Appendix 3 – Australian Museum genetics report

Australian Centre for Wildlife Genomics
Australian Museum Research Institute

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

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Executive Summary

In April 2015, the Australian Centre for Wildlife Genomics (ACWG), Australian Museum Research Institute was engaged by the Roads and Maritime Service (RMS) to provide a genetic assessment of a Koala (*Phascolarctos cinereus*) population found within and around the Wardell area of the mid-north coast of New South Wales, with the aim of providing information on four project deliverables.

On the 17th June 2015, 38 Koala tissue samples were received by the ACWG at the Australian Museum in Sydney. Using standard operating procedures, DNA was successfully extracted from all 38 samples and a portion of the mitochondrial DNA (mtDNA) Control Region was sequenced. All 38 samples were successfully genotyped for 15 microsatellite loci (at the Australian Genome Research Facility, Melbourne).

Standard population genetics diversity indices for both the mtDNA and microsatellite data were generated for the Wardell Koala population and compared to data generated from four other sites in northern NSW and southern Queensland. The results of this analysis suggest that the levels of genetic diversity present in Wardell Koalas is comparable to that found at other locations in the region.

Analysis of the genetic structure among the Koalas sampled from Wardell revealed no pre-existing population sub-structure (Project deliverable 1). In a regional context there was evidence for gene flow across the populations sampled in the region (for both mtDNA and microsatellites), but with some genetic differentiation associated with geographic distance. This pattern indicates that populations that are geographically closer to one another are more closely related genetically than those that are geographically further apart. This is a pattern of differentiation often seen in wildlife population of species with some limitations to dispersal and therefore gene flow (Project deliverable 2)

Specific locus allele frequencies for the Wardell Koala population are given in Appendix 1 (Project deliverable 3)

Details of laboratory methods, data validation and analysis are given on pages 4-7 (Project deliverable 4).

Project Background

As part of its flagship research project, The Koala Genome Project, (http://koalagenome.org/drupal/) the Australian Centre for Wildlife Genomics (ACWG), at the Australian Museum Research Institute has an established research programme investigating the population genetics of northern New South Wales (NSW) Koala populations. The primary aim of this research is to understand the genetic diversity and structure of Koala populations in the Port Macquarie and Coffs Harbour regions. These populations have been examined to identify causes of population structuring, including natural biogeographic barriers or more recent barriers to gene flow such as major roads. This research project has recently expanded to also include populations from southern Queensland, Victoria as well as captive NSW Koalas, with the aim of collating one of the largest population genetics assessments of this species to date. Thus far over 250 individual Koalas have been sampled from northern NSW and south eastern Queensland (i.e. Port Macquarie, Coffs Harbour, Tyagarah, and Coomera) and analysed using both microsatellite markers and mitochondrial DNA (mtDNA).

Project Methods

Samples and DNA extraction:

A total of 38 tissues samples of Wardell Koalas were received by the ACWG on the 17th June 2015. Genomic DNA was extracted from the 38 tissue samples using the high salt method (Sunnucks and Hales, 1996) following standard protocols.

For comparison, additional samples held by the ACWG as part of an ongoing research programme investigating the population genetics of Koalas across their distribution (as outlined in Project Background), were also extracted using the protocol above and included in subsequent analyses. For microsatellite analyses, a total of 231 Koala samples from four surrounding locations – Port Macquarie, Coffs Harbour, Tyagarah and Coomera were genotyped and included as described in the Microsatellite section below. For mtDNA analyses a total of 454 Koala samples were included from across the species' distribution, including 243 from the four sites included in the microsatellite analyses as outlined in the Mitochondrial DNA section below.
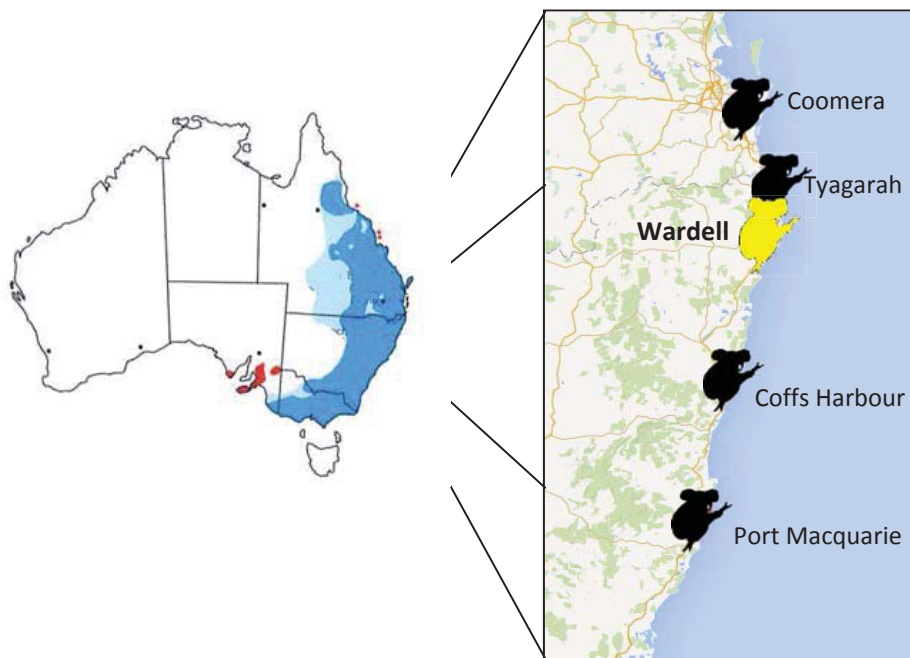
Figure 1: Map of Koala distribution, showing natural distribution (blue) and reintroduced sites (red). Insert shows the focal site (Wardell in yellow) and surrounding sampling locations in north-eastern NSW and south-eastern Qld (in black). Distribution map adapted from Van Dyck & Strahan (2008).

Microsatellite markers:

Development and amplification: To screen for tetra-, tri- and di-nucleotide microsatellite loci in the Koala, DNA was extracted from muscle tissue of one Koala specimen (Australian Museum Mammals #M.35147.004) using the DNeasy blood and tissue DNA extraction kit (QIAGEN). A total of 15.0µg of RNAse-treated genomic DNA was used in 1/8 of a plate for pyrosequencing by an external service provider, the Australian Genome Research Facility (www.agrf.com.au), on a Roche GL FLX (454) system. Using QDD (Meglécz et al. 2010), a program designed for both microsatellite detection and primer design for large data sets, we identified 1482 sequences that contained putative microsatellite motifs with five or more repeats and which had sufficiently-long flanking regions free of nanosatellites for which primers could be designed. 17 loci were identified that consistently and reliably amplified; these were optimised and used for genotyping.

Polymerase Chain Reaction (PCR) amplification was conducted using the following conditions: 94 °C for 5 min followed by 11 cycles at 94 °C (30 s), 60–55 ° (45 s; dropping 0.5 °C per cycle), and 72°C (45 s); followed by 19 cycles at 94°C (30 s), 55 °C (45 s), and 72 °C (45 s); followed by 8 cycles at 94 °C (30 s), 53 °C (45 s), and 72 °C (45 s); and a final extension step at 72 °C for 10 min. Multiplexed PCR products using labelled primers

were run on a AB3730xl Sequencer by AGRF (Melbourne) and scored using GENEMAPPER 4.1 (Applied Biosystems). Any ambiguous genotypes were re-amplified. To ensure amplification and scoring consistency, at least 10% of samples at each locus were independently rerun and genotyped (Project deliverable 4). MICROCHECKER 2.2 (Van Oosterhout et al. 2004) was used to check for the presence of null alleles (Project deliverable 4).

Population genetic analyses:

Genetic diversity: To assess the levels of genetic diversity within the Wardell population and surrounding locations we calculated standard population genetics diversity indices, including allelic diversity (N_A), allelic richness (A_R; which allows for comparisons between sites comprising different sample sizes), private alleles (A_P; the average number of alleles only found in that population) and levels of expected (H_E) and observed heterozygosity (H_O). Exact tests for Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium (LD) were conducted in GenAlEx 6.5 (Peakall & Smouse, 2012; 2006), GENEPOP 4.2 (Rousset 2008) and FSTAT 2.9 (Goudet 2001; Project deliverable 4).

Population genetic structure: The degree of genetic structure within the Wardell population (Project deliverable 1 & 3), and among surrounding Koala sampling localities (Project deliverable 2; i.e. Port Macquarie, Coffs Harbour, Coomera and Tyagarah – see Figure 1) was assessed using six different analytical approaches: STRUCTURE, DAPC, F-statistics, AMOVA, assignment tests, IBD (details provided below).

We calculated F_{ST} to assess levels of differentiation between sampling localities following the Weir & Cockerham (1984) approach in FSTAT 2.9.

An analysis of molecular variance (AMOVA) was implemented in GENALEX 6.5 to investigate if significant genetic partitioning occurred among our sampling locations, with significance calculated via bootstrapping (999 permutations).

To test for isolation by distance (IBD), a Mantel test was performed to test for a correlation between genetic and geographic distance matrices using genetic distances calculated in GENALEX 6.5.

To provide an indication of movement/gene flow between Wardell and surrounding sites, we undertook an assignment test in GENALEX 6.5. This test determines the likelihood of an individual originating from the population it was sampled in, based on allele frequencies within each population.

Spatial autocorrelation, implemented in GENALEX 6.5, was used to investigate spatial genetic structure within the sampled Wardell population. The SinglePops model was

used to calculate average pairwise relatedness at various distance classes ranging from 50 m – 15 km, with significance values calculated by bootstrapping with 999 permutations.

To assess the levels of genetic structure both within the sampled Wardell population and surrounding populations within north-eastern NSW and south-eastern Qld, STRUCTURE 2.3 (Pritchard et al. 2000) was used to assess genotypic clustering and calculate assignment probabilities. Within Wardell, we examined values of K = 1-5, using the 'admixture' model with correlated allele frequencies, running ten replicates for each value of K, with 10^5 Markov Chain Monte Carlo (MCMC) iterations and 10^4 burn-in iterations. When examining all the sampled populations, analyses were run for values of K = 1-10 (twice the number of localities sampled). Two analyses were run using the 'admixture' model, one assuming correlated and one assuming independent allele frequencies. Again, ten replicates were run for each value of K, with 10^5 MCMC iterations and 10^4 burn-in iterations. The most likely number of genetic clusters (K) within Wardell and among sample localities was determined by first manually examining log-likelihood values and using the ΔK method of Evanno et al. (2005).

In addition to STRUCTURE 2.3, we used discriminant analysis of principal components (DAPC) to describe the genetic relationship among sampling localities. DAPC is a multivariate method that uses principal components analysis (PCA) to transform data into uncorrelated components, which are then analysed using a linear discriminant method (Jombart et al. 2010). This method is ideal for assessing small and potentially fragmented populations because, unlike STRUCTURE, it does not assume HWE and linkage equilibrium, which are assumptions often violated in natural populations. We used the R package, adegenet (Jombart 2008), implemented in R 2.12 (R development core team 2013; www.r-project.org) to run DAPC. Missing data were replaced with the mean, following Horne et al. (2011) and Dennison et al. (2015). The number of genetic clusters was selected using the find.clusters function and Bayesian Information Criterion (BIC). In DAPC, retaining too many principal components (PCs) as predictors with respect to sample size can result in over-fitting the model, while retaining too few can reduce statistical power. The a.score function in the R package, adegenet (Jombart 2008), suggests the optimal number of PCs to retain taking into account this trade off, so following a.score's recommendation, a conservative 8 PCs were retained for our DAPC analysis.

Mitochondrial DNA:

Amplification: A ~800 base pair (bp) fragment of Domain 1 of the mitochondrial DNA Control Region (*CR*) was also amplified from each individual using primers L15999M and H16498M (Fumagalli et al. 1997). PCRs were carried out in 25 µl reactions with 1000 ng

of genomic DNA; BiolineMyTaq buffer; 2 pmol corresponding primers. Negative controls were included in each PCR. PCRs were performed on an Eppendorf Mastercycler EpS under the following conditions: initial denaturation (94 °C for 3 min); 36 cycles of denaturation (94 °C for 20 s); annealing (60 °C for 40 s) and extension (72 °C for 40 s) followed by a final extension (5 min at 72 °C). PCR products were cleaned using ExoSap-IT[©] (USB Corporation, Cleveland, Ohio, USA). Sequencing was resolved on an AB 3730xl Sequencer at AGRF Sydney, and verified. Any individuals with ambiguous sequence data or unique singleton haplotype (i.e. it was the only individual possessing that sequence) were re-amplified and verified. Five samples were re-amplified and sequenced as controls to ensure data integrity. Once verified, all sequences were aligned using the CLUSTAL X algorithm implemented in MEGA 6 (Tamura et al. 2013). Generated haplotypes were compared to existing published Koala haplotypes (available on Genbank; accession numbers AJ005846 to AJ005863; KJ530551 to KJ530556; KC505325; GQ851933 to GQ851940; AJ012057 to AJ012064; KF745869 to KF745875)

Data analysis: To estimate the levels of mtDNA *CR* diversity, haplotypic diversity (*h* – the number of different sequences/haplotypes present) and nucleotide diversity (π – the degree of differences between sequences; Tajima. 1983) were calculated within sites using ARLEQUIN 3.5.2.1 (Excoffier & Lischer 2010). These measures of genetic diversity were also calculated for surrounding localities (Port Macquarie, Coffs Harbour, Tyagarah and Coomera) for comparison.

The levels of differentiation at the mtDNA *CR* between Wardell and surrounding sampled populations were assessed via pairwise Φ_{ST}. Analysis of molecular variance (AMOVA), which utilises the distribution of, and sequence divergence between, haplotypes was implemented in ARLEQUIN 3.5.2.1 to assess how mtDNA diversity was partitioned (either within or between populations). We also assessed the influence of geographic distance by comparing the extent of differentiation (Φ_{ST}) with the geographic distance between populations. The significance was estimated using Mantel's test, with 10000 permutations in ARLEQUIN 3.5.2.1.

For the mtDNA *CR* haplotypes found in Wardell we constructed a haplotype network using the TCS procedure (Clement et al. 2002) implemented in PopART (http://popart.otago.ac.nz) to show how the different haplotypes were related. We then constructed a network containing all existing Koala mtDNA haplotypes to place Wardell in a broader distributional context.

Project Results

Genomic DNA was successfully extracted from all 38 samples provided from Wardell. All individuals were also successfully genotyped at 17 microsatellite loci and sequenced for

a ~800 bp portion of the mtDNA *CR*. Two microsatellite loci were removed from subsequent analyses; locus Pcin01 was found to be monomorphic at all sampling localities and locus Pcin13 showed evidence of null alleles at Wardell and three out of the other four sampled populations. The probability of identity, which is the probability of obtaining two identical microsatellite genotype profiles by chance using this combination of loci was 1.7×10^{-15}, indicating that this is a powerful marker set. Comparisons of the duplicate individuals used to assess consistency of genotype scoring showed that all individuals were consistently scored across all loci. All of the remaining 15 microsatellite loci were found to be in HWE and there was no evidence for LD (Project deliverable 4). Two out of the five localities, Port Macquarie and Coffs Harbour were significantly out of HWE. This in not surprising, given sampling at these localities covered a broader geographical area than other sites and this disequilibrium is possibly due to a Wahlund effect from some within-site spatial structure (Wahlund 1928).

A summary and explanation of the results of each analysis undertaken to assess genetic diversity and structure (see Project deliverables 1 & 2) is provided below; separated into four sections; 1. Levels of genetic diversity, 2. Genetic sub-structuring within Wardell, 3. Gene flow between Wardell and surrounds, and 4. Genetic structure within north-eastern NSW/south-eastern Qld (i.e. Wardell in the context of the surrounding area).

1. Levels of genetic diversity

A summary of standard genetic diversity indices can be found in Table 1. Based on the samples provided, the Wardell population contained an average of 5 (±0.4) alleles per locus (range 3 – 9 alleles per locus), with an average expected heterozygosity of 0.62 (±0.03; Table 1). Two mtDNA *CR* haplotypes were identified, Pc7 and Pc13, which were separated by 11 base pairs and correspond to published haplotypes H5/Q1 and H2/Q8 respectively (Houlden et al. 1999; Fowler et al. 2000). These levels of diversity are similar to those found in other Koala populations in north-eastern NSW and southern Queensland (Table 1). However, compared with populations in the southern parts of the range, such as French Island and Western Victoria, Wardell exhibits high levels of genetic diversity (Houlden et al. 1999; ACWG unpublished data 2015). In general, while measures of genetic diversity obtained from different microsatellite loci are not directly comparable these indices of genetic diversity suggest that Koalas contain moderate levels of genetic diversity compared with other marsupials (Eldridge et al. 2010). For example, levels of genetic diversity appear lower in Koalas compared with highly mobile macropodids (e.g. western grey kangaroo; Neaves et al. 2009; 2012) and some arboreal species (e.g. ring-tailed possum, Lancaster et al. 2011) but are similar to those observed in other species with similar dispersal tendencies/habitat requirements, particularly in fragmented landscapes (e.g. long-nosed potoroo (Frankham et al. 2015) and spotted-tailed quoll (Firestone et al. 1999)).

