UniSC Australia | Detection Dogs for Conservation

Genetic analysis of koala scats from Coffs Harbour and Bellingen Shire local government areas

Prepared for Jaliigirr Biodiversity Alliance and Canines for Wildlife

Prepared by Detection Dogs for Conservation, University of the Sunshine Coast - 2024

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List of abbreviations

Acronym	Meaning
DArT	Diversity Arrays Technology®, Canberra
DDC	Detection Dogs for Conservation
DNA	Deoxyribonucleic acid
LGAs	Local government areas
NSW	New South Wales
PCA	Principal component analysis
QLD	Queensland
SE	Standard Error
UniSC	University of the Sunshine Coast

Executive summary

Jaliigirr Biodiversity Alliance contracted the University of the Sunshine Coast's (UniSC) Detection Dogs for Conservation team (DDC) to genetically analyse a set of koala scat samples collected across Coffs Harbour and Bellingen Shire, local government areas (LGAs) of New South Wales (NSW), between June 2022 and December 2023, and report on genetic relatedness, sex, prevalence of *Chlamydia* and genetic diversity. Koala scat samples were collected by Jaliigirr Biodiversity Alliance and Canines for Wildlife and provided to the DDC in mid-February 2024. Here, we report on analyses and results for

1. Samples collected in 2022-23 and
2. Combined data from samples collected in 2022-23 and samples collected in 2020-22 for a previous analysis from the same region, also collected by Jaliigirr Biodiversity Alliance and Canines for Wildlife.

A total of 109 scat samples collected in 2022–23 were delivered to the UniSC laboratory, where DNA was extracted. For 107 samples, two or more scats were present in the sample tube, hence DNA was extracted in two replicates using two different scats from the sample tube. The remaining two samples only had one scat in each sample tube allowing for only single DNA extractions. Together, a total of 216 DNA extractions were sent for genotyping to Diversity Arrays Technology® (DArT) in Canberra.

Of the 109 samples, 20 samples had to be excluded because data quality was insufficient for genetic fingerprinting and further analyses. The samples likely failed due to highly degraded DNA. Out of the remaining 89 samples, 69 unique koalas were identified. Only unique koalas are used for estimating genetic relatedness, sex ratio, *Chlamydia* prevalence, and population genetic parameters. Samples of four individuals failed quality control thresholds for sex detection; the remaining 65 unique individuals included 37 males and 28 females with a sex ratio of 1.0:0.76 male to female.

Among 69 unique individuals, 10 pairs (dyads) consisting of 14 individuals showed high relatedness values between 0.4 and 0.64, indicating parent-offspring or full-sibling relationships. Another 28 dyads consisting of 24 individuals showed moderate relatedness values between 0.20 and 0.35, indicating half-sibling, grandparent-grand-offspring or aunt/uncle/niece/nephew relationships.

Chlamydia pecorum was detected in 23 of the 63 koalas, while six samples failed the quality control for the pathogen detection. Overall, this equates to a 36.5% *Chlamydia* infection prevalence. Of importance, *Chlamydia* infection does not necessarily develop into disease.

There were 59 unique koalas with samples of sufficient data quality for genetic diversity estimates. Population structure analyses indicated a panmictic group of koalas, i.e. all koalas are one breeding population. However, a group of koalas from the Fernbrook region showed some genetic differentiation from the rest. Altogether, the koalas analysed here showed high levels of heterozygosity and a low inbreeding coefficient, which are both positive results.

In a second analysis, we combined data from the current (2022–23) sample collection with data from a previous sample collection (2020–22) which increased the total number of individuals to 92. Of those, all but two presented sufficient data quality for relatedness analyses. No duplicates or re-sampling were found *between* sample collections. Considering all 90 unique individuals, 13 dyads consisting of 21 individuals showed high relatedness values between 0.4 and 0.64, indicating parent-offspring or full-sibling relationships. Another 38 dyads consisting of 36 individuals showed moderate relatedness values between 0.20 and 0.38, indicating half-sibling, grandparent-grand-offspring or aunt/uncle/niece/nephew relationships. Among these 90 individuals, four samples failed quality control threshold for sex detection and 47 males and 39 females were identified, resulting in a sex ratio of 1.0:0.83 male to female. Eight samples failed the quality control for *Chlamydia* detection, and *Chlamydia* prevalence was 32.9% with 27 individuals positive for *C. pecorum.* Among 90 koalas, 80 were with sufficient data quality for genetic diversity estimates, and population structure analyses again indicated a panmictic group of koalas, again with Fernbrook koalas

standing out from the rest. Overall, a high level of heterozygosity and a low inbreeding coefficient was observed for the 80 unique koalas from combined data, suggesting a genetically healthy group of koalas.

1 Background

Jaliigirr Biodiversity Alliance contracted the University of the Sunshine Coast's Detection Dogs for Conservation team (DDC) to genetically analyse a set of koala scat samples and report on genetic relatedness, sex, prevalence of *Chlamydia* and genetic diversity. Koala scat samples were collected by Jaliigirr Biodiversity Alliance and Canines for Wildlife across Coffs Harbour and Bellingen Shire local government areas (LGAs) in New South Wales (NSW) between June 2022 and December 2023. Samples were provided to the DDC in mid-February 2024. Data from further 23 samples which were identified as unique koalas from a previous scat collection by Jaliigirr Biodiversity Alliance and Canines for Wildlife between September 2020 and February 2022 in the Coffs Harbour region (Coffs Harbour Koala Survey – Genotyping from Scats report by DDC) were also included for co-analyses.

2 Methods

2.1 Koala scat samples

A total of 109 koala scat samples collected between 27/06/2022 and 21/12/2023 across Coffs Harbour and Bellingen Shire LGAs in NSW were received by DDC from Canines for Wildlife (see Figure 1 for scat collected locations and Appendix 1, Table A1 for further details). The samples were delivered frozen by Canines for Wildlife in mid-February 2024 and transferred to a –20 °C freezer immediately on arrival and stored until processing for DNA extractions.

Genetic data from further 23 unique individuals from 2020–22 scat collection within the Coffs Harbour region (Figure 1) were available and included for co-analyses as described in section 2.5.

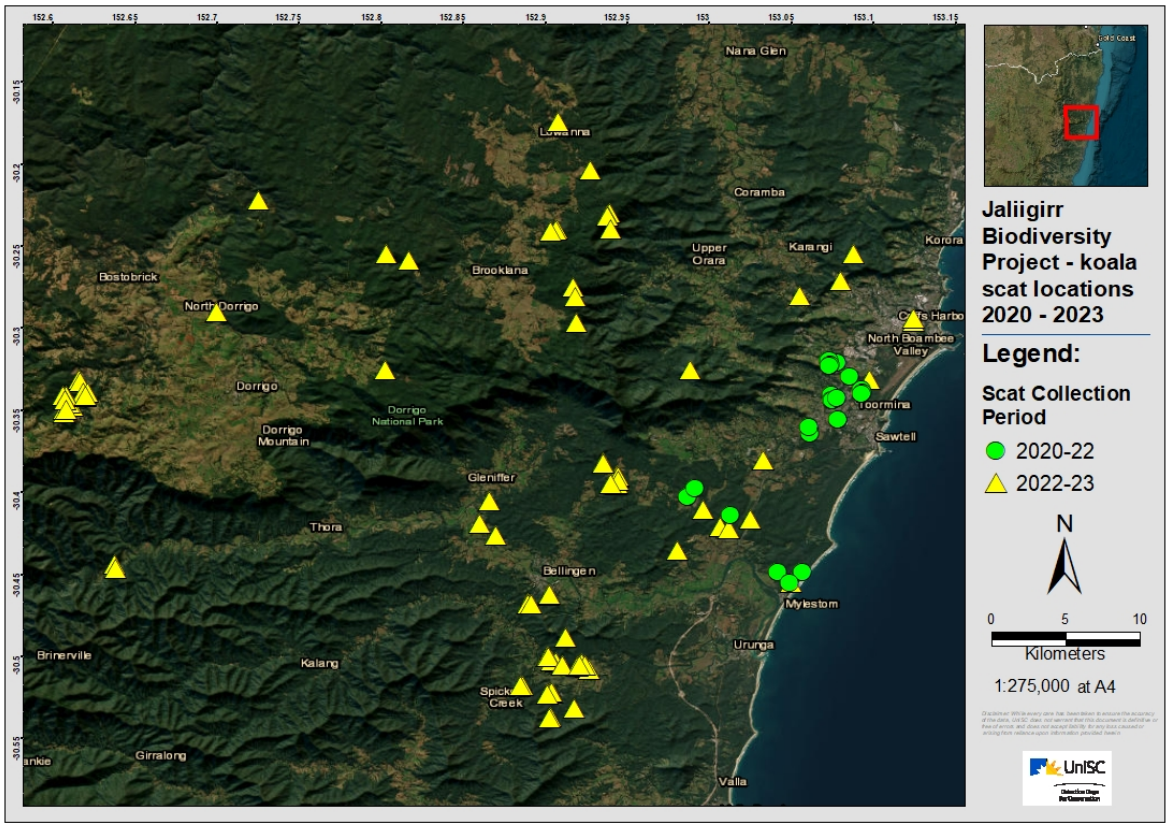

Figure 1. Distribution of 109 scats collected from Coffs Harbour and Bellingen Shire local government areas of New South Wales in 2022–23 (yellow triangles) and 23 scat samples from identified unique individuals in the 2020–22 scat collection (green circles).

DNA quality is generally higher when extracted from fresh koala scats (Schultz, Cristescu et al. 2018). Fresh scats (i.e. when the scat age is estimated to be less than one week old, categories 1 and 2, Table 1) present a shiny mucus layer and a strong smell. For the current set of samples, the records of scat age at collection are given in Appendix 1, Table A1.

Table 1. General guide used to age koala scats in the field

Scat age categories	Age	Characteristics
1	One day old or less	Very fresh (covered in mucus, wet)
2	Couple of days old	Fresh (shine and smell)
3	Couple of weeks old	Medium fresh (shine or smelly when broken)
4	Months old	Old (no shine, no smell)
5	More than a few months old	Very old and discoloured

2.2 DNA extraction

All 109 samples were processed for DNA extraction. Two samples (namely BA_6.1 and JBAKG_60.0) only had one scat in the sampling tube, only allowing for one DNA extraction per sample. For all others (N = 107) DNA extractions were replicated with a second scat from the sampling tube. In total, 216 DNA extractions were performed. This was done so that each sample could be genotyped twice in order to maximise availability and quality of genetic data for analyses. We followed the protocol of Schultz, Cristescu et al. (2018) to extract DNA from koala scats. However, instead of scraping the outer layer off the scats, we used a lysis wash to rinse the DNA off the surface of the scats. This faecal sample wash was then processed using the QIAamp PowerFecal Pro DNA Kit (Qiagen), with the following modification to the manufacturer's protocol. After adding the buffer to the faecal sample wash, a one-hour incubation step (65 °C) was added, and samples were vortexed for seven minutes at maximum speed using Genie 2 Vortex Mixer (Scientific Industries). Finally, DNA was eluted in 200 µl of elution buffer and concentrated down to a volume of ~30 µl. Extracted DNA was stored at –20°C until it was shipped on dry ice to Diversity Arrays Technology® (DArT) in Canberra for genotyping.

2.3 Genotyping

DNA aliquots were genotyped using a next-generation sequencing protocol for detecting Single Nucleotide Polymorphisms (SNPs) by DArT (Jaccoud, Peng et al. 2001, Kilian, Wenzl et al. 2012). A targeted approach was chosen (DArTag), where specifically designed molecular probes (i.e. koala-specific capture probes) select small target regions containing sequence variants. A total of 4,393 koala SNPs were genotyped. In addition, sex and *Chlamydia pecorum* markers were also genotyped from the same DNA extractions, using sex- and *Chlamydia* -specific probes. Further, a possum-specific marker was integrated to the same DArTag panel, which helps to identify whether a sample failed due to being possum rather than koala scat.

2.4 Data analysis

2.4.1 Filtering of genetic data

Genetic data were analysed using the R package *dartR* (Gruber, Unmack et al. 2019) in the R environment using R v4.1.0 (R Core Team 2018), unless specified. Genotyped data were filtered to improve the quality of the dataset by removing samples with too little data (i.e. those with low individual call rate) as well as SNP loci that were not called across most samples (i.e. those with low locus call rate). We applied a stepwise increasing locus call rate threshold, from 0.2 to 0.8 – only retaining those SNPs with at least 80% data. When filtering for individual call rate, different filtering regimes were applied, depending on the analysis. This is because only 200 high-quality loci are needed to identify unique individuals (Schultz, Cristescu et al. 2018); however, many high-quality loci are required to measure genetic diversity. Therefore, to identify unique individuals, where the focus was on maximising the number of individuals that could be used while retaining sufficient high-quality SNPs, samples were filtered for an individual call rate threshold of 0.2. On the other hand, for genetic diversity analyses, where the focus was on maximising the number of high-quality loci while maintaining as many individuals as possible, samples were filtered using a stepwise approach, increasing individual call rate threshold from 0.2 to 0.5 – resulting in only retaining samples with at least 50% data.

