Population genetic structure of Sea Cucumbers (bêche-de-mer) in northern Australia

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Non-Technical Summary

2008/733 Population genetic structure of Sea Cucumbers (bêche-de-mer) in northern Australia.

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PROJECT OBJECTIVES:

1. To characterise the genetic population structure of *Holothuria scabra* within the range fished by Tasmanian Seafoods.

2. To characterise the genetic diversity of the hatchery broodstock and progeny arrays relative to the natural populations.

3. To refine and/or recommend polices and strategies for the sustainable management and enhancement, through ranching, of *H. scabra* fisheries.

OUTCOMES ACHIEVED

The project has achieved the following:

- A suite of genetic tools that can be used to understand and monitor population structure of *H. scabra* in the wild and manage genetic diversity of hatchery stocks.
- A detailed understanding (among both commercial and regulatory participants) of the genetic stock structure of *H. scabra* in fished areas in N/T and the genetic implications of planned commercial scale ranching of Sea Cucumbers in the region.

These achievements will lead to:

- A genetic management strategy for hatchery production and ranching of *H. scabra* in N/T
- A sustainable ranching and stock enhancement activity for Sea Cucumbers in NT with minimal impact on genetic diversity of wild populations.

LIST OF OUTPUTS PRODUCED

- *H. scabra* specific primer sequences for sequencing a segment of a mitochondrial DNA gene

- Primer sequences for 18 microsatellite marker loci for H. scabra.
- Microsatellite genotypes and mtDNA haplotypes of wild caught *H. scabra* from 16 sites across NT.
- A suite of microsatellite genetic markers for parentage assignment in *H. scabra*.
- An understanding of the genetic stock structure of *H. scabra* across fished areas in NT.
- Provisional data on the genetic implications of pooled spawning in Sea Cucumbers.

Sandfish (*Holothuria scabra*), a commercially important species of Sea Cucumber, are fished off the northern Australian coast. Elsewhere in the Asia-Pacific region, where they are commonly fished, they have been found to be particularly vulnerable to over-exploitation. Currently, as little is known about their biology and population structure, they are fished off the Northern Territory using conservative methods, by limited access, area restrictions based on an arbitrary line on the water and trigger limits based on historical data. With a view to improving their management and the sustainability of the fishery, a propagation and stock enhancement program is under development. Basic research including knowledge of the population genetic structure is required to enable informed decisions on the sustainable management of existing fisheries and to develop appropriate policies and strategies for the ranching program planned by Tasmanian Seafoods.

During the course of this project we developed a panel of twenty microsatellite markers (eighteen of which were useable) and primers to sequence a segment of a mitochondrial DNA gene. These were then used to investigate the genetic structure of the sandfish within the fished area and to assess parentage of hatchery stock.

We found that the sandfish could be grouped into two genetic clusters, or populations, roughly divided east to west and corresponding to the Gulf of Carpentaria (or eastern population) and the Arafura sea (or western population). These results suggest that dispersal of the sandfish larvae is limited to these two areas with little gene flow between them. Given this, it is recommended that the sandfish are managed as two separate, genetically distinct stocks with captive broodstock and progeny from the two areas kept separately and only released back to the areas containing individuals from the same genetic stock that their parents originated from.

We also found that a subset of our microsatellite markers could be used to assign parentage confidently amongst the hatchery progeny and, from a limited spawning trial, that a small number of the broodstock tended to dominate the successful spawning. This potentially low genetic diversity amongst the progeny from captive spawning will need to be taken into account through a genetic management strategy when establishing the breeding program to produce juveniles for ranching.

KEYWORDS: *Holothuria scabra*, sandfish, bêche-de-mer, stock structure, sustainable fishery.

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1. Introduction

The Sea Cucumber *Holothuria scabra*, commonly called the sandfish, is a commercially important species found throughout the Asia-Pacific region. As a food, Sea Cucumbers have a high nutritional value due to their high protein and low fat content, amino acid profile and rich trace elements and are, in addition to food, used in traditional medicine (Wen *et al.*, 2010). The main use for Sea Cucumbers is for the consumption of the body wall, mostly as dried product (known as bêche-de-mer or trepang) of which Chinese are the main consumers (Wen *et al.*, 2010). With the economic development of the People's Republic of China and improved living standards over the last 20 years the demand for bêche-de-mer has increased both in China and in Chinese communities around the world (Chen, 2004; Friedman *et al.*, 2011). *Holothuria scabra* is easily harvested from inshore habitats and is vulnerable to over-exploitation, as has been shown for commercially important species of Sea Cucumbers around the world (D'Silva, 2001; Battaglene and Bell, 2004; Gamboa *et al.*, 2004; Uthicke and Purcell, 2004; Hamilton and Lokani, 2011). Friedman *et al.* (2011) reviewed the status of Sea Cucumber stocks from a number of Pacific Island countries, some of which having had a moratorium on exports for up to ten years, and found marked declines in coverage and abundance. Even after extended periods of moratorium, they found the density of some species to be markedly low with resilience and stock recovery, following cessation of fishing, varying greatly among both locality and species.

Holothuria scabra is a benthic detritus feeder, preferring inshore, sandy areas to coral reefs and is often found in seagrass beds (Hamel *et al.*, 2001). Sexual reproduction occurs by broadcast spawning of eggs and sperm during the warmer months of December to February in the southern hemisphere (Uthicke and Benzie, 2001). This species has a relatively long planktonic larval duration of 10-14 days in the water column before settlement (Uthicke and Purcell, 2004), so there is the potential for larval dispersal. Smaller individuals seem to prefer shallow waters close to the coast while distributional data suggest that larger individuals migrate out to deeper water. One view is that larvae settle in shallow water, and as they grow, migrate out to deeper water to spawn (Hamel *et al.*, 2001). *Holothuria scabra* are not known to reproduce asexually in the wild (Hamel 2001), however, initial trials, in the laboratory, to induce transverse fusion by constriction have been successful (Lokani *et al.*, 1996).

The Northern Territory (NT) of Australia has been home to Sea Cucumber fishing since the 1700's when the Macassans, from what is now known as Sulawesi, travelled across the Timor Sea to interact with the local Aborigines and to fish. Minimal data is available from the 1800's to estimate the extent of the fishing, or total catch, however, estimates based on historical data, indicate annual catches of around 800 tonnes were common. Fishing for *H. scabra*, in the NT, then underwent a significant period of low or no commercial exploitation until the 1980's when six licences were issued that covered the entire NT coastline. Two management areas currently exist: east of Cape Grey to the Queensland border (originally further broken down into two zones) and west of Cape Grey to the Western Australian border. Three licences operate within each management zone. Controls are also in place which limits the number of divers or collectors. Fishing is permitted from the coastline to three nautical miles seaward from the fisheries baselines. Currently all six fishing licenses for Sea Cucumber in the NT are owned by Tasmanian Seafoods.

Background information on the fisheries biology and recruitment patterns of *H. scabra*

is scarce and thus current regulations governing Sea Cucumber fisheries tend not to be well informed. With a view to improving the management and sustainability of the *H.* scabra fishery in the NT a propagation and restocking (ranching) program is currently under development by Tasmanian Seafoods. The Northern Territory's Department of Resources (DoR) has, in conjunction with all stakeholders including Tasmanian Seafoods, already developed a draft ranching and translocation policy which is in the final stages of review. It is not clear at this stage if it will be necessary to develop species or fishery specific policies (such as a Sea Cucumber policy) under this generic policy. These policies were developed through the Ministerial Advisory Committee on Aquaculture in the Northern Territory (MACANT). The policies are based around current knowledge of *H. scabra* stock delineation and have utilised arbitrary lines on the water based on areas of commercial activity.

Basic research including knowledge of population structure is required to enable informed decisions on the sustainable management of existing fisheries and to develop appropriate policies and strategies for the planned stock enhancment program.

1.1 Need

There is a broad need for population discrimination and dispersal information for the long-term viability of both the wild-harvest fishery and for the successful development of a sustainable sea ranching program. Internationally Sea Cucumber fisheries have shown themselves to be vulnerable to over-fishing if not effectively managed. With little or no information on stock delineation or population dispersal successful management must rely on very risk adverse management measures. This greatly limits the viability and productivity of the commercial wild-harvest fishery. Furthermore, continuing long-term to manage the stock on the assumption that it is a single entity poses considerable environmental risk if this management assumption is wrong. Understanding the population structure of *H. scabra* will allow for the development of more specific management controls and also inform industry in relation to managing fishing programs towards efficient and sustainable production. Successful management of the wild-harvest fishery long-term is dependent on identifying the stocks being managed.

Tasmanian Seafoods is the current Sea Cucumber licensee in the NT and is committed to the development of *H. scabra* aquaculture and sea ranching. In addition to the fisheries management objectives outlined above, there is a recognised need to understand the population genetic structure of *H. scabra* from the currently fished areas in order to inform the planned restocking program as part of the sustainable management of the fishery. This will ensure that the genetic structure of wild populations is taken into account when releasing hatchery produced progeny into the wild populations and is reflected in an appropriate genetic management strategy for hatchery stocks. The potential risk of not considering the genetic makeup of the wild populations and the interbreeding of different stocks is outbreeding depression. Outbreeding depression describes when hybridisation of two genetically distinct populations results in reduced fitness and a potential reduction in a species' ability to adapt to a changing environment, therefore, potentially increasing its vulnerability to extinction over time.

Accurate information on dispersal and population isolation will be crucial to the effective management and development of the industry and to protect genetic integrity if genetically divergent localised populations exist along the Northern Territory coastline. If all sites where the Sea Cucumbers are currently fished are found to comprise one genetic stock then broodstock would be able to be caught from any bay

and the progeny released anywhere within the current range, however, the other extreme would be if each site represents a different genetic stock. If each site was significantly genetically divergent then broodstock would need to be collected from each site, kept segregated and progeny only released back to the site their broodstock originated from.

1.2 Objectives

1. To characterise the genetic population structure of *H. scabra* within the range fished by Tasmanian Seafoods.

2. To characterise the genetic diversity of the hatchery broodstock and progeny arrays relative to the natural populations.

3. To refine and/or recommend polices and strategies for the sustainable management and enhancement, through ranching, of *H. scabra* fisheries.

2. Methods

2.1 Study Sites and Sampling

Tissue samples from a total of 737 *H. scabra* individuals were collected from 16 locations ranging along the NT coast from Popham Bay to Groote Eylandt between August-October 2009 (Table 1 and Figure 1). Two sites: McPherson Point, Warruwi Bay, South Goulburn Island and Little Lagoon, Groote Eylandt, were selected for repeated sampling to assess for possible temporal genetic variation within the study area. McPherson Point was sampled a total of three times; initially in September 2009, after 12 months in September 2010 and finally, after a further 11 months, in August 2011. Little Lagoon was sampled twice; initially in September 2009 and then again, after 18-19 months, in March-April of 2011 (Table 1).

Individuals were collected by hand, by walking along the shore and in shallow water at low tide, or by diving with hookah units, from the 16 sites. A small section of body wall and/or longitudinal muscle was sampled and immediately placed in a labelled 5 ml tube containing 100% ethanol before being transferred to the laboratories at the South Australian Regional Facility for Molecular Evolution and Ecology (SARFMEE), Adelaide, South Australia, Australia.

Table 1: Geographical sites off the Northern Territory coast sampled for *Holothuria scabra* and the number of individuals at each site sampled. Numbers in brackets represent the temporal samples collected.

