

Appendix 2 – Southern Cross University genetics report

DRAFT

**Genetic profiling of koalas:
Woolgoolga to Ballina Pacific Highway Upgrade
(Section 10–Wardell to Coolgardie)**

Southern Cross University

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Executive summary

The southern sector of the Ballina Local Government Area (LGA) has been identified as supporting an important population of around 200 koalas: the Wardell Koala Metapopulation (Wardell KMP) for *Environment Protection and Biodiversity Conservation Act 1999* purposes. Resident koala populations occur near Coolgardie, Wardell, Bagotville and Meerschaum Vale in two large tracts of remnant sclerophyll woodland on the south-eastern boundary of the former Big Scrub habitat. The Wardell KMP may be a significant source population for koala dispersal into this area which has been colonised following extensive logging of the native rainforest vegetation and conversion to agricultural land.

Section 10 of the conditionally-approved Pacific Highway upgrade will traverse the Wardell KMP and bisect the Bagotville area previously identified as containing important remnant habitat for koala dispersal. Consequently, there is potential for the realignment to disrupt dispersal within the Wardell KMP and impact regional koala population dynamics.

Genetic profiling of the Wardell KMP was undertaken by Southern Cross University (SCU) as part of the project Genetics and Population Demographics of Koalas Inhabiting Coastal Lowlands in the Ballina Local Government Area – Stage 10 Pacific Highway Upgrade. Genetic profiling was conducted using microsatellites in order to describe patterns of population structure and dispersal within the Wardell KMP and determine its regional significance as a source population.

The Wardell KMP was found to be spatially structured. Dispersal, estimated from genetic neighbourhood size and the distribution of first-order relatives (FOR), largely conformed to an isolation-by-distance model. However, we found evidence of more substantial genetic differentiation between the Northern and Southern subpopulations of the Wardell KMP with higher genetic variation and lower mean relatedness (for both males and females) in the north. This was unexpected given the geographic proximity of these subpopulations and evidence of ongoing dispersal between them. The most likely explanation is that the Northern subpopulation receives immigrants from koala populations in surrounding areas. Immigration into the Southern subpopulation appears to be limited in comparison.

These findings emphasise the potential significance of the Southern subpopulation in the vicinity of Bagotville as the main source population for habitats to the west in the area of the former Big Scrub rainforest. Although multiple source populations are indicated, spatial patterns of dispersal suggest substantially higher levels of connectivity are maintained with the Southern Wardell subpopulation. Dispersal is most likely asymmetric, occurring predominantly from the Southern Wardell subpopulation into the adjacent areas of the former Big Scrub habitat.

The Southern subpopulation occurs in an area of remnant sclerophyll woodland that will be bisected by the conditionally-approved Section 10 Pacific Highway upgrade. Genetic profiling confirmed dispersal of koalas across the proposed route. The Pacific Highway upgrade will also traverse areas occupied by the Northern subpopulation, between Wardell and Meerschaum Vale, with potential dispersal of koalas across the intervening valley floor indicated. These findings highlight the need for road-crossing structures to enable koala dispersal.

The findings presented in this report are intended to inform a concurrent Population Viability Assessment (PVA) undertaken as part of the project Genetics and Population Demographics of Koalas Inhabiting Coastal Lowlands in the Ballina Local Government Area – Stage 10 Pacific Highway Upgrade.

Acknowledgements

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Acronyms and abbreviations

CI	confidence interval
DNA	deoxyribonucleic Acid
FoK	Friends of the Koala Inc.
FOR	first-order relatives
GPA	generational persistence assessment
ha	hectare
IP	Important Population
km	kilometre(s)
KMP	Koala Metapopulation
LGA	Local Government Area
NSW	New South Wales
PVA	population viability assessment
SCU	Southern Cross University

Glossary of genetic terms

Coefficient of relatedness: a measure of the percentage of genes shared by two individuals. For first-order relatives (e.g. a mother and her offspring) this coefficient is expected to be 0.5 as the offspring obtains half their genes from the mother and half from the father. In natural populations the estimated value for first-order relatives can vary depending on the extent of inbreeding and the variability of the genetic markers examined.

F-statistics: describe the extent of genetic differentiation between populations caused by a reduction in observed heterozygosity. Heterozygosity is measured at three hierarchical levels (within individuals in each subpopulation, within subpopulations and across the total population). When a population is structured heterozygosity within subpopulations is lower than estimated for the total population. Values can range from 0.0 (no differentiation) to 1.0 (complete differentiation). In the latter case subpopulations would not share any genetic variants at the surveyed microsatellites.

Hardy-Weinberg equilibrium: in a randomly mating population genetic variants are expected to be maintained in a particular ratio of homozygotes and heterozygotes. Departures from this ratio may indicate that the genetic markers being examined are under selection which can limit their usefulness in population studies.

Stepwise mutation model: assumes that variation in microsatellites arise from a step-wise increase or decrease in repeat length. In this model repeats of similar length are considered closely related. The alternative model assumes that changes in repeat length are unconstrained such that repeats of similar size may be unrelated.

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Introduction

1.1 Project background

This report presents the results of the genetic profiling component of a project which examined the distribution, density, demographics and genetics of a koala population known to be present within approximately 8,250 ha of coastal lowlands in the Ballina Local Government Area (LGA). The project was commissioned by the New South Wales Roads and Maritime Services as part of koala preconstruction surveys associated with the conditionally-approved Woolgoolga to Ballina Pacific Highway Section 10 upgrade. Biolink Ecological Consultants and Ecosure Pty Ltd completed the koala demographic study (Phillips et al. 2015) which provided the genetic material that forms the basis of the present study. The genetic profiling project has been undertaken by Southern Cross University (SCU).

Section 10 will be traversing an area of koala habitat in the southern sector of the Ballina LGA identified as supporting an important koala population; the Wardell Koala Metapopulation (Wardell KMP) for *Environment Protection and Biodiversity Conservation Act 1999* purposes (Phillips & Chang 2013; Ecosure 2014). The LGA supports multiple landuse types including native forests, plantations, agricultural landscapes and residential areas. The northern extent occurs in highly modified habitat that was formerly part of the Big Scrub subtropical rainforest whereas southern areas are dominated by heathland and areas schlerophyll woodland and forest. Preferred koala habitat across the LGA is largely fragmented and was estimated to comprise 1,500 – 2,000 ha (Phillips & Chang 2013) of the total area.

Population distribution modelling based on Generational Persistence Assessments (GPA) and koala activity patterns confirm the long-standing presence of resident koala populations associated with two large tracts of woodland in the vicinity of Coolgardie, Wardell, Bagotville and Meerschaum Vale (Phillips & Chang 2013). The Bagotville area bisected by the conditionally-approved Section 10 Pacific Highway upgrade has been identified as containing important remnant habitat for koala dispersal (Ecosure 2014, Phillips & Chang 2013). Resident koala populations are also present in the vicinity of Uralba and Lynwood in highly modified agricultural landscapes to the north and north-west of these forested areas. For the purposes of this study and an associated PVA the Wardell KMP encompasses these resident

populations along with habitat in the vicinity of Dalwood, a north-western extension of the original Important Population (IP) boundary defined by Phillips & Chang (2013).

Koala density data reported by Phillips & Chang (2013) enable a population estimate of approximately 200 animals for the Wardell KMP. Based on the long-term persistence of this metapopulation, and increases in the extent of area occupied by koalas in the adjacent Lismore and Byron LGA's, it has been suggested that the Wardell KMP may be an important source population for these areas (Phillips & Chang 2013). The Wardell KMP is already subject to ongoing threats from road mortality, habitat loss, dog attacks and disease. There is potential for Section 10 to have significant additional impacts (Phillips & Chang 2013) to the detriment of regional koala population dynamics.

1.2 Scope of work

This project uses genetic profiling to describe patterns of population structure and gene dispersal within the Wardell KMP and determine its regional importance as a source population for surrounding areas. The results are intended to inform a concurrent PVA and provide baseline information on levels of genetic variation for future monitoring. Two main issues were considered:

1. Is the Wardell KMP spatially structured?

For species which have limited dispersal capabilities, populations are expected to be spatially structured. Where dispersal into new territories is spatially uniform this leads to a pattern of isolation-by-distance in which related individuals occur in close proximity and the degree of relatedness declines with distance. Physical barriers to dispersal, or the presence of dispersal corridors, can lead to departures from a strict isolation-by-distance model of population structure over relatively small spatial scales. Dispersal away from the natal site is considered to be somewhat limited in koalas (~3.5 km) and sex biased, with a higher proportion of males than females migrating to new territories (Dique et al 2003). For the purposes of this study we used estimates of genetic relatedness to determine if limited dispersal leads to patterns of population substructure within the Wardell KMP. The results will assist in identifying areas of high natural connectivity that may be impacted by the

conditionally-approved Section 10 upgrade. The presence of population substructure and inferred dispersal patterns should also be accounted for in the associated PVA.

2. Is the Wardell KMP an important source population for surrounding areas?

The Wardell KMP is located on the south-eastern boundary of the former Big Scrub, an area that previously supported large tracts of subtropical rainforest. The Big Scrub was extensively logged and converted to agricultural use by the early 20th century (Lott & Duggin 1993). Since then much of the area has been colonised by koalas, presumably a result of the planting of windbreaks with koala food tree species such as Tallowwood (*Eucalyptus microcorys*) that may have functioned to facilitate dispersal from surrounding areas (Phillips & Chang 2013). For the purposes of this study we used genetic profiling to test whether koala populations in the adjoining habitat to the west are related to those in the Wardell KMP, or to other regional koala populations (SCU unpublished data). If the Wardell KMP is a significant source population for these surrounding areas the conditionally-approved Section 10 upgrade could potentially impact regional koala population dynamics.

Methods

2.1 Tissue samples

Tissue samples for DNA analysis were collected from ear punches of 40 koalas captured (or found deceased) during Roads and Maritime preconstruction surveys of the Wardell KMP. An additional 11 samples were made available by SCU from samples collected on their behalf by the Friends of the Koala Inc. (FoK). Post-mortem ear biopsies were obtained from these animals which had died as a result of disease, injury or unknown causes.

Six koala scats were also collected but time constraints precluded their inclusion in the present study. Extraction of DNA from scats is more difficult and requires modifications to standard extraction protocols to concentrate the trace amounts of DNA that may be present and eliminate inhibitory substances. Furthermore, the quality of DNA obtained from scats is very dependent on the length and type of environmental exposure prior to collection and it is recommended to perform genetic profiling on freshly collected scats (Wedrowicz et al. 2013). Genetic profiles obtained from scat DNA are also subject to a high error rate. Although reliable profiles can be obtained by running each sample in triplicate (Wedrowicz et al. 2013) this imposes considerable additional time and costs that were not available.

To investigate regional koala population dynamics genetic profiles of an additional 88 koalas were made available (SCU, unpublished data). This included genetic profiles of 42 koalas from habitat areas to the west of Lismore and the Wardell KMP. Genetic profiles were also provided for koalas inhabiting outlying areas to the northeast (30) and southwest (16) of Lismore.

2.2 Genetic profiling

Genetic profiling was performed at the Australian Genome Research Facility using DNA extracted at SCU from koala tissue samples using the QIAGEN DNeasy Blood and Tissue Kit. To obtain unique genetic profiles for each sample sections of DNA that contained *short tandem repeats* (e.g. CACACACA) were examined. These regions are known as *microsatellites* and are characterised by variation in repeat length. Each animal carries two copies of a microsatellite, one inherited from their sire (father) and one from their dam (mother). The copies may be identical in repeat

length (homozygous) or may be a different length (heterozygous). Microsatellites that are heterozygous within individuals are the most useful for genetic profiling.

For this study we utilised 14 published koala microsatellites with average observed heterozygosities in the range 0.255 to 0.894 (mean 0.631) for the Wardell KMP. Together, these fourteen microsatellites have an exclusion probability when one parent is known of 99.97546, and an exclusion probability when both parents are unknown of 98.95176. The single known example of a parent-offspring pairing returned a relatedness coefficient (R) of 0.405, close to the expected mean of 0.5 for first order relatives (FOR; parent-offspring, full-siblings). All microsatellites except one were found to be in Hardy-Weinberg equilibrium with post-hoc testing showing this departure was due to spatial structure within the sample. We determined that this set of microsatellites are able to detect the presence of genetic differentiation amongst populations with a power of 0.975 or higher after 10 generations and assuming an effective population size of 50-200 (approximately the number of reproductive adults per population which is smaller than the census population size). Thus, the 14 microsatellites were confirmed as suitable for analysis of local and regional population structure, estimation of genetic diversity, and analysis of the relatedness of individuals within and between populations.

Analyses of the Wardell KMP that follow are based on the genetic profiles obtained for 47 of the 51 koalas sampled; three FoK samples being excluded from the analysis as genetic profiling showed that they were among the koalas that had been sampled earlier during the Roads and Maritime surveys. The single joey of a deceased female was also excluded from the population analysis to avoid bias.

2.3 Data analysis

2.3.1 Spatial structure within the Wardell KMP

To determine if there was evidence of spatial structure within the Wardell KMP we first employed the distance measure A_{ij} (Roussett 2000) to estimate the size (geographic extent in km^2) of genetic neighbourhoods in the study area. This approach defines the geographic range over which gene dispersal occurs and identifies the likely occurrence of clusters of closely related individuals. Geospatial data was provided as decimal degrees and the analysis run under different assumptions of koala population density to simulate a population ranging in size from

93-930 individuals. This approach broadly encompasses the current estimate of approximately 200 koalas in the Wardell KMP.

Population substructure was subsequently assessed from the spatial distribution of FOR identified using the relatedness coefficient (R) of Wang (2002) which performs well in structured populations. The 51 koala DNA samples were assigned to eight sample groups defined on the basis of habitat type, focal areas of koala activity (Phillips & Chang 2013) and patterns of intergenerational persistence (Phillips & Chang 2013) (Table 1, figure 1). Partitioning of the samples in this way allows the following questions to be addressed:

1. Do focal areas of koala activity support discrete subpopulations?
2. Are samples to the east and west of the Section 10 upgrade related and connected by high levels of contemporary dispersal?
3. Do alternative patterns of spatial structure exist within the Wardell KMP?

Table 1. Assignment of Wardell KMP DNA samples to groups based on geographic proximity, habitat type and ecological attributes. * data sourced from Phillips & Chang (2013). # habitat occupied by koalas in sample group 2b is bisected by the conditionally-approved Section 10 upgrade.

