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Extensive gene flow in a threatened bat (*Rhinonicteris aurantia*) in an arid landscape

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ABSTRACT

The bat fauna of Australia comprises some 25% of all terrestrial species, yet we know very little of the demography, dispersal, and movement dynamics of most bat species. The Pilbara leaf-nosed bat (*Rhinonicteris aurantia* Pilbara form) is a threatened microbat that roosts exclusively in caves that occur in mineral rich deposits in the Pilbara region. Due to their specific roost microclimate requirements these bats cannot survive for long without a suitable roost and are sensitive to roost disturbance. Understanding the connectivity of roosts throughout the Pilbara is crucial for informed decisions to mitigate potential impacts to persistence of this species in areas under economic development. Along with mitochondrial DNA (mtDNA) markers, we used reduced representation genomic sequencing of over 150 individuals from eight roost sites throughout the Pilbara and tested for landscape-scale population differentiation associated with the two major subregions — the Hamersley and Chichester, as well as finer-scale among roost variation. We found evidence of high rates of dispersal and low population structure within the Pilbara, indicating one panmictic population, with mtDNA results suggesting evidence of some female philopatry. Our results highlight the ongoing need for detailed genetic studies to provide critical insight to species dispersal, particularly in multi-use landscapes. We discuss the importance of identifying and retaining connectivity of key habitat to maintain genetic diversity and gene flow throughout panmictic populations.

1. Introduction

Bats (order Chiroptera) are the only group of mammals to have evolved true flight and comprise over 20% of all mammal species on Earth (Voigt and Kingston, 2015). Bats are cryptic by nature and often overlooked despite their role as pollinators, seed dispersers, predators of flying insects, and disease vectors, all which impact human health, primary industries, and the environment (Voigt and Kingston, 2015). Bats exhibit a wide range of social structures, mating systems, roost preferences and migratory capabilities; however, these aspects of bat biology are understudied in arid biomes, particularly in Africa and Australia (Lisón et al., 2020). Further,

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understanding the biology of bats in fragmented landscapes is critical to assess their conservation status and management in the wake of anthropogenic change (Frick et al., 2020).

Bat populations typically exhibit high levels of gene flow and low geographic population structure due to high dispersal rates, particularly in migratory species (Moussy et al., 2013). Maximum dispersal distance is influenced by morphological characteristics, such as wing loading and aspect ratio, with the smaller, more agile bats being less suited to long distance migration (Burns and Broders, 2014). Many bat species also exhibit female philopatry as females remain at, or return to, maternity roosts (Flanders et al., 2016). The genetic patterns of most Australian bat species are relatively unknown, with the limited studies to date finding a variety of responses from low genetic structure in widespread tree-roosting species (Fuller, 2013; Prada, 2020), evidence for male-mediated dispersal and female philopatry in cave roosting bats (Armstrong, 2006; Worthington-Wilmer et al., 1999), and isolation driven by habitat availability or fragmentation in forest-dwelling bats (Campbell et al., 2009; Fuller, 2013). Given the variety of patterns, utilising molecular data to better understand genetic relationships of bat populations is crucial to species management (Ripperger et al., 2013).

The Pilbara biogeographic region (178,000 km^2) in the north-west of Western Australia is a biodiversity hotspot with ancient and complex geology that has resulted in a multitude of habitats and mesic refuges in an otherwise heavily eroded arid landscape (McKenzie et al., 2009; Pepper et al., 2013). The bioregion is of significant economic importance, containing the largest iron ore deposits in Australia, extensive deposits of gold and other minerals, and 60% of the bioregion is under pastoral lease (McKenzie et al., 2009). The Pilbara leaf-nosed bat, *Rhinonicteris aurantia* unnamed Pilbara form is listed as Vulnerable (DoE, 2016), and is a small (8–10 g) insectivorous bat endemic to the Pilbara and adjacent bioregions (Armstrong, 2008). This species roosts in mixed social groups with mating occurring mid-year and young born in December (Churchill, 1995), and all roosts that are occupied year-round are considered maternity roosts (Cramer et al., 2016). Due to their small size and inability to enter torpor (Kulzer et al., 1970), Pilbara leaf-nosed bats roost in complex caves with stable temperatures and high humidity during the day to avoid desiccation (Baudinette et al., 2000) in a region characterized by frequent temperature extremes and highly variable rainfall (Churchill, 1991). However, caves with suitable microclimates are patchily distributed and tend to occur in strata bearing ore of economic interest throughout the Pilbara uplands (Cramer et al., 2016). Exploration and extraction activities have the potential to damage or alter caves rendering them unsuitable as diurnal roosts (Armstrong, 2010; Bullen and Creese, 2014), thus potentially impacting the connectivity and future viability of the Pilbara population (Cramer et al., 2016).

Given the potential impact of rapidly expanding mining activity in the Pilbara there has been a concerted effort to locate additional diurnal roosts and to assess the species' habitat use and movement patterns, in line with research priorities identified by Cramer et al. (2016). Over 50 roosts in the Pilbara are now known, including six in abandoned underground mines in the north-east of the region (Bat Call WA, 2021), and the movement and roost use by Pilbara leaf-nosed bats is being studied at local scales through the use of radio transmitter and GPS tracking data (Bullen and Reiffer, 2020, 2019). Whilst there is some evidence of the capacity for demographic connectivity amongst roosts over large scales (Bullen and Reiffer, 2019), little is known about the frequency of movement between roosts and the capacity for gene flow. A previous study of the phylogeographic patterns of *R. aurantia* using two mitochondrial loci found some evidence of isolation between the northern and southern roosts in the Pilbara, possibly due to female philopatry (Armstrong, 2006).

Genetic markers can help elucidate both historical and contemporary patterns of gene flow to identify past vicariance events and investigate current connectivity amongst spatially distinct populations, including contrasting patterns of dispersal amongst the sexes. Here, we build on the previous genetic data in Armstrong (2006) by sequencing the mitochondrial D-Loop from additional locations within the Pilbara and incorporating a large nuclear DNA single nucleotide polymorphism (SNP) dataset to assess dispersal and roost site connectivity of Pilbara leaf-nosed bats.

Pilbara leaf-nosed bats may display genetic structuring amongst roosts for several reasons. Firstly, due to their small size and strict physiological requirements, we anticipate long-distance dispersal to be infrequent in this species. Suitable roosting habitat is patchily distributed in the Pilbara and the species may be vulnerable to desiccation when flying long distances between roosts. Secondly, evidence from radio-tracking studies suggest that bats may make nightly foraging bouts covering 20–30 km (Bat Call WA, 2021) before returning to a centralised roost site (Bullen and Reiffer, 2020), and also show site fidelity throughout the year (Bullen and Reiffer, 2019). Further, previous genetic studies have indicated that the Fortescue Valley, which dissects the Pilbara region east-west and is 20–100 km wide, acts as a barrier to dispersal for species preferring rocky habitats (Pepper et al., 2013; Shoo et al., 2008; Umbrello et al., 2020). Considering this information, we predict gene flow may be limited at the regional scale with differentiation across the Fortescue Valley. Hence, we examined the genetic data for both a pattern of genetic isolation-by-distance due to generally restricted dispersal, and isolation-by-barrier due to restricted dispersal owing to a lack of suitable habitat in the Fortescue Valley. Similar to other bat species, we predict Pilbara leaf-nosed bats will exhibit some male-mediated dispersal (Bullen and Reiffer, 2019), with females showing more mitochondrial genetic structure and higher relatedness within roosts than males. Understanding genetic connectivity of Pilbara leaf-nosed bats will assist with locating regions that are important for sustaining genetic diversity and population connectivity which will be critical for the conservation management of this species under pressure from habitat loss and destruction.