Other constant thresholds were applied to remove potentially erroneous loci. This included filtering for allele read depth (minimum threshold of five), minor allele frequency (MAF,

minimum threshold of 0.01) and loci appearing on the same contig as another (secondary loci). Because filtering can result in previously polymorphic loci becoming monomorphic, a filter to remove all monomorphic loci was applied at the end of the filtering protocol.

2.4.2 Genetic fingerprinting and estimates of genetic relatedness

Genetic fingerprinting allows for the allocation of scat samples to individual koalas, i.e. it enabled the identification and elimination of multiple samples originating from the same individual koala, which would have otherwise biased those estimates. The unique individuals identified with this technique were used for estimates of sex, relatedness, *Chlamydia* prevalence, and genetic diversity.

SNPs filtered for an individual call rate threshold of 0.2 and a locus call rate threshold of 0.8 were used for genetic fingerprinting. Relatedness values typically range from 0 (no kinship relationship) to 1 (a duplicate individual, 100% relatedness). Based on this theoretical frame, as well as previous testing on known duplicates and related individuals, any pairwise sample set (dyad) that indicated a genetic relatedness value ≥0.75 using the 'dyadml' method (Milligan 2003) from the *related* R package (Pew, Muir et al. 2015) was considered a duplicate sample and eliminated from further analyses.

The list of unique individuals identified through genetic fingerprinting was used for estimating pairwise genetic relatedness. Theoretical classification of kinship relationships are:

- 0.5 – indicative of either parent-offspring (PO) or full sibling (FS) relationships,
- 0.25 – indicative of half-siblings (HS) or grandparent-grand-offspring (GG), or aunt/uncle/niece/nephew and
- 0.125 – indicative of first cousin (FC) relationships or avuncular relationships (Taylor Helen 2015, Wang 2017)

However, such simple categorisations of kinship are difficult to apply, because relatedness is a continuous parameter and does not present strict cutoffs (Städele and Vigilant 2016). The proportion of genome shared between two individuals does not necessarily meet

theoretically expected values (Blouin 2003). For instance, theoretically, full siblings share on average 50% of their genome (indicated as 0.5 relatedness), however, some may share much more or less due to e.g. crossover rates (Hill and Weir 2011). Taylor (2015) found that realised relatedness, using a similar estimator (TrioML), sometimes varied greatly from theoretical values. For instance, first cousins/avuncular relationships appear as high as 0.25 instead of the theoretical 0.125. Therefore, whilst we use the theoretical values as presented above as guidance for interpretation, realised relatedness is conceptually and empirically different (Städele and Vigilant 2016) and thus cutoff values to strictly differentiate between kinship classifications cannot be presented.

2.4.3 Sex and sex ratio

Sex of individual koalas was determined through sex-linked genetic markers integrated into the DArTag panel. Sex ratio, which is the relationship between number of males to number of females, was calculated. A typical sex ratio in natural, healthy populations is expected to be close to 1:1. However, a good representation of the population, i.e. large sample size and good geographic spread of samples, is required to get a reliable value.

2.4.4 *Chlamydia* detection

Chlamydia pecorum detection in scats was based on the same DNA extraction described above. *Chlamydia*-specific probes developed and integrated into the DArTag panel were used to determine the presence or absence of chlamydial DNA. The prevalence of chlamydial infection was then calculated based on the number of individual koalas for which the presence or absence of chlamydial DNA was detected.

2.4.5 Population genetic structure and genetic diversity

Data filtered for a locus call rate threshold of 0.8 and an individual call rate threshold of 0.5 were used to measure the population genetic structure and genetic diversity indices. To identify the presence of population structure within the data set, principal component analysis (PCA) and genetic structure analysis were conducted using *dartR* package and fastStructure (Raj, Stephens et al. 2014), respectively. For the latter, the number of genetic

clusters (K) was set to vary between 1 to 5 with 10 iterations and the most likely number of clusters was determined based on the 'chooseK.py' script in fastStructure (Raj, Stephens et al. 2014).

Genetic diversity was calculated using GenAlEx v6.5 (Peakall and Smouse 2012). We calculated three values: observed heterozygosity H_O, which is the level of heterozygosity from the allele frequencies of the population under study; expected heterozygosity H_E (adjusted for small sample size), which is the level of heterozygosity that could be expected based on observed allele frequencies if the population was at the Hardy-Weinberg equilibrium (panmictic population with constant genetic variation across generations); and lastly F_{IS}, also called inbreeding coefficient, which is the proportion of the variance in the subpopulation contained in an individual and which can range from –1 to 1 (the closer to 1, the higher the degree of inbreeding). Note that inbreeding can not only result from non-random mating but also from small, isolated populations, where all individuals are more closely related than in large populations. Given the increasingly fragmented landscape koalas have to navigate, this second cause of inbreeding is becoming more common and important to investigate.

Effective population size refers to the size of a breeding population or the number of individuals that effectively participate in producing the next generation. Contemporary effective population size (Ne) and associated parametric 95% confidence intervals were estimated using NeEstimator v2 (Do, Waples et al. 2014), implementing linkage disequilibrium method with random mating model with 0.05 as the lowest allele frequency.

2.5 Analyses for combined data from 2022–23 and 2020–22 scat collections

Genetic data from 23 identified individuals were available from a previous scat collection, which were collected between September 2020 and February 2022 in the Coffs Harbour region (see Figure 1 for scat collected locations and Appendix 3, Table A2 for sample details) by Jaliigirr Biodiversity Alliance and Canines for Wildlife. These 23 individuals were identified by the DDC in a previous scat analyses report (Coffs Harbour Koala Survey – Genotyping from Scats report by DDC - 2022). For the co-analyses, data from these 23 koalas were combined

and analysed together with the unique individuals identified in the present sample set. Co-analyses included genetic fingerprinting to test re-sampling of previously identified koalas, genetic relatedness, sex ratio, *Chlamydia* prevalence and population genetic estimates including population structure, heterozygosity, inbreeding and effective population size following the methods described in section 2.4.

2.6 Limitations

Genotyping was conducted non-invasively from genetic material contained on the surface of koala scats. This allows for large-scale, relatively cheap, unbiased sampling of DNA compared to other available methods (e.g., catching koalas, anaesthetising them and collecting high-quality samples such as blood or biopsies, or relying on wildlife hospital samples). However, compared to high-quality blood/biopsy samples, DNA present in scat is of lower quantity and quality, which yields lower numbers of high-quality SNPs. DDC was able to optimise scat genotyping for koalas by developing a specific-probe approach, i.e. the DArTag method, which increased genotyping success, and the quality of data. However, data quality of non-invasive samples can only be improved to a certain degree, with some samples still containing insufficient data to be included in further analyses. To maximise data derived from the non-invasive samples, all samples were extracted twice, in all instances where a minimum of two scats per sample (tube) was available.

Presence of duplicate samples (i.e. two or more samples originating from the same individual) can falsely inflate data, and collection of duplicate samples is common in non-invasive sampling methods. These samples need to be identified and removed to avoid producing skewed results. For example, if a koala with *Chlamydia* infection is sampled multiple times, it would artificially inflate *Chlamydia* prevalence, or if duplicate samples were kept, as they are genetically identical, they would falsely inflate measures of inbreeding in the population. Here, care has been taken to remove duplicate samples identified through genetic fingerprinting, retaining only the best quality sample from each cluster of duplicate samples for further analyses.

The prevalence of *Chlamydia* (i.e. the percentage of unique koalas with the pathogen) is an important population characteristic for informing conservation management. However, the presence and severity of chlamydial disease varies greatly between individual koalas, as well as between populations (Ellis, Girjes et al. 1993, Waugh, Hanger et al. 2016). Notably, individual koalas can shed large numbers of *Chlamydia* organisms without clinical signs of disease (Wan, Loader et al. 2011), and populations can have high *Chlamydia* prevalence with minimal detectable health impacts. For instance, in the Mt Lofty ranges, 90% of koalas were *Chlamydia* positive but there was a low prevalence of clinical (symptomatic) disease (Polkinghorne, Hanger et al. 2013); see also Weigler, Girjes et al. (1988). Therefore, quantifying *Chlamydia* pathogen prevalence is only the first step in understanding the threat that this pathogen presents to an individual and a population.

Sample collection was conducted by Jaliigirr Biodiversity Alliance and Canines for Wildlife, therefore DDC had no influence over the scat collection and initial storage methods. However, care has been taken to ensure no contamination or damage occurred to the samples once they had been received.

3 Results

3.1 Extraction, quality control and unique individuals

All samples were genotyped using DArTag. However, DNA quality varied, which is common when using non-invasive samples, and samples below analysis-specific quality thresholds were excluded from the analyses. Of the 109 samples, 20 samples were excluded from the analyses due to insufficient data (see Appendix 1, Table A1). Based on the possum-specific genetic markers integrated into DArTag SNP panel, one sample, namely JBAKG_17 was excluded as a potential possum scat.

Data filtration for identifying unique individuals (genetic fingerprinting) retained a total of 1,745 SNPs with an average of 11.3% missing data. Twenty samples were found to be duplicates (i.e. scats collected from the same, already identified individuals) and were subsequently removed from further analyses, retaining only the best sample from each unique koala (see Appendix 2, Figure A1 for different locations of scats collected from same individuals). From the remaining 89 samples, 69 unique koalas were identified (Table 2).

Table 2. List of unique and duplicate samples, as determined by genetic fingerprinting

Sample Name	Duplicate ID1	Duplicate ID2	Duplicate ID3	Duplicate ID4	Duplicate ID5
BA_6.2					
BA_6.3	BA_6.6				
BA_6.5	BA_6.4				
CA_1.1					
CA_1.4					
CA_1.5					
CA_1.6					
CA_1.7					
JBAKG_1.0					
JBAKG_2.0					
JBAKG_3.0					
JBAKG_4.0					
JBAKG_5.0					
JBAKG_6.0					
JBAKG_7.0					
JBAKG_8.0					

Sample Name	Duplicate ID1	Duplicate ID2	Duplicate ID3	Duplicate ID4	Duplicate ID5
JBAKG_9.0					
JBAKG_10.0					
JBAKG_12.0	JBAKG_11.0	JBAKG_41.0	JBAKG_50.0	JBAKG_51.0	JBAKG_93.0
JBAKG_15.0					
JBAKG_16.0					
JBAKG_19.0	JBAKG_13.0	JBAKG_14.0	JBAKG_18.0		
JBAKG_20.0					
JBAKG_21.0					
JBAKG_22.0					
JBAKG_23.1	JBAKG_23.0				
JBAKG_26.0					
JBAKG_27.0					
JBAKG_29.0					
JBAKG_30.0					
JBAKG_31.0					
JBAKG_32.0					
JBAKG_34.0					
JBAKG_35.0					
JBAKG_36.0					
JBAKG_37.0	JBAKG_24.0	JBAKG_25.0	JBAKG_38.0		
JBAKG_39.0					
JBAKG_40.0					
JBAKG_43.0					
JBAKG_44.0					
JBAKG_45.0					
JBAKG_46.0					
JBAKG_47.0					
JBAKG_52.0					
JBAKG_54.0	JBAKG_33.0				
JBAKG_58.0					
JBAKG_61.0					
JBAKG_62.0					
JBAKG_63.0					
JBAKG_64.0	JBAKG_65.0				
JBAKG_66.0					
JBAKG_67.0	JBAKG_68.0	JBAKG_69.0			
JBAKG_70.0					
JBAKG_71.0					
JBAKG_73.0					
JBAKG_74.0					

Sample Name	Duplicate ID1	Duplicate ID2	Duplicate ID3	Duplicate ID4	Duplicate ID5
JBAKG_76.0					
JBAKG_77.0					
JBAKG_78.0	JBAKG_79.0				
JBAKG_81.0					
JBAKG_82.0					
JBAKG_83.0					
JBAKG_84.0					
JBAKG_85.0					
JBAKG_87.0	JBAKG_88.0				
JCBIN2022					
Lowanna_6					
Lowanna_8					
Mylestrom					

3.2 Genetic relatedness among unique individuals

Genetic relatedness was tested among the 69 unique individuals, using 2,077 SNP with an average of 10.9% missing data. Table 3 shows the relatedness values: 10 pairs (dyads) consisting of 14 individuals showed high relatedness values between 0.4 and 0.64, indicating parent-offspring or full-sibling relationships. Another 28 dyads consisting of 24 individuals showed moderate relatedness values between 0.20 and 0.35, indicating half-sibling, grandparent-grand-offspring or aunt/uncle/niece/nephew relationships, based on the theoretical values for kinship relationship classification (Taylor Helen 2015, Wang 2017). Please note that relatedness is a continuous parameter and does not present strict cutoffs, hence simple categorisations of kinship are not possible.

