Australian Marine Mammal Centre

Final Report

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

- **Project No**. –
- **Title** - Genetic evidence for distinctiveness and connectivity among Australian Dugongs
- **Chief Investigator** – David Blair
- **Organisation** – James Cook University (School Marine and Tropical Biology)

Activity Period – entire span of project

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1. Activity Summary

A clear summary of approximately 500 words outlining the work undertaken and any significant findings (for publication on the Department's web site)
The dugong is a species of conservation concern. Populations along the E coast of Queensland, in particular, have suffered great reductions in recent years. However, much is not known about the demographics and population biology of dugongs – information vital for conservation and management planning in Australian waters. Genetic methods can be used to provide information not available by other means. We obtained DNA sequences from the maternally inherited mitochondrial d-loop region from almost 300 Australian dugongs, and from a number from outside Australia. We also genotyped 221 Australian dugongs using microsatellite markers that are biparentally inherited. The mitochondrial sequence data indicate that Australian dugongs are distinct from those in other parts of the world and have separated into two main mitochondrial lineages in Australia. One of these is found almost exclusively along the E coast of Queensland, the other throughout the entire Australian range of the species. Long periods of isolation during Pleistocene glacial cycles are likely drivers of this separation. Australian dugongs are genetically diverse, likely more so than populations in some other countries. The most genetically diverse populations are in the Torres Strait and North Queensland regions. Rather unexpectedly, strong population structure was detected using both mitochondrial sequence data and microsatellites. Despite the known ability of individual dugongs to travel long distances, genetic structure was apparent at scales of several hundred km. A particularly marked genetic break occurs between about Shoalwater Bay in Central Qld, and Townsville, some 600km to the north. Movement of animals across this region has been less than across comparable distances elsewhere. The region from Shoalwater Bay to Moreton Bay may need to be managed separately from that to its north. We were unable to obtain enough samples from Western Australia to

demonstrate the presence of any breaks there. Despite our expectations to the contrary, we could not demonstrate male-biased dispersal. In fact, there are hints of female-biased dispersal in the data. Analysis of this large data set is ongoing and interpretations will continue to be refined.

2. The Outcomes/Objectives

The degree to which the Activity has achieved the objectives

Objectives (See detailed text in section 6, at end of report)

The overriding objective was to identify regions in the Australian range of the dugong with high levels of genetic identity or distinctiveness, and understand the nature of that distinctiveness. We propose that these regions may then form the basis of improving management strategies to preserve the genetic diversity of this species.

Four points were listed as objectives.

l). measuring genetic diversity from dugongs across the range in Australia and also overseas, in order to establish how distinct at a global scale the Australian populations are.

This has been achieved. Microsatellite and mitochondrial d-loop data are now available for 221 and 336 dugongs respectively. Diversity is discussed in detail in Section 6 of this report.

2). further evaluating the differing evolutionary histories of the identified Australian dugong lineages and estimating trends in effective population size in each lineage.

The large number of d-loop sequences now available has made this possible. Full analysis will take some time yet. Interpretation to date is in Section 6.

3). inferring recent connectivity within and among regions in Australia to identify locations of any possible barriers, and seek explanations for such barriers.

Insufficient samples were obtained from Western Australia to facilitate recognition of any barriers to connectivity there. However, an excellent data set for Queensland provides evidence of strong population structure along the E coast of Queensland (including Torres Strait). In particular, there is a marked genetic discontinuity between populations in SE/ Central Queensland (Moreton Bay to Shoalwater Bay) and those further north. This break is a major finding, implying that management of the southern populations needs to be considered separately from that of the northern ones. See Section 6.

4). using knowledge of the gender of individual dugongs to assess if there is gender-biased movement that might be influencing our data interpretations.

Despite our initial suspicion that there may be gender-biased dispersal in the dugong, we could find no evidence for male-biased dispersal, and marginal evidence for female-biased dispersal. Further analysis of the data is taking place.

3. Appropriateness

The appropriateness of the approaches used in the development and implementation of the Activity
We know of no better approaches that could be used. The genetic markers were ones that we had used in earlier studies (mitochondrial d-loop sequences), or had been published in recent years (microsatellite loci). We contacted as many stakeholders as possible to establish a network of people able to provide samples. Of greatest importance among these were officers of the Qld Dept of Environment and Resource Management, who were able to supply many samples through the stranding network in Queensland. See Section 6 of this report for more details.

4. Effectiveness

The degree to which the Activity has effectively met its stated objectives
We consider that the activity has met most of its stated objectives. We failed to obtain sufficient samples from Western Australia to test our initial suggestion that breaks in dugong distribution/ barriers to dugong movement, might occur there. This was the principal deficiency.

6. Main Text, References and Appendix

Objectives/Outcomes

The overriding objective was to identify regions in the Australian range of the dugong with high levels of genetic identity or distinctiveness, and understand the nature of that distinctiveness. We propose that these regions may then form the basis of improving management strategies to preserve the remaining genetic diversity of this species.

To answer the questions posed, we used DNA sequence data from a portion of the mitochondrial genome (obtained from 299 dugongs of Australian origin, and a further 36+ from overseas sources) and genotypic data from 10 nuclear microsatellite loci (obtained from 221 dugongs of Australian origin).

Mitochondrial sequence data can be used to infer some population-genetic parameters, and also to provide phylogeographic information that helps illuminate historical processes shaping the genetic architecture of modern populations. Mitochondria are inherited maternally. Consequently they can only provide information on female-mediated gene flow. Microsatellite loci used are in the nuclear genome and therefore biparentally inherited. Such loci provide information on contemporary/ recent gene flow, nicely complementing mitochondrial information. Discordance between results from nuclear and mitochondrial studies are often attributed to sex-biased dispersal and gene flow.

Comparisons of published data from two complete mitochondrial genomes, representing the two main Australian lineages, showed that the 410 base-pair portion of the mitochondrial genome used, the "d-loop", contains virtually all the useable variation present in the control region. Another highly variable region was identified in the *nad5* gene. This was sequenced for about 80 individuals, demonstrating that there are strong fixed differences between the lineages in this gene region, but very little intra-lineage variation. No further work was done on this region.

The microsatellites used were the ten most informative loci reported in Hunter at al (2010). These were amplified in multiplex reaction formats.

Gender was not initially known for all of the animals sampled. A molecular sexing approach has been published (McHale et al 2008). In our hands, this was not always reliable, a view echoed by Jenny Seddon (UQ Veterinary School: pers comm). However,

we were able to establish the gender of 56 animals using the molecular approach, bringing our total for known gender to 247.

