

# A Major Highway Acts to Genetically Structure a Sugar Glider (*Petaurus Breviceps*) Population

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## Research Article

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## Abstract

Arboreal gliders are vulnerable to habitat fragmentation and to barriers that extend their glide distance threshold. Habitat fragmentation through deforestation can cause population isolation and genetic drift in gliding mammals which in turn can result in a loss of genetic diversity and population long-term persistence. This study utilised next generation sequencing technology to call 11, 292 genome-wide SNPs from 90 adult sugar gliders (*Petaurus breviceps*). Samples were collected from 12 locations in the Lake Macquarie Local Government Area (New South Wales), with two of these locations west of the Pacific Motorway, a potential major barrier to their dispersal. Overall, Lake Macquarie sugar gliders appeared to have high levels of gene flow and little genetic differentiation, however spatial least cost path analyses identified the Pacific Motorway as a barrier to their dispersal. This Motorway is still relatively new (< 40 years old), so man-made crossing structures should be erected as a management priority to mitigate any long-term effects of population isolation by assisting in the dispersal and gene flow of the species. This study provides further insight into the sugar glider after it was classed as three separate species in 2020 and could potentially be used as a model for its threatened congener in the area, the squirrel glider (*Petaurus norfolcensis*).

## Introduction

Eucalypt forests have experienced the greatest rate of deforestation in Australia, with 80% of the remaining forests modified by human activity (Bradshaw 2012). This habitat loss and fragmentation is a known major threat to arboreal marsupials that are dependent on forest food resources and require tree hollows for nesting, shelter and predator avoidance (Braithwaite *et al.* 1988; Gibbons and Lindenmayer 2000; Lindenmayer 2002). Also, arboreal marsupials have adaptations for climbing and gliding between trees that leave them slow and vulnerable to predators and vehicles when forced to cross their habitat on the ground (Bassarova *et al.* 2009; Warburton *et al.* 2012; Rupert *et al.* 2014). Because of this, there is a real and ongoing risk that populations may become isolated due to habitat fragmentation, resulting in reduced gene flow and genetic diversity (Frankham *et al.* 2002). This presents a problem for the long-term viability and persistence of populations, as genetic diversity facilitates their ability to adapt to stochastic changes in their environment (Mahoney and Springer 2009).

Conservation genetic research has examined the effect of straight-line distance on genetic distance of populations, with a non-significant result indicative of features in the landscape that may present barriers or challenges to gene flow and dispersal (Whitlock and Mccauley 1999). Over time, researchers have incorporated spatial data into Mantel tests to examine the effect of least cost path on genetic distances of populations (Wang *et al.* 2009; Milanesi *et al.* 2016). This concept of “landscape genetics” has proved extremely useful for pinpointing barriers to gene flow such as roads (Keller and Largiadèr 2003) and biogeographic barriers (Pérez-Espona *et al.* 2008; Wei *et al.* 2012). Additionally, least cost path analyses have identified corridors for priority conservation when paired with genetic data (Wang *et al.* 2009; Etherington *et al.* 2014). When investigating the effect of habitat fragmentation on population genetics, it is essential to combine spatial and genetic data to gain a better understanding of genetic structure (Storfer *et al.* 2007).

While studies have used microsatellites to investigate the effect of Australian habitat fragmentation on gliding mammals in the past (Pavlova *et al.* 2010; Taylor *et al.* 2011; Goldingay *et al.* 2013; Malekian *et al.* 2015), researchers are yet to utilise the power of next generation sequencing and genome-wide single nucleotide polymorphisms (SNPs). SNPs are single base pair nucleotide changes along the genome that vary for individuals, representing the most common form of sequence variation (Brumfield *et al.* 2003). Thanks to advances in next generation sequencing, thousands of bi-allelic, co-dominant SNP markers can be produced (Kumar *et al.* 2012). SNPs have wider genome coverage than microsatellites, making them more precise and informative when answering questions regarding genetic diversity and structure in non-model organisms (Liu *et al.* 2005). As a result, SNPs have been successfully used for a vast array of Australian mammal studies in recent years (Kjeldsen *et al.* 2016; White *et al.* 2018; Schultz *et al.* 2018; Kjeldsen *et al.* 2019; Martin *et al.* 2019; Wright *et al.* 2019).

Despite having similar life histories and being congeners, sugar gliders (*Petaurus breviceps*) are currently listed as common, while squirrel gliders (*Petaurus norfolcensis*) are listed as threatened in New South Wales (Threatened Species Conservation Act 1995). It is possible that these congeners may respond to habitat fragmentation differently, warranting further investigation into the effects of urbanisation on them. On the other hand, if similar trends are observed, then sugar glider populations could be used as a 'model' for its threatened congener. While they do not often share habitat, certain locations in their distribution present a unique opportunity to examine this. One such location is in central New South Wales (ie. Lake Macquarie Local Government Area (LGA)) where the two species occur in high densities with some overlap (Smith 2002; Smith and Murray 2003; Knipler unpublished raw data).

Although sugar gliders are listed as a common species, threats to populations from ongoing urbanisation and land clearing remains a concern for their ongoing survival. In addition to this, sugar gliders were recently divided into three separate species: the sugar glider (*Petaurus breviceps*, found in Lake Macquarie and east of the Great Dividing Range), krefft's glider (*Petaurus notatus*, found in eastern Australia, though west of the Great Dividing Range and in Tasmania where it is an introduced species) and savanna glider (*Petaurus ariel*, found in northern Australia) (Cremona *et al.* 2020). Because of this division, the conservation status of the species' perhaps need revision since there has likely been an overestimation of its range and effective population size by past studies that considered these as a single species. Cremona *et al.* (2020) call for targeted research on the three separate species to better understand their ecology and identify any areas of conservation concern. Here we use genome-wide SNP markers to investigate the effect of habitat fragmentation on sugar glider (*P. breviceps*) population genetics in the Lake Macquarie LGA. We report on their current genetic diversity and structure and thus provide the first baseline data available on their population genetics. Finally, we investigate barriers to their historical and ongoing dispersal and in doing so hypothesised that biogeographical barriers and habitat fragmentation would reduce genetic diversity and influence population genetic structure. Least cost path analyses were used to examine this in detail and subsequently direct conservation measures with the intended outcome of contributing to data driven conservation outcomes for sugar gliders.