Table 1: Summary of genetic diversity indices for Koala sampled from five sites in the north-eastern NSW and south-eastern Queensland

Locality	Microsatellites							Mitochondrial DNA			
	n	$N_a \pm SE$	$A_R \pm SE$	$P_a \pm SE$	$H_o \pm SE$	$H_e \pm SE$	F_{IS}	n	H	$h \pm SD$	$\pi \pm SD(\%)$
Wardell	**38**	**5.0 ± 0.4**	**3.8 ± 0.3**	**0.13 ± 0.09**	**0.61 ± 0.04**	**0.62 ± 0.03**	**0.025**	**38**	**2**	**0.06 ± 0.04**	**0.07 ± 0.06**
Port Macquarie	149	6.1 ± 0.5	4.1 ± 0.3	0.87 ± 0.26	0.63 ± 0.03	0.65 ± 0.03	0.027	142	3	0.44 ± 0.03	0.10 ± 0.08
Coffs Harbour	57	6.1 ± 0.3	4.5 ± 0.3	0.47 ± 0.13	0.65 ± 0.03	0.67 ± 0.03	0.043	40	2	0.05 ± 0.04	0.02 ± 0.03
Tyagarah	17	4.3 ± 0.3	3.8 ± 0.3	0.27 ± 0.12	0.57 ± 0.06	0.59 ± 0.05	0.063	15	1	-	-
Coomera	8	3.3 ± 0.3	3.3 ± 0.3	0.13 ± 0.09	0.64 ± 0.07	0.56 ± 0.05	-0.082	8	1	-	-
Overall NE NSW/SE Qld	269	8.2 ± 0.6	5.0 ± 0.3	-	0.62 ± 0.02	0.62 ± 0.02	-	243	5	0.76 ± 0.02	0.66 ± 0.35

n = no. individuals sampled, N_a = no. alleles averaged over 15 loci, A_R = allelic richness averaged over 15 loci, P_a = private alleles averaged over 15 loci, H_o = observed heterozygosity, H_e = expected heterozygosity, F_{IS} = inbreeding coefficient. H = number of haplotypes, h= haplotypic diversity, π = nucleotide diversity

2. Genetic sub-structuring within Wardell

We investigated patterns of genetic structure within the sampled Wardell population to determine if there was any sub-structuring present (Project deliverable 1). Multiple analyses (PCA, DAPC and STRUCTURE) all showed a single genetic unit within the focal area. That is, no genetic structure was detected. Spatial autocorrelation showed no significant genetic structure at all distance classes assessed within Wardell (Figure 2), and STRUCTURE and DAPC analyses (graphs not shown) assigned all individuals from Wardell to one genetic cluster. Furthermore, as Figure 3 shows individuals sampled from 'eastern' and 'western' regions of the focal area (based on data provided) form a single cluster. This lack of spatial genetic structure within the focal site is expected given the potential for Koalas to move large distances (Dique et al. 2003). Mitochondrial DNA also revealed no sub-structuring within Wardell, with 37 of the 38 individuals sampled sharing a haplotype.
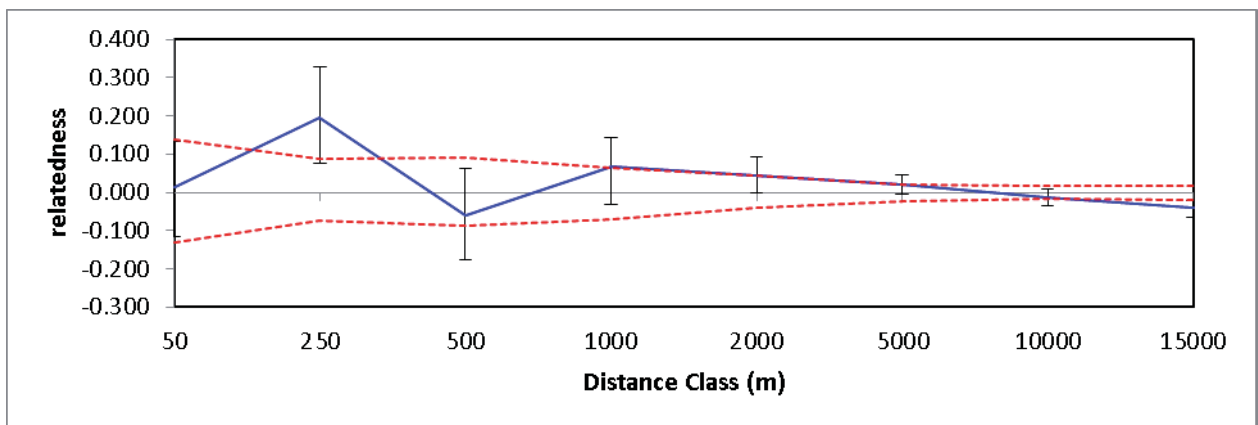

Figure 2. Spatial autocorellogram of Koalas sampled at Wardell. Average pairwise relatedness (± SE) is given at a range of distance classes. Red dotted lines represent the 95% confidence interval around which relatedness is effectively zero (i.e. random mating with no genetic structure).
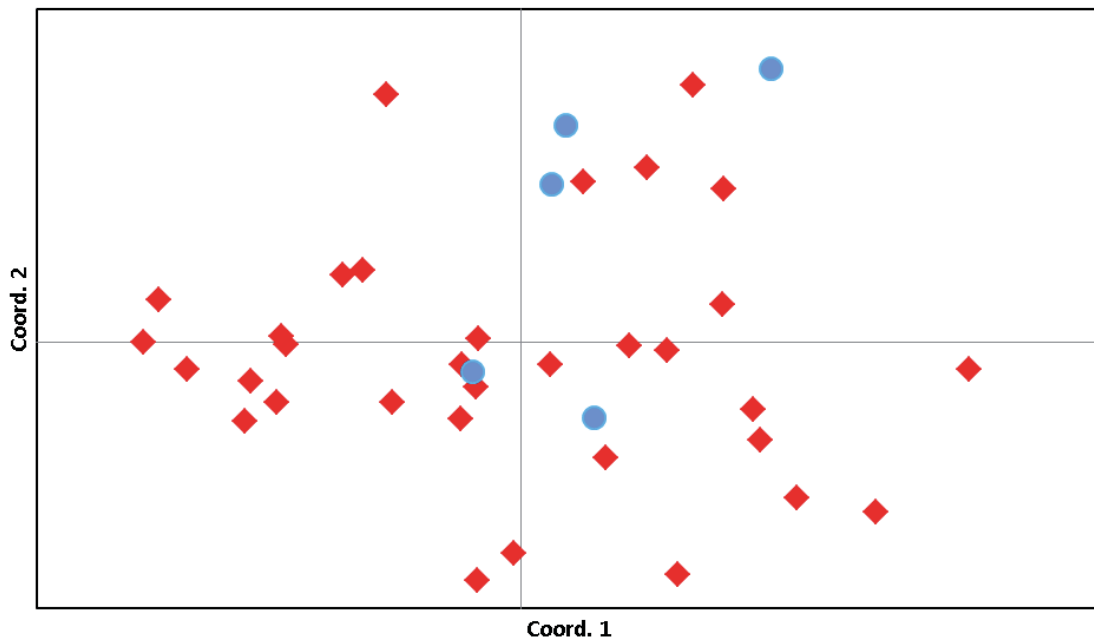
Figure 3: PCA plot showing the genetic distance between individuals within the Wardell focal area. Red diamonds represent individuals sampled in the west, while blue circles represent those sampled in the east of the focal area.

3. Gene flow between Wardell and surrounding locations

To determine how genetic diversity is partitioned among Wardell and surrounding sites we used an AMOVA. This analysis revealed that the genetic diversity present in the region is found primarily within sites, rather than partitioned between them (Table 2). This is reflected in the low numbers of private alleles (those found only within a single population) in Wardell or any other sampled population in the surrounding area (Table 1). A detailed summary of the by-locus allele frequency can be found in Appendix 1 (Project deliverable 3).

Mitochondrial DNA *CR* diversity showed a different pattern, reflecting the fact that mtDNA is maternally inherited. The AMOVA showed that mtDNA diversity was primarily partitioned between our sampled populations within the region surrounding Wardell, with only a small portion of the diversity found within populations. This result, likely reflects more limited movement in female Koalas, compared to males, leading to differences in the maternal lineages found at each site. On a larger landscape scale, however, the two haplotypes present at Wardell have also been recorded elsewhere in the range (see section 4 for details).

Table 2: AMOVA showing the partitioning of genetic diversity between and within Koala 'populations' for **a**. microsatellite markers and **b**. mitochondrial DNA

Source	df	SS	MS	Est. Var.	%
a. Microsatellite					
Among Pops	4	620.727	155.182	3.456	25%
Within Pops	264	2717.198	10.292	10.292	75%
Total	268	3337.926		13.748	100%
b. Mitochondrial DNA					
Among Pops	4	576.555		4.157	92.71
Within Pops	230	75.152		0.327	7.29
Total	234	651.707		4.484	100%

The levels of genetic differentiation varied between 0.101 and 0.217 among the sampled sites for microsatellites (F_{ST}) and between 0.000 and 0.991 for mtDNA data (Φ_{ST}) (Table 3). For the microsatellite data, there were significant levels of differentiation between all the sampled populations. Similarly, significant differentiation was found between all the sampled populations for mtDNA, with the exception of Tyagarah and Coomera. At this scale, these levels of population differentiation are expected given the geographic separation of our sampling sites and *do not* indicate an absence of gene flow between these locations. There was a significant positive correlation between genetic distance and the geographic distances separating sampling sites (Figure 4), an effect common in wildlife populations and known as isolation by distance. Hence, the further sampled sites were away from one another the more likely they were to be different, but each site is connected to others by gene flow from nearby locations. These data indicate gene flow occurs between Wardell and the surrounding areas.

Estimating numbers of animals moving between sites accurately is not possible without additional samples from nearby locations and, ideally, intensive tagging (either physical or genetic) and monitoring of individuals. However, to provide an idea of the movement of individuals between our sties we assessed the likelihood of individuals originating locally within Wardell. This analysis revealed evidence that at least two individuals appeared to originate from elsewhere (possibly the Coffs Harbour and Tyagarah areas), suggesting relatively recent immigration into the Wardell population. One of these individuals also possessed a distinct mtDNA haplotype to other Koalas in Wardell, further suggesting a non-local origin for this Koala. Additionally, examination of the other sampling localities suggested movement out of Wardell, with two individuals from

Coffs Harbour being more similar to Koalas found in Wardell than Coffs Harbour. Together these data indicate that gene flow has historically occurred amongst populations of Koalas throughout this region and maintenance of this gene flow (i.e maintenance of habitat corridors to facilitate this gene flow) is likely to be critical to maintaining genetic diversity within these populations in the future.

Table 3: Levels of differentiation between Wardell and surrounding locations. F_{ST} values calculated from microsatellite data are given above the diagonal and Φ_{ST} calculated from mitochondrial data are given below. * denotes significance at the 5% level.

	Wardell	Port Macquarie	Coffs Harbour	Tyagarah	Coomera
Wardell	-	0.180*	0.140*	0.115*	0.181*
Port Macquarie	0.933*	-	0.101*	0.217*	0.204*
Coffs Harbour	0.960*	0.204*	-	0.171*	0.155*
Tyagarah	0.961*	0.940*	0.991*	-	0.174*
Coomera	0.955*	0.937*	0.0990*	0.000	-
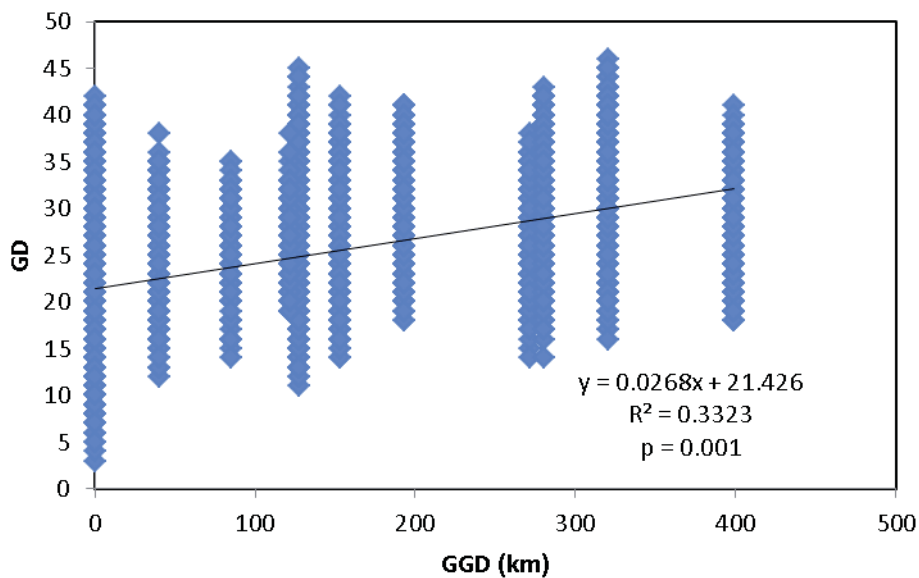
Figure 4: Relationship between genetic distance (GD; y-axis) and geographic distance (GGD; x-axis) between locations in north-eastern New South Wales and south-eastern Queensland based on microsatellite data. (Isolation by distance)

4. Genetic structure within north-eastern NSW and south-eastern Qld (i.e. Wardell in the context of surrounding locations

To place Wardell in a wider context we investigated the presence of genetic structure across the region for microsatellites, and across the distribution for mtDNA *CR* haplotypes. Figure 5 shows a PCA plot based on microsatellite genotypes, which provides a detailed picture of how genetically similar/dissimilar individual sampled Koalas are to one another. As the figure shows, the five sampled locations show some differences, reflecting the geographic distances that separate them, but there is no evidence of major genetic breaks within the region. This pattern is indicative of isolation by distance (as described above) and further sampling of intervening locations is likely to bridge the gaps between our localities.
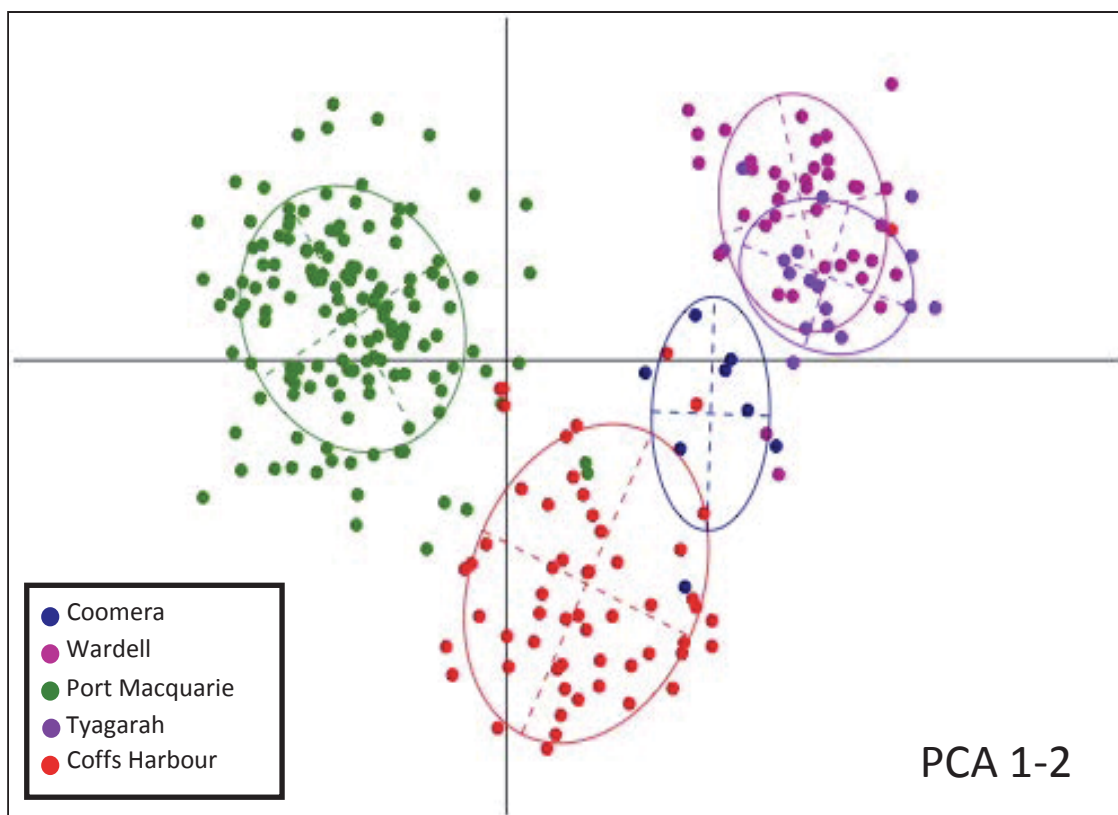
Figure 5: PCA plot showing Wardell and the four surrounding sampling sites. Each dot represents an individual Koala, and colours indicate the location from which they were sampled. The open circles represent the 95% confidence ellipses for each group.

