Phylogeography of Koalas (*Phascolarctos Cinereus*) in southeastern Australia and their relationship to other Koala populations

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Abstract

Koalas have limited genetic diversity and much of this has been blamed on the fur trade across their range and the reintroduction of koalas from island populations across Victoria. It has been hypothesized, however, that their low genetic diversity may have preceded the fur trade and could reflect prehistoric bottlenecks. To begin to test this hypothesis and, at the same time, identify koala populations with high conservation value, new sequences were obtained from the control region of the mitochondrial DNA (MT DNA) of koalas from the Sydney region, southeastern New South Wales (NSW) and central Victoria (VIC) and this data was compared to previously published sequences. Our analyses indentified 26 MT DNA haplotypes. Using this data, it was shown that on a regional basis: 1. A remnant genotype that survived the fur trade is present in Cape Otway, VIC.; 2. That Koalas from the Strathboggie Ranges, VIC appear to exclusively be descendents of translocated Koalas; 3. Koalas in the South Gippsland area have a minimum of 4 haplotypes that represent an overlap of western Victorian and southeastern NSW populations; 4. That Koalas from the coastal and highland regions of extreme southeastern NSW do not share haplotypes and should be managed separately; 5. Despite limited microsatellite diversity, the Campbelltown, NSW population contains a relatively high level of genetic diversity at the mitochondrial level, matching that previously found in MHC of these animals. On a broader scale we propose that modern Koalas are composed of a northern and a southern genetic clade that developed as the result of ancient range contractions. Also, widely dispersed remnant haplotypes suggest that additional prehistoric bottlenecks in combination with the fur trade have contributed to the paucity of Koala genetic diversity in the modern koala.
Keywords: Diversity, control region, Koala, management units, mitochondrial DNA, population structure.

Introduction

The term ESU was first used by Ryder (1986) to characterize significantly divergent groups of populations and was defined as a set of historically isolated conspecific populations with a distinct and long-term evolutionary history (Ryder 1986). Moritz (1994) proposed two criteria to distinguish ESUs: 1. Reciprocal monophyly of mtDNA haplotypes, and 2. Significant differences for allele frequencies at nuclear loci. Moritz also recommended that a divergent population should be managed to guarantee the viability of the larger ESU - Management Unit (MUs).

The Koala (*Phascolarctos cinereus*) is an Australian icon that is known worldwide. Three subspecies of Koalas (*P. c. cinereus* in New South Wales [NSW], *P. c. adustus* in Queensland [QLD] and *P. c. victor* in Victoria [VIC]) are recognized based on morphological characteristics of their skin and skulls (reviewed by Ireland and Troughton, 1934). However, there is no biological basis for recognizing the taxonomic units (Houlden *et al.* 1999). Houlden *et al.* (1999) based on analysis of sequences of the control region of the mitochondrial DNA (MT DNA) suggest that Koalas do not comprise three separate ESUs, but as a single ESU across their range and that this ESU is comprised of highly differentiated populations rather than subspecies. Genetic studies of Koala population structure in Australia, however, are limited (Houlden *et al*. 1996, 1999; Lee *et al*. 2010, 2011; Fowler *et al*. 2000) and more intensive studies need to be undertaken to identify genetically important populations and to determine the current and past relationships between populations across their geographic range.

Current genetic structure of the koala will have been impacted by hundreds of thousands of years of evolution that has occurred since it became a species, as well as, massive harvesting that occurred during the fur trade and subsequent translocation programs from remnant island populations in Victoria. It is known that the koalas range in historical times was far greater than it is today (Price, 2008) and it has recently been proposed that range contraction during the period of the last glacial maximum may have resulted restriction of Koala populations to isolated remnant habitats and this may also have impacted the genetic diversity of the modern Koala. This hypothesis is supported by a recent study of a small number of samples collected from koala skins obtained prior to the fur trade (Tsangaras *et al*. 2012).

The first comprehensive studies of Koala genetics found only a single MT DNA haplotype (French Island haplotype) among Koala populations on islands off of Victoria and on mainland Western, VIC. These findings are consistent with the theory that animals from western Victoria have completely extirpated by the fur trade and that the only animals found there were descendents of animals translocated from the genetically depauperate island populations. In addition to the French Island haplotype, other haplotypes were found in South Gippsland animals suggesting that this population was an admixture of descendants of translocated animals and animals that survived the fur trade (Houlden *et al*.1996, 1999). Houlden *et al*., (1996 and 1999) also found Queensland animals to be genetically distinct from Victorian animals and a range of unique haplotypes present in populations from NSW. Unexpectedly, some haplotypes found in NSW, though separated by many hundreds of kilometres from Victorian animals, were genetically more closely related to those found in Victorian animals than other NSW haplotypes.