Sample grouping	Location	Sample size	Habitat Type	Focal Area*	GPA*
1a	Lynwood	6	Agriculture	A	High
1b	Dalwood	7	Agriculture	-	Low
2a	Wardell	6	Forest	B	High
2b#	East Bagotville	10	Forest	B	high
3a	Coolgardie	4	Forest	C	High
3b	Meerschaum Vale	4	Agriculture/Forest	D	High
4a	West Bagotville 1	3	Forest fragment	D	Low
4b	West Bagotville 2	7	Forest fragment	D	Low

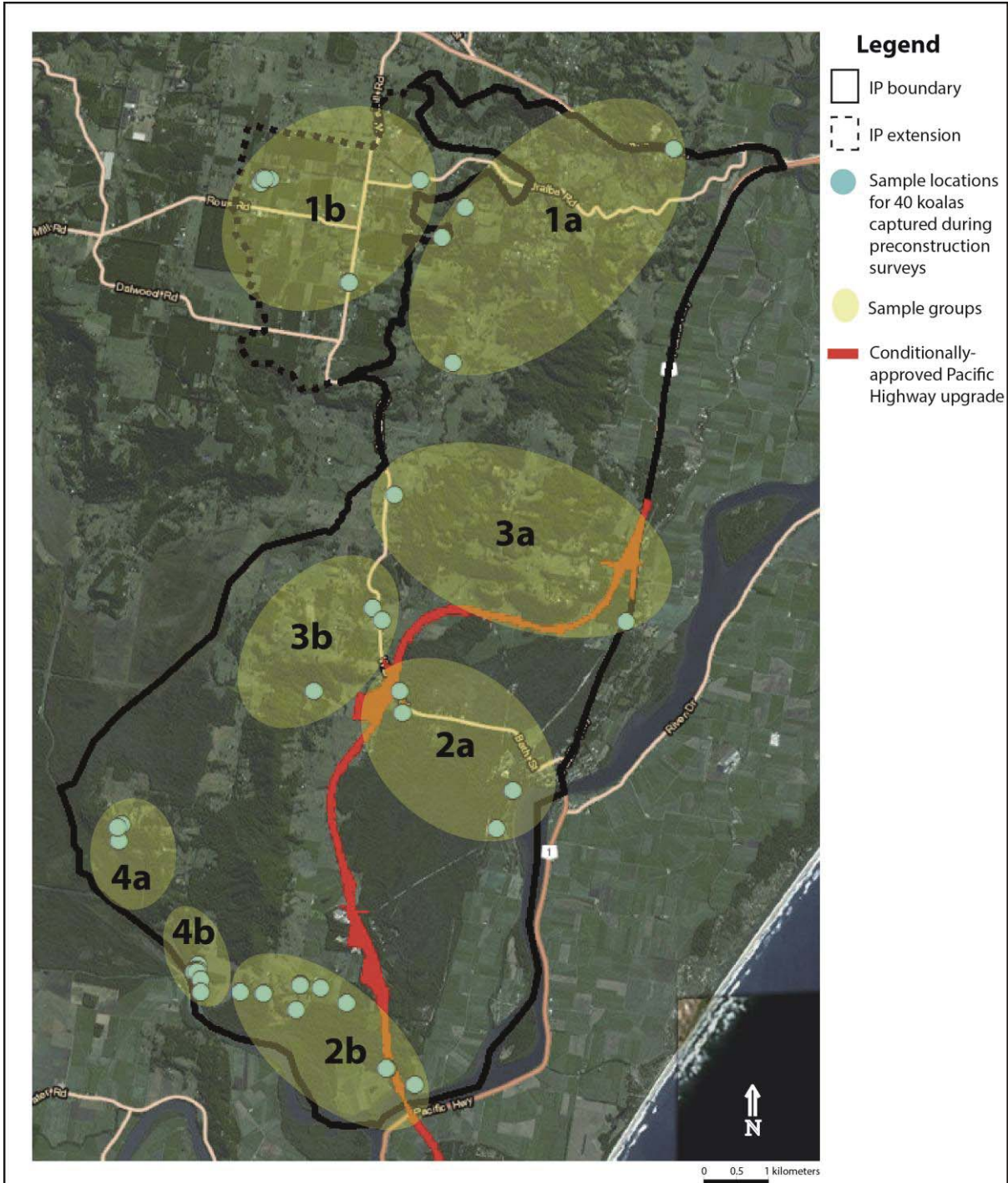


Figure 1. Wardell koala metapopulation boundary, sample locations and sample groups used to infer dispersal patterns

Pairwise relatedness (R) was estimated for all individuals within and amongst the eight groups. Potential FOR were identified using the theoretical mean R of 0.5 ± 1 standard deviation (range 0.378 – 0.622) following Blouin et al. (1996). Under limited dispersal FOR are expected to occur in close proximity, within groups or between adjacent groups as defined in the present analysis. Distant groups are not expected to share FOR unless occasional long-distance dispersal occurs. The spatial distribution of FOR was mapped to show likely patterns of contemporary dispersal within the Wardell KMP.

From the inferred patterns of dispersal sample groups were aggregated into logical clusters and the difference in mean R for each pair of clusters determined. For the purpose of this study clusters were considered to be different if this value lay outside the 95% confidence interval (CI) for the cumulative frequency of R values obtained by bootstrap resampling of the data. A significant difference indicates underlying differences in the demographic structure of the sampled populations which affect levels of koala relatedness (e.g. the extent of inbreeding and levels of immigration and/or emigration). We extended this approach to test for differences in mean R between males and females across the Wardell KMP. Sex-biased dispersal, in which males disperse further than females, has been reported for koalas (Dique et al 2003). This should lead to female koalas being more closely related than males within a population or subpopulation.

2.3.2 Regional population structure and the significance of the Wardell KMP as a source population

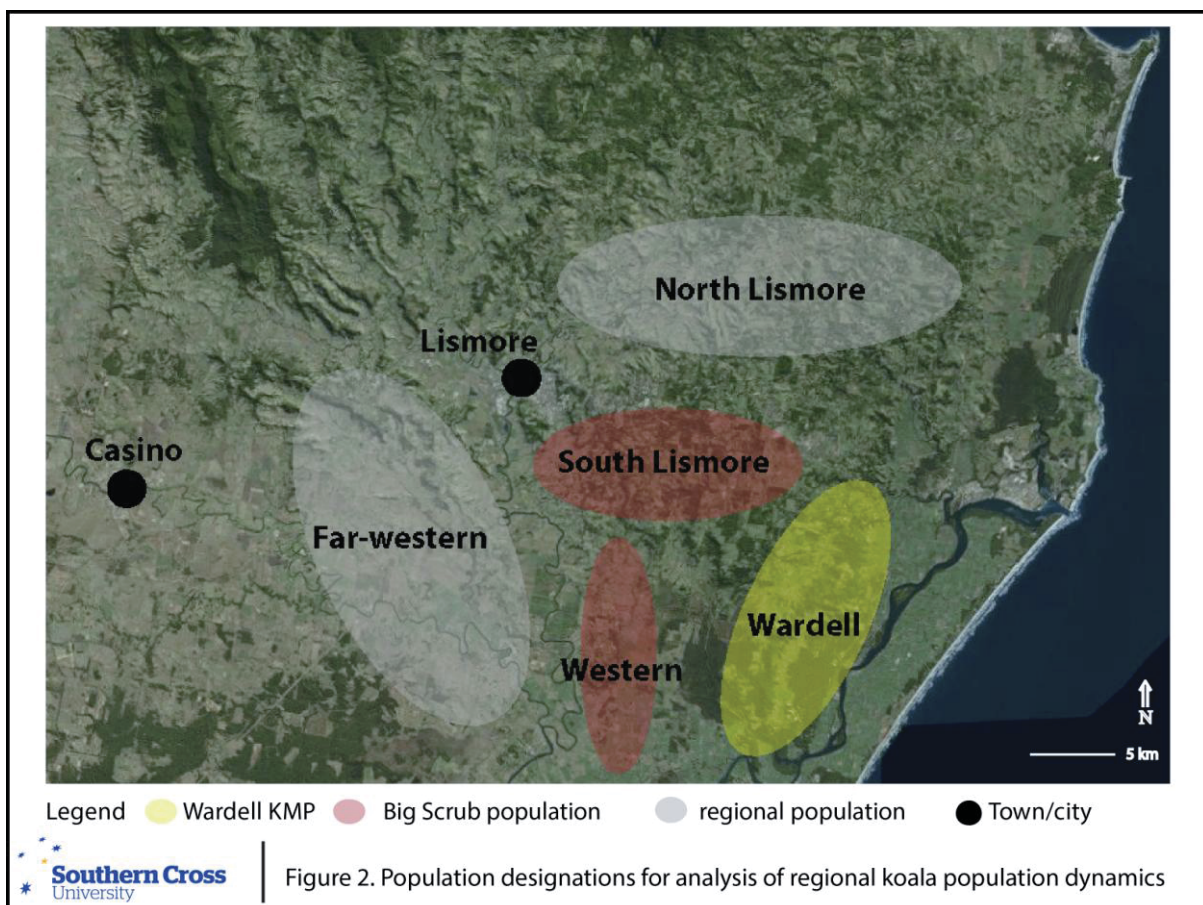
We used pairwise estimates of R (as described above) to test for evidence of dispersal between the Wardell KMP and populations present in areas of the former Big Scrub habitat to the west. Samples from the Big Scrub were provided by SCU and grouped into a South Lismore population (South Lismore-Alstonville) and a Western population (Wyrallah-East Coraki) (Figure 2).

The extent of genetic differentiation amongst regional populations and subpopulations was also assessed using pairwise F -statistics; F_{st} (Weir & Cockerham 1984) and R_{st} (Michalakis & Excoffier 1996). F -statistics quantify the extent to which population subdivision leads to a reduction in observed heterozygosity. R_{st} is a more suitable estimator for microsatellites as it assumes a

stepwise mutation model. However, previous koala genetic studies have employed *Fst* to estimate levels of population differentiation. We have included values for both estimators to allow comparison with published estimates of genetic differentiation amongst regional koala populations in New South Wales and South-east Queensland. Outlying populations to the north (North Lismore) and west of Lismore (Far-western) (SCU, unpublished data; Figure 2) were also included in the analysis. The North Lismore population occurs in an area of the Big Scrub rainforest colonised by southward dispersal of koalas from South-east Queensland (Lee et al. 2013).

2.3.3 Genetic variation

Summary statistics that describe levels of genetic variation were calculated for the Wardell KMP: observed (H_o) and expected (H_e) heterozygosity, an unbiased estimator of the effective number of alleles (AE), and an inbreeding coefficient (F_i).



Results

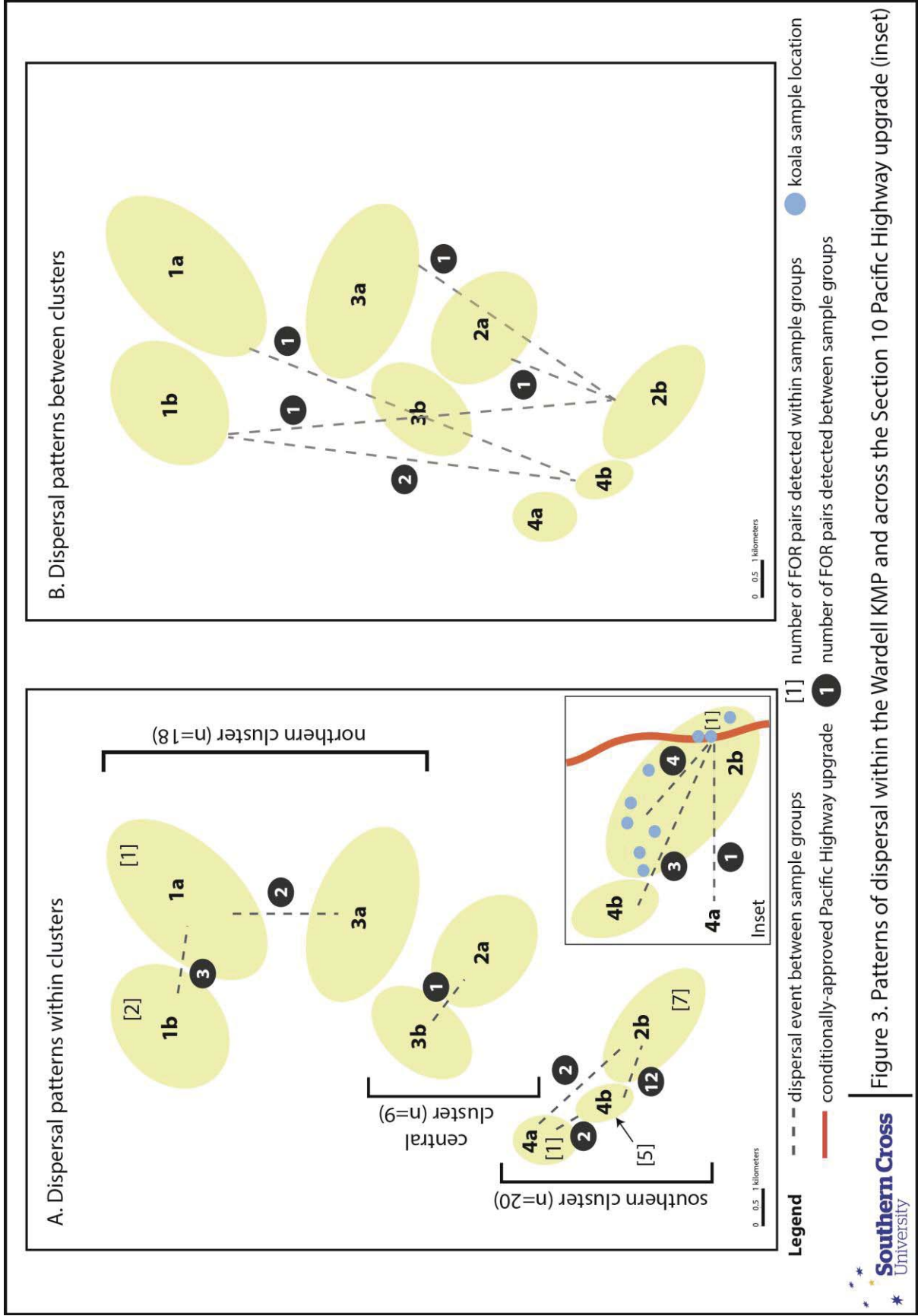
3.1 Spatial structure within the Wardell KMP

Genetic neighbourhood size in the Wardell KMP was estimated to be 21-30 Km². This confirms a pattern of limited dispersal across the study area and the likely presence of multiple subpopulations. The estimated genetic neighbourhood size suggests that koalas within the Wardell KMP typically disperse distances of 4.5-5.5 km from the natal site. This is broadly consistent with published dispersal estimates of 3.5 km for dispersing young in a peri-urban landscape in south-east Queensland (Dique et al 2003).

Analysis of the spatial distribution of FOR amongst the eight sample groups approximates an isolation-by-distance model with a high proportion of FOR occurring within (37.8%) or between adjacent groups (48.9%) (Figure 3a). This included a small number of comparisons in which R values exceeded the upper threshold of 0.622. This could reflect our application of a conservative threshold for FOR or indicate occasional mating between related animals. In the Bagotville area a large number of FOR were shared amongst the three sample groups (2b, 4a and 4b). We also detected evidence of dispersal across the area where the conditionally-approved Section 10 upgrade bisects this large tract of remnant habitat (Figure 3a inset). Potential dispersal across the valley floor was detected in the vicinity of Wardell and Meerschaum Vale (Figure 3a), an area also bisected by the conditionally-approved Section 10 upgrade.

Based on the observed patterns three potential subpopulation clusters were identified; a northern cluster comprising koalas sampled from Lynwood (1a), Coolgardie (3a) and Dalwood (1b), a central cluster comprising koalas from Wardell (2a) and Meerschaum Vale (3b), and a southern cluster comprising koalas from the Bagotville area (2b, 4a and 4b) (Figure 3a). FOR were also detected between these three clusters (13.3%) (Figure 3b) indicating the occurrence of infrequent long-distance dispersal events across the study area of up to 15 kilometres.

The significance of the observed spatial patterns were assessed using the 95% CI of the cumulative mean frequency obtained from pairwise comparisons of R for the three clusters. Only two of the three tests were significant; the mean difference in R for the northern and central clusters falling within (but close to) the 95% CI (Table



2). Based on these results a hierarchical population structure is indicated with the Wardell KMP comprising two main subpopulations:

1. a Northern subpopulation provisionally containing the northern and central clusters;
2. a Southern subpopulation located in the Bagotville area.