STRUCTURE (using both correlated and independent allele frequencies) and DAPC analyses both indicated three genetic clusters are present in our dataset, which corresponded geographically to Port Macquarie, Coffs Harbour and the three northern-most sites – Wardell, Tyagarah and Coomera (Figure 6). Unlike the analyses undertaken to assess gene flow between sites (described in section 3), these methods are not reliant

on sampling information and identify genetic clusters based on the genetic similarity between individuals (regardless of their geographic location). There is however, some overlap in the genetic clusters (DAPC scatterplot Figure 6a) suggesting gene flow occurs (or has occurred recently) between them and it is likely that additional sampling between the five sites will show further evidence of the gene flow between these clusters. These results highlight the importance of maintaining habitat connectivity between Koala populations to prevent isolation of any individual population or restricting gene flow along the coast, which may lead to the loss of genetic diversity and decline in population health.

a
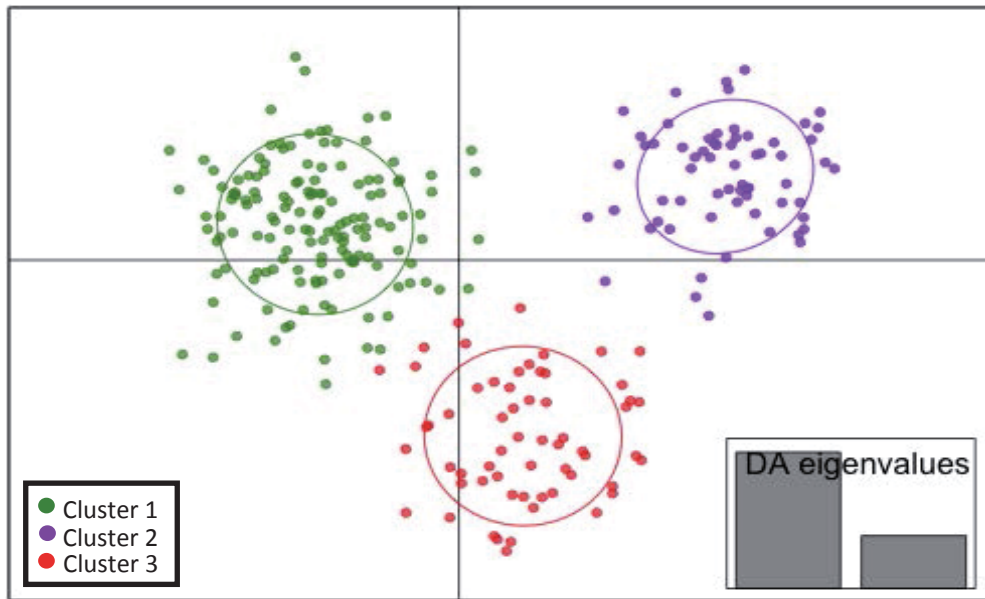
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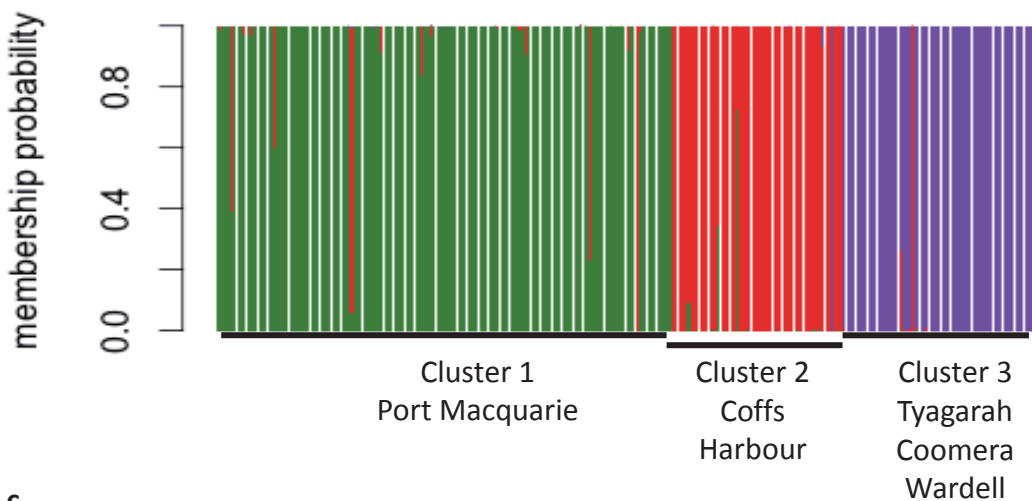
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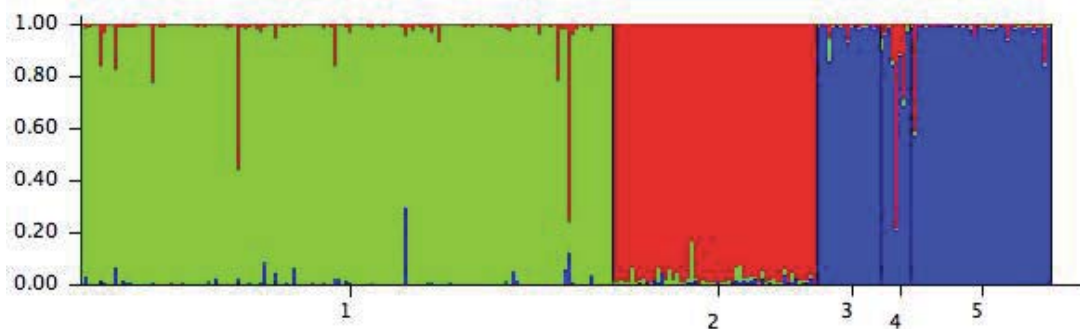
Figure 6. a. DAPC plot showing the three genetic clusters (encompassed by 95% confidence ellipses) identified in north-eastern NSW and south-eastern QLD. **b.** Plot showing the probability of assignment to the three potential genetic clusters generated by DAPC. Individuals are grouped according to the location they were sampled from. Cluster 1 corresponds to Port Macquarie, cluster 2 to Coffs Harbour and cluster 3 contains individuals from Tyagarah, Coomera and Wardell. **c.** Equivalent population assignment plot generated in STRUCTURE using the

"admixture" model with independent allele frequencies; 1 corresponds to Port Macquarie, 2 to Coffs Harbour, 3 to Tyagarah, 4 to Coomera and 5 to Wardell.

An analysis of mtDNA *CR* haplotypes from individuals distributed throughout the range, encompassing Qld, NSW, Vic and reintroduced Koala populations in South Australia (derived from samples held by ACWG and published data) revealed that overall there is little evidence of mtDNA genetic structure in Koalas. Within the north-eastern NSW/south-eastern Qld region, five haplotypes were identified, one (Pc7) was shared among Wardell, Tyagarah and Coomera, while Pc2 was shared between Coffs Harbor and Port Macquarie and the remaining three were unique to the populations they were sampled from (e.g. Pc13 in Wardell; Figure 7a). However, at the broader distributional scale for Koalas, very few haplotypes were restricted to a single location and several were present in multiple states (Figure 7b). For example, both haplotypes present in Wardell (Pc7 and Pc13) were also found elsewhere in NSW (e.g. Kyogle, Lismore, Narrandera, Tyagarah, Iluka Round Mountain, Tanglewood area) and Qld (e.g. Coomera, Gold coast and Mutdapilly; Figure 7b). Additionally, the haplotypes identified within the examined populations in north-eastern NSW and south-eastern Qld were present throughout the distribution (Figure 7a). The patterns observed in mtDNA data reflects a long history of gene flow across Koala populations but with some localized structure reflecting the more limited short-term dispersal of females.
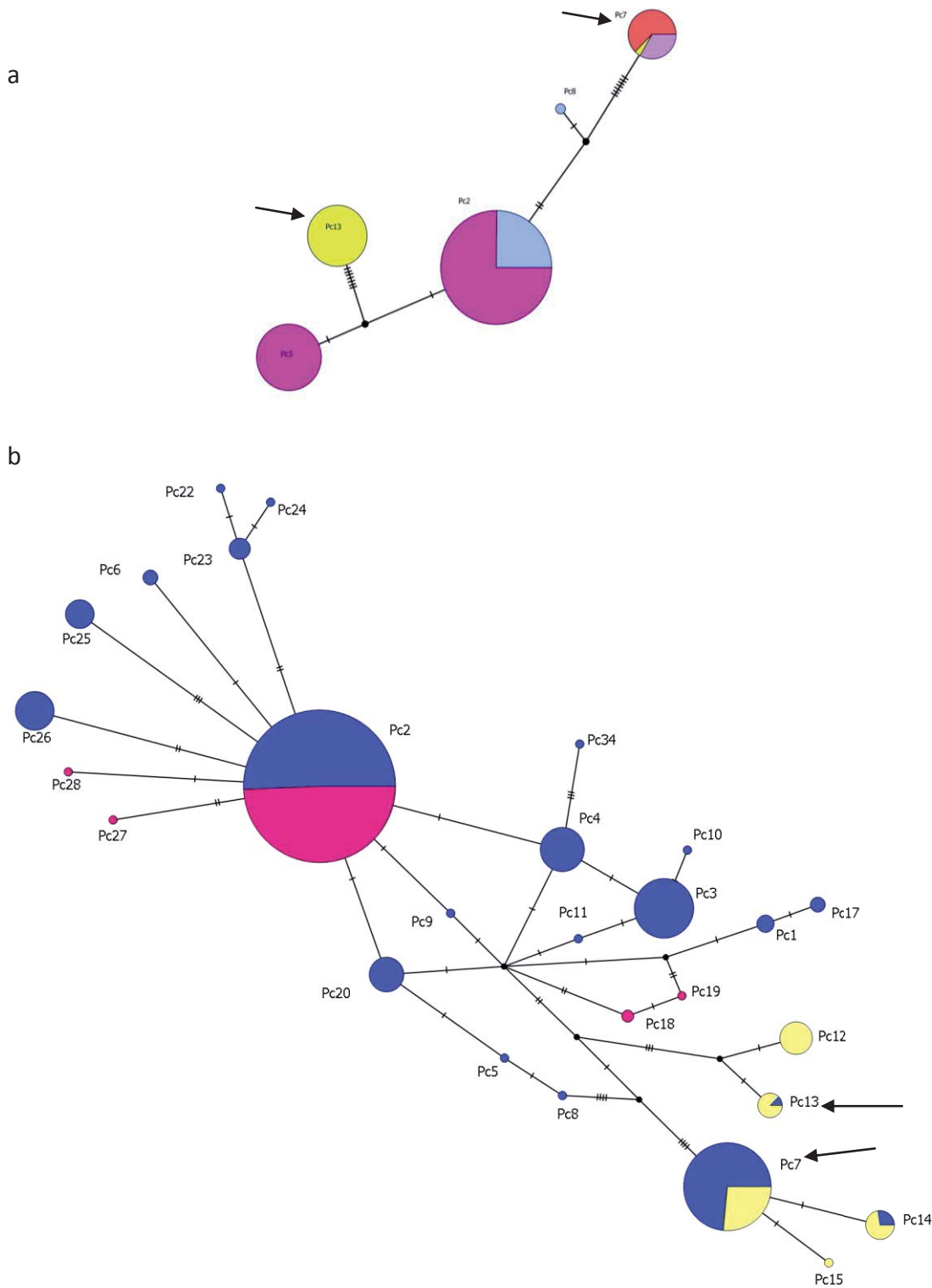
Figure 7: Mitochondrial DNA Control Region haplotype TCS networks for **a.** north-eastern NSW and south-eastern Queensland sites and **b.** all Koalas across the range. Hatches represent single base pair mutations, black circles are unsampled haplotypes and coloured circles are sampled haplotypes. The size of each circle reflects the number of times that haplotype was sampled and is divided according to the proportion of samples from each sampling location. **a.** Haplotypes from Wardell are shown in yellow, Tyagarah in red, Coomera in purple, Port Macquarie in pink and Coffs Harbour in blue **b.** Haplotypes from NSW are shown in blue, Qld in yellow and SA and Vic are shown in pink. Haplotype Pc7 and Pc13 were found in Wardell, indicated by the black arrows.

Summary

- Wardell is a genetically diverse Koala population, exhibiting similar levels of diversity to other sites sampled in this region (and higher than southern Koala populations)

- Based on the samples provided, there is no evidence of sub-structuring or spatial genetic structure within Wardell

- All sites sampled showed some degree of genetic differentiation consistent with isolation by distance.

- Gene flow is apparent between Wardell and other sites sampled in the surrounding area. In addition, there is evidence of recent movements of individuals, with two Wardell individuals appearing to have originated from outside Wardell.

- Genetically, Wardell is most similar to sampling locations further north (i.e. Tyaragah and Coomera) but there is evidence of a long history of gene flow (both nuclear and mitochondrial) throughout the region, and across the species' entire distribution.

Project Deliverables

The requested project deliverables are referred to throughout the report, but a summary is also provided below:

1. *Analyses of population structure and gene flow within the focal area will be conducted.* The results of these analyses revealed there was no evidence of genetic structure within the focal area. See section 2 for details.

2. *Analysis will be conducted to determine whether the focal population appears to be connected to populations in the surrounding area. For this analysis, animals from neighbouring regions closer to Ballina will be compared.* Gene flow connects the focal population to other sampled locations within the surrounding area (i.e. Port Macquarie, Coffs Harbour, Tyagarah and Coomera). See sections 3 and 4 for details

3. *By-locus allele frequency information for the population as a whole, and, if subpopulation structure is present, allele frequencies for each subpopulation will be reported.* A summary of the allele frequencies for each locus for all Wardell samples can be found at the end of this report. These values are for all individuals sampled at Wardell as there was no evidence of sub-structure.

4. *Summary of procedures undertaken for data validation will be reported (e.g. replicates and controls). Including testing for Hardy-Weinberg equilibrium and null alleles.* A summary of all the validation procedures can be found in the methods section of this report. In brief, this included the use of positive and negative controls in all laboratory protocols and at least 10% of all samples were replicated to ensure consistency in genotyping and sequencing. For microsatellite loci, tests for Hardy-Weinberg equilibrium, linkage disequilibrium and null alleles were conducted. The results of these analyses are described at the start of the results section.

References

Clement M, Snell Q, Walker P, Posada D, Crandall K (2002). TCS: Estimating gene genealogies. Parallel and Distributed Processing Symposium, *International Proceedings*, **2**, 184.

Dennison S, McAlpin S, Chapple DG, Stow AJ (2015) Genetic Divergence among Regions Containing the Vulnerable Great Desert Skink (*Liopholis kintorei*) in the Australian Arid Zone. *PloS one*, **10**, e0128874.

Dique DS, Thompson J, Preece HJ, Villiers DL de, Carrick FN (2003) Dispersal patterns in a regional koala population in south-east Queensland. *Wildlife Research*, **30**, 281.

Eldridge MDB, Piggott MP, Hazlitt SL (2010) Population genetic studies of the Macropodoidea: a review. *Macropods: the biology of kangaroos, wallabies and rat-kangaroos. CSIRO Publishing, Melbourne*, pg.: 35-51.

Evanno G, Regnaut S, Goudet J (2005) Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. *Molecular Ecology*, **14**, 2611–2620.

Excoffier L, Lischer HEL (2010) Arlequin suite ver 3.5: a new series of programs to perform population genetics analyses under Linux and Windows. *Molecular Ecology Resources*, **10**, 564–7.

Firestone KB, Elphinstone MS, Sherwin WB, Houlden BA. (1999). Phylogeographical population structure of tiger quolls *Dasyurus maculatus* (Dasyuridae: Marsupialia), an endangered carnivorous marsupial. *Molecular Ecology*, **8**, 1613-1625.

Frankham GF, Handasyde KA, Eldridge MDB. (2015). Evolutionary and contemporary responses to habitat fragmentation detected in a mesic zone marsupial, the long-nosed potoroo (*Potorous tridactylus*) in southeastern Australia. *In press*

Fowler E V., Houlden BA, Hoeben P, Timms P (2000) Genetic diversity and gene flow among southeastern Queensland koalas (*Phascolarctos cinereus*). *Molecular Ecology*, **9**, 155–164.

Fumagalli L, Pope LC, Taberlet P, Moritz C (1997) Versatile primers for the amplification of the mitochondrial DNA control region in marsupials. *Molecular Ecology*, **6**, 1199–1201.