## Materials And Methods

### *Study area*

Sugar gliders require tree hollows for sleeping and nesting and primarily rely on the sap, gum and nectar of eucalypt, acacia, and banksia species (Smith 1982; Lindenmayer 2002). Together the Lake Macquarie LGA and neighbouring Newcastle LGA contains 47, 100 ha of native forest (55% of total area), the majority of which is comprised of medium open eucalypt forest that is suitable for glider species (Department of Agriculture Water and Environment 2021). Due to the eucalypt, acacia and banksia habitat spread across the landscape, Lake Macquarie LGA holds the most abundant squirrel glider population in New South Wales (NSW) (Smith 2002) and similarly holds a large population of sugar gliders (Smith and Murray 2003). This location is recognized as being the most genetically diverse for squirrel gliders in Australia, and is located 130 km north of Sydney, NSW (Pavlova *et al.* 2010) (Fig. 1). Additionally, Lake Macquarie LGA contains the largest coastal saltwater lake in Australia, (120 km<sup>2</sup>) a potential biogeographical barrier to glider gene flow.

### *Live trapping and DNA collection*

Live trapping was conducted at 32 sites from 2017 to 2020, with 113 sugar glider individuals caught across 12 of the sites (Fig. 1). A combination of Mawbey traps, Elliot B traps, cage traps and Winning and King pipe traps were used to live trap gliders (Mawbey 1989; Quin 1995; Winning and King 2008). The Winning and King pipe traps were secured one metre from the base of the tree and the bottom was filled with leaves for bedding and a bait ball made from a mixture of peanut butter, honey and oats (Winning and King 2008). The other traps were instead secured to wooden planks that were drilled into tree trunks two metres above the ground. Each of these trap types contained a mix of leaves for bedding and a bait ball. A 1:4 ratio of honey water was then sprayed up and down the tree to a height of six metres as well as around the entrance to

each trap as an olfactory attractant (Sharpe and Goldingay 2007). Each site was subject to at least one week of live trapping using 12 traps per site (two rows of six with each trap spaced 50 meters apart), and further focus was given to locations that required a larger sample size for intended genetic analyses. Trapping ceased when no new individuals were detected after one week or when a previously marked individual was recaptured three times within a week.

Traps were checked each morning at sunrise. When a sugar glider was caught, body measurements were recorded. These measurements included weight, tail length, right hind foot length, head width, head length and sex to monitor the body condition of any recaptured animals. Individuals were given a unique identifying number in the form of a metal ear tag or a unique ear marking combination. Before being released, their DNA was collected in the form of an ear biopsy. A 2 mm metal ear punch (Able Scientific, Australia) was sprayed with 70% ethanol and flamed for sterilization. Once cooled, a small clipping was taken from the outside edge of the ear and stored in sterilized vials containing 95% ethanol. These were kept at  $-20^{\circ}\text{C}$  prior to DNA extraction. The processing of each sugar glider was limited to five-minutes to avoid unnecessary disturbance and following approved animal ethics protocol (AE15/11 and AE19/02). When the processing of an individual was complete, the glider was safely released onto the tree where it was caught.

### *SNP genotyping and filtering*

Genomic DNA was extracted from ear tissue using DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) as per the manufacturer's instructions. A set of 96 samples were selected for analysis after DNA concentration and purity were assessed using agarose gel electrophoresis and a NanoDrop 2000 Spectrophotometer (Thermo Scientific). These samples were plated and transported to Diversity Arrays Technology (DARTseq), University of Canberra, Australia, for concentration and sequencing (Kilian *et al.* 2012). DARTseq technology has effectively assisted with the research of Australian marsupials in recent years, including koalas (*Phascolarctos cinereus*) (Schultz *et al.* 2018; Kjeldsen *et al.* 2019), bare-nosed wombats (*Vombatus ursinus*) (Martin *et al.* 2019) and Tasmanian devils (*Sarcophilus harrisii*) (Wright *et al.* 2019). DARTseq utilises next generation sequencing and genome complexity reduction methods to call bi-allelic, codominant SNP markers (Kilian *et al.* 2012). For this species, a double digest was conducted with *Pst*I and *Sph*I restriction enzymes. The *Pst*I overhang was compatible with the barcode adapter while the *Sph*I overhang was compatible with the reverse adaptor and the flowcell attachment region (Elshire *et al.* 2011; Kilian *et al.* 2012). The resulting DNA fragments were then amplified with PCR and sequenced on Illumina HiSeq2500. Reads were cleaned, barcodes were removed, and sequences were aligned to the Leadbeater's possum reference genome (*Gymnobelideus leadbeateri*, GCA\_011680675.1 LBP\_v1") using DARTseq analytical pipelines. Once the process was complete, genome-wide SNPs were retained with a minimum sequence identity of 70%. For additional information regarding the DARTseq process, refer to Kilian *et al.* 2012.

Final filtering was conducted in R 4.0.2 (R Core Team 2015) using the *dartR* package (Gruber *et al.* 2018). SNPs were filtered on call rate (0.95 threshold) and hamming distance (0.2 threshold). Monomorphic loci and linked loci were removed, as well as those that significantly deviated from Hardy-Weinberg Equilibrium ( $p < 0.05$ ).

### *Population structure and genetic diversity analyses*

All analyses were undertaken in R 4.0.2 (R Core Team 2020) unless otherwise stated. For sampling locations with two or more individuals, average observed ( $H_{\text{obs}}$ ) and expected ( $H_{\text{ex}}$ ) heterozygosity was calculated at each SNP using the package *hierfstat* (Goudet 2005). These values were used to assess the level of inbreeding ( $F_{\text{IS}} = (H_{\text{ex}} - H_{\text{obs}}) / H_{\text{ex}}$ ) at different geographic locations. Additionally, F-statistics in the form of average  $F_{\text{IS}}$  and  $F_{\text{ST}}$  were generated for the overall region.

A Principal Component Analysis (PCOA) ordination enabled visualisation of genetic differentiation between individuals and between sampling locations (Gower 1966). This was generated with the *gl.pcoa.plot* function in *dartR* (Gruber *et al.* 2018). Similarly, Pairwise  $F_{\text{ST}}$  and an Analysis of Molecular Variance (AMOVA) were used to examine population structure

and population differentiation. Pairwise  $F_{ST}$  and the corresponding p values were calculated between the 12 sampling locations using the *gl.fst.pop* function in the R package *dartR* 1.1.11. 100 bootstraps were performed across loci to generate the p-values. The AMOVA was conducted through the R package *poppr* 2.9.0 using 9,999 permutations (Zhian Kamvar 2021).

