Appendix 21

Dasyurus hallucatus Population Genetics: Final Report September 2013

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Report compiled by Dr Gabriel Conroy and Dr Robert Lamont

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The genetic structure of Northern Quoll (*Dasyurus hallucatus*) populations centred around Mt. Emerald, Atherton Tablelands.

Introduction

Dasyurus hallucatus is the largest extant marsupial carnivore in northern Australia, with a range spanning northern Queensland, the Northern Territory and Western Australia. Although listed as being of Least Concern in Queensland (*Nature Conservation Act 1992), D. hallucatus* is listed as Endangered nationally (*Environment and Biodiversity Conservation Act 199*9). Key threatening processes include habitat destruction, fragmentation and degradation stemming from agricultural and urban development (DSEWPC 2013).

When anthropogenic pressures lead to habitat loss, fragmentation or modification, the persistence of a species may become compromised at either a local or regional level. On the Atherton Tablelands, the negative effects of habitat fragmentation are well documented for a number of taxa (Laurance 1994; Cunningham & Moritz 1998; Bowyer *et al.* 2002; Sumner *et al*. 2004). Due to the proposed wind-farm on Mt Emerald, the University of the Sunshine Coast has been commissioned to undertake a population genetic analysis of *D. hallucatus* to determine population relationships between the development site and surrounding areas. While previous research exists on the population genetics and phylogenetic structure (Firestone 2000) of the northern Quoll, these studies have been restricted to Western Australia (How *et al.* 2009) and the Northern Territory (Cardoso *et al.* 2009).

The quantification of genetic diversity facilitates an interpretation of the manner in which diversity is partitioned within and among remnant populations of threatened species (England *et al.* 2002; Frankham 2002). Establishing these patterns can also lead to an understanding of the effects of inbreeding, gene flow, selection, mutation and genetic drift (Frankham 2002). The population genetic structure of a species can provide insights into historical patterns of landscape level processes such as gene flow, and in turn, can detect

how a species may be affected by interruptions to these processes (Frankham 2005; Lowe *et al.* 2009). Familiarity with the amount and distribution of genetic variability within a species prior to the impacts of threatening processes will therefore increase the precision and effectiveness with which the main priorities for conservation management are identified and executed (England *et al.* 2002; Frankham 2005; How *et al.* 2009).

In contemporary population genetics analysis, codominant microsatellites, or simple sequence repeats (SSRs) are the marker of choice, due to a high level of polymorphism among individuals (Bhargava & Fuentes 2010). This study used SSRs to investigate the genetic importance of the *D. hallucatus* population on Mt Emerald in relation to proximate populations, particularly with regard to its contribution to regional genetic diversity and its potential status as a source or linkage for the movement of genes through associated subpopulations comprising the area's broader metapopulation.

This is the final USC report on the population genetic structure of *D. hallucatus* subpopulations in the vicinity of Mt. Emerald, and will cover DNA extraction, PCR amplification of microsatellites, genotyping, and data analysis. Implications for the future of the Mt Emerald quoll population and potential avenues of conservation in light of the proposed development are discussed.