Table 3. Genetic relatedness between unique individuals based on 'dyadml' method (Milligan 2003). Colour decodes the relatedness, from high (darker), over moderate (lighter) to low (no colour). Dyads with genetic relatedness values <0.12 are not listed

Koala 1	Koala 2	Relatedness value
JBAKG_21.0	JBAKG_20.0	0.64
JBAKG_30.0	JBAKG_23.1	0.63
JBAKG_23.1	JBAKG_62.0	0.59

Koala 1	Koala 2	Relatedness value
JBAKG_12.0	JBAKG_83.0	0.56
BA_6.2	JBAKG_7.0	0.51
JBAKG_30.0	JBAKG_62.0	0.50
JBAKG_85.0	JBAKG_84.0	0.47
JBAKG_21.0	JBAKG_31.0	0.42
JBAKG_26.0	JBAKG_37.0	0.41
JBAKG_23.1	JBAKG_20.0	0.40
JBAKG_62.0	JBAKG_20.0	0.35
JBAKG_36.0	JBAKG_47.0	0.35
JBAKG_23.1	JBAKG_27.0	0.35
JBAKG_32.0	JBAKG_54.0	0.34
JBAKG_37.0	JBAKG_31.0	0.32
JBAKG_37.0	JBAKG_27.0	0.30
JBAKG_30.0	JBAKG_20.0	0.29
JBAKG_37.0	JBAKG_20.0	0.29
JBAKG_20.0	JBAKG_31.0	0.29
JBAKG_26.0	JBAKG_30.0	0.28
JBAKG_27.0	JBAKG_20.0	0.28
JBAKG_73.0	JBAKG_74.0	0.27
JBAKG_26.0	JBAKG_20.0	0.27
JBAKG_40.0	JBAKG_83.0	0.26
JBAKG_26.0	JBAKG_62.0	0.25
JBAKG_27.0	JBAKG_31.0	0.25
JBAKG_12.0	JBAKG_40.0	0.25
JBAKG_37.0	JBAKG_21.0	0.24
JBAKG_26.0	JBAKG_31.0	0.24
JBAKG_27.0	JBAKG_21.0	0.24
JBAKG_23.1	JBAKG_21.0	0.23
JBAKG_39.0	JBAKG_16.0	0.23
JBAKG_29.0	JBAKG_27.0	0.22
JBAKG_23.1	JBAKG_37.0	0.22
JBAKG_23.1	JBAKG_31.0	0.21
JBAKG_62.0	JBAKG_27.0	0.21
JBAKG_35.0	JBAKG_32.0	0.21
JBAKG_15.0	JBAKG_58.0	0.20
JBAKG_39.0	JBAKG_15.0	0.19
JBAKG_40.0	JBAKG_15.0	0.19
JBAKG_30.0	JBAKG_27.0	0.18
JBAKG_77.0	JBAKG_70.0	0.18
JBAKG_32.0	JBAKG_36.0	0.18

Koala 1	Koala 2	Relatedness value
JBAKG_29.0	JBAKG_37.0	0.18
JBAKG_29.0	JBAKG_62.0	0.18
JBAKG_29.0	JBAKG_23.1	0.18
JBAKG_36.0	JBAKG_52.0	0.17
JBAKG_30.0	JBAKG_21.0	0.17
JBAKG_73.0	JBAKG_19.0	0.17
JBAKG_66.0	JBAKG_64.0	0.17
JBAKG_30.0	JBAKG_37.0	0.17
JBAKG_26.0	JBAKG_27.0	0.16
JBAKG_26.0	JBAKG_23.1	0.16
JBAKG_54.0	JBAKG_52.0	0.16
JBAKG_37.0	JBAKG_62.0	0.16
JBAKG_26.0	JBAKG_21.0	0.16
JBAKG_8.0	JBAKG_58.0	0.16
JBAKG_84.0	JBAKG_70.0	0.16
JBAKG_32.0	JBAKG_34.0	0.15
JBAKG_15.0	JBAKG_19.0	0.15
JBAKG_46.0	BA_6.3	0.15
JBAKG_78.0	JBAKG_77.0	0.15
JBAKG_43.0	JBAKG_44.0	0.15
JBAKG_35.0	JBAKG_36.0	0.14
JBAKG_54.0	JBAKG_47.0	0.14
JBAKG_29.0	JBAKG_31.0	0.14
JBAKG_30.0	JBAKG_31.0	0.13
JBAKG_35.0	JBAKG_54.0	0.13
JBAKG_29.0	JBAKG_20.0	0.13
JBAKG_52.0	JBAKG_47.0	0.13
BA_6.3	JBAKG_4.0	0.13
JBAKG_66.0	JBAKG_29.0	0.12
JBAKG_81.0	JBAKG_82.0	0.12
JBAKG_32.0	JBAKG_47.0	0.12
JBAKG_36.0	JBAKG_34.0	0.12
JBAKG_34.0	JBAKG_54.0	0.12
Mylestrom	JBAKG_1.0	0.12
JBAKG_35.0	JBAKG_34.0	0.12
JBAKG_8.0	JBAKG_39.0	0.12
JBAKG_8.0	JBAKG_19.0	0.12
JBAKG_40.0	JBAKG_16.0	0.12

3.3 Sex of unique individuals and sex ratio

Of the 69 unique individuals, four samples failed quality control threshold for sex detection. Based on the sex-linked markers, of the remained 65 individuals, 37 (56.9%) were males and 28 (43.1%) were females (Table 4 and see Figure 2 for locations of each individual), translating to a sex ratio of 1.0:0.76 male to female, which denotes a male biased sample set.

Table 4. Sex and *Chlamydia* status of unique individuals (N = 69). 'QC-failed' represent the sample that failed the quality control threshold for the sex/*Chlamydia* detection

Sample Name	Sex	*Chlamydia* status
BA_6.2	F	Negative
BA_6.3	M	Positive
BA_6.5	F	Negative
CA_1.1	M	Positive
CA_1.4	M	Positive
CA_1.5	F	Positive
CA_1.6	M	Positive
CA_1.7	F	QC-failed
JBAKG_1.0	M	QC-failed
JBAKG_2.0	M	Positive
JBAKG_3.0	M	Negative
JBAKG_4.0	M	Positive
JBAKG_5.0	M	Negative
JBAKG_6.0	F	Negative
JBAKG_7.0	M	Negative
JBAKG_8.0	F	QC-failed
JBAKG_9.0	F	Negative
JBAKG_10.0	M	Positive
JBAKG_12.0	F	Positive
JBAKG_15.0	F	Negative
JBAKG_16.0	F	Negative
JBAKG_19.0	M	Negative
JBAKG_20.0	F	Negative
JBAKG_21.0	F	Negative
JBAKG_22.0	M	Positive
JBAKG_23.1	F	Negative
JBAKG_26.0	QC-failed	Negative
JBAKG_27.0	F	Negative
JBAKG_29.0	M	Negative

Sample Name	Sex	*Chlamydia* status
JBAKG_30.0	M	Negative
JBAKG_31.0	M	Negative
JBAKG_32.0	M	Negative
JBAKG_34.0	QC-failed	Positive
JBAKG_35.0	M	Negative
JBAKG_36.0	F	Positive
JBAKG_37.0	M	Negative
JBAKG_39.0	M	Negative
JBAKG_40.0	F	Negative
JBAKG_43.0	M	Positive
JBAKG_44.0	F	Positive
JBAKG_45.0	F	Positive
JBAKG_46.0	M	Negative
JBAKG_47.0	M	QC-failed
JBAKG_52.0	F	Negative
JBAKG_54.0	F	Negative
JBAKG_58.0	M	Negative
JBAKG_61.0	M	Negative
JBAKG_62.0	M	Negative
JBAKG_63.0	M	Negative
JBAKG_64.0	F	QC-failed
JBAKG_66.0	M	Negative
JBAKG_67.0	M	Positive
JBAKG_70.0	M	Negative
JBAKG_71.0	F	Positive
JBAKG_73.0	M	Positive
JBAKG_74.0	M	Positive
JBAKG_76.0	F	Negative
JBAKG_77.0	QC-failed	Negative
JBAKG_78.0	M	Negative
JBAKG_81.0	F	Positive
JBAKG_82.0	M	QC-failed
JBAKG_83.0	QC-failed	Positive
JBAKG_84.0	F	Negative
JBAKG_85.0	F	Negative
JBAKG_87.0	M	Negative
JCBIN2022	M	Negative
Lowanna_6	F	Positive
Lowanna_8	M	Negative
Mylestrom	F	Positive

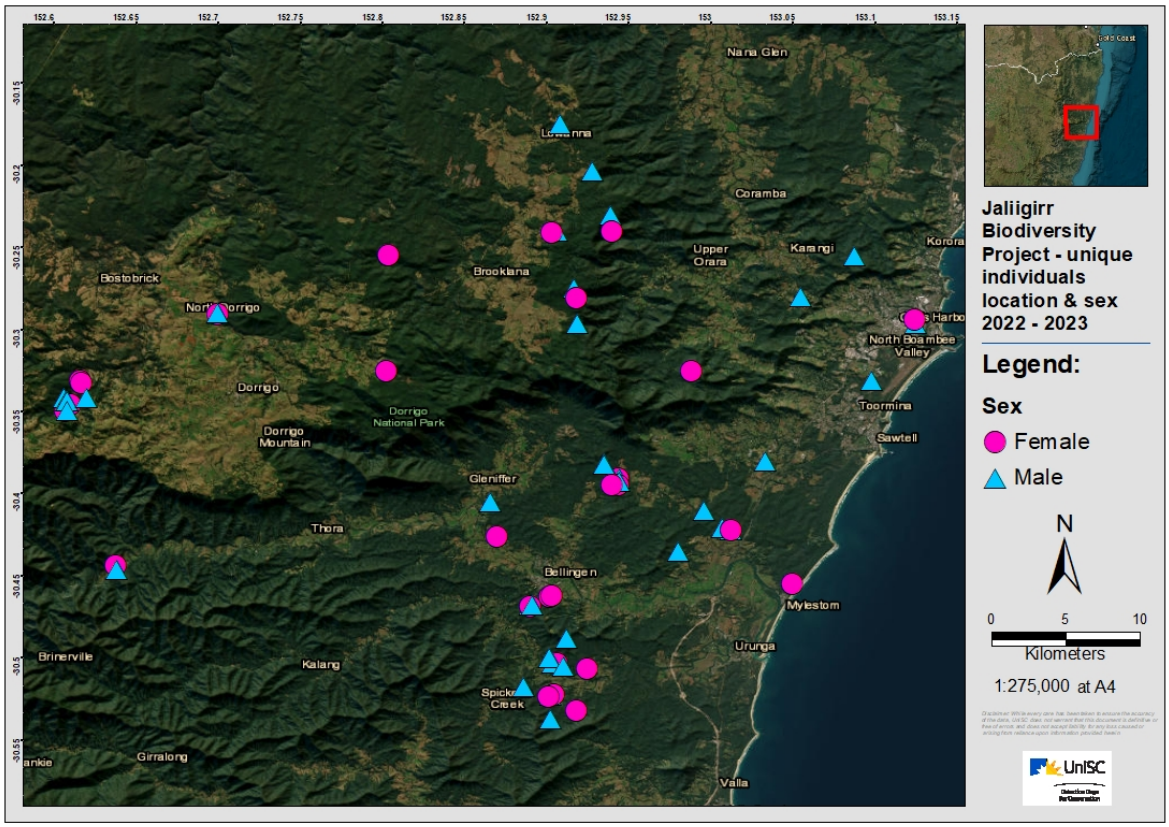

Figure 2. Distribution and sex of unique individuals from 2022–23 sample collection (N = 65), four samples that failed the data quality control threshold for sex detection were not included.

3.4 *Chlamydia* prevalence

Of the 69 unique individuals, six samples failed the quality control threshold for the *Chlamydia* detection, and 23 (36.5% prevalence) were positive for *Chlamydia* (Table 4 and see Figure 3 for sample locations by sex). However, it should be noted that the presence of the *Chlamydia* pathogen does not necessarily equate to clinical signs of disease.

