Australian Centre for Applied Marine Mammal Science
2007 Funded project report

PROJECT TITLE: Gene-tagging the dugongs of southern Queensland to determine population dynamics, relatedness and movements

INVESTIGATOR: Janet Lanyon
School of Integrative Biology
University of Queensland
St Lucia QLD 4072

PROJECT SUMMARY
This project gene-tagged dugongs in Moreton, Hervey and Shoalwater Bays to obtain key information that will be used to determine structure, dynamics, connectedness and stock size of these 'urban' dugong populations in southern Queensland. Microsatellite DNA from skin was used to individually identify dugongs in each area and sex specific primers were developed and used to determine gender. We also trialled the utility of faecal biopsies for DNA extraction. These remote sampling techniques allowed us to rapidly identify and characterise a significant proportion of each population. Further population genetic analysis will be used to define regional management units for dugongs in southern Queensland, identify migration between these major populations and determine stock size(s) and composition.

PROJECT OBJECTIVES
1. Sample DNA from dugongs in Moreton, Hervey & Shoalwater Bays via skin biopsies.

2. Apply genetic techniques to dugong DNA to determine identity (using microsatellite markers) and sex (using sex-linked DNA markers).
(a) use existing microsatellite library to screen tissue samples &
(b) develop sex-specific primers for use on skin biopsies to determine sex / gender of individuals.

3. Elucidate stock structure among dugong populations in Moreton, Hervey & Shoalwater Bays.

4. Test the utility of faeces as a potential source of DNA in genetic mark recapture studies.

OBJECTIVE 1: Sample DNA from dugongs in Moreton, Hervey & Shoalwater Bays via remote skin biopsies

Field trips to sample dugongs in Moreton Bay were conducted throughout 2007. DNA and faecal samples from a total of 97 dugongs were collected.

A 6 day field trip to Hervey Bay and the Great Sandy Straits region was conducted from 8-13 July 2007 inclusive, with Sea World providing logistical support. During this period, 61 dugongs were sampled by skin biopsy and their DNA extracted. 260 faecal samples were collected.

A 5 day field trip to Shoalwater Bay was conducted from 26-30 August 2007 inclusive. During this period, 29 dugongs were sampled on the 2.5 good weather days. 142 faecal samples were collected. Note: this was the only window available for fieldwork because the Defence Force closed the Shoalwater Bay Military area for the remainder of the year for international military exercises (live-firing). Sea World was unable to provide support on these limited dates, so that access to the area was organised using a commercial charter boat.

All dugong skin samples were genotyped and even the smallest tissue samples gave good results (see below).

OBJECTIVE 2: Apply genetic techniques to dugong DNA to determine identity (using microsatellite markers) and sex (using sex-linked DNA markers).

(a) Use existing microsatellite library to screen tissue samples

Dugong tissue samples collected from Moreton, Hervey and Shoalwater Bays were screened against a panel of 26 dugong-specific polymorphic microsatellite loci that we have developed (Broderick *et al*. 2007). This panel is suitable for individual identification of dugongs so that we were able to identify new dugongs captured in 2007, and also match dugongs captured in 2007 to dugongs caught in previous years in Moreton and Hervey Bays (2007 was our first fieldtrip to Shoalwater Bay). By the end of 2007 we had genetags on >500 dugongs in Moreton Bay, 110 dugongs in Hervey Bay and 29 dugongs in Shoalwater Bay.

Broderick D, Ovenden J, Slade R and Lanyon JM (2007) Characterisation of 26 new microsatellite loci in the dugong (*Dugong dugon*). Molecular Ecology Notes. DOI 10.1111/j.1471-8286.

(b) Develop sex-specific primers for use on skin biopsies to determine sex / gender of individuals

Introduction

Dugongs (*Dugong dugon*) and West Indian manatees (*Trichechus manatus*) are large herbivorous marine mammals listed as vulnerable to extinction (IUCN 2006). Dugongs and West Indian manatees are found respectively in the tropical and sub-tropical west Indo-Pacific and from Florida (U.S.A) to the north coast of Brazil (Vianna *et al.* 2006). Recent population and ecological studies of these species require gender information to determine important parameters (Lanyon *et al.* 2002; Vianna *et al*. 2006).