Goudet, J (2001) *FSTAT, a program to estimate and test gene diversities and fixation indices* (version 2.9.3)

Horne JB, Momigliano P, Welch DJ, Newman SJ, Van Herwerden L (2011) Limited ecological population connectivity suggests low demands on self-recruitment in a tropical inshore marine fish (*Eleutheronema tetradactylum*: Polynemidae). *Molecular Ecology*, **20**, 2291–306.

Houlden BA, Costello BH, sharkey D *et al.* (1999) Phylogeographic differentiation in the mitochondrial control region in the koala, *Phascolarctos cinereus* (Goldfuss 1817). *Molecular Ecology*, **8**, 999–1011.

Jombart T (2008) adegenet: a R package for the multivariate analysis of genetic markers. *Bioinformatics*, **24**, 1403–5.

Jombart T, Devillard S, Balloux F (2010) Discriminant analysis of principal components: a new method for the analysis of genetically structured populations. *BMC Genetics*, **11**, 94.

Lancaster ML, Taylor AC, Cooper SJB, Carthew SM (2011) Limited ecological connectivity of an arboreal marsupial across a forest/plantation landscape despite apparent resilience to fragmentation. *Molecular Ecology*, **20**, 2258–71.

Meglécz E, Costedoat C, Dubut V *et al.* (2010) QDD: a user-friendly program to select microsatellite markers and design primers from large sequencing projects. *Bioinformatics*, **26**, 403–4.

Neaves LE, Zenger KR, Prince RIT, Eldridge MDB (2012) Impact of Pleistocene aridity oscillations on the population history of a widespread, vagile Australian mammal, *Macropus fuliginosu*s. *Journal of Biogeography*, **39**, 1545–1563.

Neaves LE, Zenger KR, Prince RIT, Eldridge MDB, Cooper DW (2009) Landscape discontinuities influence gene flow and genetic structure in a large, vagile Australian mammal, *Macropus fuliginosus*. *Molecular Ecology*, **18**, 3363–3378.

Peakall R, Smouse P (2006) genalex 6: genetic analysis in Excel. Population genetic software for teaching and research. *Molecular Ecology Notes*, **6**, 288–295.

Peakall R, Smouse PE (2012) GenAlEx 6.5: genetic analysis in Excel. Population genetic software for teaching and research--an update. *Bioinformatics*, **28**, 2537–9.

Pritchard JK, Stephens M, Donnelly P (2000) Inference of Population Structure Using Multilocus Genotype Data. *Genetics*, **155**, 945–959.

Rousset F (2008) genepop'007: a complete re-implementation of the genepop software for Windows and Linux. *Molecular Ecology Resources*, **8**, 103–6.

Sunnucks P, Hale P (1996) Numerous transposed sequences of mitochondrial cytochrome oxidase I-II in aphids of the genus *Sitobion* (Hemiptera: Aphididae). *Molecular Biology and Evolution*, **13**, 510–524.

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S (2013) MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. *Molecular Biology and Evolution* , **30** , 2725–2729.

Van Dyck S, Strahan R (2008) *The Mammals of Australia*., 3rd edn.(Reed New Holland: Sydney.).

Van Oosterhout C, Hutchinson WF, Wills DPM, Shipley P (2004) micro-checker: software for identifying and correcting genotyping errors in microsatellite data. *Molecular Ecology Notes*, **4**, 535–538.

Wahlund S (1928) Zusammensetzung Von Populationen Und Korrelationserscheinungen Vom Standpunkt Der Vererbungslehre Aus Betrachtet. *Hereditas*, **11**, 65–106.

Weir BS, Cockerham CC (1984) Estimating F-Statistics for the Analysis of Population Structure. *Evolution*, **38**, 1358.

Appendix 1

Population allele frequencies for Koalas sampled in Wardell (n=38)

Locus	Alleles	Frequency
Pcin3		
	137	0.105
	141	0.013
	145	0.263
	149	0.039
	153	0.474
	157	0.105
Pcin5		
	176	0.342
	180	0.066
	188	0.276
	192	0.276
	200	0.039
Pcin6		
	153	0.447
	157	0.250
	161	0.303
Pcin7		
	133	0.434
	137	0.276
	141	0.158
	145	0.132
Pcin8		
	138	0.263
	142	0.566
	146	0.118
	158	0.026
	162	0.026
Pcin9		
	123	0.395
	125	0.250
	127	0.118
	129	0.013
	131	0.145
	135	0.079
Pcin10		
	129	0.197
	131	0.013
	141	0.763
	143	0.026

Pcin11		
	129	0.421
	133	0.053
	135	0.316
	139	0.013
	143	0.013
	145	0.066
	147	0.013
	149	0.105
Pcin14		
	132	0.224
	140	0.408
	142	0.013
	144	0.355
Pcin15		
	138	0.039
	140	0.105
	142	0.250
	144	0.105
	152	0.013
	154	0.158
	156	0.197
	158	0.118
	162	0.013
Pcin19		
	88	0.618
	97	0.289
	100	0.092
Pcin20		
	253	0.566
	256	0.421
	259	0.013
Pcin21		
	215	0.237
	221	0.079
	227	0.579
	230	0.092
	233	0.013
Pcin22		
	310	0.039
	313	0.013
	316	0.105
	319	0.066
	322	0.303

	325	0.474
Pcin23		
	87	0.026
	90	0.118
	96	0.776
	99	0.039
	102	0.039

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13th October 2015

Australian Centre for Wildlife Genomics Results Report

Addendum Report

This is an addendum to the previous report by Neaves *et al.* (2015), relating to the genetic assessment of a Koala (*Phascolarctos cinereus*) population in the Wardell area of the mid-north coast of New South Wales, which was conducted by the Australian Centre for Wildlife Genomics (ACWG), Australian Museum Research Institute and submitted to the Roads and Maritime Service (RMS) on the 12th August 2015.

Addendum details:

On the 7th of October 2015 RMS requested further assistance in the interpretation of the results of genetic analyses, including undertaking two additional analyses:

(1) calculation of the genetic distance measure Aij (Rousett 2000) to estimate the size of the genetic neighbourhood, and;

(2) calculation of the relatedness coefficient (r) (Wang 2002);

These analyses were to be completed prior to the Koala Expert Advisory Committee workshop held on 14th October 2015.

Using the same 38 Koala samples provided by RMS from the Wardell area and 17 microsatellite markers described in the original report (Neaves *et al.* 2015), we calculated pairwise Wang's (2002) relatedness coefficient (hereafter, *r*) and Rousset's '*Aij'* (hereafter, *Aij*) using SPAGEDI v.1.5 (Hardy & Vekemans 2002).
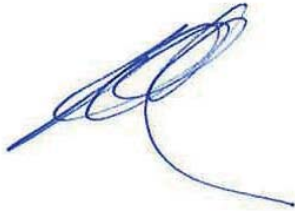
The average *r* was -0.012 (SE = 0.008), the average *Aij* was 0.025 (SE = 0.005) and the average geographic distance between individuals was 6.5 km (SE = 0.16). No relationship between geographic distance and either of the genetic variables (*r* or *Aij*) was evident (Fig. 1), which is consistent with our initial findings (See Fig. 2 Neaves *et al.* 2015). A reliable estimate of genetic neighbourhood could not be calculated from the available data. This was most likely due to the limited spatial genetic structure detected (see below for an explanation).

The distances between potential first-order relatives (FOR = parent-offspring, full siblings) averaged 2.7 km, but ranged from 3 m to 11 km. This suggests substantial movement of

Koalas across the site and is consistent with the lack of genetic structure previously reported by Neaves *et al.* 2015 in Wardell.

These additional analyses are consistent with the patterns of dispersal previously reported in Neaves *et al.* (2015). The details of the analyses and results are described below and a complete list of the pairwise values is appended at the end of this report.

Yours sincerely,

Dr Rebecca Johnson, on behalf of the co-authors listed below.

Dr Linda Neaves, Siobhan Dennison, Dr Greta Frankham, and Dr Mark Eldridge

Details of Analyses and Results:

1. *Distance measure Aij (Rousett 2000) - estimate the size of the genetic neighbourhood*

Using the genotypic data generated from the initial analyses (Neaves *et al.* 2015), we calculated pairwise Rousset's '*Aij*' (hereafter, *Aij*) for the 38 Koala samples from Wardell provided by RMS using SPAGEDI v.1.5 (Hardy & Vekemans 2002). The average geographic distance between individuals was 6.5 km (SE = 0.16), and average *Aij* was 0.025 (SE = 0.005).

Genetic neighbourhood size (*Nb*) is calculated by regressing the slope of *Aij* and log-geographic distance over a restricted geographic range (Hardy & Vekemans 2002). We tested a range of geographic distance classes and densities based on those reported by Phillips and Chang (2013) and Phillips *et al.* (2015). However, the models failed to converge under all tested scenarios and therefore no reliable estimate of *Nb* (or gene dispersal; *σ*) could be calculated (Hardy *et al.* 2006). This can be attributed to (1) low marker polymorphism, (2) too narrow a sampling scale, or (3) weak spatial genetic structure (Hardy et al. 2006). Each of these limitation is likely to contribute to the lack of convergence in this instance, particularly point (3), as our previous analyses indicated Wardell Koalas exhibit little spatial genetic structure (Neaves *et al.* 2015). This is further demonstrated in the additional analyses reported here, as evidenced by the lack of a relationship between genetic and geographic distance (Fig. 1).

2. *Relatedness coefficient (R) (Wang 2002)*

Using the genotypic data generated from the initial analyses (Neaves *et al.* 2015), we calculated pairwise Wang's (2002) related coefficients (hereafter, *r*) for the 38 Koala samples from Wardell provided by RMS using SPAGEDI v.1.5 (Hardy & Vekemans 2002).

Relatedness values ranged from -0.59 (unrelated) to 0.69 (likely FOR), averaging -0.012 (SE = 0.008). As there were no data available on known relationship swe determined the range of potential relatedness values for FOR via a simulation analysis performed in CONANCESTRY (Wang, 2011; using the known allele frequencies for Wardell and simulating 1000 Koalas for 5 relatedness classes – parent-offspring, fullsibs, halfsibs, first cousins and unrelated). Based on this analysis we determined the relatedness values for FOR to be those greater than 0.38. This indicated 38 pairs (out of 703 pairwise comparisons) of Koala within Wardell were likely FOR. It should also be noted that based on the simulated dataset there is a 26% chance of misassignment of second-order relatives to FOR (and 10% for third-order relatives). The geographic distance between these likely FOR averaged 2.7 km and ranged from 3 m to 11 km. The proximity of some closely related Koalas may reflect daughters remaining near their mothers, as is observed in other marsupials (e.g. Frankham *et al.* 2012), but without associated data on the sex of Koalas this is uncertain. Regardless, these data indicate substantial movement of Koalas within Wardell consistent with previous finding of a lack of genetic structure (Neaves *et al.* 2015).
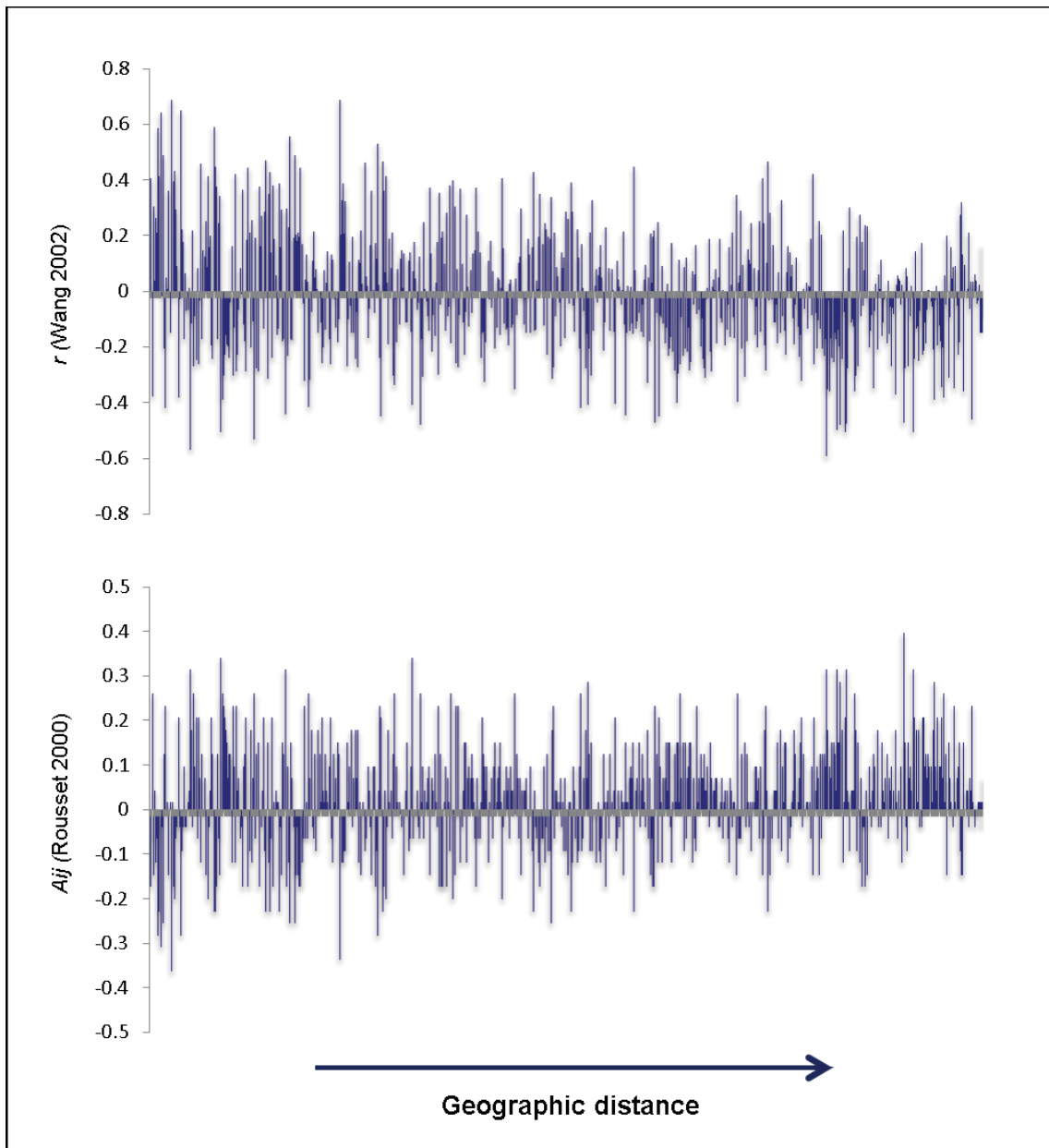
Fig. 1. The levels of genetic relatedness (top) and genetic distance (bottom) do not change substantially as geographic distance increases.

References

Frankham, G.J., Reed, R. L., Eldridge M.D.B. & Handasyde, K.A. 2012. The genetic mating system of the long-nosed potoroo (*Potorous tridactylus*) with notes on male strategies for securing paternity. *Australian Journal of Zoology* **60**: 225-234.

Hardy, O.J., Maggia, L., Bandou, E., Breyne, P., Caron, H., Chevallier, M., *et al.* (2006) Fine-scale genetic structure and gene dispersal inferences in 10 Neotropical tree species. *Molecular Ecology* **15**: 559-571.

Hardy, O.J. & Vekemans, X. (2002) SPAGeDi: a versatile computer program to analyse spatial genetic structure at the individual or population levels. *Molecular Ecology Notes* **2**: 618-620.

Neaves, L.E., Dennison, S.B. Frankham, G.J., Eldridge M.D.B. & Johnson, R.N. (2015) *Koala population genetics management.* Report to Roads and Maritime Services, NSW.

Phillips, S., & Chang, M. (2013) K*oala Habitat & Population Assessment: Ballina Shire Council LGA.* Final Report to Ballina Shire Council, NSW. Biolink Ecological Consultants. Uki, NSW.

Phillips, S., Brearly, G. &Callaghan, J. (2015) *Assessment of koala population distribution and demographics: Woolgoolga to Ballina Pacific Highway Upgrade (Section 10 Wardell to Coolgardie).*Report to Roads and Maritime Services, Biolink Consultants and Ecosure Pty Ltd.

Rousset, F. (2000) Genetic differentiation between individuals. *Journal of Evolutionary Biology* **13**: 58-62.

Vekemans, X. & Hardy, O.J. (2004) New insights from fine-scale spatial genetic structure analyses in plant populations. *Molecular Ecology* **13**: 921-935.

Wang, J. (2002) An estimator for pairwise relatedness using molecular markers. *Genetics* **160**:1203-1215.

Wang, J. (2011) COANCESTRY: A program for simulating, estimating and analysing relatedness and inbreeding coefficients. *Molecular Ecology Resources* **11**: 141-145.