Site	Location	Latitude	Longitude	Number of samples	
A	Popham Bay	11˚15'13 S	131˚48'46 E	50	
B	Trepang Bay	11˚12'38 S	131˚54'54 E	50	
C	Middle Point, Port Essington	11˚21'18 S	132˚11'17 E	51	
D	Raffals Bay	11˚13' S	132˚25' E	32	
E	Malay Bay 1	11˚21'35 S	132˚53'16 E	50	
F	Malay Bay 2	11˚22'34 S	132˚53'56 E	50	
G	Warruwi Bay, South Goulburn Island 1	11˚38'44 S	133˚23'32 E	49	
H	McPherson Point, Warruwi Bay, South Goulburn Island 2	11˚38'53 S	133˚25'22 E	50	(49, 50)
I	Warruwi Bay, South Goulburn Island 3	11˚38'51 S	133˚25'17 E	30	
J	Inverelli Bay and Middle Bay, Gove Harbour	12˚12'98 S	136˚42'13 E	31	
K	Little lagoon, Groote Eylandt	13˚50'49 S	136˚47'14 E	49	(40)
L	Guion Point	11˚47'24 S	133˚40'25 E	50	
M	Bowen Straight	11˚11'43 S	132˚29'45 E	49	
N	Pearl Farm Bay, Croker Island	11˚20'26 S	132˚34'51 E	51	
O	Nalwrung Strait	12˚02'32 S	136˚20'09 E	46	
P	Millingimbi	12˚06'27 S	134˚55'34 E	49	

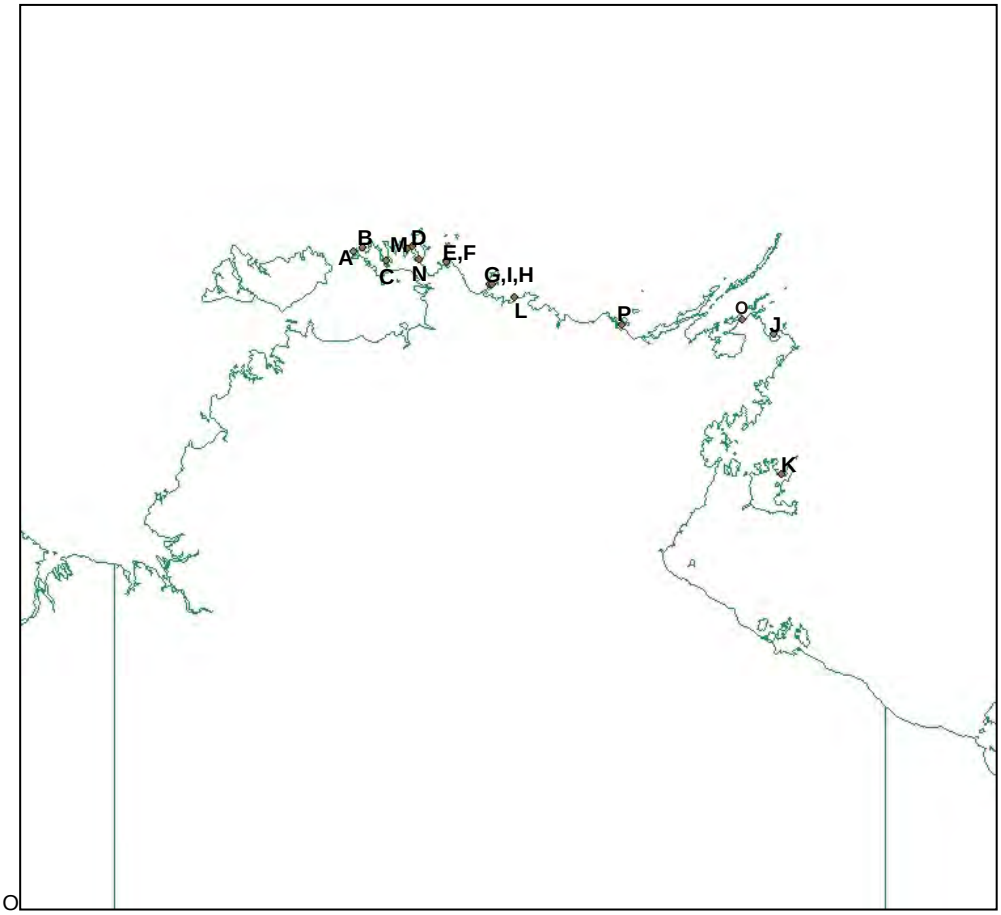


Figure 1: Locations of sites sampled for *Holothuria scabra*. A - Popham Bay; B - Trepang Bay; C - Port Essington; D - Raffals Bay; E - Malay Bay 1; F - Malay Bay 2; G - South Goulburn Island 1; H - South Goulburn Island 2; I - South Goulburn Island 3; J - Gove Harbour; K - Groote Eylandt; L - Guion Point; M - Bowen Straight; N - Croker Island; O - Nalwrung Strait; P - Millingimbi.

To examine genetic variation within hatchery produced juveniles, and to determine the genetic implications of current breeding patterns, 45 broodstock, all collected from Groote Eylandt, and 125 juveniles were sampled from a single spawning event on the 23rd of September 2011. Furthermore, to assess inheritance of our microsatellite loci 17 broodstock and 50 juveniles from two spawning events on the 10th and 11th of May 2011 were sampled and analysed.

2.2 DNA Extraction

Initial DNA extraction trials found that genomic DNA could be successfully extracted from bodywall (dermis), longitudinal muscle and gastrointestinal tissues. For trials of existing microsatellite primers and mitochondrial DNA (mtDNA) marker development trials DNA was extracted from the body wall of six Sea Cucumbers collected from Trepang Bay (3) and Groote Eylandt (3). DNA was extracted using the Gentra Puregene Tissue Kit (Qiagen), following the manufacturer's instructions. DNA extracted and sent for 454 sequencing, and subsequently used for microsatellite loci development, was extracted from the tube feet of two *H. scabra* individuals also using the Gentra Puregene Tissue Kit (Qiagen), following the manufacturer's instructions. All subsequent genomic DNA extractions, for the population, temporal and hatchery studies were done with a modified cetyltrimethylammonium bromide (CTAB) method

(Doyle and Doyle, 1987) with a chloroform precipitation step. All DNA from adult individuals was extracted from body wall and/or longitudinal muscle tissue whilst from juvenile, progeny samples DNA was extracted from the internal organs.

2.3 Marker Development

2.3.1 Microsatellite Marker Development

Initially we trialled 25 existing microsatellite loci, developed in two different species of Sea Cucumber, S*tichopus japonicus* (Kanno *et al.*, 2005; Chang *et al.*, 2009) and *Parastichopus californicus* (Nelson *et al.*, 2002) to determine if these markers could be used in our target species *H. scabra* (Table 2). We trialled the existing markers in six *H. scabra* individuals, three from Trepang Bay, NT and three from Groote Eylandt, NT. The polymerase chain reaction (PCR) protocol consisted of an initial denaturing step of 94 ^{0}C for 9 min; followed by 5 cycles of 94 ^{0}C for 30 s, annealing temperature (see Table 2) for 30 s, 72 ^{0}C for 30 s with the annealing temperature decreasing by 1 ^{0}C per cycle; followed by 30 cycles of 94 ^{0}C for 30 s, annealing temperature 5 ^{0}C lower than the annealing temperature used in step 2 for 30 s, 72 ^{0}C for 30 s; followed by a final extension of 7 min at 72 ^{0}C, as recommended by Xiaoxu Li (personal communication). Unfortunately, we found these markers were not sufficiently conserved in *H. scabra* for use in our study as none successfully amplified any product despite a number of attempts with varying PCR conditions so we developed novel microsatellite markers specific to *H.* scabra using next generation sequencing methods.

Table 2: Existing microsatellite loci trialled for use in *Holothuria scabra*. Annealing temperatures as recommended by Xiaoxu Li (personal communication).

Locus	Primer sequences (5' → 3')	Annealing temperature (^{0}C)	Reference
SCZ01	F: AACATCGACTTCTCACTCCAGG R: ATGATACAAGAGTTGGGGCAGG	64	(Chang *et al.*, 2009)
SCZ02	F: TGCAACGTTGATGTCATGAGC R: GAGACCTAGGCACTATAATTCC	62	(Chang *et al.*, 2009)
SCZ03	F: TTTGGTCAGCTTGCGGCTTTG R: ATTGCATCGAAGGAGGCGATC	60	(Chang *et al.*, 2009)
SCZ04	F: AATTGGAAGTTCCCTGACCCC R: GTAAAATTTGCCTCAGCGAGGG	62	(Chang *et al.*, 2009)
SCZ05	F: CCCTCATTATGGATCTGCCATG R: TTCTCTCCCTACCTCAACTACCC	55	(Chang *et al.*, 2009)
SCZ06	F: TTTCCATTCGCTCCTGCAAACC R: CCGGCCACAAAACTCTCCTATAAG	50	(Chang *et al.*, 2009)
Psc 1	F: CACACGAAAACAAACTAGAACACAT R: TCGTTTGTTGCCCATTTGTA	50	(Nelson *et al.*, 2002)
Psc 2	F: TCTAGGCTAGCCAAACCAAAA R: GATCAAAATTGCATCCACCA	50	(Nelson *et al.*, 2002)
Psc 3	F: AAATCTCCCACCGAAAAGTGA R: TTCGCAAACTATTTGTGGTG	55	(Nelson *et al.*, 2002)
Psc 4	F: TAGAGGATCGTAAGGGTTACACAGG R: TTCGTGTTCGCTGATGAAAAGT	60	(Nelson *et al.*, 2002)
Psc 5	F: ACCGCCCTACATCCTCTC R: AGACTGGCATTAAAATTAGACAAAC	60	(Nelson *et al.*, 2002)
Psj 1828	F: CAAACGCATACAATTACACA R: CGATCGATAGTCCTCAATC	60	(Kanno *et al.*, 2005)
Psj 2031	F: ACTAAAAAGTCATGGACACCC R: AATCATAGCCCATTTTTCTCG	60	(Kanno *et al.*, 2005)
Psj 2062	F: AGTATTTGTTGGTAAGGTGTG R: GAACTTCCTTCATTTCTCT	60	(Kanno *et al.*, 2005)
Psj 2212	F: CAACGACTTACTCCCCTACTC R: GAAACAATATGGCACAAAAGA	55	(Kanno *et al.*, 2005)
Psj 2270	F: TCAAGTGCGGAGATAAAAGT R: TGTGTAGTTGTAGCCGAGATT	55	(Kanno *et al.*, 2005)
Psj 2368	F: GCTAGTCGTGTGGACTTCTC R: TAGGTATTGATGCATTTAGGG	55	(Kanno *et al.*, 2005)

Table 2 continued.

Locus	Primer sequences (5' → 3')	Annealing temperature (^{0}C)	Reference
Psj 2575	F: GCCTCGAGAGCTTATTCAATG R: GCTCGCTTGGAGAGTAAACAC	65	(Kanno *et al.*, 2005)
Psj 2642	F: ATAGTCCTGATTTTTGGTTTC R: GAGATACCGTGTTTACAAGC	60	(Kanno *et al.*, 2005)
Psj 2643	F: TAAATTTTCGAGCTTTGAT R: TACGGTATTTACAAGCAAGT	55	(Kanno *et al.*, 2005)
Psj 2844	F: CAAAACGATAGGGACCATCTA R: TTAACATTTTCTGCCCACTTC	60	(Kanno *et al.*, 2005)
Psj 2889	F: CGAGACGTTTACTTCCACTG R: AGAGGTTGCTGGCTTTACTC	60	(Kanno *et al.*, 2005)
Psj 2969	F: TTCCTGCCCCTTACAAAATAG R: GCAGCAGAATGATGAGTGTG	60	(Kanno *et al.*, 2005)
Psj 3072	F: TGTTGCTGGTCGGAGCTACTG R: AAGAGTTGGGGCAGGTGATGT	55	(Kanno *et al.*, 2005)
Psj 3088	F: CGTATTTACAAGCCCCCAACA R: GGCGTAGAAAGCAAGGGAAAG	60	(Kanno *et al.*, 2005)

Once extracted from the tube feet of two *H. scabra* individuals the genomic DNA was sent to the Australian Genomic Research Facility (AGRF, Brisbane, Australia) for next generation sequencing on a Titanium GS-FLX (454 Life Sciences/Roche FLX). The sample occupied 12.5% of a plate and produced 184,954 individual sequences, with an average fragment size of 369 bp, 3.3% of which were found to contain microsatellites. Sequences were screened for di-hexanucleotide repeat classes with ≥ 8 tandem repeats using MSATCOMMANDER v0.8.2 (Faircloth, 2008) and primers were designed using Primer3 (as automated within MSATCOMMANDER) (Rozen and Skaletsky, 2000). To identify and exclude loci with flanking region similarities, which may represent loci which are not necessarily unique, sequences for which primers had been designed were then analysed in the program MICROFAMILY v1.2 (Meglécz, 2007).

Following the procedure outlined in Gardner *et al.*, (2011), 65 loci were chosen for further development. Initially, the loci were trialled for amplification in three individuals of *H. scabra* using the multiplex-ready PCR (MRT) method as described in (Hayden *et al.*, 2008). Forty-eight (74%) loci amplified an unambiguous product of the expected size. These 48 loci were then tested for polymorphism in six individuals selected from across the sampled geographic range on a 5% polyacrylamide gel. Of the 48 loci, 22 (46%) loci were polymorphic, 1 (2%) was monomorphic, 9 (19%) did not amplify, and alleles in 16 (33%) loci were unclear. Initially, 20 polymorphic loci were selected for genotyping and analysis; however, two loci were subsequently dropped due to inconsistent amplification, resulting in a panel of 18. Each forward primer was labelled with a fluorescent tag at the 5′ end: FAM (GeneWorks), NED, PET or VIC (Applied Biosystems) (Table 3). To verify the selected panel of 18 microsatellite loci samples from one population, Crocker Island, were selected and the number and range of alleles, observed and expected heterozygosity, polymorphic information content, estimated null allele frequencies (using CERVUS v3.0.3 (Kalinowski *et al.*, 2007), deviation from Hardy-Weinberg Equilibrium (HWE) and tests of linkage disequilibrium were estimated (using GENEPOP v4.1.10 (Raymond and Rousset, 1995) (Table 3). *P* values from HWE tests were adjusted for multiple tests of significance using the sequential Bonferroni method (Rice, 1989).