A breakdown of data obtained, arranged by type of genetic marker, geographical origin and mitochondrial lineage, is in Table 1. Genetic analysis requires that animals are placed into populations, usually on a geographical basis. Given the extensive range of dugongs around the Australian coast, and given the largely opportunistic method of sampling. This requirement creates some difficulties. Samples from Hervey Bay (including the Bundaberg area and in some cases Gladstone) were placed together within "Hervey Bay". Samples from the region between Bowen and Cardwell were grouped as the "Townsville" population, and those from between Mourilyan and Starke/Hopevale were placed in the "Cairns" population. In some cases, "Townsville" and "Cairns" samples were lumped into a single "NQ" population. Samples from the Western Australian coastline were all lumped into a single population for the analyses reported here. Numbers remained insufficient for more detailed analysis of geographical variation within WA.

A database of all samples has been completed, with new records being entered as samples became available. The database lists 607 animals, although usable tissue samples were not available for all of these. This database does not include the samples from bone material held in the Museum of Tropical Queensland (see below). Remaining DNA samples have been archived in the Molecular Ecology and Evolution Laboratory at James Cook University.

All new samples (usually as small pieces of skin in alcohol or DMSO) were made available from stranding networks (e.g. that operated by Qld Dept of Environment and Resource Management) and Indigenous hunters. During the funded period, around 120 new samples were obtained. Sample quality did vary considerably: some were unusable or yielded insufficient quantities of high-quality DNA for all analyses.

One important highlight of this work was the acquisition of many samples from the region between Hervey Bay and Cairns (Fig. 1 and Table 1). Numbers of samples available previously from, in particular, the Townsville region were insufficient to allow confidence in results. Another highlight is our success at genotyping from 221 animals at 10 microsatellite loci. Thanks to the dedication of Brendan Jones in the laboratory, genotyping was even possible from many old and poorly preserved samples. We were also able to (almost) double the number of d-loop sequences available for analysis.

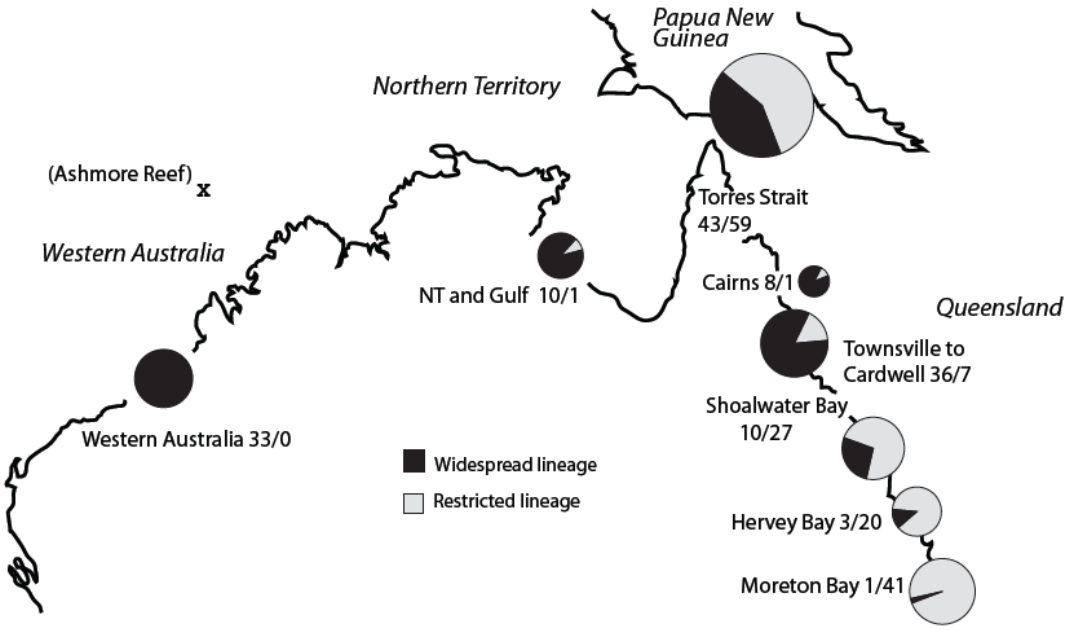


Figure 1: Geographical distribution of samples from which mitochondrial d-loop sequences were obtained. The breakdown into different mitochondrial lineages is shown.

Table 1: Summary of data obtained (d-loop sequences, microsatellites, and gender), broken down by type of data, mitochondrial lineage and geographical region.

	d-loop only (gender known)	d-loop+msats (gender known)	Msats only (gender known)	d-loop (subtotal)	Msats (subtotal)	Widespread Lineage (# of haplotypes)	Restricted Lineage (# of haplotypes)
MB	14 (6)	28 (24)	9 (0)	42	37	1	41 (4)
HB	2 (0)	13 (13)	2 (1)	15	15	2 (2)	13 (2)
GL	0	8 (8)	1 (0)	8	9	1	7 (2)
SWB	22 (1)	15 (15)	6 (3)	37	20	10 (5)	27 (5)
TSV	16 (11)	27 (27)	2 (2)	43	28	36 (7)	7 (3)
CNS	2 (0)	7 (5)	1 (0)	9	8	8 (2)	1
TSI	30 (27)	72 (72)	5 (1)	102	77	43 (16)	59 (12)
MNI	1 (0)	2 (2)	0	3	2	3 (3)	0
NT	2 (1)	7 (7)	0	8	7	7 (4)	1
WA	22 (4)	11 (11)	5	33	16	33 (15)	0
O	36 (7)	ongoing	ongoing	36	NA		
				336[1]	220	143	156

[1]Additional sequences from the literature and GenBank bring the total to 363 d-loop sequences. Of these, 299 are from Australian dugongs.

Key to localities: MN – Moreton Bay; HB – Hervey Bay and environs; GL – Gladstone (often placed within the "Hervey Bay" population); SWB – Shoalwater Bay; TSV – Townsville region; CNS – Cairns region; TSI – Torres Strait islands; MNI – Mornington Island (often grouped with "NT"); NT – Northern Territory; WA – Western Australia; O – outside Australia.

The analyses presented here are largely basic and standard ones that can be performed relatively quickly. **We must emphasise that raw data were still being accumulated until a week before the submission of this report**. More complex analyses remain to be done over the next few months during preparation of the work for publication. In particular, analyses that require days to weeks of computer time (e.g. Bayesian analyses exploring divergence time and population size change) cannot be reported at this stage.

