

low densities relative to the other habitat types. The ecotone supported only 0.182 ± 0.132 brushtails per hectare, which may suggest some potential for the mosaic ecotone habitat to restrict gene flow between the two morphs by limiting the frequency of interactions between them, or by deterring migration through this habitat type. Rainforest had a population density 18.6 times greater, with 3.392 ± 0.132 brushtail possums per hectare. . Dry sclerophyll forest supported 8.7 times more brushtails than ecotone habitat, with 1.588 ± 0.686 brushtails per hectare. The differences in population density between rainforest and dry sclerophyll forest is likely a result of there being more resources in the rainforest (Taitt, 1981), whether in the form of more tree hollows for possum dens or increased food productivity.

Coppery and grey brushtail possums were morphologically distinct in body shape as well as fur colour, but not in body mass, as was historically suspected (Winter, 1984). On average, coppery brushtail possums had ears that were 8mm shorter and 3.4mm thinner, legs that were 3.6mm shorter from knee to heel, and tails that were 34mm longer than grey brushtail possums of the Atherton Tablelands. There was no sexual dimorphism in brushtail possums along the habitat gradient, for body size or colour. Both grey and coppery brushtail possums were also similar in that they both appeared to reproduce seasonally and were reproductively synchronous along a habitat gradient.

4.2. Gene flow along the habitat gradient

Gene flow was restricted, but not absent between coppery and grey brushtail possums. Across the broad scale of the Atherton Tablelands coppery and grey brushtails were not genetically distinct. These two morphs have evolved together in multiple distantly related clades. Their morphological distribution is not the product of secondary contact between reciprocally monophyletic populations. Grouping populations by morphology did not explain the genetic differences between them. However the mean F_{st} values for each population demonstrated that genetic divergence was greatest among the populations closest to rainforest, and that brushtail populations become genetically more

similar with increasing remoteness from rainforest. The identification and separate grouping of the four populations that were closest to rainforest explained 30.1% of mitochondrial DNA variation. These included coppery and grey brushtail possums found along the habitat gradient, which were distinct from each other. Yet measurements of gene flow between these two populations indicated that approximately two brushtails per generation move from dry sclerophyll forest to rainforest and *vice versa*. These findings demonstrate that while gene flow is restricted along a habitat gradient, reproductive isolation has not occurred.

4.3. The role of selection

Natural selective pressures may have helped to shape the distribution, divergence and evolution of the coppery brushtail possum. As dimorphic body size is characteristic of sexual selection in brushtail populations (Isaac and Johnson, 2003), and there is a lack of sexual dimorphism both by colour and body-shape in Atherton Tablelands brushtail possums it seems that sexual selection is not a significant influence on these traits at this time (Lande, 1980b). Using the mitochondrial clade groups to investigate morphology I found that variation in body-shape morphology was not associated with genetic similarity, but with fur colour; genetically distant brushtails with the same fur colour also shared a common body-shape morphology, and genetically similar brushtails that were morphologically different by colour, also differed in body size. If brushtail fur colour is genetically determined, then this together with the dichotomous distribution of morphs along habitat gradients suggests that natural selection is acting upon these morphological traits to produce the phenotypes and distribution observed (Endler, 1977; Slatkin, 1987).

If brushtail fur colour is not genetically determined, but instead is phenotypically plastic and determined by environmental effects, then this association between colour and body-shape morphologies may derive from a common influence (Scheiner *et al*., 1991; Roulin *et al*., 2006). However each morph displayed increased structural development in few and differing traits, thus the correlation between colour and non-colour morphological traits is not indicative of a general trade off being made between brushtail development and colour expression.

The associations between colour, ear size, tail and leg length could be the product of linkage disequilibrium between separate genes, or they might all be connected to a similar developmental pathway that responds consistently to the environmental conditions experienced by each colour morph (Scheiner *et al*., 1991). Although both modes of development may account for the correlation between colour and body-shape morphologies without invoking selection, the correlation of traits across geographic and genetic distance suggests a history of selection on the combination of traits uniquely associated with each brushtail morph (chapter three, Fig. 3.6; Scheiner *et al*., 1991). The strong habitat affinities of each colour morph further suggest that the morphologies and distributions of these two forms are in part products of selective pressures acting upon them (Nosil, 2004). Not one coppery brushtail along the habitat gradient was observed in dry sclerophyll forest, nor were any grey brushtails observed in rainforest (chapter two, 2.4.4). The documented dispersal of male brushtails as they reach maturity (Clinchy *et al*., 2004; Isaac and Johnson, 2003), even allowing for a preferential dispersal of juvenile males into habitats akin to their natal ranges (Haughland and Larson, 2004; Mabry and Stamps, 2008), together considered with the typical longevity of brushtail possums (Winter, 1980; Isaac, 2005), suggests that a low frequency of migrant possums should be detected in atypical habitat. Their absence is likely indicative of selection against coppery brushtail possums occupying dry sclerophyll forest and grey brushtails inhabiting rainforest (Darwin, 1859; Nosil, 2004).

4.4. But is coppery brushtail fur colour genetically determined?

A model of genetic inheritance of colour did not adequately account for the morphological and genetic distributions observed. With selection acting on morphology and gene flow restricted along a habitat gradient, then at the same distance of separation I expected that relatedness between possums of different colour morphs would be less than the relatedness between possums of the same colour (Wright, 1943; Slatkin, 1987; Smith *et al.*, 1997). This was not observed. Across pairwise comparisons of all individuals there was a significant negative linear correlation between relatedness and the base ten logarithm of geographic distance. When this isolation by distance was accounted for,

possums of different colour morphologies could be just as closely related to each other as two grey brushtails. Coppery brushtail possums were likely more closely related to other coppery brushtails, whether geographically close or distant. I believe this to be a consequence of their higher population densities, which may mean that relatives in rainforest possess smaller home ranges and live closer (Trewhella *et al*., 1988; Johnson and Gaines, 1990; Bowman *et al.*, 2002; Glessner and Britt, 2005; Dahle *et al*., 2006), thus increasing relatedness over the distance sampled, and making the detection of such a familial effect easier than for populations with smaller population densities were we to increase the survey area. Ultimately however, current models of genetic inheritance were inadequate in explaining the distribution observed. If fur colour is a genetically determined trait, then brushtail colour is inherited and determined via an unprecedented mechanism. While this can not be discounted, I suggest that the morphological and genetic distributions are better accounted for by a model in which brushtail fur colour is a phenotypically plastic trait.

4.5. A theory of selection and phenotypic plasticity

Fur colour can be phenotypically plastic. Climate and diet can determine fur colour, though usually only temporarily (Iljin and Iljin, 1930; Fox, 1962; Hill, 1993; Lyons *et* al., 2005; Vage *et al.*, 2005; Hays *et al.*, 2006). In these brushtail possums fur colour appears to be permanently determined in early development. The presence and fine-scale distribution of coppery and grey brushtails in rainforest fragments suggested that climatic effects are highly unlikely to be determining fur colour. There has been one previous demonstration of diet permanently determining fur colour *in utero* in laboratory mice, but never in a wild population (Cropley et al., 2006). I suggest that determination of brushtail fur colour by maternal diet and selection upon this trait better explains the observed morphological distribution and genetics in Atherton Tablelands brushtail possums, though further experimental research is needed to conclusively demonstrate this effect: to explore how widespread this capacity for coppery brushtail colour is, and discover which foods are involved in affecting fur colour expression. This mechanism of colour determination may

also explain the distribution of Tasmanian and New Zealand brushtail colour morphs, which like those on the Atherton Tablelands, are distinguished by the more melanistic form inhabiting wetter, denser habitat, and the grey morph having an affinity for drier, less dense forest despite small distances between habitat types (Guiler and Banks, 1958; Kean, 1971). This type of congenital change would be highly significant in evolutionary research as it allows without mutation the widespread single generation adaptation of offspring to the environmental conditions experienced by the parental generation. This would have far reaching implications for our understanding of evolutionary mechanisms, particularly our assumptions about the capacities for species to adapt to changing environments and the role of habitat in directly determining phenotypic expression.

4.6. Recommendations for further research and ecological management

The coppery brushtail possum is at present classified *Trichosurus vulpecula johnsonii*, a subspecies of *Trichosurus vulpecula* (Kerle and How, 2008). While the coppery and common brushtail possums are morphologically and behaviourally dichotomous, genetically they are indistinguishable, and should be considered a single subspecies. Until we have experimentally demonstrated the ability of Atherton Tablelands brushtail possums to produce opposite coloured offspring as a result of the environmental conditions experienced, and explored whether this ability is limited to brushtail possums of this region, I would advocate that the coppery brushtail possum continue to be regarded as a unique Wet Tropics endemic subspecies. Furthermore, the likely role of habitat in determining fur colour makes the conservation and management of these forest types on the Atherton Tablelands particularly important. The mosaic ecotone along these gradients is crucial not just for brushtails; in providing a zone able to be inhabited by both colour morphs, facilitating colour change and migration between them; but also more broadly, ecotone habitats are important in speciation and in generating rainforest biodiversity (Smith *et al.*, 1997; Schneider *et al.,* 1999; Moritz *et al*., 2000). Thus such habitat gradients are especially valuable and their successful management is vital.

