Outline of the technical aspects of dugong genetics work being done at James Cook University in Australia.

Concern about the plight of the dugong in many parts of its range has led to the development of a Memorandum of Understanding on the Conservation of Dugongs and their Habitat, which is administered by the UNEP/ Convention on Migratory Species (CMS) office in Abu Dhabi. The UNEP/CMS Dugong MOU Secretariat (the Secretariat) has recognised the value of a study on dugong genetics across the range states. Such a study will provide information on the recent evolutionary history of the species (where they lived in the past and where they live now), on the extent of gene flow mediated by movement of individuals between dugong populations and localities, and on the genetic diversity remaining in different dugong populations.

The Secretariat is supporting the development of a network of scientists in range states who can obtain samples suitable for this work (usually small pieces of skin) and who are willing to contribute information to a range-wide study. Researchers at James Cook University in Townsville, Australia, have already established a set of methods that are being used to obtain genetic information from Australian dugongs. Samples from other range states can be sent to Australia for analysis. However, it is important to build capacity in other countries and this is being encouraged by the Secretariat. This document is intended to provide technical guidance for geneticists in countries where dugongs occur and who wish to do the genetic analysis in their own country. If further discussion about methods is needed, contact the following: david.blair@jcu.edu.au, lynne.vanherwerden@jcu.edu.au,

Preservation of samples

We normally collect a piece of skin (the coloured part of the skin, not the underlying white dermis or fat) about 10 x 10 mm and 1-2 mm thick. The trailing edge of the tail is an easy place to sample for skin. This can be preserved in various ways. The preserving fluid we prefer to use is 20% DMSO (dimethylsulphoxide), 0.25 M EDTA Ethylenediaminetetraacetic acid) pH 7.5 in a saturated salt (NaCl) solution. This is classified as non-hazardous for shipping by air. However, it is necessary to pack and seal tubes to eliminate the possibility of leakage: DMSO is unpleasant and should not be allowed to come in contact with skin if possible.

It is also possible to preserve samples in ethanol (90% solution is probably best). Ethanol is flammable and not regarded as safe for shipment by air.

Important: Have a ratio of about 1 volume of tissue to at least 10 volumes of preserving fluid. Remember to label each sample with date and location, as well as any other relevant information. Write labels using a pencil, not a pen.

For further information about preservation and/or shipment of samples, contact us (email details above) and/or see Williams et al (2007). A copy of the Williams paper can be sent to you.

Genetic markers

Two classes of genetic markers are used: mitochondrial DNA sequences and microsatellites.

Mitochondrial DNA sequencing

We have been sequencing a portion of the control region of the mitochondrial genome using the primers;
DL_f: CATATTACAACGGTCTTGTAAACC
DL_r: GTCATAAGTCCATCGAGATGTC

To date, the lab in Townsville has well over 300 partial control region sequences (each about 500 bp). Most are from Australian dugongs, but a few are from other countries and demonstrate some broad-scale groupings of populations (Fig. 1 – at end).

Microsatellite Genotyping

The following is a summary of the method that we have developed for screening dugong DNA for 10 microsatellite loci in 2 multiplex reactions. We use the Qiagen Type-It Microsatellite PCR Kit for this work. It is relatively simple and mostly follows the instructions outlined in the Qiagen Type-It microsatellite PCR handbook. You should obtain a copy of this protocol booklet (available online http://www.qiagen.com/products/type-itmicrosatellitepcrkit.aspx#Tabs=t2).

The primers used were selected from the papers by Broderick *et al.* (2007) and Hunter *et al.* (2010) – see references at end. Initially, 11 loci were selected based on their reported variability, repeat type and their suitability for multiplex PCR in a capillary electrophoresis machine (we use a MegaBACE with 4 dye channels (ladder + 3), but different labs may have different machines and therefore use different dyes). The 11 loci were amplified in 2 multiplex PCRs, a 6-plex and a 5-plex. The loci, multiplex and the dye tags we used, along with other relevant information, can be seen in Tables 1 and 2 below.

Table 1: loci included in each multiplex PCR.