Results and interpretation.

This section is framed around the four points listed in Section 2.

1). Assessment of genetic diversity from dugongs across the range in Australia and also overseas, in order to establish how distinct at a global scale the Australian populations are.

Microsatellite data: Most loci in most populations do not deviate significantly from Hardy-Weinberg expectations. Pairs of loci are not consistently linked. Two, perhaps

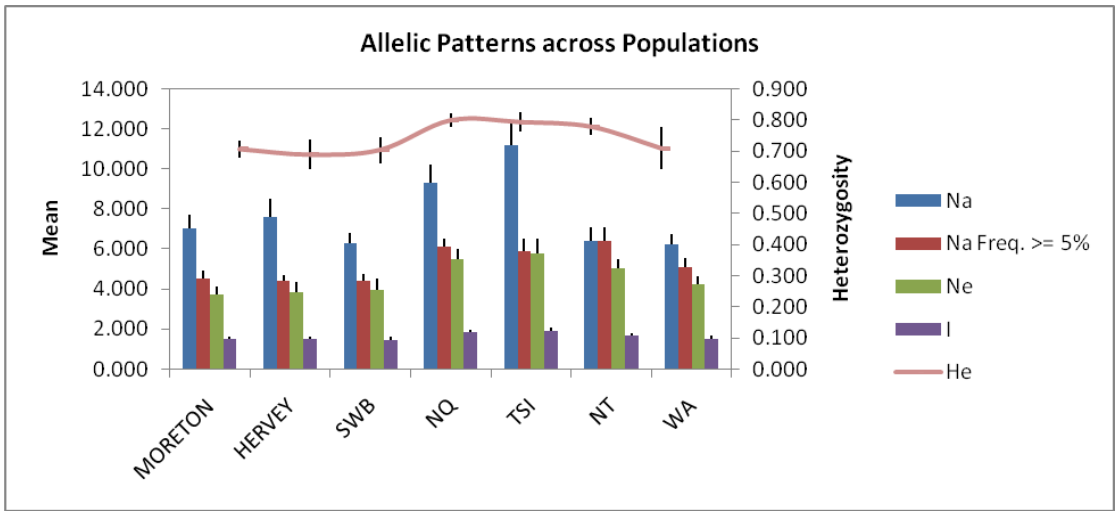
three, loci require further attention because of the possible presence of null alleles. Additional summary information is provided in Table 2.

Table 2: summary of diversity data for microsatellites from Australian dugongs (Analyses in GenAlEx and Arlequin).

Population	MORETON	HERVEY	SWB	NQ	TSI	NT	WA
Sample size	37	24	21	37	78	8	16
Na	7.000	7.600	6.300	9.300	11.200	6.400	6.200
Na Freq. >= 5%	4.500	4.400	4.400	6.100	5.900	6.400	5.100
Ne	3.710	3.846	3.965	5.481	5.756	5.005	4.213
I	1.495	1.510	1.473	1.844	1.893	1.665	1.511
Ho	0.703	0.670	0.710	0.719	0.766	0.788	0.680
He	0.716	0.703	0.721	0.810	0.798	0.830	0.731

Na = No. of Different alleles:
Na Freq. >= 5% no. of different alleles with frequency greater than 5%
Ho observed heterozygosity
He expected heterozygosity

Figure 2: Allelic patterns across populations based on microsatellites from 221 Australian dugongs (Analyses in GenAlEx). Abbreviations as in Table 1.



D-loop data:

Analysis of mitochondrial (mt) sequence data is complicated by the historical constraints imposed by the non-recombining nature of the mt genome and its maternal inheritance. We are confident that the two mt lineages identified in Australian waters have different demographic histories and geographic origins (Blair et al, in review). Therefore the lineages are analysed separately here. The "restricted" lineage occurs along the eastern seaboard of Queensland, from Moreton Bay to Torres Strait. A single representative of this lineage has been found west of Torres Strait – from Blue Mud Bay in the NT (Fig. 1; Table 1). The "widespread" lineage occurs throughout the entire Australian range of the dugong, but is very rare in SE Queensland (Fig. 1; Table 1). Explanations are discussed further in the next section.

The restricted lineage includes 17 haplotypes (different sequences) and the restricted lineage 41 haplotypes (if a haplotype from New Caledonia is included) (Table 3).

Diversity, expressed as haplotypic and nucleotide diversity, is far greater in the widespread lineage, despite this lineage being represented by fewer individuals. Average numbers of nucleotide differences between sequences are similarly much higher in the widespread lineage (Table 3). Strikingly few haplotypes occur in SE Queensland. Among the 41 individuals from Moreton Bay belonging to the restricted lineage, only 4 different haplotypes occur (Table 1).

Table 3: summary statistics relating to mitochondrial sequences in the "restricted" and "widespread" lineages (prepared using GenAlEx).

	Widespread	Restricted
No of individuals	143[1]	156
No of haplotypes	41	17
Nucleotide diversity	0.0079	0.0056
Haplotype diversity	0.940	0.818
Av no. nucleotide differences	3.267	2.272
Tajima's D[2]	-1.205 (ns)	-0.665 (ns)
Fu's Fs[2]	-30.174**	-4.231 (ns)
R2 statistic[2]	0.0528 (ns)	0.0665 (ns)