Consistent with this arrangement we also found differences in R when comparing the demographic profiles of the two subpopulations. Both males and females in the Southern subpopulation are, on average, more closely related than those in the Northern subpopulation (Table 3). Within the Southern subpopulation the mean R for females (~0.20) was twice that of males (~0.09) a pattern consistent with more limited dispersal of females away from the natal site.

Table 2. Mean difference in relatedness (R) between sample clusters in the Wardell KMP. Differences are significant if the mean falls outside the 95% CI (2.5-97.5% quantiles).

Cluster 1	Cluster 2	Mean difference	2.5% quantile	97.5% quantile
northern	central	0.0676	-0.0618	0.0685
central	southern	0.1939	-0.0713	0.0653
northern	southern	-0.1121	-0.0607	0.0639

Table 3. Mean difference in relatedness (R) between males and females in the Wardell KMP. Differences are significant if the mean falls outside the 95% CI (2.5-97,5% quantiles)

Comparison: (A cf. B)	Mean R A	Mean R B	Δ Mean R	95% CI
Across Wardell KMP: males cf. females	-0.0068	-0.0234	0.0166	-0.0422 – 0.0399
Males: Northern cf. Southern	-0.0974	0.0887	0.1862	-0.1719 – 0.1835
Females: Northern cf. Southern	-0.0289	0.1973	0.2262	-0.0809 – 0.0830

The proposed subdivision of the Wardell KMP into a Northern and Southern subpopulation does not support models of population structure based solely on areas of focal activity, habitat type or landuse. The Northern subpopulation occupies both agricultural landscapes and contiguous native forest, contains three focal areas of activity, and encompasses areas of high and low generational persistence (Table 2). The Southern subpopulation is largely confined to fragmented and contiguous forest habitats and also contains multiple focal areas of activity and areas of variable generational persistence. We also reject a model in which the Wardell KMP is divided into an eastern and western subpopulation corresponding to the two large tracts of remnant sclerophyll woodland and forest. Both the Northern and Southern subpopulations are transected by the proposed Section 10 upgrade (Figure 1).

3.2 Regional population structure

Analysis of the spatial distribution of FOR amongst regional koala populations indicate that populations inhabiting the former Big Scrub habitat to the west of the Wardell KMP may be derived from multiple source populations (Figure 4). Spatial patterns indicate higher levels of contemporary dispersal between populations in the former Big Scrub habitat and the Wardell KMP (64%), than between outlying regional populations (Far-western 30%; North Lismore 6%). Spatial patterns also indicate variable levels of contemporary dispersal between populations in the Big Scrub habitat, and the Southern (52%) and Northern (12%) subpopulations of the Wardell KMP. The extent to which these patterns reflect historical patterns of colonisation is, however, uncertain.

Pairwise estimates of genetic differentiation amongst regional populations ranged from 0.03 to 0.071 (*Rst*) and 0.027 to 0.099 (*Fst*) in comparisons with the Wardell KMP subpopulations (Table 4; SCU unpublished data). Both estimators indicate that the Northern and Southern subpopulations of the Wardell KMP are genetically differentiated (*Rst* = 0.081, *Fst* = 0.058) and share closer genetic affinities with populations inhabiting the former Big Scrub habitat than each other.

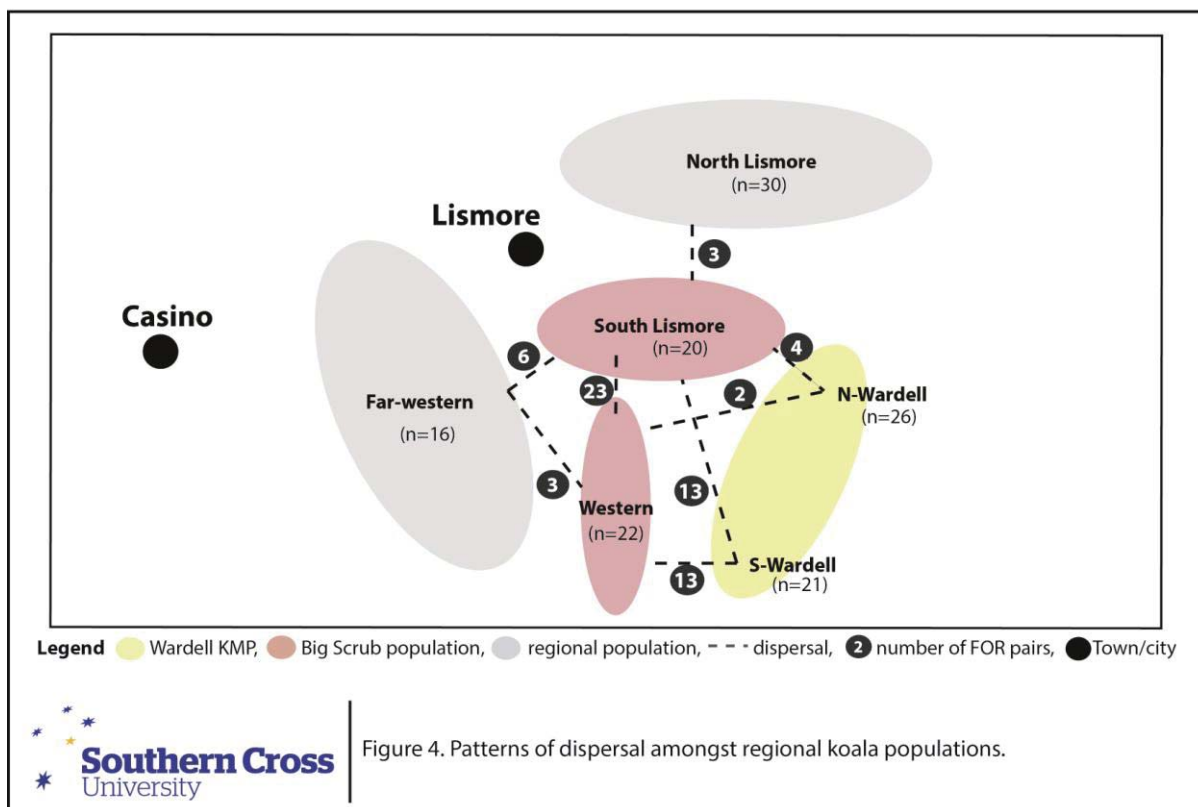


Table 4. Genetic differentiation between subpopulations of the Wardell KMP and regional koala populations (SCU, unpublished data). F_{st} values greater than zero but less than 0.05 indicate low genetic differentiation; values between 0.05 and 0.15 indicate moderate genetic differentiation.

Populations	Rst		Fst	
	N-Wardell	S-Wardell	N-Wardell	S-Wardell
N-Wardell	-	0.081	-	0.058
S-Wardell	0.081	-	0.058	-
South Lismore	0.043	0.042	0.034	0.027
Western	0.032	0.030	0.042	0.030
North Lismore	0.031	0.068	0.053	0.099
Far-western	0.050	0.071	0.052	0.062

3.3 Genetic variation

Descriptive statistics for both the Northern and Southern subpopulations of the Wardell KMP, as well as the combined sample are presented in Table 5. Levels of genetic variation, as measured by H_o , are within the range reported for populations in northern NSW (0.47-0.78), central NSW (0.5-0.66) and southeast Queensland (0.39-0.73) but exceed those reported for populations in Victoria (0.38-0.56) (from Lee et al 2012). These comparisons are *indicative only* as the number of microsatellites used to estimate H_o varied across the studies. Both A_E and H_o indicate higher levels of genetic variation are present in the northern subpopulation of the Wardell KMP. For both subpopulations the average level of inbreeding is negligible with randomisation tests indicating that the observed values are not statistically different from 0.

Table 5. Summary statistics of genetic variation in the Wardell KMP. A_E = unbiased estimator of the effective number of alleles; H_o = average observed heterozygosity; H_e = average expected heterozygosity; F_i = individual inbreeding coefficient.

Subpopulation	Sample size	A_E	H_o	H_e	F_i
Northern	26	3.6	0.668	0.676	0.012
Southern	21	2.76	0.587	0.587	-0.001
Combined*	47	3.33	0.630	0.654	0.038

Discussion and recommendations

4.1 Discussion

This study has shown that the Wardell KMP is spatially structured. Dispersal, estimated from genetic neighbourhood size and the distribution of first-order relatives, largely conforms to an isolation-by-distance model with adjacent areas more likely to share close relatives than distant ones. Of particular relevance, koala dispersal was detected between habitats bisected by the conditionally-approved Section 10 Pacific Highway upgrade in the vicinity of Bagotville, an area previously identified as containing important habitat for dispersal and the main focus of proposed mitigation efforts (Phillips & Chang 2013, Ecosure 2014). Koala dispersal was also detected between habitats in the vicinity of Wardell and Meerschaum Vale suggesting possible dispersal across the valley floor in an area also bisected by the conditionally-approved Section 10 upgrade. These dispersal events were detected with relatively small sample sizes for each location (Table 1) suggesting that dispersal amongst adjacent sites is relatively common.

There is strong evidence that the Wardell KMP, as currently defined, is comprised of a distinctive Northern and Southern subpopulation with detectable level of dispersal between them. The extent of genetic differentiation between them exceeds that observed between populations in the adjacent Big Scrub habitat south of Lismore and outlying regional populations. This is unexpected given their close geographic proximity and evidence of ongoing dispersal between them. The most likely explanation is that the Northern subpopulation receives immigrant koalas from surrounding populations. This is supported by evidence of higher levels of genetic variation in the Northern subpopulation, measured from both observed heterozygosity and the effective number of alleles (Table 2), with 10 of the 14 surveyed microsatellites containing a greater diversity of repeat lengths than observed in the Southern subpopulation. Immigration of koalas into the Northern subpopulation is also supported by significant differences in the average relatedness (R) of males and females in the two subpopulations. This was especially pronounced for females with those in the Northern subpopulation having fewer FOR relatives living in close geographic proximity. This is unexpected for a species in which dispersal away from the natal site is limited.

In contrast, the genetic and demographic profile of the Southern subpopulation appears to be consistent with that expected in a functional koala metapopulation. This includes a high proportion of FOR occurring in close geographic proximity, a decrease in relatedness with distance (isolation-by-distance effects), an absence of detectable inbreeding effects and females, on average, being more closely related than males consistent with a known sex-bias in dispersal rates (Dique et al. 2003) and the structuring of koala populations along matriarchal lines.

It is unclear from the present analysis whether the Northern subpopulation is part of the Wardell KMP historically but has received higher levels of immigration from surrounding areas, or if it has been recently colonised along with other areas of the former Big Scrub habitat south of Lismore. A third possibility is that the boundary of the Wardell KMP, as currently defined, does not accurately reflect the natural (historical) population boundary and the inclusion of samples from peripheral populations of mixed origins has biased the analysis. Unfortunately, current sample sizes were insufficient to enable a more detailed analysis of this issue. Irrespective of the historical processes, these results emphasise the potential significance of the Southern subpopulation of the Wardell KMP as the remaining relatively pure gene pool for koalas in this region.

Spatial patterns of dispersal indicate that koalas inhabiting areas of the former Big Scrub rainforest to the west of the Wardell KMP are derived from multiple source populations including the Wardell KMP and those to the north and west of Lismore. Initial assessments indicate that the greatest number of dispersal events occur between the Southern Wardell subpopulation and those in the former Big Scrub rainforest habitat. Dispersal is most likely asymmetric, occurring predominantly from the Southern Wardell subpopulation into the former Big Scrub habitat. This is supported by the observation of lower levels of genetic variation in the Southern subpopulation compared to the Northern subpopulation (Table 5), as well as regional populations, especially South Lismore (SCU, unpublished data). If substantial levels of immigration were occurring into the Southern subpopulation of the Wardell KMP we would expect more uniform levels of genetic variability across all populations in the region. Asymmetric dispersal is most likely influenced by a combination of demographic, behavioural and landscape factors (e.g., density-dependent regulation, territoriality and the spatio-temporal configuration of dispersal corridors) and a

greater understanding of these is required to fully discern the role of the Wardell KMP in regional koala population dynamics.

4.2 Limitations

- & Spatial patterns in the distribution of FOR have been used to identify koala dispersal *events* within the Wardell KMP, and between regional koala populations, but we cannot infer dispersal *routes* or directly infer the *direction* of dispersal in most cases.
- & It is beyond the scope of this study to discern the factors that facilitate long-distance dispersal events across the Wardell KMP or proposed asymmetric dispersal between regional populations. Demographic, behavioural and landscape factors are likely to play a role.
- & Due to limited sample sizes the inclusion of the central cluster (Wardell and Meerschaum Vale) in the Northern Wardell subpopulation is considered provisional only.

4.3 Recommendations

To model the impacts of the conditionally-approved Section 10 Pacific Highway upgrade on the Wardell KMP, and the effectiveness of proposed mitigation strategies, the PVA should incorporate information from this study with respect to the presence of population substructure within the Wardell KMP, potential impacts from disrupting observed patterns of dispersal, and relationships with regional koala populations.

Specifically, the PVA should;

- & Be conducted under different assumptions of population structure within the Wardell KMP; a null model with the Wardell KMP treated as a single panmictic population and compared to an alternative model with isolation-by-distance effects incorporated, and a model in which the Wardell KMP is represented by a distinctive Northern and Southern subpopulation as defined here.
- & Examine the effects of disrupting patterns of dispersal in the Bagotville area and across the valley floor and adjacent woodland in the vicinity of Wardell and Meerschaum Vale.

- & Consider the effects of regional koala population dynamics on the viability of the Wardell KMP in which dispersal approximates a model with net emigration from the Southern Wardell subpopulation (into adjacent habitats to the west), and net immigration into the Northern Wardell subpopulation (most likely from the west and/or north).

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Appendices

**Genetic profiling of koalas:
Woolgoolga to Ballina Pacific Highway Upgrade
(Section 10–Wardell to Coolgardie)**

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October 2015

Executive summary

These Appendices detail additional analyses and information requested by NSW Roads and Maritime Services as a supplement to the report *Genetic profiling of koalas: Woolgoolga to Ballina Pacific Highway Upgrade (Section 10-Wardell to Coolgardie)* (Norman et al 2015) undertaken by Southern Cross University (SCU). The analyses were commissioned in response to comments obtained during the external review process of this report and comparisons with a parallel report submitted by the Australian Museum Research Institute (Neaves et al 2015). These analyses aim to provide some standardisation of the information content of the two reports. Key findings are summarised below.

Spatial autocorrelation, principal component analysis (PCA) and Mantel tests all provide evidence of isolation-by-distance effects within the Wardell KMP.

Model-based clustering using PARTITION-ML confirmed the presence of two regional populations; one located to the north of Lismore, the other encompassing areas to the south and west of Lismore including the WKMP.

Assignment tests and model-based clustering indicate some movement of animals between these two regional populations is likely.

These results support the findings of our initial report (Norman et al. 2015) that there is fine-scale genetic structure with the WKMP and weak but detectable genetic differentiation at larger spatial scales. These effects reflect asymmetric levels and patterns of dispersal across the region and should be accounted for in the accompanying population viability analysis (PVA) of the WKMP.

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Genetic diversity data for the Wardell Koala Metapopulation

These Appendices detail additional genetic diversity data for the 14 microsatellite loci used to estimate the Coefficient of Relatedness (R), Rousett's distance (A_{ij}) and F-statistics (F_{st} and R_{st}) for animals in the Wardell Koala Metapopulation (Norman et al. 2015). Primer sequences and details of the 14 loci examined were sourced from the literature: Phc11, Phc13, Phc25 (Houlden et al. 1996a); K2.1, K10.1, Pcv2, Pcv6.1, Pcv25.1, Pcv25.2, Pcv30, Pcv31 (Cristescu et al. 2009); Phci5, Phci9, Phci15 (Ruiz-Rodriguez et al. 2014).