Looking ahead, I believe a definitive demonstration of the effects of maternal diet on offspring phenotype is needed and could be undertaken using an experiment in captivity, controlling the diet of females during pouch young development to produce young of different colours. The types and quantities of different rainforest compounds could also be tested to find how they affect the ability of the mother to produce a coppery offspring, as Cropley *et al.*'s study (2006) suggested that there may thresholds to colour change. This sort of study would also allow experimental testing of the timing at which colour is determined by manipulating temporal access to key diets during pouch young development. However there are several practical difficulties associated with such an experiment. Firstly, although there has been one study examining coppery brushtail diet (Kerle, 1984), we know very little about the full extent of their dietary intake, and do not know what plants or chemicals among the vast array of rainforest plants would trigger the development of coppery offspring. Investigation of rainforest fragments and comparison with non-fragmented rainforest, together with further behavioural studies may help to narrow the range of candidate species. The similar morphological distribution of colour morphs in Tasmania (Guiler and Banks, 1958) and New Zealand (Kean, 1971) may also provide an opportunity to find common dietary characteristics associated with increased melanin expression, even to test directly with possums expressing the black colour phenotype. Secondly, brushtails kept in captivity less than two years do not have high reproductive success (Kerle *et* al., 1991; Baker and Gemmell, 1999). This leads to the third difficulty in such an experiment: in order to achieve good reproductive success a long acclimatization period of several years is required for brushtails, meaning that animals must be mated in captivity. Even ignoring the difficulties of breeding animals in captivity, this increases the number of brushtails needed for such an experiment and thus demands a greater outlay in resources; large cages and appropriate food for every possum. Despite these challenges, there are considerable advances to be made from investigating and conclusively demonstrating the processes behind coppery brushtail possum evolution. There is the potential for such a study to increase our understanding of the role of environment on phenotypic change, of diet on appearance, of phenotypic

change to occur across populations without mutation, of the inheritance of traits, and to challenge our current theories of how quickly species can adapt to a changed environment.

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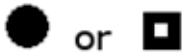
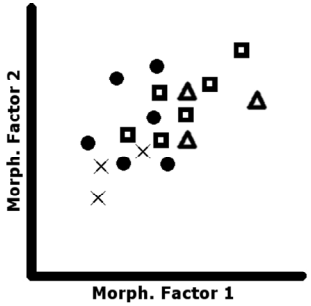
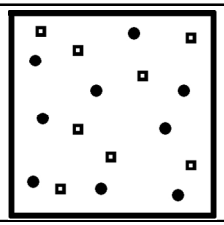
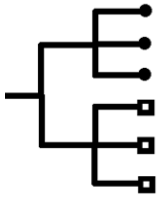
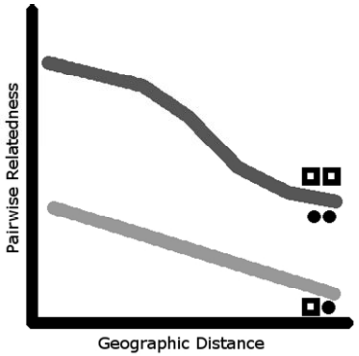
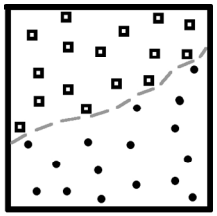
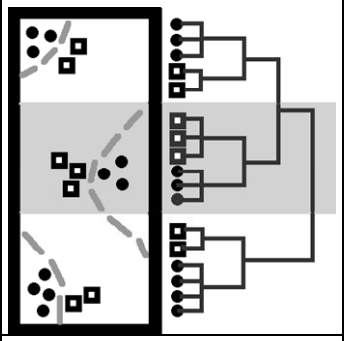
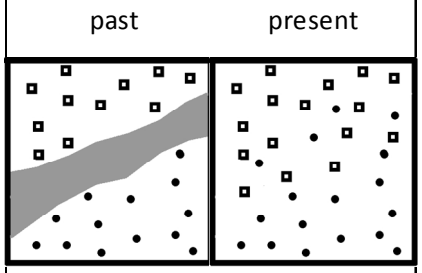
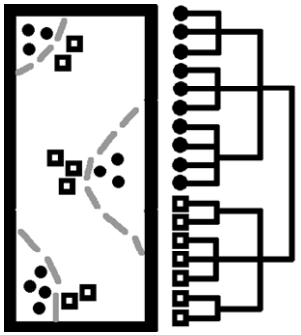
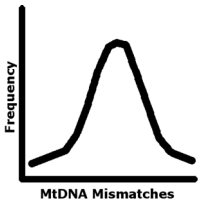
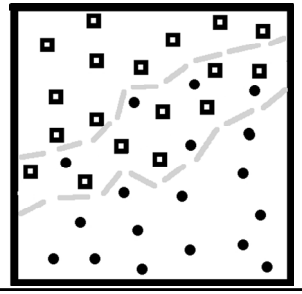
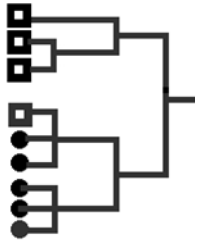
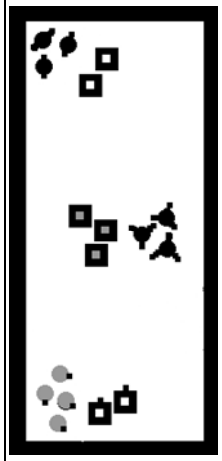
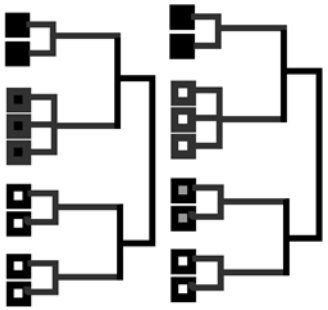
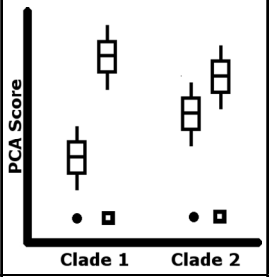
APPENDICES

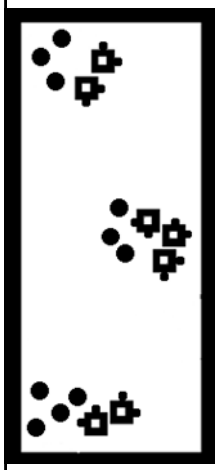
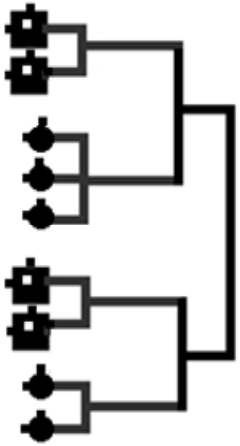
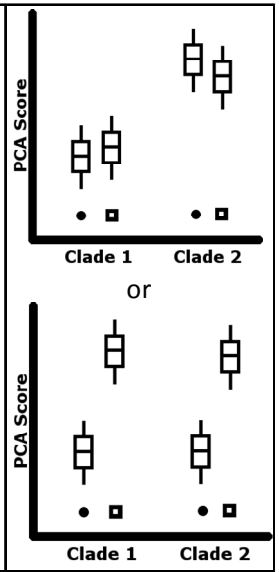
Appendix 1.0 Table of hypotheses and predictions

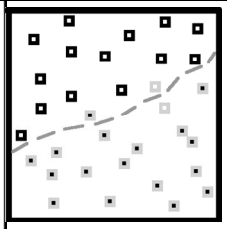
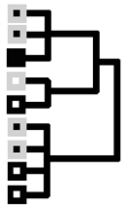
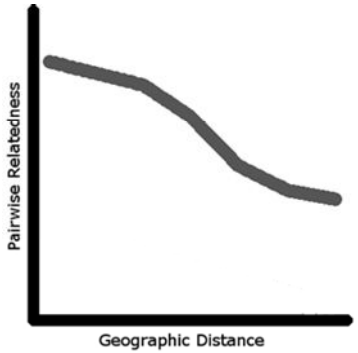
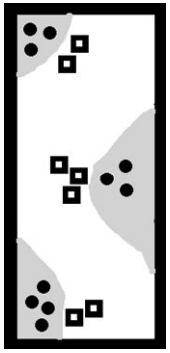
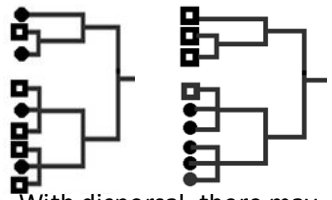
The following table illustrates the expected morphological, mitochondrial, and microsatellite distribution patterns for different evolutionary hypotheses relevant to the case of the coppery brushtail possum. Square and circle symbols denote different morphological groups, with shading and shape 'appendages' representative of additional unlinked morphological characteristics an individual expresses and how these traits correlate across populations. The dashed lines in the distribution diagrams symbolise geographic or environmental boundaries. In graphs showing expected correlations between factors such as relatedness and geographic distance, the notation of two squares or two circles is representative of pairwise comparisons between individuals of the same morphology (here they overlap, and so one line may have two lables), and an annotation of one square and one circle denotes comparisons between individuals of different morphology.

	Hypothesis	Predicted observations	
		Distribution	Morphological measurements
Are there seperate morphs?	morphs can be identified	Eg. 	 clustering by morphology into two seperate groups. There may be variation within these groups but it is less than the divide between the two morphotypes
	No clear morph groups	Eg. 	 morphology not clustered. Variation within these groups indistinguishable from that between two morphotypes

Hypothesis	Predicted observations			
	Distribution	MtDNA	Microsatellites	Other
sympatry			Across a habitat gradient individuals of the same morph are expected to be more closely related than individuals of the opposite morph, though hybridisation possible in close proximity 	
parapatry				
allopatry and secondary contact				 expansion following deterioration of physical barrier

Has full speciation occurred?	**geneflow between populations**	For there to be gene flow, opposite morphs must be able to interact and mate eg. in parapatry: 	 with geneflow, alleles for a particular morphology are shared		NeM values calculated from genetic diversity using multiple markers. NeM>1 is a high level of gene flow.
Mechanisms of reinforcing divergence	**genetic drift**	 populations may share one morphological feature, but others will have 'drifted' and vary from one population to the next	genetically & morphologically similar or genetically different with variation in many morphological traits 		 or 

Mechanisms of reinforcing divergence	**natural selection**	 populations that share one morphological feature, may share others also	genetically different but similar for several morphological traits 		
	sexual selection				sexual dimorphism

<table>
<tr><td rowspan="4">Is fur colour genetically or environmentally determined?</td><td>fur colour is genetically determined</td><td colspan="4">the morphological distribution, phylogeny, and pairwise relatedness at a given distance along a habitat gradient would be products of the processes outlined above, ond would conform to these expectations</td></tr>
<tr><td>fur colour is temporary</td><td>  fur colour will change in captivity or with migration</td><td>  there may be no correlation between lineage and colour (dependent on dispersal)</td><td rowspan="2">relatedness and geographic distance relationship the similar among individuals of the same morph and between individuals of different morphs  </td><td>when subject to the same environment, fur colour should be uniform</td></tr>
<tr><td>fur colour is permanent and determined by climate</td><td rowspan="2">  the potential for individuals to cross habitat types depends on the relative amounts of migration and strength of selection</td><td rowspan="2">  With dispersal, there may be no correlation between lineage and colour (eg. above left). If there is no dispersal or if it is male-biased, there may be a correlation (avove right). This correlation is also more likely if there is selection against dispersal into new environments</td><td>fur colour will not change in captivity or with migration. When subject to the same climate, fur colour should be uniform</td></tr>
<tr><td>fur colour is permanent and determined by diet</td><td>Fur colour will not change in captivity or with migration. When subject to the same diet, fur colour should be uniform</td></tr>
</table>

Appendix 2.0 Macro 'RandomMorphDist'

This macro randomizes the observations of brushtail colour morphs along a habitat gradient, and subsamples the number of possums seen in each habitat 5000 times. This then allowed me to calculate the likelihood that distribution of the two brushtail colour morphs along a habitat gradient is the product of chance.