Gender assignment for some aquatic mammals in the field is difficult but molecular sexing from tissue biopsies is possible as males are heterogametic. Gender assignment by detection of male markers requires controls to detect

experimental failure that may falsely assign female sex. Co-amplification of gametologues (highly homologous non-recombining regions on the sex chromosomes) overcomes this problem by targeting both sex chromosomes (Berube & Palsboll 1996). We have developed primers to detect the male specific SRY gene and differentiate the male specific ZFY gene from its gametologue ZFX. The multiplex PCR assay is robust to experimental failure and accurately assigns gender of these two sirenian species.

Methods

DNA was isolated from ~10 mg of skin by salting out (Miller *et al.* 1988). Sequencing template was prepared in 20 or 50 µL PCR reactions containing 10 - 20 ng of DNA, 0.5x Q-Solution, 0.2 µM primers and 1x QIAGEN Multiplex Master Mix containing 3mM $MgCl_2$. PCR cycling conditions were 94°C for 15 minutes and 35 cycles of 94°C for 30 s, 58°C for 45 s, 72°C for 60 s with a final extension at 72°C for 10 minutes. Amplicons were purified using QIAGEN's QIAquick PCR Purification Kit. Cycle sequencing was conducted in 10 µL reactions using ABI's Big Dye Terminator v3.1. Cycle sequencing conditions were 94°C for 60 s and 25 cycles of 94°C for 10 s, 58°C for 5 s, 60°C for 60 s with a final extension at 60°C for 5 minutes. All capillary electrophoresis was performed on an ABI 3130xl Genetic Analyser and sequence traces were analysed using Staden Package (Staden *et al.* 2000). Primers were designed using Primer 3 (Rozen & Skaletsky 2000).

Sequence was obtained for 1014 bp of ZFX (#AXXXXXX-X) in both species with primers ZFYX0097F and ZFYX1204R (Palsboll *et al.* 1992). Sequence from eight males and females of each species were compared to identify heteroplasmic sites and determine ZFY sequence (#AXXXXXX-Y). Primers were designed to anneal to each gametologue and 3' mismatching ensured gametologue specific amplification (Table 1).

Sequence was also obtained for 181 bp of *SRY* (#AXXXXXX-Y) in both species with primers ESRY3-F (GTCAAGCGACCCATGAA, derived from #AF180946) and ESRY-R (GTTCGGGTATTTCTCTCGGTGCA, (Gupta *et al.* 2006)). Due to poor amplification, 1 µL of the initial PCR was re-amplified and agarose gel purified with QIAGEN's QIAquick Gel Extraction Kit prior to cycle sequencing. A forward primer, DSRY-F (CAACTGCGAAACTCAGAGAT) was designed with a M13 sequence tag (see Table 1).

Our sexing assay targeted SRY, ZFX and ZFY in 6 uL multiplex PCR reactions comprising 0.01uM ZFX-F, 0.05uM ZFY-F, 0.01uM ZFXY, 0.02uM DSRY-F, 0.2uM ESRY-R, 0.11uM $M13^{TET}$, primers, 10 ng of genomic DNA, 0.5x Q-solution and 3ul of QIAGEN's multiplex master mix containing 3 mM $MgCl_2$. Cycling conditions were 94°C for 15 minutes and 35 cycles of 94°C for 30 s, 58°C for 30 s, 72°C for 30 s with a final extension at 72°C for 10 minutes. Amplicons were diluted 25-fold with ultrapure Milli-Q water and 6-fold with ABI Hi-Di Formamide before capillary electrophoresis. Alleles were sized against an internal size standard (GeneScan — 500 LIZ) and scored using GeneMapper® software (v3.7, ABI). One hundred animals from each species were assayed. Males were readily distinguishable from females by presence of three amplicons (155bp from SRY, 230bp from ZFX and 242bp from ZFY)

compared to a single product in females (230bp from ZFX). This assay has potential to assign gender in other sirenians and closely related species. Gender assignment primers have been incorporated into microsatellite panels (Broderick *et al.* 2007) that genetically identify individuals in a long term mark-recapture program (Lanyon *et al.* 2002, 2006).

This work has been published as:

- McHale M, Broderick D, Ovenden JR & Lanyon JM. (2007) A PCR assay for gender assignment in dugong (*Dugong dugon*) and West Indian manatee (*Trichechus manatus*) Molecular Ecology Notes. Doi 10.1111/j1471-8286.

A further publication is in progress and will be completed in April 2008, when Lanyon travels to the USA to meet with colleague, RK Bonde:

- Lanyon JM, McHale M, Ovenden JR & Bonde RK. (2007) Sexing sirenians: validation of field techniques to determine sex against molecular sex determination in wild dugongs (*Dugong dugon*) and Florida manatees (*Trichechus manatus latirostris*).