Appendix

Table A1. Table of pairwise geographic distance (in km), relatedness (Wang 2002), and genetic distance (Rousset 2000) calculated from 38 Koala samples from Wardell.

Individual1	Individual2	Geographic distance (km)	Relatedness	
			r (Wang 2002)	*Aij (Rousset 2000)*
AM161_001	AM161_002	0.237397	-0.381073	0.205714
AM161_001	AM161_003	12.926400	-0.390896	0.287143
AM161_001	AM161_004	1.861320	0.291838	-0.147143
AM161_001	AM161_005	0.646394	0.123779	0.070000
AM161_001	AM161_006	5.150700	-0.354003	0.260000
AM161_001	AM161_007	0.175913	0.291358	-0.065714
AM161_001	AM161_008	0.130168	0.360076	-0.147143
AM161_001	AM161_009	2.706850	0.105006	0.097143
AM161_001	AM161_010	1.555270	0.444454	-0.174286
AM161_001	AM161_011	3.191660	0.167136	-0.038571
AM161_001	AM161_012	1.537140	0.184017	-0.011429
AM161_001	AM161_013	3.230370	0.003577	0.097143
AM161_001	AM161_014	2.496780	-0.184191	0.151429
AM161_001	AM161_015	2.606190	-0.269831	0.151429
AM161_001	AM161_016	0.197806	0.090056	-0.038571
AM161_001	AM161_017	2.281970	-0.074766	0.178571
AM161_001	AM161_018	1.536220	-0.288812	0.205714
AM161_001	AM161_019	4.818350	-0.150762	0.205714
AM161_001	AM161_020	10.369100	-0.088946	0.097143
AM161_001	AM161_021	12.715400	-0.243904	0.205714
AM161_001	AM161_022	6.375840	0.107413	-0.065714
AM161_001	AM161_023	8.632660	-0.006720	0.015714
AM161_001	AM161_024	5.204250	-0.119914	0.042857
AM161_001	AM161_025	0.990095	-0.230178	0.205714
AM161_001	AM161_026	0.152577	0.394991	-0.174286
AM161_001	AM161_027	5.506360	-0.090293	0.042857
AM161_001	AM161_028	5.673190	-0.044258	0.015714
AM161_001	AM161_029	6.306640	0.003732	-0.011429
AM161_001	AM161_030	14.885400	0.022034	0.015714
AM161_001	AM161_031	8.121760	0.164990	-0.092857
AM161_001	AM161_032	0.155072	0.432837	-0.201429
AM161_001	AM161_033	12.465200	-0.001226	0.042857
AM161_001	AM161_034	12.532900	0.058614	-0.038571
AM161_001	AM161_035	12.520800	-0.210821	0.178571
AM161_001	AM161_036	12.520500	0.024965	-0.011429
AM161_001	AM161_037	12.153000	-0.137376	0.151429

Individual1	Individual2	Geographic distance (km)	Relatedness	
			r (Wang 2002)	Aij (Rousset 2000)
AM161_001	AM161_038	11.101000	-0.206679	0.097143
AM161_002	AM161_003	12.712000	-0.504883	0.314286
AM161_002	AM161_004	1.933280	-0.233333	0.097143
AM161_002	AM161_005	0.784948	-0.244582	0.124286
AM161_002	AM161_006	5.085370	-0.193714	0.070000
AM161_002	AM161_007	0.105615	-0.420337	0.232857
AM161_002	AM161_008	0.122604	0.008976	0.015714
AM161_002	AM161_009	2.481030	-0.130472	0.070000
AM161_002	AM161_010	1.678970	-0.240822	0.205714
AM161_002	AM161_011	3.335850	-0.451579	0.205714
AM161_002	AM161_012	1.610290	-0.288506	0.151429
AM161_002	AM161_013	3.373430	-0.191304	0.178571
AM161_002	AM161_014	2.275880	-0.318379	0.070000
AM161_002	AM161_015	2.377360	-0.203407	0.042857
AM161_002	AM161_016	0.434100	-0.090117	0.097143
AM161_002	AM161_017	2.371020	-0.258360	0.205714
AM161_002	AM161_018	1.604470	-0.109267	0.070000
AM161_002	AM161_019	4.616770	-0.113879	0.042857
AM161_002	AM161_020	10.168600	-0.203877	0.042857
AM161_002	AM161_021	12.507700	-0.348821	0.124286
AM161_002	AM161_022	6.181630	-0.039225	-0.011429
AM161_002	AM161_023	8.515110	-0.288095	0.070000
AM161_002	AM161_024	5.045130	-0.114167	0.042857
AM161_002	AM161_025	1.105470	-0.200222	0.070000
AM161_002	AM161_026	0.089674	-0.205037	0.124286
AM161_002	AM161_027	5.341920	-0.139407	-0.038571
AM161_002	AM161_028	5.590990	-0.199520	0.070000
AM161_002	AM161_029	6.119810	-0.140639	-0.011429
AM161_002	AM161_030	14.702900	-0.463096	0.232857
AM161_002	AM161_031	7.920510	-0.400455	0.151429
AM161_002	AM161_032	0.138172	-0.149330	0.015714
AM161_002	AM161_033	12.235200	-0.481311	0.287143
AM161_002	AM161_034	12.303000	-0.276786	0.042857
AM161_002	AM161_035	12.291400	-0.475834	0.314286
AM161_002	AM161_036	12.290700	-0.506319	0.205714
AM161_002	AM161_037	11.943500	-0.361659	0.097143
AM161_002	AM161_038	10.881000	-0.237784	0.070000
AM161_003	AM161_004	12.703300	-0.269202	0.151429
AM161_003	AM161_005	12.969100	-0.180369	0.097143
AM161_003	AM161_006	10.185900	0.074332	0.070000

Individual1	Individual2	Geographic distance (km)	Relatedness	
			r (Wang 2002)	Aij (Rousset 2000)
AM161_003	AM161_007	12.812800	-0.213436	0.205714
AM161_003	AM161_008	12.834200	-0.112622	0.097143
AM161_003	AM161_009	11.077800	-0.077185	0.042857
AM161_003	AM161_010	13.088000	0.057986	0.015714
AM161_003	AM161_011	13.869300	-0.347533	0.232857
AM161_003	AM161_012	12.711900	0.018631	0.042857
AM161_003	AM161_013	13.867700	0.086094	-0.038571
AM161_003	AM161_014	11.323000	-0.231957	0.124286
AM161_003	AM161_015	11.055100	-0.262528	0.205714
AM161_003	AM161_016	13.115700	-0.049553	0.124286
AM161_003	AM161_017	12.893100	0.004545	0.124286
AM161_003	AM161_018	12.678000	-0.473011	0.395714
AM161_003	AM161_019	8.149120	-0.083852	0.232857
AM161_003	AM161_020	2.903550	-0.244123	0.178571
AM161_003	AM161_021	0.816022	0.374444	-0.174286
AM161_003	AM161_022	6.713650	-0.154331	0.151429
AM161_003	AM161_023	7.585600	-0.129379	0.151429
AM161_003	AM161_024	8.318540	0.009804	0.097143
AM161_003	AM161_025	12.930400	-0.033764	0.097143
AM161_003	AM161_026	12.800200	-0.229608	0.205714
AM161_003	AM161_027	7.980910	-0.231424	0.151429
AM161_003	AM161_028	9.630090	0.148984	0.015714
AM161_003	AM161_029	6.893510	-0.047067	0.124286
AM161_003	AM161_030	3.956840	-0.141236	0.097143
AM161_003	AM161_031	4.938590	-0.003229	0.097143
AM161_003	AM161_032	12.846800	-0.186972	0.124286
AM161_003	AM161_033	2.468570	-0.005368	0.124286
AM161_003	AM161_034	2.446410	-0.263416	0.205714
AM161_003	AM161_035	2.336440	0.050970	0.124286
AM161_003	AM161_036	2.425330	0.030868	0.042857
AM161_003	AM161_037	0.959783	-0.304004	0.232857
AM161_003	AM161_038	1.958270	-0.171633	0.151429
AM161_004	AM161_005	1.241420	0.419715	-0.120000
AM161_004	AM161_006	3.692660	-0.147401	0.097143
AM161_004	AM161_007	1.975520	0.190830	-0.038571
AM161_004	AM161_008	1.936510	0.228325	-0.120000
AM161_004	AM161_009	4.004550	0.135324	-0.011429
AM161_004	AM161_010	0.504648	0.084208	-0.065714
AM161_004	AM161_011	1.612110	0.375924	-0.174286
AM161_004	AM161_012	0.324409	0.175828	-0.038571

Individual1	Individual2	Geographic distance (km)	Relatedness	
			r (Wang 2002)	Aij (Rousset 2000)
AM161_004	AM161_013	1.639540	0.268806	-0.038571
AM161_004	AM161_014	3.870870	-0.306610	0.097143
AM161_004	AM161_015	3.866390	-0.172969	0.015714
AM161_004	AM161_016	1.851120	0.388085	-0.228571
AM161_004	AM161_017	0.458860	-0.247513	0.205714
AM161_004	AM161_018	0.328876	-0.172602	0.097143
AM161_004	AM161_019	4.632130	-0.076497	0.151429
AM161_004	AM161_020	9.958690	0.244259	-0.092857
AM161_004	AM161_021	12.380400	-0.303768	0.178571
AM161_004	AM161_022	5.990310	-0.154050	0.070000
AM161_004	AM161_023	7.422360	0.092206	-0.120000
AM161_004	AM161_024	4.480130	0.397074	-0.201429
AM161_004	AM161_025	0.897089	-0.173790	0.124286
AM161_004	AM161_026	1.925980	0.295415	-0.174286
AM161_004	AM161_027	4.817430	-0.241748	0.015714
AM161_004	AM161_028	4.298120	0.282796	-0.174286
AM161_004	AM161_029	5.829090	-0.019651	-0.065714
AM161_004	AM161_030	14.192000	0.094931	-0.011429
AM161_004	AM161_031	7.771090	-0.164700	-0.038571
AM161_004	AM161_032	1.978960	0.489574	-0.255714
AM161_004	AM161_033	12.602100	-0.155295	0.070000
AM161_004	AM161_034	12.666600	0.024184	-0.120000
AM161_004	AM161_035	12.638300	-0.370298	0.178571
AM161_004	AM161_036	12.651100	0.057801	-0.065714
AM161_004	AM161_037	11.854200	-0.351791	0.178571
AM161_004	AM161_038	11.010100	0.014284	0.015714
AM161_005	AM161_006	4.714100	-0.127354	0.097143
AM161_005	AM161_007	0.792157	0.446450	-0.228571
AM161_005	AM161_008	0.746916	0.087625	-0.011429
AM161_005	AM161_009	3.194890	0.362380	-0.147143
AM161_005	AM161_010	0.908882	0.245490	-0.065714
AM161_005	AM161_011	2.553490	0.688620	-0.337143
AM161_005	AM161_012	0.922235	0.343835	-0.147143
AM161_005	AM161_013	2.591520	0.321637	-0.092857
AM161_005	AM161_014	3.008480	0.111752	-0.120000
AM161_005	AM161_015	3.078430	0.027448	-0.011429
AM161_005	AM161_016	0.610070	0.147572	-0.011429
AM161_005	AM161_017	1.644900	-0.025274	0.042857
AM161_005	AM161_018	0.927262	-0.506780	0.341429
AM161_005	AM161_019	4.821380	-0.147947	0.124286

Individual1	Individual2	Geographic distance (km)	Relatedness	
			r (Wang 2002)	Aij (Rousset 2000)
AM161_005	AM161_020	10.339000	0.325711	-0.092857
AM161_005	AM161_021	12.718600	-0.126956	0.015714
AM161_005	AM161_022	6.330370	-0.405063	0.205714
AM161_005	AM161_023	8.307440	-0.277627	0.124286
AM161_005	AM161_024	5.026860	0.154241	-0.038571
AM161_005	AM161_025	0.348953	-0.568531	0.314286
AM161_005	AM161_026	0.746955	0.414911	-0.201429
AM161_005	AM161_027	5.345340	0.036613	-0.065714
AM161_005	AM161_028	5.271180	0.131530	-0.011429
AM161_005	AM161_029	6.228450	0.049552	-0.065714
AM161_005	AM161_030	14.765900	0.033308	0.015714
AM161_005	AM161_031	8.101960	0.060382	-0.065714
AM161_005	AM161_032	0.785811	0.589788	-0.228571
AM161_005	AM161_033	12.629800	-0.082797	0.042857
AM161_005	AM161_034	12.696500	0.054111	-0.092857
AM161_005	AM161_035	12.678900	-0.275841	0.151429
AM161_005	AM161_036	12.683100	0.083688	-0.038571
AM161_005	AM161_037	12.167400	-0.197607	0.151429
AM161_005	AM161_038	11.184200	0.253474	-0.147143
AM161_006	AM161_007	5.175110	0.122635	0.070000
AM161_006	AM161_008	5.157390	-0.017600	0.070000
AM161_006	AM161_009	5.815390	-0.050998	0.097143
AM161_006	AM161_010	4.197290	0.097788	0.015714
AM161_006	AM161_011	4.113140	-0.046609	0.124286
AM161_006	AM161_012	3.914610	0.007499	0.042857
AM161_006	AM161_013	4.095500	-0.300310	0.232857
AM161_006	AM161_014	5.848460	0.221504	-0.120000
AM161_006	AM161_015	5.669360	0.283920	-0.092857
AM161_006	AM161_016	5.238720	-0.015335	-0.011429
AM161_006	AM161_017	3.584790	0.011623	-0.011429
AM161_006	AM161_018	3.892520	0.246722	-0.065714
AM161_006	AM161_019	3.555510	0.079763	0.015714
AM161_006	AM161_020	7.288470	0.246391	-0.120000
AM161_006	AM161_021	9.697210	0.114791	-0.038571
AM161_006	AM161_022	3.950980	-0.090568	0.070000
AM161_006	AM161_023	3.800730	-0.068402	0.042857
AM161_006	AM161_024	2.313940	0.108608	-0.038571
AM161_006	AM161_025	4.444320	0.379409	-0.092857
AM161_006	AM161_026	5.130430	0.043442	0.097143
AM161_006	AM161_027	2.555820	0.201345	-0.011429

Individual1	Individual2	Geographic distance (km)	Relatedness	
			r (Wang 2002)	Aij (Rousset 2000)
AM161_006	AM161_028	0.652245	0.252932	-0.147143
AM161_006	AM161_029	3.646730	0.108463	-0.092857
AM161_006	AM161_030	10.953000	-0.158865	0.151429
AM161_006	AM161_031	5.431980	-0.124185	0.097143
AM161_006	AM161_032	5.196540	-0.024150	0.042857
AM161_006	AM161_033	10.716800	-0.049384	0.124286
AM161_006	AM161_034	10.771700	-0.127854	0.015714
AM161_006	AM161_035	10.715300	-0.190915	0.178571
AM161_006	AM161_036	10.751400	0.121719	-0.011429
AM161_006	AM161_037	9.253760	-0.307226	0.151429
AM161_006	AM161_038	8.815350	-0.163410	0.070000
AM161_007	AM161_008	0.047460	0.208937	-0.065714
AM161_007	AM161_009	2.532380	-0.101112	0.124286
AM161_007	AM161_010	1.697340	0.187610	0.015714
AM161_007	AM161_011	3.345140	0.415166	-0.201429
AM161_007	AM161_012	1.651360	0.470529	-0.228571
AM161_007	AM161_013	3.383350	0.081482	0.042857
AM161_007	AM161_014	2.321290	0.214134	-0.065714
AM161_007	AM161_015	2.432870	0.143337	0.015714
AM161_007	AM161_016	0.358886	-0.116428	0.178571
AM161_007	AM161_017	2.406550	0.077066	0.015714
AM161_007	AM161_018	1.647550	-0.135129	0.205714
AM161_007	AM161_019	4.720650	0.075082	0.070000
AM161_007	AM161_020	10.272400	0.100566	0.042857
AM161_007	AM161_021	12.610300	-0.057709	0.042857
AM161_007	AM161_022	6.286370	-0.143753	0.124286
AM161_007	AM161_023	8.615710	-0.111591	0.070000
AM161_007	AM161_024	5.150230	-0.120048	0.042857
AM161_007	AM161_025	1.126060	-0.039970	0.124286
AM161_007	AM161_026	0.049937	0.585616	-0.282857
AM161_007	AM161_027	5.447290	0.222436	-0.038571
AM161_007	AM161_028	5.684710	-0.011657	0.015714
AM161_007	AM161_029	6.225120	0.167086	-0.120000
AM161_007	AM161_030	14.808300	0.036784	-0.011429
AM161_007	AM161_031	8.024320	0.119925	-0.065714
AM161_007	AM161_032	0.034071	0.405756	-0.174286
AM161_007	AM161_033	12.328000	0.301132	-0.092857
AM161_007	AM161_034	12.395900	-0.050507	-0.038571
AM161_007	AM161_035	12.384700	-0.089622	0.070000
AM161_007	AM161_036	12.383700	0.275238	-0.120000