In setting up data for this macro to operate successfully in Microsoft Excel (Microsoft Corporation, 2003), column A held the records of grey or coppery brushtail colour, with each morph represented by either a '1' or '2'. The number of replicates can be adjusted by altering the text 'For i=1 to 5000' such that '5000' is replaced by the number of iterations required. The formula used and its parameters can be adjusted with modifications to the line '"=COUNTIF(RC[-3]:R[8]C[-3],2)"', which in this case counts the number of grey brushtail possums (designated by the value '2' in column A) that were among a random selection of nine brushtail possums from each replicate. Nine brushtail possums were selected from each randomisation replicate as this was the number observed in the dry sclerophyll forest, thus allowing a calculation of the probability that all grey brushtail possums were distributed together by random chance.

```
Sub RandomMorphDist()
'
' RandomMorphDist Macro
' Macro recorded 15/06/2009 by Sarah Emily Kerr
'

'

    Dim i As Integer
    For i = 1 To 5000
    Columns("A:A").Select
```

```
    Application.Run "PERSONAL.XLS!RandomSort"
    Range("D1").Select
    ActiveCell.FormulaR1C1 = "=COUNTIF(RC[-3]:R[8]C[-3],2)"
    Range("D1").Select
    Selection.Copy
    Selection.PasteSpecial Paste:=xlPasteValues, Operation:=xlNone,
SkipBlanks _
        :=False, Transpose:=False
    Application.CutCopyMode = False
    Selection.Insert Shift:=xlDown
    Next i
End Sub
```

Macro ‘RandomSort’ (rbrhodes, 2007); subroutine within the macro ‘RandomMorphDist’ which randomly reassigns the cells of a single column; column A; to new positions.

```
Sub RandomSort()

    Dim rnum
    Dim arr()
    Dim i As Long
    Dim x As Long
    Dim col As Long
    Dim nrow As Long
    Dim srow As Long
    Dim countt As Long

     'check one col and more than 1 row
    If Selection.Rows.Count = 1 Or Selection.Columns.Count > 1 Then Exit Sub
```

```
    'turn off screen for  speed
    Application.ScreenUpdating = False

    'get start row number
    srow = Selection.Rows.Row

    'get current col #
    col = Selection.Column

    'get number of rows
    countt = Selection.Rows.Count

    'sort
    Selection.Sort Key1:=Cells(srow, col), Order1:=xlAscending,
Header:=xlGuess, _
    OrderCustom:=1, MatchCase:=False, Orientation:=xlTopToBottom

    'eliminate blanks
    Range(Cells(srow, col), Cells(srow, col).End(xlDown)).Select

    'check number of rows remaining
    If Selection.Rows.Count < countt Then
        'was blanks, reset  count
       countt = Selection.Rows.Count
    End If

    'set array length
    ReDim arr(countt)

    'arr index
    x = 1

    'row index
```

```
    nrow = srow

     'load array with values
    For i = 1 To countt
        arr(x) = Cells(nrow, col)
        x = x + 1
        nrow = nrow + 1
    Next i

     'reset i
    i = 1
    nrow = srow

     'repopulate selection 'randomly'
    Do Until i = countt + 1
        rnum = Int((countt - 1 + 1) * Rnd + 1)
        If arr(rnum) <> "" Then
            Cells(nrow, col) = arr(rnum)
            arr(rnum) = ""
            i = i + 1
            nrow = nrow + 1
        End If
     Loop

    Cells(srow, col).Select

     'turn on screen
    Application.ScreenUpdating = True

End Sub
```

Appendix 2.1: Calculation of brushtail possum population densities

Brushtail possum density; calculated using Buckland *et al.* (2004).

x=perpendicular distance from the transect line

Pr=probability

Pa=probability of detection

g(x)=detection function

L=effective length of the transect line

w= maximum detection distance

$f(x)\,dx = \Pr\{\text{object in } x, x+dx \Pr\{\text{object in } x,x+dx)|\text{ object detected}\}$

$$= \frac{\Pr\{\text{object in } (x, x+dx) \text{ and object detected}\}}{\Pr\{\text{object detected}\}}$$

$$= \frac{(\Pr\{\text{object detected} \mid \text{object in } (x, x+dx))\cdot(\Pr\{\text{object in } (x, x+dx)\}}{Pa}$$

$$= \frac{g(x)\cdot(dx\cdot L)/(w\cdot L)}{Pa}$$

$$= \frac{\text{detection function}\cdot dx\cdot \text{effective line length}/(\text{maximum detection distance}\cdot \text{effective line length})}{\text{probability of detection}}$$

$$\therefore f(x) = \frac{g(x)}{w\cdot Pa}$$

w_{dry}= 50 meters

w_{eco}= 40 meters

w_{rf}= 30 meters

$g_{dry}(x)= 1.0285-7.33E^{-2}x +2.22E^{-3}x^2-2.26E^{-5}x^3$

$R^2=0.964$, $F_{3,7}=61.913$, $P<0.0001$

$g_{eco}(x)= 0.973+2.77E^{-3}x -1.69E^{-3}x^2+2.5E^{-5}x^3$

R^2=0.958, $F_{3,7}$=53.8748, P<0.0001

$g_{rf}(x)$= 0.9366-6.83E^{-2}x +1.70E$^{-3}x^2$-1.42 E$^{-5}x^3$

R^2=0.978, $F_{3,7}$=102.369, P<0.0001

μ= area under the curve in the relationship between x and g(x)

$$\int_0^w f(x)dx = 1$$

$$\therefore \mu = \int_0^w g(x)dx$$

$$\mu_{dry} = \int_0^{50} -5.65 \cdot 10^{-6}(x-77.616)x((x-53.358)x+2345.33)$$

$$= 16.987$$

$$\mu_{eco} = \int_0^{40} 6.25 \cdot 10^{-6}(x+34.465)((x-124.598)x+4515.83)$$

$$= 21.072$$

$$\mu_{rf} = \int_0^{30} -3.55 \cdot 10^{-6}(x-81.145)x((x-78.48)x+3251.51)$$

$$= 9.788$$

μ= w· Pa

$$Pa = \frac{\mu}{w}$$

$$Pa_{dry} = \frac{16.987}{50}$$

$$= 0.3398$$

$$Pa_{eco} = \frac{21.072}{40}$$

$$= 0.5268$$

$$Pa_{rf} = \frac{9.788}{30}$$

$$= 0.3263$$

μ= w· Pa

μ= maximum detection distance · probability of detection

μ= effective transect half width

∴ area surveyed = a = 2wL

effective area surveyed = A

A = 2μL· Pa

L_{dry}= 6000 meters

L_{eco}= 4950 meters

L_{rf}= 6000 meters

A_{dry} = 2·16.987·6000·0.3398

= 69266.1912 m^2

A_{eco} = 2·21.072·4950·0.5268

= 109897.223 m^2

A_{rf} = 2·9.788·6000·0.3263

= 38325.89 m^2

N= observed number of animals

N_{dry} = 11

N_{eco} = 2

N_{rf} = 13

E(s) = clustering coefficient

E(s) = 1

∴ Density = $\frac{N}{A}$

D_{dry} = $\frac{11}{69266.191}$

= 1.588 possums per 10000m²

= 1 possum per 6297.23 m²

$D_{eco} = \frac{2}{109897.223}$

= 0.182 possums per 10000m²

= 1 possum per 54945.05 m²

$D_{rf} = \frac{13}{38325.89}$

= 3.392 possums per 10000m²

= 1 possum per 2948.11 m²

Calculation of standard error:

Using N for each transect.

$\frac{\text{Ndry transect} * \text{Ldry}}{\text{L dry transect}}$ = projected N for L_{dry}=N_{proj}

$D_{\text{dry transect}} = \frac{\text{N proj}}{\text{Adry}}$

Standard error of D from the six surveys of each habitat was then calculated:

SE_{dry}=±0.686 brushtail possums per 10000m²

SE_{eco}=±0.132 brushtail possums per 10000m²

SE_{rf}=±0.559 brushtail possums per 10000m²

Appendix 2.2: Source locations of brushtail possums sampled

N	Habitat	55K	UTM	Colour morph	Status
4	dry sclerophyll	336530	8086200	4 greys	Roadkills
1	dry sclerophyll	342900	8121470	1 grey	Roadkills
1	dry sclerophyll	334480	8074590	1 grey	live capture
21	dry sclerophyll	331670	8081790	21 greys	live capture
6	dry sclerophyll	333550	8111340	6greys	Roadkills
6	dry sclerophyll	314850	8156730	6 greys	live capture (Winter)
1	dry sclerophyll	339210	8051320	1 grey	Roadkills
1	dry sclerophyll	334880	8106790	1 grey	live capture(Winter)
1	dry sclerophyll	335920	8071110	1 grey	live capture
4	ecotone	332080	8083120	3 coppery, 1 grey	live capture
9	rainforest	332100	8084310	9 coppery	live capture
8	rainforest/urban	352820	8063430	2 greys, 6 coppery	live capture
4	rainforest frag.	340850	8086670	4 coppery	live capture
1	rainforest frag.	338340	8093580	1 coppery	Roadkills
1	rainforest frag.	346250	8084800	1 coppery, 2 greys	Roadkills

Appendix 2.3: Mean body shape dimensions of each colour morph.