More recent work by Lee *et al.,* (2010) using microstatellite markers strongly argued that the South Gippsland population of Koalas is clearly distinct from the translocated island populations and are not of French Island population descent but represent descents of animals from remnant populations that survived the fur trade. Carpenter *et al.,* (2010) found a unique haplotype from a sample obtained from a Koala in Cape Otway, VIC, suggesting that other populations of Koalas in Victoria may also contain genes from remnant Koala populations.

Lee *et al.* (2010) also used microsatellites to study genetic diversity and structure in Koala populations in the Greater Sydney area and found that genetic diversity had been significantly reduced in a population of Koalas found near Campbelltown, NSW and to a lesser extent in the Southern Highlands south of Sydney. This work also suggested that the population found in the Blue Mountains to the west of Sydney had maintained its genetic diversity despite the fur trade. Low density Koala populations of threatened and unknown status extend from the Southern Highlands along the coast to the Victorian state line. In extreme southern New South Wales, Koalas are found along the coast and also in the highlands. Genetic studies on these animals have not been done but are critical if these populations are to be managed appropriately.

Population genetics have also been studied in Koalas using diversity found in the cellular MHC II DAB and DBB genes (Lau *et al*. 2012). MHC II genes, as adaptive loci, play an important functional role in immunity and response to pathogen-driven selection (Hughs *et al.*1998). Lau *et al*. (2012) examined the MHC II DAB and DBB genes variant diversity of northeastern and southeastern Koala population. Their result showed low MHC II DAB and DBB diversity in southeastern relative to northeastern with varying degrees of diversity in between. The authours hypothesize that this pattern could have been the result of pathogen-driven local evolution due to positive selection in the northern population or loss diversity in the south resulting from genetic bottlenecks or a combination of both. Unexpectedly, they found a high level of diversity in animals from Campbelltown, a population that showed little diversity when examined by microsatellites.

The objective of this study is to investigate the hypothesis that Koala populations retracted and isolated in separate refuge areas in pre history and help to define management units for Koala population in Victoria and SE NSW. This paper also aims to identify demographically independent populations, and investigate the genetic diversity, population structure and relationship within and among Victoria and SE NSW Koalas' population based on analyzing MT DNA control region sequences.

Materials and methods

Samples

DNA obtained swabs from Koalas from the Srathboggie Ranges in Victoria (n=13) where kindly provided by Damien Higgens. DNA samples from Campbelltown, (n=27), the Southern Highlands (n=8), Blue Mountains (n=9) and South Gippsland were the same samples used by Lee *et al.* (2010) and Lee *et al.* (2012). Samples from South Gippsland were selected so that they covered a range of ecosystems. The towns closest to where the animals were collected are Mt St Gwinear (n=1); Moondarra (n=1); Rawson (n=1); Fish Creek (n=1); Yinnar (n=2); Hazelwood (n=2); Woodside (n=1); Traralgon (n=1); Mt.Best (n=1); Boolarra (n=1); Mirboo (n=1); and Jeeralang (n=1). Faecal samples and tissue samples from

Koalas in National Forests and Parks in southeast NSWwere provided by Chris Allen. These samples came from Mt.Clifford (n=1); West Numeralla (n=3); Black Ridge (n=2); and Mt Dowling (n=1) State Forests in the highlands and Kooraban National Park, Bermagui, and Murrah and Mumbulla State Forests on the coast. DNA was extracted from faeces using the QIAamp DNA Stool Mini Kit (Qiagen) following the kit protocol. Additional sequences obtained by Houlden *et al*. (1999) were downloaded from Genbank. The sequence of the Cape Otway haplotype was provide by Carpenter *et al*. (2010) (n=1).