[1] 149 if sequences from New Caledonia are included

[2] A significant value implies population growth

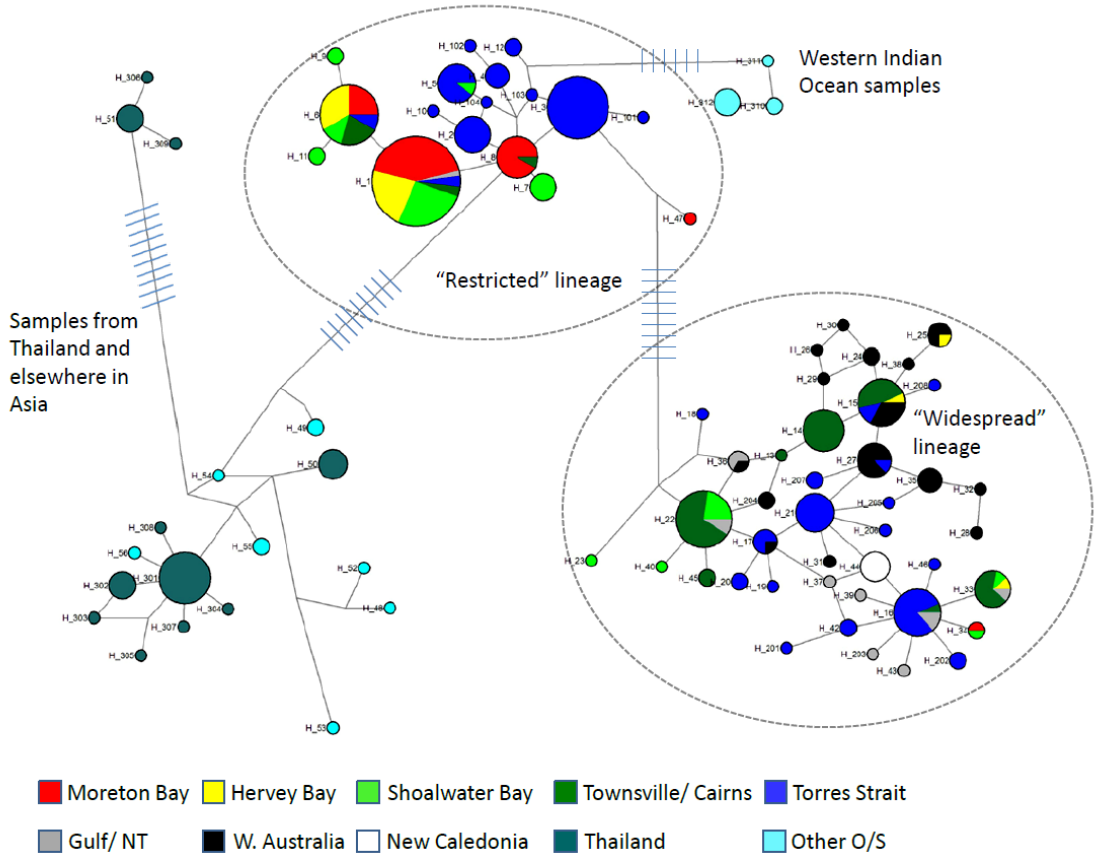


Figure 3 (previous page): Haplotype network (constructed using NETWORK) showing relationships between the mt haplotypes found, frequencies of each and geographical origins. Lengths of branches connecting haplotypes are proportional to the numbers of mutations inferred as occurring along that branch. Most internal branches in the "restricted" and "widespread" clusters have a length of 1. Slashes across some of the longer branches indicate the numbers of changes along those branches. Number of individuals having a particular haplotype is indicated by the area of each circle. For example, haplotype 1(restricted lineage) was shared by 54 animals, haplotype 301 (Thailand) by 18 and haplotype (widespread lineage) 22 by 22 dugongs.

Comparisons with populations outside Australia:

We have limited data to make such comparisons as yet. We have microsatellite data from several individuals from Abu Dhabi, but have not yet analysed these. Leslee Parr in the US is genotyping a set of samples from Thailand, and Marc Oremus is working on samples from New Caledonia where allelic diversity appears to be much lower than in Australia (Marc Oremus, pers. comm.). Diversity in mitochondrial d-loop sequences appears limited in at least some populations outside Australia. Few haplotypes exist in New Caledonia (Marc Oremus, pers. comm.) and few in the western Indian Ocean (Fig. 3). Such patterns suggest a small founding population of females in these localities. The situation in Thailand (Palmer, 2005 and Fig. 3) is more complicated and will be discussed in the next section.

Interpretation: Microsatellite diversity is greatest in the North Queensland and Torres Strait populations. Mitochondrial d-loop diversity is also greatest in these areas, although the results have not been corrected for differing sample size. As will be discussed in the next section, the SE Queensland populations are at the edge of the species' range and seem to have been founded from a relatively small number of individuals. These factors, if gene flow with populations to the north is limited, can explain the relatively low genetic diversity of the southern populations. It is also possible, although unlikely, that selection pressure at the edge of the species' range might restrict genetic diversity there. It is important to stress that there is no reason to suppose the lower genetic diversity in SE Queensland is a cause for concern. Indeed, historically these populations have been very large and apparently healthy. We have too few samples from Shark Bay, at the southern limit of the dugong in WA, to allow comment. The greatest genetic diversity is in Torres Strait, where populations are large and extensive and where gene flow from several directions (E Qld coast, Gulf of Papua, Gulf of Carpentaria), even at low levels, could contribute to genetic diversity.

Phylogenetically, Australian mitochondrial lineages are distinct from those in other countries (Fig. 3). No member of the restricted lineage has been found outside eastern or northern Australia. Outside Australia, the widespread lineage is represented only by dugongs in New Caledonia. Phylogenetic comparisons using nuclear data, such as microsatellites, remain to be done.

2). Evaluation of the differing evolutionary histories of the identified Australian dugong lineages and estimating trends in effective population size in each lineage,

Interpreting a much smaller set of mitochondrial sequence data, Blair et al (in review) proposed that glacial cycles had a profound influence on dugong populations. At the last glacial maximum (LGM) (~18ka), sea levels were about 130m lower than today. The Great Barrier Reef did not exist and habitat suitable for seagrasses and dugongs on the east coast of Australia would have been rare or non-existent. Torres Strait was exposed as

a landbridge from ~115 ka until ~7 ka. Elsewhere in the region, seagrass habitat was probably limited or non-existent in New Caledonia. The Sunda Shelf was exposed (Voris, 2000), separating animals in the Andaman Sea from those in the South China Sea by an immense number of sea-miles. Off the east coast of Australia, dugongs probably existed in relictual populations, perhaps on the offshore Queensland and Marion Plateaux. Rising sea levels after the LGM and establishment of shallow habitat for seagrasses, would have allowed dugongs to re-establish in their present localities in Qld from about 10ka. Along the Western Australian coastline, much more shallow-water habitat was available and dugongs would have been able to persist in many places. Blair et al (in review) proposed that during the long closure of Torres Strait, mitochondrial sequences diverged between the large western and the relictual eastern populations of dugongs. A population starting to expand from a narrow genetic base is expected to have a haplotype network pattern similar to that for the restricted lineage in Fig. 3. We consider this lineage to be descendants of one or more relictual populations isolated off the E Australian coast during the LGM. The widespread lineage we regard as descended from the dugong populations to the west of Torres Strait. These populations would have been much larger than relictual eastern populations, and would have had substantial and increasing areas of habitat available to them since the LGM.