Appendix A provides information on allele frequency distributions for the 14 loci with data presented for the combined WKMP sample as well as the Northern and Southern subpopulations. As outlined in our accompanying report (Norman et al. 2015) there are marked differences in allele frequency distributions with the Northern subpopulation having a greater number of alleles at most loci. We concluded that this was most likely due to higher levels of immigration (or dispersal) of koalas from areas to the north and west leading to greater genetic diversity in the Northern subpopulation (Norman et al. 2015). This is supported by the results of our PARTITION-ML analysis detailed at Appendix I.

Appendix B provides more detailed results of the tests we conducted to evaluate Hardy-Weinberg equilibrium (HWE) (summarised on p.5; Norman et al 2015). Departures from HWE indicate the possible presence of selection at a locus, or can occur as a result of population admixture, non-random mating or population substructure (Wahlund effects). We used GENEPOP 4 to test for heterozygote deficiencies (estimated as F_{is} , the inbreeding coefficient) (Weir & Cockerham 1984) with significance evaluated using a Markov chain randomisation procedure. Significant departures from HWE were detected at three loci (K10.1, Pcv30 and Phci5) in the Northern subpopulation and are most likely a consequence of non-random mating and population admixture associated with the immigration or dispersal of koalas from surrounding areas.

Appendix C provides results of several tests we performed to check for null alleles. Null alleles result from the failure of one allele to amplify and may lead to errors in genotyping. The presence of null alleles is inferred where there is a deficit of heterozygotes or evidence of large allele dropout, however, heterozygote deficits can also result from non-random population processes (see above).

We first used MICROCHECKER to test for null alleles and genotyping errors due to stutter bands. To minimise errors arising from stuttering all reverse primers used in this study were

PIG-tailed (Brownstein et al 1996) with a seven base pair extension (GTTTCTT). MICROCHECKER did not detect evidence of null alleles or genotyping errors at any locus.

As our earlier tests of HWE identified heterozygote deficits at three loci we also employed a maximum likelihood test for the presence of null alleles performed using ML-Null with null frequencies calculated using GENEPOP 4. This analysis indicated the presence of null alleles in the Northern subpopulation at the three loci exhibiting heterozygote deficiencies (K10.1, Pcv30 and Phci5). Additional analyses presented here and in our report (Norman et al. 2015) indicate that observed heterozygote deficits are more likely to be the result of non-random population processes than PCR artefacts. Consequently, genotyping errors resulting from the presence of null alleles are considered to be negligible or absent in this dataset.

Appendix D provides results of tests for linkage disequilibrium amongst the 14 microsatellite loci performed using GENEPOP 4 with Bonferonni correction for multiple tests. There was no evidence of LD in the Southern Wardell subpopulation although LD was detected for two pairs of loci in both the Northern Wardell subpopulation and the combined WKMP sample. As patterns of LD were not consistent across populations it is most likely that the observed disequilibrium has arisen through population-specific processes rather than physical linkage of the loci on a chromosome. LD can arise through genetic drift and is not unexpected in small populations or where inbreeding occurs. Immigration can also lead to increased levels of LD where the source populations are genetically differentiated and is the most likely explanation for the LD observed in the Northern Wardell subpopulation of the WKMP. Population-specific processes are also supported by the absence of LD detected in other studies employing these loci (Cristescu et al. 2009, Lee et al. 2010, Lee et al. 2012, Ruiz-Rodriguez et al. 2014). Conversely, Houlden et al. (1996b) found LD at multiple loci but was able to attribute this to the effects of genetic drift after partitioning the variance components of the disequilibrium coefficients within and between populations. Thus, the detection of LD at a small number of loci in the present study is unremarkable.

Appendix E presents several estimators of genetic diversity (\pm SE) for the WKMP calculated using GENALEX.

Appendix A. Microsatellite allele sizes, repeat number and frequency data for the combined WKMP samples and the Northern and Southern subpopulations

Locus	Alleles		Frequencies		
	Size	Repeats	WKMP	Northern	Southern
Phc11	155	15	0.01	-	0.03
	157	16	0.21	0.22	0.20
	159	17	0.07	0.13	-
	161	18	0.05	0.04	0.08
	165	20	0.05	0.09	-
	169	22	0.01	0.02	-
	177	26	0.01	0.02	-
	179	27	0.17	0.11	0.25
	181	28	0.35	0.30	0.43
	183	29	0.05	0.07	0.03
Phc13	115	23	0.03	0.06	-
	117	24	0.14	0.11	0.18
	119	25	0.49	0.39	0.63
	121	26	0.12	0.19	0.03
	123	27	0.13	0.11	0.15
	125	28	0.05	0.07	0.03
	131	31	0.02	0.04	-
	133	32	0.01	0.02	-
	135	33	0.01	0.02	-
Phc25	125	31	0.53	0.50	0.58
	129	33	0.07	0.07	0.08
	135	36	0.01	0.02	-
	137	37	0.02	0.04	-
	147	42	0.16	0.19	0.13
	151	44	0.05	0.06	0.05
	153	45	0.13	0.09	0.18
	155	46	0.02	0.04	-
K2.1	149	13	0.05	0.02	0.10
	155	16	0.19	0.28	0.08
	159	18	0.27	0.31	0.20
	161	19	0.01	0.02	-
	163	20	0.07	0.09	0.05
	173	25	0.36	0.24	0.53
	175	26	0.04	0.04	0.05
K10.1	126	10	0.17	0.06	0.33
	128	11	0.38	0.31	0.48
	130	12	0.03	0.06	-
	132	13	0.11	0.17	0.03
	138	16	0.01	-	0.03
	140	17	0.02	0.04	-
	142	18	0.01	0.02	-
	146	20	0.10	0.09	0.10
	152	23	0.06	0.09	0.03
	154	24	0.11	0.17	0.03

Locus	Alleles		Frequencies		
	Size	Repeats	WKMP	Northern	Southern
Pcv2	121	14	0.01	0.02	-
	131	19	0.27	0.11	0.28
	133	20	0.28	0.26	0.30
	135	21	0.32	0.39	0.23
	137	22	0.12	0.20	-
	139	23	0.01	0.02	-
Pcv6.1	211	17	0.05	0.09	-
	213	18	0.02	0.04	-
	215	19	0.01	0.02	-
	217	20	0.20	0.28	0.10
	221	22	0.20	0.15	0.28
	223	23	0.23	0.30	0.15
	225	24	0.28	0.13	0.48
Pcv25.1	72	1	0.01	-	0.03
	74	2	0.72	0.67	0.80
	82	6	0.05	0.06	0.05
	88	9	0.07	0.11	0.03
	90	10	0.11	0.11	0.10
	94	12	0.03	0.06	-
Pcv25.2	172	17	0.85	0.81	0.90
	174	18	0.14	0.17	0.10
	178	20	0.01	0.02	-
Pcv30	183	47	0.01	0.02	-
	195	53	0.45	0.39	0.53
	197	54	0.13	0.30	0.03
	199	55	0.38	0.33	0.45
	201	56	0.02	0.04	-
	203	57	0.01	0.02	-
Pcv31	213	14	0.40	0.54	0.23
	223	19	0.18	0.17	0.20
	233	24	0.19	0.04	0.40
	239	27	0.22	0.26	0.18
Phci5	146	11	0.09	0.02	0.19
	150	12	0.01	0.02	-
	154	13	0.08	0.06	0.11
	158	14	0.14	0.13	0.17
	162	15	0.19	0.20	0.17
	166	16	0.49	0.57	0.36
Phci9	164	15	0.65	0.61	0.70
	167	16	0.14	0.15	0.13
	170	17	0.20	0.22	0.18
	173	18	0.01	0.02	-
Phci15	208	10	0.10	0.06	0.15
	212	11	0.01	0.02	-
	216	12	0.53	0.41	0.70
	220	13	0.35	0.50	0.15
	224	14	0.01	0.02	-

Appendix B. Tests of Hardy-Weinberg equilibrium for the combined WKMP samples and the Northern and Southern subpopulations. *, denotes loci showing significant departures from HWE with a deficit of heterozygotes (exact P-value ≤ 0.05).

Locus	WKMP combined		Northern		Southern	
	Fis	P-value	Fis	P-value	Fis	P-value
Phc11	-0.123	0.95	-0.159	0.90	-0.101	0.84
Phc13	0.017	0.14	-0.027	0.17	0.035	0.69
Phc25	0.020	0.09	-0.046	0.10	0.131	0.17
K2.1	0.105	0.36	0.038	0.35	0.121	0.38
K10.1	0.091	0.06	0.071	0.03*	0.035	0.53
Pcv2	0.116	0.15	0.000	0.37	0.157	0.29
Pcv6.1	0.115	0.12	0.170	0.06	-0.100	0.56
Pcv25.1	0.031	0.32	0.031	0.41	0.015	0.58
Pcv25.2	0.015	0.62	0.057	0.55	-0.086	1.00
Pcv30	0.075	0.00*	0.112	0.01*	-0.029	0.65
Pcv31	0.002	0.39	-0.186	0.94	0.052	0.45
Phci5	0.046	0.12	0.106	0.03*	-0.063	0.58
Phci9	-0.179	0.98	-0.183	0.96	-0.161	0.91
Phci15	0.137	0.27	0.125	0.28	-0.049	0.73

Appendix C. Estimated null allele frequencies and P-values for the 14 microsatellite loci. Tests were computed for the combined WKMP sample and the Northern and Southern subpopulations using GENEPOP 4 (null frequencies per locus) and ML-Null (randomisation tests). *, denotes loci showing a significant deficit of heterozygotes (exact P-value ≤ 0.05) and the possible presence of null alleles. As we have shown that these heterozygote deficits are most likely due to non-random population processes we have not computed corrected allele frequency distributions.

Locus	WKMP combined		Northern		Southern	
	Null Freq.	P-value	Null Freq.	P-value	Null freq.	P-value
Phc11	0	0.95	0	0.90	0	0.83
Phc13	0.019	0.15	0.216	0.17	0	0.65
Phc25	0.042	0.10	0.047	0.09	0.044	0.15
K2.1	0.004	0.34	0	0.37	0	0.37
K10.1	0.153	0.56	0.203	0.02*	0	0.46
Pcv2	0.036	0.15	0	0.36	0	0.28
Pcv6.1	0.038	0.12	0.063	0.06	0	0.54
Pcv25.1	0.011	0.29	0.019	0.41	0	0.25
Pcv25.2	0.006	0.36	0.021	0.55	0	0.15
Pcv30	0.057	<0.01*	0.072	<0.01*	0	0.50
Pcv31	0.004	0.39	0	0.94	0	0.41
Phci5	0.027	0.09	0.05	0.03*	0	0.47
Phci9	0	0.98	0	0.96	0	0.73
Phci15	0.038	0.25	0.03	0.28	0	0.60

Appendix D. Tests of linkage disequilibrium linkage for the 14 microsatellite loci after Bonferroni correction (significant LD if $P \leq 0.0036$). No LD was observed amongst loci in the Southern Wardell subpopulation.

WKMP			Northern			Southern		
Locus1	Locus2	P-value	Locus1	Locus2	P-value	Locus1	Locus2	P-value
Phc11	Pcv6.1	0.002	Pcv30	Pcv31	0.003	-	-	-
Pcv25.2	Phci15	<0.001	Pcv25.2	Phci15	0.003	-	-	-

Appendix E. Genetic diversity estimates averaged over the 14 microsatellite loci in the WKMP. Statistics were calculated for the Northern and Southern subpopulations as well as the combined WKMP sample. *n*, sample size; *Na*, number of alleles; *Ne*, effective number of alleles, *Pa*, private alleles, *Ho*, observed heterozygosity; *He*, expected heterozygosity; *F*, fixation index; SE, standard error.

Sub-population	<i>n</i>	<i>Na</i> ± SE	<i>Ne</i> ± SE	<i>Pa</i> ± SE	<i>Ho</i> ± SE	<i>He</i> ± SE	<i>F</i> ± SE
Northern	27	6.29 ± 0.5	3.44 ± 0.3	2.14 ± 0.3	0.67 ± 0.04	0.66 ± 0.04	-0.01 ± 0.03
Southern	20	4.29 ± 0.4	2.60 ± 0.2	0.14 ± 0.1	0.58 ± 0.04	0.57 ± 0.04	-0.03 ± 0.02
Combined	47	6.43 ± 0.6	3.26 ± 0.3	6.43 ± 0.6	0.63 ± 0.04	0.65 ± 0.04	0.02 ± 0.02

Population genetic analysis of the Wardell Koala Metapopulation

Appendices F-H provide details of additional population genetic analyses of the WKMP. The first (Appendix F) provides two estimates of the effective population size (N_e) of the combined WKMP sample and the two subpopulations (Northern and Southern) calculated using the program Ne ESTIMATOR. The first uses the linkage disequilibrium method and assumes random mating while the second employs the molecular co-ancestry method which is appropriate for population samples comprising a single cohort. As our data does not meet the assumptions of either model we urge caution in interpreting these outputs. Furthermore, estimates of N_e cannot be compared directly with estimates of census population size obtained from survey data. The larger estimated N_e for the Northern Wardell subpopulation is consistent with other data presented herein and Norman et al (2015) that immigration/dispersal contributes to higher genetic diversity, heterozygote deficiencies and genetic disequilibrium in this sample.

Appendix G provides the results of a Mantel test for isolation-by-distance (IBD) performed by regressing both (a) Relatedness and (b) genetic distance on geographic distance. A significant relationship (P (rxy random \geq rxy data) was observed for both analyses ($P = 0.001$ for relatedness, $P = 0.01$ for genetic distance). This is consistent with our previous analyses in which IBD effects were inferred from estimates of genetic neighbourhood size (21-30 km²) and spatial patterns in the distribution of First Order Relatives. We found closely related individuals were spatially clustered, occurring within or between adjacent sampling areas within the WKMP. Nevertheless, instances of long-distance dispersal across the WKMP study area were also detected but at a much lower frequency.

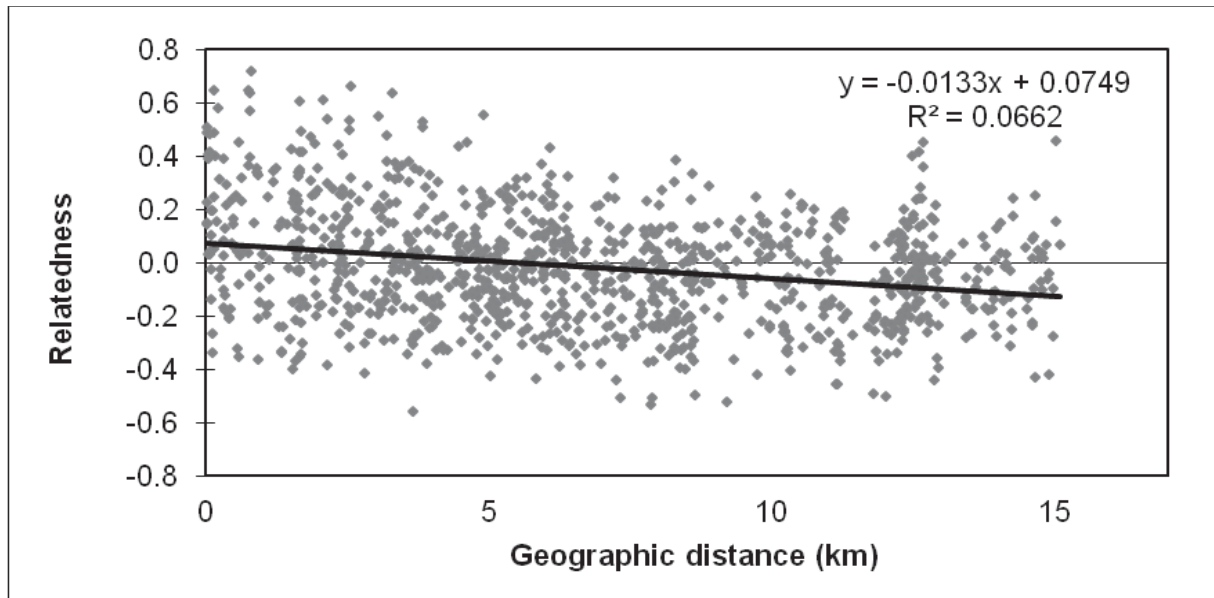
Spatial structuring within the WKMP was also apparent from the spatial autocorrelation analysis (Appendix H) calculated using Nei's genetic distance. The failure of Neaves et al. (2015) to detect spatial structuring using this approach may be a function of the smaller sample size (38 compared with 47) used in their analysis resulting in relatively few comparisons for some distance classes.