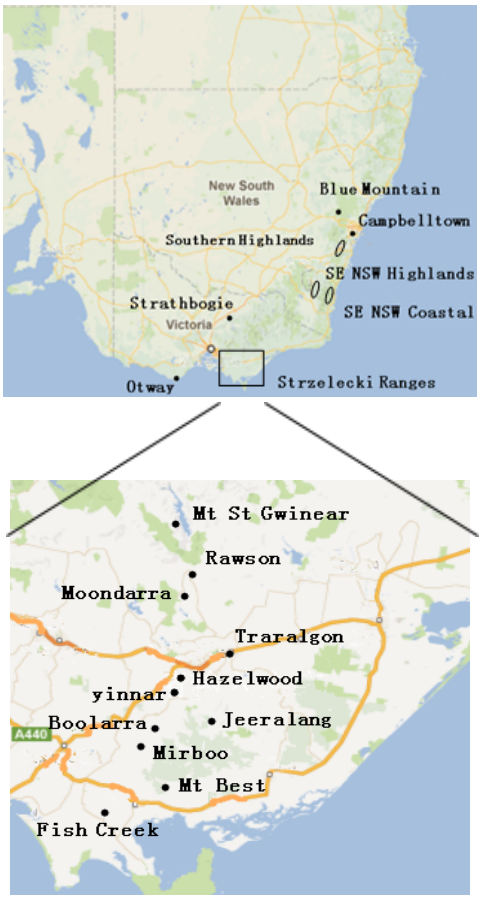

Fig. 1. (*A*) Map of southeastern Australia showing sampling location (black note and circled) for animals from South Gippsland.

(*B*) Inset. Map of South Gippsland showing the closest town to where koalas that were sampled in this study were found.

Samples collection and DNA extract

DNA primer sets

A single primer set was used to amplify the majority of the mitochondrial control

region. The reverse primer (KMTH2: GAACCAGTAGCCAGTTAAAG) was developed by Houlden et al (1999). The forward primer (199: CTACCATCAACACCCAAGC) was developed using CLC workbench 5.7 (CLC Bio) using the complete mitochondrial genome from *Phascolarctos cinereus* (NC_008133.1.).

DNA amplification

DNA was amplified by PCR in 20µL volumes, each containing 4µL 5 x PCR buffer(Bioline 5x MyTaq™ Reaction Buffer),0.65 units of Bioline MyTaq™ DNA Polymerase, 1 µM forward primer, 1 µM reverse primer and sterile water to 20µL. PCR was performed as a touchdown PCR protocol as follows; initial 94°C denaturation for 3 min, followed by six touchdown cycles of 94°C for 30 s, annealing temperature at 60°C for 45 s, and extension step of 72°C for 60 s. On completion of the final touchdown cycle, a further 30 cycles were performed at the 50°C annealing temperature, followed by a final extension of 72°C for 10 min. Amplification products were visualized on an 2% agarose gel containing ethidium bromide using UV light. The amplified fragments were purified with AMICON ULTRA 0.5 centrifugal filter devices following the manufacture's instruction. The purified fragments were sequenced by the Australian Genome Research Facility at the Westmead Millennium Institute.

Sequence analysis

Haplotypes found in this study were based on a 560 bp sequence of the control region of the MTDNA. Sequences were aligned using CLC main workbench 5.7 (CLC Bio). Published sequences AJ005846-63 from Houlden *et al.* (1999), AJ012057-64 from Fowler *et al.* (2000), and also from Phalen *et al.* (unpublished data) were compared to the sequences from this study. Median-joining networks were constructed with Network 4.6.1.0 (Bandelt *et al.* 1999). Neighbor-joining trees (Saitou and Nei 1987) were generated based on the haplotypes found in this study and the published haplotypes using MEGA 4.