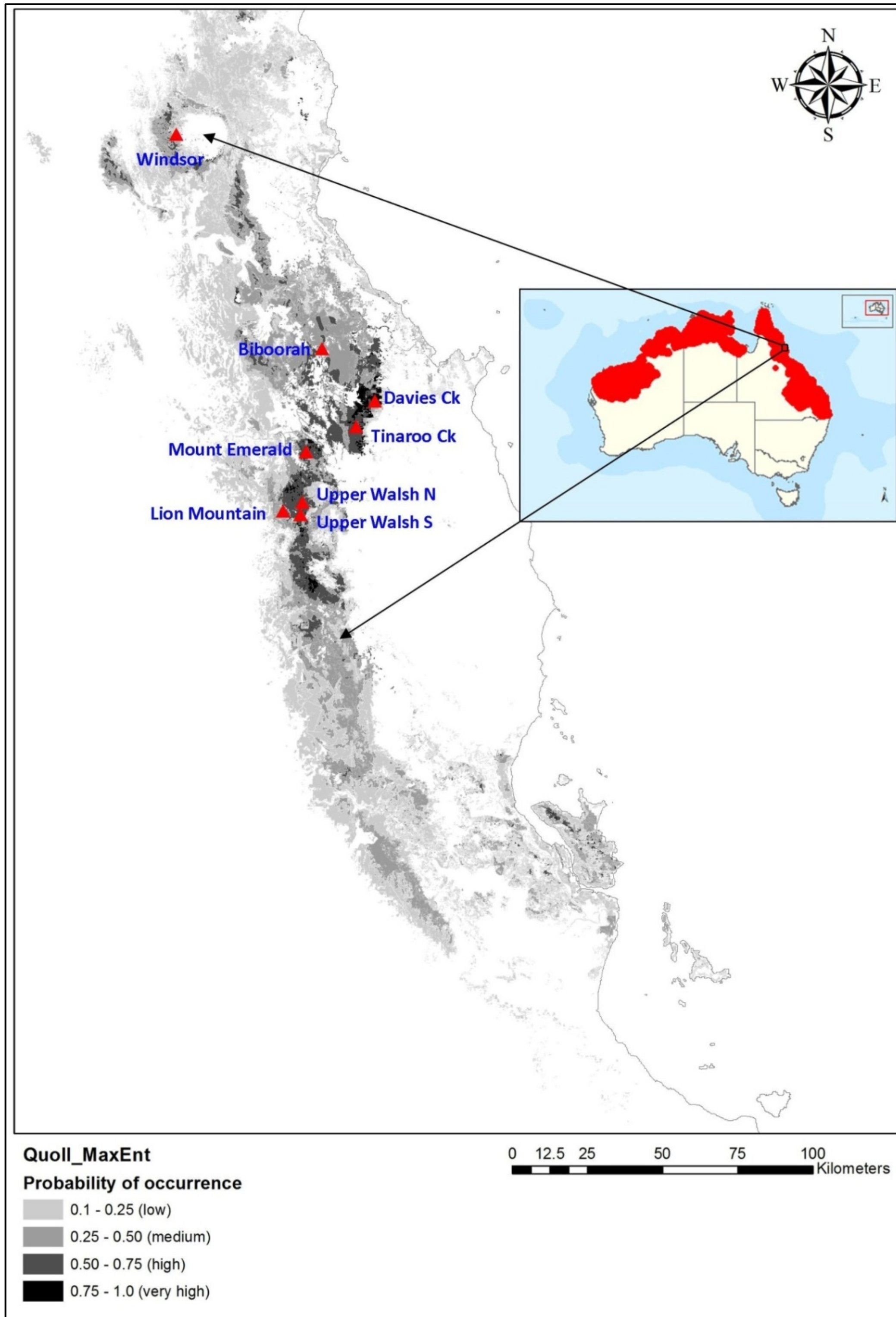


Figure 1: Sampling locations (▲) and modelled probability of *Dasyurus hallucatus* occurrence as a function of suitable habitat within an approximate 200km radius of Mt Emerald. Probability of occurrence as modelled by Shimizu and Burnett (2013) is indicated by the degree of shading as per legend. Inset: Geographic distribution of *D. hallucatus* across northern Australia (DSEWPC, 2013).

Methods

Field Methods

A total of 379 sticky traps were set on Mt Emerald and surrounds to collect hairs from foraging *D. hallucatus*, however only 34 of these were successful in obtaining hair samples. Initial extraction trials focussed on ascertaining the most appropriate method for extracting DNA from sticky-trap samples, most of which consisted of a single hair, or hairs without follicles. Two extraction techniques, Qiagen's DNeasy Blood and Tissue Kit and the Chelex method were tested. The Qiagen kit was unsuccessful due to insufficient quantities of DNA, and although some extractions were successful using Chelex, DNA yield from these samples was very low. During subsequent PCR and genotyping, many of the genetic markers employed were found to amplify inconsistently, suggesting that the integrity of the DNA had been compromised by the length of time spent in the field under tropical conditions prior to trap retrieval.

As a result, the study proceeded with directly-plucked hair samples from 81 trapped individuals, sourced from nine 'populations' (Table 1). Although sufficient samples (n-33) were collected from Mt Emerald, samples from proximate localities were pooled in order to achieve a more statistically-robust dataset (Table 1). Individuals from Upper Walsh North (12), Upper Walsh South (10) and Lion Mountain (1) were combined to create a subpopulation of 23 samples, and individuals from Tinaroo Creek (11), Davies Creek (6), and Biboohra (1) were pooled to create a sample size of 18 (Table 1; Figure 1). Two distant populations, Windsor (2; ~250km) and Weipa (5; ~650km), were included as outgroups for genetic comparison and treated as distinct entities (Table 1).