Table 3. Characterisation of 18 microsatellite loci developed in *Holothuria scabra* in individuals collected from Croker Island. Given are sample size (*N*); Observed alleles (*Na*); proportions of observed and expected heterozygosity (*Ho* and *He* respectively); polymorphic information content (PIC); Null allele frequency (*F*(null)) and Hardy-Weinberg equilibrium (HWE). * indicates significance after Bonferroni correction. Superscripts F, N, V, and P indicate primers were labelled with the dyes 6-FAM, NED, VIC and PET respectively.

Locus	Primer sequence (5'-3')	PCR multiplex	Primer conc^{n} (μM)	Repeat motif	*N*	Allele size range	*Na*	*Ho*	*He*	PIC	*F*(null)	HWE
Hsc1	F: ^{N}CTTAGTCTGGTACGGTTGTCC R: GTTTACTAAGCAAGTGTCACAAAC	2	0.15	$(GT)_8$	24	79-105	5	0.250	0.682	0.607	+0.452	0.000*
Hsc4	F: ^{F}AGAGCATGTATGTATCATCGAAACC R: AAACGGAACGGAACAAGCC	4	0.20	$(GT)_{11}$	38	111-127	8	0.263	0.676	0.624	+0.448	0.000*
Hsc11	F: ^{F}TGTTCATAGAGGGAATGTGAGG R: CGTTGAGTTAGAGCGTACCG	1	0.15	$(GT)_{12}$	50	150-168	6	0.400	0.396	0.374	+0.007	0.408
Hsc12	F: ^{V}TGAGGTCATCTGTTCGCCC R: CCTACGCATTTCGATCCGTC	1	0.15	$(GT)_{14}$	46	158-164	3	0.043	0.085	0.082	+0.288	0.022
Hsc14	F: ^{N}GGGCATGTAGGCAAACTCTTC R: TATTGCTCCAGTTCGCCCC	1	0.20	$(AC)_{13}$	18	154-164	2	0.000	0.108	0.099	+0.765	0.029
Hsc17	F: ^{P}AGATTCATTTGGGAACTTTGGC R: AGGGTTGATGTAAGCTGCG	4	0.20	$(AC)_{11}$	38	185-199	7	0.211	0.729	0.674	+0.547	0.000*
Hsc20	F: ^{F}TGCGTGTGGTGATTTTGAC R: ACCATTCTACAGCTCGTCCC	2	0.15	$(AC)_{12}$	47	188-228	17	0.872	0.930	0.914	+0.025	0.139
Hsc24	F: ^{V}TCCTTCGTCGCAGCATGAC R: TTCTTGTATTCCTTTGCAGGC	4	0.20	$(AC)_{18}$	43	193-219	11	0.628	0.849	0.821	+0.142	0.056
Hsc28	F: ^{P}TTCTGGTCTCGACTGGCAC R: TCAGTATCGGCTCCACAGG	5	0.30	$(GAT)_{15}$	41	217-484	28	0.317	0.952	0.937	+0.499	0.000*
Hsc31	F: ^{V}TGTAGGTAGGTAGGTAGGTATGTATG R: CAGCAGTGGGTTTGGACAC	5	0.30	$(ATGT)_{11}$	40	170-176	3	0.925	0.515	0.391	-0.295	0.000*
Hsc40	F: ^{P}GCATTGATCATGTGGAATTTGCG R: CACCATAGACCTGGCTTGC	3	0.35	$(ATGT)_{13}$	49	233-345	26	0.959	0.954	0.941	-0.008	0.723
Hsc42	F: ^{N}TCCTTCGTCGCAGCATGAC R: TTCTTGTATTCCTTTGCAGGC	5	0.25	$(AC)_{18}$	47	248-254	4	0.191	0.251	0.236	+0.118	0.107
Hsc44	F: ^{F}GACGGTACGTCACCAGAGG R: TTCTTCGTCTTTTGGCGGG	5	0.30	$(AAAC)_9$	50	170-198	8	0.760	0.797	0.760	+0.019	0.648
Hsc48	F: ^{V}ACAATGCGGACGACAATGG R: ATCGTGTTTACAAGCGGGC	4	0.15	$(GT)_8$	48	151-163	5	0.375	0.619	0.579	+0.226	0.000*
Hsc49	F: ^{P}TGAGCACGGTGTATTGTCC R: TGATGTGAGCCACTGCG	2	0.20	$(ACAG)_8$	46	157-237	18	0.674	0.930	0.914	+0.152	0.000*
Hsc54	F: ^{F}AGACAGTTGTGGGAAGGGC R: TGGATGGAATAACAATAGGTGTCC	3	0.20	$(CTGT)_8$	48	190-206	3	0.125	0.192	0.178	+0.202	0.039
Hsc59	F: ^{F}AGAGCACACGTATCCCCAC R: GGGGCAGGATAGAGCACATAG	3	0.25	$(AC)_8$	49	234-264	13	0.816	0.837	0.812	+0.010	0.720
Hsc62	F: ^{V}AGCTAGCAGGGAAAAGAAGAAAG R: AGAGGCGGATGCTCTTACC	2	0.20	$(AT)_8$	31	245-255	6	0.484	0.739	0.687	+0.188	0.003*

2.3.2 Mitochondrial DNA Marker Development

To assess variation in mtDNA, primers were trialled for a number of mitochondrial genes, including the control region, cytochrome c oxidase 1 (*CO1*), 16s ribosomalRNA (*16s rRNA*) and NADH dehydrogenase subunit 4 (*ND4*) (Table 4). Sequences for *ND4* from *H. scabra* were recovered from the 454 shotgun run by examining Blast hits following Bertozzi et al. (2012) and primers were designed with Primer3. Neither the 16s*RNA* nor *CO1* gene regions showed significant variation whilst the control region would not sequence reliably, so we proceeded with amplifying and sequencing the mtDNA *ND4* gene.

Table 4: Mitochondrial DNA primers trialled for amplification in *Holothuria scabra*.

Mitochondrial gene	Primer	Primer Sequence	Reference
Control region	SrRNAb	F: GATCGAGGTGCAGCTAATAAG	(Arndt and Smith, 1998)
	Metb	R: CATTCTTGGGATATGAGCC	
CO1	CO1e-F	F: ATAATGATAGGAGGRTTTGG	(Arndt *et al.*, 1996)
	CO1e-R	R: GCTCGTGTRTCTACRTCCAT	
16s rRNA	16Sar	F: CGCCTGTTTATCAAAAACAT	(Palumbi *et al.*, 1991)
	16Sbr	R: CTCCGGTTTGAACTCAGATCA	
ND4	ND4F6	F: AGCCATAAGAGGAGCACTG	This study
	ND4R662	R: GGTCTTGTTTCGTTATTATTG	

2.4 Marker Amplification

2.4.1 Microsatellite Loci

Multiplex PCR amplifications (between 3-4 loci per reaction, Table 3) were performed in 10 μL volumes containing template DNA, 1X Qiagen Mulitplex PCR Master Mix and 0.15-0.35 μM of each primer (Table 3). Amplifications were performed using the following cycling protocol: 95 ^{0}C for 15 min; 35 cycles of 94 ^{0}C for 30 s, 57 ^{0}C for 90 s, 72 ^{0}C for 1 min; 60 ^{0}C for 30 min. PCR products from each individual were first pooled and then separated on an AB3730 DNA Analyser (Applied Biosystems). Microsatellite alleles were sized according to an internal standard, GeneScan 500LIZ(-250) in the program GENEMAPPER v4.0 (Applied Biosystems).

To calculate genotyping error rates, we repeat-genotyped approximately 15% of samples for each marker and calculated scoring errors per allele and per reaction for each locus and summarised across all loci (Hoffman and Amos, 2005). Furthermore, a number of samples were examined for Mendelian transmission of alleles between parents (n=17) and offspring (n=50) by comparing genetic data with known pedigree data in CERVUS 3.0.3 (Kalinowski *et al.*, 2007).

2.4.2 Mitochondrial DNA

An approximately 650 bp fragment of the mitochondrial *ND4* gene was amplified and sequenced in 251 *H. scabra* individuals, selected from across all 16 sites, using the forward primers ND4F6 with the reverse primer ND4R662 designed for this study from sequences generated by the next generation sequencing (Table 4).

PCR amplifications for *ND4* were carried out in a final volume of 25 μL using AmpliTaq Gold DNA polymerase (Applied Biosystems) and containing template DNA, 1X PCR

Gold buffer, 2 mM magnesium, 200 uM of each dNTP and 0.2 µM of each primer, with the following conditions: an initial denaturing step of 94 ^{0}C for 9 min; followed by 40 cycles of 94 ^{0}C for 45 s, 50 ^{0}C for 45 s, 72 ^{0}C for 1 min; followed by a final extension of 6 min at 72 ^{0}C. PCR products were purified using MultiScreen® PCR Plates (Millipore) following the manufacturer's instructions and then sequenced, using the same forward primer as that used for PCR amplification, using the BigDye Terminator Cycle Sequencing kit v3.1 (Applied Biosystems). Sequencing products were then purified using MultiScreen$_{384}$-Seq Filter Plates (Millipore) and run on an ABI Prism 3730*xl* capillary sequencer at AGRF (Adelaide). Sequences were edited and aligned using Geneious v5.4.3 (Biomatters). Nucleotide sequence data will be deposited in the NCBI Genbank nucleotide sequence database upon acceptance of publications arising from this report.

2.4.3 Hatchery Stock

DNA for all 45 broodstock, who contributed to a spawning on the 23rd September 2011, and 125 progeny from the same spawning event, was extracted and microsatellites and mtDNA amplified and scored as described above.

2.5 Microsatellite Data Analyses

2.5.1 Temporal Data

To check for differences in population structure over time the temporal samples collected from both McPherson Point and Little Lagoon were analysed separately using STRUCTURE v2.3.3 (Pritchard *et al.*, 2000), a clustering program which assigns individual multilocus genotypes into potential genetic clusters minimising deviation from HWE. Both analyses were run with a 200,000 burn-in period and 1,000,000 Markov chain Monte Carlo (MCMC) replications under the admixture model and the assumption of correlated allele frequencies among samples, as suggested in (Falush *et al.*, 2003). We searched for 1-5 potential genetic clusters (*K*) within each site with five iterations of each run. Following the recommendations of (Evanno *et al.*, 2005), the ad hoc statistic Δ*K* was calculated, based on the rate of change of the log-likelihood of the present data set between consecutive *K* values, as implemented in STRUCTURE HARVESTER v0.6.91 (Earl and vonHoldt, 2011).

2.5.2 Population Data

All assessment of population genetic structure was performed on the samples collected from the 16 sites from between August-October 2009.

The number of alleles per locus, observed and expected heterozygosity and null allele frequencies were estimated using CERVUS v3.0.3 (Kalinowski *et al.*, 2007) and the inbreeding coefficient (F_{is}) estimated using FSTAT v2.9.3.2 (Goudet, 2001). Significance of departure from Hardy-Weinberg equilibrium (HWE) with 10,000 dememorisation steps, batches and iterations, was estimated using GENEPOP v4.0.10 (Raymond and Rousset, 1995) for each locus and location. Tests of linkage disequilibrium were performed using a likelihood-ratio test with a level of significance determined by permutation (10,000 dememorisation steps, batches and iterations) also in GENEPOP v4.0.10 (Raymond and Rousset, 1995). Levels of statistical significance were adjusted according to a sequential Bonferroni correction for multiple comparisons (Rice, 1989). The program MICROCHECKER v2.2.3 (Van Oosterhout *et al.*, 2004) was used to check for null alleles, scoring errors and large allele drop out. The data

were also analysed for evidence for recent bottleneck events using the program BOTTLENECK v1.2.02 (Cornuet and Luikart, 1996). Deviations from expected heterozygosity were estimated according to a two-phase model of mutation with the proportion of stepwise mutation model set at 95% and the variance among multiple steps at 5%, as recommended by (Cornuet and Luikart, 1996). Significance of deviations was tested using the Wilcoxin sign-rank test with 1000 iterations.

Assessment of structure within studied genotypes, without *a priori* assumptions regarding populations, was performed using a Bayesian model-based clustering approach, as implemented in STRUCTURE v2.3.3 (Pritchard *et al.*, 2000). The software was run first with the total dataset and the number of potential genetic clusters (*K*) assessed using a value of *K* ranging from 1 to 20 to determine potential strong trends for population subdivision, followed by two secondary runs (with *K*=1-13) using only the genotypes corresponding to the two clusters found in the first run respectively, to detect any potential substructure. For each run, uninformed priors were used with a 100,000 burn-in period and 1 million MCMC replications under the admixture ancestry model and the assumption of correlated allele frequencies among samples. Ten iterations of each run were performed. The ad hoc statistic ΔK was calculated, as implemented in STRUCTURE HARVESTER v0.6.91 (Earl and vonHoldt, 2011).