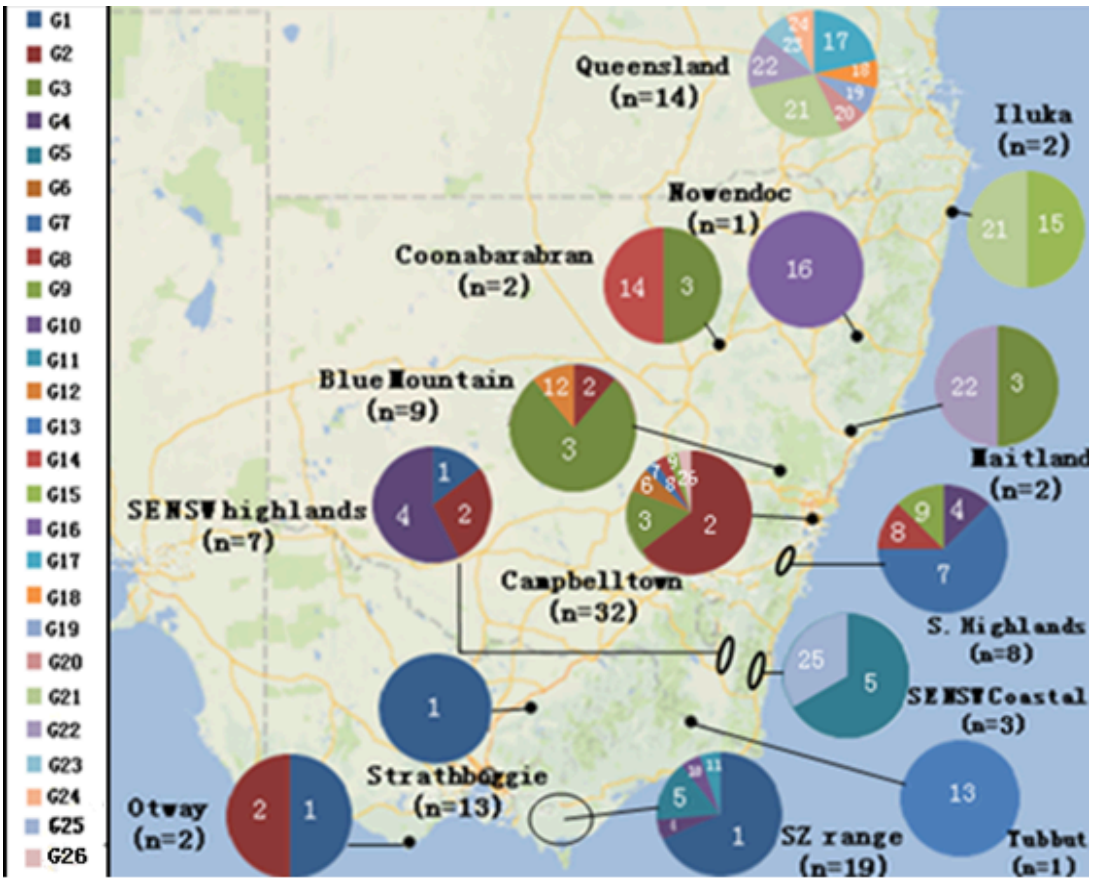

Fig. 2. Graphical illustration of geographic distribution of mtDNA haplotypes used in this study.

Results

Geographical distribution of mitochondrial haplotypes

A total 26 mtDNA control region haplotypes were identified, including those reported by Houlden *et al.* (1999) and Fowler *et al.* (2000). The geographic distribution of the haplotypes is shown (Figure 2).

Victorian animals

Haplotype G1 is the French Island genotype and Houlden previously reported it to be the only genotype found in the island populations and Mornington Peninsula. Carpenter *et al*. (2010) found this genotype in 6/7 of the animals tested from Cape Otway, VIC. Haplotype G1 was the only haplotype found in the samples from the Strathboggie Ranges and was the predominate genotype found in South Gippsland samples. It was also found in one sample from the SE NSW highlands population.

Haplotype G2 was found in a Cape Otway animal was also found in populations as far north as the Blue Moutaians. As suggested by microsatalite analsysis and analysis of MHC DAB genes, the South Gippsland population is genetically diverse and in addition to haplotype G1, it contains haplotypes shared with the SE NSW coastal and highlands populations and contains 2 unique genotypes. The Tubbut, VIC haplotype G13 was also unique.

New South Wales Animals

There was no overlap of haplotypes from the highland and coastal populations of

southeastern New South Wales. However, both populations contained haplotypes that were found in Victorian animals and the highland population had haplotypes that were found in the greater Sydney populations.

The 3 populations in the Greater Sydney area shared haplotyes with each other. Each also contained unique haplotypes and there was overlap between haplotypes in this population and populations as far south as Victoria. The Blue Mountain and Campbelltown population also shared genotypes with the two populations from the central North Coast of NSW. In contrast to a previous study by Lee *et al.* (2010), the Campbelltown population was found to have one of the highest levels of genetic diversity of any of the populations studied.

Only small numbers of animals were available from northern NSW. The haplotypes found in these animals suggest that the Queensland haplotypes extend into northern NSW (G21 Iluka and G22 Maitaland) but that these regions contain their own unique haplotypes.