Table 1: Sampling location, population code for combined sites, number of individual quolls sampled per site (*n*), and number of individuals per combined population (*N*) for the five demes.

Location	Popn Code	*n*	*N*
Mt Emerald	**MTE**	**33**	**33**
Upper Walsh N	**UWL**	**12**	
Upper Walsh S	**UWL**	**10**	**23**
Lion Mtn	**UWL**	**1**	
Tinaroo Crk	**DTB**	**11**	
Davies Crk	**DTB**	**6**	**18**
Biboohra	**DTB**	**1**	
Windsor	**WIN**	**2**	**2**
Weipa	**WEI**	**5**	**5**
Total			**81**

Laboratory Methods

DNA Extraction

Total genomic DNA was isolated from the hair follicles of captured quolls using the DNeasy Blood and Tissue Kit (Qiagen) according to the manufacturer's instructions. Ten hairs from each individual were trimmed to approximately 1cm in length from the follicle, placed in 1.7mL microcentrifuge tubes and lysed overnight in 180μl of ATL lysis buffer and 20μl of 10 mg/mL Proteinase-K at 56°C. DNA was eluted in 100μl of AE buffer and compared with known concentrations (5ng/μl, 10ng/μl, and 20ng/μl) of Lambda EcoR1/*Hind*III digest molecular-weight marker (Fisher-Biotec) to estimate yields using agarose gel electrophoresis.

Microsatellite analysis

Eleven Dasyurid microsatellite primers (Firestone 1999; Jones *et al.* 2003; Spencer *et al.* 2007) were selected for use and end-labelled with either VIC, NED, PET or FAM fluorescent dyes (Applied Biosystems; Table 2). PCR amplification was performed using reaction volumes of 12.5µl containing ~20ng of template DNA, 1 x reaction buffer (67mM Tris-HCl (pH 8.8), 16.6mM (NH4)2 SO4, 0.45% Triton X-100, 0.2mg/mL gelatine), 200µM of each

dNTP, 1.5mM $MgCl_2$, 0.4µM of each primer and 0.5U *Taq* F1 DNA polymerase (Fisher Biotech). Initial gradient trials using a Mastercycler® gradient thermocycler (Eppendorf) were conducted to establish the most appropriate annealing temperature for each primer pair, resulting in an optimised annealing temperature of 55°C for all primers except Q4.4.2 (59°C). PCR was performed using an initial denaturation at 95°C for 3 min, followed by 40 cycles of 94°C for 30s, annealing at either 55°C or 59°C for 30s, extension at 72 °C for 45s; with a final extension at 72 °C for 10 min. Prior to genotyping, PCR products were run on 1.5% agarose 0.6xTBE check gels at 140V for 40min, and visualised using ethidium bromide and UV light. Amplification products were separated by capillary electrophoresis on an AB 3500 Genetic Analyser (Applied Biosystems) with fragment sizes determined relative to an internal lane standard (GS-600 LIZ; Applied Biosystems) using GENEMARKER V1.95 software (SoftGenetics) and double-checked manually to ensure accuracy.

Data Analysis

Allelic frequencies were calculated for each population using GenAlEx v6.4 (Peakall & Smouse 2006) to derive standard genetic diversity and inbreeding measures including the mean number of alleles per locus (A), the number of private alleles (A_P) unique to a particular population, observed heterozygosity (H_O), expected heterozygosity (H_E), and the allelic fixation index (F) as a measure of inbreeding (Wright 1965). Polymorphism information content (PIC) was computed in CERVUS v3.0 (Kalinowski *et al*. 2007). To account for differences in sampling intensity, FSTAT Version 2.9.3 (Goudet 2001) was used to calibrate allelic richness (A_R) among populations.

Nei's standard genetic distance measures (Nei 1972; 1978) were calculated to examine patterns of genetic differentiation among individuals and populations and used to perform frequency based population assignment tests to designate a percentage 'self' or 'other' assignment by assigning samples to sites with the highest likelihood of genetic similarity using GenAlEx v6.4 (Peakall & Smouse 2006). A pairwise squared genetic distance matrix was generated in GenAlEx v6.4 (Peakall & Smouse 2006) prior to carrying out an hierarchical cluster analysis (UPGMA - unweighted pair group method with arithmetic averaging) using 1000 permutations in PRIMER 5 software (Clarke & Gorley 2001). The matrix was then used