A STRUCTURE analysis examined individual-based structure and admixture. The program STRUCTURE 2.3.4 was employed to determine the appropriate number of clusters (K) and to visualise the ancestry proportions per individual (Pritchard *et al.* 2000; Pritchard *et al.* 2003). The program tested genetic clusters K = 1 to 15 with 8 repeats of K. Each run had a 10,000-length burn-in period followed by 10,000 Monte Carlo Markov Chain replications. The results were then uploaded to Structure Harvester (Web v0.6.94) and the most probable K was chosen based on the Evanno method and the largest Delta K value (Evanno *et al.* 2005; Earl and VonHoldt 2012). Individuals with q values > 0.8 were considered pure to a cluster while individuals with q values < 0.8 were considered admixed.

#### *Isolation by distance and least cost path analyses*

An isolation by distance (IBD) Mantel test with 9,999 permutations assessed whether there was a relationship between geographic distance and genetic distance of populations. This analysis took the log of straight-line distances between populations using the *gl.ibd* function in the *dartR* package and compared them to genetic distances in the form of pairwise  $F_{ST}$  (that is,  $F_{ST}/(1-F_{ST})$ ).

Additionally, three least cost path analyses were performed using the *gl.genleastcost* function in *dartR* to account for habitat suitability and barriers. All rasters (friction matrixes) were created in ARCMAP 10.7.1. The first friction matrix accounted for biogeographical barriers by assigning "NoData" values to the ocean and lake from a land use spatial layer (NSW Government 2007), indicating a complete barrier to dispersal. Everything else was assigned a cell value of 1 to allow easy dispersal. This matrix was used in a Mantel test and will be hereon referred to as Mantel test B (biogeographical barriers).

The second friction matrix built on Mantel test B by assigning a cell value of 200 to four-lane highways obtained from the NSW Department of Industry, indicating unsuitable habitat and high effort for dispersal. This was used in a Mantel test and given the name Mantel test BH (biogeographical barriers and highways). The third and final friction matrix was created with cells ranging from optimal habitat (1) to unsuitable habitat (200) and given the name BHH (biographical barriers, highways and habitat). First, Plant Community Type (PCT) spatial layers were taken from Bell *et al.* (2016) and Eco Logical Australia Pty Ltd (2003) and vegetation types were divided into five categories with unique cell cost values based on the habitat and dietary preferences of sugar gliders (Smith 1982; Smith and Murray 2003). These included highly suitable vegetation (e.g. *Eucalyptus haemastoma* or *racemose*, *Angophora costata*, understorey of *Banksia spp* and *Xanthorrhoea spp*, cell cost value 1), suitable vegetation (10), moderately suitable vegetation (20), moderately unsuitable vegetation (75) and unsuitable vegetation (e.g. saltmarsh, sedgeland, cell cost value 100). Next, land use spatial layers were taken from the NSW Government (2007) and divided into the following classes and cell cost values: Urban residential (80), industrial (100), roads (100), railways (80), rural residential without agriculture (70), rural residential with agriculture (100), native/exotic pasture matrix (100), grazing irrigated modified pastures (100), irrigated turf farming (100), mines/quarries and large cleared areas (200) and the Lake and Ocean ('NoData', complete biogeographical barrier to dispersal). Highly suitable vegetation was given a 25-meter buffer so that two patches either side of a barrier (e.g., road/river) would intersect with a glide distance threshold of 50 meters, indicating crossing potential. Major highways were overlaid with a value of 200 with the assumption that sugar gliders would avoid busy four-lane highways (Pacific Motorway, Pacific Highway).

New geographic distances were calculated using the three friction matrices (cell factor = 15, function = mean) and the least cost paths between spatial coordinates of individuals. The new pairwise geographic distances were then tested for

correlation with genetic distances and number of neighbours = 8. The results from the four spatial analyses (IBD and three least cost paths) were compared to find the best fit, as recommended by Milanese *et al.* (2016).

## Results

### *SNP loci summary statistics*

Many gliders were recaptured at the same site they were originally trapped, including one female sugar glider that was trapped 50 meters from where it was caught as a juvenile three years prior (glider ear tag#041: 1<sup>st</sup> year 85 grams, 3<sup>rd</sup> year 130 grams). None were trapped at a different site sampled. This suggests that female adult gliders demonstrate site philopatry. Additionally, a male sugar glider was trapped on both sides of a 30-meter powerline easement during one week of live trapping, thus indicating crossing structure potential of wooden power poles and powerline easements.

25,241 genome-wide SNPs were identified through DArTseq's analytical pipeline. Three sugar glider samples were excluded during the DArTseq process and two sugar glider samples were excluded during the final filtering stage. A sixth sample was also excluded as it was identified as a squirrel glider/sugar glider hybrid. In total, 11,292 SNPs were retained from 90 sugar glider individuals sampled from 12 different sites spanning 20 km of the landscape (Table 1). The 11,292 filtered genome-wide SNPs were used to undertake population genetics statistics for the sugar gliders sampled.

### *Population genetic diversity and structure estimates*

To gain insight into the genetic diversity of sugar gliders, observed and expected heterozygosity were compared. Observed heterozygosity was lower than expected heterozygosity for all locations except BEC. This was reflected in the  $F_{IS}$  values, with all sampling locations displaying values from 0.017 to 0.119, except for BEC and CP which had values close to 0 (Table 1). Average overall  $F_{ST}$  was 0.0566 and average  $F_{IS}$  was 0.050.

Next, PCoA analyses were conducted to visualise genetic differentiation between individuals and between sampling locations. Population differentiation was evident (yet minimal) in the PCoA plots, with populations separating out on different axes. The cumulative percentage of genetic variation explained by the first eight axes was 20.4%, with axis 1 accounting for only 3.7% of the variation (Fig. 2). The clearest patterns noted in the PCoA plots included the separation of sampling location CP on the first axis, OR on the second axis, AD on the fourth axis, and BEC on the sixth.

The AMOVA examined population structure and population differentiation and showed that a high proportion of the genetic variation was contained within sugar glider samples (89.2%) while only 5.4% of the genetic variation was observed between populations and 5.4% of the genetic variation was observed between samples within populations (Table 2).