Appendix F. Estimates of effective population size (N_e , plus 95% confidence intervals) for the WKMP and the two subpopulations. N_e was calculated using the linkage disequilibrium model (LD model) with a lower bound allele frequency of 0.02, and the molecular coancestry model (MC model).

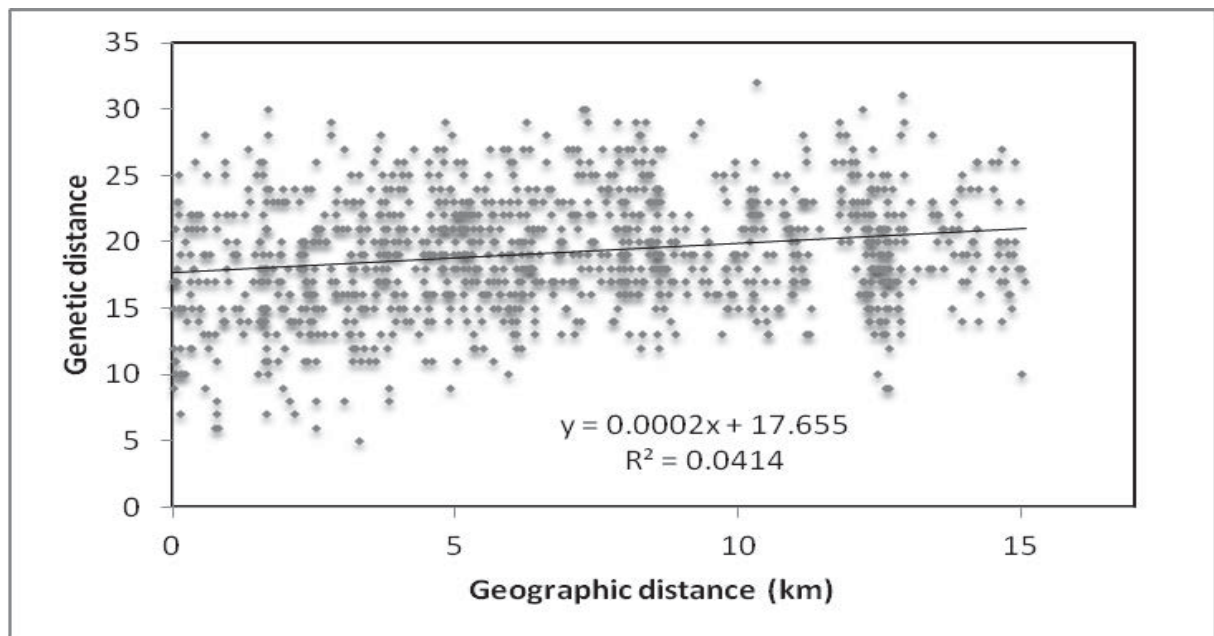
	LD model		MC model	
Population	N_e	95% CI	N_e	95% CI
Northern	65.2	38.4 – 168.8	13.7	3.3 – 31.4
Southern	14.9	10.3 – 23.0	8.3	2.5 – 17.5
Combined	47.4	36.8 – 63.3	9.6	5 – 15.7

Appendix G. Mantel tests of isolation-by-distance in the WKMP. **a.** Relationship between Wang's relatedness coefficient (R) and geographic distance. **b.** Relationship between Nei's genetic distance and geographic distance. km, kilometres.

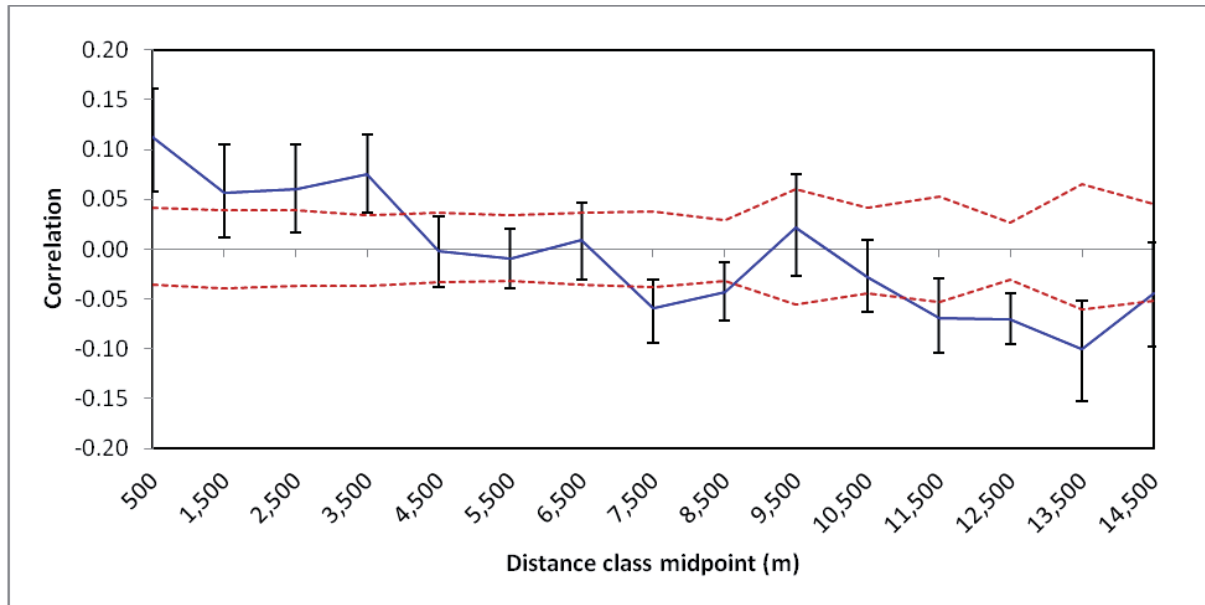
a.



b.



Appendix H. Spatial autocorrelogram for the WKMP. The blue line shows the correlation of average pairwise relatedness (\pm SE) as a function of geographic distance classes in metres (m). Red dotted lines show the 95% confidence interval around a null hypothesis of no spatial structure.



Genetic analysis of regional koala population structure

These Appendices detail additional analyses of regional koala population structure that are most appropriate for detecting genetic patterns arising through the action of long-term (evolutionary) processes over large spatial scales. These analyses are not suitable for discerning aspects of fine-scale population structure within the WKMP that were required to inform the concurrent Population Viability Analysis.

Appendix I presents results of a model-based clustering analysis of the 134 koala samples from the WKMP, areas of the former Big Scrub rainforest (South Lismore, Western and North Lismore) and habitat to the west of Lismore. These populations have been analysed previously using the program STRUCTURE (Lee et al. 2013) which identified the presence of two populations; one occurring to the north of Lismore and extending into south-east Queensland (QLD), the other occurring to the south and west of Lismore. To complement this analysis we employed an alternative program (PARTITION-ML) which implements a maximum likelihood model to discern population structure. In this analysis samples are assigned to populations with the number of populations (k) iterated over the range 1-5 with the resulting likelihoods used to identify the best fit model *via* a Likelihood Ratio test. PARTITION-ML also identified a distinct northern (North Lismore samples) and southern (Wardell, South Lismore, Western and Far-western samples) population. Both studies also found evidence of low level dispersal/immigration between the two regional populations. In our PARTITION-ML analysis eleven (11) koalas sampled to the south of Lismore were assigned to the northern population. Five (5) of these animals were from the Northern Wardell subpopulation, three (3) from the Western population and three (3) from the South Lismore population. No samples from the Southern Wardell subpopulation were identified as being from North Lismore. These results are consistent with our previous analyses that revealed higher genetic diversity in the Northern Wardell subpopulation and F_{st}/R_{st} values that aligned it more closely with the North Lismore population.

Recent studies have shown that programs such as STRUCTURE and PARTITION-ML that employ model-based clustering to identify population structure perform poorly when there is weak genetic differentiation (Putman & Carbone 2014) such as that expected to occur at finer spatial scales. The lower limit of detection for STRUCTURE was found to be F_{st} 0.03, although accurate assignment of individuals to populations requires F_{st} to be 0.05 or above (Latch et al. 2006). Given that our previous analyses show levels of population differentiation ranging from F_{st} 0.027-0.062 (Norman et al. 2015) model-based clustering programs are at

the lower limits for detecting regional population structure in this dataset and are unsuitable for detecting fine-scale population structure within the WKMP.

Appendix J shows results of the AMOVA analysis for the 134 regional koala samples when assigned to (a) two or (b) six populations. In both analyses the among population component of molecular variance was relatively small (6-7%) with most variance attributable to genetic differences within individuals (90%).

Appendix K presents results of an Assignment test conducted in GENALEX which indicate that 5% of koalas were sampled at sites other than their population of origin. This is consistent with results of other analyses that indicate some immigration/dispersal between the North Lismore population and those to the south (WKMP, South Lismore, Western and Far-western).

Appendix L presents the results of a principal component analysis (PCA) of genetic dissimilarity amongst 134 koalas sampled from the WKMP and surrounding areas. There is evidence of weak genetic structure consistent with IBD effects across the region. Neaves et al. (2015) found a similar pattern of weak genetic structuring amongst populations in northern NSW (Wardell and Tyagarah) and south-east Queensland (Coomera) when analysed using PCA and discriminant analysis of principal components (DAPC, in which these three populations collapsed to a single cluster) with strongly demarcated populations only detected at Port Macquarie and Coffs Harbour to the south. Lee et al. (2013) in an earlier study utilising 6 microsatellite loci also found weak genetic structure amongst koala populations from across this region. Weak genetic structure is expected to arise within and between populations when there are restrictions on the level and direction of gene flow (migration-dispersal) across the landscape. Discerning these patterns to inform the concurrent PVA was the main objective of our report (Norman et al. 2015). Strong genetic differentiation of populations is only likely where there are (i) long-term barriers to dispersal with limited or no genetic exchange amongst populations, and (ii) sufficient time has elapsed for random drift, mutation, selection and non-assortative mating to cause detectable changes in the genetic makeup of the populations.

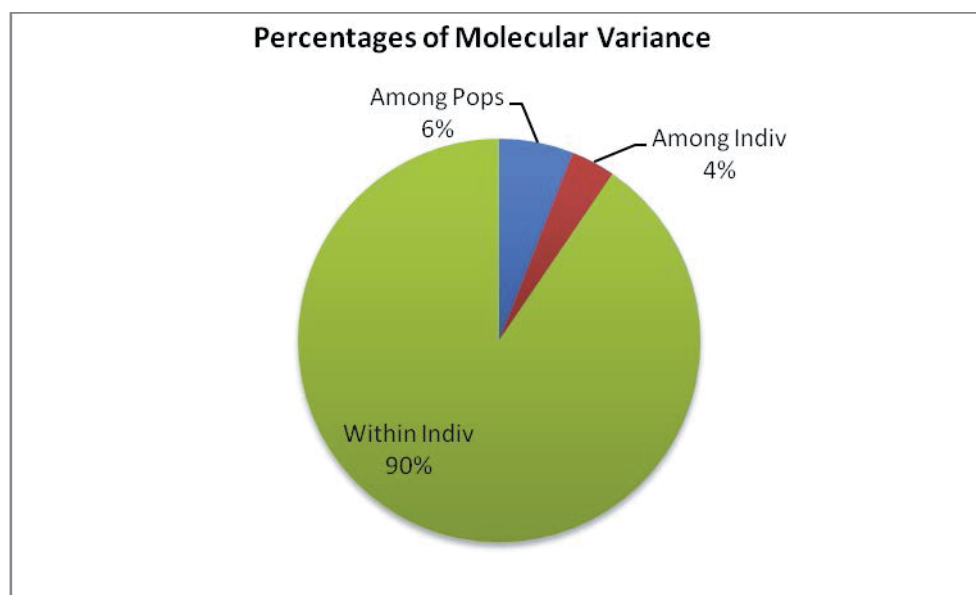
Appendix I. Number of regional populations identified using model-based clustering as implemented by PARTITION-ML. k , number of populations; L, likelihood for the model; df, degrees of freedom; CV, critical value for the chi-squared test (χ^2). Significance was tested using the Likelihood Ratio test for k_n+1 with partitions identified as significant at the $P = 0.05$ level indicated (*).

PARTITION-ML Model Parameters (df = 111, CV = 87.68)		
k	L	χ^2
1-null model	-3892.43	
2	-3833.54	117.78*
3	-3818.39	30.3
4	-3807.20	22.38
5	-3789.24	35.92

Appendix J. AMOVA partitioning of genetic diversity among and within regional koala populations. **a.** Samples partitioned into two populations based on the results of the PARTITION-ML analysis. **b.** Samples partitioned into six regional populations following Norman et al (2015). For this analysis within population diversity was also partitioned into the among and within individual (ind.) components. **c.** Partitioning of molecular variance for the six regional populations shown graphically.

Source	df	SS	MS	Est. Var.	%
a. Regional-2					
Among pops	1	40.16	40.16	0.32	7
Within pops	266	1213.03	4.56	4.56	93
Total	267	1253.19		4.88	100
b. Regional-6					
Among pops	5	85.70	17.14	0.29	6
Within pops	262	1167.50	4.46	4.46	94
(among ind.)	(128)	(592.50)	(4.63)	(0.17)	(4)
(within ind.)	(134)	(575.00)	(4.29)	(4.29)	(90)
Total	267	1253.19		4.74	100

c.