to conduct a principal coordinates analysis (PCoA) to look for genetic relationships between individuals, both within and among populations, while the hierarchical partitioning of genetic variation among sampled populations (*PhiPT* - the correlation between individuals within a population relative to all individuals within a species) was tested for statistical significance using an analysis of molecular variance (AMOVA; Excoffier *et al.* 1992). Measures of gene flow (*Nm*) based on *PhiPT* values were used to construct a pairwise population *PhiPT* matrix with associated estimates of N_m using GenAlEx v6.4 (Peakall & Smouse 2006). Tests for correlations between pairwise geographic and genetic distance matrices (Mantel 1976) were performed in PRIMER v6.1.5 (Clarke & Gorley 2001) using 1000 permutations.

To determine the likelihood of recent bottlenecks, Wilcoxon's sign rank tests and sign tests for mutation-drift equilibrium were applied to the allelic frequency data using BOTTLENECK v1.2.02 (Piry *et al.* 1999). These tests were conducted under the assumptions of the infinite alleles model (IAM), the stepwise mutation model (SMM), and the intermediate two-phased model (TPM), with results reported for the latter, due to its suitability for microsatellite data.

Table 2: Characteristics of the 11 microsatellite loci used to quantify genetic diversity in *Dasyurus hallucatus* populations in the vicinity of Mt. Emerald. *PIC*, polymorphic information content; N_A, number of alleles per locus; H_O, observed and H_E, expected heterozygosities; primers *pDG1A1*, *pDG1H3*, *pDG5G4*, and *pDG6D5* from Spencer *et al.* (2007); *3.1.2*, *3.3.1*, *3.3.2*, *4.4.2*, and *4.4.10* from Firestone (1999); Sh3o and Sh6e from Jones *et al*. (2003).

Locus GenBank	Repeat motif	Primer sequences (5'-3')	Size range (bp)	*PIC*	N_A	H_O	H_E
pDG1A1	$(AC)_{20}$	F: **NED**-ATTTGCTTCTTGCTCCCTACAGC	208-220	0.465	7	0.432	0.488
EF077168		R: TTTCACTCCTTCTGAGTTTATCACC					
pDG1H3	$(TG)_{17}$	F: **VIC**-GTGGATTGACACAATCAGAGTGG	184-200	0.801	9	0.827	0.828
EF077169		R: GCAATTCCATCTTTATTGCATGC					
pDg5G4	$(AC)_{24}$	F: **PET**-TAGATTCCTTCAATGGCTATCCC	109-111	0.348	2	0.481	0.451
EF077170		R: GCTCCTGACATAGAGTGATGATGG					
pDG6D5	$(AC)_{22}$	F: **NED**-CCTCCAGACAAATGCAACC	133-151	0.576	7	0.630	0.640
EF077171		R: TCTCTGAATTTACTGATAGTATCTTTGG					
3.1.2	$(CA)_{18}$	F: **FAM**-AGGAAACTTCACAAGTGTCGA	177-189	0.760	7	0.741	0.794
AF124212		R: ATTAATGACTCATCTGTTGTTGG					
3.3.1	$(CA)_{20}$	F: **NED**-CAGCCCTTGAGTCTTGAGATT	128-157	0.822	10	0.864	0.846
AF124213		R: CATACCACCCCAGGAGTTTC					
3.3.2	$(CA)_{21}$	F: **FAM**-GCATATTGGAGATTAAAACAGAGC	152-184	0.721	11	0.580	0.762
AF124214		R: CTCCGCGCACTCAGATCTAT					
4.4.2	$(CA)_{19}$	F: **VIC**-GAAATCCAAGCTCATTTTAG	117-133	0.751	8	0.580	0.786
AF124215		R: AATCAACTCTGGAATGCATC					
4.4.10	$(CA)_{29}$	F: **FAM**-AATGCTAGATTTCACTCCC	216-220	0.111	3	0.123	0.117
AF124216		R: CCTCACATTTCTGGAACTG					
Sh3o	$(CA)_{22}$	F: **PET**-CTCAATGCCAAAGGTATCTTT	203-223	0.729	10	0.728	0.767
AJ515733		R: CATAGTTCCAAATCACTCTCCAG					
Sh6e	$(CA)_{18}$	F: **PET**-GATTCTAGAAGGGATAGCAAGC	169-183	0.691	8	0.790	0.731
AJ515737		R: GACACTCCATAGAAATGCACTG					