2. Materials and methods

2.1. Sample collection

Tissue samples were collected in 2017–2019 from eight diurnal roost sites considered maternity roosts (all roosts that sustain colonies of this species throughout the year are considered maternity roosts, see Cramer et al., 2016), located in the Hamersley and Chichester subregions of the Pilbara (Table 1; Fig. 1). The two subregions represent distinct geological units of the Pilbara up lands

(DoE 2012) that contain multiple and complex caves, and are separated by the low-lying Fortescue River Valley, which has been identified as a biogeographic barrier for multiple species (Pepper et al., 2008; Shoo et al., 2008; Umbrello et al., 2020). Many of the roosts in the Chichester subregion are in abandoned gold mines that bats have colonised in the past 100 years (Armstrong, 2001; Churchill et al., 1988). Our sampling included three roosts in disused mines in the Chichester — Lalla Rook, Bow Bells, and Copper Hills — and five cave roosts in the Hamersley — Cane River, Duck Creek, Ratty Spring, Gudai-Darri (previously Koodaideri) Adit, and Kalgan Creek. Whilst colony size estimates are large at some sites, we were restricted in the number of bats able to be captured due to animal ethics considerations.

This collection included 288 samples of which 220 were taken from wing-membrane of live-caught bats using sterile 3 mm biopsy punches, and 68 samples were scats collected opportunistically from roosts. Bats were captured using mist nets erected at the entrances of roost caves that were monitored for two hours, bats were implanted with Passive Integrated Transponder tags and released afterwards (see Bullen and Reiffer, 2020). Samples were placed in 1.5 mL vials of 100% ethanol and kept cold while in field, then frozen at −18 °C. Total genomic DNA was extracted from wing tissue using a standard salting out extraction protocol (Sunnucks and Hales, 1996) with the addition of 3 μL 10 mg/mL RNase to the TNES buffer to remove RNA contamination. DNA was extracted from scat samples using Omega Biotek Mag-Bind® Stool DNA 96 Kits (www.omegabiotek.com) following the manufacturer's protocol. The concentration of DNA extractions was obtained using a Qubit™ fluorometer and Qubit™ dsDNA BR Assay kit (Invitrogen, Thermo Fisher Scientific) according to manufacturer's instructions using 1 μL of DNA for each sample.

2.2. Mitochondrial DNA sequencing

We amplified part of the mitochondrial D-loop region from 91 samples (87 wing tissue and four scats) using the primers P (5'-TCCTACCATCAGCACCCAAAGC-3') and E (5'-CCTGAAGTAGGAACCAGATG-3') from Wilkinson and Chapman (1991). Mitochondrial DNA was amplified via PCR in 25 μL reaction volumes using the Qiagen AllTaq Master Mix Kit with 5 μL AllTaq, 2.5 μL of each primer (at 5 μM), 1–2 μL template DNA, and remaining volume deionised H_2O, under the following PCR conditions; 95 °C for 5 min followed by 80 °C for 5 min, then 30–40 cycles of 95 °C for 45 s, 55 °C for 45 s and 65 °C for 90 s, after which a final extension at 65 °C for 5 mins was run. PCR product was visualised by running 4 μL PCR product with 3 μL 5x loading buffer on 1.5% agarose gel with 1 x TAE. Samples of 20 μL of PCR product were sent to the Australian Genome Research Facility, Perth, for bi-directional sequencing using the P and E primers.

Raw forward and reverse sequence reads returned were processed in *Geneious* v.10 (Kearse et al., 2012; https://www.geneious.com) following Umbrello et al. (2017). Nucleotide BLAST® (https://blast.ncbi.nlm.nih.gov/) was used to identify sequences from non-target species extracted from scat samples, including *Macroderma gigas* and *Taphozous georgianus* as both species are known to roost with Pilbara leaf-nosed bats. These sequences were removed from the alignments prior to analyses. All D-loop sequences were uploaded to Genbank, accession numbers MZ020332–MZ020419 and MZ571159–MZ571161.

2.3. Mitochondrial DNA analyses

To visualise geographic patterns in mtDNA we used *PopART* 1.7 to construct TCS haplotype networks (Clement et al., 2000; Leigh and Bryant, 2015). Haplotype networks were created for two alignments, the 88 D-loop sequences from this study (length = 754 bp), and a second with these aligned to published sequences of *R. aurantia* (Armstrong, 2006). The second alignment was trimmed to the shorter length of the published sequences, and a 104 bp repeat region, described in Armstrong (2006) was removed, as *PopART* excludes gaps and missing regions (final length = 458 bp). Individuals were coloured based on sample location, either by roost or region (Pilbara — Chichester, Hamersley, Barlee — Kimberley and NT). All subsequent analyses were conducted on the alignment with only the samples generated in this study (n = 88). Summary statistics and neutrality tests, Tajima's *D* (Tajima, 1989) and Fu's *Fs* (Fu, 1997), for each roost and subregion were obtained in *DnaSP* v.6 (Rozas et al., 2017). Hierarchical pairwise comparisons of genetic distance (ϕ_{ST}) and analysis of molecular variance (AMOVA) between roost sites within subregions were conducted in *Arlequin* 3.5 using 10,000 permutations (Excoffier and Lischer, 2010). Differences in genetic differentiation across the landscape between the male and female datasets were visualised by spatial interpolation of ϕ_{ST} comparisons between roosts in *QGIS* 2.18.16 (QGIS Development Team, 2016)

Table 1
Collection information for the genetic samples used in this study and colony size estimates (observational) for the sampling period (2017–2019) at Lalla Rookh (LR), Bow Bells (BB), Copper Hills (CH), Cane River (CR), Duck Creek (DC), Kalgan Creek (KC), Gudai-Darri Adit (GA) and Ratty Spring (RS) roosts. The number of samples for which mtDNA and SNP genotypes were successfully sequenced and used in analyses are provided, males and females are indicated in parentheses. For full sample metadata refer to Supplementary Information.

Bioregion	Roost	Latitude	Longitude	Estimated colony size	No. of tissue samples	No. mtDNA sequences	No. SNP genotypes
Chichester	LR	− 21.1°S	119.3°E	1500	17	12 (5 m:7 f)	12 (5 m:7 f)
	BB	− 21.3°S	119.9°E	1500	53	15 (10 m:5 f)	40 (22 m:18 f)
	CH	− 21.7°S	120.0°E	500	7	5 (4 m:1 f)	4 (3 m:1 f)
Hamersley	CR	− 22.2°S	116.3°E	12,000	35	15 (7 m:7 f: 1 scat)	28 (11 m:17 f)
	DC	− 22.4°S	116.9°E	100	20	1 (m)	0
	KC	− 23.2°S	119.8°E	500	27	13 (9 m:4 f)	23 (15 m:8 f)
	GA	− 22.5°S	119.0°E	500	21	12 (6 m:6 f)	11 (6 m:5 f)
	RS	− 23.2°S	117.5°E	150	53	15 (7 m:8 f)	37 (18 m:19 f)

