Australian Marine Mammal Centre

Final Report

(subclause 9 and Schedule Item 5 of the Funding Agreement)

- **Project No**. – 0809/12
- **Title** - Microsatellites, mating systems and males influence on management units in the Australian sea lion
- **Chief Investigator** – Prof Rob Harcourt
- **Organisation** – Macquarie University

Activity Period – 30 January 2009 – 29 May 2010

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1. Activity Summary

The Australian sea lion (ASL) is Australia's only endemic and rarest seal species and was listed as *Threatened* under the EPBC Act in February 2005. A risk-assessment by Goldsworthy & Page (2007) determined that within South Australia subpopulations are at risk from even low-levels of fisheries bycatch and a similar situation exists in Western Australia (Campbell 2003). They have an unusual life history, unique among pinnipeds with a non-annual breeding cycle that is also temporally asynchronous across its range and within any one colony the breeding period is prolonged (Gales et al. 1994, Gales & Costa 1997).The evolutionary determinants of this atypical life-history are unknown but the implications are profound. Recent population genetic studies have shown that the Australian sea lion has a level of female population structure unparalleled in marine vertebrates with little or no interchange of females among breeding colonies, even those separated by short (20 km) distances (Campbell et al. 2007).

The important management implication of extreme philopatry is that each colony could represent a closed population. But male mediated gene flow between colonies remains likely, and maybe critical to the identification of management units. Preliminary studies by Campbell (2003) suggested male dispersal of the order of 250-300 km, but were based on limited samples and few microsatellite loci. Moreover, the non-annual, asynchronous breeding system provides for the possibility that, in contrast to all other pinnipeds, males may not be restricted to breeding at single colony during any one breeding cycle.

High quality DNA was extracted from 50 male skin samples collected from five different islands and used to develop a microsatellite library. Microsatellite development was enhanced by the use of pyrosequencing technologies with a 454 sequencer. From these sequence data 200+ potential microsatellite markers were isolated and characterised. From this pool of candidate markers 20 loci were selected on the basis of microsatellite length and the characteristics of the nucleotide motif. Primers were developed and conditions to optimise Polymerase Chain Reaction identified. A library of 17 microsatellite markers has been developed (Table 1) and the library will be published shortly. A further eight loci are in the process of being optimised. This should provide us with a library of 20-25 markers.

Analysis of male mtDNA haplotypes shows unequivocally that they are shared over a significantly larger

range than are females, with males on Olive Island showing overlap with those breeding in Lilliput and Blefuscu in the Nuyts archipelago, despite being 6 months out of synchrony with that island group. This has implications for paternity across regions with the sharing of haplotypes opening the possibility of males being successful sires at multiple colonies across different temporal scales. At the same time a degree of differentiation from the Liguanea Island males suggests that regional differentiation is likely even if male dispersal exceeds female dispersal by some orders of magnitude.

2. The Outcomes/Objectives

The degree to which the Activity has achieved the objectives

1) Develop and optimize a microsatellite library for the Australian sea lion *Neophoca cinerea*

The microsatellite library developed for *Neophoca cinera* includes 14 species-specific markers and 6 cross-amplified from other species. A publication is being prepared and will be submitted shortly, ensuring that these markers will be available to the scientific community in general. The innovative use of the pyrosequencing technologies with a 454 sequencer (the 454 sequencer identified 93901 clones of which 2778 contains repeats) and this has provided a large number of potential microsatellite markers (200+) that have been isolated and characterised. Primers for PCR have been designed for each of these and further microsatellite markers can be optimised as required in a rapid and cost-effective manner.

A scientific publication describing the microsatellite library (Table 1) is being prepared and a copy will be submitted to the AMMC once it has been accepted.