	COLOUR MORPH					
	Coppery			Grey		
MEASUREMENT TYPE	N	mean	SE	N	mean	SE
body mass (g)	20	1854.70	80.20	34	1836.32	63.73
head length (mm)	22	82.11	0.96	37	82.41	1.11
head width (mm)	21	49.04	0.61	27	48.69	1.64
ear length (mm)	23	44.00	0.69	39	52.01	1.33
ear width (mm)	22	29.67	0.63	30	33.04	0.77
tail length (mm)	24	339.67	4.58	42	305.26	4.46
hind leg heel-knee length (mm)	23	96.31	1.09	39	99.89	0.96
pes (foot) length (mm)	20	58.32	0.64	37	58.49	0.46

Appendix 2.4: Pouch young birth dates for reproductive synchronicity assessment.

Colour morph	Date observed	Head length (mm)	Body length (mm)	Date of birth	number of days from 1st June
Grey	01/11/2005	51		08/07/2005	38
Grey	28/10/2005		150	26/06/2005	26
Grey	21/09/2005	44		16/06/2005	16
Grey	28/10/2005	58		16/06/2005	16
Coppery	21/10/2006	54		19/06/2006	19
Coppery	30/11/2006		200	13/06/2006	13
Coppery	01/12/2006		200	14/06/2006	14
Coppery	07/11/2007		200	21/05/2007	-11
Coppery	07/11/2007		150	06/07/2007	36

Appendix 3.0: Macro 'RandomizedSlopes'

This macro calculates the value of the slope in each linear relationship for 5000 randomly generated combinations of 2 variables: in this case pairwise relatedness between individuals (Queller and Goodnight, 1989) and pairwise geographic distance between brushtails.

Used in Microsoft Excel (Microsoft Corporation, 2003), column A holds the values of the dependent variable and column B holds the values of the independent variable. The number of combinations can be adjusted by altering the text 'For i=1 to 5000' such that '5000' is replaced by the number of iterations required. The formula used and its parameters can be adjusted with modifications to the line '"=LINEST(RC[-3]:R[560]C[-3],RC[-2]:R[560]C[-2],TRUE,FALSE) "'. See Appendix 2.0 for detail of the subroutine macro 'RandomSort' (rbrhodes, 2007).

```
Sub RandomizedSlopes()
'
' RandomizedSlopes Macro
' Macro recorded 16/05/2009 by Sarah Emily Kerr
'

'
    Dim i As Integer
    For i = 1 To 5000
    Columns("A:A").Select
    Application.Run "PERSONAL.XLS!RandomSort"
    Range("D1").Select
    ActiveCell.FormulaR1C1 = _
        "=LINEST(RC[-3]:R[560]C[-3],RC[-2]:R[560]C[-2],TRUE,FALSE)"
    Range("D1").Select
    Selection.Copy
    Selection.PasteSpecial Paste:=xlPasteValues, Operation:=xlNone,
SkipBlanks _
```

```
        :=False, Transpose:=False
    Application.CutCopyMode = False
    Selection.Insert Shift:=xlDown
    Next i
End Sub
```

Appendix 3.1: Mitochondrial DNA alignment

The alignment of all Tablelands sequences, including 4739, which is the young of 782D, together with New Zealand and New South Wales individuals as outgroups (NZ outgroup: Genbank accession number AF357238; Phillips *et al.*, 2001; NSW outgroup: Genbank accession number EF166067, Eymann *et al.*, 2007)

```
                    10        20        30        40        50
               ....|....|....|....|....|....|....|....|....|....|
4739           ATAAATCATTTAG--ATTCATGAATCATTGACTGTAATG-TCTATGTAAC
782D           ............--.........................-.........
0E61           ............--.........................-.........
0022           ............--.........................-.........
115A           ............--.........................-.........
0574           ............--.........................-.........
624E           ............--.........................-.........
0674           ............--.........................-.........
1123           ............--................A......-.........
1936           ............--.........................-.........
1949           ............--.........................-.........
2182           ............--.........................-.........
6764           ............--.........................-.........
RKM192         ........C....--.........................-.........
6126           ............--.........................-.........
347A           ............--.........................-.........
6D53           ............--.........................-.........
12.9.07RK      ........C....--.........................-.........
RK29.1.07      ............--.........................-.........
1176           ............--.........................-.........
6721           ............--.........................-.........
2702           ............--.........................-.........
HenrysMum      ............--.........................-.........
4DOC           ............--.........................-.........
0902           ............--.........................-.........
JW12B-2        ........C....--.........................-.........
4A35           ............--.........................-.........
1D28           ............--.........................-.........
6609           ............--.........................-.........
BT04F          ............--.........................-.........
0774           ........C....--.........................-.........
454F           ............--.........................-.........
2717           ............--.........................-.........
BT70F          ............--.........................-.........
BT74M          ............--.........................-.........
5002           ............--.........................-.........
2F2E           ............--.........................-.........
7E76           ............--.........................-.........
RKBT           ........C....--.........................-.........
13.7.06RK      ............--.........................-.........
581B           ............--.........................-.........
BT10M          ............--.........................-.........
JW12B-3        ............--.........................-.........
JW10C-8        ............--.........................-.........
BT70M          ............--.........................-.........
75005          ............--.........................-.........
3968           .....C.......--.........................-.........
51D13          ............--.........................-.........
```

```
7613B          .....C.......--.........................-..........
4972           ............--.........................-..........
2353           ............--.........................-..........
4440           .....C.......--.........................-..........
71D27          ............--.........................-..........
4770           ............--.........................-..........
RK03           ............--.........................-..........
JW12B-10       ............--.........................-..........
JW11C-2        ............--.........................-..........
RKBT1          ............--.........................-..........
RK1M           .......G.....--....GC-..................-.T........
7D29           ............--.........................-..........
JW17C-6        ............--......A..................-..........
RKBT2          ............--.........................-..........
RKM5.4.08      ............--.........................-..........
JW12C-9.2      ............--.........................-..........
NZ Outgroup    .C.T..A...C.ATAG.A.TA..GA.TA.A.-ATC....ACT...AGG.T
NSW Outgroup   .C.T..A...C.ACAG.A.TA..GA.TA.A.-ATC....ACT...AGG.T

                         60        70        80        90       100
                         ....|....|....|....|....|....|....|....|....|
4739           CCAACAATAT-ATGTATTATGTAAAATTATG------ATATTAATGTATT
782D           ..........-....................------.............
0E61           ..........-....................------.............
0022           ..........-....................------.............
115A           T.........-....................------.............
0574           ..........-....................------.............
624E           ..........-....................------.............
0674           ..........-....................------.............
1123           ..........-....................------.............
1936           ..........-....................------.............
1949           ..........-....................------.............
2182           ..........-....................------.............
6764           ..........-....................------.............
RKM192         ..........-....................------.............
6126           ..........-....................------.............
347A           ..........-....................------.............
6D53           ..........-....................------.............
12.9.07RK      ..........-....................------.............
RK29.1.07      T.........-....................------.............
1176           T.........-....................------.............
6721           ..........-....................------.............
2702           T.........-....................------.............
HenrysMum      T.........-....................------.............
4DOC           ..........-....................------.............
0902           ..........-....................------.............
JW12B-2        ..........-....................------.............
4A35           T.........-....................------.............
1D28           ..........-....................------.............
6609           T.........-....................------.............
BT04F          ..........-....................------.............
0774           ..........-....................------.............
454F           ..........-....................------.............
2717           ..........-....................------.............
BT70F          ..........-....................------.............
BT74M          ..........-....................------.............
5002           ..........-....................------.............
2F2E           ..........-....................------.............
7E76           ..........-....................------.............
RKBT           ..........-....................------.............
13.7.06RK      ..........-....................------.............
```

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581B          T.........-..................------.............
BT10M         ..........-..................------.............
JW12B-3       T.........-..................------.............
JW10C-8       ..........-..................------.............
BT70M         ..........-..................------.............
75005         T.........-..................------.............
3968          T.........-..................------.............
51D13         ..........-..................------.............
7613B         T.........-..................------.............
4972          ..........-..................------.............
2353          T.........-..................------.............
4440          T.........-..................------.............
71D27         T.........-..................------.............
4770          T.........-..................------.............
RK03          ..........-..................------.............
JW12B-10      T.........-..................------.............
JW11C-2       T.........-..................------.............
RKBT1         ..........-..................------.............
RK1M          T.........-..................------...A.........
7D29          ..........-..................------.............
JW17C-6       T.........-..................------.............
RKBT2         ..........-..................------.............
RKM5.4.08     ..........-..................------.......C.....
JW12C-9.2     ..........-..................------.............
NZ Outgroup   AT..TTG...T..A..A..CA....TA.G.ATATAGT.C......A....
NSW Outgroup  AT..TTG...T..A..A..CA....TA.G.ATATAGT.C......A....