Individual1	Individual2	Geographic distance (km)	Relatedness	
			r (Wang 2002)	Aij (Rousset 2000)
AM161_007	AM161_037	12.045600	-0.253631	0.151429
AM161_007	AM161_038	10.979700	-0.065062	-0.011429
AM161_008	AM161_009	2.576790	0.206376	-0.092857
AM161_008	AM161_010	1.653160	0.348292	-0.120000
AM161_008	AM161_011	3.299380	0.117012	-0.092857
AM161_008	AM161_012	1.612190	0.162545	-0.011429
AM161_008	AM161_013	3.337660	0.359330	-0.174286
AM161_008	AM161_014	2.366640	-0.113324	0.015714
AM161_008	AM161_015	2.476490	-0.030880	0.015714
AM161_008	AM161_016	0.318874	0.222415	-0.092857
AM161_008	AM161_017	2.365640	-0.014310	0.124286
AM161_008	AM161_018	1.608950	-0.278854	0.124286
AM161_008	AM161_019	4.736800	-0.077001	0.124286
AM161_008	AM161_020	10.288500	-0.033685	-0.011429
AM161_008	AM161_021	12.629000	-0.061137	-0.011429
AM161_008	AM161_022	6.300130	0.080746	0.015714
AM161_008	AM161_023	8.609000	-0.096115	0.015714
AM161_008	AM161_024	5.153530	0.043531	-0.011429
AM161_008	AM161_025	1.082610	-0.189427	0.151429
AM161_008	AM161_026	0.034535	0.305931	-0.147143
AM161_008	AM161_027	5.452060	-0.229818	0.124286
AM161_008	AM161_028	5.670330	0.261202	-0.147143
AM161_008	AM161_029	6.236500	-0.113799	0.015714
AM161_008	AM161_030	14.818600	-0.043451	-0.011429
AM161_008	AM161_031	8.040580	-0.285516	0.151429
AM161_008	AM161_032	0.042952	0.262577	-0.120000
AM161_008	AM161_033	12.357100	-0.101224	0.042857
AM161_008	AM161_034	12.424900	-0.143381	0.015714
AM161_008	AM161_035	12.413400	-0.027160	0.042857
AM161_008	AM161_036	12.412700	0.236443	-0.174286
AM161_008	AM161_037	12.065100	-0.150916	0.124286
AM161_008	AM161_038	11.003500	-0.135023	0.015714
AM161_009	AM161_010	3.961080	0.373802	-0.147143
AM161_009	AM161_011	5.594140	0.139803	-0.038571
AM161_009	AM161_012	3.737660	0.176948	-0.011429
AM161_009	AM161_013	5.625730	0.082687	0.015714
AM161_009	AM161_014	0.257676	-0.031388	-0.038571
AM161_009	AM161_015	0.151475	-0.026078	0.015714
AM161_009	AM161_016	2.886840	0.195307	-0.038571
AM161_009	AM161_017	4.458310	-0.187893	0.260000

Individual1	Individual2	Geographic distance (km)	Relatedness	
			r (Wang 2002)	Aij (Rousset 2000)
AM161_009	AM161_018	3.715550	-0.410643	0.341429
AM161_009	AM161_019	3.631910	-0.113335	0.151429
AM161_009	AM161_020	8.840130	0.211345	-0.038571
AM161_009	AM161_021	11.004400	0.017454	-0.011429
AM161_009	AM161_022	5.154250	-0.085830	0.124286
AM161_009	AM161_023	8.436780	-0.217744	0.151429
AM161_009	AM161_024	4.701740	0.275589	-0.120000
AM161_009	AM161_025	3.432410	-0.339283	0.260000
AM161_009	AM161_026	2.556010	0.328571	-0.120000
AM161_009	AM161_027	4.883630	-0.029666	0.015714
AM161_009	AM161_028	6.103580	0.327387	-0.092857
AM161_009	AM161_029	5.226900	-0.149179	0.070000
AM161_009	AM161_030	13.584900	0.159551	0.015714
AM161_009	AM161_031	6.656140	-0.116504	0.070000
AM161_009	AM161_032	2.558500	0.388513	-0.120000
AM161_009	AM161_033	10.252400	-0.145517	0.097143
AM161_009	AM161_034	10.321800	-0.111559	0.042857
AM161_009	AM161_035	10.325100	-0.188791	0.151429
AM161_009	AM161_036	10.312500	-0.113786	0.042857
AM161_009	AM161_037	10.415000	-0.230475	0.151429
AM161_009	AM161_038	9.156390	0.094396	-0.011429
AM161_010	AM161_011	1.665560	0.138899	-0.065714
AM161_010	AM161_012	0.376092	0.218699	-0.038571
AM161_010	AM161_013	1.701620	0.057960	0.042857
AM161_010	AM161_014	3.800300	0.045711	-0.011429
AM161_010	AM161_015	3.832030	0.123652	-0.065714
AM161_010	AM161_016	1.500710	0.364850	-0.174286
AM161_010	AM161_017	0.777857	0.199307	0.042857
AM161_010	AM161_018	0.410033	-0.269924	0.260000
AM161_010	AM161_019	4.974850	0.043438	0.097143
AM161_010	AM161_020	10.370000	-0.013429	0.015714
AM161_010	AM161_021	12.783700	0.171356	-0.038571
AM161_010	AM161_022	6.382720	0.042942	0.042857
AM161_010	AM161_023	7.921810	-0.297938	0.151429
AM161_010	AM161_024	4.917410	0.070341	-0.065714
AM161_010	AM161_025	0.573541	-0.263712	0.205714
AM161_010	AM161_026	1.650610	0.286758	-0.092857
AM161_010	AM161_027	5.251930	0.186495	-0.065714
AM161_010	AM161_028	4.801680	0.137375	-0.065714
AM161_010	AM161_029	6.236350	-0.213403	0.124286

Individual1	Individual2	Geographic distance (km)	Relatedness	
			r (Wang 2002)	Aij (Rousset 2000)
AM161_010	AM161_030	14.649600	0.035352	0.042857
AM161_010	AM161_031	8.166150	-0.021502	0.015714
AM161_010	AM161_032	1.693000	0.377728	-0.174286
AM161_010	AM161_033	12.917200	-0.208320	0.178571
AM161_010	AM161_034	12.982500	-0.204061	0.070000
AM161_010	AM161_035	12.957500	-0.193607	0.205714
AM161_010	AM161_036	12.967600	-0.122822	0.097143
AM161_010	AM161_037	12.250600	-0.243296	0.178571
AM161_010	AM161_038	11.367500	-0.173622	0.097143
AM161_011	AM161_012	1.856930	0.158250	-0.038571
AM161_011	AM161_013	0.042931	0.036597	0.042857
AM161_011	AM161_014	5.446330	0.243212	-0.120000
AM161_011	AM161_015	5.459610	0.195908	-0.092857
AM161_011	AM161_016	3.105180	-0.008078	0.015714
AM161_011	AM161_017	1.192700	-0.304366	0.232857
AM161_011	AM161_018	1.880010	-0.181660	0.151429
AM161_011	AM161_019	5.989020	-0.275660	0.178571
AM161_011	AM161_020	11.045300	0.421859	-0.147143
AM161_011	AM161_021	13.482100	-0.312331	0.151429
AM161_011	AM161_022	7.191430	-0.470855	0.232857
AM161_011	AM161_023	7.898090	-0.285030	0.124286
AM161_011	AM161_024	5.551060	0.052948	0.015714
AM161_011	AM161_025	2.235300	-0.322949	0.232857
AM161_011	AM161_026	3.300390	0.531339	-0.282857
AM161_011	AM161_027	5.889440	0.168064	-0.092857
AM161_011	AM161_028	4.765350	0.148451	-0.065714
AM161_011	AM161_029	6.983330	0.050341	-0.065714
AM161_011	AM161_030	15.020600	0.166032	-0.038571
AM161_011	AM161_031	8.938160	0.031529	-0.065714
AM161_011	AM161_032	3.337210	0.466636	-0.228571
AM161_011	AM161_033	13.954200	0.092311	0.015714
AM161_011	AM161_034	14.016700	0.131106	-0.147143
AM161_011	AM161_035	13.981300	-0.228015	0.097143
AM161_011	AM161_036	14.000000	0.273101	-0.092857
AM161_011	AM161_037	12.984000	-0.382875	0.260000
AM161_011	AM161_038	12.266400	0.216844	-0.120000
AM161_012	AM161_013	1.888150	-0.173789	0.124286
AM161_012	AM161_014	3.593890	0.135567	-0.147143
AM161_012	AM161_015	3.602730	-0.113198	0.042857
AM161_012	AM161_016	1.530580	-0.119402	0.097143

Individual1	Individual2	Geographic distance (km)	Relatedness	
			r (Wang 2002)	Aij (Rousset 2000)
AM161_012	AM161_017	0.766276	0.158859	-0.038571
AM161_012	AM161_018	0.034137	-0.377977	0.260000
AM161_012	AM161_019	4.600350	0.368402	-0.120000
AM161_012	AM161_020	9.995630	0.081645	0.015714
AM161_012	AM161_021	12.408400	0.077063	-0.038571
AM161_012	AM161_022	6.007000	0.211645	-0.092857
AM161_012	AM161_023	7.605540	-0.231641	0.151429
AM161_012	AM161_024	4.549540	0.080206	-0.011429
AM161_012	AM161_025	0.583554	-0.035184	0.070000
AM161_012	AM161_026	1.601870	0.256135	-0.120000
AM161_012	AM161_027	4.883060	0.110388	-0.120000
AM161_012	AM161_028	4.504570	0.304861	-0.147143
AM161_012	AM161_029	5.861890	0.120805	-0.092857
AM161_012	AM161_030	14.287400	0.208894	-0.038571
AM161_012	AM161_031	7.790470	0.171651	-0.120000
AM161_012	AM161_032	1.654620	0.429096	-0.228571
AM161_012	AM161_033	12.547600	0.113572	-0.038571
AM161_012	AM161_034	12.612700	-0.269867	0.097143
AM161_012	AM161_035	12.587100	-0.015864	0.070000
AM161_012	AM161_036	12.597800	0.037737	-0.065714
AM161_012	AM161_037	11.874900	-0.031904	-0.011429
AM161_012	AM161_038	10.992400	0.030986	0.015714
AM161_013	AM161_014	5.479010	-0.313587	0.178571
AM161_013	AM161_015	5.490840	-0.275397	0.232857
AM161_013	AM161_016	3.144870	0.461117	-0.092857
AM161_013	AM161_017	1.215650	-0.131239	0.097143
AM161_013	AM161_018	1.910820	-0.441919	0.314286
AM161_013	AM161_019	5.997280	-0.407241	0.287143
AM161_013	AM161_020	11.041400	-0.085883	0.097143
AM161_013	AM161_021	13.478300	0.198689	-0.147143
AM161_013	AM161_022	7.192900	-0.077540	0.124286
AM161_013	AM161_023	7.878010	-0.216816	0.015714
AM161_013	AM161_024	5.549230	0.088008	0.042857
AM161_013	AM161_025	2.272100	-0.415771	0.260000
AM161_013	AM161_026	3.338470	0.067849	0.042857
AM161_013	AM161_027	5.887340	-0.419156	0.260000
AM161_013	AM161_028	4.747740	0.135720	0.070000
AM161_013	AM161_029	6.983230	-0.330843	0.178571
AM161_013	AM161_030	15.007900	-0.148505	0.015714
AM161_013	AM161_031	8.937670	-0.396378	0.260000

Individual1	Individual2	Geographic distance (km)	Relatedness	
			r (Wang 2002)	Aij (Rousset 2000)
AM161_013	AM161_032	3.375610	0.186558	0.015714
AM161_013	AM161_033	13.960200	-0.051994	0.070000
AM161_013	AM161_034	14.022700	-0.360790	0.151429
AM161_013	AM161_035	13.986900	-0.183380	0.151429
AM161_013	AM161_036	14.005800	0.319123	-0.147143
AM161_013	AM161_037	12.981300	-0.343281	0.205714
AM161_013	AM161_038	12.268600	-0.074580	0.015714
AM161_014	AM161_015	0.277524	0.649825	-0.282857
AM161_014	AM161_016	2.672050	-0.100154	0.015714
AM161_014	AM161_017	4.327620	-0.055672	0.015714
AM161_014	AM161_018	3.573340	0.116777	-0.011429
AM161_014	AM161_019	3.808080	-0.040080	-0.038571
AM161_014	AM161_020	9.066780	0.056663	-0.065714
AM161_014	AM161_021	11.243200	0.203289	-0.065714
AM161_014	AM161_022	5.347920	0.009265	-0.011429
AM161_014	AM161_023	8.563790	0.001846	0.042857
AM161_014	AM161_024	4.829940	-0.326257	0.097143
AM161_014	AM161_025	3.260280	-0.078719	0.097143
AM161_014	AM161_026	2.347570	0.007811	-0.011429
AM161_014	AM161_027	5.024630	0.405210	-0.201429
AM161_014	AM161_028	6.161190	0.079985	-0.120000
AM161_014	AM161_029	5.409740	-0.000776	-0.065714
AM161_014	AM161_030	13.803100	0.043571	-0.011429
AM161_014	AM161_031	6.874180	-0.005764	-0.038571
AM161_014	AM161_032	2.346050	0.080183	-0.092857
AM161_014	AM161_033	10.508900	0.067678	-0.011429
AM161_014	AM161_034	10.578300	0.051256	-0.038571
AM161_014	AM161_035	10.581300	0.095928	0.015714
AM161_014	AM161_036	10.568900	0.140460	-0.065714
AM161_014	AM161_037	10.654700	-0.098125	0.042857
AM161_014	AM161_038	9.404450	-0.000512	-0.038571
AM161_015	AM161_016	2.789440	-0.052984	-0.011429
AM161_015	AM161_017	4.319110	-0.089750	0.070000
AM161_015	AM161_018	3.580130	0.147927	-0.038571
AM161_015	AM161_019	3.538010	-0.185030	0.097143
AM161_015	AM161_020	8.790180	0.157955	-0.120000
AM161_015	AM161_021	10.970800	0.005978	0.124286
AM161_015	AM161_022	5.073060	-0.138064	0.097143
AM161_015	AM161_023	8.317700	-0.028822	0.042857
AM161_015	AM161_024	4.582600	-0.085532	-0.011429

Individual1	Individual2	Geographic distance (km)	Relatedness	
			r (Wang 2002)	Aij (Rousset 2000)
AM161_015	AM161_025	3.308610	-0.238378	0.232857
AM161_015	AM161_026	2.454480	0.132456	-0.065714
AM161_015	AM161_027	4.770350	0.370448	-0.174286
AM161_015	AM161_028	5.962240	0.012597	-0.065714
AM161_015	AM161_029	5.137800	-0.129343	0.042857
AM161_015	AM161_030	13.525600	-0.091996	0.070000
AM161_015	AM161_031	6.596700	-0.141476	0.070000
AM161_015	AM161_032	2.459980	0.113191	-0.120000
AM161_015	AM161_033	10.262900	-0.193399	0.178571
AM161_015	AM161_034	10.332100	0.066672	-0.065714
AM161_015	AM161_035	10.333800	-0.149155	0.178571
AM161_015	AM161_036	10.322500	-0.047354	0.042857
AM161_015	AM161_037	10.382800	-0.141567	0.151429
AM161_015	AM161_038	9.139600	-0.205603	0.124286
AM161_016	AM161_017	2.252260	0.130862	0.015714
AM161_016	AM161_018	1.534050	-0.180127	0.124286
AM161_016	AM161_019	5.001110	-0.158024	0.151429
AM161_016	AM161_020	10.550400	0.161585	-0.120000
AM161_016	AM161_021	12.900800	0.002231	0.097143
AM161_016	AM161_022	6.553760	0.018837	0.015714
AM161_016	AM161_023	8.757330	-0.047065	-0.065714
AM161_016	AM161_024	5.359970	0.349469	-0.174286
AM161_016	AM161_025	0.958012	-0.389599	0.260000
AM161_016	AM161_026	0.346615	0.013153	0.042857
AM161_016	AM161_027	5.665450	-0.167043	0.070000
AM161_016	AM161_028	5.773400	0.285269	-0.120000
AM161_016	AM161_029	6.479970	-0.446548	0.151429
AM161_016	AM161_030	15.054900	-0.085155	0.070000
AM161_016	AM161_031	8.303670	-0.245185	0.070000
AM161_016	AM161_032	0.330000	0.065511	-0.038571
AM161_016	AM161_033	12.661900	-0.050085	0.097143
AM161_016	AM161_034	12.729600	-0.071464	-0.038571
AM161_016	AM161_035	12.717300	-0.133556	0.178571
AM161_016	AM161_036	12.717200	0.142221	-0.011429
AM161_016	AM161_037	12.339500	-0.110559	0.042857
AM161_016	AM161_038	11.293300	-0.206500	0.124286
AM161_017	AM161_018	0.777945	-0.195782	0.205714
AM161_017	AM161_019	4.890150	0.182102	-0.011429
AM161_017	AM161_020	10.114900	-0.157804	0.097143
AM161_017	AM161_021	12.545200	0.006164	-0.011429