2) Determine how male mating strategies effect the formation of management units and in particular effective population size for Australian sea lions in two regions subject to significant bycatch in gill net fisheries (Nuyts Archipelago, and Olive Is, SA and Jurien Bay, WA)

By sharing resources with another AMMC Project *AMMC 2009/38 Structure and subdivision of the Australian sea lion - defining species-wide management units using ecological and genetic information (Goldsworthy, Donnellan and Harcourt CIs)* samples have been collected from a wider region than that outlined in the original project at no cost to 0809/12. In South Australia samples of males, females and pups were collected from the Nuyts (Olive, Lilliput and Blefuscu) as part of 0809/12 and from Liguanea and West Waldegrave as part of 2009/ 38. DNA was extracted from adult male skin samples from these five SA colonies and polymerase chain reaction (PCR) used to selectively amplify 645bp of mtDNA. 17 Haplotypes were identified and a haplotype network (Figure 1) was constructed by statistical parsimony in TCS 1.21 to examine genealogical relationships (Clement *et al.* 2000). The implications of this analysis extend the findings of Campbell (2003) who suggested that mtDNA sequences should be used to assess male fidelity to natal breeding sites in order to assess male dispersal, inbreeding coefficients and male mediated gene flow. These results show that mtDNA haplotypes are shared over a significantly larger range than are females, with males on Olive Island showing a degree of overlap with those breeding in Lilliput and Blefuscu in the Nuyts archipelago, despite being 6 months out of synchrony with that island group. This has implications for paternity across regions with the sharing of haplotypes opening the possibility of males being successful sires at multiple colonies across different temporal scales. A fortuitous outcome of the additional sampling undertaken with AMMC 2009/38 is that samples outside the Nuyts but still within west coast, South Australia, were collected. The degree of differentiation from animals from the furthermost island, ie Liguanea Island males, suggests that regional differentiation is likely even if male dispersal exceeds female dispersal by some orders of magnitude.

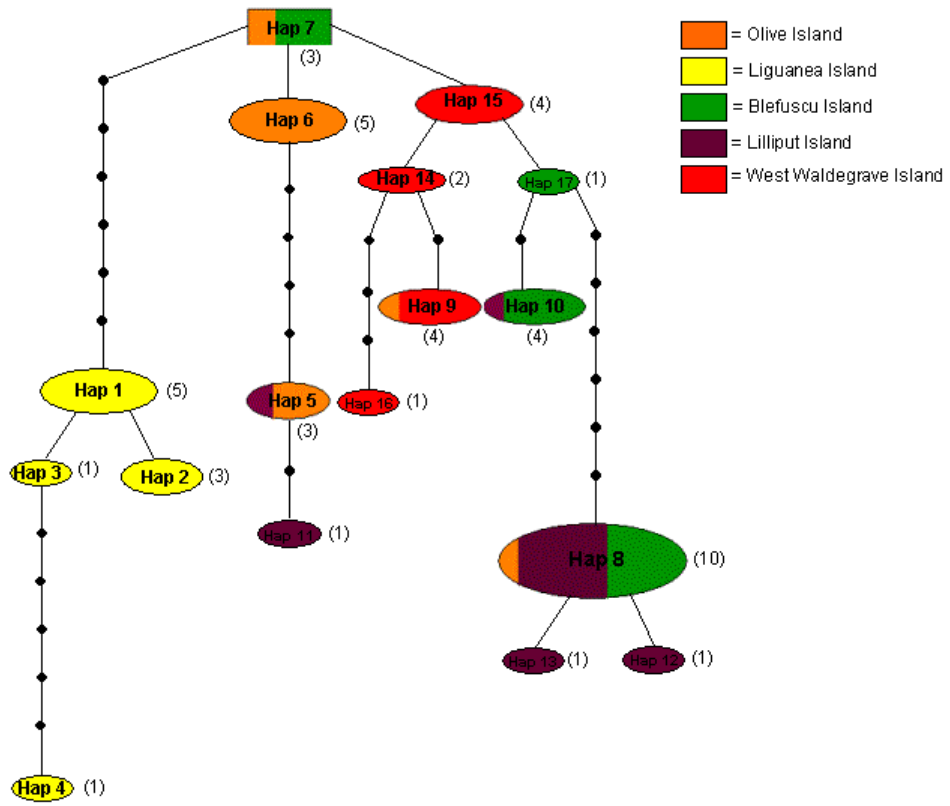

Figure 1 Haplotype network for mtDNA control region. Each line indicates one mutation between haplotypes, and small shaded circles between connecting lines represent missing haplotypes. The colours represent each sampling island and the numbers in brackets how many individuals in total have this haplotype.