Population pairwise  $F_{ST}$  values allowed a clear comparison of populations to detect differentiation (Table 3). Values were mostly all significant except for the comparisons between locations HAD and SP, and locations WYC and SP. Significant pairwise  $F_{ST}$  values ranged from low genetic differentiation (0.011, HAD vs WR) to moderate genetic differentiation (0.131, SP vs BR). The pairwise  $F_{ST}$  values indicated genetic similarity between populations within a large bushland patch in a 5 km radius (WR, WRW and HAD) and moderately high genetic differentiation when comparing the locations on the western side of the pacific motorway (BEC and BR) with most of the sites on the eastern side ( $F_{ST}$  average of west compared to east = 0.083). Additionally, CP which is located on a peninsula, had moderate genetic differentiation from most other sites sampled (Table 3).

### *STRUCTURE*

The Structure Harvester results for individual-based structure and admixture showed that  $\Delta K$  had the highest value at  $K = 5$  (Fig. 3). Therefore, it was understood that the sugar glider samples collected from within the Lake Macquarie LGA were

derived from five ancestral genetic clusters. When  $K = 5$ , unique, pure clusters appeared in populations CP and OR in the form of cluster three and cluster five respectively (Fig. 4).

Cluster admixture was present in all sampling locations except for population AD (Fig. 4). In population AD, 100% of the individuals were considered pure to cluster 1. In BEC, 75% of the individuals were pure to cluster 2. In CP, 70% of the individuals were pure to cluster 3. In FDW, 27% of the individuals were pure to cluster 4. In OR, 11% of the individuals were pure to cluster 1, 22% were pure to cluster 4 and 22% were pure to cluster 5. In WR, 27% of the individuals were pure to cluster 2. And finally, 89% and 55% of individuals were pure to cluster 1 in WYB and WYC consecutively. The rest of the individuals (including all of those in populations BR, HD, SP and WRW) were admixed (Fig. 4).

### *Results of least cost path analyses*

The IBD analysis produced a significant result, with a correlation between genetic distance and euclidian geographic distance (IBD Mantel's  $r = 0.389$ ,  $p = 0.011$ ). The proportion of genetic variation explained by geographic distance did not increase when the lake was included as a biogeographical barrier (Mantel test "B", Mantel's  $r = 0.334$ ,  $p = 0.098$ ), however it did significantly increase once highways were included alongside the biogeographical barriers (Mantel test "BH", Mantel's  $r = 0.619$ ,  $p = 0.001$ ) (Fig. 5a). When habitat was incorporated, the Mantel  $r$  statistic only slightly increased (Mantel test "BHH", Mantel's  $r = 0.647$ ,  $p = 0.001$ ) (Fig. 5b), indicating that the highways explained the most genetic variation out of the three factors examined: biogeographical barriers, highways and habitat (Table 4).

## **Discussion**

Habitat fragmentation can inhibit gene flow of arboreal gliders, particularly when gaps exceed their glide distance threshold and contain dangers to their survival in the form of high-speed vehicles or large bodies of water. In the current study, genome-wide SNPs proved useful in identifying fine-scale genetic structure and population differentiation of the sugar glider in the Lake Macquarie LGA.

Locations FDW, HAD, WR and WRW had little to no differentiation from each other and had expansive remnant bushland between them. On the other hand, location CP appeared moderately differentiated from other locations in the pairwise  $F_{ST}$  and the STRUCTURE analysis results. One plausible explanation is biogeographic isolation since CP is on a peninsula, however the lake did not significantly explain genetic variation in the least cost path analysis. Overall, subtle genetic variation was detected in the PCoA and the AMOVA. This combined with the initial significant isolation-by-distance result suggests moderately high connectivity and random mating, and little structure in the overall sugar glider population. This contrasts with research by Goldingay *et al.* (2013) who found no isolation-by-distance effect for squirrel glider populations in Mackay and Brisbane. They assumed this was due to heavy fragmentation and genetic drift. The difference in results here could be because Lake Macquarie has retained a greater proportion of vegetation in the urban matrix (promoting dispersal), or perhaps sugar gliders are less susceptible to urbanisation (Caryl *et al.* 2013). Population genetic analysis of the Lake Macquarie squirrel gliders could put this speculation to rest (Pavlova *et al.* 2010).

Despite the low genetic structure, pairwise  $F_{ST}$  and the least cost path analyses found a significant highway effect on the genetic distances of populations. In this case, populations BEC and BR West of the Pacific Motorway were differentiated from populations to the East. The Pacific Motorway was completed in 1987 and 1988 in Lake Macquarie (Stubbs 2015). Annual average daily traffic is between 40,000 to 50,000 vehicles a day (north and south bound combined) (Roads and Maritime Services 2018), generating edge effects from noise and light pollution (Pocock and Lawrence 2005). Furthermore, the highway exceeds the glide distance threshold of sugar gliders (70 m) despite containing trees in some parts of the median strip. These trees are not currently mature (ie. tall) enough to support the crossing of sugar gliders that require an average glide angle of  $29.69^\circ$  (Jackson 2000).

In Lake Macquarie LGA the Pacific Motorway has been a dispersal barrier for the last 30 – 35 years, impeding gene flow for approximately ten generations of sugar gliders. This is consistent with microsatellite research by Goldingay *et al.* (2013) who found genetic differentiation in populations of squirrel gliders within 30 years of landscape change in Queensland. It is important to catch this effect early on (as this study has done) to mitigate any long term effects on the genetic structure and viability of populations. The installation of rope bridges and glider poles would act as an asset to conservation management of sugar and squirrel gliders, as well as conserving any trees in the median strip to allow them to reach an optimal height for sugar gliders to successfully utilise them as stepping stones. Taylor and Rohweder (2013) observed sugar gliders using mature trees to cross a median strip when they were tall enough to support a successful glide (tall enough to support average glide angle of 29.69°, Jackson 2000). Additionally, rope bridges and glider poles have successfully encouraged squirrel gliders and sugar gliders to cross highways across the east coast of Australia. These species were observed using the man-made crossing structures through radio-tracking, hair-trapping and camera trapping (Ball and Goldingay 2008; Taylor and Goldingay 2012; Soanes *et al.* 2015; Soanes *et al.* 2018; Goldingay *et al.* 2019). Research in Port Macquarie concluded that glider poles were preferred over rope bridges for squirrel gliders and sugar gliders, while rope bridges were utilised by non-gliding mammals (Goldingay *et al.* 2019). With this in mind, Lake Macquarie should prioritise glider poles or install both options along the Pacific Motorway to facilitate dispersal of the species across the landscape and thus mitigate the effects of genetic drift. Future research should focus on the western side of the Pacific Motorway to identify additional locations where sugar gliders are present. This would assist with identifying optimal locations for man-made crossing structures and subsequently improve genetic connectivity of populations.