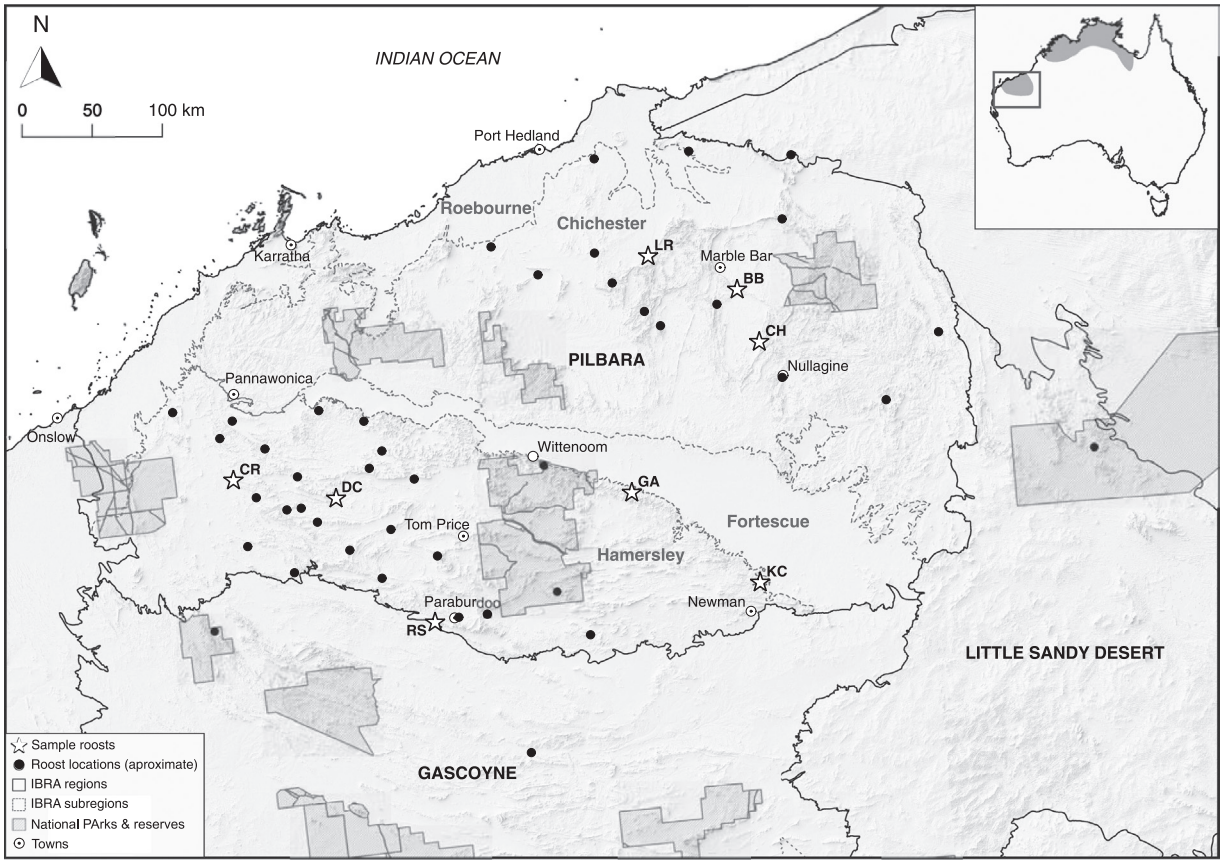

Fig. 1. Map of sample roost sites used in this study (white stars) Kalgan Creek (KC), Ratty Spring (RS), Gudai-Darri Adit (GA), Cane River (CR), Duck Creek (DC), Lalla Rookh (LR), Copper Hills (CH), Bow Bells (BB), and approximate locations of all known Pilbara leaf-nosed bat roosts (black circles). Inset map of Australia identifying Pilbara region and showing range of the species across northern Australia.

taking a similar approach to Vandergast et al. (2011). We used genetic differentiation (ϕ_{ST}) between non-overlapping nearest-neighbour population pairs to interpolate areas of connectivity and divergence using the QGIS 'interpolation' plugin.

2.4. SNP preparation and sequencing

A total of 188 individuals were selected for SNP genotyping ensuring spatial representativeness and even sex ratio per roost where possible, based on suitable DNA concentrations (Supp. material table S1), given that minimum sample sizes of four to eight individuals are required to estimate F_{ST} and heterozygosity, respectively (Nazareno et al., 2017; Willing et al., 2012). This included 179 samples taken from wing membrane and nine samples extracted from scats (scat samples DNA concentration, mean = 2.73 ng, range = 1.06–7.29 ng). Aliquots of DNA were taken for each sample and DNA concentrations were standardised to an average of 295 ng per sample (range = 82–596 ng) and dried using the Savant DNA SpeedVac® Concentrator (Thermo Scientific). Library preparation and sequencing of SNP genotypes was carried out by Diversity Arrays Technologies (DArT Pty Ltd, Canberra, Australia), following the protocols of Kilian et al. (2012); Sansaloni et al. (2011). The restriction enzymes PstI and *Nla*III (V4) were used for medium density sequencing.

Sequences were processed using proprietary DArT Pty Ltd analytical pipelines as described by Georges et al. (2018) to build and genotype SNP loci. Due to the very low sequence coverage of scat samples, the resulting SNP data was re-analysed to exclude samples with low sequencing coverage (n = 22).

2.5. Filtering of SNP dataset

Filtering of SNP loci was performed in R (R Core Team, 2013) using *dartR* 1.8.3, (Gruber et al., 2018), by reading in the DArT SNP genotype file to a genlight object (*adegenet* 2.1.3; Jombart, 2008). First, we removed scat samples as the results from the mtDNA sequences indicated scats were most likely to be non-target species, then we excluded individuals with loci call rates below 0.4, which removed seven individuals (Supp. material table S1). Following this, we excluded loci with a call rate below 0.95, to ensure only loci with high coverage across all remaining samples were retained. We then excluded loci with very low (<5) or very high (>60) average

read depths, as these can indicate sequence errors or paralogues respectively. We filtered on the *dartR* specific filter 'reproducibility', a quality index of reproducibility of repeated runs per loci, using a threshold of 0.95. We then applied a minor allele frequency cut-off of 0.02. We filtered for only one SNP per locus using 'filter.secondaries' in *dartR* and used a Hamming distance of 0.2. Monomorphic loci were removed at each filtering step and only polymorphic loci were included in the final dataset. All remaining loci conformed to Hardy Weinberg expectations. The filtered dataset included 155 individuals and was also divided by sex to examine sex-specific patterns.

2.6. Analysis of SNP dataset

To visualise potential population structure, we used principal coordinate analysis (PCoA) first on the raw and then final filtered SNP dataset using 'gl.pcoa' in *dartR*, and categorised samples in the resultant PCoA plot by roost and subregion (Hamersley or Chichester). Diversity estimates (allelic richness, number of private alleles, observed and expected heterozygosity and inbreeding coefficient) were calculated in the packages *adegenet*, *poppr* (Kamvar et al., 2014), *hierfstat* (Goudet, 2005) and *PopGenReport* 3.0.4 (Adamack and Gruber, 2014). Genetic diversity metrics were estimated for both individual roosts and pooled at the subregional level (Hamersley vs. Chichester). We estimated effective genetic population size (N_e) for the all individuals as no population structure was detected (see Results), using the linkage-disequilibrium method with a random mating system as implemented in *NeEstimator* 2 (Do et al., 2014), using one SNP per locus (Nadachowska-Brzyska et al., 2021). We reduced the dataset to 2000 random loci with 0.29% missing data as confidence interval estimates in *NeEstimator* fail with high (>2 million) independent comparisons. Input files were created by converting an.str file created in *dartR* to genepop format in *PGDSpider* 2.1.1.5 (Lischer and Excoffier, 2012).

2.6.1. Relatedness and spatial autocorrelation

We checked for the presence of highly related individuals, as these can overinflate the results of genetic clustering analyses when present, using *related* 1.0 (Pew et al., 2015). We calculated the relatedness values using the Lynch-Li estimator (Li et al., 1993; Lynch, 1988), which is considered one of the least biased estimators for small sample sizes (Wang, 2017). Where highly related individuals were identified, one from each pair was removed prior to downstream clustering analyses. Relatedness within and between roosts was compared and a simulation test (with 1000 iterations) to check if observed relatedness within roosts differed significantly from the expected relatedness.

Spatial autocorrelation was examined on the Euclidean genetic and geographic distance matrices in *GenAlEx* 6.5 (Peakall and Smouse, 2012, 2006). We used five distance classes at 100 m, 100 km, 200 km, 300 km, and 400 km to capture relatedness within roosts and allow for suitable sample size comparisons.