In Jurien Bay, Western Australia samples were collected during a major expedition between Feb 19 and March 22 2010. Breeding for these 'synchronous' colonies was expected to peak over February / March. However there was an unusually delayed breeding season for both North Fisherman and Buller Island (as of time of writing Buller Island has still not commenced breeding). To compensate for the delays from these two sites, additional visits were made to sample. Fortuitously R. Harcourt was in Perth on other business in April and so an extra sampling visit was made to North Fisherman by R.Harcourt who then trained DEC WA MPA employees in sampling techniques while collecting samples and conducting a mark-recapture census on 17 April which was then repeated by MPA personnel on May 12, and from Beagle Island 26 April. 172 samples have now been collected, but breeding is yet to occur on Buller, a worrying development.

Due to the delayed breeding activities in WA, while DNA has been extracted, analysis of WA samples is of necessity also delayed. Therefore this objective is only partially achieved at this point. However, through cost sharing and immense effort by the two PhD students on this project the sampling effort has been significantly greater than originally planned and ultimately the results will be more robust. The South Australian work outlined above strongly suggest that male mediated flow will be highly influential in determining Management Units for this species.

3) Assess male determinants of gene flow within regions by comparing variance in male breeding success and paternity across clusters of colonies that are either synchronous or asynchronous in breeding period

Objective 3, comparing variance in male breeding success and paternity across the two archipelagos is also of necessity delayed until the analysis of WA colonies is complete.

3. Appropriateness

The appropriateness of the approaches used in the development and implementation of the Activity

The approach used, was highly effective and refined new techniques which have widespread applicability, namely biopsy sampling of adult male and female sea lions and photo-identification of male Australian sea lions. The scope of the study was expanded significantly through resource sharing with other projects, and this approach will be used in continuance of this project if funded (an AMMC proposal was submitted in 2010). The utilisation of the 454 sequencer for identification of microsatellite repeats has ensured that there will be significant time and cost savings for future genetic studies on this species. Considerable flow on to management agencies including training of State Government personnel in monitoring protocols and safe sea lion handling have been an effective byproduct of this work.

4. Effectiveness

The degree to which the Activity has effectively met its stated objectives

We consider that this project has been highly effective in meeting its stated objectives, with the exception of completing the final objectives due to time constraints in part imposed by delays in the breeding cycle of these unique animals. By utilisation of state of the art technology we have greatly exceeded objective 1 and have developed a suite of powerful tools for genetic analysis applicable within the scope of this project but also beyond it. We have provided evidence that male dispersal exceeds that of females and this supports our contention that male mediated gene flow is an important factor in population structure for this species. The time frame of this project was hampered by the delayed breeding season for the WA colonies but the techniques developed ensure that the latter aims will be achieved shortly, as they constitute the major part of a PhD program.

Table 1 Characterisation of 12 new microsatellite loci for the Australian sea lion (*Neophoca cinerea*) and 5 cross-species amplified microsatellite loci from the Galápagos sea lion (*Zalophus californianus wollebaeki*).