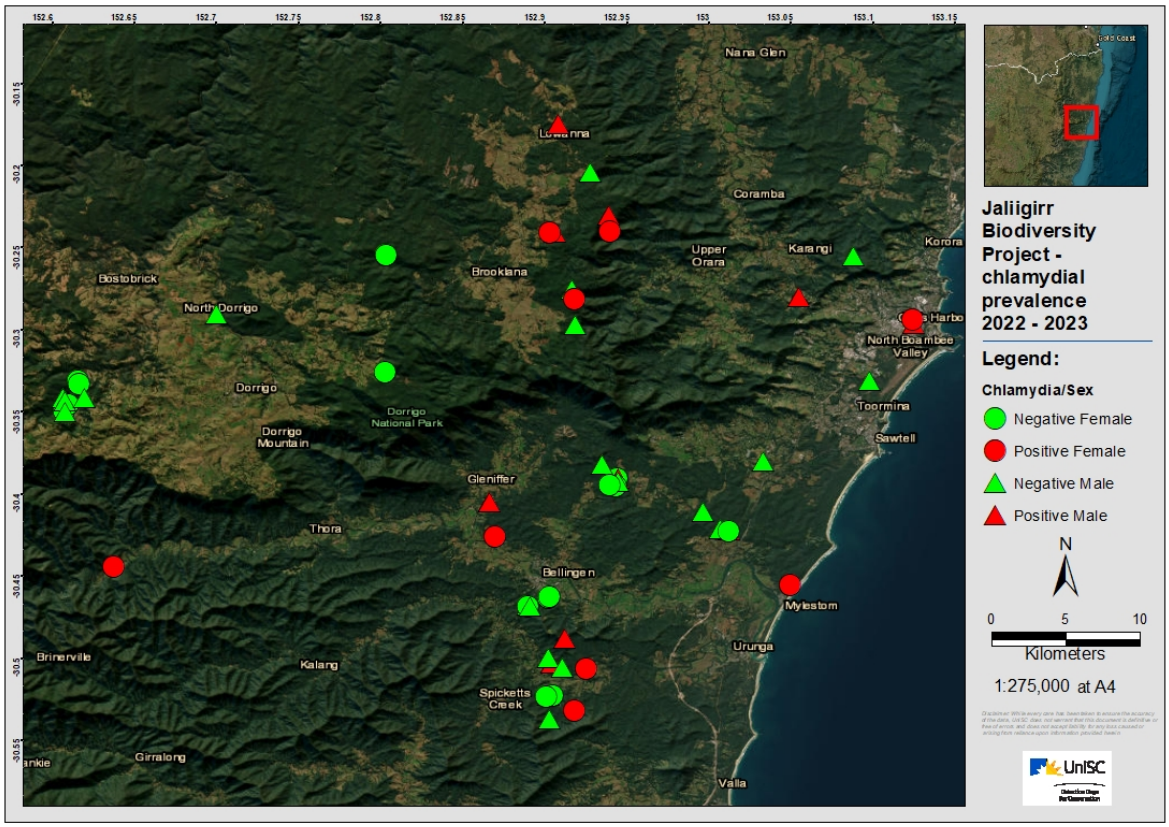

Figure 3. Location and *Chlamydia* status of unique individuals by sex from 2022–23 sample collection (N = 59), ten samples that failed the data quality control threshold for either sex detection or *Chlamydia* detection were not included.

3.5 Population genetic structure and genetic diversity

After the application of more stringent filtering for the individual call rate, a further 10 samples were removed from the 69 unique individuals due to insufficient data, retaining 59 samples for population specific analyses (see Appendix 1, Table A1 for the list of samples that passed filtering for population genetic analyses). A total of 2,737 loci were retained with 5.2% missing data.

Principal component analysis did not indicate strong clustering among the individuals. However, seven samples from Fernbrook (namely, JBAKG_20.0, JBAKG_21.0, JBAKG_23.1, JBAKG_26.0, JBAKG_27.0, JBAKG_31.0 and JBAKG_37.0) showed signs of differentiation

(Figure 4). Nonetheless, results from the population structure analysis showed presence of only a single population (Appendix 4, Figure A2).

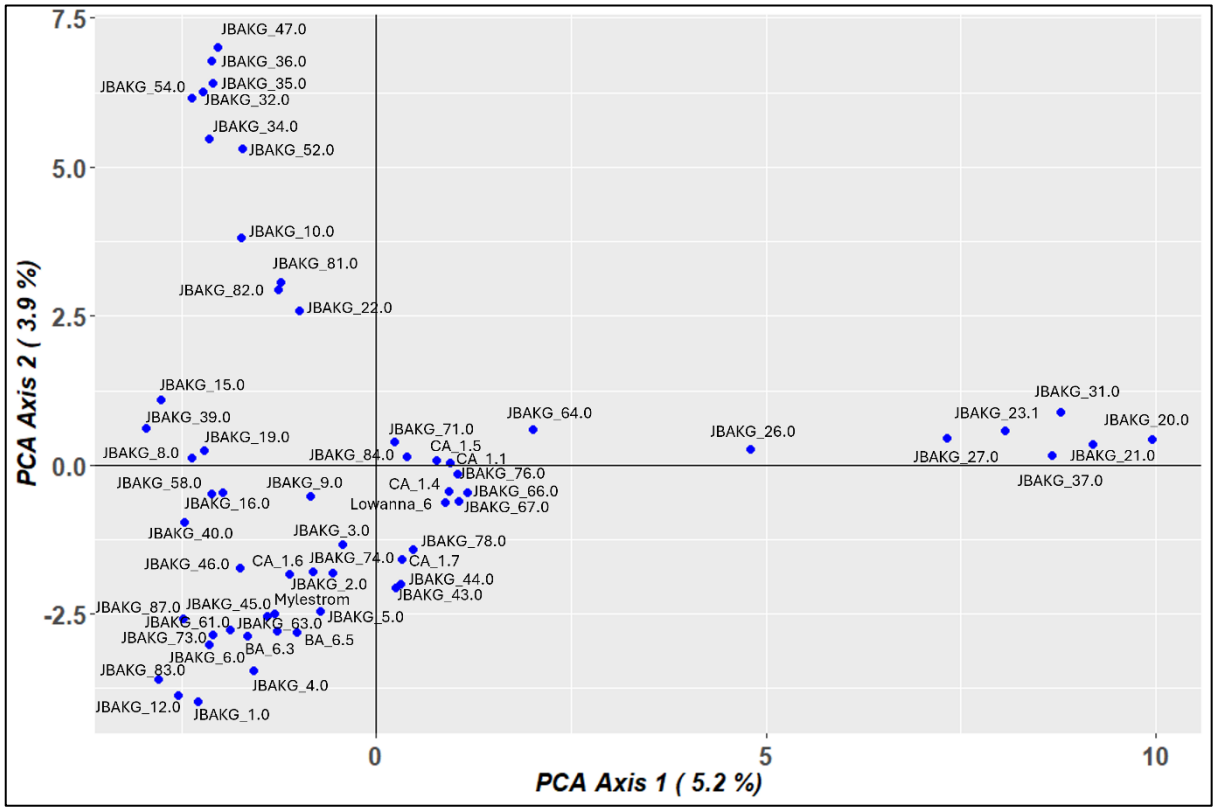

Figure 4. Results of the principal component analysis (PCA) for the 59 unique koalas, indicating one panmictic population with seven individuals from the Fernbrook region (JBAKG_20.0, JBAKG_21.0, JBAKG_23.1, JBAKG_26.0, JBAKG_27.0, JBAKG_31.0 and JBAKG_37.0) showing a small degree of differentiation.

Three genetic diversity indices including observed heterozygosity (H_O), expected heterozygosity (H_E) and inbreeding coefficient (F_{IS}) were calculated and indicated a high level of heterozygosity and a low level of inbreeding (Table 5). The estimated effective population size for the 59 unique koalas was 121.2 (95% CI = 117.8 – 122.8). These values were compared to those of other koala populations in the discussion (i.e. section 4).

Table 5. Genetic diversity indices for the 59 unique koalas: SE: standard error

Parameter	Mean	SE
Observed heterozygosity (H_O)	0.276	0.003
Expected heterozygosity (H_E)	0.297	0.003
Inbreeding coefficient (F_{IS})	0.074	0.003

3.6 Analyses for 2022–23 and 2020–22 combined data

A total of 92 samples, including the 69 unique individuals from the current (2022–23) sample collection and 23 unique individuals from the previous (2020–22) sample collection, were collated for co-analyses. All samples were genotyped using the same DArTag panel. Data filtration for identifying unique individuals (genetic fingerprinting) retained a total of 90 individuals and 1,733 SNPs with an average of 12.3% missing data. No duplicate samples were identified between two sample collections; therefore all 90 samples were confirmed unique individuals for further analyses.

3.6.1 Genetic relatedness among unique individuals

Genetic relatedness was tested among 90 unique individuals using the combined and filtered SNP data. Table 6 lists relatedness dyads of samples from 2020–22 and 2022–23 collections but excluding the dyads already observed and listed for the 2022–23 samples (3.2, Table 3). No dyads had relatedness value ≥0.5 (theoretical value for FS/PO relationships) for individuals within 2020–22 but one dyad between 2020–22 and 2022–23 sample collections (Table 6). Considering all 90 unique individuals, 13 dyads consisting of 21 individuals showed high relatedness values between 0.4 and 0.64, indicating parent-offspring or full-sibling relationships. Another 38 dyads consisting of 36 individuals showed moderate relatedness values between 0.20 and 0.38, indicating half-sibling, grandparent-grand-offspring or aunt/uncle/niece/nephew relationships, based on the theoretical values for kinship relationship classification (Taylor Helen 2015, Wang 2017). Among 90 unique individuals, 22 individuals showed low relatedness values (less than 0.12, theoretical value for first cousin relationships). Please note again that relatedness is a continuous parameter and does not present strict cutoffs, hence simple categorisations of kinship are not possible.

Table 6. Genetic relatedness of unique individuals for combined data, excluding the dyads listed in Table 3 for unique samples collected in 2022–23. Colour decodes the relatedness, from high (darker), over moderate (lighter) to low. Dyads with genetic relatedness values <0.12 are not listed

Koala 1	Koala 2	Relatedness value
JBAKG_9.0	Coffs_19.2	0.52
Coffs_13.3	Coffs_13.1	0.42
JBAKG_44.0	Coffs_6.13	0.42
Coffs_6.5	Coffs_6.6	0.38
Coffs_6.1	Coffs_6.5	0.35
JBAKG_43.0	Coffs_2.1.3	0.32
Coffs_2.1	Coffs_2.1.3	0.29
JBAKG_3.0	Coffs_22.1	0.29
Coffs_13.1	Coffs_13.5	0.28
Coffs_13.3	Coffs_13.5	0.26
Coffs_2.1	Coffs_13.1	0.22
Coffs_2.1	Coffs_13.3	0.21
Coffs_2.1.3	Coffs_6.13	0.20
Coffs_6.1	Mylestrom_1.1	0.19
JBAKG_44.0	Coffs_2.1	0.19
JBAKG_43.0	Coffs_2.1	0.18
JBAKG_63.0	Coffs_6.13	0.18
Coffs_2.1.3	Coffs_6.1	0.17
JBAKG_63.0	Coffs_2.1.3	0.16
BA_6.3	Coffs_6.1	0.16
Coffs_2.1	Coffs_13.5	0.16
JBAKG_44.0	Coffs_2.1.3	0.15
Coffs_13.3	Coffs_2.1.3	0.15
Coffs_13.3	Coffs_6.1	0.15
Coffs_4.1	Coffs_6.1	0.15
BA_6.2	Coffs_19.1	0.14
JBAKG_77.0	Coffs_6.1	0.13
JBAKG_63.0	Coffs_2.1	0.13
JBAKG_77.0	Coffs_1.7	0.13
JBAKG_4.0	Coffs_19.1	0.13
Coffs_2.1	Coffs_6.1	0.13
CA_1.6	Coffs_13.3	0.13
Coffs_19	Coffs_22.1	0.12
JBAKG_3.0	Coffs_19.2	0.12

Koala 1	Koala 2	Relatedness value
JBAKG_43.0	Coffs_6.13	0.12
Coffs_13.1	Coffs_2.1.3	0.12
Coffs_2.1	Coffs_6.6	0.12
Coffs_19	Coffs_19.2	0.12
Coffs_1.7	Coffs_6.1	0.12
JBAKG_61.0	Coffs_7.1	0.12

3.6.2 Sex of unique individuals and sex ratio

Of the 90 unique individuals, four samples failed quality control threshold for sex detection. Of the 86 individuals, 47 (54.7%) were males and 39 (45.3%) were females (see Figure 5 for locations of each individual by sex and Table 4 and Appendix 3, Table A2 for details by individual), translating to a sex ratio of 1.0:0.83 male to female, which is a small bias towards males.