2.6.2. Population differentiation and clustering

Population differentiation, F_{ST} and Nei's G_{ST}, between roosts pairs were calculated in *StAMPP* 1.6.1 (Pembleton et al., 2013), and AMOVA calculated in *poppr*. We used three clustering-type analyses to identify genetic clusters. First, TESS (*tess3r* 1.1.0; Caye et al., 2016), was used as it incorporates spatial information and can produce better admixture estimates in species with weak genetic structure. Second, LEA (*LEA* 2.8.0; Frichot and François, 2015), was implemented as, like TESS, it employs a sparse non-negative matrix factorization algorithm to effectively process large SNP datasets. Third, a discriminate function analysis (DAPC), was employed using *adegenet*. All three analyses use K-means to infer genetic clusters and we assessed K-values ranging from 1 to 10 and determined the most suitable value of K using cross-validation, cross-entropy, and the Bayesian Information Criterion where appropriate for each analysis, settings and parameters used for each analysis are listed in the Supplementary Information.

2.6.3. Spatial genetic structure

We tested for isolation-by-distance (IBD) vs. the null hypothesis of panmixia on samples grouped by roost, and at the individual

Table 2
Diversity statistics and neutrality test results for 754 bp of D-loop sequence data from *Rhinonicteris aurantia*, statistics were calculated on all samples, by subregion (Chichester and Hamersley) and by roost site; Lalla Rookh (LR), Bow Bells (BB), Copper Hills (CH), Cane River (CR), Ratty Spring (RS), Gudai-Darri Adit (GA) and Kalgan Creek (KC; note Duck Creek excluded as $n = 1$). Number of individuals (n), number of unique haplotypes (H_n), haplotype diversity ($H_d \pm$ sd), nucleotide diversity ($\pi \pm$ sd), Tajima's D (D), and Fu's Fs (Fs).

Locality	n	H_n	H_d	π	D	Fs
Pilbara (all)	88	42	0.970 ± 0.007	0.016 ± 0.008	−0.380	−9.042
Chichester	32	19	0.964 ± 0.015	0.017 ± 0.009	0.279	−1.311
Hamersley	56	31	0.960 ± 0.012	0.015 ± 0.008	−0.561	−6.441
LR	12	10	0.970 ± 0.044	0.017 ± 0.009	−0.194	−0.798
BB	15	13	0.971 ± 0.040	0.017 ± 0.009	−0.431	−2.406
CH	5	4	0.900 ± 0.161	0.019 ± 0.012	0.598	2.232
CR	15	9	0.905 ± 0.054	0.008 ± 0.004	−1.786*	−0.682
RS	15	13	0.981 ± 0.031	0.013 ± 0.007	−0.554	−3.299
GA	12	12	1.000 ± 0.034	0.016 ± 0.009	−0.517	−4.258
KC	13	7	0.885 ± 0.064	0.020 ± 0.011	1.438	3.747

* P-value = 0.023

level. For analysis by roost, we used 'gl.ibd' in *dartR* that uses the genetic distance from $F_{ST}/(1 - F_{ST})$ (Rousset, 1997) and the log of Euclidean geographic distance. For the individual level IBD analysis, we calculated individual genetic distance using two methods, Euclidean genetic distance using 'dist()' in R, and Nei's G_{ST} (Nei, 1972) using the package *StAMPP*, with both distances producing the same results. We calculated the Euclidean geographic distance matrix using 'dist()', and used the log of that distance. Relationships between the genetic and geographic distance matrices were evaluated by Mantel tests and plotted with a line of best fit to visualise patterns of IBD, based on each type of genetic (population and individuals), and distance matrix combination (Euclidean and log

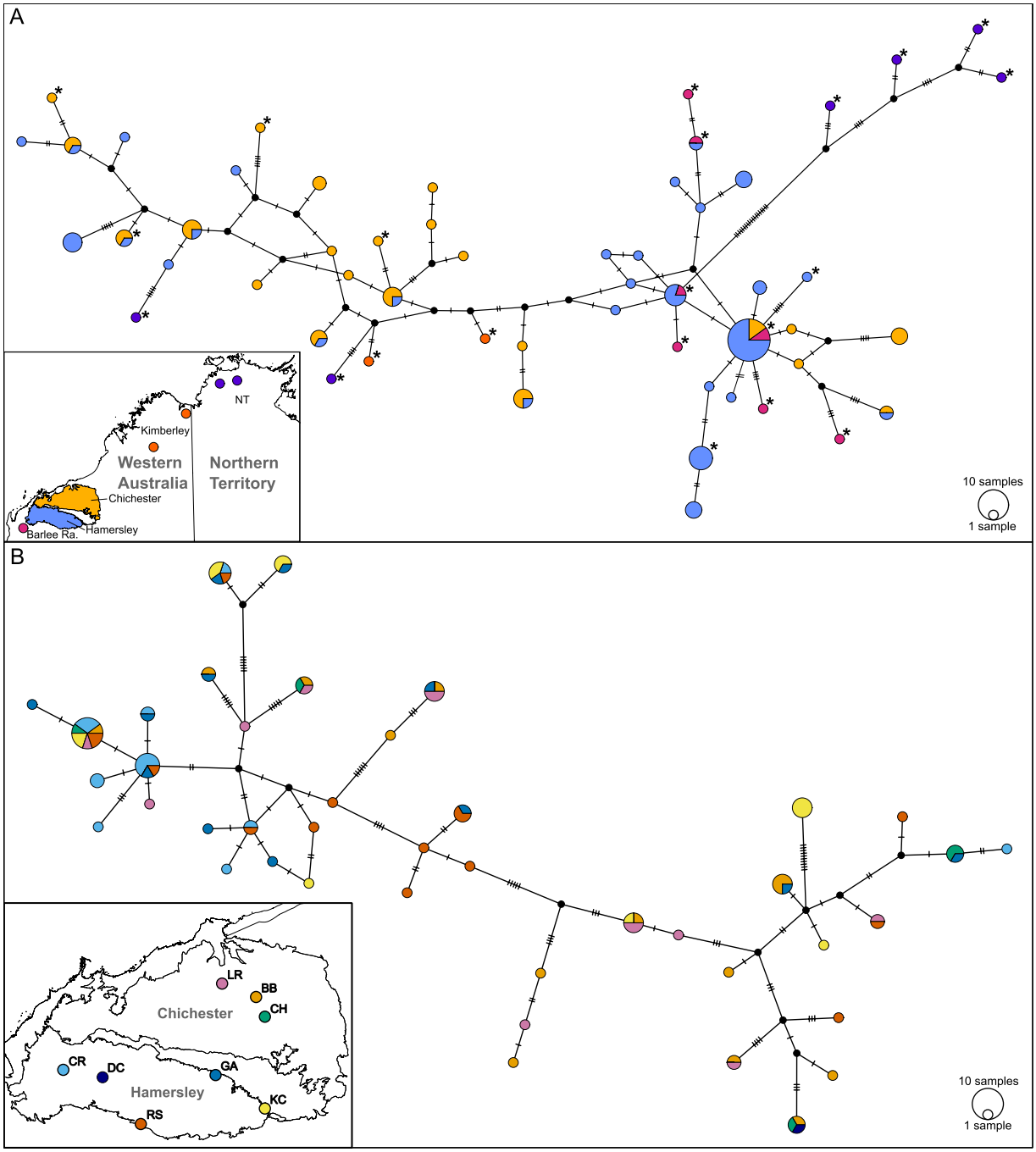

Fig. 2. Mitochondrial haplotype networks of D-loop sequence data from *Rhinonicteris aurantia*. Colours indicate regions or roosts where samples were collected. A) network includes sequences from this study (n = 88,) and sequences from Genbank, denoted by asterisks (n = 22, length = 458 bp). B) network of new D-loop sequence data generated in this study (n = 88, length = 754 bp). Haplotype networks on male and female data in Supplementary Information (Fig. S1).

geographic distance). Analyses were conducted on the full data, and on male and female samples separately, to test for evidence of female philopatry and difference in gene flow between each sex.