Estimates of population size change and of effective population size can be made from mitochondrial sequence data. Neutrality indices and related statistics can provide an indication of population change. Examples of these are in Table 3. As was also the case for Blair et al (in review), the results are equivocal. Plots of pairwise differences between mt sequences are expected to have characteristic appearances for populations undergoing growth (or remaining stable etc). These plots can be compared with those expected based on models of e.g. population growth. Examples are in Fig 4 and suggest that pairwise mismatch plots for each lineage closely resemble those expected in an expanding population.

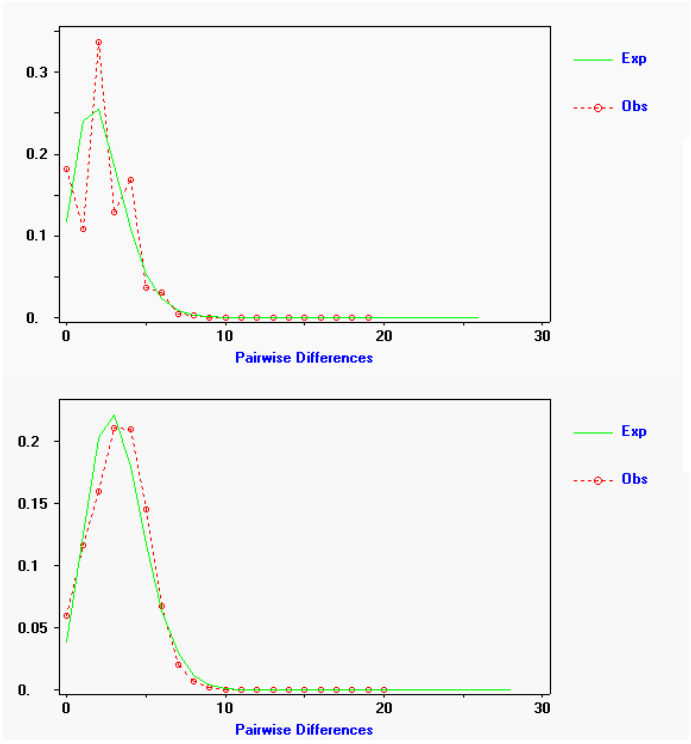


Figure 4. Plots of pairwise mismatches between sequences for the restricted lineage (upper plot) and the widespread lineage (lower plot). Frequency is on the Y-axis and pairwise differences on the X-axis. The green line shows the frequency distribution modelled under the assumption of population growth.

Blair et al (in review) also used Bayesian skyline plots to estimate effective population sizes (along with confidence intervals) at different time intervals. These suggested a ten-fold increase in effective population size in the widespread lineage since the LGM but were not very informative about the restricted lineage, except to indicate a far lower effective population size. Running these analyses takes a very long time. We now have almost twice as many sequences as were available for the Blair et al (in review) study and anticipate similar but more robust results.

Before moving on, a few more points from Fig 3 are worth noting. Although rather few in number, it is clear that sequences from Thailand fall into two widely separated clusters in the network. It is likely that this is also a historical signal, the two having diverged during the long period of low sea levels, when what is now the Gulf of Thailand was separated from the Andaman Sea by a huge distance. Today, these bodies of water are separated by the narrow Isthmus of Kra and connected by the coastline of the Malay Peninsula. At the LGM, there was little habitat suitable for dugongs in New Caledonia. It is likely that the modern population was derived from a small relictual or founding population (Oremus, pers. comm.). What is perhaps surprising is that this population should have mt sequences of the widespread lineage. Another surprise is that the small group of sequences available from the Western Indian Ocean (India, Persian Gulf and Mauritius), should have sequences more similar to those of the restricted lineage than to any other group. We won't speculate on these findings here.

The geographical distribution of sequences from the restricted lineage is not as expected under a simple scenario such as expansion from a single post-glacial population re-colonising SE Queensland. Whereas representatives of the restricted lineage constitute most of the samples from SE Qld, and over 50% of those from Torres Strait (Fig. 1), they form a small minority of samples from the Townsville and Cairns regions (8 out of 52 sequences). Furthermore, population-genetic analyses of restricted lineage sequences suggest significant differentiation between Torres Strait and southern members of the lineage. It might be that the restricted lineage occurred in dugongs in two major relictual populations that themselves diverged prior to recolonising Queensland waters. One such population could have been on the southern Marion Plateau, and the other on the Queensland Plateau further north.

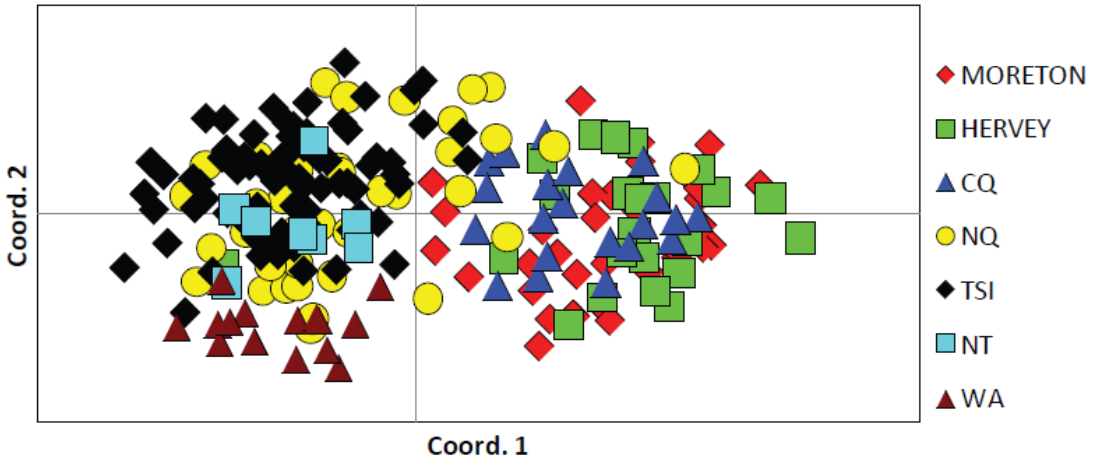
The flooding of Torres Strait ~7ka meant that animals bearing mitochondrial sequences of either Australian lineage could mix geographically. That such mixing has not proceeded to geographical homogenisation in the span of ~7000 years is puzzling: the widespread lineage is very poorly represented in Moreton Bay, and the restricted lineage is rare west of Torres Strait. One possible explanation is that female dugongs are strongly philopatric. Now that we have data from nuclear markers, this will be explored in more detail in the next two sections.