OBJECTIVE 3: Population genetic structure of dugongs in south-east Queensland

Samples for genetic analysis have been obtained from three populations in south-east Queensland (Moreton (MB), Hervey (HB) & Shoalwater (SWB) Bays) and assayed using microsatellite markers and control region sequence data. The microsatellite dataset used to genetically identify individual dugongs is also suited to determine the level of population genetic subdivision between Moreton and Hervey Bays. To generate the sequence data set it was necessary to design novel primers to amplify a fragment of control region mitochondrial DNA.

Methods & results

Microsatellite data

The full microsatellite panel consists of 26 loci and two gender determination markers, however, not all animals have been successfully genotyped at all loci. Here we used 23 loci dataset of fully genotyped animals (n=466) to assess population genetic structure (n=25, 368 & 73 from SWB, MB & HB respectively). We found slight (F_{ST}=0.05) but significant (P < 0.01) population genetic structuring overall among sampling locations. That is, most of the genetic variation was found within populations (95%) rather than among (5%, Figure 1). Genetic distance and geographic distance appear to be correlated with nearby locations (e.g. MB v HB, F_{ST}=0.04) being more genetically similar than those more distant (e.g. MB v SWB, F_{ST}=0.072; Table 1).

Table 1: Pairwise F_{ST} values (below diagonal) for Moreton, Hervey and Shoalwater Bays inferred from 23 loci. All comparisons were significant ($p<0.01$).

	SWB	MB	HB
SWB			
MB	0.072		
HB	0.051	0.040	

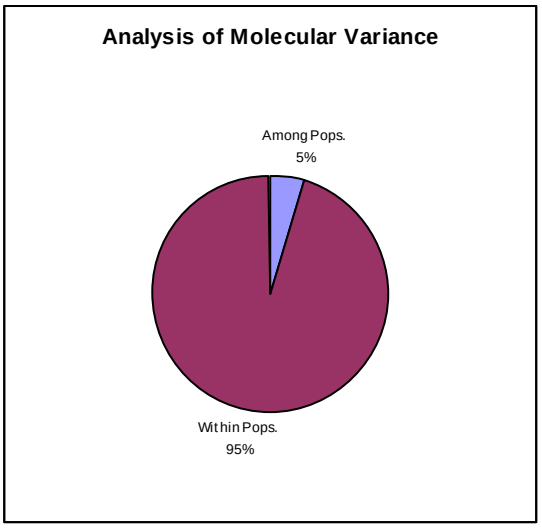

Figure 1: Analysis of Molecular variance among MB, HB and SWB dugongs inferred from 23 microsatellite loci.

Sequence data

Previous work on dugong mtDNA control region sequence data considered a fragment of approximately 400bp. We took the opportunity to design novel primers to amplify a larger fragment (~800bp) to increase sensitivity. We sequenced 184 dugongs describing 12 haplotypes; two haplotypes were shared between MB and HB (HapA & HapB), one haplotype was shared between HB and SWB (HapC), three haplotypes were shared between MB and SWB (Hap D-F) and 6 haplotypes occurred in SWB only (HapI-N) (Table 2). Haplotypes were clustered into two clades that differered by an average of 14 nucleotide changes (range 15-22 changes, 2.5% net sequence divergence), haplotypes within each clade differed by 1-6 nucleotide changes (Figure 2). Despite sharing some haplotypes at high frequency we detected significant population genetic structuring among Moreton, Hervey and Shoalwater bays (MB v HB $F_{ST}=0.16$, $p < 0.001$; MB v SWB $F_{ST}=0.33$, $p < 0.001$; HB v SWB $F_{ST}=0.38$ $p < 0.001$). Averaging across all 3 populations, 74% of the genetic variation was found within populations ($F_{ST}=0.26$ $p < 0.001$)

Table 2: MtDNA haplotype frequencies observed in Moreton, Hervey and Shoalwater Bays.