To estimate the level of genetic differentiation, between all sampled geographic sites and between the two clusters found by STRUCTURE, pairwise F_{ST} values were estimated (Weir and Cockerham, 1984), in GENEPOP v4.0.10 (Raymond and Rousset, 1995), with the significance level α set to 0.05 and 10,000 permutations. Additionally, Jost's (2008) actual measure of differentiation, D_{est}, using 1,000 bootstraps, was calculated using SMOGD v1.2.5 (Crawford, 2010). Analyses of molecular variance (AMOVAs) were used to partition genetic variation at three hierarchical levels: among sites and clusters, among individuals within sites and clusters, and within individuals using ARLEQUIN v3.5 (Excoffier and Lischer, 2010) with 16,000 permutations.

Isolation by distance was analysed by testing the correlation between pairwise $F_{ST}/(1-F_{ST})$ values and the logarithm of the geographic distances between locality pairs. The significance of the relationship between geographical and genetic distance was evaluated with a Mantel test in GENEPOP v4.0.10 (Raymond and Rousset, 1995), with 1000 permutations. Finally, to estimate recent migration rates between the two clusters suggested by STRUCTURE we used a Bayesian method implemented in the software BAYESASS v3.0.1 (Wilson and Rannala, 2003). BAYESASS computes the immigrant ancestry of each individual and the generation in which immigration occurred. This allows us to detect presumed recent immigrants and their recent descendants. BAYESASS was run with 5 million MCMC iterations, sampled every 2,000, the first 1 million of which were discarded as burn-in. To ensure the acceptance rates were within recommended limits (20-40%) (Wilson and Rannala, 2003) the mixing parameter coefficient values for migration rate, allele frequency and inbreeding were set at 0.04, 0.12 and 0.12 respectively (resulting in acceptance rates of 0.30, 0.31 and 0.28 respectively). Five independent runs, started with different random number seeds, were run and compared for consistency.

2.6 Mitochondrial DNA Data Analyses

A fragment of the *ND4* gene was amplified and sequenced in 251 *H. scabra* individuals, selected from the 16 sampled sites as detailed above. Haplotype and nucleotide diversity were calculated using DnaSP v5.10.01 (Librado and Rozas, 2009).

The variation among and within the 16 sites and two clusters was assessed using AMOVAs, implemented in ARLEQUIN v3.5 (Excoffier and Lischer, 2010), with 16,000 permutations and a median-joining haplotype network was estimated using the program NETWORK v4.6 (Bandelt and Forster, 1997). The demographic history of the sampled range and the two clusters found by STRUCTURE was inferred using mismatch distribution analyses implemented in DnaSP v5.10.01 (Librado and Rozas, 2009). Mismatch distribution analyses, under the assumption of selective neutrality, were also used to evaluate possible historical events of population growth and decline (Rogers and Harpending, 1992). Theoretical distributions under the assumption of constant population size and the sudden expansion model were compared to the observed data. The goodness-of-fit of the observed data to a simulated model of expansion was tested with the sum of squared deviations and the raggedness index (Harpending 1994), Tajima's *D* test (Tajima, 1989) and Fu's F_s (Fu, 1997) tests, using 1,000 simulated resamplings.

2.7 Hatchery Data

Genetic diversity and parentage assignments of hatchery brood stocks were assessed using the program CERVUS 3.0.3 (Kalinowski *et al.*, 2007). This program calculates the *a priori* polymorphic information content (*PIC*) for every locus from each broodstock and total exclusionary power. In addition, the program creates simulations for parental assignments. Total exclusionary power is defined as the probability of excluding an arbitrarily unrelated parent. As soon as multiple candidate parents remain non-excluded the exclusionary approach is inadequate (Marshall *et al.*, 1998). To minimise the amount of missing data and thus increase the reliability of the analysis only nine loci were included in the parentage analyses, Hsc4, 11, 12, 20, 31, 42, 48, 49 and 54. The parentage assignment simulations were carried out taking into account the number of breeders per broodstock. Five potential broodstock were excluded from the analysis due to missing data: T9, T25, T27, T28 and T41. Ten thousand cycles of simulated assignations were carried out using 95% confidence intervals. All offspring were assigned to most likely candidate parent pairs with sexes unknown. In the assignment procedures, we allowed for typing errors (6%), since this dramatically reduces the impact of two other possible causes of mismatches in parent-offspring relationships, mutations and null alleles (Marshall *et al.*, 1998) and we also specified that only 89% of the candidate parents had been sampled to allow for the five excluded broodstock.

3. Results

3.1 Population Structure

3.1.1 Microsatellite Data

Repeat genotyping of samples revealed scoring errors for all loci except Hsc20. Amongst the remaining loci, scoring error rates per allele ranged from 0.011 (Hsc42 and Hsc44) to 0.147 (Hsc1) and per reaction from 0.011 (Hsc44) to 0.158 (Hsc1). Summarised across all loci this equates to an error rate of 0.059 per allele and 0.058 per reaction. All loci confirmed to a Mendelian pattern of inheritance and no genetic structure was found among the temporal samples. All microsatellite loci used in this study were polymorphic except for Hsc12 and Hsc14 which were monomorphic at 5 and 12 sites respectively. The number of alleles per locus ranged from 1 (Hsc12 and 14) to 33 (Hsc28) and averaged 9.03 alleles per locus per site. Observed heterozygosities ranged from 0.00 (Hsc12 and 14) to 1.00 (Hsc20 and Hsc31) (Table 5). No pairs of microsatellite loci showed significant genotypic linkage, after Bonferroni correction (P>0.05), across all sites. The bottleneck analysis revealed no significant evidence for a recent event, indicating all studied populations were under mutation-drift equilibrium. Probability values for H_E excess were >0.95 for all populations, except Malay Bay 1 (P=0.90), and plots of allelic frequency distributions were L-shaped, which is consistent with normal frequency class distribution ranges.

Based on the exact test of HWE three loci (Hsc4, 28 and 31) significantly deviated from equilibrium, after sequential Bonferroni correction (P<0.05), at all sites, whilst Hsc1, 17, 24 and 49 were out of HWE at most sites. MICROCHECKER found significant evidence of null alleles but no evidence of scoring errors or large allele drop out. Deviation from HWE due to null alleles appears to be fairly common in Sea Cucumbers and other marine invertebrates (Addison and Hart, 2005; Plutchak *et al.*, 2006; Whitaker, 2006; Chang *et al.*, 2009; Yasuda *et al.*, 2009; Dailianis *et al.*, 2011; Kang *et al.*, 2011) and the existence of null alleles is the most probable explanation for the deviation from HWE of a number of our loci, although other population level processes such as subpopulation structure (Wahlund effect) and deviation from panmixia are possible. Carlsson (2008) simulated the effect of null alleles on assignment testing and concluded that microsatellite loci affected by null alleles would probably not alter the overall outcome of assignment testing and could, therefore, be included in these studies.

Posterior probability values from the STRUCUTRE analysis suggested the data were best explained by two genetic groupings or clusters of *H. scabra* individuals (K=2, Figure 2). Ten independent runs at K=2 produced the same clustering solution illustrated in Figure 2 and Figure 3, corresponding to geographical groupings of localities into regions; cluster 1 (Popham Bay, Trepang Bay, Port Essington, Raffals Bay, Malay Bay, South Goulburn Island, Guion Point, Bowen Straight, Croker Island and Millingimbi) and cluster 2 (Gove Harbour, Groote Eylandt and Nalwrung Strait). Additional hierarchical STRUCTURE runs performed on the initial K=2 clusters found no further substructure within the initial two clusters.

On the whole the genetic structure inferred from STRUCTURE analysis was similar to that inferred from the pairwise F_{ST}. Estimates of F_{ST} between sites ranged from less than 0.001 to 0.060 (Table 6, below diagonal). The highest F_{ST} value was found between samples from Popham Bay and Groote Eylandt. Pairwise estimates of F_{ST} were highest for comparisons between Gove Harbour, Groote Eylandt and Nalwrung

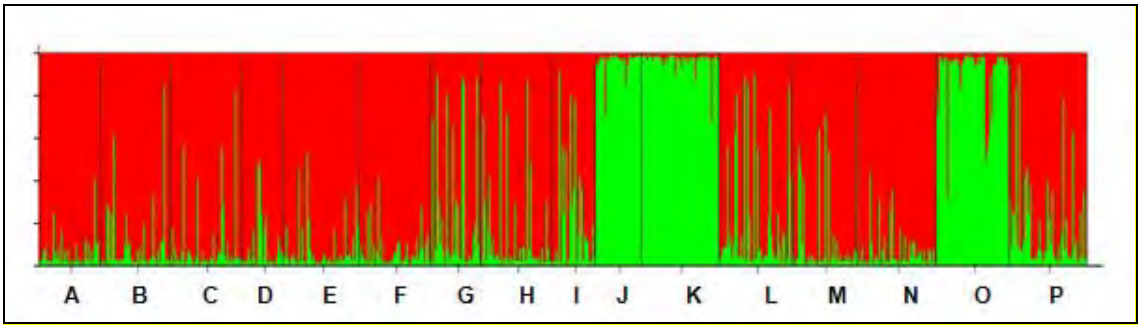


Figure 2: Proportional memberships of *Holothuria scabra* individuals from sequential cluster analysis using the program STRUCTURE. The two initial clusters are shown with vertical bars representing each individual broken into coloured segments based on the proportion of the genome estimated to have originated from each of the two clusters. A - Popham Bay; B - Trepang Bay; C - Port Essington; D - Raffals Bay; E - Malay Bay 1; F - Malay Bay 2; G - South Goulburn Island 1; H - South Goulburn Island 2; I - South Goulburn Island 3; J - Gove Harbour; K - Groote Eylandt; L - Guion Point; M - Bowen Straight; N - Croker Island; O - Nalwrung Strait; P - Millingimbi.

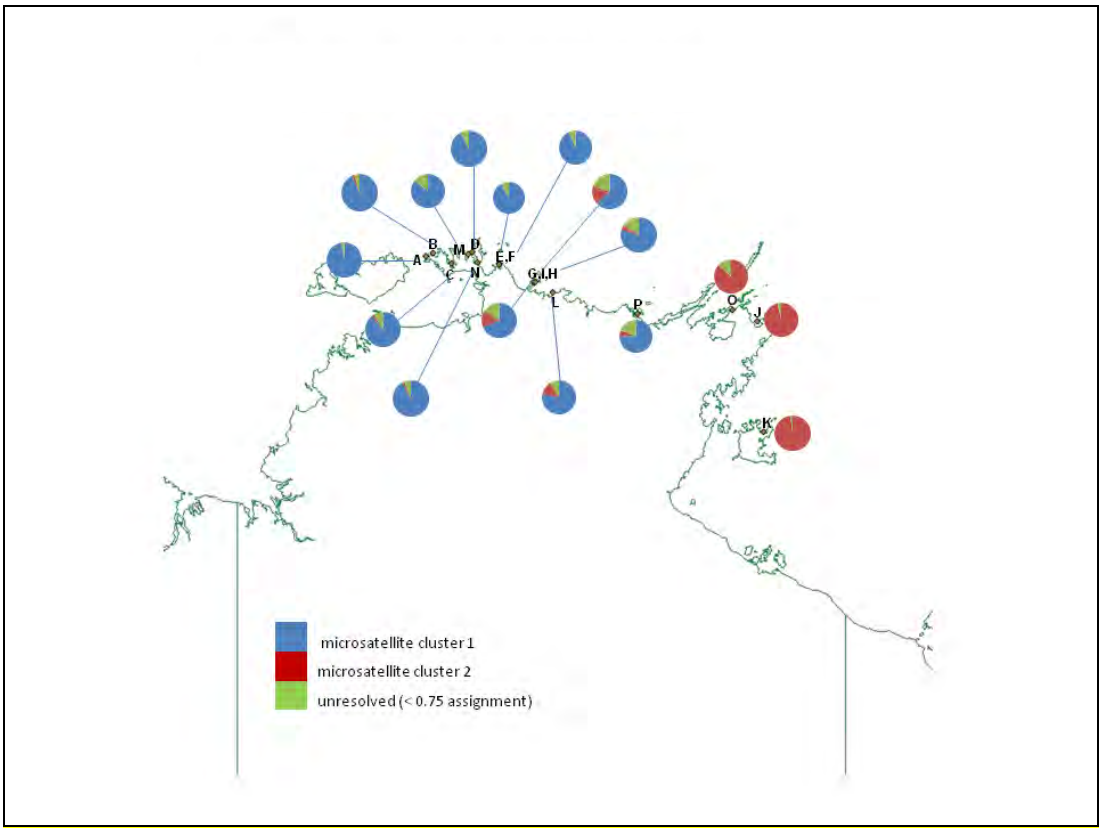


Figure 3. Proportional memberships of *Holothuria scabra* individuals to STRUCTURE cluster 1 and 2 by sampling locality. A - Popham Bay; B - Trepang Bay; C - Port Essington; D - Raffals Bay; E - Malay Bay 1; F - Malay Bay 2; G - South Goulburn Island 1; H - South Goulburn Island 2; I - South Goulburn Island 3; J - Gove Harbour; K - Groote Eylandt; L - Guion Point; M - Bowen Straight; N - Croker Island; O - Nalwrung Strait; P - Millingimbi.