Queensland
As observed by others (Houldan *et al.* 1999; Fowler *et al*. 2000), overall the Queensland population contains a high level of genetic diversity with 8 haplotypes.

Population structure analysis
Haplotypes network analysis was used to infer the relationship within and between Koala populations across their range. Results of the Median- Joining Network Analysis are shown in Figure 3 and Neighbor-Joining Analysis in Figure 4a and b. Together they illustrate that Australian koalas fall into two genetically distinct clades, a Northern Clade that is confined to Queensland and northern New South Wales and a Southern Clade, that is composed of the Victorian and the majority of the New South Wales haplotypes. No geographical partitioning was found for the haplotyes identified in the South Gippsland animals (Fig 4b). Re-examination of the mircostatellite analysis of the South Gippsland Koalas versus the Mornington Peninsula and French Island Koalas only using animals from South Gippsland with the French Island haplotype still showed them to be two distinct populations (data not shown).

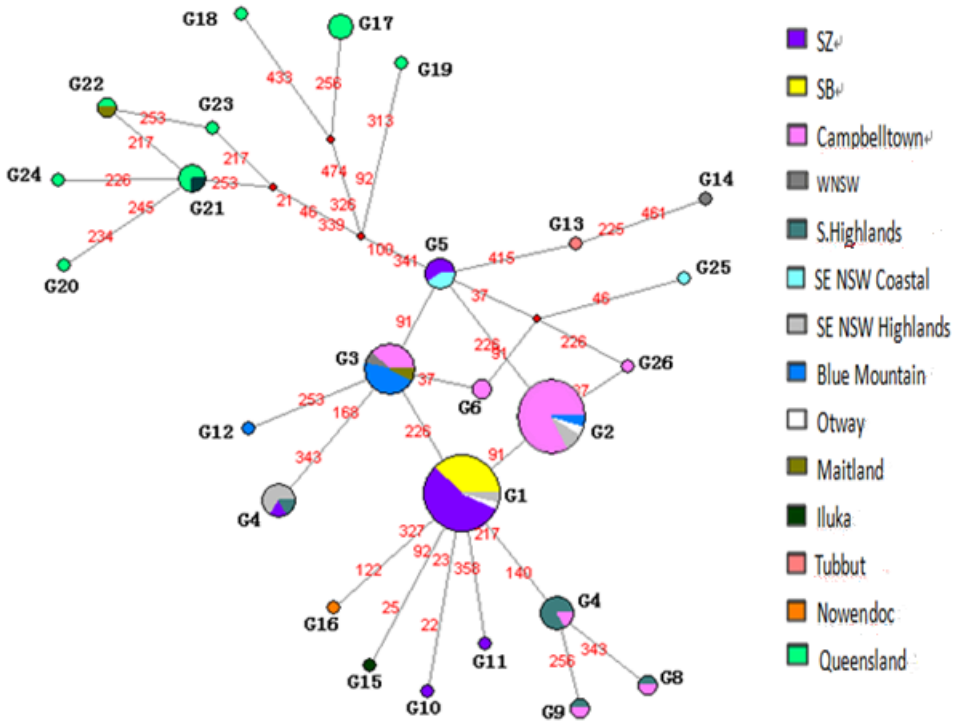

Fig. 3. Median-joining networks showing phylogeographic structure in Koalas. Red numbers above lines refer mutation positions separating the haplotypes. G1-G26 represent the haplotypes indentified in this study.

The Median Joining Analysis and Neighbor Joining analsysis also predicts that many of the haplotypes found in New South Wales are actually more closely related to the French Island haplotype then they are other New South Wales haplotypes.