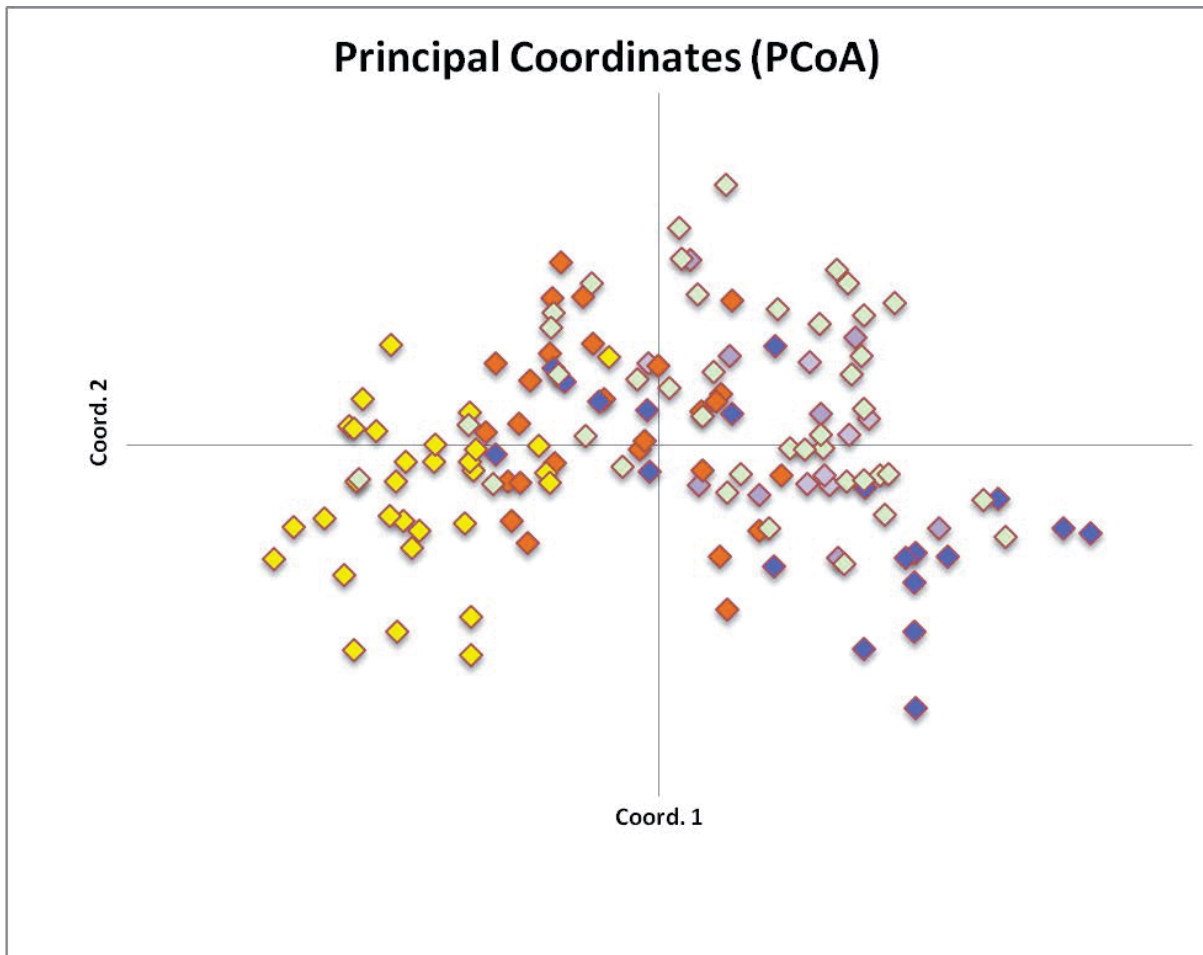


Appendix K. Assignment test for regional koala populations. Samples were assigned to a northern (North Lismore) and southern population (WKMP, South Lismore, Western and Far-western). 95% were detected in their assigned population of origin whereas 5% were sampled outside their assigned population of origin.

Population	Original pop.	Other pop.
Northern	30	1
Southern	97	6
Total	127	7
Percent	95%	5%

Appendix L. Principal component plot of genetic distances between koalas from the WKMP and surrounding regions.

◆ N-Wardell ◆ S-Wardell ◆ West+S-Lismore ◆ Far-western ◆ N-Lismore



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Supplementary Appendices 1

Genetic profiling of koalas: Woolgoolga to Ballina Pacific Highway Upgrade (Section 10–Wardell to Coolgardie)

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October 2015

Executive summary

Supplementary Appendices S1a-S1d detail additional analyses and information requested by NSW Roads and Maritime Services as a supplement to the report *Genetic profiling of koalas: Woolgoolga to Ballina Pacific Highway Upgrade (Section 10-Wardell to Coolgardie)* (Norman et al. 2015) undertaken by Southern Cross University (SCU), and the Appendices to that report. The analyses herein provide genetic estimates of dispersal, estimated as the number of migrants per generation (Nm) calculated using Shannon's Mutual Information Index and assuming symmetrical migration. Nm was calculated for;

- (i) populations of koalas occupying habitat to the east and west of the conditionally-approved Section 10 Pacific Highway upgrade, and
- (ii) between the Wardell Koala Metapopulation (WKMP) and surrounding areas.

Key findings and recommendations are:

The estimated mean migration rate for koalas between habitats to the east and west of the conditionally-approved Section 10 Pacific Highway upgrade ranged from 9.5 to 273 migrants per generation (S1d). Assuming a generation time of 6.02 – 7.8 years it is estimated that between 1.2 and 45 koalas/year migrate between these habitats.

The estimated mean migration rate for koalas between the WKMP and surrounding regional populations ranged from 4.9 to 51 migrants per generation for the Northern population, and 9.9 to 115 for the Southern population (S1d). Assuming a generation time of 6.02 – 7.8 years it is estimated that 0.6 to 8.5 koalas/year migrate between WKMP and the Northern population with migration between WKMP and the Southern population higher at 1.3 to 19.1 koalas/year.

Estimates of Nm presented here are likely to be lower than the number of dispersing koalas as migration rates do not account for transient dispersal by koalas which includes sub-adults seeking to establish new territories, or resident koalas traversing their normal home ranges.

Due to numerous sources of variability associated with the estimation of migration rates we advise caution when using Nm as input for Population Viability Analysis. We recommend that estimated values of Nm presented herein should serve as a guide for the levels of migration to be included in sensitivity analyses for the PVA rather than absolute values of migration.

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Estimation of koala dispersal

These Supplementary Appendices provide estimates of the number of koalas exchanged between regional populations and dispersing across the conditionally-approved Section 10 Pacific Highway upgrade. For the purposes of this report, dispersal is expressed as the number of migrants per generation (Nm) where N is the effective size of each population and m is the proportion of individuals that migrate. Nm was calculated using Shannon's Mutual Information Index (Sherwin et al. 2006, Rossetto et al. 2008) as implemented in GENALEX. This approach aims to estimate the number of koalas that are able to disperse across a boundary and breed on both sides. Nm is likely to be smaller than the total number of dispersing koalas as it does not account for transient animals or those undertaking foraging movements within their home range.

Supplementary Appendix S1a provides estimates of Nm for each of the 14 microsatellite loci and the chi-square significance of the G-test for allele frequency differences at each locus. This analysis assumes an effective population size of 500 for each population, an unrealistic assumption for the WKMP (Appendices to Norman et al. 2015) and regional populations (see S1b below). Thus, for subsequent estimation of migration rates we first calculated effective population sizes (N_e) from the data and used these as input into the analysis.

Supplementary Appendix S1b provides details of N_e estimated for each population using the Linkage Disequilibrium (LD) and Molecular Coancestry (MC) methods implemented in NE ESTIMATOR. We computed N_e for different population partitions to determine how sampling strategies and population assignments might affect subsequent estimates of migration. For the WKMP we partitioned the available samples into those occurring east (E-Wardell) and west (W-Wardell) of the conditionally-approved Section 10 Pacific Highway upgrade. Due to the small number of koalas sampled to the east of the upgrade ($n = 10$), and possible isolation-by-distance effects over larger spatial scales to the west, we chose to calculate N_e for the entire western sample (W-Wardell^b, $n=37$) and a smaller subset (W-Wardell^a, $n=15$) restricted to those animals sampled in forest fragments immediately to the west of the upgrade in the vicinity of Bagotville, Meerschaum Vale and Coolgardie. We also conducted a hierarchical analysis of regional populations with N_e estimated for each of the four (4) regional populations analysed by Norman et al. (2015), a combined Sth. Lismore and Western population, the Southern and Northern population clusters identified in STRUCTURE and PARTITION-ML analyses (Lee et al. 2013, Appendices to Norman et al. 2015) and the combined regional populations (All Regional).

All methods of N_e estimation are subject to various forms of error, bias and assumptions that may be violated by real data. For LD it is recommended that the sample size (n) should be

equal to the estimated N_e for reliable results to be obtained (Russell & Fewster 2009). Over half (0.55) of our populations do not meet this criterion (S1b), including E-Wardell and W-Wardell^a. Nevertheless, estimates of N_e calculated using the LD method were used in subsequent computations to determine koala migration as the MC method assumes samples are taken from a single cohort making this a less appropriate method. Both methods, however, failed to provide reliable estimates of N_e for some populations returning a point estimate or upper confidence interval of infinity. For LD, this occurs when the observed value is less than expected due to sampling error. In this case there is no evidence of disequilibria caused by genetic drift due to sampling a finite number of individuals – it can all be explained by sampling error (Do et al. 2014). For the LD method we also used critical values of 0.01 and 0.02 to define the frequency at which rare alleles were excluded from the analysis. This led to substantial variation in N_e for some populations (S1b) and is an additional source of imprecision. We used LD estimates of N_e using the critical value of 0.01 as input for estimation of Nm .

Supplementary Appendix S1c presents Nm estimates for the WKMP (east and west of the conditionally-approved Pacific Highway upgrade) and surrounding populations. Nm was estimated using empirical values of N_e calculated by the LD method (S1b) as input. As reliable estimates of N_e could not be calculated for E-Wardell and the Northern population we used the lower CI's as input which were not dissimilar to the point estimates of N_e obtained for other populations. As our initial analysis also revealed substantial variation in Nm estimates obtained for individual loci (S1a) we applied three cutoff values for $^S H_{UA}$ (0.0001, 0.001 and 0.01) to provide an objective criteria for eliminating anomalous values. Loci exhibiting heterozygote deficiencies or genetic disequilibrium were retained in the analysis as we have previously shown that these were due to population-specific processes and not general features of those loci (Appendices to Norman et al 2015). Additionally, loci exhibiting heterozygote deficits or disequilibria accounted for only a few of the anomalous Nm values determined using the $^S H_{UA}$ cutoff of 0.01 suggesting that their effect on estimation of Nm was negligible.

Our analyses show that N_e , sampling design, the assignment of population partitions and choice of $^S H_{UA}$ can substantially influence estimates of Nm . For the WKMP, estimates of koala dispersal across the conditionally-approved Section 10 Pacific Highway upgrade ranged from a minimum estimate of 9.5 to a maximum of 273 with a three-fold difference in Nm observed between the two W-Wardell partitions (S1c). Given that the WKMP is estimated to comprise ~200 koalas the upper Nm value of 273 calculated using a $^S H_{UA}$ cutoff of 0.0001 appears to overestimate dispersal. Given these factors, and other sources of imprecision in the estimation of Nm , it is recommended that sensitivity analyses employing a

range of ecologically plausible Nm values be conducted to determine the effect of koala dispersal on the viability of the WKMP. For the purposes of the PVA, our estimates of Nm were converted to migrants/year assuming a generation time in koalas of 6.02 or 7.8 years (Phillips 2000) (S1d).

Despite the difficulties inherent in estimating Nm the results presented here are consistent with the levels and patterns of dispersal we inferred from the spatial distribution of First-Order Relatives and other analyses (Norman et al. 2015). This includes isolation-by-distance effects, high levels of migration between WKMP and populations to the west (South Lismore and Western), and low but detectable migration between WKMP and the Northern population.

Appendix S1a. Migrants per generation ($Nm \pm SE$) calculated for each of the 14 loci assuming an effective population size >500 for each population. A cutoff value of 0.0001 was used for $^5H_{UA}$ and a log base=2 transformation applied for diploids. W-Wardell^a is restricted to those koalas sampled from forest fragments immediately to the west of the conditionally approved Pacific Highway upgrade (n=15). W-Wardell^b includes all koalas sampled to the west of the upgrade (n=37) including those sampled to the north and northwest in areas of the former Big Scrub rainforest. All other populations are as defined in Norman et al. (2015) and Appendices. *, significance of G-test for allele frequency differences calculated for $^5H_{UA}$ where $P \leq 0.05$.

Pop1	Pop2	K10.1	K2.1	Pcv2	Pcv25.1	Pcv25.2	Pcv30	Pcv31	Pcv6.1	Phc11	Phc13	Phc25	Phc15	Phc5	Phc9	$Nm \pm SE$
E-Wardell	W-Wardell ^a	1.53	62.10	6.93	361	866	28.25	2.32*	5.19	1.47	4.52	10.77	1.96*	2.73	-	104 ± 69
E-Wardell	W-Wardell ^b	3.36	74.61	9.38	834	1632	10.4	9.83	31.14	2.75	6.34	9.64	8.96	6.91	543	227 ± 127
<i>Nm between Wardell and Southern populations</i>																
Wardell	Sth Lismore	2.97	1.45*	12.54	4.25*	853	5.09*	98.66	0.81*	1.30*	3.10*	0.52*	21.03	6.03	2954	283 ± 214
Wardell	Western	2.14*	6.67	7.70	39.51	156	6.18*	136	0.83*	2.13*	1.34*	0.97*	34.29	2.74*	60.57	33 ± 14
Wardell	Far-western	2.76	0.45*	1.07*	4.42*	536	5.81*	9.96*	0.26*	1.73*	2.52*	0.84*	13.33	1.18*	15.99	43 ± 38
Wardell	S.Lis+West	2.35*	3.65*	10.92	11.29*	221	3.69*	339	0.66*	1.66*	1.82*	0.66*	25.75	5.67*	1762	171 ± 125
Wardell	All Southern	6.06	2.16*	5.07*	13.86*	591	3.21*	199	0.47*	1.72*	3.29*	0.69*	27.15	5.36*	207	76 ± 44
<i>Nm between Wardell and the Northern population</i>																
Wardell	Northern	0.76*	0.89*	1.52*	1.57*	183	2.92*	2.59*	0.24*	3.79*	3.35*	0.11*	1.32*	1.52*	9.01*	15 ± 13
<i>Nm between Wardell and the combined regional populations</i>																
Wardell	Regional	5.86*	3.30*	8.03*	6.96*	1434	8.56*	232	0.51*	6.95*	10.98*	0.54*	31.77*	74.45	141	140 ± 101

Appendix S1b. Effective population size (N_e) for koalas in the WKMP and surrounding areas. N_e calculated using the linkage disequilibrium (LD) and molecular coancestry (MC) methods with 95% confidence intervals (CI). N, sample size. *, denotes sample sizes that are within 10% of the estimated N_e . LD critical values for exclusion of rare alleles in brackets. inf, infinity.

Population	n	LD (0.02)	95% CI	LD (0.01)	95% CI	MC	95% CI
Population partitions and N_e for Wardell							
E-Wardell	10	148.0	20.3-inf.	148.0	20.3-inf.	inf.	inf.
W-Wardell ^a	15	21.8	14.9-35.3	21.8	14.9-35.3	inf.	inf.
W-Wardell ^b	37*	37.9	28.8-52.5	20.6	17.1-25.0	12.7	4.4-25.3
Wardell	47*	47.4	36.8-63.6	24.4	20.8-28.9	9.6	5-15.7
Population partitions and N_e for regional populations							
S Lismore	19	43.6	24.9-123.0	43.6	24.9-123.0	19.8	0-99.5
Western	23*	19.0	13.7-28.2	19.0	13.7-28.2	2.5	1.4-3.8
S Lis.+Western	42*	73.9	48.8-134.7	41.5	32.4-55.4	4.0	1.8-7.0
Far-western	16	28.6	15.3-92.8	28.6	15.3-92.8	9.0	3.3-17.4
Southern	57	115	74.6-221.8	79.2	58.7-115.6	4.2	2.4-6.4
Northern	31	inf.	193.4- inf.	271.6	98.9- inf.	inf.	inf.
All Regional	88*	49.7	42.5-58.8	67	56.8-80.3	27.2	7.4-59.7

Appendix S1c. Migrants per generation (Nm) calculated for each of the 14 loci using empirical estimates of effective population size (N_e) calculated by the LD method. Cutoff values for ${}^5H_{UA}$ used in the estimation of Nm were set at 0.0001^A (dash), 0.001^B and 0.01^C , with those loci falling below this value omitted from the calculation of mean Nm . W-Wardell^B is restricted to those koalas sampled from forest fragments immediately to the west of the conditionally approved Pacific Highway upgrade ($n=15$). W-Wardell^B includes all koalas sampled to the west of the upgrade ($n=37$) including those sampled to the north and northwest in areas of the former Big Scrub rainforest. All other populations are as defined in Norman et al. (2015) and Appendices.