                       110       120       130       140       150
                      ....|....|....|....|....|....|....|....|....|
4739          A---TGTAATGTTATAGTTATTACTTATTATGTAATATTA-TAGTTTAAT
782D          .---.....................................-.........
0E61          .---.....................................-.........
0022          .---.....................................-.........
115A          .---...................T..G.............-.........
0574          .---.....................................-.........
624E          .---.....................................-.........
0674          .---.....................................-.........
1123          .---.....................................-........A
1936          .---...................T.................-.........
1949          .---.....................................-.........
2182          .---.....................................-.........
6764          .---.....................................-.........
RKM192        .---......A..............................-.........
6126          .---.....................................-.........
347A          .---.....................................-.........
6D53          .---......A..............................-.........
12.9.07RK     .---......A..............................-.........
RK29.1.07     .---.....................................-.........
1176          .---...................T.................-.........
6721          .---.....................................-.........
2702          .---.....................................-.........
HenrysMum     .---.....................................-.........
4DOC          .---.....................................-.........
0902          .---.....................................-.........
JW12B-2       .---......A..............................-.........
4A35          .---...................T..G.............-.........
1D28          .---.....................................-.........
6609          .---...................T.................-.........
BT04F         .---.....................................-.........
0774          .---......A..............................-.........
454F          .---.....................................-.........
```

```
2717           .---.....................................-.........
BT70F          .---.....................................-.........
BT74M          .---.....................................-.........
5002           .---.....................................-.........
2F2E           .---.....................................-.........
7E76           .---.....................................-.........
RKBT           .---......A..............................-.........
13.7.06RK      .---.....................................-.........
581B           .---....................T..G.............-.........
BT10M          .---.....................................-.........
JW12B-3        .---.....................................-.........
JW10C-8        .---......A..............................-.........
BT70M          .---.....................................-.........
75005          .---.....................................-.........
3968           .---.....................................-.........
51D13          .---......A..............................-.........
7613B          .---.....................................-.........
4972           .---.....................................-.........
2353           .---.....................................-.........
4440           .---....................T..G.............-.........
71D27          .---....................T..G.............-.........
4770           .---....................T..G.............-.........
RK03           .---.....................................-.........
JW12B-10       .---.....................................-.........
JW11C-2        .---.....................................-.........
RKBT1          .---......A..............................-.........
RK1M           .---....................T................-.........
7D29           .---.....................................-.........
JW17C-6        .---......A............T..G.............-.........
RKBT2          .---......A..............................-.........
RKM5.4.08      .---......A..............................-.........
JW12C-9.2      .---......A..............................-.........
NZ Outgroup    TACC.C..GCA.ATA...AGA...A..C...TC..C....C..AAA..CA
NSW Outgroup   TACCCC..GCA.ATA...AGA...A..C...TC..C....C..AAA..CA

                        160       170       180       190       200
                       ....|....|....|....|....|....|....|....|....|
4739           GTATGTTAGATATGCTATTATATAAATCACTATATAGTAATT-GTTTAAT
782D           ........................................-........
0E61           ........................................-........
0022           ........................................-....G..
115A           ....A.............C...C......T......A.GG..-A....G.
0574           ........................................-....G..
624E           ........................................-........
0674           ........................................-........
1123           ........................................-........
1936           ....A.................C......T......A.GG..-A....G.
1949           ........................................-........
2182           ........................................-....G..
6764           ........................................-....G..
RKM192         ....A...............CG.......T......A.G...-A......
6126           ........................................-........
347A           ........................................-........
6D53           ....A...............C........T......A.G...-A......
12.9.07RK      ....A...............CG.......T......A.G...-A......
RK29.1.07      ........................................-........
1176           ....A.............C..........T......A.GG..-A....G.
6721           ........................................-....G..
2702           ....A...................................-....G..
HenrysMum      ....A...................................-....G..
4DOC           ........................................-........
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0902            ........................................-....G..
JW12B-2         ....A.............CG.......T......A.G...-A......
4A35            ....A............C...C......T......A.GG..-A....G.
1D28            ........................................-.......
6609            ....A............C.........T......A.GG..-A....G.
BT04F           ........................................-....G..
0774            ....A.............CG.......T......A.G...-A......
454F            ........................................-.......
2717            ........................................-....G..
BT70F           ........................................-....G..
BT74M           ........................................-....G..
5002            ........................................-....G..
2F2E            ........................................-.......
7E76            ........................................-....G..
RKBT            ....A.............CG.......T......A.G...-A......
13.7.06RK       ........................................-.......
581B            ....A............C...C......T......A.GG..-A....G.
BT10M           ........................................-....G..
JW12B-3         ....A........................T........G...-A......
JW10C-8         ....A.............CG.......T......A.G...-A......
BT70M           ........................................C....G..
75005           ....A...................................-.......
3968            ....A........................T...........-.......
51D13           ....A..................C......T........G...-.......
7613B           ....A........................T...........-.......
4972            ........................................C....G..
2353            ....A...................................-.......
4440            ....A............C...C......T......A.GG..-AA.....
71D27           ....A............C...C......T......A.GG..-AA.....
4770            ....A............C.........T......A.GG..-A....G.
RK03            ........................................-.......
JW12B-10        ..................C.....................-.......
JW11C-2         ....A...................................-....G..
RKBT1           ....A..................C......T...........-.......
RK1M            ....A........................T......A.G...-A.....-
7D29            ........................................-....G..
JW17C-6         ....A....................T...T...........-AC....-
RKBT2           ....A..................C......T...........-.......
RKM5.4.08       ....A.............CG.......T......A.G...-A......
JW12C-9.2       ....A.............CG.......T......A.G...-A......
NZ Outgroup     T..GA...A...-AT..C..C....TGATT......A..GCATA.C....
NSW Outgroup    T..GA...A.C.-AT..C......GTGATT..C...A..GCATA.C....

                        210       220       230       240       250
                ....|....|....|....|....|....|....|....|....|....|
4739            TT-TGATGTATTTTAGTA----ATGTTGAATACTATGTATCTACTTATAT
782D            ..-.............----.........................
0E61            ..-.............----.........................
0022            ..-.............----.........................
115A            ..-.A...........----.........................
0574            ..-.............----.........................
624E            ..-.............----.........................
0674            ..-.............----.........................
1123            ..-.............----.........................
1936            ..-.A...........----.........................
1949            ..-.............----.........................
2182            ..-.............----.........................
6764            ..-.............----.........................
RKM192          C.-.A...........----.........................
6126            ..-.............----.........................
347A            ..-.............----.........................
```

```
6D53          C.-.A.............----.........................
12.9.07RK     C.-.A.............----.........................
RK29.1.07     ..-...............----.........................
1176          ..-.A.............----.........................
6721          ..-...............----.........................
2702          ..-...............----.........................
HenrysMum     ..-...............----.........................
4DOC          ..-...............----.........................
0902          ..-...............----.........................
JW12B-2       C.-.A.............----.........................
4A35          ..-.A.............----.........................
1D28          ..-...............----.........................
6609          ..-.A.............----.........................
BT04F         ..-...............----.........................
0774          C.-.A.............----.........................
454F          C.-...............----.........................
2717          C.-...............----.........................
BT70F         C.-...............----.........................
BT74M         ..-...............----.........................
5002          ..-...............----.........................
2F2E          ..-...............----.........................
7E76          ..-...............----.........................
RKBT          C.-.A.............----.........................
13.7.06RK     ..-...............----.........................
581B          ..-.A.............----.........................
BT10M         ..-...............----.........................
JW12B-3       C.-.A.............----.........................
JW10C-8       C.-.A.............----.........................
BT70M         ..-...............----.........................
75005         ..-...............----.........................
3968          ..-...............----.........................
51D13         C.-.A.............----.........................
7613B         C.-...............----.........................
4972          ..-...............----.........................
2353          ..-...............----.........................
4440          ..-.A.............----.........................
71D27         ..-.A.............----.........................
4770          ..-.A.............----.........................
RK03          ..-...............----.........................
JW12B-10      ..-......G........----.........................
JW11C-2       ..-...............----.........................
RKBT1         C.-.A.............----.........................
RK1M          CC-.A.............----.........G...............
7D29          ..-...............----.........................
JW17C-6       C.-.A.............----.........G...............
RKBT2         C.-.A.............----.........................
RKM5.4.08     C.-.A.............----.........................
JW12C-9.2     C.-.A.............----.........................
NZ Outgroup   A.ACAT.AA.C.A..A..TTAC..AACA....A..AC...AACA...C..
NSW Outgroup  A.ACAT.AA.C.A.....TTAC..AACA...GA..AC...AACA...C..

                       260       270       280       290       300
                      ....|....|....|....|....|....|....|....|....|
4739          GCTAGAG-GTAAATATATTAATGTACTATATACATATTTATGTATTATAT
782D          .......-.........................................
0E61          .......-.........................................
0022          .......-.........................................
115A          .....G.-.........................................
0574          .......-.........................................
624E          .......-.........................................
0674          .......-.........................................
```

```
1123           .......-.....................................
1936           .......-.....................................
1949           .......-.....................................
2182           .......-.....................................
6764           .......-.....................................
RKM192         .....G.-.....................................
6126           .......-.....................................
347A           .......-.....................................
6D53           .....G.-.....................................
12.9.07RK      .....G.-.....................................
RK29.1.07      .....G.-.....................................
1176           .......-.....................................
6721           .......-.....................................
2702           .....G.-.............C.......................
HenrysMum      .......-.............C.......................
4DOC           .......-.....................................
0902           .......-.....................................
JW12B-2        .....G.-.....................................
4A35           .......-.....................................
1D28           .......-.....................................
6609           .......-.....................................
BT04F          .......-.....................................
0774           .....G.-.....................................
454F           .......-.....................................
2717           .......-.....................................
BT70F          .......-.....................................
BT74M          .......-.....................................
5002           .......-.....................................
2F2E           .......-.....................................
7E76           .......-.....................................
RKBT           .....G.-.....................................
13.7.06RK      .......-.....................................
581B           .....G.-.....................................
BT10M          .......-.....................................
JW12B-3        .....G.-.....................................
JW10C-8        .....G.-.....................................
BT70M          .......-.....................................
75005          .......-.....................................
3968           .......-.....................................
51D13          .......-.....................................
7613B          .......-.....................................
4972           .......-.....................................
2353           .......-.....................................
4440           .....G.-.....................................
71D27          .....G.-.....................................
4770           .....G.-.....................................
RK03           .......-.....................................
JW12B-10       .....G.-.....................................
JW11C-2        .......-.............C.......................
RKBT1          .......-.............C.......................
RK1M           .......-.A.....................G.........C.....
7D29           .......-.....................................
JW17C-6        .......-.....................................
RKBT2          .......-.....................................
RKM5.4.08      .....G.-.....................................
JW12C-9.2      .....G.-.....................................
NZ Outgroup    AA..C.TTA.T.TC...A.CT.AC.TA...C.T.....GT..AG...C..
NSW Outgroup   AA..C.TTA.T.TC...A.CT.AC.TA...C.T.....GT..G....C..