3). Assessment of levels of recent connectivity within and among regions in Australia to identify locations of any possible barriers, and seek explanations for such barriers,

All analyses point to the existence of considerable genetic structure in the dugong around the Australian coastline. Approaches we used include a principal coordinates analysis, measures of population differentiation such as Fst and Rst (tested for significance by permutation), analysis of molecular variance (not reported here) and assignment tests. Yet to be done are formal tests of isolation-by-distance and spatial autocorrelation.

A principal coordinates analysis (PCA) provides a visual representation of similarities (or dissimilarities) of data and uses a distance matrix as its starting point. While not providing statistical rigour, it is a useful way to start exploring complex data.

Figure 5. Principal Coordinates Analysis (in GenAlEx) based on microsatellite data. The first two axes explain 55% of variation. Each symbol represents a single animal.



The PCA in Fig. 5 suggests that dugongs in the three southern Queensland groups (Moreton, Hervey and Shoalwater Bays – the last called CQ in Fig. 5) share genetic similarities to the exclusion of those from Torres Strait, NT and Western Australia. Animals from NQ (Townsville and Cairns regions) are mostly allied with the Torres Strait and NT populations, but some overlap with the southern Queensland populations. The WA animals are rather distinct.

F-statistics (and the similar R-statistics, developed for microsatellites and assuming a particular mode of mutation) have long been used to provide a measure of population differentiation. Tables 4 and 5 present pairwise population Fst and Rst values respectively for the microsatellite data. Tables 6 and 7 provide pairwise population Fst values for the mitochondrial lineages, restricted and widespread respectively. Absolute values of Fst and Rst are of little interest. Focus instead should be on whether any given population is significantly differentiated from any other (based on a data permutation procedure).

Table 4: Pairwise population Fst values for microsatellites (from Arlequin). An asterisk indicates significant differentiation from other populations (1023 permutations).

	Moreton	Hervey	SWB	NQ	TSI	NT
Hervey	0.02248*					
SWB	0.03293*	0.01458*				
NQ	0.09729*	0.10083*	0.07782*			
TSI	0.11932*	0.1286*	0.10815*	0.01809*		
NT	0.11789*	0.13219*	0.10532*	0.01742	0.01956*	
WA	0.17782*	0.19427*	0.16737*	0.10042*	0.10308*	0.05431*

Table 5: Pairwise population Rst values for microsatellites (from GenAlEx). An asterisk indicates significant differentiation from other populations (9999 permutations).

	Moreton	Hervey	SWB	NQ	TSI	NT
Hervey	0.014					
SWB	0.028*	0.011				
NQ	0.069*	0.082*	0.035*			
TSI	0.123*	0.147*	0.096*	0.050*		
NT	0.141*	0.187*	0.122*	0.087*	0.040[1]	
WA	0.173*	0.173*	0.086*	0.091*	0.076*	0.051[1]

[1] P-value marginally greater than 0.05.

Table 6: Population Pairwise Fst values (Arlequin) for restricted mitochondrial lineage, 10100 permutations.

	MB	HB_GL	SWB	TSV_CNS
HB_GL	0.12547*			
SWB	0.01351	0.05212		
TSV_CNS	0.24318*	0.01894	0.09673	
TSI	0.30979*	0.42743*	0.29579*	0.42461*

Table 7: Pairwise Fst values (Arlequin) for widespread mitochondrial lineage, 10100 permutations.

	SEQ	TSV	CNS	TSI	Gulf_NT
TSV	0.07183				
CNS	0.03237	0.06749			
TSI	0.1079*	0.19584*	0.30054*		
Gulf_NT	0.00486	0.18357*	0.27178*	0.06333	
WA	0.26518*	0.14427*	0.30869*	0.27723*	0.3537*

Tables 4 and 5 show that almost every population is significantly differentiated from every other on the basis of the microsatellite data. The lack of significant differentiation between NT and NQ in Table 4 is unexpected. This might be a consequence of the small number of NT samples. It also surprising that SWB, Moreton Bay and Hervey Bay are significantly distinct. This pattern of distinction in SE Queensland is less apparent in Table 5, where all other population pair comparisons are significant, or closely approach the threshold for significance. Tables 6 and 7 show comparable figures based on mt sequences, the lineages treated separately. Small numbers of representatives in the restricted lineage from the Townsville/Cairns region and of the widespread lineage in SE Queensland (Table 1) do not help the analysis. Nevertheless, most pairs of populations are significantly differentiated, much as in Tables 4 and 5.

Assignment tests are useful in demonstrating the coherence/ distinctiveness of a particular population with respect to others. Such tests examine each genotype in turn, "forget" where it came from and place it within a population deemed most likely on genetic

grounds to be its origin. The assignment test provided in GenAlEx (Table 8) strongly supported the general groups suggested by the PCA. Of 221 animals, only six were assigned outside the groupings suggested by the PCA. Thus, of the 82 animals genotyped from Moreton Bay to Shoalwater Bay, only one was assigned to a population outside this region. Of the 123 animals genotyped between Townsville and the NT, only five were assigned to a population outside this region. All WA samples were assigned to WA.

Table 8: Summary results of assignment test in GenAlEx.

Pop	Assigned to popn. of origin	Assigned to another popn.	Assigned to any popn. within MB_SWB Group	Assigned to any popn. within NQ_TSI_NT Group
MORETON	27	10	37	0
HERVEY	14	10	23	1
SWB	14	7	21	0
NQ	27	10	2	35
TSI	55	23	2	76
NT	3	5	1	7
WA	16	0	0	0
Total	156	65		
Percent	71%	29%		

Of 37 animals genotyped from the Townsville and Cairns regions, only one was assigned to a more southerly population (Hervey Bay) and of 21genotyped from Shoalwater Bay and environs, none was assigned to a more northerly population but several were assigned to the Moreton or Hervey Bays to the south. The distance between Shoalwater Bay and Townsville is 600 km, and between Shoalwater Bay and Moreton Bay about 890 km.

STRUCTURE is a model-based clustering method for inferring genetic structure from markers such as microsatellites. Typical analyses involve specifying different numbers of clusters in separate runs and deciding on the most likely number based on statistics generated by the program. Two or three clusters are most likely (Figs 6 and 7).

Figure 6 (next page): Results of analysis in STRUCTURE, specifying that all populations should fall into two clusters. Each vertical line represents data from a single animal. The proportion of each line with a particular colour indicates the extent to which that individual may have membership of one or more of the clusters. Population groups we have used are indicated, but are not considered by the program in its calculations.