Pop1	Pop2	K10.1	K2.1	Pcv2	Pcv25	Pcv25	Pcv30	Pcv31	Pcv6.	Phc1	Phc1	Phc1	Phc2	Phc1	Phc5	Phci9	^A $Nm \pm SE$	^B $Nm \pm SE$	^C $Nm \pm SE$
<i>Nm</i> between east (E-) and west (W-) Wardell																			
E-Wardell	W-Wardell ^A	1.27	47.33	4.72	287 ^C	675 ^C	28.64	1.57	4.97	1.01	4.14	6.77	1.63	2.02	-	82 ± 54	82 ± 54	9.5 ± 4.5	
E-Wardell	W-Wardell ^B	4.15	152	13.38	2085 ^C	909 ^C	20.75	16.75	60.87	2.82	10.04	7.30	27.40	12.64	505 ^C	273 ± 156	273 ± 156	29.8 ± 13.1	
<i>Nm</i> between Wardell and Southern populations																			
Wardell	Sth Lismore	1.72	1.40	10.65	2.54	430 ^C	2.54	69.42	0.72	0.83	2.32	0.41	19.24	3.81	2755 ^C	235 ± 196	235 ± 196	9.6 ± 5.7	
Wardell	Western	1.50	5.37	5.67	27.29	110	4.01	101	0.63	1.55	0.99	0.74	25.39	2.01	45.04	23.7 ± 9.9	23.7 ± 9.9	23.7 ± 9.9	
Wardell	Farwestern	1.31	0.26	0.38	2.55	239	2.38	5.37	0.18	0.70	1.11	0.49	4.98	0.51	8.91	19.2 ± 16.9	19.2 ± 16.9	19.2 ± 16.9	
Wardell	S.Lis+West	2.25	4.67	12.18	11.36	210	3.25	382 ^C	0.74	1.72	1.98	0.74	29.22	5.41	2027 ^C	192 ± 144	192 ± 144	23.6 ± 17.1	
Wardell	Southern	8.78	4.71	8.82	20.35	704 ^C	3.95	390 ^C	0.88	2.55	5.43	1.21	44.99	7.26	410 ^C	115 ± 59	115 ± 59	9.9 ± 3.7	
<i>Nm</i> between Wardell and the Northern population																			
Wardell	Northern	1.30	1.97	2.75	3.26	645 ^C	5.93	4.31	0.47	5.46	6.87	0.32	3.59	3.67	23.21	51 ± 46	51 ± 46	4.9 ± 1.6	
<i>Nm</i> between Wardell and the combined regional populations																			
Wardell	Regional	7.40	4.71	10.67	8.83	2088 ^C	11.06	326 ^C	0.69	8.22	14.26	0.74	47.06	103	199	202 ± 147	202 ± 147	34.6 ± 17.1	

Appendix S1d. Estimated dispersal between koala populations. Point estimates of N_e (N_e -Pop1/ N_e -Pop2) calculated using the LD method were used as input except those values marked with an asterisk (*) which could not be reliably estimated and the lower CI was used. Mean N_m is given as migrants per generation calculated using $^5H_{UA}$ cutoff values of 0.0001 and 0.001. Migrants/generation is converted to migrants/year assuming a generation time for koalas of 6.02 or 7.8 years.

Pop1	Pop2	N_e	$N_m \pm SE$ ($^5H_{UA}$ cutoff 0.0001)			$N_m \pm SE$ ($^5H_{UA}$ cutoff 0.01)		
			(per gen.)	(6.02 y/gen.)	(7.8 y/gen.)	(per gen.)	(6.02y/gen.)	(7.8 y/gen.)
Mean N_m between east (E-) and west (W-) Wardell								
E-Wardell	W-Wardell ^a	20*/22	82 ± 54	13.6 ± 9	10.5 ± 6.9	9.5 ± 4.5	1.6 ± 0.7	1.2 ± 0.6
E-Wardell	W-Wardell ^b	20*/21	273 ± 156	45 ± 26	35 ± 20	29.8 ± 13.1	5 ± 2.2	3.8 ± 1.7
Mean N_m between Wardell and Southern populations								
Wardell	S-Lismore	24/43	235 ± 196	39 ± 32	30 ± 25	9.6 ± 5.7	1.6 ± 0.9	1.2 ± 0.7
Wardell	Western	24/19	23.7 ± 9.9	3.9 ± 1.6	3.0 ± 1.3	23.7 ± 9.9	3.9 ± 1.6	3.0 ± 1.3
Wardell	Far-western	24/29	19.2 ± 16.9	3.2 ± 2.8	2.5 ± 2.2	19.2 ± 16.9	3.2 ± 2.8	2.5 ± 2.2
Wardell	S-Lismore+Western	24/42	192 ± 144	32 ± 24	24.6 ± 18.5	23.6 ± 17.1	3.9 ± 2.8	3.0 ± 2.2
Wardell	Southern	24/79	115 ± 59	19.1 ± 9.8	14.7 ± 7.6	9.9 ± 3.7	1.6 ± 0.6	1.3 ± 0.5
Mean N_m between Wardell and the Northern population								
Wardell	Northern	24/99*	51 ± 46	8.5 ± 7.6	6.5 ± 5.9	4.9 ± 1.6	0.8 ± 0.3	0.6 ± 0.2
Mean N_m between Wardell and the combined regional populations								
Wardell	All regional	24/67	202 ± 147	34 ± 24	26 ± 19	34.6 ± 17.1	5.7 ± 2.8	4.4 ± 2.2

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PEER REVIEW OF TWO REPORTS:

"AMRI"

"Koala population genetics management. A report to the Roads and Maritime Service (RMS)" 12th August 2015. Linda E. Neaves, Siobhan B. Dennison,, Greta J. Frankham, Mark D. B. Eldridge and Rebecca N. Johnson. Australian Museum Research Institute

and

"SCU"

"Genetic profiling of koalas: Woolgoolga to Ballina Pacific Highway Upgrade (Section 10-Wardell to Coolgardie)". August 2015. Dr J.A. Norman, Dr C. Blackmore, Assoc. Prof. R. Goldingay & Prof L. Christidis. Southern Cross University.

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SUMMARY and RECOMMENDATIONS

In the Wardell area of northern NSW, a planned upgrade to the Pacific Highway passes through an area inhabited by koalas. NSW Roads and Maritime Services wishes to assess the possible impact of the upgrade on the koala population, and have commissioned two genetic reports: AMRI (by Australian Museum Research Institute) and SCU (by Southern Cross University).

The question “Could the upgrade disrupt koala dispersal sufficiently to reduce the viability of the koala population?” is expressed as a series of deliverables, plus the apparent intention to follow up with a Population Viability Analysis (PVA). I presume that PVA will be used comparatively, examining chance of population persistence over multiple generations, with various levels of dispersal between sub-populations. Such comparative use of PVA is called “sensitivity analysis”.

To set the baseline for such a PVA, genetic methods can be used to assess the natural amount of dispersal between areas. The margins of areas to be investigated can be set for various reasons including habitat suitability or human alteration (eg the highway upgrade).

AMRI and SCU each used a number of different methods to assess genetic subdivision, without converting them to dispersal estimates. It is currently not possible to make a direct comparison between the results of AMRI and SCU at Wardell, because there is insufficient corresponding geographic information. This should be clarified with detailed geographic information for each individual at Wardell and immediate surrounds. However, in both studies, there was a general pattern of increasing genetic similarity at decreasing separation (“Isolation by Distance”), but there were no clear boundaries where one locality was genetically isolated from another.

But what is the dispersal across these boundaries? In each report only one method specifically targeted dispersal, by identifying particular koalas who might have dispersed in the most recent generation: those that were genetically assigned to a location other than the one in which they were sampled (AMRI); or those with first-order relatives in a location other than the one in which they were sampled (SCU). Only a small number of individuals were identified as potential dispersers, but it is worth noting that only small numbers are needed to halt genetic differentiation by chance processes in transmission. Thus the areas within Wardell, appear to be important for mutual support of each other, and adjacent areas.

Both studies indicated that dispersal was relatively high both between subpopulations within Wardell, and between Wardell and adjacent areas. This dispersal probably does two things: opposes loss of genetic variation within subpopulations, and forestalls immediate extinction. Therefore, a precautionary conservation management approach would be to avoid any reduction of the measured level of dispersal, pending results of the PVA-sensitivity analysis.

There are two limitations to the use of AMRI’s and SCU’s genetic dispersal estimates as base-line dispersal for the PVA-sensitivity analysis. The first limitation is that sample sizes are unavoidably small, so that the estimates are subject to high error rates. This is compounded by the fact that these particular analyses only tell us about dispersal in the most recent generation. AMRI and SCU were correct to avoid methods that convert their genetic subdivision estimates into dispersal rates, because this has been criticised on various grounds.

However, there exists a Mutual Information method that avoids the problems that beset other genetic dispersal measures, and can deal with the widest possible range of population sizes and dispersal rates. The data from AMRI and SCU should be used in this way, to produce dispersal assessments as a baseline in the PVA-sensitivity analysis, to investigate how the koala population’s viability might be affected if the Pacific Highway upgrade reduces dispersal below this baseline.

AMRI and SCU also produce estimates of Genetic variation within subpopulations, which can be included in some PVA programs.

Finally, in commissioning the analysis of sensitivity of population extinction to altered dispersal, I encourage the Roads and Maritime Services to require information on not only the most likely outcome, but also the worst-case outcome, to facilitate precautionary management.

GENERAL COMMENTS

In the Wardell area of northern NSW, a planned upgrade to the Pacific Highway passes through an area inhabited by koalas. NSW Roads and Maritime Services wishes to assess the possible impact of the upgrade on the koala population, and have commissioned two genetic reports: AMRI (by Australian Museum Research Institute) and SCU (by Southern Cross University).

The scope of the two reports appears to be slightly different. The question “Could the upgrade disrupt koala dispersal sufficiently to reduce the viability of the koala population?” is expressed as deliverables that are listed by AMRI and SCU. These deliverables are abbreviated as follows.

1. CONNECTIVITY WITHIN WARDELL. AMRI 1: Analyses of population structure and gene flow within the focal area. Also SCU 1: Is the Wardell KMP spatially structured?
2. CONNECTIVITY BETWEEN WARDELL AND SURROUNDS. AMRI 2: Determine whether the focal population appears to be connected to populations in the surrounding area. Also SCU 2: Is the Wardell KMP an important source population for surrounding areas?
3. WITHIN-LOCALITY GENETIC VARIATION. AMRI 3. Allele frequency information for the population as a whole (and for each subpopulation if relevant).
4. VALIDATION. AMRI 4. Summary of procedures undertaken for data validation.

The SCU report indicated the intention to follow these reports with a formal Population Viability Analysis (PVA). I presume that PVA will be used comparatively, examining chance of population persistence over multiple generations, with various levels of dispersal between sub-populations. Such comparative use of PVA is called “sensitivity analysis” (Penn *et al.* 2000; Reed 2009).

To set the baseline for such a PVA, genetic methods can be used to assess the natural amount of dispersal between areas. The margins of areas to be investigated in this way might be set for various reasons including habitat suitability, human alteration (eg the highway upgrade), or intrinsic differences such as genetic incompatibilities (the latter are unlikely in the case of koalas, Sherwin *et al.* 2000). In these reports, the AMRI and SCU are asked to assess dispersal between areas within Wardell KMP (deliverable 1), and between Wardell KMP and adjacent areas (deliverable 2).

The spatial arrangement of sampling within Wardell was evident in the SCU study, but not in the AMRI study – perhaps AMRI did not have access to detailed location data.

There are many different methods that use genetic data to estimate average levels of dispersal between areas for a PVA. The AMRI and SCU reports each use a number of different methods to assess genetic subdivision, most of which were not converted to dispersal estimates. In both studies, there was a general pattern of increasing genetic similarity at decreasing separation (“Isolation by Distance”), but there were no clear boundaries where one locality was genetically isolated from another.

In each report only one method specifically targeted dispersal, by identifying some individuals who might have dispersed in the most recent generation: koalas that were genetically assigned to a location other than the one in which they were sampled (AMRI); or koalas with first-order relatives in a location other than the one in which they were sampled (SCU).

Both studies indicated that dispersal was relatively high both between subpopulations within Wardell, and between Wardell and adjacent areas. As the authors point out, this dispersal probably does two things: opposes loss of genetic variation within subpopulations (thus potentially aiding future adaptability, Frankham *et al.* 2010), and forestalls immediate extinction, which is a risk in small isolated populations, including koalas (Lunney *et al.* 2002).

Therefore, a precautionary conservation management approach would be to avoid any reduction of the measured level of dispersal, unless there had been assessment of the potential effects of such reduction. However, if PVA-sensitivity analysis shows that a certain reduction of dispersal (x%) is not

likely to significantly affect population viability, then it would be reasonable to implement particular management options, if it could be reliably demonstrated that these options would reduce dispersal by no more than x%.

SCU recommends that the genetic measures of dispersal be incorporated into the future PVA. There are two limitations to the use of AMRI's and SCU's genetic dispersal estimates as base-line dispersal for the PVA-sensitivity analysis. The first limitation is that sample sizes are unavoidably small, so that the estimates are subject to high error rates. This is compounded by the fact that these particular analyses only tell us about dispersal in the most recent generation. AMRI and SCU were correct to avoid methods that purport to assess dispersal over tens of generations, but have been criticised on various grounds.

However, there exists a Mutual Information method that can successfully assess dispersal over tens of generations, from genetic data (Sherwin 2006, 2010, Dewar et al 2011, Chao et al 2015). This method avoids the problems that beset other genetic diversity and dispersal measures, and, unlike the other methods, can deal with the widest possible range of population sizes (10 upwards) and dispersal rates (from one in a thousand, to one-third of the population, per generation; Sherwin 2006). All the data from AMRI and SCU should be analysed by the Mutual Information method, to produce robust multigeneration dispersal assessments; the Mutual Information calculations can be carried out in the freeware GENALEX (<http://biology.anu.edu.au/GenALEX>) which was used for other tasks by AMRI. The GENALEX website also contains a guide for conversion of Mutual Information for microsatellites to a dispersal estimate. For the mitochondrial DNA, such a conversion could be achieved by following either Dewar (2011, equation 8) for each variable site, or Chao et al. (2015 supplement equations B5-7) for entire haplotypes.

These dispersal assessments from Mutual Information could then be used as baseline in the PVA-sensitivity analysis to investigate how the viability of the koala population might be affected if the upgrade of the Pacific Highway reduces dispersal below this baseline level. The PVA-sensitivity analysis should assess the effect of the highway upgrade, including any measures likely to increase or decrease the road corridor's permeability to koalas, such as fences or overpasses.