                        310       320       330       340       350
                       ....|....|....|....|....|....|....|....|....|
```

```
4739          AATACAATTATATCCTATAAGTCATTGATTTATAGTCTTTAG-TACTGTT
782D          .......................................-.......
0E61          ...G...................................-.......
0022          .......................................-.......
115A          .......................................-.......
0574          .......................................-.......
624E          .......................................-.......
0674          .......................................-.......
1123          .......................................-.......
1936          .......................................-.......
1949          .......................................-.......
2182          .......................................-.......
6764          .......................................-.......
RKM192        .......................................-.......
6126          .......................................-.......
347A          .......................................-.......
6D53          .......................................-.......
12.9.07RK     .......................................-.......
RK29.1.07     .......................................-.......
1176          .......................................-.......
6721          .......................................-.......
2702          .......................................-.......
HenrysMum     ...................................C...-.......
4DOC          .......................................-.......
0902          .......................................-.......
JW12B-2       .......................................-.......
4A35          .......................................-.......
1D28          .......................................-.......
6609          .......................................-.......
BT04F         .......................................-.......
0774          .......................................-.......
454F          .......................................-.......
2717          .......................................-.......
BT70F         .......................................-.......
BT74M         .......................................-.......
5002          .......................................-.......
2F2E          .......................................-.......
7E76          .......................................-.......
RKBT          .......................................-.......
13.7.06RK     .......................................-.......
581B          .......................................-.......
BT10M         .......................................-.......
JW12B-3       .......................................-.......
JW10C-8       .......................................-.......
BT70M         .......................................-.......
75005         .......................................-.......
3968          .......................................-.......
51D13         .......................................-.......
7613B         .......................................-.......
4972          .......................................-.......
2353          .......................................-.......
4440          .......................................-.......
71D27         .......................................-.......
4770          .......................................-.......
RK03          .......................................-.......
JW12B-10      .......................................-.......
JW11C-2       .......................................-.......
RKBT1         .......................................-.......
RK1M          ................T..................C...-.......
7D29          .......................................-.......
JW17C-6       .......................................-....A..
```

```
RKBT2          ........................................-.......
RKM5.4.08      ........................................-.......
JW12C-9.2      ........................................-.......
NZ Outgroup    .G-...T.-.C.GT.A..G.T....GA..C..A.CGA....CCC...AGC
NSW Outgroup   .G-.....-.C.GT.A..G.T....GA..C..A.CGA....CCC...AGC

                        360       370       380       390       400
                       ....|....|....|....|....|....|....|....|....|
4739           GAATTAT----ATGTACTTACCTCTCGCTATGACTT---GCAAGATTGTA
782D           .......----.........................---...........
0E61           .......----.........................---...........
0022           .......----.........................---...........
115A           .......----..........CG.T..........---...........
0574           .......----.........................---...........
624E           .......----.........................---...........
0674           .......----.........................---...........
1123           .......----.........................---...........
1936           .......----..........CG.T..........---...........
1949           .......----.........................---...........
2182           .......----.........................---...........
6764           .......----.........................---...........
RKM192         .......----............T..........---...........
6126           .......----.........................---...........
347A           .......----.........................---...........
6D53           .......----............T..........---...........
12.9.07RK      .......----............T..........---...........
RK29.1.07      .......----.........................---...........
1176           .......----..........CG.T..........---...........
6721           .......----.........................---...........
2702           .......----.........................---...........
HenrysMum      .......----.........................---...........
4DOC           .......----.........................---...........
0902           .......----.........................---...........
JW12B-2        .......----............T..........---...........
4A35           .......----..........CG.T..........---...........
1D28           .......----.........................---...........
6609           .......----..........CG.T..........---...........
BT04F          .......----.........................---...........
0774           .......----............T..........---...........
454F           .......----.........................---...........
2717           .......----.........................---...........
BT70F          .......----.........................---...........
BT74M          .......----.........................---...........
5002           .......----.........................---...........
2F2E           .......----.........................---...........
7E76           .......----.........................---...........
RKBT           .......----............T..........---...........
13.7.06RK      .......----.........................---...........
581B           .......----..........CG.T..........---...........
BT10M          .......----.........................---...........
JW12B-3        .......----..........C..T..........---...........
JW10C-8        .......----............T..........---...........
BT70M          .......----.........................---...........
75005          .......----.........................---...........
3968           .......----.........................---...........
51D13          .......----..........C..T...G......---...........
7613B          .......----.........................---...........
4972           .......----.........................---...........
2353           .......----.........................---...........
4440           .......----..........CG.T..........---..........G
71D27          .......----..........CG............---..........G
```

```
4770            .......----..........CG.T.........---..........
RK03            .......----..............................---..........
JW12B-10        .......----..............................---..........
JW11C-2         .......----..............................---..........
RKBT1           .......----..........C..T...G......---..........
RK1M            .......----.............T..........---........A..
7D29            .......----..............................---..........
JW17C-6         .......----...........T.T..C...T..----....A......
RKBT2           .......----..........C..T...G......---..........
RKM5.4.08       .......----.............T..........---..........
JW12C-9.2       .......----.............T..........---..........
NZ Outgroup     AT..C..CACC...AGT.AT..CT.AATC.CC..C.CAC.AG....CAC-
NSW Outgroup    AT..C..CACC...AGT.AT..CT.AATC.CC..C.CAC.AG....CAC-

                         410        420        430        440        450
                ....|....|....|....|....|....|....|....|....|....|
4739            CATGTTTGAATATTGATGGTTGATATAATATATAGGAAAT---TAGGGTA
782D            ...........................................---.......
0E61            ...........................................---.......
0022            ...........................................---.......
115A            .........G.................................---.......
0574            ...........................................---.......
624E            ...........................................---.......
0674            ...........................................---.......
1123            ...........................................---.......
1936            .........G.................G.............---.......
1949            ...........................................---.......
2182            ...........................................---.......
6764            ...........................................---.......
RKM192          .........G.............................G.---..A....
6126            ...........................................---.......
347A            ...........................................---.......
6D53            .........G.............................G.---..A....
12.9.07RK       .........G.............................G.---..A....
RK29.1.07       ...........................................---.......
1176            .........G.................G.............---.......
6721            ...........................................---.......
2702            ...........................................---.......
HenrysMum       ...........................................---.......
4DOC            ...........................................---.......
0902            ...........................................---.......
JW12B-2         .........G.............................G.---..A....
4A35            .........G.................................---.......
1D28            ...........................................---.......
6609            .........G.................G.............---.......
BT04F           ...........................................---.......
0774            .........G.............................G.---..A....
454F            ...........................................---.......
2717            ...........................................---.......
BT70F           ...........................................---.......
BT74M           ...........................................---.......
5002            ...........................................---.......
2F2E            ...........................................---.......
7E76            ...........................................---.......
RKBT            .........G...........A.................G.---..A....
13.7.06RK       ...........................................---.......
581B            .........G.................................---.......
BT10M           ...........................................---.......
JW12B-3         .........G.................................---.......
JW10C-8         .........G.............................G.---..A....
BT70M           ...........................................---.......
```

```
75005          ....................................---.......
3968           ....................................---.......
51D13          .........G.....................G.....---.......
7613B          ....................................---.......
4972           ....................................---.......
2353           ....................................---.......
4440           .........G..........................---.......
71D27          .........G..........................---.......
4770           .........G..........................---.......
RK03           ....................................---.......
JW12B-10       ....................................---.......
JW11C-2        ....................................---.......
RKBT1          .........G.....................G.....---.......
RK1M           ......................C.G.G......G...---.......
7D29           ....................................---.......
JW17C-6        ........................G...........---.......
RKBT2          .........G.....................G.....---.......
RKM5.4.08      .........G.........................G.---..A....
JW12C-9.2      .........G.........................G.---..A....
NZ Outgroup    ...CCCGCC..C..A.G.CACATC..CC.TC.G..C..GCCCA.TAAC.G
NSW Outgroup   ...CCCGCC..C..A.G.CACATC..CC.TC.G..C..GCCCA.TAAC.G

                        460       470       480       490       500
                       ....|....|....|....|....|....|....|....|....|
4739           TGA-CATAACAGTAAGGTTTGG-TTTCAGGAAGTAGTTTATTTAGAATGT
782D           ...-...................-..........................
0E61           ...-...................-..........................
0022           ..G-T..............A--..........................
115A           .A.-T.........G.....A--..........................
0574           ..G-T..............A--..........................
624E           ...-...................-..........................
0674           ...-...................-..........................
1123           ...-...................-..........................
1936           ...-T.........G.....A--..........................
1949           ...-...................-..........................
2182           ..G-T..............A--..........................
6764           ..G-T..............A--..........................
RKM192         ...-..........G.....A--..........................
6126           ...-...................-.....................A.....
347A           ...-...................-..........................
6D53           ...-..........G.....A--............T........A.....
12.9.07RK      ...-..........G.....A--..........................
RK29.1.07      ...-...............A--..........................
1176           ...-T.........G.....A--..........................
6721           ..G-T..............A--..........................
2702           ...-...............A--..........................
HenrysMum      ...-...............A--....TTT..-..C............A
4DOC           ...-...................-..........................
0902           ..G-T..............A--..........................
JW12B-2        ...-..........G.....A--..........................
4A35           ...-T.........G.....A--.....................-.....
1D28           ...-...................-..........................
6609           ...-T.........G.....A--..........................
BT04F          ..G-T..............A--..........................
0774           ...-..........G.....A--..........................
454F           ...-...................-..........................
2717           ..G-T..............A--..........................
BT70F          ..G-T..............A--..........................
BT74M          ..G-T..............A--..........................
5002           ..G-T..............A--..........................
2F2E           ...-...................-..........................
```