Table 5: Summary statistics for genetic variation across 16 localities at 18 microsatellite loci in *Holothuria scabra*. Given are numbers of observed alleles (*Na*); proportions of observed and expected heterozygosity (H_O and H_E respectively), the inbreeding coefficient (F_{IS}) and the frequency of null alleles (*F*(null)). NA is shown where data is not available due to a monomorphic locus.

Locality		Locus																		
		Hsc1	Hsc11	Hsc12	Hsc14	Hsc17	Hsc20	Hsc24	Hsc28	Hsc31	Hsc4	Hsc40	Hsc42	Hsc44	Hsc48	Hsc49	Hsc54	Hsc59	Hsc62	Mean
A	*Na*	13	4	3	2	5	15	11	24	2	6	23	4	11	5	18	4	14	6	9.440
	H_O	0.531	0.167	0.038	0.063	0.211	0.861	0.469	0.5	1	0.138	0.971	0.375	0.9	0.4	0.794	0.139	0.861	0.65	0.504
	H_E	0.887	0.159	0.112	0.063	0.694	0.898	0.795	0.973	0.511	0.671	0.921	0.327	0.825	0.612	0.941	0.205	0.86	0.683	0.619
	F_{IS}	0.405	-0.047	0.662	0	0.702	0.042	0.414	0.492	-1	0.797	-0.055	-0.15	-0.092	0.35	0.158	0.327	-0.002	0.05	0.189
	F(null)	0.243	-0.035	0.435	-0.008	0.528	0.013	0.264	0.311	-0.333	0.656	-0.035	-0.096	-0.056	0.215	0.077	0.231	-0.008	-0.017	0.133
B	*Na*	12	5	3	1	5	16	12	26	2	7	23	4	9	5	20	4	12	7	9.610
	H_O	0.472	0.341	0.029	0	0.167	0.683	0.658	0.379	1	0.261	0.897	0.342	0.923	0.436	0.718	0.205	0.875	0.593	0.499
	H_E	0.816	0.326	0.136	0	0.732	0.898	0.865	0.953	0.509	0.624	0.942	0.305	0.833	0.724	0.934	0.327	0.828	0.696	0.636
	F_{IS}	0.425	-0.049	0.793	NA	0.776	0.241	0.242	0.606	-1	0.588	0.048	-0.124	-0.109	0.401	0.234	0.376	-0.057	0.151	0.219
	F(null)	0.258	-0.03	0.586	NA	0.633	0.134	0.127	0.426	-0.333	0.4	0.017	-0.085	-0.061	0.253	0.125	0.242	-0.039	0.051	0.159
C	*Na*	13	5	1	2	4	14	9	28	2	5	25	4	8	5	17	3	16	5	9.220
	H_O	0.368	0.357	0	0.056	0.143	0.881	0.611	0.344	1	0.241	0.905	0.333	0.738	0.525	0.75	0.098	0.786	0.677	0.490
	H_E	0.85	0.37	0	0.056	0.731	0.91	0.762	0.961	0.507	0.76	0.942	0.335	0.811	0.659	0.918	0.299	0.865	0.637	0.631
	F_{IS}	0.57	0.035	NA	0	0.808	0.032	0.2	0.646	-1	0.686	0.04	0.004	0.091	0.205	0.185	0.676	0.093	-0.065	0.228
	F(null)	0.395	0.007	NA	-0.01	0.665	0.011	0.102	0.471	-0.33	0.516	0.017	0.021	0.04	0.101	0.097	0.499	0.045	-0.07	0.152
D	*Na*	10	4	3	1	6	14	9	17	2	3	19	5	9	5	14	5	12	5	7.940
	H_O	0.5	0.4	0.056	0	0.227	0.96	0.455	0.435	1	0.091	0.92	0.458	0.72	0.417	0.75	0.391	0.72	0.714	0.512
	H_E	0.9	0.464	0.16	0	0.652	0.901	0.821	0.94	0.511	0.506	0.931	0.4	0.829	0.56	0.924	0.349	0.82	0.643	0.629
	F_{IS}	0.452	0.14	0.66	NA	0.657	-0.067	0.452	0.543	-1	0.828	0.013	-0.15	0.134	0.26	0.191	-0.125	0.124	-0.116	0.190
	F(null)	0.273	0.085	0.453	NA	0.463	-0.042	0.279	0.359	-0.333	0.681	-0.003	-0.116	0.068	0.109	0.092	-0.098	0.062	-0.098	0.131
E	*Na*	11	5	2	2	5	16	9	29	2	9	24	5	9	5	18	3	13	7	9.670
	H_O	0.378	0.34	0	0	0.086	0.979	0.444	0.231	1	0.114	0.936	0.326	0.957	0.511	0.783	0.143	0.766	0.633	0.479
	H_E	0.825	0.371	0.151	0.085	0.73	0.916	0.807	0.961	0.506	0.649	0.942	0.294	0.833	0.72	0.933	0.329	0.819	0.757	0.646
	F_{IS}	0.545	0.084	1	1	0.884	-0.07	0.452	0.762	-1	0.826	0.006	-0.108	-0.152	0.293	0.162	0.569	0.065	0.166	0.261
	F(null)	0.373	0.049	0.862	0.695	0.785	-0.04	0.29	0.61	-0.33	0.704	0	-0.08	-0.08	0.164	0.083	0.384	0.027	0.07	0.254
F	*Na*	11	4	3	1	6	14	12	26	2	8	23	4	9	5	17	4	12	7	9.330
	H_O	0.515	0.366	0.091	0	0.286	0.881	0.526	0.351	1	0.206	0.951	0.205	0.81	0.524	0.634	0.244	0.69	0.519	0.489
	H_E	0.851	0.343	0.144	0	0.721	0.902	0.839	0.954	0.507	0.653	0.945	0.214	0.827	0.585	0.922	0.336	0.81	0.74	0.627
	F_{IS}	0.399	-0.067	0.373	NA	0.608	0.024	0.376	0.635	-1	0.688	-0.007	0.044	0.021	0.105	0.315	0.276	0.149	0.303	0.223
	F(null)	0.238	-0.04	0.214	NA	0.425	0.004	0.219	0.457	-0.33	0.515	-0.01	0.049	0.006	0.058	0.181	0.139	0.078	0.139	0.138
G	*Na*	8	4	2	4	5	16	11	21	2	9	21	4	10	5	18	4	11	6	8.940
	H_O	0.36	0.643	0.048	0	0.259	0.897	0.5	0.619	1	0.12	0.897	0.125	0.862	0.552	0.577	0.321	0.69	0.667	0.508
	H_E	0.744	0.567	0.136	0.549	0.755	0.915	0.784	0.962	0.512	0.856	0.94	0.16	0.861	0.673	0.931	0.389	0.747	0.685	0.676
	F_{IS}	0.521	-0.137	0.655	1	0.661	0.021	0.366	0.362	-1	0.862	0.046	0.225	-0.001	0.182	0.385	0.176	0.077	0.028	0.254
	F(null)	0.352	-0.07	0.444	NA	0.478	0	0.202	0.207	-0.33	0.749	0.014	0.205	-0.01	0.092	0.229	0.067	0.037	-0.04	0.154
H	*Na*	8	5	1	2	6	19	10	26	3	6	19	5	9	5	18	4	15	5	9.220
	H_O	0.444	0.405	0	0	0.323	0.842	0.758	0.444	1	0.077	0.892	0.294	0.842	0.568	0.694	0.194	0.816	0.684	0.515
	H_E	0.741	0.483	0	0.177	0.714	0.931	0.774	0.938	0.529	0.722	0.937	0.294	0.83	0.662	0.942	0.228	0.84	0.696	0.635
	F_{IS}	0.407	0.163	NA	1	0.553	0.097	0.021	0.53	-0.926	0.895	0.049	0	-0.015	0.145	0.265	0.148	0.029	0.017	0.191
	F(null)	0.254	0.098	NA	0.897	0.369	0.045	0.004	0.354	-0.32	0.804	0.017	0.006	-0.01	0.088	0.146	0.063	0.012	-0.03	0.165

Table 5 continued.

Locality (N)		Locus Hsc1	Hsc11	Hsc12	Hsc14	Hsc17	Hsc20	Hsc24	Hsc28	Hsc31	Hsc4	Hsc40	Hsc42	Hsc44	Hsc48	Hsc49	Hsc54	Hsc59	Hsc62	Mean
I	*Na*	6	4	3	1	3	15	8	16	2	5	18	5	7	5	17	3	14	6	7.670
	H_O	0.333	0.64	0.045	0	0.208	1	0.35	0.55	0.9	0.19	0.88	0.458	0.88	0.64	0.565	0.08	0.88	0.474	0.504
	H_E	0.771	0.531	0.132	0	0.414	0.918	0.654	0.936	0.508	0.653	0.942	0.5	0.804	0.749	0.928	0.079	0.904	0.632	0.614
	F_{IS}	0.583	-0.209	0.661	NA	0.502	-0.091	0.471	0.419	-0.81	0.713	0.067	0.085	-0.097	0.148	0.396	-0.011	0.027	0.255	0.184
	F(null)	NA	-0.12	0.446	NA	0.321	-0.05	0.297	0.249	-0.29	0.536	0.024	0.001	-0.05	0.061	0.231	-0.01	0.006	0.112	0.110
J	*Na*	7	5	1	1	5	14	8	17	2	6	26	3	8	5	9	5	7	7	7.560
	H_O	0.818	0.464	0	0	0.304	0.964	0.821	0.529	1	0.4	0.926	0.6	0.714	0.536	0.75	0.038	0.821	0.368	0.559
	H_E	0.749	0.397	0	0	0.629	0.886	0.819	0.93	0.512	0.739	0.952	0.515	0.779	0.655	0.771	0.476	0.779	0.663	0.625
	F_{IS}	-0.098	-0.172	NA	NA	0.522	-0.091	-0.002	0.439	-1	0.464	0.028	-0.169	0.084	0.185	0.028	0.921	-0.056	0.451	0.108
	F(null)	-0.08	-0.12	NA	NA	0.337	-0.05	-0.01	0.265	-0.33	0.289	0.004	-0.12	0.042	0.086	0.005	0.846	-0.03	0.28	0.088
K	*Na*	7	5	1	2	7	15	12	29	2	7	27	3	10	5	12	4	8	8	9.110
	H_O	0.714	0.438	0	0.026	0.308	0.917	0.8	0.553	1	0.396	0.938	0.578	0.792	0.521	0.667	0.051	0.75	0.48	0.552
	H_E	0.712	0.495	0	0.026	0.786	0.911	0.834	0.956	0.507	0.728	0.954	0.494	0.805	0.657	0.842	0.495	0.768	0.804	0.654
	F_{IS}	-0.004	0.116	NA	0	0.612	-0.007	0.042	0.425	-1	0.459	0.017	-0.172	0.017	0.209	0.21	0.898	0.024	0.408	0.159
	F(null)	0	0.058	NA	0	0.426	-0.01	0.007	0.262	-0.33	0.296	0.005	-0.1	0.013	0.125	0.12	0.813	0.016	0.239	0.114
L	*Na*	5	5	2	1	6	19	10	23	2	7	26	4	9	5	18	4	14	5	9.170
	H_O	0.368	0.386	0.023	0	0.212	0.818	0.622	0.412	1	0.097	0.977	0.395	0.886	0.705	0.692	0.119	0.791	0.667	0.509
	H_E	0.586	0.48	0.023	0	0.717	0.919	0.823	0.919	0.506	0.646	0.954	0.378	0.85	0.671	0.904	0.238	0.833	0.706	0.620
	F_{IS}	0.378	0.197	0	NA	0.707	0.111	0.248	0.556	-1	0.852	-0.024	-0.045	-0.043	-0.05	0.236	0.502	0.051	0.056	0.180
	F(null)	0.232	0.115	0	NA	0.541	0.056	0.139	0.379	-0.33	0.732	-0.02	-0.02	-0.03	-0.04	0.128	0.323	0.027	0	0.131
M	*Na*	8	4	3	2	6	15	12	27	2	7	23	5	8	5	17	4	14	7	9.390
	H_O	0.267	0.342	0.071	0.077	0.222	0.846	0.594	0.303	1	0.182	0.923	0.289	0.821	0.538	0.639	0.353	0.872	0.667	0.500
	H_E	0.802	0.419	0.071	0.077	0.748	0.916	0.813	0.961	0.508	0.586	0.944	0.308	0.826	0.591	0.939	0.468	0.858	0.714	0.642
	F_{IS}	0.675	0.185	-0.009	0	0.707	0.078	0.272	0.688	-1	0.693	0.023	0.062	0.006	0.09	0.322	0.249	-0.016	0.068	0.224
	F(null)	0.496	0.11	-0.01	-0.01	0.537	0.035	0.154	0.516	-0.33	0.521	0.005	5E-04	-0.01	0.067	0.183	0.145	-0.01	0.002	0.133
N	*Na*	5	6	3	2	7	17	11	28	3	8	26	4	8	5	18	3	13	6	9.610
	H_O	0.25	0.388	0.044	0	0.216	0.87	0.628	0.317	0.925	0.263	0.958	0.196	0.755	0.383	0.689	0.128	0.813	0.484	0.462
	H_E	0.682	0.389	0.087	0.108	0.733	0.93	0.849	0.952	0.515	0.676	0.953	0.256	0.799	0.627	0.928	0.16	0.831	0.739	0.623
	F_{IS}	0.638	0.002	0.491	1	0.708	0.066	0.262	0.67	-0.815	0.614	-0.005	0.239	0.056	0.392	0.26	0.205	0.022	0.349	0.262
	F(null)	0.452	0.016	0.289	0.765	0.539	0.027	0.142	0.499	-0.29	0.448	-0.01	0.117	0.025	0.222	0.141	0.102	0.008	0.188	0.204
O	*Na*	7	6	2	1	5	15	11	28	2	7	29	3	7	5	14	3	13	6	9.110
	H_O	0.571	0.61	0.049	0	0.306	0.923	0.865	0.69	0.951	0.321	0.897	0.4	0.78	0.512	0.595	0.108	0.8	0.364	0.541
	H_E	0.839	0.597	0.048	0	0.582	0.884	0.849	0.972	0.505	0.766	0.961	0.378	0.793	0.49	0.869	0.198	0.774	0.641	0.619
	F_{IS}	0.327	-0.022	-0.013	NA	0.478	-0.045	-0.019	0.294	-0.905	0.585	0.067	-0.058	0.016	-0.046	0.319	0.458	-0.034	0.438	0.128
	F(null)	0.174	-0.01	0	NA	0.308	-0.03	-0.02	0.161	-0.31	0.395	0.028	-0.06	0.002	-0.07	0.188	0.284	-0.03	0.261	0.075
P	*Na*	8	5	2	1	7	15	8	33	2	8	20	5	8	5	17	4	14	8	9.440
	H_O	0.524	0.468	0.023	0	0.405	0.83	0.439	0.488	1	0.156	0.894	0.533	0.83	0.596	0.705	0.136	0.8	0.625	0.525
	H_E	0.783	0.456	0.023	0	0.75	0.913	0.759	0.966	0.506	0.734	0.938	0.458	0.839	0.706	0.914	0.21	0.856	0.777	0.644
	F_{IS}	0.336	-0.026	0	NA	0.463	0.092	0.424	0.498	-1	0.79	0.048	-0.167	0.011	0.158	0.231	0.353	0.066	0.199	0.187
	F(null)	0.19	-0.02	0	NA	0.303	0.045	0.27	0.324	-0.33	0.642	0.02	-0.11	1E-04	0.073	0.121	0.198	0.033	0.083	0.108