3. Results

3.1. Mitochondrial DNA patterns

The P and E primers produced over 780 bp of D-loop sequence, which was trimmed to 754 bp in the final alignment of 88 individuals with 69 variable sites and high haplotype diversity ($H_d = 0.97$, Table 2). No evidence of population size changes was detected for the Pilbara population, each subregion or roost site, except for Cane River that had a significant negative Tajima's *D* value, which could indicate a recent selective sweep or population expansion, although Fu's *Fs* — the more sensitive of the two tests — was not significant for this roost.

The TCS haplotype networks showed a subtle pattern of Chichester-Hamersley isolation, which was more apparent in the trimmed

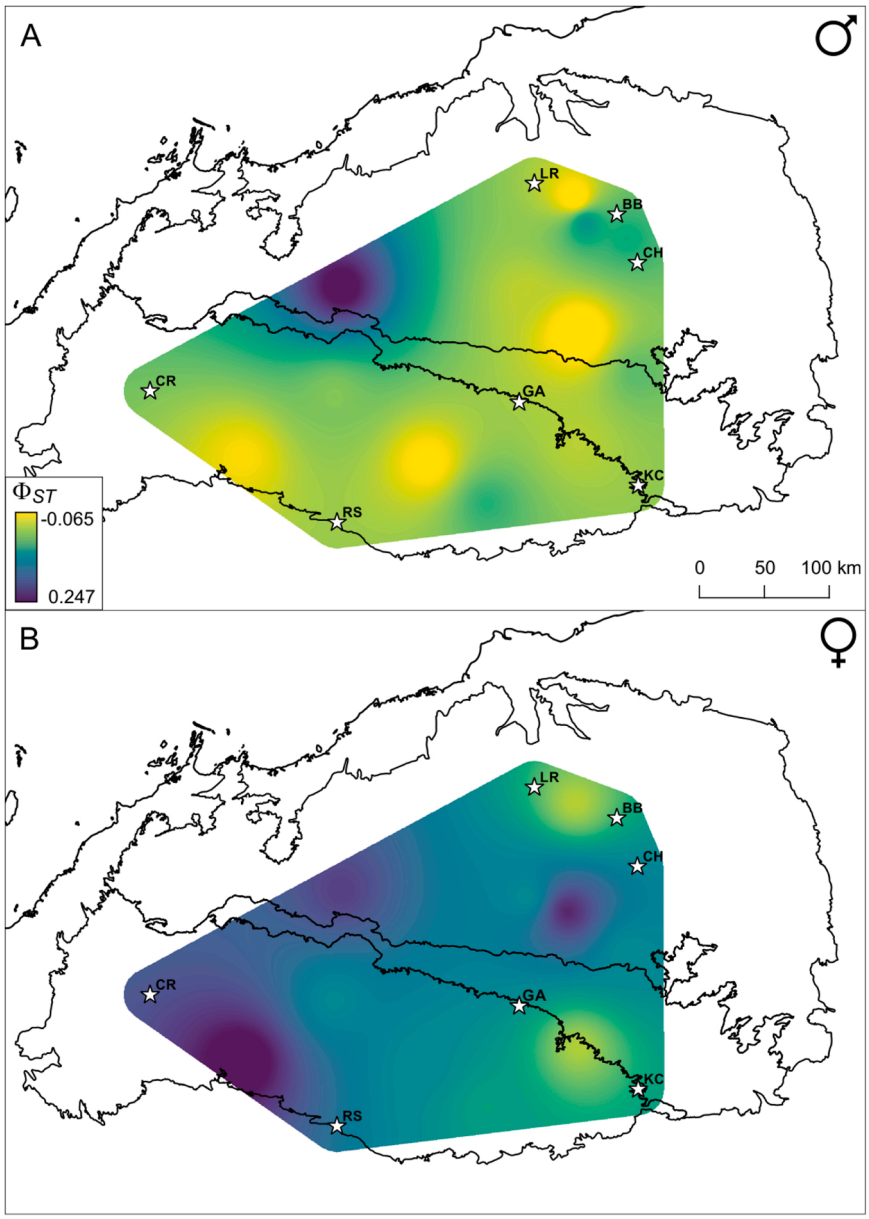

Fig. 3. Spatial interpolation of D-loop ϕ_{ST} for male (A) and female (B) Pilbara leaf-nosed bats at roost sites in the Pilbara. Yellow indicates areas of high connectivity between roosts (lower ϕ_{ST}) and dark blue areas are those with low connectivity (higher ϕ_{ST}).

dataset aligned to the Genbank sequences that included individuals from the Kimberley and NT (Fig. 2). When aligned and trimmed to the Genbank sequences our data included 34 additional unique haplotypes to the 20 published sequences, and we had samples from five of the 12 published haplotypes from the Pilbara and Barlee Range locations (Fig. 2).

Tests for population differentiation (ϕ_{ST}) showed that Cane River was the most differentiated (ϕ_{ST} ranging from 0.05 to 0.33; Supplementary Information Table S2), followed by Bow Bells. Genetic differentiation was greater for females than for males, and both datasets had significant differentiation between Cane River and the northern roosts, Lalla Rookh and Bow Bells (Fig. 3; Supplementary Information Table S3).

Genetic differentiation between the Chichester and Hamersley subregions was low, but significant (global $\phi_{ST} = 0.115$; *P*-value 0.001). The hierarchical AMOVA conducted on all samples showed that most of the variation (85%) was present within roost sites, rather than between roosts or subregions, which was the same in the female and male data (Table 3). Females showed the highest degree of variance between the two subregions (14%) and between roosts (10%), while males had very low ϕ-statistics, all of which were not significant, and with very high variation within roosts (93%).

3.2. Nuclear SNP data, contemporary patterns

The assembled SNP loci dataset contained 42,875 unfiltered loci with 20.56% missing data from 166 individuals. After filtering, 9088 loci from 155 individuals remained with 1.82% missing data (individuals retained after filtering are reported in Supplementary Information Table S1). Genetic diversity statistics were similar across the seven Pilbara roosts, and observed heterozygosity was consistently slightly lower than expected heterozygosity, indicating some level of inbreeding in all locations (F_{IS} range = 0.086–0.143; Table 4). Private alleles were only detected at Bow Bells, and there was some variation in inbreeding coefficients, with two Chichester roosts (LR and CH) and one Hamersley roost (RS) showing higher levels of inbreeding than other roosts.

The PCoA on the filtered SNP sequence data indicated a lack of population structure with low variation explained by the first two PC axes and a gradual decrease in eigenvalues from PC1 (Fig. 4). A pair of highly related individuals formed a slight outgroup to the remaining samples, and one individual (T8238, Bow Bells) from the pair was removed. The resultant PCoA had 1.1% variation explained by PC1 and 0.9% explained by PC2 with all Chichester roosts samples below 0 on PC1. This pattern was similar when female and male data was examined separately, with more overlap in the Hamersley and Chichester samples in the male dataset (Supplementary Information Fig. S2).

Genetic differentiation between roosts was very low for all pairwise comparisons of F_{ST} and G_{ST} (F_{ST} range = 0.0001–0.009), with comparisons between the subregions yielding the highest values and those within subregions with the lowest (Supplementary Information Table S4). The F_{ST} values in the female data were slightly greater than the male data (Supplementary Information Table S5). Analysis of molecular variance showed that over 99% of the total variation within the SNP data was found within roosts, with only a very small percentage being found between roosts or subregions, and with very little difference between the sexes (Table 3).

Relatedness estimates were extremely low with most values being below zero, and average relatedness within roosts ($r = -0.126 \pm 0.0015$) was similar to between roosts ($r = -0.137 \pm 0.0008$; Fig. 5). Average relatedness estimates were similar for each roost, with male relatedness being lower than females except Bow Bells and Gudai-Darri Adit, and Cane River had the highest relatedness that was driven by the female data (Fig. 5). Observed within roost relatedness did not differ from expected, except for Cane River where the observed relatedness was higher than expected (*P*-value < 0.01), but still negative ($r = -0.1$). Pairwise relatedness between individuals was very low except for two pairs with relatedness values close to 0.2; T7337 and T7338 ($r = 0.21$) a male and female sampled at Kalgan Creek at the same date, and T8244 and T8238 ($r = 0.18$), two males sampled at Bow Bells at the same date.