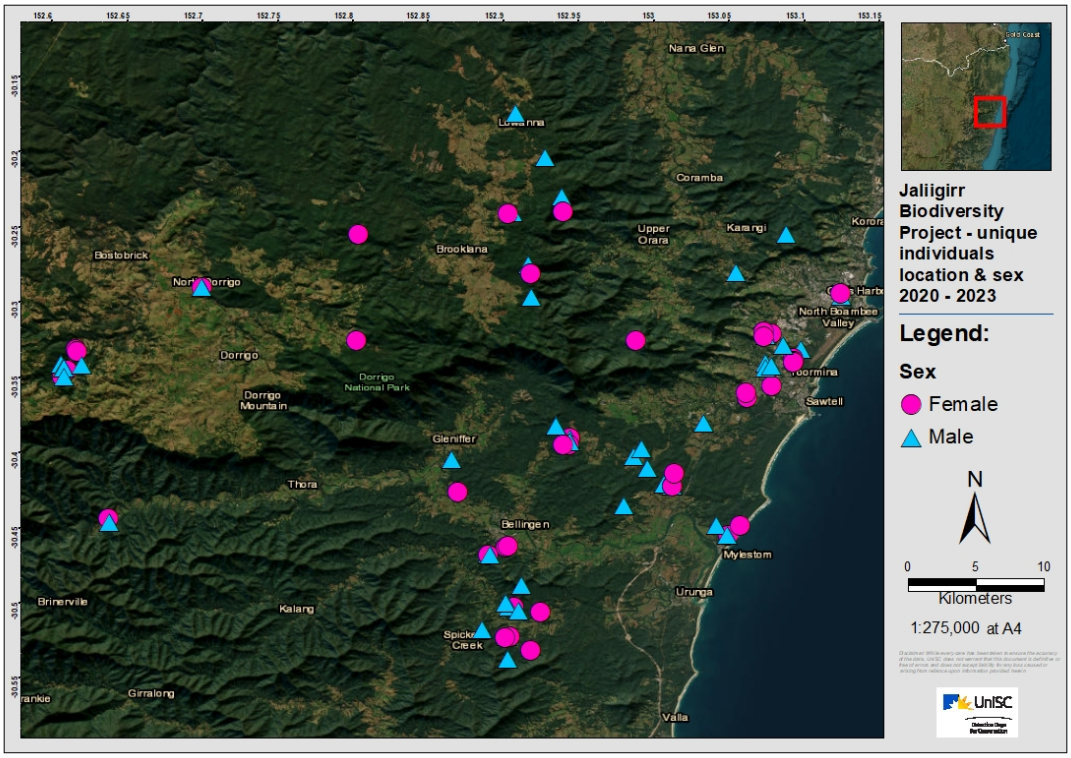

Figure 5. Distribution and sex of unique individuals for combined data (N = 86), four samples that failed the data quality control threshold for sex detection were not included.

3.6.3 *Chlamydia* prevalence

Of the 90 unique individuals, eight samples failed the quality control threshold for the *Chlamydia* detection (six samples from the 2022–23 sample set and two samples from 2020–22 sample set). A total of 27 (32.9%) individuals were positive for *Chlamydia* (see Figure 6 for locations of individuals by sex *Chlamydia* status and Table 4 and Appendix 3, Table A2 for details by individual). However, it should be noted again that the presence of the *Chlamydia* pathogen does not necessarily equate to disease.

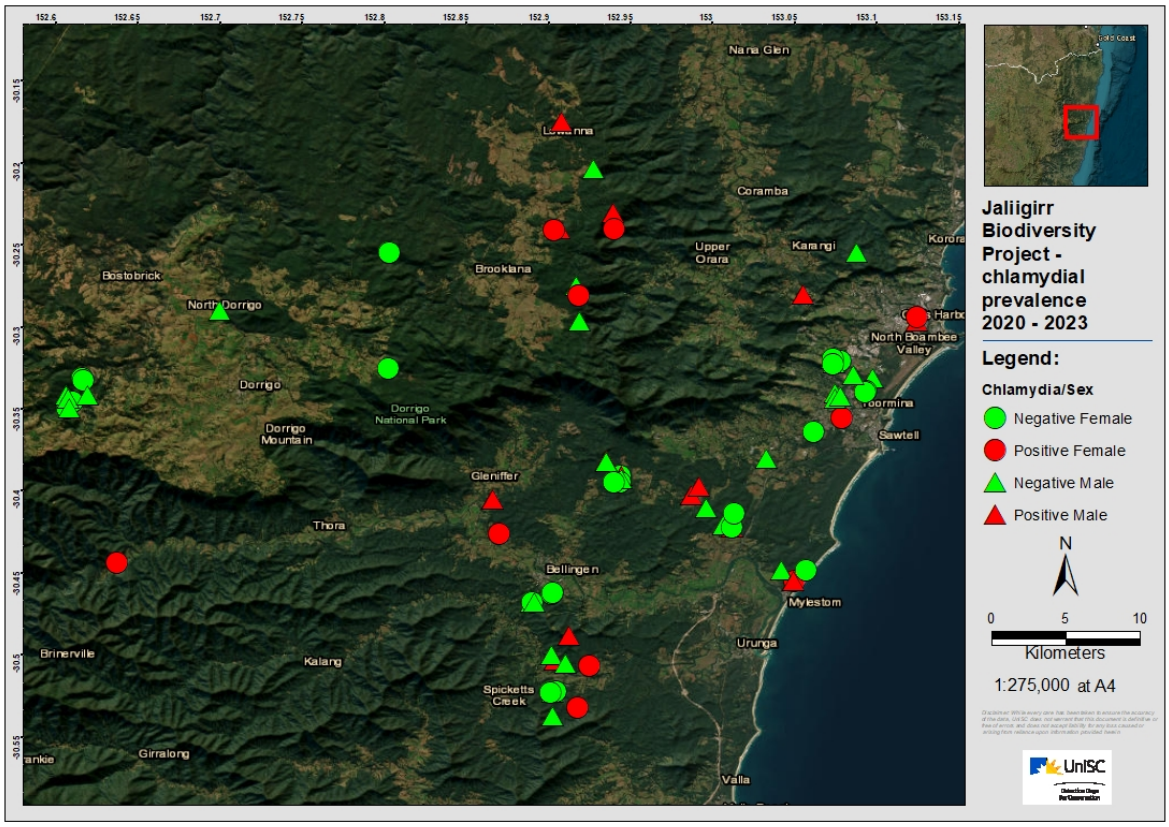

Figure 6. Location and *Chlamydia* status of unique individuals by sex for combined data (N = 78), 12 samples that failed the data quality control threshold for either sex detection or *Chlamydia* detection were not included.

3.6.4 Population genetic structure and genetic diversity

Out of the 90 unique individuals, 80 passed the data filtering criteria for population genetic diversity estimates with a total of 1,896 loci. Again, Principal Component Analysis did not show strong clustering, though the seven Fernbrook samples (namely, JBAKG_20.0, JBAKG_21.0, JBAKG_23.1, JBAKG_26.0, JBAKG_27.0, JBAKG_31.0 and JBAKG_37.0) again stood out from the rest, consistent with the previous results. Results from population structure analyses again indicated one single population (Appendix 5, Figure A3).

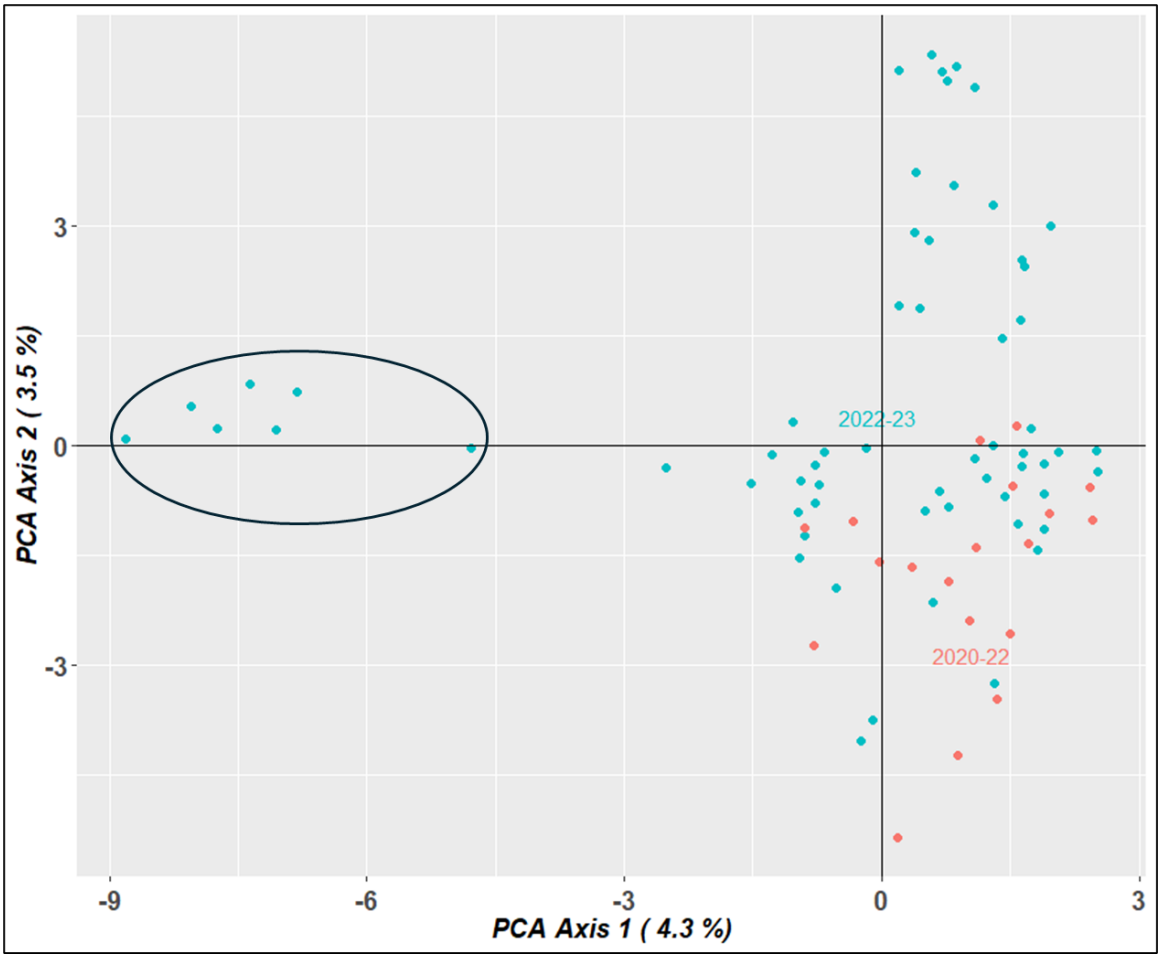

Figure 7. Results of the principal component analysis (PCA) for the unique koalas in 2020–22 (red dots) and 2022–23 (blue dots) sample collections. Seven individuals from Fernbrook (JBAKG_20.0, JBAKG_21.0, JBAKG_23.1, JBAKG_26.0, JBAKG_27.0, JBAKG_31.0 and JBAKG_37.0) are shown inside the circle.

For the 80 unique samples, high level of heterozygosity and low level of inbreeding were observed (Table 7). The estimated effective population size for the 80 unique koalas was Ne = 139.2 (95% CI = 136.3 – 141.2).

Table 7. Genetic diversity indices for the 64 unique koalas: SE: standard error

Parameter	Mean	SE
Observed heterozygosity (H_O)	0.290	0.004
Expected heterozygosity (H_E)	0.312	0.004
Inbreeding coefficient (F_{IS})	0.072	0.004

4 Discussion

4.1 Genetic relatedness among unique individuals

Among the unique identified individuals (N = 69), a small proportion (N = 9, 13%) indicated close genetic relatedness (relatedness values over 0.4). When analysing all unique koalas from both 2020–22 and 2022–23 sample collections together, this proportion was 12.2% (11 out of 90 individuals). The proportion of related individuals by itself does not lead to any conclusions, as these include naturally close relatives for example mum and joey pairs.

4.2 Sex of unique individuals and sex ratio

Overall, the male to female sex ratio indicated a small male bias in the current sample set (1:0.76) as well as for the combined data (1:0.8). Generally, while the sex ratio of a natural, healthy population is expected to be close to 1:1, a small bias toward females may be advantageous for conservation purposes, as larger female cohorts are associated with larger number of offspring, and therefore a larger population in the next generation. It is important to monitor the dynamic of sex ratio of this group of koalas in long-term as females drive population growth, and if the male biased sex ratio gets severe which can have detrimental consequences for the conservation and management.

4.3 *Chlamydia* prevalence

We observed 36.5% prevalence of chlamydial infection in the current sample set, with 23 of the koalas positive for *Chlamydia* presence. We observed an increase in chlamydial infection prevalence between two sample collections (19% in 2020–22 vs 36.5% in 2022–23), though the sample size for 2020-2022 was a lot smaller and from a smaller geographic area. Furthermore, the two collections do not necessarily reflect two separate time points, but rather a continuous effort (2020-2023). Hence, the best estimate for prevalence might be coming out of the combined data with 32.9% *Chlamydia* positive koalas. Overall, the current prevalence was less than what has been found in some other populations for *C. pecorum* urogenital infections, including Mutdapilly (52%) in Queensland (QLD), Mount Lofty Ranges (47%) in South Australia and DDC surveyed site of Ngunya Jargoon Indigenous Protected Area in NSW (58%) in 2022, and similar to proportions observed for Redland City Council (mainland) in Southeast Queensland (38%) in 2020–21, surveyed by DDC. However, the prevalence is higher than for instance in Coombabah (10%) and Moreton Bay (27%) in QLD (Jackson, White et al. 1999, Nyari, Waugh et al. 2017, Fabijan, Caraguel et al. 2019).