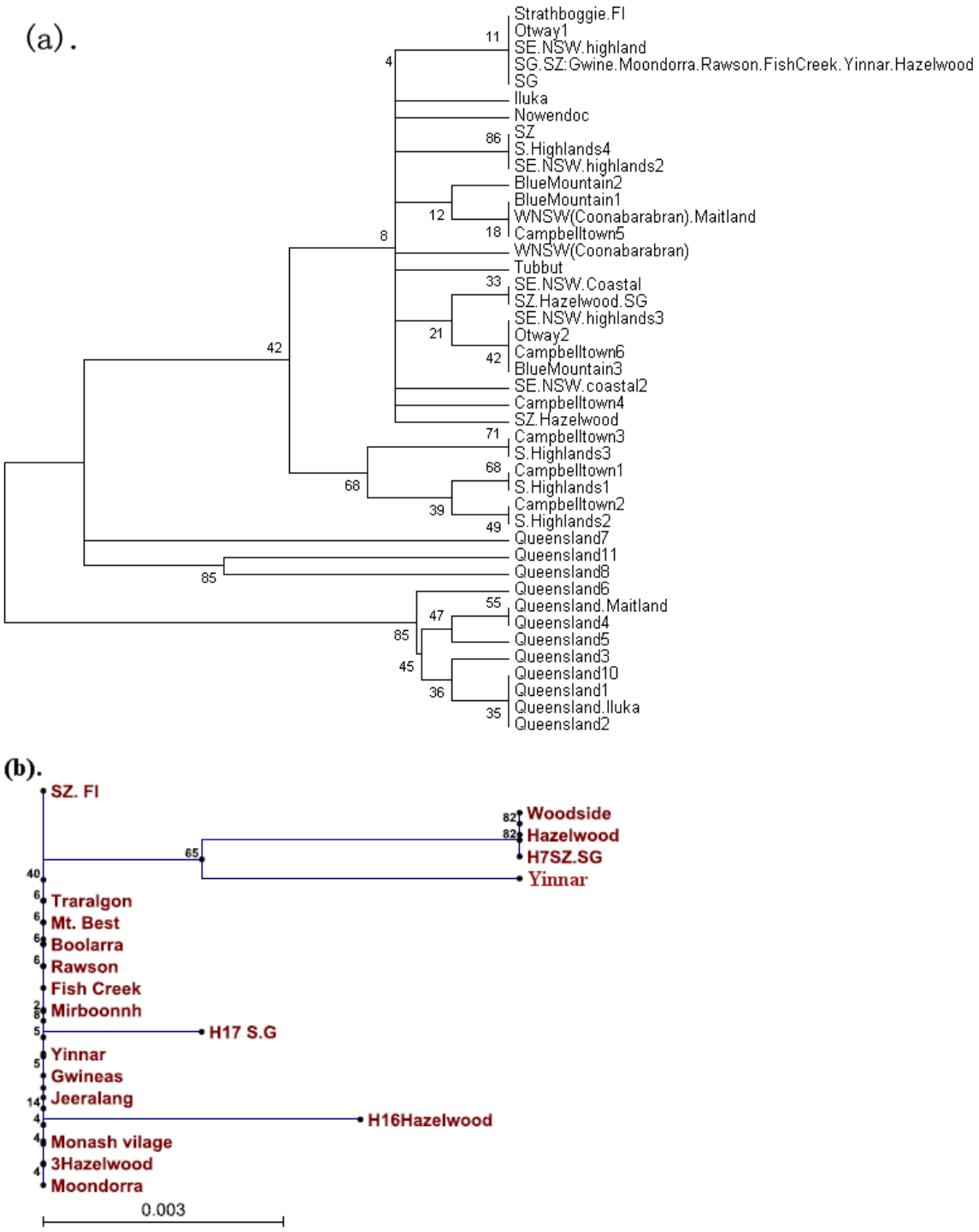

Fig. 4. (*A*) Neighbour-Joining Tree using UPGMA method, number of bootstrap replication=500,anlaysising haplotypes from Houlden *et al.* (1999), Fowler *et al.* (2000) and Phalen *et al.* (unpublished data) and haplotypes from this study. (*B*) South Gippsland region and Strzelecki range region Neighbour-joining tree done by CLC.

Discussion

This study should be considered to be a preliminary study for several reasons. Only a

portion of the mitochondrial control region was sequenced and thus other mutations that might have given this analysis greater power were not included in this study. Also, there were only limited data sets available for some populations. Never-the-less these findings provide important new insights into Koala genetic diversity on a regional and national basis.

Locally in Victoria, this data confirms the work of Houlden *et al*. (1999), Carpenter *et al.* (2010), and Lee *et al.* (2012) that translocation of koalas from Island populations has resulted in the Koalas in the studied areas of Western Victoria being of only a single haplotype. Our data suggests that this is also the case for animals in the Strathboggie Ranges, although comparison of microsatellite diversity between this population and Island animals will be necessary to prove this.