Haplotype	HB	MB	SWB
HapA	44	37	0
HapB	12	12	0
HapC	18	0	10
HapD	0	34	1
HapE	0	1	1
HapF	0	1	1
HapI	0	0	2
HapJ	0	0	1
HapK	0	0	4
HapL	0	0	1
HapM	0	0	2
HapN	0	0	4
Total	74	85	25

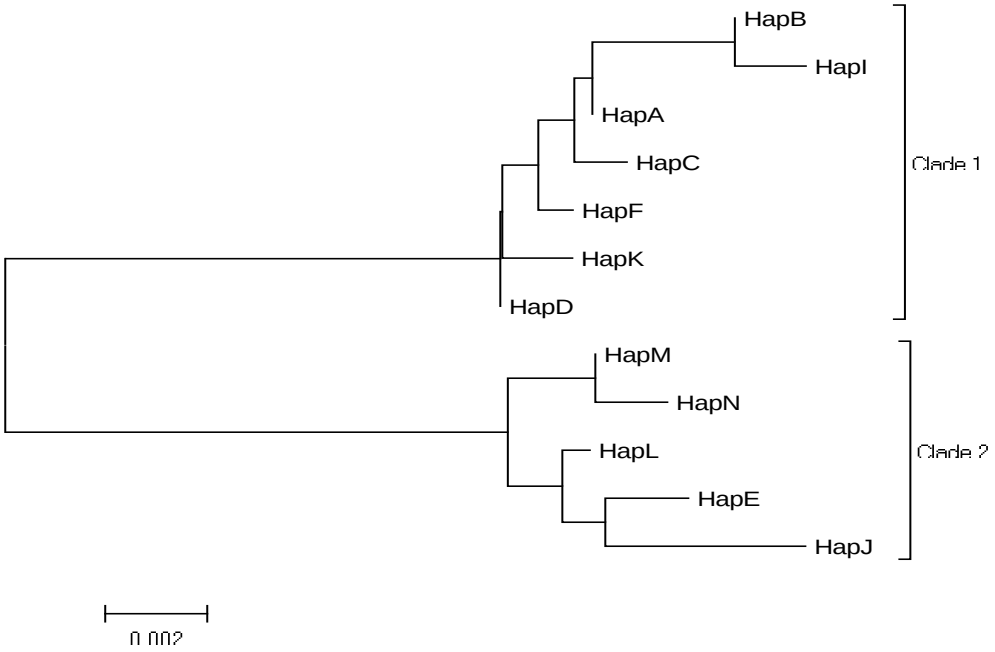

Figure 2: Unrooted neighbourhood joining tree describing the relationships among mtDNA sequences observed in SE QLD dugong populations.

Discussion

Preliminary analysis has detected significant population genetic structuring between MB, HB and SWB for both nuclear (microsatellite) and mitochondrial (sequence) markers. This is surprising given the anecdotal evidence of recent connectivity post flooding in Hervey Bay and from informal conversations with previous workers (e.g. Tikel, McDonald) indicating that they have not detected population genetic structuring. This work will be continued as part of an ARC-Linkage funded project 2008-2010.

Objective 4: Dugong faeces as a source of mitochondrial but not nuclear DNA

Introduction

Dugongs (*Dugong dugon*) are large, herbivorous marine mammals that inhabit often murky habitats in the tropical and sub-tropical west Indo-Pacific and are listed as vulnerable to extinction (Marsh 1982, IUCN, 2006). Capture or collection of tissue biopsies can be difficult and population genetics studies would benefit greatly from less invasive and labour intensive sampling strategies such as stool collection.

Dugongs typically feed in large herds and their feeding trails are easily recognisable. Additionally their faeces float and often collect in flotsam trails, bays and intertidal zones allowing the collection of many stool samples with minimal effort.

Faeces contains DNA from an organism's food, bacteria in its gut and a small amount of DNA from the organism itself derived from sloughed endothelial cells along the digestive tract. DNA is susceptible to degradation in the digestive tract, may co-extract with PCR inhibitors and further, the sloughed endothelial cells are significantly less abundant than food and microbial cells (Frantzen, 1998).

Amplification of 194bp of Dugong mitochondrial sequence from remotely collected stool samples was demonstrated by Tikel *et al*. (1996). In this study we endeavoured to amplify larger (549 and 812bp) fragments of mtDNA as well as short (150-500bp) microsatellite markers from nuclear DNA.

Methods

Samples were collected from the intertidal seagrass beds in Hervey and Shoalwater Bays and placed on ice for approximately three hours until they could be frozen. Whole stools were collected from the exposed seagrass meadow at low tide following local feeding at high tide, and from the surface of the water after an unknown period of exposure and placed into plastic zip-lock bags. A single "fresh" stool was collected immediately when the dugong defecated during pursuit and some samples were collected from Moreton Bay dugongs by inserting a flexible tube into the lower digestive tract. From other stools a small amount of faeces was split and stored in two tubes, one with 98% ethanol and another with buffer ASL.