```
7E76           ..G-T.............A--.........................
RKBT           ...-.........G.....A--.........................
13.7.06RK      ...-.................-.........................
581B           ...-T.........G.....A--.........................
BT10M          ..G-T.............A--.........................
JW12B-3        ...-T.........G.....A--.........................
JW10C-8        ...-.........G.....A--.........................
BT70M          ..G-T.............A--.........................
75005          ...-.............A--.........................
3968           ...-.............A--.........................
51D13          ...-T.........G.....A--.........................
7613B          ...-.............A--.........................
4972           ..G-T.............A--.........................
2353           ...-.............A--.........................
4440           ...-T.........G.....A--.........................
71D27          ...-T.........G.....A--.........................
4770           .A.-T.........G.....A--.........................
RK03           ...-.................-.........................
JW12B-10       ...-.............A--.........................
JW11C-2        ...-.............A--.........................
RKBT1          ...-T.........G.....A--.........................
RK1M           ...-.....T..C.-.....--.........................
7D29           ..G-T.............A--.........................
JW17C-6        ...-...G.T-...G.......G.........................
RKBT2          ...-T.........G.....A--.........................
RKM5.4.08      ...-.........G.....A--.........................
JW12C-9.2      ...-.........G.....A--.........................
NZ Outgroup    ..GT.G..C.TAA.CA.C..T--.ACTG.CT.C.G.G.GC.ACTTC.G..
NSW Outgroup   ..GT.G..C.TAA.CA.C..T--.ACTG.CT.C.G...----------


               .
4739           C
782D           .
0E61           .
0022           .
115A           .
0574           .
624E           .
0674           .
1123           .
1936           .
1949           .
2182           .
6764           .
RKM192         .
6126           .
347A           .
6D53           .
12.9.07RK      .
RK29.1.07      .
1176           .
6721           .
2702           .
HenrysMum      .
4DOC           .
0902           .
JW12B-2        .
4A35           .
1D28           .
6609           .
```

```
BT04F          .
0774           .
454F           .
2717           .
BT70F          .
BT74M          .
5002           .
2F2E           .
7E76           .
RKBT           .
13.7.06RK      .
581B           .
BT10M          .
JW12B-3        .
JW10C-8        .
BT70M          .
75005          .
3968           .
51D13          .
7613B          .
4972           .
2353           .
4440           .
71D27          .
4770           .
RK03           .
JW12B-10       .
JW11C-2        .
RKBT1          .
RK1M           .
7D29           .
JW17C-6        .
RKBT2          .
RKM5.4.08      .
JW12C-9.2      .
NZ Outgroup    .
NSW Outgroup   -
```

Appendix 3.2: Fst values for pairwise population comparisons: MtDNA

The table below shows the Fst and P values for each pairwise population comparison (Fig. 3.1) calculated from mitochondrial DNA sequences using the Arlequin analysis software (Schneider *et al.*, 2000). Statistically significant results are shaded grey, as are the populations for which at least half of the individuals have coppery colour morphology.

		Population						
Pop.		**1C**	**1G**	**2**	**3**	**4**	**5**	**6**
1G	Fst	0.406						
	P	<0.00001						
2	Fst	0.369	0.306					
	P	<0.00001	0.009					
3	Fst	0.912	0.751	0.399				
	P	<0.00001	<0.00001	<0.00001				
4	Fst	0.665	0.212	-0.131	0.428			
	P	0.135	0.541	0.631	0.117			
5	Fst	0.605	0.371	-0.023	0.517	0.039		
	P	<0.00001	0.018	0.468	<0.00001	0.387		
6	Fst	0.578	0.421	0.065	0.262	-0.098	0.101	
	P	<0.00001	<0.00001	0.171	0.045	0.514	0.162	
7	Fst	0.606	0.418	0.017	0.518	0.082	-0.168	0.179
	P	<0.00001	0.009	0.342	0.009	0.414	0.991	0.117

Appendix 3.3: Microsatellite diversity summary by population and locus

All individuals						
Locus	**N**	**Observed heterozygosity**	**Expected heterozygosity**	**P**	**SD**	**Fis**
Tv12	65	0.523	0.557	0.831	0.000	0.061
Tv16	65	0.892	0.908	0.099	0.000	0.018
Tv19	64	0.797	0.920	0.001	0.000	0.134
Tv27	64	0.844	0.882	0.015	0.000	0.043
Tv53	65	1.000	0.927	0.196	0.000	-0.079
Tv54	65	0.292	0.789	0	0	0.63
Tv58	65	0.769	0.884	0.259	0.000	0.130
Tv64	64	0.891	0.914	0.393	0.000	0.025
Population 1C						
Tv12	12	0.667	0.565	0.752	0.001	-0.179
Tv16	12	0.750	0.873	0.394	0.001	0.141
Tv19	12	0.500	0.826	0.022	0.000	0.395
Tv27	11	0.818	0.831	0.277	0.001	0.016
Tv53	12	1.000	0.870	0.268	0.001	-0.150
Tv58	12	0.750	0.848	0.159	0.001	0.115
Tv64	12	0.667	0.837	0.066	0.001	0.203
Population 1G						
Tv12	21	0.381	0.553	0.098	0.001	0.311
Tv16	21	0.905	0.890	0.128	0.001	-0.017
Tv19	21	0.857	0.878	0.712	0.001	0.024
Tv27	21	1.000	0.878	0.390	0.001	-0.139
Tv53	21	1.000	0.943	0.767	0.001	-0.060
Tv58	21	0.762	0.861	0.409	0.001	0.115
Tv64	21	0.857	0.930	0.094	0.000	0.079
Population 2						
Tv12	7	0.429	0.604	0.436	0.001	0.291
Tv16	7	1.000	0.923	1.000	0.000	-0.083
Tv19	6	0.833	0.924	0.507	0.001	0.098
Tv27	7	0.857	0.824	0.775	0.001	-0.040
Tv53	7	1.000	0.846	0.957	0.001	-0.182
Tv58	7	0.857	0.890	0.813	0.001	0.037
Tv64	7	1.000	0.912	0.623	0.001	-0.096
Population 3						
Tv12	4	0.500	0.429	1.000	0.000	-0.167
Tv16	4	1.000	0.929	1.000	0.000	-0.077
Tv19	4	0.750	0.786	1.000	0.000	0.045
Tv27	4	0.750	0.893	0.432	0.001	0.160
Tv53	4	1.000	1.000	1.000	0.000	0.000
Tv58	4	0.750	0.929	0.308	0.001	0.192
Tv64	3	1.000	0.933	1.000	0.000	-0.071

Population 4						
Tv12	Monomorphic locus: no calculation made					
Tv16	2	1.000	1.000	1.000	0.000	0.000
Tv19	2	1.000	0.833	1.000	0.000	-0.200
Tv27	2	0.500	0.833	0.332	0.001	0.400
Tv53	2	1.000	1.000	1.000	0.000	0.000
Tv58	2	1.000	1.000	1.000	0.000	0.000
Tv64	2	1.000	1.000	1.000	0.000	0.000
Population 5						
Tv12	4	0.500	0.429	1.000	0.000	-0.167
Tv16	4	0.750	0.857	0.653	0.002	0.125
Tv19	4	1.000	0.929	1.000	0.000	-0.077
Tv27	4	1.000	0.893	1.000	0.000	-0.120
Tv53	4	1.000	0.929	1.000	0.000	-0.077
Tv58	4	0.750	0.857	0.659	0.001	0.125
Tv64	4	1.000	0.893	1.000	0.000	-0.120
Population 6						
Tv12	8	0.750	0.658	0.771	0.001	-0.139
Tv16	8	0.875	0.842	0.700	0.001	-0.040
Tv19	8	0.875	0.883	0.894	0.001	0.009
Tv27	8	0.750	0.858	0.330	0.001	0.126
Tv53	8	1.000	0.867	1.000	0.000	-0.154
Tv58	8	0.500	0.808	0.083	0.001	0.381
Tv64	8	1.000	0.917	1.000	0.000	-0.091
Population 7						
Tv12	6	0.667	0.485	1.000	0.000	-0.375
Tv16	6	1.000	0.955	1.000	0.000	-0.048
Tv19	6	0.833	0.939	0.444	0.001	0.113
Tv27	6	0.667	0.848	0.459	0.001	0.214
Tv53	6	1.000	0.939	1.000	0.000	-0.065
Tv58	6	1.000	0.848	1.000	0.000	-0.179
Tv64	6	1.000	0.970	1.000	0.000	-0.031

Heterozygosity and associated P and SD values were calculated using Arlequin (Schneider *et al.*, 2000). Fis calculated as per Nei (1977).

Private Alleles			
Population	**Tv Locus**	**Allele**	**Freq**
1C	19	278	0.042
1C	27	199	0.091
1G	19	285	0.024
1G	19	300	0.024
1G	27	169	0.024
1G	53	241	0.024
1G	53	263	0.048
1G	53	267	0.024
1G	58	157	0.024
1G	64	189	0.048
1G	64	198	0.071
1G	64	202	0.024
1G	64	204	0.048
2	19	288	0.167
2	19	290	0.167
2	58	147	0.071
2	64	168	0.071
3	53	252	0.125
5	19	294	0.100
5	64	175	0.100
5	64	206	0.100
6	19	270	0.063
6	64	170	0.063
7	19	295	0.083
7	19	299	0.083
7	27	175	0.167
7	53	239	0.083
7	58	163	0.083
7	64	172	0.083
7	64	192	0.083

Appendix 3.4: Fst values for pairwise population comparisons: microsatellites

The table below shows the Fst and P values for each pairwise population comparison (Fig. 3.1) calculated from microsatellite data using the Arlequin analysis software (Schneider *et al.*, 2000). Statistically significant results are shaded grey, as are the populations for which at least half of the individuals have coppery colour morphology.

		Population						
Pop.		**1C**	**1G**	**2**	**3**	**4**	**5**	**6**
1G	Fst	0.038						
	P	<0.00001						
2	Fst	0.030	0.011					
	P	0.027	0.171					
3	Fst	0.043	0.019	0.005				
	P	0.054	0.108	0.360				
4	Fst	0.084	0.004	0.026	0.024			
	P	0.027	0.468	0.432	0.676			
5	Fst	0.088	0.019	0.037	0.043	-0.014		
	P	<0.00001	0.081	0.099	0.099	0.676		
6	Fst	0.047	0.020	0.022	0.034	0.021	0.038	
	P	<0.00001	0.036	0.099	0.081	0.270	0.108	
7	Fst	0.038	0.017	0.006	0.002	0.007	0.010	0.019
	P	0.027	0.099	0.306	0.369	0.351	0.225	0.180

Appendix 3.5: MC1R sequence for Atherton Tablelands brushtail possums

The 642bp MC1R Sequence for Atherton Tablelands brushtail possums, *Trichosurus vulpecula* is shown in alignment (Thompson *et al.,* 1997; Hall, 1999) with bases 177-821 of the full *Mus musculus* MC1R gene (Genbank accession number: AB306322.1). Four individuals with grey morphology and four of the coppery morph were tested, with no base pair differences between any of the eight individuals.

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                ..../..../ ..../..../ ..../..../ ..../..../ ..../..../
                      10         20         30         40         50
Mus musculus    ATGTCCACTC AGGAGCCCCA GAAGAGTCTT CTGGGTTCTC TCAACTCCAA
T.vulpecula     ---------- ---------- ---------- ---------- ----------

                ..../..../ ..../..../ ..../..../ ..../..../ ..../..../
                      60         70         80         90        100
                TGCCACCTCT CACCTTGGAC TGGCCACCAA CCAGTCAGAG CCTTGGTGCC
                ---------- ---------- ---------- ---------- ----------

                ..../..../ ..../..../ ..../..../ ..../..../ ..../..../
                     110        120        130        140        150
                TGTATGTGTC CATCCCAGAT GGCCTCTTCC TCAGCCTAGG GCTGGTGAGT
                ---------- ---------- ---------- ---------- ----------

                ..../..../ ..../..../ ..../..../ ..../..../ ..../..../
                     160        170        180        190        200
                CTGGTGGAGA ATGTGCTGGT TGTGATAGCC ATCACCAAAA ACCGCAACCT
                ---------- ---------- ------GGCC ATCATCAAGA ACCGCAACCT

                ..../..../ ..../..../ ..../..../ ..../..../ ..../..../
                     210        220        230        240        250
                GCACTCGCCC ATGTATTACT TCATCTGCTG CCTGGCCCTG TCTGACCTGA
                CCATTCACCC ATGTACTATT TTGTCTGCTG CTTGGCTTTA TCAGATCTTC

                ..../..../ ..../..../ ..../..../ ..../..../ ..../..../
                     260        270        280        290        300
                TGGTAAGTGT CAGCATCGTG CTGGAGACTA CTATCATCCT GCTGCTGGAG
                TGGTGAGTGT CAGCAACCTG CTGGAGACCT TGGTGTTGCT ACTGCTGGAG
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                    ....|....| ....|....| ....|....| ....|....| ....|....|
                          310        320        330        340        350
                    GCGGGCATCC TGGTGGCCAG AGTGGCTTTG GTGCAGCAGC TGGACAACCT
                    AAAGGGGTGC TGGTGATGCA GGCGCCTATG GTGCAACAGC TTGACAATGT

                    ....|....| ....|....| ....|....| ....|....| ....|....|
                          360        370        380        390        400
                    CATTGACGTG CTCATCTGTG GCTCCATGGT GTCCAGTCTC TGCTTCCTGG
                    CATTGATGTG TTGATCTGTG GTTCCATGAT GTCCTCAATT TCCTTCCTAG

                    ....|....| ....|....| ....|....| ....|....| ....|....|
                          410        420        430        440        450
                    GCATCATTGC TATAGACCGC TACATCTCCA TCTTCTATGC GCTGCGTTAT
                    GAGCCATCGC GGTTGATCGC TACATCAGTA TCTTCTATGC CCTTCGCTAC

                    ....|....| ....|....| ....|....| ....|....| ....|....|
                          460        470        480        490        500
Mus musculus        CACAGCATCG TGACGCTGCC CAGAGCACGA CGGGCTGTCG TGGGCATCTG
T.vulpecula         CACAGCATTG TCACCCCTTG TCGAGCTCAG GGAGTCCTTG CTGGCATCTG

                    ....|....| ....|....| ....|....| ....|....| ....|....|
                          510        520        530        540        550
                    GATGGTCAGC ATCGTCTCCA GCACCCTCTT TATCACCTAC TACAAGCACA
                    GGTGTCCAGT GCCCTCTCTG GTACCCTCTT CATCTCCTAT TACAACCATG

                    ....|....| ....|....| ....|....| ....|....| ....|....|
                          560        570        580        590        600
                    CAGCCGTTCT GCTCTGCCTC GTCACTTTCT TTCTAGCCAT GCTGGCACTC
                    TTGCAGTCCT GCTCTGTCTC ATTGCCTTCT TCTTGTCTAT GTTGGGGCTT

                    ....|....| ....|....| ....|....| ....|....| ....|....|
                          610        620        630        640        650
                    ATGGCGATTC TGTATGCCCA CATGTTCACG AGAGCGTGCC AGCACGCTCA
                    ATGGTGGTCC TCTACATTCA CATGTTTATC CAAGCATGCC AGCATGCCAG

                    ....|....| ....|....| ....|....| ....|....| ....|....|
                          660        670        680        690        700
```