Individual1	Individual2	Geographic distance (km)	Relatedness	
			r (Wang 2002)	Aij (Rousset 2000)
AM161_017	AM161_022	6.183160	0.057525	0.015714
AM161_017	AM161_023	7.363950	-0.084358	0.097143
AM161_017	AM161_024	4.612250	0.097485	-0.038571
AM161_017	AM161_025	1.295990	-0.288274	0.232857
AM161_017	AM161_026	2.357600	-0.149004	0.178571
AM161_017	AM161_027	4.952210	-0.206019	0.151429
AM161_017	AM161_028	4.213930	-0.144164	0.097143
AM161_017	AM161_029	6.001980	-0.206019	0.124286
AM161_017	AM161_030	14.261500	0.008716	0.042857
AM161_017	AM161_031	7.954700	-0.264113	0.260000
AM161_017	AM161_032	2.407490	-0.136878	0.124286
AM161_017	AM161_033	12.868700	-0.066755	0.070000
AM161_017	AM161_034	12.932400	0.018916	0.042857
AM161_017	AM161_035	12.900900	-0.039062	0.097143
AM161_017	AM161_036	12.916400	-0.054854	0.015714
AM161_017	AM161_037	12.029200	-0.168677	0.070000
AM161_017	AM161_038	11.234700	-0.133448	0.124286
AM161_018	AM161_019	4.566220	-0.272966	0.232857
AM161_018	AM161_020	9.962240	-0.028256	0.015714
AM161_018	AM161_021	12.374800	-0.360820	0.260000
AM161_018	AM161_022	5.973290	-0.072085	0.070000
AM161_018	AM161_023	7.579400	-0.186819	0.070000
AM161_018	AM161_024	4.517250	-0.259720	0.232857
AM161_018	AM161_025	0.592732	0.458700	-0.120000
AM161_018	AM161_026	1.597930	-0.200865	0.178571
AM161_018	AM161_027	4.850620	-0.005394	0.097143
AM161_018	AM161_028	4.480780	-0.027725	-0.011429
AM161_018	AM161_029	5.828490	-0.147874	0.042857
AM161_018	AM161_030	14.255800	-0.021785	0.042857
AM161_018	AM161_031	7.756770	-0.204480	0.151429
AM161_018	AM161_032	1.651510	-0.315749	0.151429
AM161_018	AM161_033	12.513600	-0.071361	0.097143
AM161_018	AM161_034	12.578700	-0.028244	0.015714
AM161_018	AM161_035	12.553100	-0.161452	0.151429
AM161_018	AM161_036	12.563700	-0.111588	0.042857
AM161_018	AM161_037	11.841100	-0.594565	0.314286
AM161_018	AM161_038	10.958300	-0.323017	0.205714
AM161_019	AM161_020	5.551790	-0.039716	0.015714
AM161_019	AM161_021	7.903900	-0.006639	0.015714
AM161_019	AM161_022	1.591030	0.211653	-0.092857

Individual1	Individual2	Geographic distance (km)	Relatedness	
			r (Wang 2002)	Aij (Rousset 2000)
AM161_019	AM161_023	4.963030	0.047975	0.070000
AM161_019	AM161_024	1.429960	-0.067892	0.070000
AM161_019	AM161_025	4.783910	-0.016413	0.070000
AM161_019	AM161_026	4.702320	0.016543	0.070000
AM161_019	AM161_027	1.441800	0.081851	-0.065714
AM161_019	AM161_028	3.466800	-0.028299	-0.011429
AM161_019	AM161_029	1.606720	0.192788	-0.065714
AM161_019	AM161_030	10.141500	-0.056448	0.097143
AM161_019	AM161_031	3.303790	0.022120	0.042857
AM161_019	AM161_032	4.754260	-0.009071	0.015714
AM161_019	AM161_033	7.978620	-0.093928	0.070000
AM161_019	AM161_034	8.042350	-0.253415	0.151429
AM161_019	AM161_035	8.011370	-0.207369	0.151429
AM161_019	AM161_036	8.026340	-0.216613	0.070000
AM161_019	AM161_037	7.348150	-0.449712	0.205714
AM161_019	AM161_038	6.394110	-0.047284	0.042857
AM161_020	AM161_021	2.436920	-0.173652	0.097143
AM161_020	AM161_022	4.008760	-0.217218	0.070000
AM161_020	AM161_023	4.839800	-0.184045	0.042857
AM161_020	AM161_024	5.506140	0.210130	-0.065714
AM161_020	AM161_025	10.264800	-0.285524	0.232857
AM161_020	AM161_026	10.254000	0.405804	-0.147143
AM161_020	AM161_027	5.165970	0.101215	-0.065714
AM161_020	AM161_028	6.727400	0.445676	-0.228571
AM161_020	AM161_029	4.133760	0.213461	-0.174286
AM161_020	AM161_030	4.794590	0.215440	-0.065714
AM161_020	AM161_031	2.248330	0.040936	-0.065714
AM161_020	AM161_032	10.306100	0.282070	-0.147143
AM161_020	AM161_033	4.090120	0.174977	-0.038571
AM161_020	AM161_034	4.120390	0.192660	-0.174286
AM161_020	AM161_035	4.027740	-0.087172	0.042857
AM161_020	AM161_036	4.095580	0.354552	-0.174286
AM161_020	AM161_037	1.965340	-0.176971	0.070000
AM161_020	AM161_038	2.043040	0.178713	-0.038571
AM161_021	AM161_022	6.403990	0.001872	0.015714
AM161_021	AM161_023	6.917260	0.039959	0.015714
AM161_021	AM161_024	7.940240	0.113533	0.015714
AM161_021	AM161_025	12.659100	0.038593	-0.011429
AM161_021	AM161_026	12.594700	-0.185984	0.124286
AM161_021	AM161_027	7.600210	0.024843	0.042857

Individual1	Individual2	Geographic distance (km)	Relatedness	
			r (Wang 2002)	Aij (Rousset 2000)
AM161_021	AM161_028	9.119810	0.290709	-0.065714
AM161_021	AM161_029	6.551280	-0.149794	0.015714
AM161_021	AM161_030	3.362640	0.030349	-0.038571
AM161_021	AM161_031	4.621700	-0.229496	0.151429
AM161_021	AM161_032	12.644200	0.022872	0.015714
AM161_021	AM161_033	3.184450	-0.062813	0.042857
AM161_021	AM161_034	3.171550	-0.170273	0.097143
AM161_021	AM161_035	3.059820	0.102314	0.015714
AM161_021	AM161_036	3.148780	0.054535	-0.065714
AM161_021	AM161_037	0.597150	-0.172780	0.124286
AM161_021	AM161_038	2.158620	-0.043488	-0.065714
AM161_022	AM161_023	3.939890	-0.037757	0.015714
AM161_022	AM161_024	1.712240	-0.156874	0.015714
AM161_022	AM161_025	6.258340	-0.006081	0.015714
AM161_022	AM161_026	6.265590	-0.016191	0.042857
AM161_022	AM161_027	1.411670	-0.230235	0.042857
AM161_022	AM161_028	3.598460	-0.021347	-0.011429
AM161_022	AM161_029	0.332182	-0.071004	-0.038571
AM161_022	AM161_030	8.551020	0.014655	-0.011429
AM161_022	AM161_031	1.783520	-0.136666	0.015714
AM161_022	AM161_032	6.319650	-0.146127	0.042857
AM161_022	AM161_033	6.849000	-0.110036	0.070000
AM161_022	AM161_034	6.907740	-0.163740	0.070000
AM161_022	AM161_035	6.861140	-0.078364	0.151429
AM161_022	AM161_036	6.889000	-0.145815	0.042857
AM161_022	AM161_037	5.867870	-0.205459	0.097143
AM161_022	AM161_038	5.077500	-0.131752	0.042857
AM161_023	AM161_024	3.735180	0.073387	-0.065714
AM161_023	AM161_025	8.084500	-0.049641	0.070000
AM161_023	AM161_026	8.577670	-0.186440	0.070000
AM161_023	AM161_027	3.570590	-0.118972	0.015714
AM161_023	AM161_028	3.155850	0.017533	-0.011429
AM161_023	AM161_029	3.672400	0.138089	-0.065714
AM161_023	AM161_030	7.409420	-0.093023	0.015714
AM161_023	AM161_031	4.089520	0.031783	0.015714
AM161_023	AM161_032	8.642840	-0.193383	0.042857
AM161_023	AM161_033	8.875790	-0.093142	0.015714
AM161_023	AM161_034	8.913110	0.345476	-0.147143
AM161_023	AM161_035	8.827720	0.022446	0.042857
AM161_023	AM161_036	8.888970	-0.166914	0.015714

Individual1	Individual2	Geographic distance (km)	Relatedness	
			r (Wang 2002)	Aij (Rousset 2000)
AM161_023	AM161_037	6.630040	0.013914	0.097143
AM161_023	AM161_038	6.809030	-0.133824	0.042857
AM161_024	AM161_025	4.891010	-0.061595	0.070000
AM161_024	AM161_026	5.119830	0.025924	-0.065714
AM161_024	AM161_027	0.340205	-0.065595	0.015714
AM161_024	AM161_028	2.096820	0.444756	-0.174286
AM161_024	AM161_029	1.455020	-0.011299	-0.038571
AM161_024	AM161_030	9.749380	0.096135	0.015714
AM161_024	AM161_031	3.394940	-0.302975	0.124286
AM161_024	AM161_032	5.181050	0.295589	-0.147143
AM161_024	AM161_033	8.560520	0.042764	0.042857
AM161_024	AM161_034	8.618990	0.005456	-0.065714
AM161_024	AM161_035	8.571470	0.079975	0.070000
AM161_024	AM161_036	8.600110	0.189828	-0.038571
AM161_024	AM161_037	7.434780	-0.114747	0.070000
AM161_024	AM161_038	6.760610	0.075115	-0.011429
AM161_025	AM161_026	1.078740	-0.156134	0.178571
AM161_025	AM161_027	5.216400	-0.029662	0.042857
AM161_025	AM161_028	5.016880	0.013344	0.015714
AM161_025	AM161_029	6.139370	0.020636	-0.011429
AM161_025	AM161_030	14.640100	-0.063001	0.070000
AM161_025	AM161_031	8.037770	-0.130047	0.097143
AM161_025	AM161_032	1.123130	-0.241107	0.124286
AM161_025	AM161_033	12.657800	0.046140	0.042857
AM161_025	AM161_034	12.724100	-0.252423	0.178571
AM161_025	AM161_035	12.703400	-0.017357	0.070000
AM161_025	AM161_036	12.710000	-0.089163	0.097143
AM161_025	AM161_037	12.114500	-0.499129	0.314286
AM161_025	AM161_038	11.169600	-0.103629	0.070000
AM161_026	AM161_027	5.418150	0.170564	-0.065714
AM161_026	AM161_028	5.641830	0.122088	-0.092857
AM161_026	AM161_029	6.202000	0.087893	-0.092857
AM161_026	AM161_030	14.784200	0.062161	-0.038571
AM161_026	AM161_031	8.006100	0.086657	-0.092857
AM161_026	AM161_032	0.066233	0.413656	-0.228571
AM161_026	AM161_033	12.324900	0.082096	0.015714
AM161_026	AM161_034	12.392700	0.177557	-0.174286
AM161_026	AM161_035	12.381100	-0.270706	0.151429
AM161_026	AM161_036	12.380400	0.194222	-0.092857
AM161_026	AM161_037	12.030800	-0.238554	0.151429

Individual1	Individual2	Geographic distance (km)	Relatedness	
			r (Wang 2002)	Aij (Rousset 2000)
AM161_026	AM161_038	10.969900	-0.004695	-0.038571
AM161_027	AM161_028	2.268520	0.032270	-0.065714
AM161_027	AM161_029	1.135190	0.163064	-0.120000
AM161_027	AM161_030	9.424090	-0.180358	0.124286
AM161_027	AM161_031	3.061740	0.218974	-0.147143
AM161_027	AM161_032	5.478560	0.339900	-0.255714
AM161_027	AM161_033	8.250010	-0.066536	0.070000
AM161_027	AM161_034	8.307840	-0.311901	0.042857
AM161_027	AM161_035	8.258710	-0.198044	0.124286
AM161_027	AM161_036	8.288700	-0.096046	0.070000
AM161_027	AM161_037	7.095530	-0.180945	0.070000
AM161_027	AM161_038	6.434230	-0.118975	0.015714
AM161_028	AM161_029	3.277650	0.172174	-0.174286
AM161_028	AM161_030	10.310000	0.167202	-0.011429
AM161_028	AM161_031	4.957350	-0.130977	0.015714
AM161_028	AM161_032	5.708060	0.390864	-0.228571
AM161_028	AM161_033	10.256200	0.242522	-0.065714
AM161_028	AM161_034	10.309100	-0.028167	-0.065714
AM161_028	AM161_035	10.248600	0.252856	-0.065714
AM161_028	AM161_036	10.288200	0.466672	-0.228571
AM161_028	AM161_037	8.690390	-0.139601	0.042857
AM161_028	AM161_038	8.318900	0.186620	-0.038571
AM161_029	AM161_030	8.585530	0.048112	-0.038571
AM161_029	AM161_031	1.955960	0.555773	-0.255714
AM161_029	AM161_032	6.257980	0.228841	-0.174286
AM161_029	AM161_033	7.115560	0.207209	-0.147143
AM161_029	AM161_034	7.173200	0.216540	-0.174286
AM161_029	AM161_035	7.123700	0.030160	-0.065714
AM161_029	AM161_036	7.153990	0.197208	-0.174286
AM161_029	AM161_037	6.028240	-0.305320	0.151429
AM161_029	AM161_038	5.306230	0.429167	-0.228571
AM161_030	AM161_031	6.929010	0.094204	-0.038571
AM161_030	AM161_032	14.841000	-0.032358	0.015714
AM161_030	AM161_033	6.416780	0.212512	-0.147143
AM161_030	AM161_034	6.389790	0.016367	-0.038571
AM161_030	AM161_035	6.281810	0.081126	-0.065714
AM161_030	AM161_036	6.369890	-0.009447	-0.092857
AM161_030	AM161_037	3.860710	-0.478864	0.260000
AM161_030	AM161_038	5.462660	0.189472	-0.092857
AM161_031	AM161_032	8.057960	0.072863	-0.120000

Individual1	Individual2	Geographic distance (km)	Relatedness	
			r (Wang 2002)	Aij (Rousset 2000)
AM161_031	AM161_033	5.299190	0.156174	-0.092857
AM161_031	AM161_034	5.351860	0.193832	-0.120000
AM161_031	AM161_035	5.291340	-0.149291	0.097143
AM161_031	AM161_036	5.330900	-0.144170	0.042857
AM161_031	AM161_037	4.084510	-0.160541	0.124286
AM161_031	AM161_038	3.390770	0.211318	-0.065714
AM161_032	AM161_033	12.361700	-0.128672	0.042857
AM161_032	AM161_034	12.429500	-0.084509	-0.038571
AM161_032	AM161_035	12.418400	-0.204161	0.097143
AM161_032	AM161_036	12.417400	0.232564	-0.147143
AM161_032	AM161_037	12.079600	-0.173795	0.042857
AM161_032	AM161_038	11.013700	0.188924	-0.065714
AM161_033	AM161_034	0.069787	-0.019921	-0.038571
AM161_033	AM161_035	0.139325	0.689120	-0.364286
AM161_033	AM161_036	0.067585	0.642866	-0.310000
AM161_033	AM161_037	2.923050	-0.273877	0.178571
AM161_033	AM161_038	2.067360	0.209643	-0.147143
AM161_034	AM161_035	0.111840	0.047827	-0.011429
AM161_034	AM161_036	0.025246	-0.007231	-0.065714
AM161_034	AM161_037	2.922460	-0.075991	0.070000
AM161_034	AM161_038	2.104080	0.167735	-0.120000
AM161_035	AM161_036	0.089139	0.487490	-0.255714
AM161_035	AM161_037	2.811660	-0.149686	0.178571
AM161_035	AM161_038	2.019730	0.203186	-0.147143
AM161_036	AM161_037	2.898110	-0.151434	0.070000
AM161_036	AM161_038	2.079960	0.196937	-0.174286
AM161_037	AM161_038	1.606120	-0.533333	0.260000

23rd October 2015

Australian Centre for Wildlife Genomics Results Report
Addendum Report II

This is a second addendum to the previous report by Neaves *et al.* (2015), relating to the genetic assessment of a Koala (*Phascolarctos cinereus*) population in the Wardell area of the mid-north coast of New South Wales, which was conducted by the Australian Centre for Wildlife Genomics (ACWG), Australian Museum Research Institute and submitted to the Roads and Maritime Service (RMS) on the 12th August 2015.