There was significant, but low, genetic spatial autocorrelation within roosts and roosts that were less than 200 km apart, and relatedness values of roosts at greater distances were not different from zero or were negative (Fig. 6). There was also no significant difference in spatial autocorrelation between males and females, but females had slightly higher relatedness than males within roosts

Table 3
Hierarchical analysis of molecular variance of the D-loop and SNP sequence data. Structural levels are defined as: two subregions, including Chichester (containing roosts Lalla Rookh, Bow Bells and Copper Hills), and Hamersley (containing roots Cane River, Ratty Spring, Duck Creek, Gudai-Darri Adit, and Kalgan Creek).

Dataset	Variance	% Total	ϕ-statistics	Variance	% Total	ϕ-statistics
	mtDNA			*SNP*		
	All samples (n = 88)			All samples (n = 154)		
Among subregions	0.67	10.05	$\phi_{CT} = 0.100$*	−4.94	−0.249	$\phi_{CT} = -0.002$
Among roosts within subregions	0.32	4.81	$\phi_{SC} = 0.053$*	14.84	0.748	$\phi_{SC} = 0.008$*
Within roosts	5.70	85.14	$\phi_{ST} = 0.149$*	1973.35	99.5	$\phi_{ST} = 0.005$*
	Females (n = 37)			Females (n = 75)		
Among subregions	0.84	13.87	$\phi_{CT} = 0.139$*	−14.41	−0.73	$\phi_{CT} = -0.007$
Among roosts within subregions	0.60	9.94	$\phi_{SC} = 0.115$*	38.67	1.97	$\phi_{SC} = 0.020$*
Within roosts	4.62	76.19	$\phi_{ST} = 0.238$	1933.47	98.76	$\phi_{ST} = 0.012$*
	Males (n = 47)			Males (n = 77)		
Among subregions	0.45	6.42	$\phi_{CT} = 0.064$	−3.98	−0.20	$\phi_{CT} = -0.002$
Among roosts within subregions	0.03	0.48	$\phi_{SC} = 0.005$	8.01	0.40	$\phi_{SC} = 0.004$*
Within roosts	6.51	93.1	$\phi_{ST} = 0.069$	1982.71	99.8	$\phi_{ST} = 0.002$*

* *P*-value < 0.05

Table 4
Diversity statistics for SNP data by IBRA subregion (Chichester and Hamersley) and roost; Lalla Rookh (LR), Bow Bells (BB), Copper Hills (CH), Cane River (CR), Ratty Spring (RS), Gudai-Darri Adit (GA) and Kalgan Creek (KC). The number of individuals (n), mean allelic richness ($A_r \pm$ SE), private alleles (A_p), expected heterozygosity ($H_e \pm$ SE), observed heterozygosity ($H_o \pm$ SE), inbreeding coefficient ($F_{IS} \pm$ SE).

Locality	n	A_r	A_p	H_e	H_o	F_{IS}
Chichester	56	1.967 ± 0.001	4	0.191 ± 0.002	0.168 ± 0.001	0.115 ± 0.002
Hamersley	99	1.982 ± 0.001	132	0.192 ± 0.001	0.169 ± 0.001	0.116 ± 0.002
LR	12	1.343 ± 0.003	0	0.182 ± 0.002	0.163 ± 0.002	0.113 ± 0.004
BB	40	1.354 ± 0.003	2	0.190 ± 0.001	0.171 ± 0.001	0.098 ± 0.002
CH	4	1.316 ± 0.004	0	0.161 ± 0.002	0.151 ± 0.002	0.143 ± 0.007
CR	28	1.350 ± 0.003	0	0.187 ± 0.002	0.171 ± 0.002	0.086 ± 0.003
RS	37	1.354 ± 0.003	0	0.190 ± 0.002	0.166 ± 0.001	0.116 ± 0.003
GA	11	1.348 ± 0.003	0	0.185 ± 0.002	0.169 ± 0.002	0.096 ± 0.004
KC	23	1.354 ± 0.003	0	0.189 ± 0.002	0.172 ± 0.002	0.096 ± 0.003

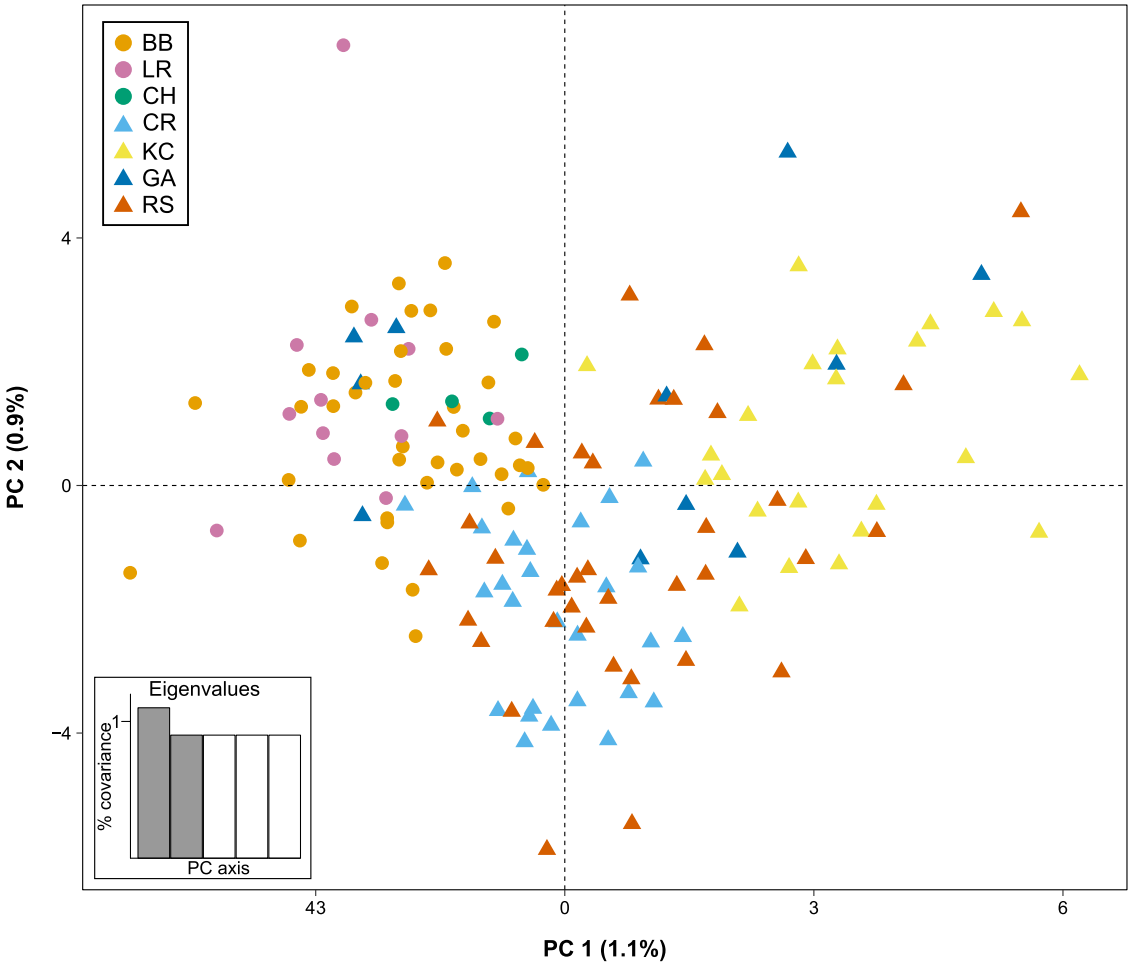

Fig. 4. Principal coordinates analysis of the filtered SNP sequence data for Pilbara leaf-nosed bats in this study (n loci = 9088). Sample roosts are indicated by colour key and Pilbara subregion are indicated by symbol shapes (Hamersley = triangles, Chichester = circles), the percent variation contributed by the first five PC is shown.