Results and Discussion

A total of 82 alleles were resolved using 11 microsatellite loci in 81 *D. hallucatus* individuals (Table 2), although not all alleles were present in every population (Table 3). The most intensively sampled population, Mt Emerald (MTE), displayed a comparable level of allelic diversity to individuals from the Upper Walsh (UWL, ~16km to the southwest), particularly after rarefaction to account for differences in sampling effort. However, a higher diversity of alleles many of which were unique, were detected within the much smaller sample size obtained from the Davies/Tinaroo Creeks area (DTB, ~19km to the northeast; Figure 1, Table 3). Estimates of observed and expected heterozygosity (Table 3) in the main study populations (UWL, MTE, DTB) were almost identical and similar to those found in previous studies of *D. hallucatus* populations in Western Australia (How *et al.* 2009) and the Northern Territory (Cardoso *et al.* 2009). Inbreeding was minimal with negligible values showing a slight excess of either homozygotes (+*F*) or heterozygotes (-*F*, Table 3), with populations close to conditions of Hardy-Weinberg equilibrium.

Table 3: Summary of genetic measures for the 81 *D. hallucatus* individuals sampled: *N***,** number of animals sampled per population; N_A, number of alleles per population resolved across 11 loci ; *A*, mean number of alleles per locus; A_P, number of private alleles per population; A_R, mean allelic richness across populations; H_O, observed heterozygosity; H_E, mean expected heterozygosity, and *F*, inbreeding coefficient. Standard deviations are shown in parentheses. Population codes are given in Table 2.

Population	N	N_A	A	A_P	A_R	H_O	H_E	F
MTE	33	57	5.18	1	1.73	0.63	0.61	-0.06
			(0.69)			(0.08)	(0.08)	(0.05)
UWL	23	52	4.73	2	2.26	0.64	0.62	-0.03
			(0.57)			(0.07)	(0.05)	(0.05)
DTB	18	60	5.46	7	3.33	0.61	0.63	0.03
			(0.71)			(0.07)	(0.07)	(0.05)
WIN	2	30	2.72	4	n/a	0.64	0.53	-0.17
			(0.27)			(0.12)	(0.07)	(0.15)
WEI	5	32	2.91	3	n/a	0.46	0.46	-0.05
			(0.37)			(0.09)	(0.08)	(0.09)

While the low sample sizes from the Windsor (~250km) and Weipa (~650km) populations have been included for comparison, the limited number of samples must necessarily preclude these sites from meaningful genetic analysis of the populations under review. Perhaps the main inference which can be drawn from their inclusion can be derived from the considerable number of private alleles (A_P) detected in only seven individuals, suggesting that the geographic partitioning of genetic diversity among *D. hallucatus* populations will be more apparent over a scale of hundreds of kilometres rather than over the distances (~20km) relevant to this study.

A high level of historical gene flow (N_m) between the MTE/UWL and the DTB populations to the northeast was detected (Table 4), as would be anticipated from a species known to travel distances of up to 10km overnight, although it is unknown to what extent connectivity has been affected by the significant fragmentation of valley habitat along the Walkamin corridor since settlement (Figure 2). The resolution of a relatively large number of private alleles in the northeast populations (DTB), not present in the Mt Emerald or Upper Walsh populations suggests that this barrier has had some effect in restricting gene flow already however, to date, this appears insufficient to be totally excluding genetic exchange.

Table 4: Estimated mean number of migrants per generation (N_m) based on *PhiPT* values of population differentiation for the three Atherton Tablelands populations of *D. hallucatus*. N_m values >1 (migrant per generation) are considered necessary to avoid inbreeding and/or genetic drift. Population codes are given in Table 2. **UWL,** Upper Walsh River population; **MTE,** Mt Emerald population; **DTB**, Lamb Range population.

	UWL	MTE	DTB
UWL	0.00		
MTE	7.28	0.00	
DTB	5.96	8.64	0.00

Both the UPGMA (Figure 3) and principle coordinates analysis (Figure 4) demonstrated the high genetic similarity of individuals within these populations and no correlation (r^2=0.045, p>0.05) was found between genetic and geographic distance matrices using Mantels tests.