While this study has been particularly insightful into the sugar glider population of Lake Macquarie LGA, future study should examine the threatened squirrel glider population in the LGA to see if they show similar responses to fragmentation. As a common species, sugar gliders may occur in higher abundance and therefore have a greater effective population size. Genetic structure is not as pronounced in larger populations, but can have strong effects in small populations (commonly the case with threatened species) (Coleman *et al.* 2018).

In conclusion, this research was particularly insightful due to the recent division of the once sugar gliders into three glider species (sugar glider, krefft's glider and savanna glider) (Cremona *et al.* 2020). The results of this study suggests that the overall sugar glider population in Lake Macquarie LGA has appropriate levels of random mating with high levels of gene flow, however there is evidence that the relatively recent installation of the Pacific Motorway (30 – 35 years ago) is starting to impose genetic structure on either side of the road. This can be mitigated with the installation of man-made crossing structures such as glider poles. Overall, the Lake Macquarie LGA sugar glider population has high levels of gene flow with potential for recovery in the area of concern (the Pacific Motorway).

## Declarations

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**Conflicts of interest/Competing interests:** The authors declare no competing interest.

**Availability of data and material:** DArTseq data is available from the authors upon request.

**Code availability:** Not applicable.

**Ethics approval:** Sugar glider genetic samples were collected under University of Wollongong Animal Ethics permit AE15/11 and AE19/02.

**Consent to participate:** Not applicable.

**Consent for publication:** All authors consent to the submission of this article to Conservation Genetics.

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## Tables

**Table 1.** Location code, latitude, longitude and the number of genotyped sugar glider samples ( $N$ ) collected from 12 locations in the Lake Macquarie Local Government Area. Mean observed heterozygosity  $H_{obs}$ , mean expected heterozygosity  $H_{ex}$  and fixation index  $F_{IS}$  are listed for each location plus/minus standard error. Total sample size of 90 individuals and 11,292 SNPs.

Code	Location	Latitude	Longitude	N	H <sub>obs</sub> (± SE)	H <sub>ex</sub> (± SE)	F <sub>IS</sub>
AD	Awabakal Drive	-33.142	151.6082	9	0.116 (0.062)	0.124 (0.060)	0.065
BEC	Becks Rd	-33.0099	151.4776	4	0.126 (0.114)	0.123 (0.099)	-0.024
BR	Bushells Ridge Road	-33.1994	151.4621	2	-	-	-
CP	Coal Point	-33.0362	151.6109	10	0.122 (0.061)	0.123 (0.057)	0.009
FDW	Freemans Drive	-33.0286	151.5006	11	0.123 (0.053)	0.134 (0.054)	0.082
HAD	Hadleigh Road	-33.0461	151.5725	2	-	-	-
OR	Rathmines Park	-33.0372	151.5895	9	0.126 (0.066)	0.128 (0.061)	0.017
SP	Sunshine Park	-33.1078	151.5616	1	-	-	-
WR	Wilton Road (Est)	-33.029	151.5548	11	0.128 (0.054)	0.136 (0.053)	0.063
WRW	Wilton Road (Wst)	-33.0233	151.5432	11	0.125 (0.053)	0.135 (0.053)	0.073
WYB	Wyee Road (Nth)	-33.133	151.4801	9	0.124 (0.064)	0.130 (0.061)	0.046
WYC	Wyee Road (Sth)	-33.1539	151.4821	11	0.120 (0.052)	0.136 (0.054)	0.119

**Table 2.** Percentages of Molecular Variance within sugar glider samples, between samples within populations and between populations. Results of 11, 292 SNPs from 90 sugar glider samples and 9,999 permutations. Degrees of freedom (df), sum of squares (SS), mean squares (MS).

Source of variance	df	SS	MS	% variation	p value
Between populations	11	30781.54	2798.322	5.40	0.01
Between samples within populations	78	121855.83	1562.254	5.40	0.02
Within samples	90	125422.32	1393.581	89.20	0.01
Total	179	278059.70	1553.406	100.00	

**Table 3.** Pairwise F<sub>ST</sub> values between sugar glider sampling locations are shown below the diagonal.

Location	AD	BEC	BR	CP	FDW	HAD	OR	SP	WR	WRW	WYB	WYC
AD	-	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
BEC	0.106	-	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
BR	0.091	0.128	-	NA	NA	NA	NA	NA	NA	NA	NA	NA
CP	0.103	0.107	0.111	-	NA	NA	NA	NA	NA	NA	NA	NA
FDW	0.063	0.070	0.055	0.066	-	NA	NA	NA	NA	NA	NA	NA
HAD	0.072	0.083	0.078	0.079	0.025	-	NA	NA	NA	NA	NA	NA
OR	0.088	0.098	0.101	0.078	0.055	0.054	-	NA	NA	NA	NA	NA
SP	0.083	0.122	0.131	0.122	0.028	<i>0.014*</i>	0.094	-	NA	NA	NA	NA
WR	0.057	0.056	0.053	0.056	0.021	0.011	0.042	0.033	-	NA	NA	NA
WRW	0.059	0.064	0.062	0.056	0.024	0.019	0.040	0.041	0.014	-	NA	NA
WYB	0.070	0.093	0.078	0.091	0.051	0.054	0.077	0.046	0.045	0.048	-	NA
WYC	0.052	0.066	0.042	0.060	0.030	0.022	0.051	<i>0.008*</i>	0.023	0.025	0.028	-