(Fig. 6).

All three clustering analyses, LEA, TESS and DAPC, indicate that K = 1 is the most likely scenario for the SNP sequence data, which was also the case when the male and female datasets were analysed separately (Supplementary Information Fig. S3). Similarly, Mantel tests for IBD were not significant at the population or individual level (p values > 0.1), indicating panmixia instead of IBD is better at explaining the spatial genetic structure of Pilbara leaf-nosed bats. Estimations of effective genetic population size were affected by the presence of linked loci, and minor allele frequency (MAF) cut-off, with a stricter MAF of 0.05 giving a slightly lower estimate of N_e (N_e

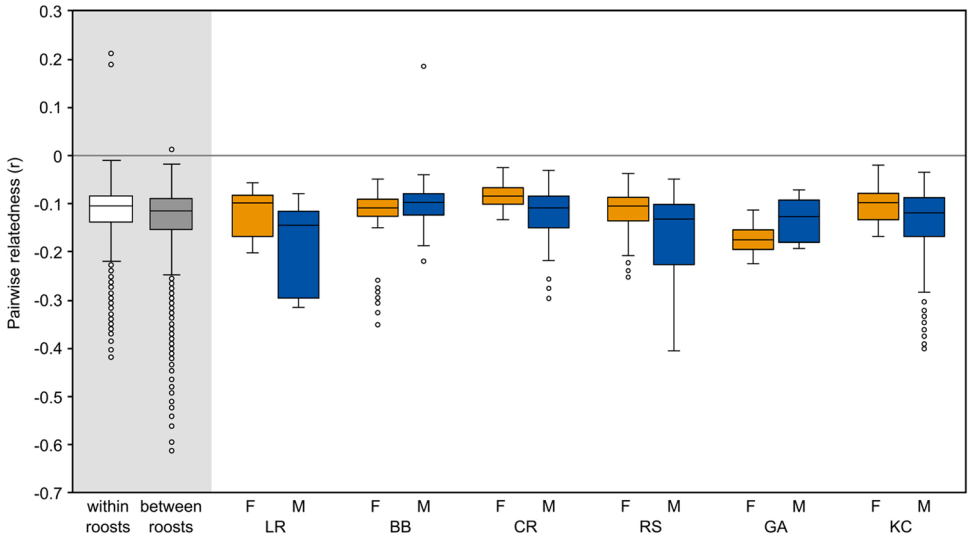

Fig. 5. Comparison of pairwise relatedness (Lynch-Li estimator) of Pilbara leaf-nosed bat sample pairs within roosts and between roosts, and for females (F) and males (M) at each roost showing outlier points.

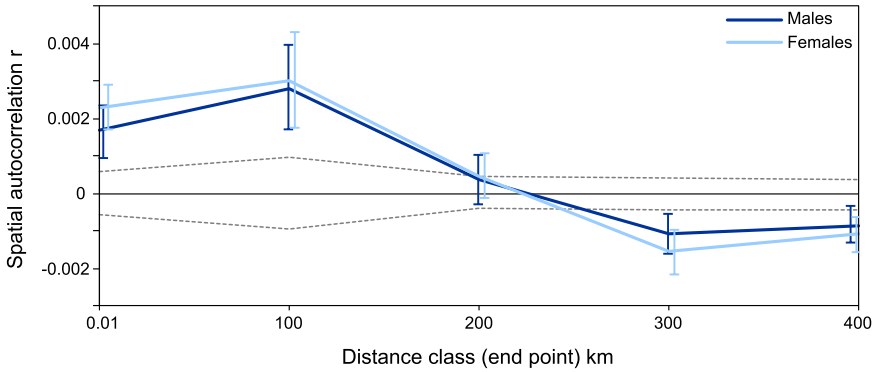

Fig. 6. Genetic spatial autocorrelation (r) of genetic and geographic distances for Pilbara leaf-nosed bat samples separated by sex at five distance classes up to 400 km. Upper and lower confidence intervals (CI) around zero shown by dashed grey lines.

= 3009.0; 95% CI 2622.9–41557.8) compared to a MAF of 0.02 (N_e = 3896.4; 95% CI 2028.3–4538.9).

4. Discussion

Our investigation of genetic structure and genetic diversity of the threatened Pilbara leaf-nosed bat at mitochondrial and nuclear SNP loci has increased our knowledge of the dispersal patterns and genetic structure of bat species inhabiting an arid biome. In contrast to our prediction of limited dispersal due to the species' small size and strict roost habitat requirements, we found extremely low population differentiation, and evidence of panmixia throughout the Pilbara, with some evidence of more restricted dispersal of female relative to male bats. Given this pattern of high genetic connectivity, future management will be required to ensure roosting and foraging habitat is adequately conserved to maintain connectivity in this vulnerable species.

4.1. Historical isolation and evidence of female philopatry from mtDNA

Initial research by Armstrong (2006) reported unique haplogroups associated with the major biogeographical subregions in the Pilbara — the Chichester and Hamersley. Having increased the geographic spread of samples, we found some evidence of a Chichester-Hamersley division with our mtDNA data, but it was less pronounced than reported by Armstrong (2006). We detected 34 additional unique haplotypes, with eight being shared between the Chichester and Hamersley. Similar to other bat mtDNA studies (Vonhof and Russell, 2015; Rakotoarivelo et al., 2019; Collevatti et al., 2020; Menchaca et al., 2020), haplotype diversity was high (range = 1–0.89). Armstrong (2006) attributed the geographic pattern to female philopatry, rather than the lowlands of the Fortescue River valley acting a barrier to dispersal as bats can likely fly across it in many places (Armstrong, 2006). However, Pilbara leaf-nosed

bats were rarely detected in the Fortescue region in a wide-scale echolocation study (McKenzie and Bullen, 2009) which could be due to a lack of suitable roost habitat in this area.

Most of the variation within our mtDNA dataset is present within roosts (and subregions) rather than between roosts, with Cane River and Bow Bells being the most differentiated. Cane River is one of the western-most and largest known natural colonies of Pilbara leaf-nosed bats (Table 1) and could act as a refuge during drought, with bats re-colonising nearby roosts when conditions are favourable. Both sexes show lowest connectivity between Cane River and the northern Chichester roosts across the lowlands of the Fortescue valley. Dispersal over this distance, approx. 330–380 km, would be difficult due the lack of suitable roost caves in the western Fortescue subregion (Cramer et al., 2016). In contrast, connectivity is higher across the eastern Fortescue region where distances between suitable cave habitats are much shorter.

Analysis of male and female datasets separately, showed stronger genetic differentiation for females, with males exhibiting a pattern closer to panmixia with very low differentiation between roosts and subregions. This is consistent with a pattern of female philopatry and potential male-mediated dispersal. Female philopatry and male-biased dispersal are common strategies in bat species (Moussy et al., 2013) and have been reported in species from a range of environments including the neotropics (Huguin et al., 2018), monsoonal-tropics of Australia (Worthington-Wilmer et al., 1999), temperate forests of Japan (Flanders et al., 2016), and temperate-arid central Oregon (Arnold and Wilkinson, 2015). These strategies tend to occur where resources for females raising young are limited, such as suitable maternity caves, and to reduce the occurrence of inbreeding (Moussy et al., 2013). It is not unexpected that we see higher structure in female Pilbara leaf-nosed bats since roost caves with suitable microclimate conditions, within the broader arid landscape, are limited (Armstrong, 2001; Churchill et al., 1988).