Table 6: Pairwise F_{ST} (below diagonal) and D_{est} (above diagonal) estimates between sites for *Holothuria scabra* based on microsatellite loci. A - Popham Bay; B - Trepang Bay; C - Port Essington; D - Raffals Bay; E - Malay Bay 1; F - Malay Bay 2; G - South Goulburn Island 1; H - South Goulburn Island 2; I - South Goulburn Island 3; J - Gove Harbour; K - Groote Eylandt; L - Guion Point; M - Bowen Straight; N - Croker Island; O - Nalwrung Strait; P - Millingimbi.

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P
A	--	0.004	0.001	0.008	0.005	0.009	0.043	0.009	0.032	0.116	0.138	0.026	0.006	0.009	0.096	0.018
B	0.000	--	0.006	0.003	0.004	0.002	0.039	0.010	0.033	0.108	0.111	0.009	0.006	0.008	0.071	0.011
C	0.000	0.002	--	0.007	0.003	0.005	0.024	0.002	0.017	0.107	0.117	0.012	0.007	0.010	0.071	0.004
D	0.003	0.001	0.002	--	0.007	0.001	0.042	0.004	0.028	0.110	0.125	0.012	0.009	0.011	0.073	0.016
E	0.003	-0.001	0.001	0.001	--	0.001	0.009	0.002	0.027	0.126	0.118	0.008	0.001	0.001	0.072	0.003
F	0.002	-0.001	0.004	-0.004	-0.002	--	0.024	0.006	0.022	0.116	0.116	0.010	0.003	0.004	0.073	0.014
G	0.022	0.015	0.013	0.018	0.008	0.012	--	0.004	0.039	0.119	0.099	0.011	0.021	0.019	0.069	0.005
H	0.006	0.003	-0.001	0.002	0.000	0.003	0.002	--	0.011	0.098	0.088	0.006	0.003	0.001	0.044	0.002
I	0.018	0.022	0.013	0.012	0.017	0.020	0.025	0.008	--	0.082	0.082	0.003	0.018	0.013	0.067	0.006
J	0.054	0.053	0.049	0.054	0.055	0.054	0.054	0.049	0.049	--	0.004	0.080	0.115	0.101	0.042	0.079
K	0.060	0.052	0.049	0.051	0.052	0.051	0.042	0.042	0.052	0.006	--	0.068	0.115	0.098	0.037	0.075
L	0.014	0.010	0.010	0.009	0.005	0.008	0.008	0.003	0.009	0.045	0.039	--	0.015	0.004	0.047	0.003
M	0.004	0.001	0.003	0.000	-0.003	0.001	0.016	0.002	0.020	0.053	0.054	0.010	--	0.004	0.068	0.005
N	0.004	0.005	0.004	0.006	-0.001	0.001	0.012	-0.001	0.012	0.055	0.052	0.003	0.003	--	0.062	0.005
O	0.049	0.041	0.037	0.038	0.044	0.042	0.036	0.027	0.041	0.027	0.024	0.032	0.044	0.041	--	0.054
P	0.009	0.007	0.003	0.007	0.001	0.007	0.006	0.000	0.008	0.040	0.040	0.001	0.003	0.002	0.035	--

Strait and other localities (0.0265-0.060, average = 0.0448). The F_{ST} estimate between STRUCTURE clusters was 0.0383. Patterns of differentiation between sites, and clusters, were similar whether estimated by F_{ST} or D_{est}, however, estimates were considerably higher using D_{est}, ranging from 0.0005 to 0.1255 (Table 6, above the diagonal). D_{est} between clusters 1 and 2 was 0.0803. Quantitative estimates of hierarchical gene diversity (AMOVA) indicated significant population structure at all levels tested with the greatest variance observed, with F_{ST}, within individuals. However, when using the R_{ST} estimate, the greatest variation was observed among individuals within sites or clusters (Table 7).

Table 7: Results of analysis of molecular variance (AMOVA), based on the microsatellite data, among geographic sites and clusters suggested by STRUCTURE for *Holothuria scabra*.

Source of Variation		F_{ST}			R_{ST}		
	df	Variation (%)	Significance (*P*-value)	F_{ST}	Variation (%)	Significance (*P*-value)	R_{ST}
16 geographic sites							
Among sites	15	2.762	<0.001	0.028	3.153	<0.001	0.032
Among individuals within sites	605	33.900	<0.001		92.300	<0.001	
Within individuals	621	63.338	<0.001		4.546	<0.001	
2 clusters							
Among clusters	1	3.972	<0.001	0.040	2.689	<0.001	0.027
Among individuals within clusters	619	34.327	<0.001		92.841	<0.001	
Within individuals	621	61.701	<0.001		4.470	<0.001	

No significant correlation between genetic $F_{ST}/(1-F_{ST})$ and geographical distances was detected across all pairs of samples (R^2=0.021, P=0.145) indicating no significant isolation by distance effects within our sampled range.

Recent migration rates between clusters, as calculated in BAYESASS, were low with the majority of migration occurring within each cluster. The number of individuals migrating from cluster 1 to 2 was estimated at 0.001 (0.001; 95% confidence interval) whilst the number migrating from cluster 2 to 1 was 0.008 (0.006; 95% confidence interval). Results were consistent across independent runs.

3.1.2 Mitochondrial DNA Data

An alignment of 608 bp of *ND4* sequence was obtained for 251 individuals selected from all 16 geographical locations. The *ND4* sequences were characterized by low nucleotide (0.004) and high haplotype (0.772) diversity. Overall, 56 different haplotypes were detected and polymorphisms were observed at 55 of the 608 (9%) sites. Of these variable sites 36%, 16% and 48% were detected at first, second and third codon positions, respectively. The AMOVA revealed significant diversity among, and within, the sites but not the clusters (Table 8). The greatest variance was found among the sites but still a significant amount was attributed to within them (Table 8).

Table 8: Results of analysis of molecular variance (AMOVA) based on the mitochondrial sequence data among geographic sites and clusters suggested by STRUCTURE for *Holothuria scabra*.

Source of Variation	Degrees of freedom	Variation (%)	Significance (*P*-value)	F_{ST}
16 geographic sites				
Among sites	15	57.50	<0.001	0.575
Within sites	112	42.50	<0.001	
2 clusters				
Among clusters	1	-125.10	NS	-1.251
Within clusters	126	225.10	NS	

The minimum spanning network of the haplotypes showed no evidence of clustering based on geographical location, showing five main haplotypes shared between most sampling locations (Figure 4). Further, individuals split into clusters 1 and 2 by STRUCTURE were found shared between three of the main haplotypes (Figure 5).

Indices of neutral evolution (Tajima's D and Fu's F_S) were both negative and significant in the population as a whole (-2.101, P<0.05 and -48.932 P<0.01 respectively). In addition, population expansion is indicated by the mismatch distribution (Figure 6). The distribution is usually multimodal in samples at populations at demographic equilibrium but unimodal in populations following recent demographic expansion (Rogers and Harpending, 1992). Furthermore population expansion is supported by the low raggedness index (0.0682).

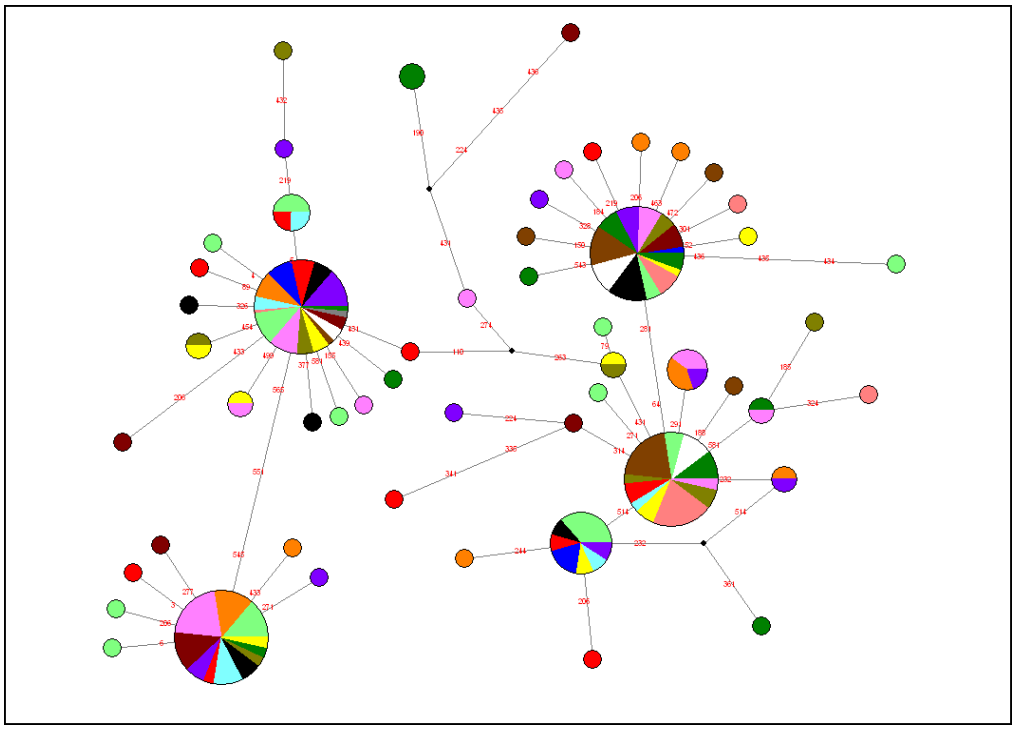


Figure 4: Mitochondrial DNA haplotype network by sampling location. Red numbers along branches represent mutations. Different colours represent different sampling localities.