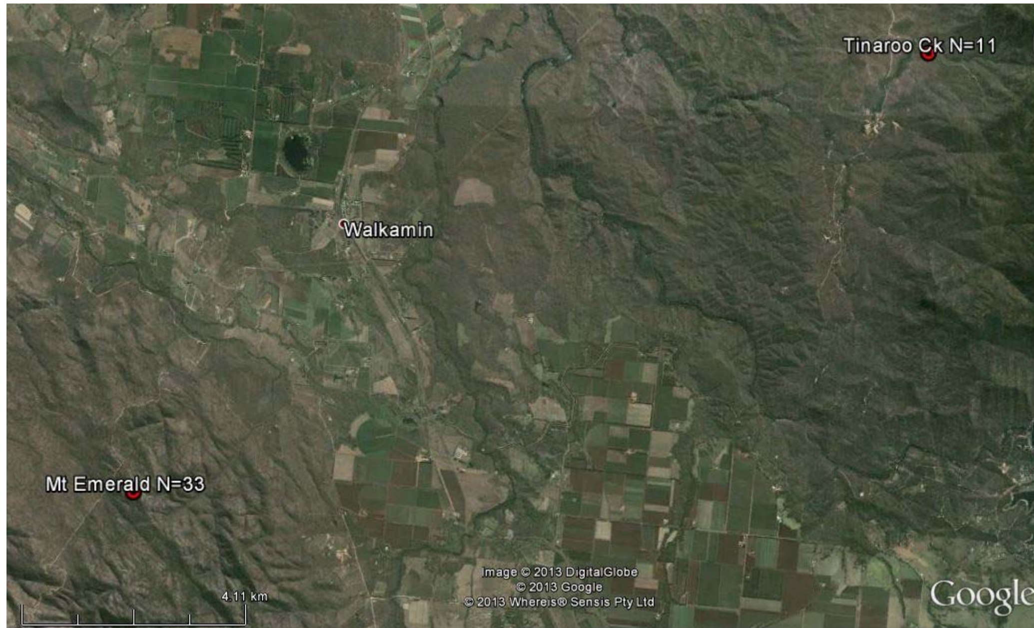


Figure 2: Aerial view of the now heavily fragmented valley habitat separating the Mt Emerald/Upper Walsh (MTE, UWL) and Davies/Tinaroo Creek (DTB) populations. **Image courtesy of Google Earth.**