4.2. Panmixia and high gene flow in Pilbara leaf-nosed bats

Bats are highly vagile, and migratory species often exhibit higher gene flow than sedentary species with natal dispersal strategies (Moussy et al., 2013), although many factors influence genetic structure such as mating systems and dispersal ability (Burns and Broders, 2014). While our mtDNA data provided some evidence of historical isolation and female philopatry, albeit weak, we found this pattern was further diluted in the nuclear SNP dataset indicating overall high levels of gene flow. We expected to find some evidence for limited gene flow, due to the patchy distribution of suitable roost caves and limited nightly flight distances; however, genetic differentiation between roosts and within subregions was not detected with any of the analyses we employed, and similar genetic diversity statistics at each roost also point to high levels of gene flow. This is in slight contrast to our mtDNA results that suggested some female philopatry, with male-mediated dispersal often leading to discrepancy between maternal and bi-parental inherited genetic markers, a common pattern in volant taxa including bats (Arnold and Wilkinson, 2015; Collevatti et al., 2020; Flanders et al., 2016; Huguin et al., 2018; Sovic et al., 2016). While we found low-moderate levels of inbreeding for this species, pairwise relatedness estimates of individuals within roosts were extremely low, indicating high gene flow between roosts. There was significant positive genetic spatial autocorrelation at roosts within 200 km distance, indicating some relatedness between individuals at shorter distances, although these values were still very low, $r < 0.003$.

Our results indicate that Pilbara leaf-nosed bats can be considered one panmictic population in the Pilbara with high levels of contemporary gene flow and little differences between the sexes. This contrasts with our hypothesis where we expected to find some evidence of restricted gene flow due to the limited distribution and availability of suitable roosts throughout the landscape and indicates Pilbara leaf-nosed bats have higher population connectivity than previously thought. Other studies on microbats using nuclear markers have reported high levels of gene flow and well connected populations in migratory (Chipps et al., 2020; Korstian et al., 2015; Vonhof and Russell, 2015), tropical island (Pinzari et al., 2020), and widely-occurring Australian tree-dwelling bat species (Fuller, 2013; Prada, 2020). Breeding behaviour, such as swarming during mating can also facilitate gene flow (Burns and Broders, 2015; Kerth et al., 2003), though it is unknown whether this phenomenon occurs in Pilbara leaf-nosed bats.

4.3. Long-distance dispersal driving genetic patterns

Life-history traits such as high mobility, random mating, and long lifespans contribute to high levels of gene flow in microbat species (Moussy et al., 2013). For the Pilbara leaf-nosed bat, one instance of long distance dispersal has been recorded; a male that dispersed 170 km from Ratty Spring to Gudai-Darri Adit within a year (Sep 2017–Sep 2018; Bullen and Reiffer, 2019). As the nightly foraging distance is estimated at 20–30 km to-and-from the roost (Bat Call WA, 2021), this individual must have rested at suitable roost caves between Ratty Spring and Gudai-Darri Adit – although only two are currently known from this area (Fig. 1). Similar to other microbats, males reach reproductive maturity in their second year, but females can reproduce within their first year (Churchill, 1995). This could give males more time to disperse from natal roosts, an inbreeding avoidance strategy, whereas females have less time to disperse before the first breeding season.

Bats are long lived, and Pilbara leaf-nosed bats likely live for eight to ten years, similar to other hipposiderid bats (Wilkinson and South, 2002), but it is unknown if they can disperse multiple times during their lifetime. While Bullen and Reiffer (2019) recorded one instance of long-distance dispersal, 17 of the 18 individuals tagged in the study remained at the same roost over 12 months (Bullen and Reiffer, 2020), suggesting high site fidelity of most individuals at least over short temporal scales. It remains unknown whether dispersal is most frequently associated with either natal dispersal or breeding seasons, or in response to roost microclimatic conditions becoming unsuitable as has been previously observed (Armstrong, 2001). Monitoring of additional sites and further research into the dispersal ecology of Pilbara leaf-nosed bats could lead to a better, mechanistic understanding of gene flow patterns in the species.

4.4. Conservation implications

Many Australian arid-zone mammals respond to cycles of resource availability by expanding, contracting, and shifting ranges to habitat refuges (Céré et al., 2015; Pavey et al., 2017). We found here that *R. aurantia* exhibits a pattern of panmixia within the Pilbara bioregion that is consistent with this observation. Whilst long-distance dispersal events potentially contribute to connectivity in the landscape, the ongoing loss and/or degradation of critical roosting habitat is of significant concern for the conservation of the species. Pilbara leaf-nosed bats are highly sensitive to changes in roost microclimatic conditions and will readily abandon roosts if they become unsuitable; therefore, preserving all possible roosts is critical in buffering this species against drought cycles and a future drying climate. Further, due to the species physiological limitations, each roost plays an integral role in maintaining connectivity throughout the landscape (Cramer et al., 2016), facilitating demographic and genetic stability of the metapopulation through time.

While the high contemporary gene flow and connectivity observed is a positive result, it also indicates that future economic development and management proposals should consider impacts to the species across the entire Pilbara population, not just at local scales. In addition to preserving critical roosting habitat, protecting and maintaining dispersal corridors between roosts is also important to maintain connectivity. Such areas are likely to be associated with permanent water as Pilbara leaf-nosed bats forage in well-vegetated riparian zones within the Pilbara ranges (McKenzie and Bullen, 2009). They are also sensitive to human induced disturbances including activities associated with mining, such as drilling, blasting and draining of underground water (Armstrong, 2010; Bullen and Creese, 2014). The use of some disused mines indicates their habitat value as bat roosts, but the longevity of these historical structures is uncertain compared to natural caves (Cramer et al., 2016).

4.5. Concluding comments

Our study provides information at the bioregion and local scales using samples from eight of the approximately 50 Pilbara leaf-nosed bat roosts. Better understanding of how roosts are being used and when dispersal is occurring will require fine-scale demographic studies, and non-invasive monitoring of scat samples (via extracted DNA) deposited below roosts has been a promising method for the larger ghost bat (Ottewell et al., 2020). We have demonstrated that opportunistic collections of Pilbara leaf-nosed bat scats are not a suitable method for obtaining sufficient DNA, for the genetic approaches reported here, but further improvements to scat collecting methodology and use of alternative genomic approaches (e.g., targeted SNP panels, Carroll et al., 2018) could allow for non-invasive demographic monitoring while reducing stress and handling of a threatened species. An important finding from this study is that our initial predictions based on the ecological knowledge of the species were not met, with much higher levels of gene flow detected than expected. This highlights the considerable utility of genetic approaches in combination with ecological understanding to reduce uncertainty in conservation decision making. Genetic information on arid species is generally lacking, particularly the bat fauna (Lisón et al., 2020), and such information is crucial to identify species diversity and connectivity in complex arid landscapes. Further research is required to understand how species in complex and multi-use landscapes are impacted by habitat loss and fragmentation to allow for more effective conservation management of habitat critical for maintaining population connectivity and longevity.

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Data statement

The mitochondrial sequence data generated and analysed during the current study are available in Genbank (accession numbers MZ020332-MZ020419 and MZ571159-MZ571161), the raw nuclear sequence reads and the filtered SNP dataset used in analyses are available from the Department of Biodiversity, Conservation and Attractions online data catalogue https://data.dbca.wa.gov.au/dataset/pilbara-leaf-nosed-bat-genetic-data.

CRediT authorship contribution statement

All authors contributed to the study conception and design. Sample and data collection was performed by Robert Bullen, material preparation was performed by Shelley McArthur, Robyn Shaw and Linette Umbrello, and analysis by Linette Umbrello. The first draft of the manuscript was written by Linette Umbrello and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Competing interests

Financial interests: Author Robert Bullen has received funding from the Department of Biodiversity, Conservation and Attractions who managed the offset funds to support the field program to collect the tissue samples. All other authors declare they have no competing interests.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.gecco.2022.e02154.

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