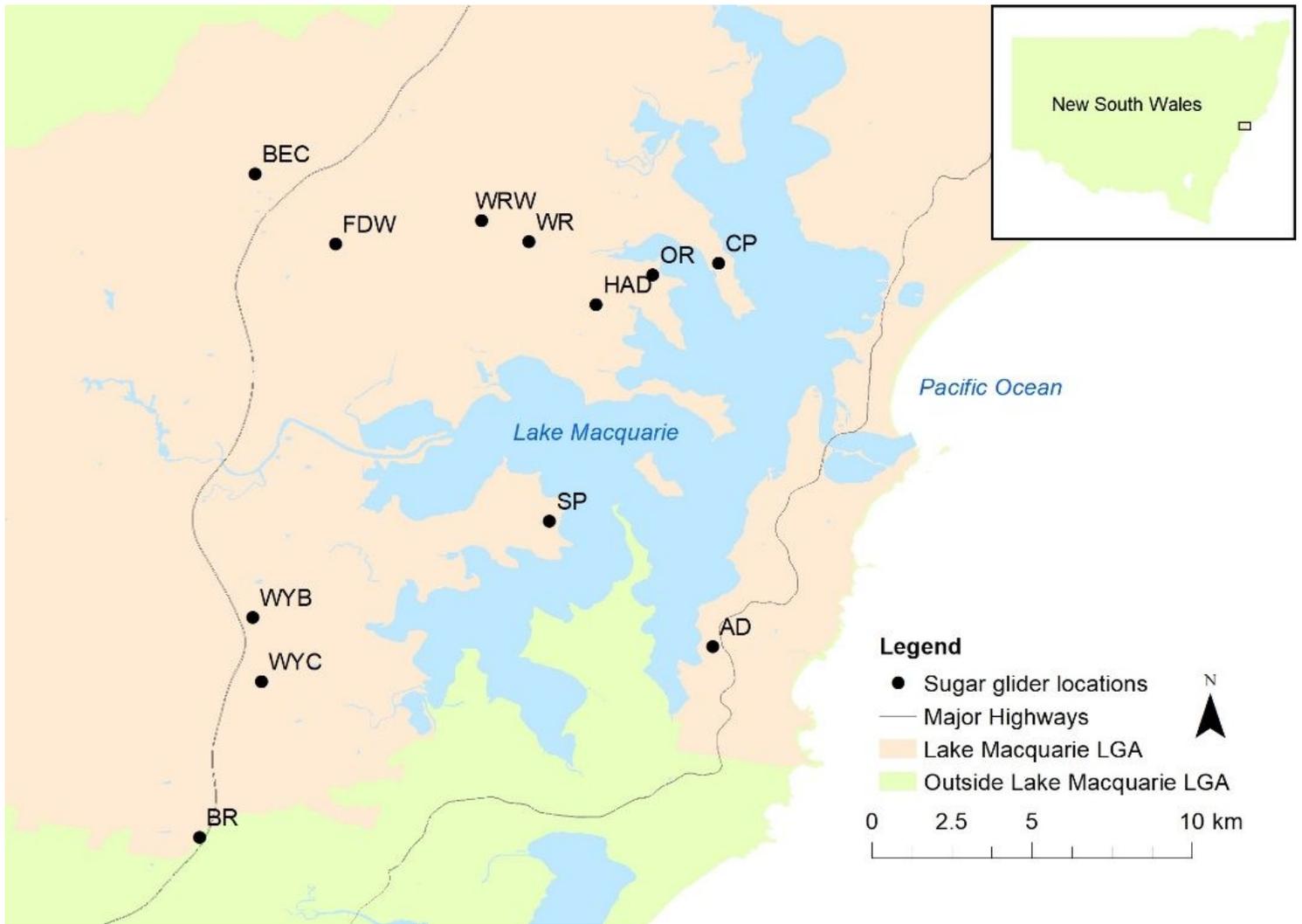
**\*NOTE:**  $F_{ST}$  values in italics are not significant according to the method proposed by Wright (1949) and updated by Weir and Cockerham (1984) ( $p > 0.05$ )

**Table 4.** Comparison of sugar glider Mantel tests for isolation by distance (IBD), biogeographical barriers (B), biogeographical barriers and four lane highways (BH), biogeographical barriers, four lane highways and habitat (BHH). Table includes Mantel statistic 'r' and significance value 'p'.

Mantel test	r	p
IBD	0.389*	0.011
B	0.334 <sup>ns</sup>	0.098
BH	0.619*	0.001
BHH	0.647*	0.001

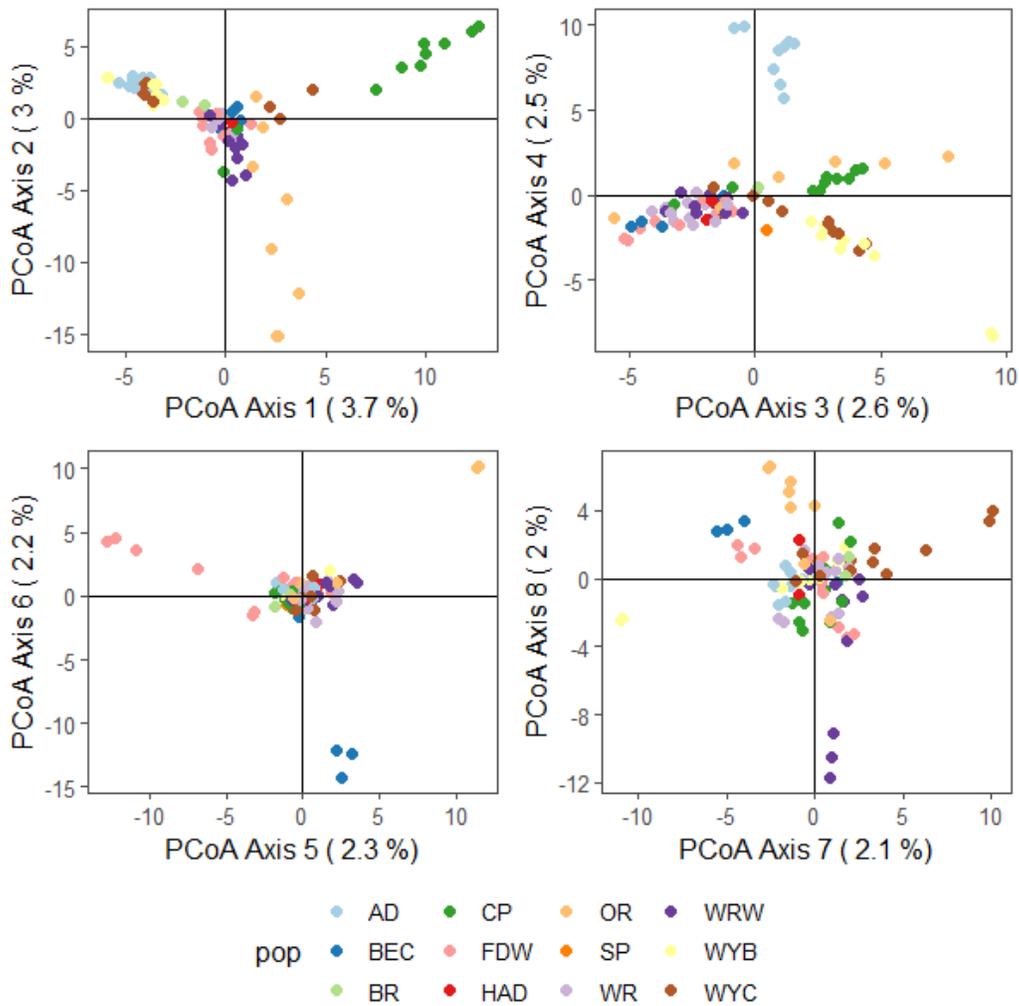
\*:  $p < 0.05$ ; ns: non-significant  $p > 0.05$