Popham Bay
Malay Bay 2
Groote Eylandt
Millingimbi
Trepang Bay
Sth Goulburn1
Guion Point
Port Essington
Sth Goulburn. 2
Bowen Straight
Raffals Bay
Sth Goulburn 3
Croker Island
Malay Bay 1
Gove Harbour
Nalwrung Strait

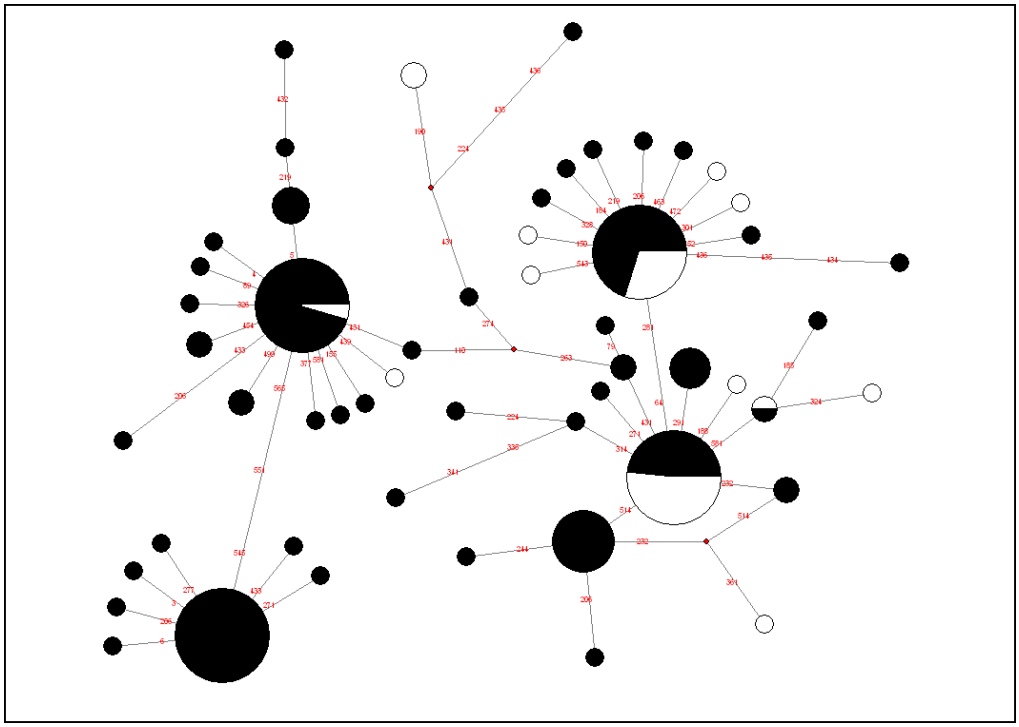


Figure 5: Mitochondrial DNA haplotype network by microsatellite cluster. Red numbers along branches represent mutations. Black represents cluster 1 (Popham Bay, Trepang Bay, Port Essington, Raffals Bay, Malay Bay, South Goulburn Island, Guion Point, Bowen Straight, Croker Island and Millingimbi) whilst white represents cluster 2 (Gove Harbour, Groote Eylandt and Nalwrung Strait).

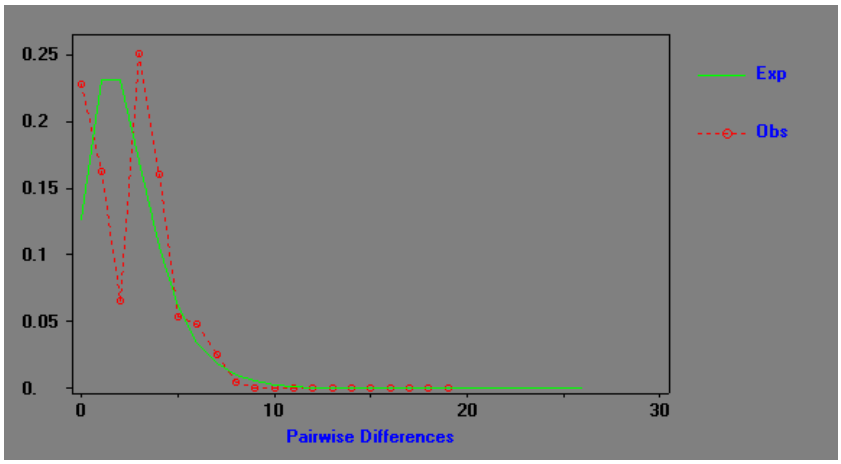


Figure 6: Mismatch distribution for ND4 haplotypes in *Holothuria scabra*. The expected frequency is based on a population growth-decline model (initial theta = 1.165, final theta = 1000, tau = 1.298).

3.2 Broodstock and Hatchery

All microsatellite loci were polymorphic except for Hsc12 which was monomorphic in both the broodstock and the wild-caught population. The number of alleles per locus ranged from 1 (Hsc12) to 29 (Hsc28) in the wild-caught population and 1 (Hsc12) to 19 (Hsc40) in the broodstock. The average number of alleles in the wild-caught population was 9.110 and 8.222 in the broodstock. Observed heterozygosities ranged from 0.00 (Hsc12) to 1.00 (Hsc31) in both the wild-caught population and the broodstock (Table 9).

Simulation studies in CERVUS indicated that when all 18 loci are used there is a very high probability of Assignment (Fig 7). This is reduced substantially when only the 9 loci that amplified in the broodstock (Fig. 8) were used. The parentage assignments revealed that the true parent pair, with unknown sexes, would be expected (by simulation) to be found 40% of the time using genotypes from nine microsatellites that amplified successfully in the majority of the broodstock. However, all 123 progeny were assigned to a parental couple without uncertainty. We determined that 30 breeders (75%) took part in reproduction with 5 breeders contributing to 71% of the offspring.

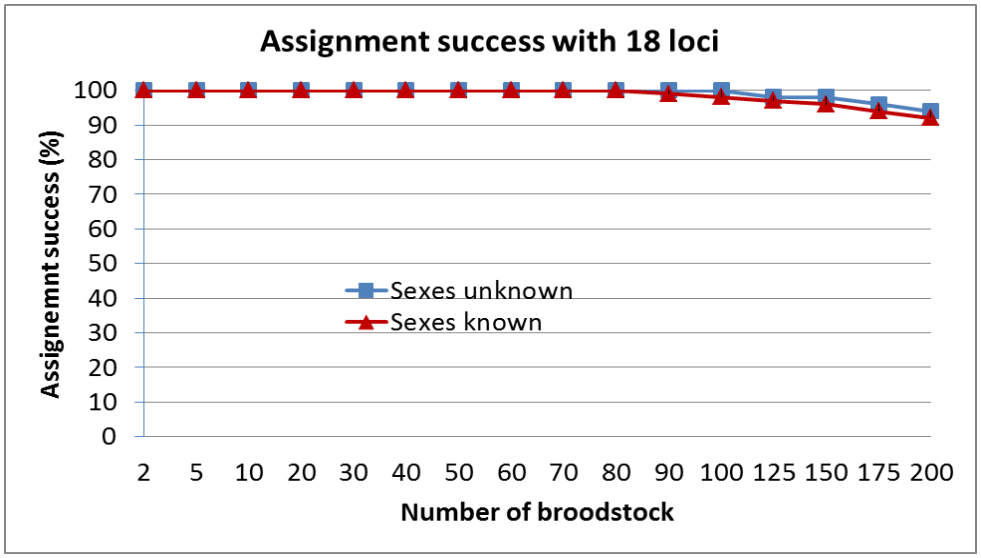


Figure 7: Simulated assignment success with the 18 microsatellite loci used in the study.

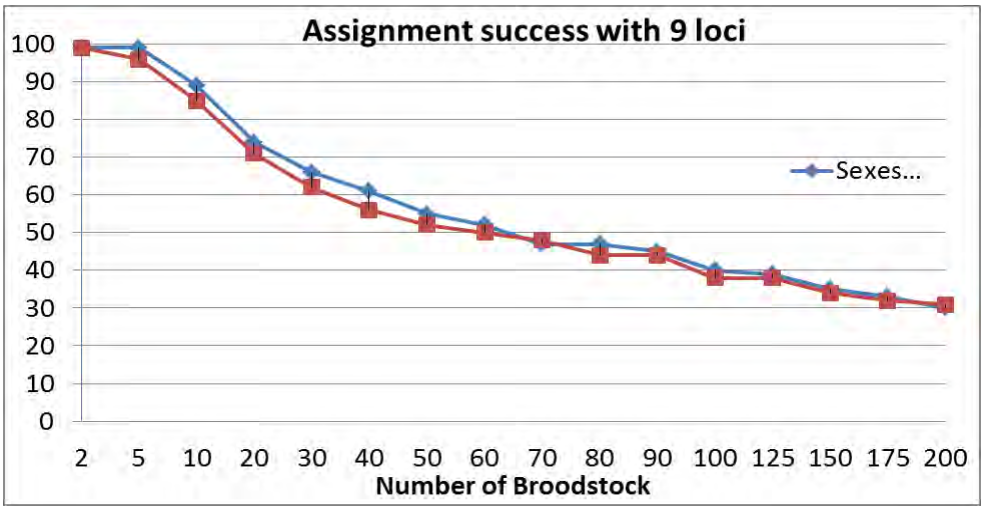


Figure 8: Simulated assignment success with the 9 loci that amplified in the broodstock.

Table 9: Summary statistics for genetic variation between the broodstock and the wild-caught population at 18 microsatellite loci. Given are numbers of observed alleles (*Na*); proportions of observed and expected heterozygosity (H_O and H_E respectively), the inbreeding coefficient (F_{IS}) and the frequency of null alleles (*F*(null)). NA is shown where data is not available due to a monomorphic locus.

		Locus																		
Locality		Hsc1	Hsc11	Hsc12	Hsc14	Hsc17	Hsc20	Hsc24	Hsc28	Hsc31	Hsc4	Hsc40	Hsc42	Hsc44	Hsc48	Hsc49	Hsc54	Hsc59	Hsc62	Mean
K	*Na*	7	5	1	2	7	15	12	29	2	7	27	3	10	5	12	4	8	8	9.110
(Groote Is.)	H_O	0.714	0.438	0	0.026	0.308	0.917	0.8	0.553	1	0.396	0.938	0.578	0.792	0.521	0.667	0.051	0.75	0.48	0.552
	H_E	0.712	0.495	0	0.026	0.786	0.911	0.834	0.956	0.507	0.728	0.954	0.494	0.805	0.657	0.842	0.495	0.768	0.804	0.654
	F_{IS}	-0.004	0.116	NA	0	0.612	-0.007	0.042	0.425	-1	0.459	0.017	-0.172	0.017	0.209	0.21	0.898	0.024	0.408	0.159
	F(null)	0	0.058	NA	0	0.426	-0.01	0.007	0.262	-0.33	0.296	0.005	-0.1	0.013	0.125	0.12	0.813	0.016	0.239	0.114
Broodstock	*Na*	11	4	1	4	5	18	8	10	2	10	19	4	7	5	17	4	13	6	8.222
	H_O	0.545	0.5	0	0.105	0.375	0.75	0.611	0.357	1	0.529	0.429	0.235	0.81	0.393	0.758	0.286	0.8	0.5	0.499
	H_E	0.855	0.416	0	0.245	0.756	0.923	0.835	0.913	0.514	0.78	0.951	0.316	0.856	0.714	0.89	0.374	0.878	0.832	0.669
	F_{IS}	0.368	-0.206	NA	0.576	0.512	0.19	0.274	0.618	-1	0.324	0.556	0.26	0.056	0.455	0.151	0.239	0.091	0.412	0.228
	F(null)	0.204	-0.130	NA	0.448	0.323	0.099	0.152	0.410	-0.333	0.190	0.373	0.118	0.017	0.282	0.077	0.103	0.035	0.234	0.153