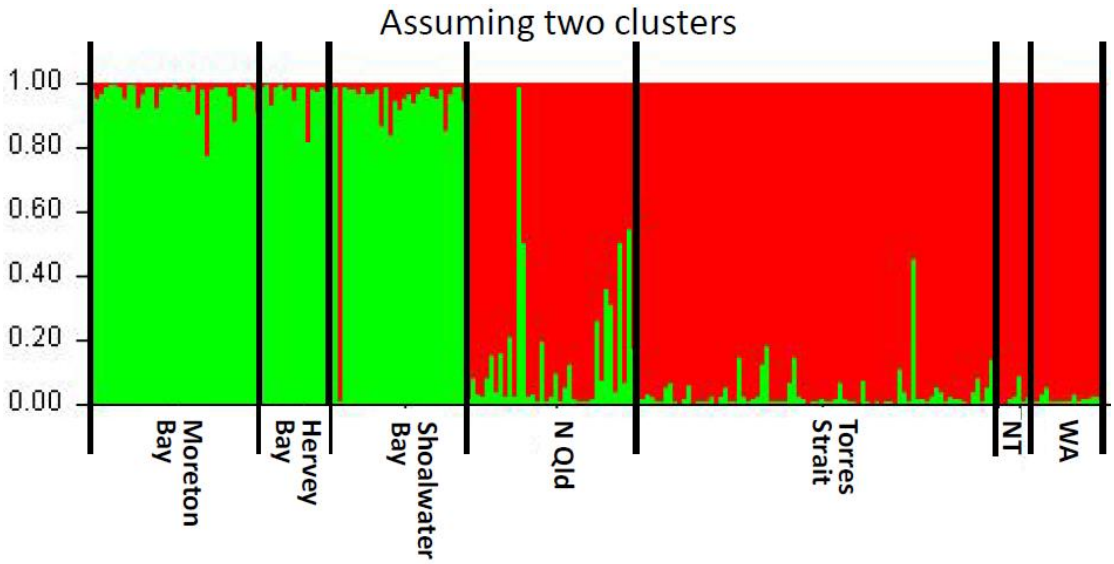
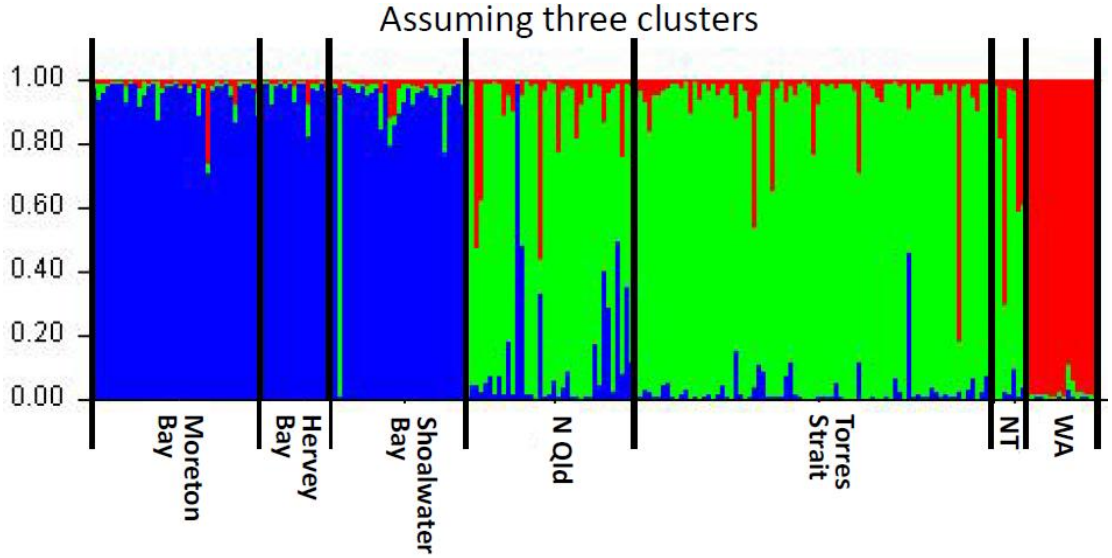


Figure 7: Results of analysis in STRUCTURE, specifying that all populations should fall into three clusters. See caption to Figure 6 for more details.



When two clusters are specified, the southern Queensland populations are placed in one and all remaining populations in the other. The pattern remains similar when three clusters are specified, the main difference being that WA samples are now separated out into a cluster of their own: the distinction between southern Queensland and more northerly populations remains.

We have not had time to do isolation-by-distance analyses. A common feature of animal and plant species is that genetic distinction between populations increases with distance between them. This has been demonstrated for dugongs in Australia using a smaller set of d-loop sequence data (Blair et al, in review). Given the results reported in this section, we are certain that microsatellite data will provide strong indication of isolation-by-distance. Indeed, the extent of population-genetic differentiation in the dugong is very surprising, given their known ability to travel long distances (Sheppard et al, 2006).

Interpretation and comments: Dugongs exhibit considerable genetic structure across their Australian range. The full extent of this cannot be assessed until very many more samples are available from the vast coastline of N and NW Australia, something we now regard as logistically impractical. However, we can say something about the east coast of Queensland, a region where management of the dugong is of considerable concern.

Statistics such as Fst and Rst suggest that most pairs of populations are genetically differentiated. PCA, assignment tests and the analyses in STRUCTURE all indicate that the major genetic break in Queensland is between Shoalwater Bay and the Townsville region. This break is more pronounced than the differentiation between populations within each cluster, which may prove to be typical isolation-by-distance.

The fact that a few individuals were assigned to a population distant from that in which they were found might represent examples of long-distance movement. Such candidates are few in number, and any long-distance movement (assuming vagrants mate when they reach their destination) has not been sufficient for genetic homogeneity. Of the six dugongs assigned outside their regional groupings, four were males and two females.

The second genetic break suggested by STRUCTURE when three clusters are specified separates WA samples from those elsewhere. However, given the immense length of coastline in WA, this distinction could be no more than isolation-by-distance.

4). using knowledge of the gender of individual dugongs to assess if there is gender-biased movement that might be influencing our data interpretations.

Our previous work (Blair et al in review), using only maternally inherited mitochondrial d-loop sequences, detected genetic structure across the Australian range of the dugong. This was regarded as surprising, given that dugongs are large animals capable of long-distance movement. One explanation is that females are philopatric and that such structure might not be apparent when nuclear markers – biparentally inherited – are analysed. We have presented such analyses here, with results similar to those for the mitochondrial marker: strong population structure exists in the dugong.