## Figures



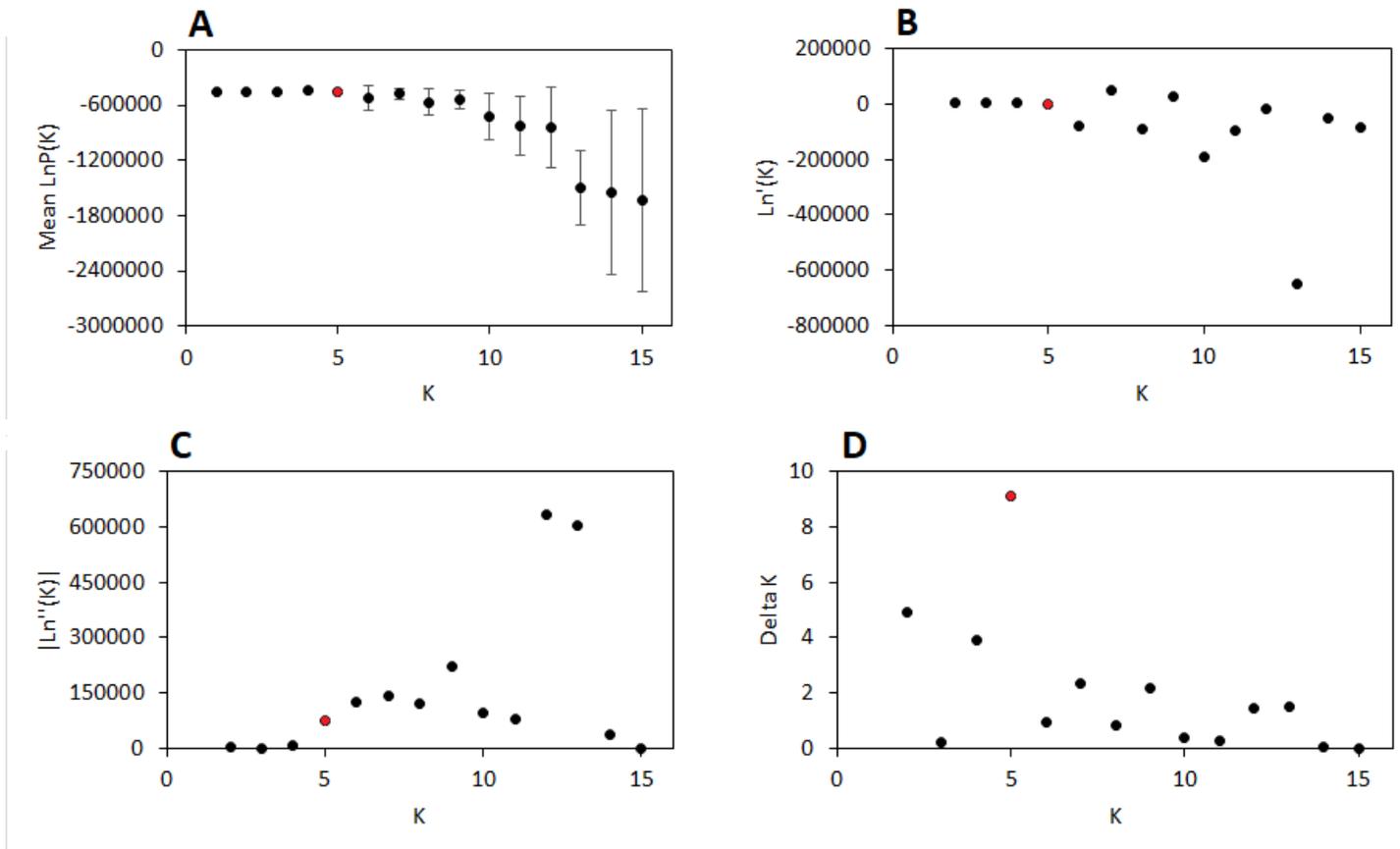
**Figure 1**

Sugar glider trapping locations from 2017 – 2020 in the Lake Macquarie Local Government Area (LGA). See Table 1 for number of individuals and location information. Note: The designations employed and the presentation of the material on this map do not imply the expression of any opinion whatsoever on the part of Research Square concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. This map has been provided by the authors.