DNA was extracted from ~200mg of faeces using the QIAamp DNA stool kit (QIAGEN) as per manufacturer's instructions. The 200mg was obtained from the surface of whole stools with a scalpel and from the unexposed faeces beneath this layer. Ethanol suspended samples were pelleted by centrifugation (1min, 20000g, 4°C) before tipping off the ethanol and drying for

5 minutes in a Speedivac. Buffer ASL suspended samples were mixed thoroughly by vortexing and 200uL of the suspension was used.

A single stool was extracted in 11 replicates from surface scraped faeces. Two replicates were extracted as above; one of these was spiked with 10uL of positive control DNA (isolated from skin tissue by salting out, ~17ng/uL) to validate the extraction. The other nine extractions were eluted into 40uL of water and pooled into a single tube to be purified by AmOAC/Ethanol precipitation and resuspended in 30uL of buffer AE.

Results

Nucleic acid content and size was assessed by spectrophotometry and gel electrophoresis for some samples. The average nucleic acid content of six surface scrapes and the fresh stool was 5ng/uL (range ~1-15ng/uL) and the pooled sample contained ~168ng/uL. Gel electrophoresis of six extracts from unexposed faeces and a sample collected with the flexible hose displayed a low molecular weight smear (<1000 bp) representing RNA and degraded DNA. Three of these also displayed a high molecular weight fraction (~20000bp); these were isolated from stools that appeared green in suspension while others were brown indicating that the source of this high molecular weight DNA could be from undigested plant material.

PCR reactions were conducted using QIAGEN's multiplex PCR kit as per manufacturer's instructions with positive and negative controls for all reactions. Dugong specific mitochondrial control region primers amplifying a 549bp product (CR5-M13F (Garcia-Rodriguez, 1998) CR-4Dug (optimised for dugong sequence from (Garcia-Rodriguez, 1998)) and an 812bp product (CR5-M13F to CRDug01-M13R (Developed from Genbank#AY075116)) were used in 0.2uM concentrations. Multiplexed microsatellite amplification and detection was conducted as per (Broderick *et al*.) though with a 1/15 dilution of PCR product before detection on the ABI 3130xl Genetic Analyser rather than 1/30 and with the addition of sexing primers (McHale *et al*.).

The 549bp mitochondrial amplicon was detected in all of six surface scrapes tested including the pooled sample, using 24uL reactions with 4uL of extract. The 812 bp mitochondrial amplicon failed to amplify in similar reactions with four of these extracts, including the pooled extract. In 6uL reactions with 1uL of extract the 549bp fragment was detected in 2/5 extracts from ethanol and 4/5 extracts from buffer ASL, only one stool was successful in both. The 812 bp fragment was detected in one ethanol and one buffer ASL stored sample, though not from the same stool.

In 6uL reactions with microsatellite primers (P1-2) and 1uL of extract from the pooled sample, six scrapes, six unexposed samples and the spiked extraction, amplicons were only detected in the spiked extraction control. In similar spiked reactions designed to test for PCR inhibition, as much as 0.975uL of extract failed to prevent amplification of 0.025uL of positive control DNA. Re-amplification in two, forty cycle PCR reactions (12uL reaction with 2uL of extract followed by a 6uL reaction with 1uL of PCR product as

template) produced non-specific amplicons. In 6, 12 and 18uL reactions with microsatellite primers (P1-1) and 1, 2 and 3uL of surface scrapes extract respectively, no products were detected. In 8 multiplexed reactions, each targeting 3-4 microsatellite loci with a total of 26 microsatellite markers and 3 sex linked markers ranging from 150-500bp, no products were detected in five ethanol and buffer ASL stored samples. In 6uL reactions targeting a single microsatellite marker (H200E11) with primers in 0.2uM concentrations no amplicons were detected, even following re-amplification as above.

Discussion

Successful amplification of a 549bp fragment of mitochondrial DNA was achieved in most cases and amplification of the larger 812bp fragment was successful though not from most samples. More frequent successes with the shorter fragment indicate degradation of the mitochondrial genome resulting in shorter fragments of available template. This success builds upon that established by Tikel *et al*. (1996).

Amplifiable genomic DNA was not obtained from dugong stools. This is most likely due to degradation of nuclear template as spiked tests failed to detect PCR inhibitors. Collection of fresh samples and alternate storage conditions failed to achieve success, suggesting very active degradation in the organism's gut and low abundance of sloughed cells. Presence of mitochondrial template in cases of complete nuclear degradation reflects the abundance of the mitochondrial genome and the relative sensitivity of the nuclear genome to degradation.