Nevertheless, sex-biased dispersal might occur and could be an important mediator of gene flow. In many mammal species, males are the dispersing sex. We have used the sex-biased dispersal test in GenAlEx for populations from Torres Strait, NQ and Shoalwater Bay. Results for the last two of these did not indicate sex-biased dispersal. For the Torres Strait population, however, results indicated significant, or near-significant **female-biased** dispersal. This is unexpected and requires further investigation using additional tests (such as those available in FSTAT). One very interesting approach for detecting sex-biased dispersal and suitable for use with the dugong data, involves spatial autocorrelation analyses (Banks and Peakall, 2012). We will be investigating this as soon as possible.

Summary: see Section 1 of this report.

Difficulties. It is unfortunate that we were not able to obtain more samples from Western Australia. Efforts to establish contacts, especially with people in the Kimberley region, were not successful. Only four samples were obtained from the Kimberley region. Other difficulties have been mentioned in progress reports: one of the PIs (Waycott) could not participate fully in the project because of illness and then departure to a new position elsewhere, limiting some aspects particularly in publicising our work to stakeholder groups through newsletters and specialised media.

Ongoing and Future Work. We have established a network of people in Australia aware of the value of genetic work in answering questions about the biology of dugongs. We are requesting that samples from dugongs continue to be accumulated for a future study to supplement this one.

Supported by a small grant from the Convention on Migratory Species office in Abu Dhabi, we have established a network of dugong researchers in the species' range states.

DB has attended two regional meetings (Malaysia and Kenya) and has made presentations on dugong genetics at these. He also distributed an information sheet (included below as an appendix) containing technical details of the work done at JCU in Townsville. Governments of range states are increasingly reluctant to allow export of samples, preferring to see analyses done in-country. The information sheet is intended to allow for compatibility of data across different laboratories. One difficulty in this regard is that microsatellite data needs to be calibrated across different machines, requiring an exchange of a few samples between labs. This has proved tricky.

Although not flagged in the original proposal, the value of "ancient DNA" samples became apparent to us as the work progressed. No data from such samples were included in the analyses presented here, but they should become available in the next few months. In collaboration with Mike Bunce from Murdoch University, bone samples have been taken from about 160 skulls held in the Museum of Tropical Queensland in Townsville. Most of these were from animals from the Townsville region, and from the Gulf of Carpentaria. Additional information from both regions is valuable. Initially, only d-loop sequences will be generated from these samples. It is hoped that some microsatellite loci can also be amplified.

Dr. Mike Bunce of Murdoch University drilling a sample from a dugong jaw held in the Museum of Tropical Queensland.

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Appendix: information sheet provided to workshop participants in Malaysia, Kenya, and distributed by email to additional researchers.

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 (it is probably safest to email all these people at the same time): **david.blair@jcu.edu.au**, **brendan.jones1@jcu.edu.au**, **lynne.vanherwerden@jcu.edu.au**,

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 over 200 partial (about 500 bp) control region sequences. Most are from Australian dugongs, but a few are from other countries and demonstrate some broad-scale groupings of populations (Fig. 1).

Microsatellite Screening

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	$(CA)_{20}$	291-313
		R:TCGGTCTCATGCTACCTCAA		
DduG11	EF078658	F:GGAGGCAAAAAGGAAAAAGC	$(TG)_{18}$	351-384
		R:GCCTTTTCCTCACTCTGTGG		
DduH04	EF078663	F:CTGAATGCCCCTCACATCTT	$(CA)_{22}$	355-365
		R:TATGCCCTTAGATGCCTTGG		

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 omitted this marker from our analysis.

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 mastermix, 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] **X** 28cycles
30min @ 60°C

As the locus TmaA04 was not scored and is not useable, it would be best if it were removed from multiplex 1 altogether. Depending on what you system you will utilise, you prefer not to use HEX (which we found to be weaker and less robust in amplification) and to remove TmaA04 from your reactions.

<u>References</u> (we can send you copies if needed)

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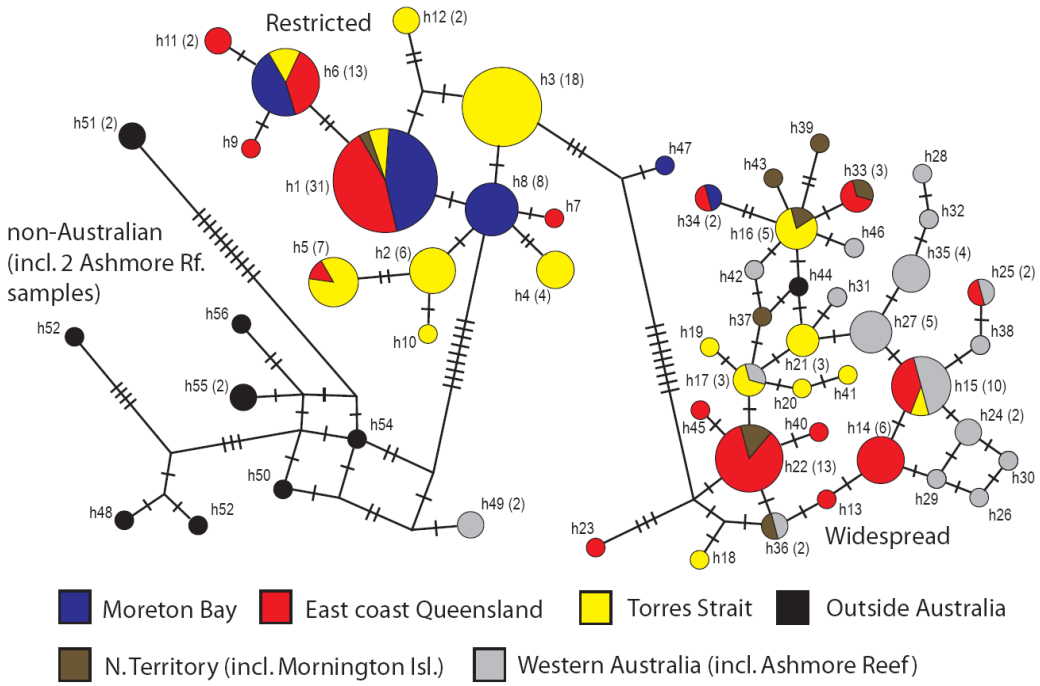

Fig 1. Median-joining network based on partial mitochondrial control-region sequences from 188 dugongs, mostly from Australia. Each circle represents a haplotype (unique sequence). The size of the circle is proportional to the number of individuals with that haplotypes. Coloured slices indicate geographical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