Our work also confirms previous work by Houlden *et al*. (1999), Carpenter *et al.* (2010), and Lee *et al.* (2012) showing that animals survived the fur trade and that not all animals in Victoria are descendants of animals translocated from Islands. This is particularly true for South Gippsland and given the identification of a unique haplotype by Houlden et al. (1999) in Tubbut, may be the case for animals in Victoria east of South Gippsland. Haplotype 1 (the French Island haplotype) clearly exists in South Gippsland. Given that animals with this genotype can be distinguished from Island animals by microsatellites, it appears that these animals represent a continuum of the Haplotype 1 population that was present prior to the fur trade and that they do not represent translocated animals. This hypothesis is further supported by translocation records which do not show introductions into South Gippsland and finding of a Haplotype 1 animal in southern New South Wales (Houldan et al., 1999). Together, these findings show that Koalas in Eastern Victoria merit special protection and further study if their valuable genetic diversity is to be maintained.

The data generated on Koala populations in southeastern New South Wales is the first for this region. It demonstrates that these populations have historically had continuity with Victorian populations and populations of Koalas from the Greater Sydney region. The coastal habitat and the highlands habitat in this area varies significantly with more rain and a more lush forest along the coast as apposed to the highlands (Chris Allen, pers. comm.). Although the numbers that we have had in this study are small, our data suggests that these two populations are genetically distinct and should be managed independently.

Analysis of the genetic structure of the Koalas in the greater Sydney area is consistent with the findings of Lau *et al*., (2012), showing that there is unexpected genetic diversity present in the Campbelltown population and that although closely related to adjacent populations unique haplotypes are present in the Southern Highlands and Blue Mountain populations. These data, however, conflict with those of Lee *et al.* (2010) who found very limited genetic diversity in the Campbelltown population using microsatellites. These findings in conjunction with those of South Gippsland show that it will be necessary to examine genetic diversity by multiple means if the full genetic structure of the population is to be accurately determined. Additional findings from the Greater Sydney populations connect them to North Coast populations and this is consistent with Lau *et al.* (21012) observed but given relatively limited data, more animals from different locations in the North Coast populations need to be examined.

On a larger scale across their range, our results show that Koalas should be considered to be of two genetic clades, a northern clade and a southern clade. Our analysis using data from Houlden *et al*. (1999) and Fowler *et al.* (2000) in conjunction with that of Lau *et al.* (2012) demonstrate that these two clades overlap in northern New South Wales, but do not represent a continuum. We hypothesize that these two clades came to be as the result of a relatively ancient event that resulted in the isolation of two small populations followed by a recolonization of habitats in between. This would also explain the very limited genetic diversity present in modern Koalas.

The finding of geographically dispersed animals with identical or similar haplotypes in the absence of a genetic continuum across the range of the southern clade also points to additional population declines since these two clades were established. There is clear evidence that the fur trade and policies on translocations impacted the genetic diversity in Western Victoria and is likely to have impacted genetic diversity in the Greater Sydney populations. However, given the relatively large number of animals that have now been genetically characterized, it is possible that climate changes following the establishment of the northern and southern clades may have resulted in additional range contractions and expansions creating in the current genetic structure observed today in modern Koalas.

Lau *et al.* (21012) suggested that their observations of a greater diversity in the MHC II DAB genes of koalas from the Northern range of the Koala, as apposed to the Southern range, may represent a response to gene selection as the result of disease pressures or a loss of variation in the south due to genetic bottlenecks and founder effects. Our data does not rule out that animals from the northern clade have increased DAB diversity as the result of gene selection, however, we found essentially identical trends in MHC II diversity and MT DNA diversity in animals from the southern clade strongly supporting a pattern of loss of MHC II diversity over time and not a selection for it.

Conclusion

The genetic structure and diversity of modern Koalas appears to be impacted by multiple historical events. An ancient event resulted in the establishment of a northern and southern clade that subsequently expanded from isolated populations and now overlap in northern New South Wales. It is possible that other climatic events then caused range contraction, loss of genetic diversity and possibly isolations of populations, but additional work will be necessary to confirm this. Added to these events, loss of genetic diversity has clearly occurred as the result of the fur trade leaving isolated populations with important genetic diversity across their entire range. Therefore, the genetic health of the koala in the future will require careful management of all populations across their range from Queensland to South Gippsland.

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