It is important to note that although the pathogen was detected in around 33-36% of the sampled koalas, this does not necessarily reflect their chlamydial disease status. For instance, some koalas could have recovered from disease but were still carrying *Chlamydia* in their gastrointestinal tracts and others could be carrying the pathogen without any clinical signs (Robbins, Hanger et al. 2019). When *Chlamydia* infection does however progress into disease, it can cause infertility and overall increased morbidity and mortality (Hulse, Beagley et al. 2021, Pagliarani, Johnston et al. 2022). This could have a large negative impact on the population and its long-term persistence.

While we can report on chlamydial infection, veterinary examinations are required to detect and confirm chlamydial disease. Given the level of infection prevalence, we suggest an investigation into *disease* prevalence through veterinary examinations could be beneficial. This would help with assessing the specific risk that this pathogen poses to this population. It should be noted that we did not detect any chlamydial infection in individuals from the

Fernbrook region. This is interesting, as it could highlight a healthy group of koalas of high conservation value, and management plans could help to safeguard this group of koalas from spreading disease. However, the sample size remains small, and this could mean chlamydia pathogen was present but due to sampling, was not detected. Further investigations into koalas from this area would be of conservation interest, in case this group of koalas is indeed pathogen free.

4.4 Population genetic structure and genetic diversity

Considering both the results from 2022–23 sample collection as well as from the combined analysis, PCA indicated a small degree of differentiation of individuals from the Fernbrook area from rest of the population. This might reflect some potentially recent constraints to geneflow/koala movements in and out of the Fernbrook region. However, population structure analyses did not show a strong differentiation or presence of more than one population and hence, we considered this group of koalas as one panmictic cluster for population genetic diversity estimates.

Theoretically, high heterozygosity means high genetic variability and diversity, and is therefore assumed to indicate higher resilience (e.g. higher chances of adapting to current and future challenges) and evolutionary potential, characterising a genetically healthy population (Orsted, Hoffmann et al. 2019). Another sign of a healthy population is low inbreeding values (Moss, Arce et al. 2007). In general, if the observed heterozygosity is lower than the expected heterozygosity, the discrepancy is attributed to inbreeding. The koalas studied in this project showed signs of a genetically healthy population, with high levels of genetic diversity (H_O = 0.290 and H_E = 0.312) and low levels of inbreeding (F_{IS} = 0.072).

The genetic diversity values are best interpreted by comparing them to other populations where diversity measures were calculated using similar methods. In a previous study in the Northern Tablelands, NSW, in 2019–2020, DDC estimated diversity for the Armidale/Uralla region and for the Inverell/Delungra region, Redland City Council (mainland), Southeast Queensland in 2020–21, and the Ngunya Jargoon Indigenous Protected Area, NSW in 2022

(Table 8). Further comparisons can be made by consulting Table 9, which was taken from Kjeldsen, Zenger et al. (2016). This table shows genetic diversity measures from other wild koala populations across Queensland, New South Wales and Victoria, using a different set of SNPs obtained through double digest restriction-associated genotyping (DArTseq).

Table 8. Genetic diversity measured through SNP sequencing in wild koala populations in NSW and QLD by DDC. N = sample size, H_O = observed heterozygosity, H_E = expected heterozygosity, F_{IS} = inbreeding coefficient

Population	N	H_O	H_E	F_{IS}
This study population (Mid North Coast)	69	0.29	0.31	0.07
Armidale/Uralla, NSW	36	0.23	0.28	0.20
Inverell/Delungra, NSW	40	0.23	0.28	0.18
Redland City Council (mainland), QLD	227	0.24	0.32	0.26
Ngunya Jargoon Indigenous Protected Area, NSW	20	0.30	0.33	0.08

Table 9. Genetic diversity in wild koala populations across QLD, NSW and Victoria. N = sample size, H_O = observed heterozygosity, H_E = expected heterozygosity, F_{IS} = inbreeding coefficient, Ne_{LD} = effective population size calculated using linkage equilibrium. Table taken from Kjeldsen et al. (2016)

State	Location	N	H_O	H_E	F_{IS} (*P* <0.01)	Ne_{LD} (95 %CI)
QLD	St Bees Island	19	0.29	0.35	0.23	Infinite (∞)
QLD	St Lawrence	19	0.26	0.30	0.20	Infinite (∞)
QLD	Koala Coast	24	0.22	0.30	0.32	Infinite (921.20-∞)
QLD	Ipswich	23	0.27	0.31	0.19	Infinite (∞)
NSW	Port Macquarie	45	0.23	0.28	0.21	116.8 (109.8-124.6)
NSW	Campbelltown	09	0.27	0.33	0.27	2.7 (2.4-3.2)
VIC	South Gippsland	19	0.24	0.30	0.27	Infinite (∞)
VIC	Cape Otway	13	0.24	0.25	0.11	46.7 (40.8-54.4)

The observed heterozygosity values in the current study were higher than many populations listed in Table 8 and Table 9 but were comparable with, for instance, koalas in Ngunya Jargoon

Indigenous Protected Area, NSW, which is geographically close. Most koala populations compared in Table 8 and Table 9 show higher inbreeding (F_{IS}) than what we found in this study, a positive sign for the studied koala population.

It should be noted, however, that measures of genetic diversity and inbreeding come with an associated time-lag (Landguth, Cushman et al. 2010) and often, signs of decline in these measures only occur after the population has already experienced a major impact. Therefore, genetic diversity measures might not reflect current issues in a population.

The effective population size (Ne) indicates the number of koalas that effectively participates in breeding and contribute to the next generation in the population, which is usually less than the census population size. Maintaining genetic diversity and evolutionary potential are often linked to the effective population size and small populations may result in increased levels of inbreeding and genetic drift. The estimated effective population size for 59 unique koalas (121.2) from the 2022–23 sample set is higher than what has been observed for 45 koalas (116.8) in Port Macquarie, NSW (Table 9). The value is further increased (139.2) for combined data from 80 unique kolas which is a positive characteristic representing a larger number of breeding animals. It is important to note that the Ne in Kjeldsen, Zenger et al. (2016) is often infinite, the authors highlighted this is due to the limitation of sample sizes that were not sufficient to obtain an accurate estimate of Ne.

Overall, measures of genetic diversity suggest a genetically healthy population. The study group of koalas had a higher level of heterozygosity than most populations we can compare it to, and a low inbreeding coefficient. However, while these are positive findings, we also found a prevalence of *Chlamydia* infections which can put the population at risk.

5 Recommendations

We recommend to monitor the *Chlamydia* infection prevalence of this population and further investigation into disease prevalence through veterinary examinations would likely be beneficial. We also recommend to continue monitoring of the sex ratio in this population.

Both disease and male skewed sex ratios can have detrimental effects on populations if they were to escalate.

It would furthermore be of interest to access more samples from the Fernbrook group of koalas to validate our finding with a larger sample size. It would be of interest to assess density through drone surveys and do further studies into genetic connectivity between Fernbrook and the remainder of the koalas. Also, if no pathogens nor signs of disease are found in this area, it would be of value to investigate potential underlying reasons for this and find ways to safeguard the Fernbrook koalas.

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Appendices

Appendix 1

Table A1. List and overview of koala scat samples collected in 2022–23 (N = 109) including quality control status for genetic analyses

Sample Name	Survey date	Location	Scat age	Latitude	Longitude	Quality control for genetic fingerprinting	Quality control for population genetic analyses
BA_6.1	27.6.22	Valery	Fresh	-30.3896	152.9436	Failed	Failed
BA_6.2	27.6.22	Valery	Fresh	-30.3906	152.9438	Passed	Failed
BA_6.3	28.6.22	Valery	Fresh	-30.3908	152.9444	Passed	Passed
BA_6.4	28.6.22	Valery	Fresh	-30.3952	152.9432	Passed	Failed
BA_6.5	28.6.22	Valery	Fresh	-30.3950	152.9434	Passed	Passed
BA_6.6	27.6.22	Valery	Fresh	-30.3900	152.9443	Passed	Failed
CA_1.1	19.9.22	Lowanna	Fresh	-30.1746	152.9080	Passed	Passed
CA_1.2	27.9.22	Karangi	Fresh	-30.2711	153.0799	Failed	Failed
CA_1.3	29.9.22	Ulong	Fresh	-30.2400	152.9068	Failed	Failed
CA_1.4	29.9.22	Lowanna	Fresh	-30.2405	152.9063	Passed	Passed
CA_1.5	30.9.22	Lowanna	Fresh	-30.2409	152.9028	Passed	Passed
CA_1.6	6.10.22	Karangi	Fresh	-30.2803	153.0548	Passed	Passed
CA_1.7	6.2.23	Karangi	Fresh	-30.3253	152.9881	Passed	Passed
JBAKG_1.0	27.6.23	Bongil Bongil NP	Fresh	-30.4353	152.9804	Passed	Passed
JBAKG_2.0	29.6.23	Bindarri (Timboon)	Fresh	-30.3818	152.9349	Passed	Passed
JBAKG_3.0	7.7.23	Bongil Bongil NP	Fresh	-30.3824	152.9349	Passed	Passed
JBAKG_4.0	7.7.23	Bongil Bongil NP	Fresh	-30.4222	153.0120	Passed	Passed
JBAKG_5.0	12.7.23	Timboon Rd Valery	Fresh	-30.3927	152.9444	Passed	Passed