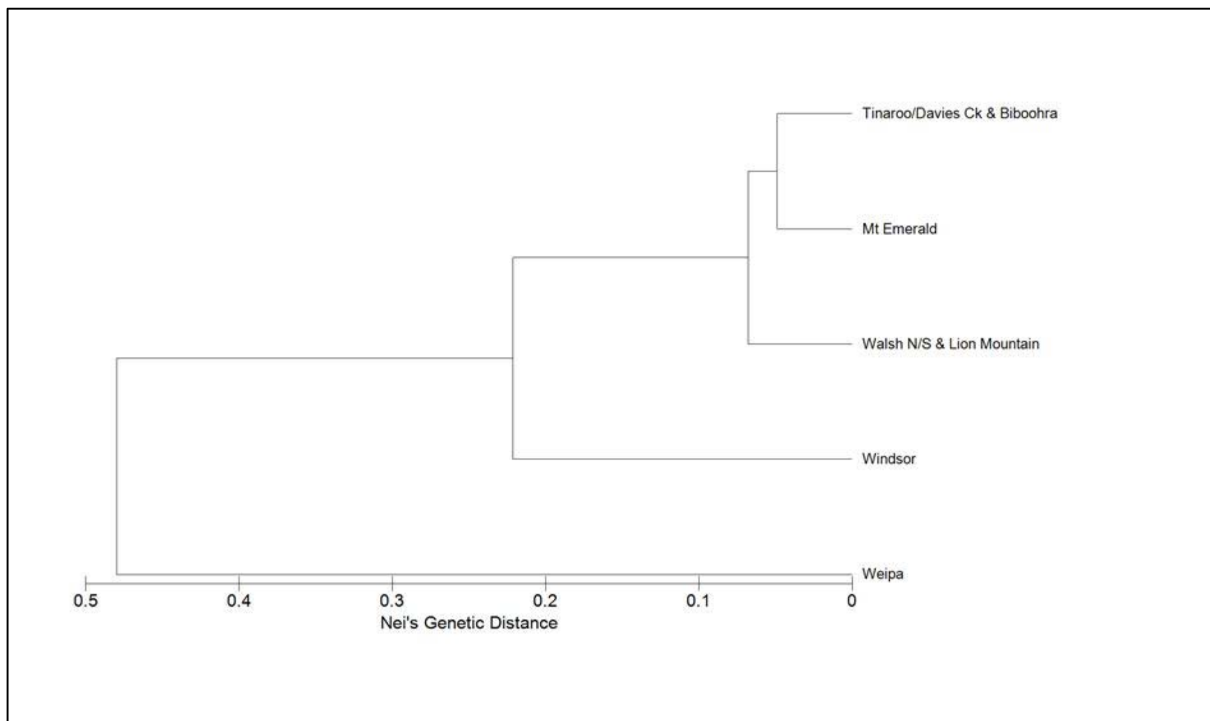


Figure 3: UPGMA cluster analysis of the five populations of *D. hallucatus* sampled, using Nei's standard (1972) genetic distance, showing >90% similarity among the Atherton Tablelands populations.

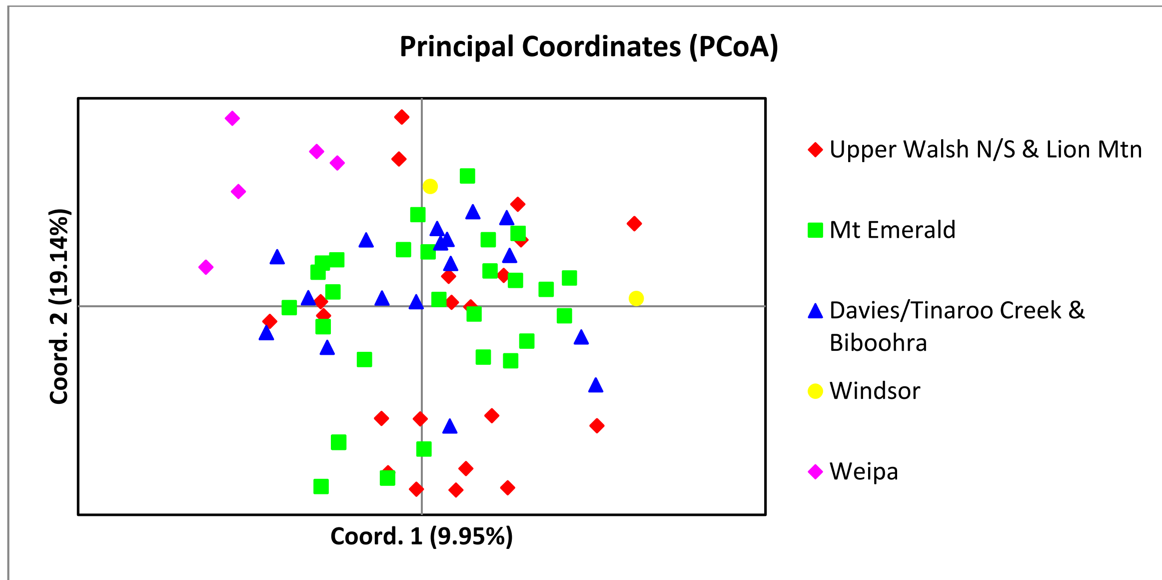


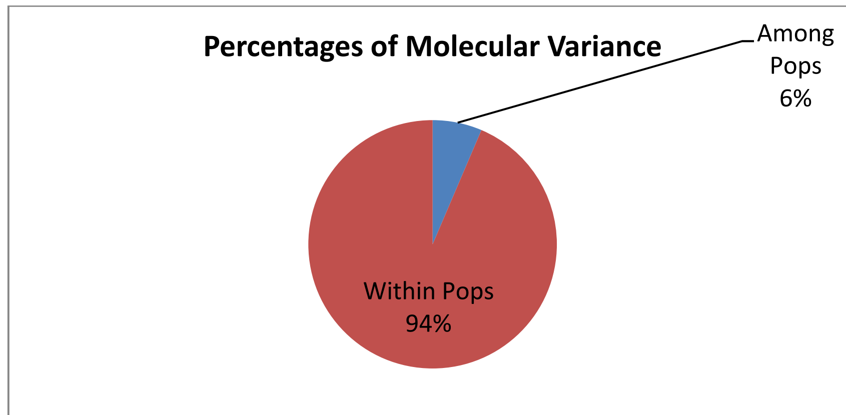
Figure 4: Principal coordinates analysis (PCoA) using genetic distance matrices with data standardisation showing the genetic relationships among *D. hallucatus* populations sampled for this assessment. Individuals from the five populations are indicated by the colours and symbols shown. Combined, the first three axes account for only 26.46% of the variation in the data.