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GGGCATTGCC CAGCTCCACA AAAGGCGGCG GTCCATCCGC CAAGGCTTCT
GAGGATTGCT CGGCTGCACA AGAGATG--- CACAATTCAC CAACTGTCAA


....|....| ....|....| ....|....| ....|....| ....|....|
        710        720        730        740        750
GCCTCAAGGG TGCTGCCACC CTTACTATCC TTCTGGGGAT TTTCTTCCTG
TCCTCAAGGG GGCTGTCACC CTCACAATCC TGTTGGGCAT CTTCTTCCTC


....|....| ....|....| ....|....| ....|....| ....|....|
        760        770        780        790        800
TGCTGGGGCC CCTTCTTCCT GCATCTCTTG CTCATCGTCC TCTGCCCTCA
TGCTGGGCCC CCTTTTTCCT GCACCTCACG CTTATTGTCC TCTGTCCCAA

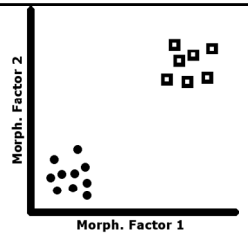
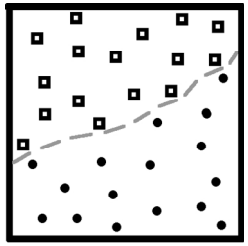
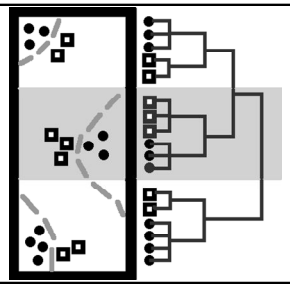

....|....| ....|....| ....|....| ....|....| ....|....|
        810        820        830        840        850
GCACCCCACC TGCAGCTGCA TCTTCAAGAA CTTCAACCTC TTCCTCCTCC
GCATCCCACA TGCAGCTGCT A--------- ---------- ----------

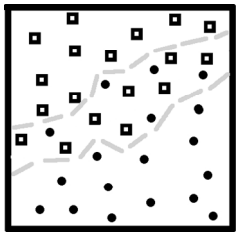
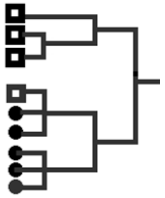
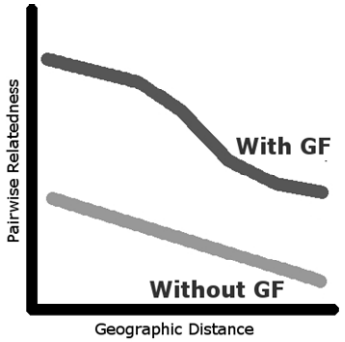
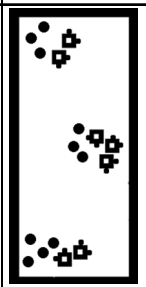
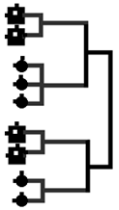
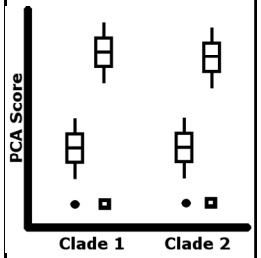
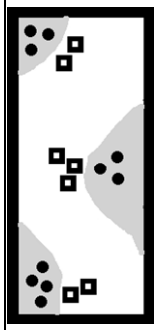
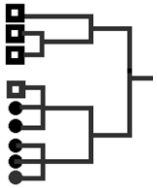
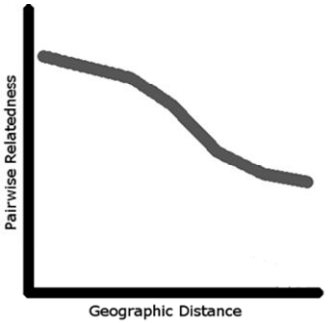

....|....| ....|....| ....|....| ....|....| ....|....|
        860        870        880        890        900
TCATCGTCCT CAGCTCCACT GTTGACCCCC TCATCTATGC TTTCCGCAGC
---------- ---------- ---------- ---------- ----------


....|....| ....|....| ....|....| ....|....| ....|....|
        910        920        930        940        950
CAGGAGCTCC GCATGACACT CAAGGAGGTG CTGCTGTGCT CCTGGTGA--
---------- ---------- ---------- ---------- ----------
```

Appendix 4.0: Illustrated summary of hypotheses not rejected

The following table illustrates the hypotheses (outlined fully in appendix 1.0, and in text) that were not rejected by the data collected. Square and circle symbols denote different morphological groups, with shading and shape 'appendages' representative of additional unlinked morphological characteristics an individual expresses and how these traits correlate across populations. The dashed lines in the distribution diagrams symbolise geographic or environmental boundaries.

Hypothesis	Predicted, confirmed observations			
morphs can be identified		clustering by colour and body shape morphology into two seperate groups. There is variation within these groups but it is less than the divide between the two morphotypes		
	Distribution	MtDNA	Microsatellites	Other
parapatry				

geneflow between populations	Opposite morphs able to interact and mate in the ecotone 	 with geneflow, alleles for a particular morphology are shared		4NeM≈1
natural selection	 populations that share one morphological feature, share others also	 genetically different but similar for several morphological traits		
fur colour is permanent and determined by diet	 the potential for individuals to cross habitat types depends on the relative amounts of migration and strength of selection	 correlation on a local scale between lineage and colour, but not across multiple populations		Fur colour does not change in captivity or with migration. When subject to the same diet, fur colour should be uniform