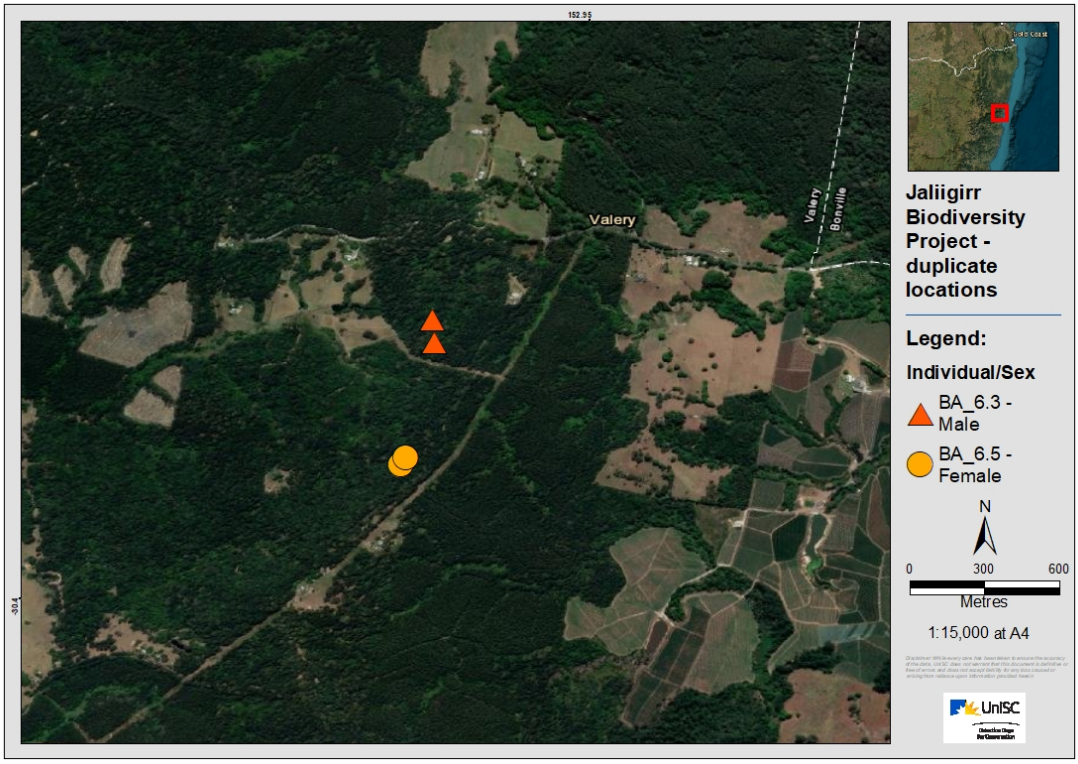
Sample Name	Survey date	Location	Scat age	Latitude	Longitude	Quality control for genetic fingerprinting	Quality control for population genetic analyses
JBAKG_6.0	12.7.23	Timboon Rd Valery	Fresh	-30.3946	152.9397	Passed	Passed
JBAKG_7.0	13.7.23	Bongil Bongil NP	Fresh	-30.4211	153.0067	Passed	Failed
JBAKG_8.0	27.7.23	South Arm Road Brierfield	Fresh	-30.5031	152.9065	Passed	Passed
JBAKG_9.0	28.7.23	Bongil Bongil NP	Fresh	-30.4225	153.0119	Passed	Passed
JBAKG_10.0	1.8.23	South Arm Road Brierfield	Medium Fresh	-30.5030	152.9037	Passed	Passed
JBAKG_11.0	9.8.23	South Arm Road Brierfield	Medium	-30.5081	152.9268	Passed	Failed
JBAKG_12.0	12.8.23	South Arm Road Brierfield	Medium Fresh	-30.5063	152.9247	Passed	Passed
JBAKG_13.0	13.8.23	South Arm Road Brierfield	Fresh	-30.5000	152.9010	Passed	Failed
JBAKG_14.0	13.8.23	South Arm Road Brierfield	Fresh	-30.5002	152.9011	Passed	Failed
JBAKG_15.0	25.8.23	Endeavour Drive Bellingen	Medium Fresh	-30.4628	152.9019	Passed	Passed
JBAKG_16.0	25.8.23	Endeavour Drive Bellingen	Medium Fresh	-30.4622	152.9027	Passed	Passed
JBAKG_17.0	2.6.22	Wallambia	Medium	-30.5063	152.9224	Failed	Failed
JBAKG_18.0	4.9.23	South Arm Road Brierfield	Fresh	-30.5001	152.9011	Passed	Failed
JBAKG_19.0	7.9.23	South Arm Road Brierfield	Fresh	-30.5000	152.9018	Passed	Passed
JBAKG_20.0	12.9.23	Johnsons Rd Fernbrook	Fresh	-30.3312	152.6158	Passed	Passed
JBAKG_21.0	12.9.23	Johnsons Rd Fernbrook	Fresh	-30.3326	152.6165	Passed	Passed
JBAKG_22.0	13.9.23	Tarkeeth SF	Fresh	-30.4883	152.9122	Passed	Passed
JBAKG_23.0	14.9.23	Johnsons Rd Fernbrook	Fresh	-30.3515	152.6067	Passed	Failed
JBAKG_23.1	14.9.23	Johnsons Rd Fernbrook	Fresh	-30.3495	152.6068	Passed	Passed
JBAKG_24.0	15.9.23	Johnsons Rd Fernbrook	Fresh	-30.3391	152.6210	Passed	Failed
JBAKG_25.0	15.9.23	Johnsons Rd Fernbrook	Fresh	-30.3389	152.6186	Passed	Failed
JBAKG_26.0	15.9.23	Johnsons Rd Fernbrook	Fresh	-30.3477	152.6118	Passed	Passed
JBAKG_27.0	15.9.23	Johnsons Rd Fernbrook	Fresh - Medium Fresh	-30.3455	152.6097	Passed	Passed

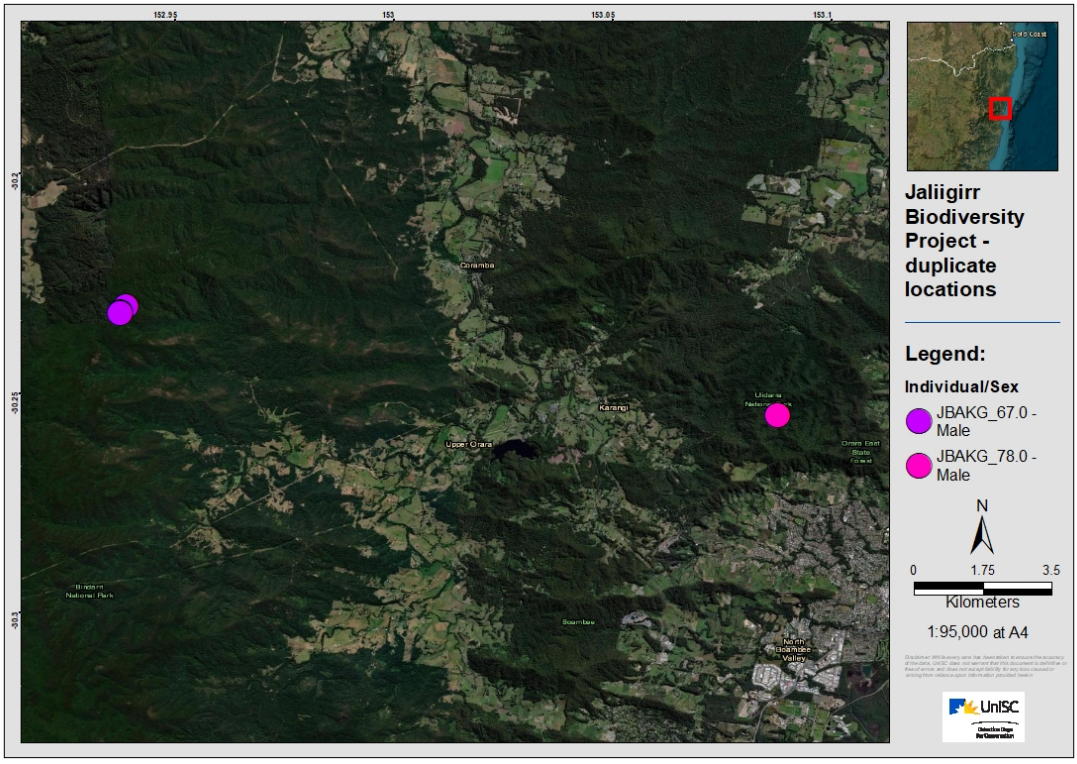
Sample Name	Survey date	Location	Scat age	Latitude	Longitude	Quality control for genetic fingerprinting	Quality control for population genetic analyses
JBAKG_28.0	21.9.23	Johnsons Rd Fernbrook	Medium Fresh	-30.3415	152.6078	Failed	Failed
JBAKG_29.0	21.9.23	Johnsons Rd Fernbrook	Medium Fresh	-30.3414	152.6062	Passed	Failed
JBAKG_30.0	21.9.23	Johnsons Rd Fernbrook	Fresh	-30.3437	152.6075	Passed	Failed
JBAKG_31.0	21.9.23	Johnsons Rd Fernbrook	Fresh	-30.3437	152.6076	Passed	Passed
JBAKG_32.0	26.9.23	Jaaningga NR	Fresh	-30.5226	152.9023	Passed	Passed
JBAKG_33.0	26.9.23	Jaaningga NR	Fresh	-30.5230	152.9011	Passed	Failed
JBAKG_34.0	26.9.23	Jaaningga NR	Fresh	-30.5371	152.9030	Passed	Passed
JBAKG_35.0	26.9.23	Jaaningga NR	Fresh	-30.5372	152.9026	Passed	Passed
JBAKG_36.0	26.9.23	Jaaningga NR	Fresh	-30.5318	152.9178	Passed	Passed
JBAKG_37.0	27.9.23	Johnsons Rd Fernbrook	Fresh	-30.3416	152.6195	Passed	Passed
JBAKG_38.0	27.9.23	Johnsons Rd Fernbrook	Fresh	-30.3414	152.6195	Passed	Failed
JBAKG_39.0	5.10.23	Bellingen Tip reserve	Fresh	-30.4677	152.8895	Passed	Passed
JBAKG_40.0	5.10.23	Bellingen Tip reserve	Fresh	-30.4683	152.8898	Passed	Passed
JBAKG_41.0	5.10.23	South Arm Road Brierfield	Fresh	-30.5064	152.9243	Passed	Failed
JBAKG_42.0	18.10.23	Bongil Bongil Overpass	Medium Fresh	-30.4166	153.0247	Failed	Failed
JBAKG_43.0	18.10.23	Coffs Botanic Garden	Fresh	-30.2963	153.1244	Passed	Passed
JBAKG_44.0	18.10.23	Coffs Botanic Garden	Fresh	-30.2940	153.1239	Passed	Passed
JBAKG_45.0	18.10.23	Roses Rd Gleniffer	Medium Fresh	-30.4260	152.8696	Passed	Passed
JBAKG_46.0	24.10.23	Bongil Bongil NP	Medium Fresh	-30.4106	152.9960	Passed	Passed
JBAKG_47.0	25.10.23	Bowerville Rd Brierfield	Fresh	-30.5175	152.8858	Passed	Passed
JBAKG_48.0	25.10.23	Bowerville Rd Brierfield	Fresh	-30.5176	152.8850	Failed	Failed
JBAKG_49.0	29.10.23	South Arm Road Brierfield	Fresh	-30.5079	152.9269	Failed	Failed
JBAKG_50.0	29.10.23	Tarkeeth SF	Fresh	-30.5041	152.9223	Passed	Failed

Sample Name	Survey date	Location	Scat age	Latitude	Longitude	Quality control for genetic fingerprinting	Quality control for population genetic analyses
JBAKG_51.0	30.10.23	Tarkeeth SF	Fresh	-30.5053	152.9240	Passed	Failed
JBAKG_52.0	6.7.23	Jaaningga NR	Fresh	-30.5225	152.9044	Passed	Passed
JBAKG_53.0	6.7.23	Jaaningga NR	Fresh	-30.5217	152.9040	Failed	Failed
JBAKG_54.0	6.7.23	Jaaningga NR	Fresh	-30.5232	152.9009	Passed	Passed
JBAKG_55.0	10.8.23	Bellingen Tip reserve	Fresh	-30.4679	152.8896	Failed	Failed
JBAKG_56.0	10.8.23	Bellingen Tip reserve	Fresh	-30.4682	152.8898	Failed	Failed
JBAKG_57.0	10.8.23	Bellingen Tip reserve	Fresh	-30.4680	152.8895	Failed	Failed
JBAKG_58.0	10.8.23	Bellingen Tip reserve	Fresh	-30.4682	152.8912	Passed	Passed
JBAKG_59.0	2.10.23	Bonville Hall	Fresh	-30.3803	153.0329	Failed	Failed
JBAKG_60.0	26.4.23	Bonville Hall	Fresh	-30.3803	153.0329	Failed	Failed
JBAKG_61.0	9.9.23	Bonville Hall	Fresh	-30.3803	153.0329	Passed	Passed
JBAKG_62.0	29.10.23	Fernbrook	Fresh	-30.3498	152.6080	Passed	Failed
JBAKG_63.0	13.7.22	Hogbin Drive WIRES	Fresh	-30.3311	153.0976	Passed	Passed
JBAKG_64.0	14.11.23	Junuy Juluum NP	Fresh	-30.2904	152.6997	Passed	Passed
JBAKG_65.0	14.11.23	Junuy Juluum NP	Fresh	-30.2904	152.6996	Passed	Failed
JBAKG_66.0	14.11.23	Junuy Juluum NP	Fresh	-30.2903	152.6997	Passed	Passed
JBAKG_67.0	15.11.23	Langleys Rd Upper Bindarri	Fresh	-30.2302	152.9389	Passed	Passed
JBAKG_68.0	15.11.23	Langleys Rd Upper Bindarri	Fresh	-30.2316	152.9376	Passed	Failed
JBAKG_69.0	15.11.23	Langleys Rd Upper Bindarri	Fresh	-30.2319	152.9375	Passed	Failed
JBAKG_70.0	15.11.23	Corfes Rd Upper Bindarri	Medium Fresh	-30.2752	152.9167	Passed	Failed
JBAKG_71.0	15.11.23	Range Rd Upper Bindarri	Fresh	-30.2811	152.9180	Passed	Passed
JBAKG_72.0	28.11.23	Bollanolla NR	Fresh	-30.6039	152.9009	Failed	Failed
JBAKG_73.0	23.10.23	Roses Rd Gleniffer	Fresh	-30.4050	152.8659	Passed	Passed