Multiplex 1	Multiplex 2
TmaA04 (TET)	DduC05 (FAM)
DduH09 (HEX)	DduE04 (FAM)
DduB02 (FAM)	DduB01 (HEX)
DduD08 (TET)	DduE09 (TET)
DduG11 (FAM)	DduG12 (FAM)
DduH04 (TET)	

Table 2: Primers, dye tags, product length range and motifs for each of the loci we used: modified from Broderick *et al*. (2007) and Hunter *et al.* (2010)

Locus name	GenBank Accession	Primers	Motif	Length range of PCR products
TmaA04	AF223652	F:GAACACAAGACCGCAATAAC-TET	$(GT)_{19}$	204-?
		R:TGGTGTATCACTCAGGGTTC		
DduH09	EF078668	F:GCTTCTCTTTTGGGGTAGGC	$(TG)_{19}$	214-220
		R:TGGACGGGTATCGTATGTCA		
DduB02	EF078600	F:AAACCCAAATCGGATCATGT	$(CA)_{26}$	222-252
		R:GCTGGGTTTTCCATTCTCAT		

DduD08	EF078625	F:TGCATTGTTCTCTTTTGAATGG R:TCGGTCTCATGCTACCTCAA	$(CA)_{20}$	291-313
DduG11	EF078658	F:GGAGGCAAAAAGGAAAAAGC R:GCCTTTTCCTCACTCTGTGG	$(TG)_{18}$	351-384
DduH04	EF078663	F:CTGAATGCCCCTCACATCTT R:TATGCCCTTAGATGCCTTGG	$(CA)_{22}$	355-365
DduC05	EF078613	F:CCATTGGCATTACATTCGTG R:TGTTGTTCCCTTCTGAAGCA	$(CA)_{27}$	242-256
DduE04	EF078631	F:TATCACAACACCCCATTCCA R:CTGTCCAGAGGGAAAGGTCA	$(CA)_{28}$	324-338
DduB01	EF078599	F:CACTGTGGTGAAAAGGGACA R:TTATTTGGCTTGGGACTTGG	$(TG)_{33}$	375-397
DduE09	EF078636	F:CCTGCCTGCTTCAGAGAATC R:CAGGAGCCAAACAGTGTCAA	$(TG)_{28}$	376-388
DduG12	EF078659	F:TGGCACTTCTGAAACTTTGC R:TCTTCTCCAGCTTTGCCATT	$(TG)_{27}$	378-406

After testing different reaction conditions we found that we could confidently score ten of the loci. The locus TmaA04 was found to be unreliable, so we will probably omit this marker from our analyses.

Primer master mixes were constructed largely using the method outlined in the Qiagen Type-It microsatellite PCR handbook (pages 13-14). Our primer stocks were 100µM. The protocol called for 10µl of each primer stock to be added and then the mixture made up to a total volume of 500µl. However after testing amounts were altered for individual primer pairs as reported in Table 3 below.

Table 3a: Primer Master Mix for Multiplex 1

Locus	Forward	Reverse
TmaA04 (TET)	10µl	10µl
DduH09 (HEX)	12µl	12µl
DduB02 (FAM)	8µl	8µl
DduD08 (TET)	8µl	8µl
DduG11 (FAM)	8µl	8µl
DduH04 (TET)	8µl	8µl
Buffer to 500µl (392µl)		

Table 3b: Primer Master Mix for Multiplex 2

Locus	Forward	Reverse
DduC05 (FAM)	8µl	8µl
DduE04 (FAM)	8µl	8µl
DduB01 (HEX)	12µl	12µl
DduE09 (TET)	8µl	8µl
DduG12 (FAM)	8µl	8µl
Buffer to 500µl (412µl)		

The reactions conditions for the PCR of both multiplex 1 and 2 were the same. Each reaction consisted of 4.5µl of H_2O, 12.5µl of PCR master mix (from the Type-it kit), 2.5µl of primer master mix, 2.5µl of Q-solution and 3.0µl of template DNA solution, which had been diluted to 3.0-4.5ng/µl (total template added per reaction 9 to 13 ng).