Locus	Primer Sequence (5'-3')	Repeat motif	T_a (°C)	Allele size range (bp)	N	N_a	H_O	H_E	F_{IS}	P-value
NCW1W	F: TACCAGCAATTGTAGGATGC	$(ATTT)_{13}$	50	223-235	30	4	0.633	0.551	-0.152	0.2835
	R: TCATAATCTCAGGGTTGTGG									
NCW1R	F: GAGCAACATGCTCAGAGG	$(AAAT)_{11}$	50	207-219	29	4	0.724	0.748	0.032	0.5556
	R: GGCAATGCAGAACTCTACC									
NCJKW	F: TAGCCCCAAAGTAGAAGAGG	$(ATTT)_{11}$	50	210-222	31	4	0.452	0.548	0.179	0.0639
	R: CTGGGGATCTAACCTATGG									
NCTUC	F: GACTGGGAGACAGAAGATAGG	$(AGAT)_{11}$	50	177-189	30	4	0.733	0.649	-0.133	0.8434
	R: ACCTTGTGATATGGTTGTAGC									
NCUYM	F: TTCCATCCATGTTGTTGC	$(ATCT)_{12}$	50	221-241	31	6	0.645	0.672	0.04	0.3967
	R: ATACCTCAGCACCTCTCTCC									
NCQDM	F: CCTTCAAGCTGAATTCTTCC	$(ATGT)_{11}$	50	259-267	28	3	0.393	0.509	0.232	0.3451
	R: GAGCACTGGGTGTTATACG									
NCLH2	F: GGGAGGTAATTAGGTCATGG	$(AGAT)_{11}$	50	207-215	27	3	0.370	0.526	0.3	0.1207
	R: CAGGTTGCTTCTGTGTGC									
NCAH9	F: CATTATCCCCACATCTAAGC	$(AAAT)_{11}$	50	162-174	30	4	0.567	0.730	0.227	0.2227
	R: CAACTGTTACTCCTGTGAAGG									
NC61H	F: CAATGGGAAGAATAACTTGG	$(ATCT)_{12}$	50	237-253	30	6	0.633	0.779	0.189	0.553
	R: GCTTTGATTTCCTCATCTCC									
NCUVP	F: GGTCCTTAGGCTAGTGTTCC	$(CTTT)_{13}$	50	212-236	30	7	0.767	0.773	0.008	0.524
	R: GGAACTTGAACAGACATTTCC									
NCMZ1	F: CATATTGGATTAGGGCTTACC	$(CTTT)_{15}$	50	154-166	27	4	0.704	0.610	-0.157	0.8863
	R: GCTTAAGATTCTCCCTCTCC									
ZcwF07[1]	F: TATTCCTAGAGGGGCAAGTCAAG	$(GT)_2TT(GT)_{20}$	50	184-190	30	4	0.767	0.714	-0.076	0.9282
	R: CATTGACTCTCTGAAATGGTGTC									
ZcwB09[2]	F: AGCCTCAGATAAGCCCCACATA	$(AC)_{20}(TC)(AC)_7(CA)_2(TA)_2CCTACAA(AC)_3$	50	138-154	29	8	0.690	0.646	-0.069	0.0624
	R: AGGCTTCTGACCTGTGCTTCTT									
ZcwE04[1]	F: GCTGCTGTTACCACCTTTGTT	(GT)20(ATCT)3	50	199-207	30	3	0.600	0.576	-0.043	0.1255
	R: TAAGAAGACCCAGGATAGAGACCAG									
ZcwG04[1]	F: TTGCTGGTGAGTTGGATGAC	$(AC)GC(AC)_{25}$	50	203-311	31	7	0.742	0.779	0.049	0.4819
	R: AGAAGAGGGTCCTGTTCACTTG									
NCNG5	F: GAATCGTTCCGTGATTACC	$(GGAT)_{11}$	50	257	24	1				
	R: GTTGTGTTGGAAAATGATGC									
ZcwC11[2]	F: CAGGTGCTGCAAGATTTCATTC	$(GT)_2(AT)_3(GT)_{22}$	50	198	31	1				
	R: TGTAGGGATCTGAAGGGGTACAT									
Total Mean							0.628	0.654	0.040	0.251

*T*a optimal annealing temperature, N number of individuals, N_a number of alleles, H_O observed heterozygosity, H_E expected heterozygosity, F_{IS}

inbreeding coefficient. None of the loci showed significant departure from Hardy–Weinberg equilibrium (P\0.05). Number of alleles, observed and expected heterozygosity and inbreeding coefficient were calculated using the program FSTAT version 2.9.3.2 (Goudet 1995). Conformation to Hardy-Weinberg was evaluated using Genepop version 4.0.10 (Raymond & Rousset 1995). Tests for linkage disequilibrium between pairs of loci (with Bonferroni corrections for multiple tests) were conducted using FSTAT version 2.9.3.2 (Goudet 1995).
Original citations of the Galápagos sea lion microsatellite loci: [1] Hoffman et al. 2007, [2] Wolf et al. 2006.

References

Goudet J (1995) FSTAT (version 1.2): a computer program to calculate F-statistics. Journal of Heredity, 86, 485–486.
Hoffman JI, Steinfartz S, Wolf JBW: Ten novel dinucleotide microsatellite loci cloned from the Galápagos sea lion (Zalophus californianus wollebaeki) are polymorphic in other pinniped species. Mol Ecol Notes 2007, 7:103-105.
Raymond M. & Rousset F, 1995. GENEPOP (version 1.2): population genetics software for exact tests and ecumenicism. J. Heredity, 86:248-249
Wolf JBW, Tautz D, Caccone A, Steinfartz S (2006) Development of new microsatellite loci and evaluation of loci from other pinniped species for the Galápagos sea lion (Zalophus californianus wollebaeki). Conservation Genetics, 7, 461–465.