Addendum details:

On the 14th October 2015, following the Koala Expert Advisory Committee PVA Workshop, RMS requested estimates of dispersal (the number of Koalas per generation; *Nm*) to be calculated using the Shannon's Mutual Information index (Sherwin et al., 2006; Rossetto et al., 2008; Dewar et al., 2011) under the assumption that dispersal was symmetrical. These analyses were to be completed by Friday the 23rd October for inclusion into the PVA undertaken by RMS.

Estimates of dispersal were requested for:

1. Between Wardell and the surrounding areas; and
2. Within Wardell, between areas East and West of the proposed upgrade
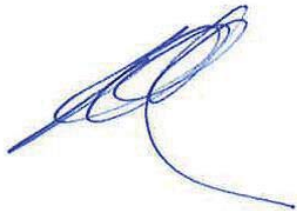
The average level of dispersal between Wardell and surrounding sites ranged from 0.199 – 0.400 individuals per generation. These levels are relatively low but reflect the geographical distance between sites. An estimate of 0.400 (per-locus range 0.076 – 23.224, Table 1) Koalas per generation from the comparison between Wardell and Tyagarah represents the most useful estimate for the PVA, as these

sites are closest geographically. Dispersal of males and females appears similar (Nm_{MALES} 0.304 Koalas per generation, $Nm_{FEMALES}$ 0.386 Koalas per generation, Table 2).

Within Wardell, 1.56 Koalas per generation dispersed between the Eastern and Western sides of the proposed upgrade, with an upper limit of 50.588 and lower limit 0.145 based on individual loci. Given the small sample sizes we did not assess differences between the sexes.

The details of the analyses and results are described below and a complete list of the pairwise dispersal estimates for each locus and overall is appended at the end of this report.

Yours sincerely,

Dr Rebecca Johnson, on behalf of the co-authors listed below.

Dr Linda Neaves, Siobhan Dennison, Dr Greta Frankham, and Dr Mark Eldridge

Details of Analyses and Results:

We generated locus-by-locus and overall estimates of dispersal between Wardell and surrounding locations (Port Macquarie, Coffs Harbour and Tyagarah), and within Wardell using the same samples and 17 microsatellite markers described in the original report (Neaves *et al.,* 2015). Pairwise estimates of dispersal (*Nm*) were calculated using the Shannon's Mutual Information index (Sherwin et al., 2006; Rossetto et al., 2008; Dewar et al., 2011) implemented in GENALEX (Peakall and Smouse 2006, 2012). Where possible data on the sex of sampled koalas was included, for Wardell samples this was obtained from Phillips *et al*. 2015 (20 females, 15 males, 3 unknown).

1. Between Wardell and the surrounding areas

Overall the estimates of dispersal in and out of Wardell were relatively low (*Nm* = 0.199 – 0.400 Koalas per generation across all sites, Table 1), but this is not surprising given the distance between Wardell and these sites. The highest level of dispersal was found between Wardell and Tyagarah (*Nm* 0.400 ; Table 1), which are closest geographically. This is consistent with the previous genetic analysis showing Wardell and Tyagarah were genetically similar (i.e. the same genetic cluster, Figure 6 of Neaves *et al*. 2015). Given the proximity of the two sites this value represents the most useful estimate for the PVA, but the level of immigration/emigration is likely to be higher between areas closer to Wardell, e.g. Lismore.

Comparisons of male and female dispersal between Tyagarah and Wardell indicate similar levels of dispersal for females (*Nm* 0.386) and males (*Nm* 0.304). These estimates of dispersal are comparable to the estimate obtained overall (Table 2).

2. Within Wardell, between areas East and West of the proposed upgrade

Comparison of Koalas sampled East and West of the proposed upgrade indicated 1.5 Koalas per generation are dispersing, with estimates for individual loci ranging from 0.145 to 50.588. These estimates should be treated with caution given the small sample size, particularly for the Eastern side of the proposed road (*n* 5). To assess variation in movement across Wardell and potential impacts of skewed sample sizes we assessed dispersal between eastern and western areas of Wardell using even sample sizes (rather than the proposed road). This indicated approximately 11.6 Koalas moved per generation across the site. Hence, while it is possible that dispersal within Wardell has been underestimated due to the skewed sample size it likely falls within the range reported above.

References

Dewar, R.C., Sherwin, W.B., Thomas, E., Holleley, C.E., Nichols, R.A. 2011. Predictions of single-nucleotide polymorphism differentiation between two populations in terms of mutual mutation. *Molecular Ecology* **20**:3156–3166.

Neaves, L.E., Dennison, S.B., Frankham, G.J., Eldridge, M.D.B., Johnson, R.N. 2015. *Koala population genetics management.* Report to Roads and Maritime Services, NSW.

Peakall, R. and Smouse, P.E. 2006. GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. *Molecular Ecology Notes* **6**:288–295.

Peakall, R. and Smouse, P.E. 2012. GenAlEx 6.5: genetic analysis in Excel. Population genetic software for teaching and research – an update. *Bioinformatics* **28**:2537–2539.

Phillips, S., Brearley, G., and Callaghan, J. 2015. *Assessment of koala population distribution and demographics: Woolgoolga to Ballina Pacific Highway Upgrade (Section 10 – Wardell to Coolgardie).* Final report to Roads and Maritime, Biolink Ecological Consultants and Ecosure Pty Ltd.

Rossetto, M., Kooyman, R., Sherwin, W., Jones, R. 2008. Dispersal limitation, rather than bottlenecks or habitat specificity, can restrict the distribution of rare and endemic rainforest trees. *American Journal of Botany* **95**:321–329.

Sherwin, W.B., Jabot, F., Rush, R., Rossetto, M. 2006. Measurement of biological information with applications from genes to landscapes. *Molecular Ecology* **15**:2857–2869.

Table 1. Locus by locus estimates of dispersal (Koalas per generation; *Nm*) between sampling locations based on Shannon's mutual information index. Overall *Nm* estimates can be found at the bottom

Port Macquarie Wardell		Coffs Harbour Wardell		Tyagarah Wardell	
Locus	Nm	Locus	Nm	Locus	Nm
Pcin3	0.256	Pcin3	0.611	Pcin3	0.920
Pcin5	0.098	Pcin5	0.451	Pcin5	0.161
Pcin6	0.131	Pcin6	0.153	Pcin6	0.168
Pcin7	1.702	Pcin7	0.720	Pcin7	23.224
Pcin8	0.326	Pcin8	0.491	Pcin8	0.451
Pcin9	0.122	Pcin9	0.330	Pcin9	0.231
Pcin10	0.036	Pcin10	0.063	Pcin10	3.298
Pcin11	0.048	Pcin11	0.089	Pcin11	0.076
Pcin14	2.314	Pcin14	0.340	Pcin14	0.492
Pcin15	0.328	Pcin15	0.230	Pcin15	0.503
Pcin19	0.793	Pcin19	1.491	Pcin19	1.077
Pcin20	0.732	Pcin20	1.848	Pcin20	0.388
Pcin21	0.812	Pcin21	0.659	Pcin21	19.085
Pcin22	0.071	Pcin22	0.093	Pcin22	0.397
Pcin23	1.252	Pcin23	0.967	Pcin23	0.152
Mean over Loci	**0.199**	**Mean over Loci**	**0.289**	**Mean over Loci**	**0.400**

Table 2. Sex-specific locus by locus estimates of dispersal (Koalas per generation; *Nm*) between Tyagarah and Wardell based on Shannon's mutual information index, with the overall *Nm* estimate at the bottom

	Tyagarah	Wardell
Locus	**Nm (males)***	**Nm (females)[#]**
Pcin3	0.790	0.651
Pcin5	0.111	0.191
Pcin6	0.098	0.277
Pcin7	3.836	2.212
Pcin8	0.353	0.375
Pcin9	0.417	0.148
Pcin10	1.644	8.009
Pcin11	0.059	0.088
Pcin14	0.435	0.235
Pcin15	0.218	0.565
Pcin19	1.131	1.175
Pcin20	0.251	0.686
Pcin21	1.690	9.562
Pcin22	0.627	0.394
Pcin23	0.148	0.145
Mean over Loci	**0.304**	**0.386**

*n   23 (8 from Tyagarah, 15 from Wardell)

[#]n   29 (9 from Tyagarah, 20 from Wardell)

Table 3. Locus by locus estimates of dispersal (Koalas per generation; *Nm*) based on Shannon's mutual information index. Overall *Nm* can be found at the bottom

East-West of proposed road (East *N* = 5, West *N* 33)	
Locus	**Nm**
Pcin3	1.060
Pcin5	18.753
Pcin6	44.696
Pcin7	0.145
Pcin8	23.346
Pcin9	1.607
Pcin10	8.781
Pcin11	0.621
Pcin14	50.588
Pcin15	0.236
Pcin19	3.382
Pcin20	5.389
Pcin21	1.532
Pcin22	9.528
Pcin23	0.809
Mean over Loci	**1.563**

From:	Rod Kavanagh
To:	RAVALLION Julie A
Cc:	WILSON Simon; LAWRENCE Scott B
Subject:	FW: Dispersal Estimates - additional information
Date:	Tuesday, 3 November 2015 4:04:53 PM
Attachments:	image001.png
	image002.wmz
	image003.gif

Hi Julie,

These are the "comparative" results received late on Friday from Siobhan from the Australian Museum – for your records.

Regards,

Rod

Dr Rod Kavanagh

Principal Research Ecologist
Sydney office
PO Box 2443, North Parramatta, NSW 1750
rkavanagh@niche-eh.com   www.niche-eh.com
Mob: 0428 637 960 **Fax:** 02 4017 0071

From: Siobhan Dennison [mailto:Siobhan.Dennison@austmus.gov.au]
Sent: Friday, 30 October 2015 4:03 PM
To: Rod Kavanagh
Cc: Linda Neaves; Greta Frankham; Rebecca Johnson; Mark Eldridge; 'William Sherwin'
Subject: Dispersal Estimates - additional information

Dear Rod,

Here is the additional information you requested following our conversation earlier today. I have re-calculated *Nm* for both within-Wardell (East vs West of the proposed upgrade), and between Wardell and Tyagarah.

After we spoke, I had some further correspondence with Bill Sherwin too, who suggested that the arithmetic mean was the correct value to use rather than the value I had reported, which was output by Genalex. I have therefore included the arithmetic mean and standard error from these new analyses in **Table 1** below for you.

In addition, I re-calculated the arithmetic mean and standard error from the original analyses in Addendum II (calculated using the *sHua* cut-off of 0.0001) for these areas:

Between Tyagarah and Wardell, Nm = 3.375 (SE = 1.886)
Within Wardell, East and West of the proposed upgrade, Nm = 11.365 (SE = 4.215)

I hope this helps! The values in the table below seem to align more with the values you gave me from the SCU report. Please do not hesitate to get back in contact if you have any further questions.

Thank you and Kind Regards,

Siobhan

Table 1. Locus by locus estimates of *Nm* between (i) eastern and western sides of the proposed upgrade within Wardell, and (ii) between Wardell and Tyagarah (*sHua* cut-off = 0.01)

Wardell East-West of proposed road (East *N = 5*, West *N = 33*)		Tyagarah & Wardell (Tyagarah *N = 17*, Wardell *N = 38*)	
Locus	**Nm**	**Locus**	**Nm**
Pcin3	4.044	Pcin3	1.198
Pcin5	26.355	Pcin5	0.200
Pcin6	5.232	Pcin6	0.232
Pcin7	5.407	Pcin7	35.732
Pcin8	47.839	Pcin8	0.664
Pcin9	31.291	Pcin9	0.324
Pcin10	6.700	Pcin10	3.506
Pcin11	5.171	Pcin11	0.103
Pcin14	-*	Pcin14	0.538
Pcin15	4.275	Pcin15	0.632
Pcin19	45.999	Pcin19	1.316
Pcin20	-*	Pcin20	0.662
Pcin21	38.103	Pcin21	26.459
Pcin22	6.545	Pcin22	0.585
Pcin23	33.159	Pcin23	0.180
Mean (SE)	**17.341 (4.538)**	**Mean** (SE)	**4.822 (2.799)**

* no estimate could be made for this locus, because *sHua* fell below the cut-off value
** arithmetic mean

--
Siobhan Dennison
Technical Officer | Australian Centre for Wildlife Genomics
Australian Museum 1 William Street Sydney NSW 2010 Australia
T 61 2 9320 6402 **M** 61 423 775 653

Facebook | **Twitter** | **Instagram** | **YouTube**

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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.

ASSESSMENT BY:

PROFESSOR WILLIAM B SHERWIN
Evolution & Ecology Research Centre
Deputy Head, School of Biological, Earth and Environmental Science
BEES, UNSW Australia, SYDNEY 2052 AUSTRALIA   FX: +61 (2) 9385 1558   PH: +61 (2) 9385 2119
EM:W.Sherwin@unsw.edu.au   Web:http://www.bees.unsw.edu.au/staff/academic/Sherwin
CRICOS Code: 00098G

UNSW DISLCAIMER

ASSESSMENT PROVIDED FOR

MR SIMON WILSON
Environment Officer
Environment | Infrastructure Development
Roads and Maritime Services
Level 2 76 Victoria Street Grafton NSW 2460
T   02 6640 1376 M   0477 315 247
E   Simon.WILSON@rms.nsw.gov.au
www.rms.nsw.gov.au

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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 Disatnce"), 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 Disatnce"), 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 BEWTEEN 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). Fst and Phi-st did show significant departures from zero, but there are many criticisms of Fst, and only partial fixes for these criticisms (Sherwin 2010, Wang 2015). Phi-st likely suffers from many of the same problems as Fst, because Phi-st is also a variance partition and an "order 2" diversity measure (Hill, 1973), the two characteristics that are at the root of Fst's many problems.

Of course, low differentiation may be due to high dispersal, and some of these measures (including Fst) 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/Phi-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 Km^2. 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 BEWTEEN 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 every 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.

REFERENCES

Dewar RC, Sherwin WB, Thomas E, Holleley CE, Nichols RA. 2011. Predictions of single-nucleotide polymorphism differentiation between two populations in terms of mutual information. Molecular Ecology 20:3156–3166.

Frankham RF Ballou JD & Briscoe DA 2010. Introduction to conservation genetics. Cambridge Univ Press Cambridge.

Hanski, I. Metapopulation Ecology Oxford University Press. 1999

Hill, M.O. Diversity and evenness: a unifying notation and its consequences. Ecology 1973, 54:427-432.

Kimura M and Crow JF 1970. An Introduction to Population genetics Theory. Harper and Row, New York.

Levins, R. 1969. Some demographic and genetic consequences of environmental heterogeneity for biological control. Bulletin of the Entomological Society of America 15: 237–240

Lunney D, O'Neill L, Matthews A and Sherwin WB 2002. Modelling mammalian extinction and forecasting recovery: koalas at Iluka (NSW Australia). Biological Conservation 106:101-113.

Penn AM, Sherwin WB, Gordon G, Lunney D, Melzer A and Lacy RC. 2000 Demographic Forecasting in Koala Conservation. Conservation Biology 14:629-638.

Reed J 2009. Vital rate sensitivity analysis as a tool for assessing management actions for the desert tortoise. Biological Conservation 142:2710–2717.

Sherwin WB, Timms P, Wilcken J, and Houlden B. 2000. Analysis and Conservation Implications of Koala Genetics. Conservation Biology 14:639-649.

Wang J 2015. Does Gst underestimate genetic differentiation from marker data? Molecular Ecology (2015) 24, 3546–3558

ANU COLLEGE OF MEDICINE, BIOLOGY & ENVIRONMENT
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Craig Moritz, FAA
Director, Centre for Biodiversity Analysis
Professor & ARC Laureate Fellow
Evolution, Ecology & Genetics
Research School of Biology
Building 116
Canberra ACT 0200 Australia

T: +61 2 6125 5651 or 9492
F: +61 2 6125 5573
E: craig.moritz@anu.edu.au

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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 (Fst; 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 neighboohood size of ~ 30 km^2 but, paradoxically, also infer a high rate of dispersal to nearby regional populations around k ismore.

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

Craig Moritz, FAA
Director, Centre for Biodiversity Analysis
Professor & ARC Laureate Fellow
Evolution, Ecology & Genetics
Research School of Biology
Building 116
Canberra ACT 0200 Australia

T: +61 2 6125 5651 or 9492
F: +61 2 6125 5573
E: craig.moritz@anu.edu.au

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,