Thermocycler conditions were as per the manual, with the “60°C protocol” being utilised.

5min @ 95°C
30sec @ 95°C
90sec @ 60°C
30sec @ 72°C
30min @ 60°C
X 28cycles

As the locus TmaA04 gave results that were hard to score, it would be best if it were removed from multiplex 1 altogether. Depending on what you system you will utilise, you may prefer not to use HEX (which we found to be weaker and less robust in amplification).

References (we can send you copies if needed)

BRODERICK, D., OVENDEN, J., SLADE, R. and LANYON, J. M. (2007), Characterization of 26 new microsatellite loci in the dugong (*Dugong dugon*). Molecular Ecology Notes, 7: 1275–1277. doi: 10.1111/j.1471-8286.2007.01853.x

HUNTER, M. K., BRODERICK, D., OVENDEN, J. R., TUCKER, K. P., BONDE, R. K., MCGUIRE, P. M. and LANYON, J. M. (2010), Characterization of highly informative cross-species microsatellite panels for the Australian dugong (*Dugong dugon*) and Florida manatee (*Trichechus manatus latirostris*) including five novel primers. Molecular Ecology Resources, 10: 368–377. doi: 10.1111/j.1755-0998.2009.02761.x

WILLIAMS, S.T. (2007) Safe and legal shipment of tissue samples: does it affect DNA quality? Journal of Molluscan Studies. 73:416-418

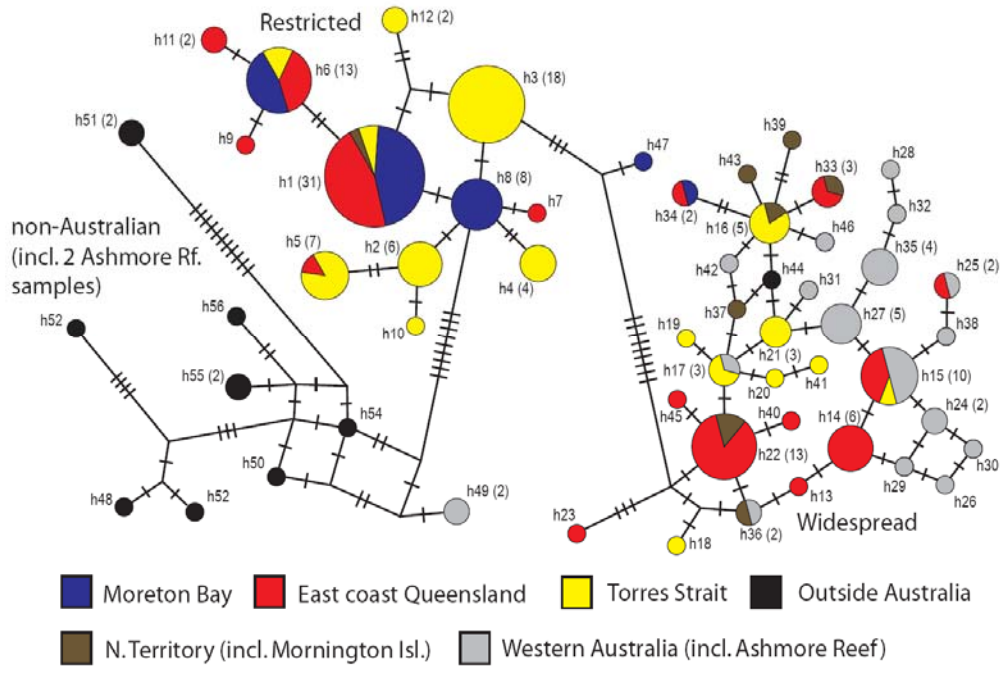

Fig 1. Median-joining network based on partial mitochondrial control-region sequences from 188 dugongs, mostly from Australia. Each circle represents a haplotype (unique sequence). The size of the circle is proportional to the number of individuals with that haplotypes. Coloured slices indicate geographical origins of each sample. Lines connect related haplotypes the numbers of mutations inferred as occurring along each branch are indicated by slash marks.