However, assignment analysis using multilocus genotypes were able to be assign the majority of individuals to their correct population of origin, with only 15% of randomly selected samples being assigned to a population other than their own (Table 5).

Table 5: Frequency-based population assignment analysis outcomes for *D. hallucatus* study populations from the Atherton Tablelands region. The percentage of samples per population assigned correctly (Self) or incorrectly (Other) using highest log likelihoods are given. **UWL,** Upper Walsh River population; **MTE,** Mt Emerald population; **DTB**, Lamb Range population.

Population	Self	Other
MTE	95.65	4.35
UWL	81.82	18.18
DTB	77.78	22.22
Total %	85	15

a)



b)

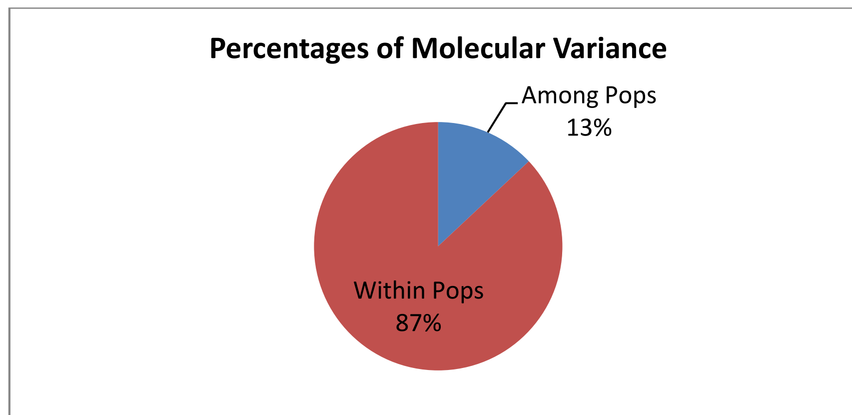


Figure 5: Partitioning of genetic diversity both within and among populations of *D. hallucatus* - a) at the Atherton Tablelands sites (UWL, MTE, DTB) only, and b) including Windsor Tableland and Weipa samples.

The majority of genetic variation was due to variation among individuals within populations, rather than differences among populations (*PhiPT* = 0.06; Figure 5). This is most likely a product of strong historical gene flow, as evidenced by the high number of migrants per generation (N_m; Table 4) and is again supported by the results of the principle coordinates analysis (Figure 4) which does not indicate clearly definable patterns between individuals from populations in the Atherton Tablelands region based on allelic composition.

Population genetic theory predicts that small isolated populations may suffer a loss of genetic diversity over time (Frankham 2002) and previous research has demonstrated genetic erosion to be an issue for isolated populations of *D. hallucatus* in the Northern Territory (Cardoso *et al.* 2009) and Western Australia (How *et al.* 2009). Cardoso *et al.*

(2009) found reduced genetic variation after just three generations in small, translocated populations. The effect was also shown to be more pronounced in island populations isolated from the mainland, although genetic bottlenecks indicating recent reductions in abundance were detected in both island and mainland *D. hallucatus* populations (Cardoso *et al.* 2009). In contrast, no evidence was found in this study to indicate recent bottlenecks in any of the Atherton Tablelands populations. There were no statistically significant (p>0.05) instances of heterozygosity-excess under the two-phase model (TPM) for any of the populations, and thus no mode-shifts that would reflect a disproportionate loss of rare alleles relative to those that occur at intermediate frequency (Luikart & Cornuet 1998; Piry *et al.* 1999). This is most likely due to the strong levels of historical gene flow detected among the three Atherton Tablelands subpopulations. Genetic bottlenecks are therefore unlikely to occur unless these populations become isolated from established avenues of gene flow, or suffer a significant reduction in population size.

Conclusions

Fragmentation has been highlighted as a key threatening process for *D. hallucatus (DSEWPC* 2013*)* and substantial anthropogenic disturbance has already occurred in the region (Cunningham & Moritz 1998; Sumner *et al*. 2004) and adjacent to the study site (Figure 2). It is unknown whether the effects of the proposed wind farm and associated infrastructure will further contribute to complete isolation of either the Mt Emerald population, or of the other populations in the immediate vicinity. However, the results of this study strongly suggest that the Mt Emerald population site spans a narrow corridor of high-value habitat, and is currently serving as an important linkage for the movement of genes between proximate *D. hallucatus* aggregations to the northeast and southwest.

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