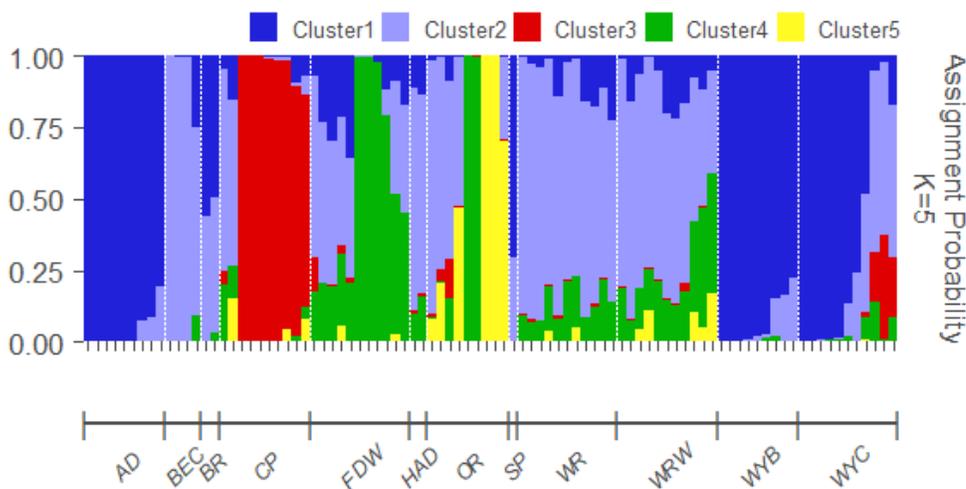
**Figure 2**

Principal Coordinates Analysis (PCoA) based on the genetic distances of sugar gliders from 12 putative populations (“pop”) and 11, 292 SNPs. Dots represent individuals and colours represent locations/putative populations. From top to bottom, left to right: graphical representation of the first two PCoA axes, the third and fourth PCoA axes, fifth and sixth PCoA axes, and 7th and 8th PCoA axes. N= 90.



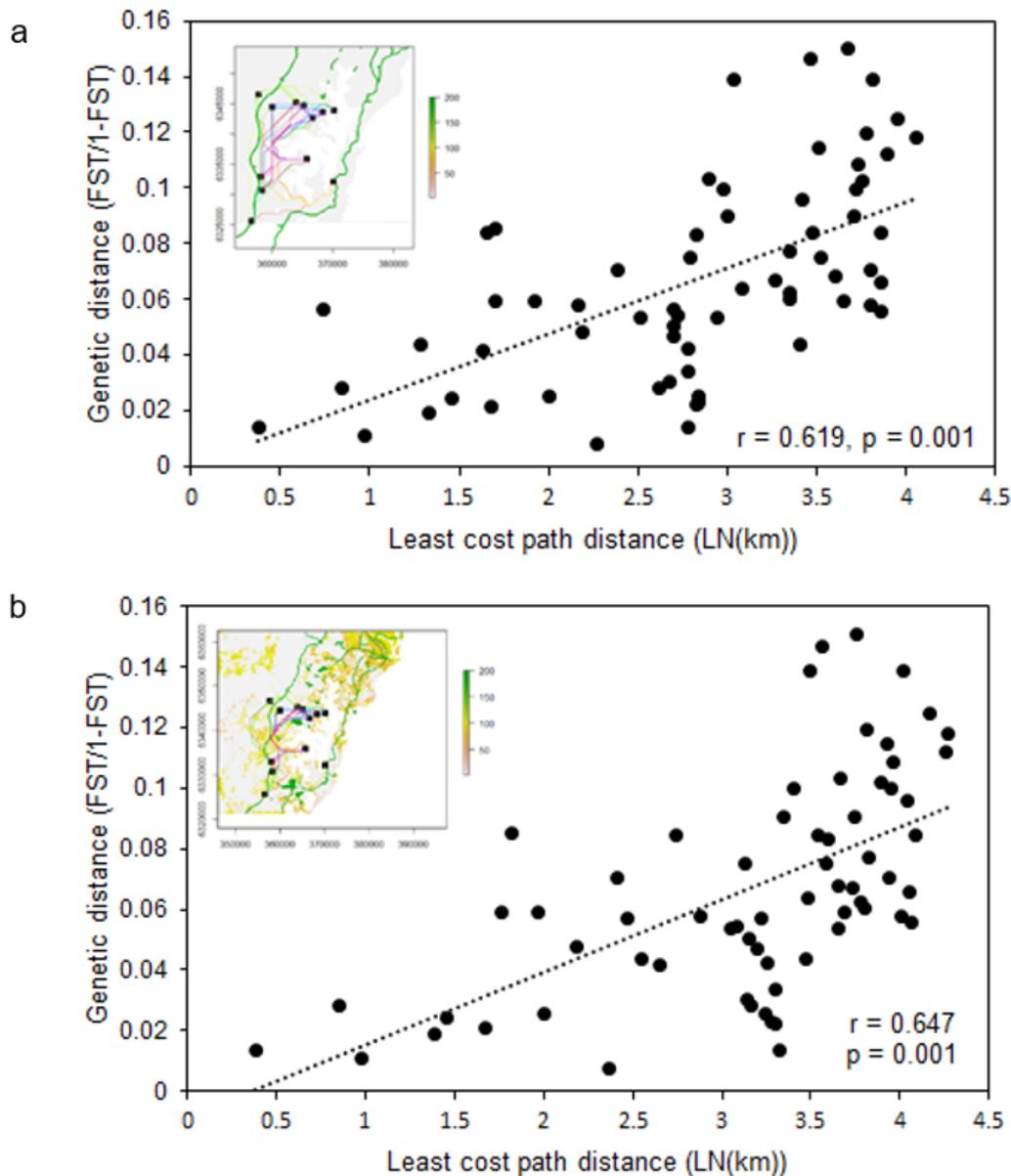
**Figure 3**

STRUCTURE tested 15 clusters ( $K = 1 - 15$ ) with 8 replicates each, and the Structure Harvester Evanno method results were graphed. The graphs display (a)  $L(K)$  mean  $\pm$  SD, (b) the mean rate of change of the likelihood distribution, (c) the mean absolute value of the 2nd order rate of change of the likelihood distribution, and (d)  $\Delta K = \text{mean}|L_n''(K)|/\text{sd}(L(K))$ .  $K = 5$  had the largest  $\Delta K$  value and is displayed in red.



**Figure 4**

STRUCTURE admixture results for 90 sugar glider individuals and  $K = 5$  clusters. Putative populations are divided by the white dashed lines and labelled below the plot. Each cluster is coloured as shown.



**Figure 5**

Relationship between genetic distances ( $F_{ST}/(1 - F_{ST})$ ) and logarithm of geographic distances (km) for 12 locations of sugar gliders in Lake Macquarie, using the results of the least cost path analysis (a) “Mantel test BH” and (b) “Mantel test BHH”. Least cost path distances were calculated for each friction matrix as seen in top left corner of the plots. Note: The designations employed and the presentation of the material on this map do not imply the expression of any opinion whatsoever on the part of Research Square concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. This map has been provided by the authors.