Sample Name	Survey date	Location	Scat age	Latitude	Longitude	Quality control for genetic fingerprinting	Quality control for population genetic analyses
JBAKG_74.0	28.11.23	Roses Rd Gleniffer	Fresh	-30.4050	152.8659	Passed	Passed
JBAKG_75.0	1.12.23	Slingsbys New England NP	Fresh	-30.3252	152.8021	Failed	Failed
JBAKG_76.0	1.12.23	Slingsbys New England NP	Fresh	-30.3255	152.8024	Passed	Passed
JBAKG_77.0	12.12.23	Ulidarra NP	Fresh	-30.2550	153.0877	Passed	Failed
JBAKG_78.0	12.12.23	Ulidarra NP	Fresh	-30.2550	153.0877	Passed	Passed
JBAKG_79.0	12.12.23	Ulidarra NP	Fresh	-30.2550	153.0877	Passed	Failed
JBAKG_81.0	15.12.23	Darkwood Rd Darkwood	Fresh	-30.4440	152.6372	Passed	Passed
JBAKG_82.0	15.12.23	Darkwood Rd Darkwood	Medium Fresh	-30.4464	152.6382	Passed	Passed
JBAKG_83.0	15.12.23	South Arm Road Brierfield	Fresh	-30.5062	152.9198	Passed	Passed
JBAKG_84.0	19.12.23	Cascade NP	Medium Fresh	-30.2547	152.8032	Passed	Passed
JBAKG_85.0	19.12.23	Cascade NP	Medium Fresh	-30.2547	152.8032	Passed	Failed
JBAKG_86.0	19.12.23	Cascade NP	Medium Fresh	-30.2586	152.8167	Failed	Failed
JBAKG_87.0	21.12.23	South Arm Road Brierfield	Fresh	-30.5055	152.9103	Passed	Passed
JBAKG_88.0	21.12.23	South Arm Road Brierfield	Fresh	-30.5055	152.9103	Passed	Failed
JBAKG_89.0	21.12.23	South Arm Road Brierfield	Fresh	-30.5053	152.9100	Failed	Failed
JBAKG_90.0	13.12.23	Roses Rd Gleniffer	Fresh	-30.2219	152.7254	Failed	Failed
JBAKG_91.0	14.11.23	Roses Rd Gleniffer	Fresh	-30.4188	152.8598	Failed	Failed
JBAKG_93.0	12.8.21	South Arm Road Brierfield	Medium	-30.5055	152.9206	Passed	Failed
JCBIN2022	8.11.22	Bindarri	NA	-30.2967	152.9187	Passed	Failed
Lowanna_6	12.10.22	Lowanna	Fresh	-30.2400	152.9395	Passed	Passed
Lowanna_8	19.10.22	Lowanna	Fresh	-30.2039	152.9276	Passed	Failed
Mylestrom	19.10.22	Mylestrom	Fresh	-30.4550	153.0494	Passed	Passed

Appendix 2


152.95
Valery
Valley
Bonville
-30.4
Jaliigirr Biodiversity Project - duplicate locations
Legend:
Individual/Sex
BA_6.3 - Male
BA_6.5 - Female
N
0
300
600
Metres
1:15,000 at A4
UniSC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153
153.05
153.1
-30.2
-30.25
-30.3
Coramba
Karangi
Upper Orara
Boambee
Jaliigirr Biodiversity Project - duplicate locations
Legend:
Individual/Sex
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JBAKG_78.0 - Male
N
0
1.75
3.5
Kilometers
1:95,000 at A4
UniSC

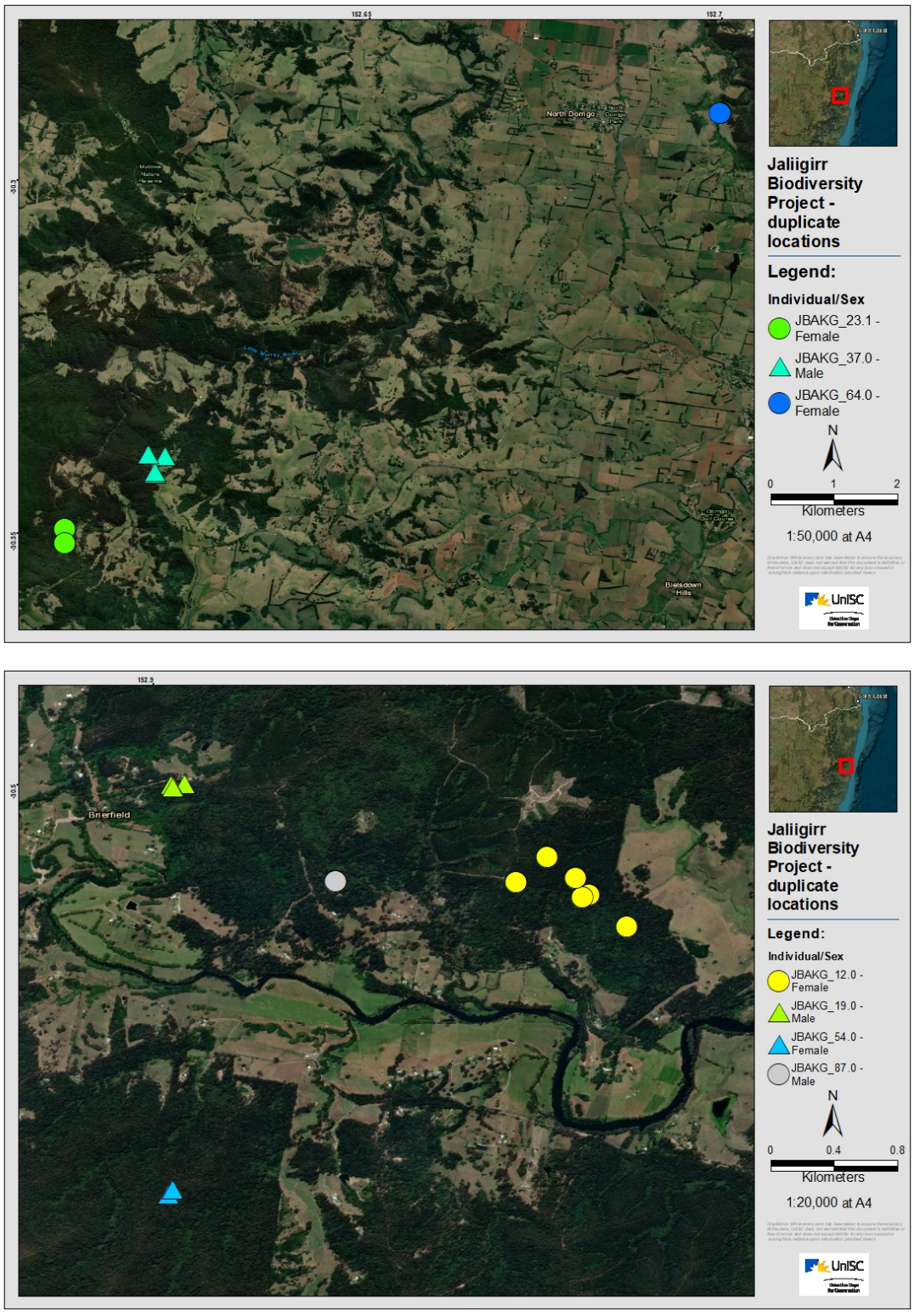

Figure A1. Locations of duplicate samples collected from 11 individuals.

Appendix 3

Table A2. List of unique koalas (N = 23) from 2020–22 scat samples collection included into co-analyses. 'QC-failed' represent the sample that failed the quality control threshold for the *Chlamydia* detection

Sample Name	Sex	*Chlamydia* status	Latitude	Longitude
Coffs_1.2	M	Negative	-30.3555	153.0784
Coffs_1.3	F	Positive	-30.3555	153.0784
Coffs_1.7	F	QC-failed	-30.3371	153.0927
Coffs_2.1	F	Negative	-30.3393	153.0935
Coffs_2.1.3	F	Negative	-30.3398	153.0928
Coffs_4.1	M	Negative	-30.3291	153.0858
Coffs_6.1	F	Negative	-30.321	153.0779
Coffs_6.11	M	Negative	-30.3193	153.0727
Coffs_6.13	F	Negative	-30.3198	153.0732
Coffs_6.5	M	Negative	-30.3223	153.074
Coffs_6.6	F	Negative	-30.3229	153.0729
Coffs_7.1	F	Negative	-30.3638	153.0617
Coffs_7.3.1	F	QC-failed	-30.3602	153.061
Coffs_13.1	M	Negative	-30.3438	153.0745
Coffs_13.2	M	Negative	-30.3441	153.0754
Coffs_13.3	M	Negative	-30.3407	153.0744
Coffs_13.5	M	Negative	-30.3425	153.0779
Coffs_19	F	Negative	-30.4138	153.0132
Coffs_19.1	M	Positive	-30.4028	152.9869
Coffs_19.2	F	Negative	-30.4483	153.0571
Coffs_19.3	M	Positive	-30.3972	152.9917
Coffs_22.1	M	Negative	-30.4482	153.0416
Mylestrom_1.1	M	Positive	-30.4549	153.0488

Appendix 4

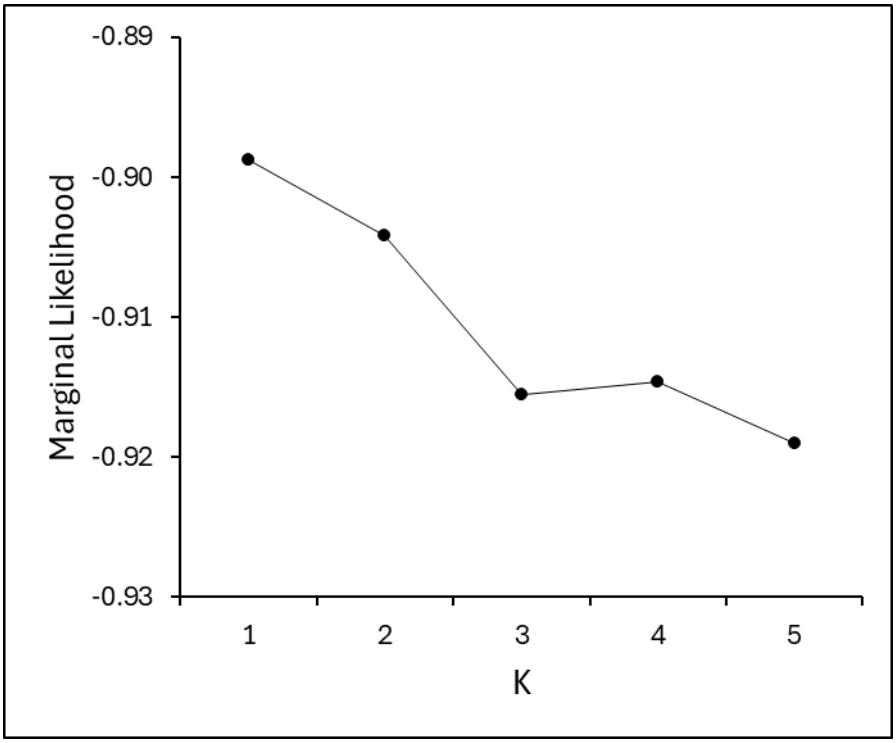

Figure A2. Marginal likelihood values determined at each K by fastStructure analyses for 59 unique koala samples collected in 2020–23. The peak indicates the most possible number of genetic clusters (K) present within the data set.

Appendix 5

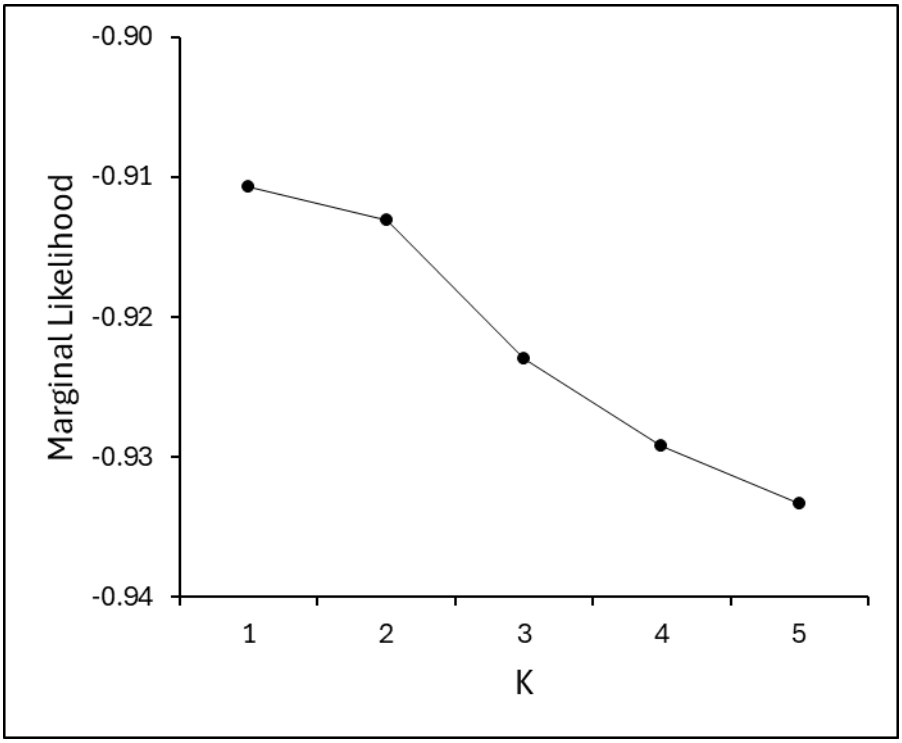

Figure A3. Marginal likelihood values determined at each K by fastStructure analyses for 80 unique koala samples used in co-analyses. The peak indicates the most possible number of genetic clusters (K) present within the data set.