The other data that can be included in the PVA is AMRI's and SCU's estimates of genetic variation within subpopulations, which can be included in some PVA programs. Its inclusion will add to realism, especially allowing assessment of when the genetic diversity at Wardell might fall below the lowest levels listed in populations of koalas and other species, reviewed by AMRI.

Finally, in commissioning the analysis of sensitivity of population extinction to altered dispersal, I encourage the Roads and Maritime Services to require information on not only the most likely outcome, but also the worst-case outcome, to facilitate precautionary management.

DETAILED COMMENT – AMRI REPORT

SAMPLING THE LOCALITIES AND INDIVIDUALS:

From Wardell there were 38 samples for microsatellite and mitochondrial DNA. The lack of identification of position for East and West Wardell makes it difficult to assess their importance relative to the proposed highway upgrade, and to compare this to the SCU report, which uses other nomenclature. Also the tiny number of samples from East makes any conclusions weak.

Four other sites near the NSW/Queensland border (Macquarie, Coffs Harbour, Tyagarah and Coomera) were sampled for both microsatellite and mitochondrial DNA, plus a sample set from the whole koala range for mitochondrial DNA only.

THE GENES USED AND THEIR VALIDATION – DELIVERABLE 4.

1. Microsatellites – biparental inheritance

The analysis was based on a good number of genes -15, and appeared suitable for the tasks of determining variability within location, and differentiation and dispersal between locations. The genes were checked carefully. Two other microsatellite genes were excluded for good reasons, and 10% of individuals were independently re-genotyped. The probability of two individuals having the same microsatellite profile was low (10^{-15}), showing that a good battery of genes had been analysed. Variants at the 15 genes appeared to be inherited independently, so that each provided useful information for the analysis (no “linkage disequilibrium”). In most cases there was no evidence of non-random mating within site (ie, there were few cases of genes out of “HWE”).

2. Mitochondrial DNA – maternal inheritance

An 800 bp portion of the mitochondrial DNA control region was sequenced, and appeared suitable for the tasks of determining variability within location, and differentiation and dispersal between locations.

WITHIN-LOCALITY GENETIC VARIATION – DELIVERABLE 3.

Microsatellite diversity within locations was summarised by a suitable array of measures: allelic diversity, allelic richness, private alleles (AP on page 6, called Pa later in Table 1) expected and observed heterozygosity, Hardy-Weinberg equilibrium (HWE–Fis Table 1) and linkage disequilibrium. These measures were not out of the ordinary for koalas (Table 1).

For mitochondrial DNA, within-locality variation was assessed by suitable statistics - haplotypic diversity and nucleotide diversity - within Wardell and the four other main sites. Wardell values were not out of the usual for koalas, although 37 out of 38 individuals had the same mitochondrial genotype (haplotype) at Wardell.

CONNECTIVITY WITHIN WARDELL _DELIVERABLE 1.

And

CONNECTIVITY BETWEEN WARDELL AND SURROUNDING AREAS – DELIVERABLE 2.

1. Microsatellites – biparental

Microsatellite geographic structure was assessed by a number of suitable methods: STRUCTURE, DAPC, F-statistics, AMOVA, Isolation-by-distance tests in Mantel, Spatial autocorrelation of pairwise relatedness in GENALEX 6.5. Many analyses were presented without saying which type of gene (microsatellite or mitochondrial) they were based upon; I believe that in all such cases, they were microsatellites.

The authors avoided specifying definitive management units, which I consider to be wise given the relatively low differentiation indicated by most measures. There was only one genetic cluster at Wardell (deliverable 1), and gradually increasing differentiation with distance from Wardell, but no sharp breaks, a pattern called “Isolation by Distance” (deliverable 2).

As well as the DAPC, there was also a PCA presented on P11 (Fig 3), but not described in the methods section. This appears to be an analysis of microsatellite data, though that is not stated. The PCA showed that the five koalas from “East of focal area” were scattered amongst those from the west of focal area. The text states that the data in Fig 3 come from within Wardell. Thus “East of focal area” appears to mean the East part of Wardell itself, rather than an area to the east of the Wardell area, which would be the interpretation in other parts of the document, where the whole of Wardell appears to be referred to as the “focal area”. Perhaps for the purposes of the PCA, the “focal area” means the proposed upgraded highway. If that interpretation is correct, then there appears to be no justification, at least with this small sample, for considering the koalas on either side of the proposed upgraded highway to be members of distinct separate populations. This should be clarified with detailed geographic information for each individual, so that there could be direct comparison with the results of SCU, which is currently not possible.

There were only two exceptions to the pattern of low differentiation, but I would not prioritise these two findings over the general consensus that there is little geographic differentiation within Wardell or between Wardell and other populations). F_{st} and Φ_{st} did show significant departures from zero, but there are many criticisms of F_{st} , and only partial fixes for these criticisms (Sherwin 2010, Wang 2015). Φ_{st} likely suffers from many of the same problems as F_{st} , because Φ_{st} is also a variance partition and an “order 2” diversity measure (Hill, 1973), the two characteristics that are at the root of F_{st} 's many problems.

Of course, low differentiation may be due to high dispersal, and some of these measures (including F_{st}) can be converted to measures of dispersal, but the authors wisely did not do so, given the criticisms mentioned already.

However, there was one assessment of dispersal in and out of Wardell by microsatellites. Microsatellite DNA is biparentally inherited, so it traces dispersal of both sexes. Microsatellites were used to assess dispersal by identifying some individuals who might have dispersed in the most recent generation: koalas that were genetically assigned to a location other than the one in which they were sampled. The assignment test used was in GENALEX 6.5. It showed that some individuals were likely to have moved between Wardell and nearby localities such as Coffs Harbour, but the authors noted that the conclusions were hampered by a lack of samples from localities immediately adjacent to Wardell. Only a small number of individuals were identified as potential dispersers, but it is worth noting that only small numbers are needed to halt genetic differentiation by chance processes in transmission (Kimura and Crow, 1970).

2. Mitochondrial DNA – female dispersal

Mitochondrial DNA generally confirmed the results of the microsatellite analysis, but indicated slightly reduced dispersal of females, relative to males

Mitochondrial DNA is maternally inherited, so traces female dispersal. Mitochondrial geographic structure was assessed by suitable methods: AMOVA/ Φ_{ST} versus distance, and a haplotype network. The AMOVA showed that 92% of mitochondrial variation was within locations. This contrasts with 75% of biparentally-inherited microsatellite variation being within locations, suggesting limited female dispersal. However, Wardell mitochondrial haplotypes do occur elsewhere, so that there must be some female dispersal. Also, mitochondrial DNA suggested that one individual was an immigrant to the Wardell area.

DETAILED COMMENT – SCU REPORT

SAMPLING THE LOCALITIES AND INDIVIDUALS:

Tables 1 and 5 show 47 samples sourced from the Wardell KMP plus two adjacent localities to the north: Lynwood and Dalwood. This group of samples will be collectively referred to as “Wardell” throughout this assessment. There were also an additional 88 koalas outside Wardell, whose locality information was somewhat scattered in the document, but from Figure 4 it seems that there were three sample sets from localities immediately to the west of Wardell, named from north to south as 30 koalas from “North Lismore”, 20 from “South Lismore” and 22 from “Western”. There were also 16 other koalas from further to the west of Wardell (“Far-western”).

THE GENES USED AND THEIR VALIDATION – DELIVERABLE 4 (not listed as such by SCU).

The SCU analysis used only microsatellite genes - fourteen of them, and adequate number. It is not clear if this set of genes overlaps the set of genes analysed by AMRI. The variation at these genes was sufficient to give a 99% chance that a non-parent would be excluded as a potential parent. Microsatellite analysis of one known parent-offspring pair gave a relatedness estimate of approximately 0.5 (the correct value for such a pair. These values give confidence to the subsequent work assigning first-order relatives (FOR – parent-offspring or full-sibling). It was stated that these microsatellites are “able to detect the presence of genetic differentiation amongst populations with a power of 0.975 or higher after 10 generations and assuming an effective population size of 50-200”. It was not explained how this power analysis was carried out.

WITHIN-LOCALITY GENETIC VARIATION – DELIVERABLE 3 (not listed as such by SCU).

Microsatellite diversity within-locations was not unusual for koalas (Table 5). In the north part of Wardell, there was marginally higher genetic variation than in the south (Table 5, with no confidence limits, so the significance of the difference cannot be evaluated). In the north part of Wardell, there was also lower mean relatedness (Table 3). If real, these two differences could indicate that the north has larger population size, or that it receives more immigration from elsewhere.

CONNECTIVITY WITHIN WARDELL _DELIVERABLE 1.

Genetic Subdivision

There appears to be mild genetic substructure within the Wardell area, but no complete isolation.

On Page 10 it is stated that “Genetic neighbourhood size in the Wardell KMP was estimated to be 21-30 Km². This confirms a pattern of limited dispersal across the study area and the likely presence of multiple subpopulations.” A genetic neighbourhood is the size of an area within which mating appears to be random. Fig 1 indicates that the Wardell area is about 6km x20km, so that multiple neighbourhoods a few km across could indeed fit into the Wardell area. However, note that neighbourhood calculations are based on the idea that the population is continuous over a much larger scale than the neighbourhood, so they give no indication of sharp boundaries – indeed they assume that no such boundaries exist.

Sharp boundaries were also not supported by the FOR analysis, which suggested that connectivity between localities within Wardell is greatest between the closest localities, and decreases with distance (called “Isolation by Distance” page 10, paragraph 2).

Fst and a related quantity Rst suggest subdivision within Wardell. These measures of genetic differentiation are relatively high between North and South Wardell, compared to their values for differentiation between Wardell and the two closest localities (South Lismore and Western) west. However, these values are presented with neither significance testing, nor confidence limits. I suspect that the latter would be so wide that the comparison is meaningless - Fst has poor statistical properties, as discussed above.

On page 12 it is stated that “We also reject a model in which the Wardell KMP is divided into an eastern and western subpopulation corresponding to the two large tracts of remnant schlerophyll woodland and forest”. It should be clarified why this model was rejected, and where on the map are

the two large remnant tracts. It is also not clear how to compare this result to AMRI's "East" and "West", but if the division is the same in the two reports, then AMRI's finding in their PCA would confirm the SCU assertion. However, the correspondence cannot be known until the geographic and genetic data for both studies are plotted on a single map.

Genetic Estimates of Dispersal

Only one method specifically targeted dispersal, by identifying some individuals who might have dispersed in the most recent generation: koalas with first-order relatives (FOR) in a location other than the one in which they were sampled. Fig 3 showed that first-order relative pairs were found to be shared between most parts of Wardell, though decreasingly so at greater distances. Most pertinently for the purpose of the study, on pages ii and 10 it is stated that the FOR analysis confirmed that dispersal occurs across the proposed highway upgrade, at two places: Bagotville in the south, as well as in the north.

CONNECTIVITY BETWEEN WARDELL AND SURROUNDING AREAS – DELIVERABLE 2.

Similarly to the pattern within Wardell, there is some evidence that localities more distant to Wardell are more genetically differentiated from Wardell (Table 4, again without significance testing or confidence limits). This is also reflected in the pattern of inferred dispersal events (Fig 4).

The authors also suggest that dispersal into the northern Wardell area is indicated by its relatively high levels of genetic variation (Table 5), however, as discussed above, the difference is marginal and has no confidence limits to allow assessment of its significance.

It was asserted several times that dispersal was asymmetrical, but few data were available to confirm this. There are programs such as MIGRATE that can attempt to fit models of asymmetric dispersal to genetic data, but I suspect that these programs would fail to converge, due to lack of data. With the existing smaller dataset, it might be possible to infer directionality of dispersal from the FOR data, if (1) there are data on ages of members of each FOR pair, (2) it is assumed that the younger member of the pair is an offspring, and (3) it is assumed that offspring are more likely to disperse. These assumptions mean that such an analysis might have only dubious value.

OTHER

Page 17 talks of "the potential significance of the Southern subpopulation of the Wardell KMP as the remaining relatively pure gene pool for koalas in this region". It is not clear how genetic purity is defined, nor is it explained why genetic purity is needed. Generally, the opposite - higher genetic variability - is good for conservation management (Frankham et al 2010), unless there are problems of genetic incompatibility between different races of koalas, which no-one has ever suggested, to my knowledge.

Page 17 says that the south part of Wardell is more like a "functional koala metapopulation" than the north. There are two definitions of "metapopulation" (Levins 1969, Hanski 1999), either of which could probably apply to both north and south Wardell. I recommend that this term should not be used without further explanation.

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Assessment of Koala genetics reports for NSW Roads & Maritime services report.

The two reports, Neaves et al. and Norman et al. each report on genetic diversity and structure of koala populations within the Wardell koala metapopulation and in relation to other regional populations. Both generate and analyse microsatellite datasets for a set of samples provided by RMS, but that is about where the congruence across project ends.

I am satisfied by the technical quality of each analysis. Norman et al. use published microsatellite loci (which ones, ref?), whereas Neaves et al. appear to have generated new marker loci. Neaves et al. use technical replicates and explicitly mention use of positive and negative PCR controls to directly assess consistency and potential for cross-contamination. No mention of this is made in the Norman et al. report. That said, based on prior experience, I do trust the quality of data provided both groups.

The scale of sampling differs between the two groups. Neaves et al include just the 34 RMS samples from the Wardell KMP, whereas Norman et al. supplement these with other samples from this region. At broader scale, Neaves et al. compare Wardell with more geographically distant populations, whereas Norman et al. have finer-scale sampling across the NE NSW region. Based on the information provided by Norman et al., most samples provided are from north or west of the proposed highway upgrade – there are very few from east of the Highway. The corollary is that the power to directly model the potential impact of the road works is rather limited.

The analytical methods differ across studies, as do their conclusions. In all cases, the methods are applied appropriately. Both studies find relatively high genetic diversity in the Wardell KMP, but superficially they come to different findings about structuring and dispersal within the region. Neaves et al. focus on summary statistics (F_{st} ; spatial autocorrelation), clustering (PCA, STRUCTURE) and assignment methods and conclude that there is little evidence for substructure within Wardell and at the larger regional scale (to SE Qld.). By contrast, Norman et al. focus on relatedness estimates, especially distances among inferred First-order relatives as a surrogate for dispersal. They conclude that there is local structuring, with a local genetic neighbourhood size of $\sim 30 \text{ km}^2$ but, paradoxically, also infer a high rate of dispersal to nearby regional populations around kismore.

These marked differences reflect the different forms of analysis, as well as scales of sampling. The Neaves et al. approach will be strongly influenced by long-term average metapopulation dynamics, possibly including colonization and density changes accompanying anthropogenic changes to habitat structure across the region (as discussed in Norman et al.). By contrast, the focus on first-order relatives by Norman et al. is better suited to analyzing recent (1-2 generation) dispersal k

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pattern, as is the finer-scale sampling across the region. Accordingly, I find the results of Norman et al. more relevant to the question at hand.

That said, I do have some reservations and recommendations:

- 1.k The rather poor sampling east of the proposed new road limits the power to test directly for potential disruption of connectivity.
- 2.k The results in Norman et al. are somewhat counter-intuitive in suggesting higher contemporary dispersal rates at large than small scale. Their analyses do not infer the direction of dispersal, so whether the southern populations are a source for those to the west remain speculation.
- 3.k To address (1) in the context of the forthcoming PVA, it should be possible to model dispersal rate (using logistic regression on FOR distances or regression of pairwise r values) as a function of linear distance and habitat heterogeneity and with or without a potential road barrier
- 4.k To address (2), methods that infer migration rates over recent generations (e.g. BayesAss) could used to test for asymmetry among north, south and adjacent regional populations. k

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Yours truly,