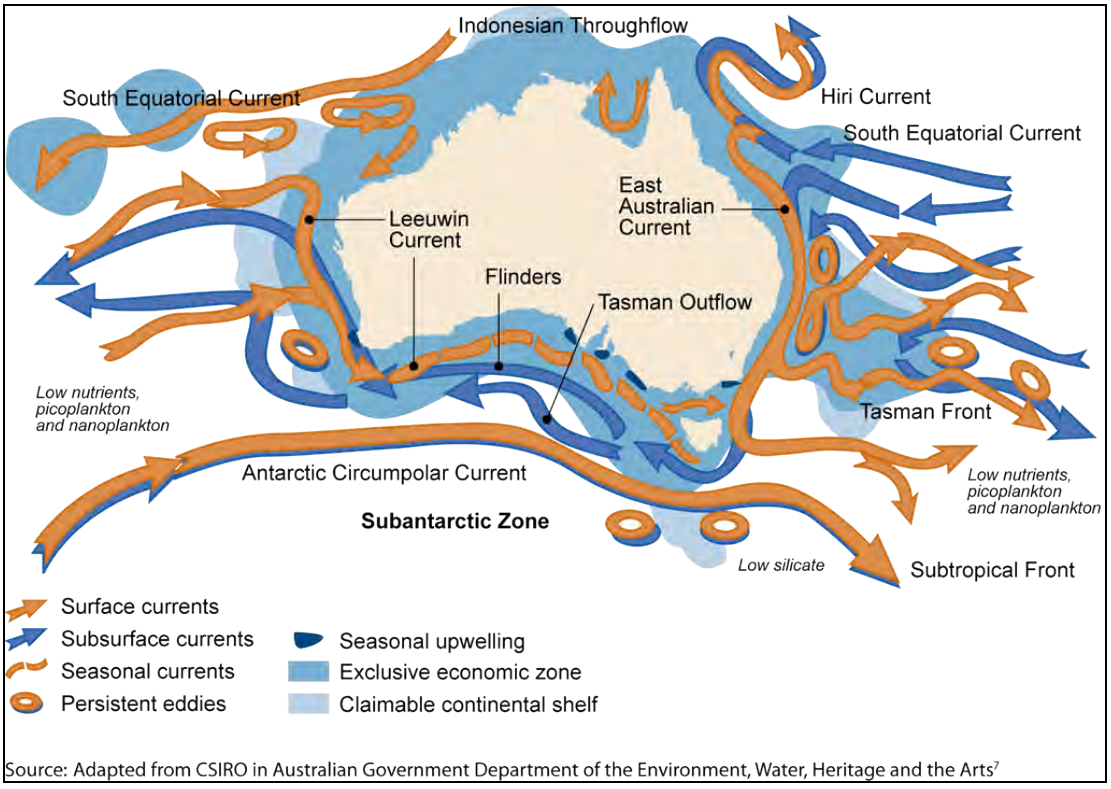
4. Discussion

This study investigated gene flow in *H. scabra* populations with a view to increasing knowledge of the population structure of this commercially important species and to assist the sustainable management of the existing fishery and planned restocking program. Previous studies in *H. scabra*, using allozyme electrophoresis, have found both high levels of gene flow between separated populations 16 km apart as well as restricted gene flow between populations greater than 800 km apart (Uthicke and Benzie, 2001). The time span of planktonic larval development is often considered an important factor in dispersal capabilities in the marine environment (Hedgecock, 1986; Doherty *et al.*, 1995). However, larval development in *H. scabra* takes 10 to 14 days, well within the range of that for other invertebrates which show little population differentiation (Uthicke and Benzie, 2001). The high dispersal capability of *H. scabra* has the potential to allow for large amounts of gene flow, however, dispersal of larvae may be effected by the existence of hydrological and ecological barriers, such as currents, temperature, salinity (Duran *et al.*, 2004) as well as length of larval life and larval behaviour (Whitaker, 2004). Therefore, it is difficult to make *a priori* judgements with regard to the population structure of marine species such as *H. scabra* that have a wide geographical range with complex hydrography.

The results of this study suggest that there are two distinct genetic clusters of *H. scabra* in the waters off the NT with generally high gene flow within each cluster. Analysis of microsatellite data indicate that *H. scabra* populations along the northern coast of Australia from Popham Bay to Groote Eylandt can be grouped into two genetically distinct stocks: cluster 1, the Arafura Sea, from Popham Bay to Millingimbi; and cluster 2, the Gulf of Carpentaria, from Nalwrung Strait to Groote Eylandt. Thus our study supports the findings of (Uthicke and Benzie, 2001) and (Uthicke and Purcell, 2004) that significant genetic differences can exist in *H.* scabra populations over relatively short distances. In contrast, however, the mtDNA sequence data does not show the same differentiation but does indicate population growth. These differences between the mtDNA and the microsatellite data may be due to the relative timeframes these two genetic markers reflect. Mitochondrial DNA generally evolves at a slower rate than microsatellite loci and so, therefore, reflects older events, such as restrictions in gene flow during the last ice-age, 22,000-19,000 years before present, when sea levels were 120m below their present levels (Grosjean *et al*., 2007). The *ND4* network showed a tight assemblage of haplotypes separated by a small number of mutations. Some haplotypes were abundant and have given rise to a large number of related and rare haplotypes, a pattern consistent with a recent population expansion (Avise, 2000), a pattern supported by the mismatch distributions. This pattern, of a few frequent and many low frequency haplotypes with few differences, has been observed in other marine invertebrates, including other species of Sea Cucumber (Uthicke and Benzie, 2003; Duran *et al.*, 2004; Lejeusne and Chevaldonne, 2006).

The Arafura Sea is a semi-enclosed, shallow, continental shelf sea, generally less than 200 m deep, between northern Australia and Indonesia (Grosjean *et al.*, 2007). Climatically, this sea is tropical and experiences the relatively stable trade winds during part of the year and intermittent monsoonal flows during the southern hemisphere summer. It has a warm-water current flowing from the Pacific into the Indian Ocean called the Indonesian Throughflow. This current has a substantial influence on the climate of the entire region because it transports heat and moisture to the Indian Ocean and adjacent land. Rivers drain onto the topographically lower lying Arafura Basin, situated just west of the Wessel Islands (Grosjean *et al.*, 2007). Present day currents are shown in Figure 9. Given the broadcast spawning behaviour of *H. scabra* it is possible that the two genetic clusters found in our study are the result of the passive dispersal of *H. scabra* larvae due to the prevailing currents. Alternatively, in general, echinoderms are considered species with low adaptation to salinity

changes (Vergara-Chen *et al.*, 2010) and, in particular, there are numerous reports of *H. scabra* being found preferentially in areas of low salinity (Hamel, 2001). The potential change in salinity, during the summer, monsoonal, spawning months, in the Arafura Basin, located between genetic clusters 1 and 2, may act as a barrier to larval dispersal and, therefore, gene flow. Further information on currents needs to be obtained to understand the contribution of currents to gene flow within the species.



Source: Adapted from CSIRO in Australian Government Department of the Environment, Water, Heritage and the Arts[7]

Figure 9: Ocean currents around Australia (CSIRO Australia).

Interestingly genetic variation was consistently high at the intrapopulation level, as indicated by the AMOVA. This is interesting as populations of *H. scabra* have been fished here for a number of years and under this pressure genetic diversity would be expected to be reduced through population bottlenecks and genetic drift (Dailianis *et al.*, 2011). The maintenance of high intrapopulation genetic variability in our data could be explained by adequate levels of population connectivity, the potential effect of regeneration of partially harvested individuals and/or the existence of robust populations that could promote recolonization of affected areas (Dailianis *et al.*, 2011).

Analyses have identified that microsatellite markers showed significant heterozygote deficiency within populations which could be the result of null alleles although other population level processes such as Wahlund effect and deviation from panmixia are also possible. Deviations from Hardy-Weinberg equilibrium is found fairly commonly in marine invertebrates (Addison and Hart, 2005; Plutchak *et al.*, 2006; Whitaker, 2006; Yasuda *et al.*, 2009; Dailianis *et al.*, 2011; Kang *et al.*, 2011) though causal links remain unclear. Regarding the possible presence of non-amplifying alleles, it is worth mentioning that a low percentage of failed amplifications were observed and that the calculated error rate was due to failed amplifications. There was also no evidence of scoring errors, of large allele drop out and markers were found to show Mendelian inheritance.

Following the development of culture methods for *H. scabra*, many countries are now considering restocking with hatchery produced juveniles as a way to accelerate the recovery of sizeable breeding populations (Uthicke and Purcell, 2004). This strategy raises concerns about altering the genetic diversity of remnant stocks as it may be desirable to translocate the hatchery-produced juveniles to sites other than where the adult broodstock were collected (Uthicke and Purcell, 2004). Release of hatchery produced animals can cause reduced genetic diversity in wild stocks by introgression of exogenous alleles, caused either by interbreeding of animals or the outcompeting of native alleles by the introduced alleles, or by outbreeding depression, when hybridisation of two genetically distinct populations results in reduced fitness.

Microsatellites, because of their high rates of variation, are a useful tool for determining parentage assignments and rates of inbreeding in aquaculture even in large commercial mass spawning tanks (Herlin *et al.*, 2007). Only six of the nine loci used here were in HWE, the other loci showed significant heterozygote deficits, however, parentage assignments using CERVUS v3.0.3 have been improved to take possible genotyping errors into account and to minimise errors (Kalinowski *et al.*, 2007).

The effect of the unequal reproductive success on the effective breeder number estimates (those taking part in reproduction) should be noted. While 30 of 40 (75%) breeders left descendants in this work, only five of 40 breeders (13%) are effectively taking part in reproduction (responsible for around 71% of the descendants). Inbreeding in culture stocks has serious consequences when used for supportive breeding programs (Utter and Epifanio, 2002). Using a small fraction of the wild parental individuals for hatchery production may favour the reproductive rate of one segment of the overall population, thus increasing the total variance of family size. While such programs may immediately increase the absolute abundance of wild populations, it may threaten genetic diversity through reduction of effective population sizes (Borrell *et al.*, 2011). One way to mitigate the potential side effects of low diversity in progeny from large contributions from a small number of brood would be to separate brood into smaller numbers of brood lots for spawning and then mix the resultant progeny before release. However the spawning was atypical (it was performed outside the main spawning season and involved collection of eggs on only one day) and further work needs to be carried out to truly asses the contribution of broodstock to progeny. Additionally, it would be of benefit to understand if there is differential survival of progeny from different broodstock.

5. Benefits and Adoption

A workshop was held on the 24th of May to disseminate the results of this report. The workshop was attended by the key stakeholders including senior representatives of Tasmanian Seafoods and the NT Dept. of Resources. A summary of this meeting is attached as Appendix A.

The results of this project will benefit Tasmanian Seafoods by proving them with information on population genetic stocks so that stock enhancement activities can take place in the future making sure progeny from broodstock taken from one wild genetic stock are released into areas where the same genetic stock exist. Having this knowledge will enable them to develop an appropriate genetic management strategy which will be critical to obtaining regulatory approval for commercial scale ranching of *H. scabra* in NT.

The information regarding the population genetic stock delineation has been delivered to the relevant agency (NT Department of Resources, Fisheries) and they will consider changing the management zones to better reflect the population genetic stocks uncovered in this study. As the current zones are fairly close to what was uncovered, the implementation of this should be relatively simple. NT DoR Fisheries will also use this knowledge of the genetic structure of *H. scabra* populations to inform their future policy development on enhancement of Sea Cucumbers and in reviewing Notifications of Intent to ranch them.

6. Further Development

1. Further genotyping of progeny arrays to more accurately represent typical spawning should be carried out to further inform the development of a genetic management strategy for a commercial scale Sea Cucumber hatchery.
2. Clarification of a target effective population size of broodstock to produce juveniles for ranching.
3. Areas for intended ranching activities should be clarified.
4. Standing stocks should be estimated in areas targeted for ranching.
5. Tasmanian Seafoods to develop a genetic management plan for hatchery reared Sea Cucumbers in consultation with NT Fisheries, Seafood CRC and Flinders University representatives.
6. Information on local currents should be sourced to identify potential drivers of population genetic structure.
7. The area (around Elcho Island NT) between the two stocks identified could be sampled more intensively to allow an understanding of how abruptly the population genetic structure changes from one stock to the next.
8. As no information was obtained from areas of the NT coastline West of Popham Bay, further work could be done to assess the western extent of the genetic populations in that region.

7. Planned Outcomes

7.1 Public Benefit Outcomes

No public benefit outcomes were identified although the maintenance of genetic diversity of wildstock and their informed management is of indirect public benefit.

7.2 Private Benefit Outcomes

Tasmanian Seafoods benefited from the knowledge of population genetic structure in the areas they currently fish *H. scabra* from. This information can be used to inform future stock enhancement programs. This information will form the basis of a genetic management plan that Tasmanian Seafoods will produce to apply for permits required to undertake ranching activities. NT Fisheries benefit by a more thorough understanding of the population genetic stock of areas currently being fished and can use this information to create an appropriate regulatory framework make changes to the management of the fishery in the NT.

7.3 Linkages with CRC Milestone Outcomes

The project linked to the following CRC milestone outcomes:

1.2.1 Milestone. Key constraints to increased production characterised and research prioritised in at least one selected fishery

This project provided key research into the population genetic structure of the Sea Cucumber fishery in NT which was identified as a constraint to the development of stock enhancement.

1.2.2 Milestone. Production interventions implemented in at least one fishery.

This project provided information for use in the ranching of Sea Cucumbers in the NT.

1.3.1 New genetic tools developed for genetic management and improvement of at least two aquaculture species

This project delivered genetic tools suitable for stock delineation and assessment of the contribution of broodstock to progeny arrays.

7.4 Planned Publications

At least two publications in peer reviewed journals are planned for the work. The first is a Primer Note outlining the development of the microsatellite loci. This manuscript is in the final stages of preparation and is titled “Development of eighteen microsatellite markers for the commercially valuable Sea Cucumber, *Holothuria scabra* (Echinodermata: Holothuriidae).” The first draft of this manuscript is in Appendix 2 and will likely be submitted to the Australian Journal of Zoology.

The second manuscript will based on the results of the stock delineation and is yet to be prepared. No journal has been selected yet for the submission of this second manuscript.

8. Conclusion

In this study the genetic structure of *H. scabra* in the waters of the NT was revealed using microsatellite markers. Two genetic populations were found, within the Arafura Sea and the Gulf of Carpentaria, with significant differentiation. These results suggest that larval dispersal is limited to within the two regions: the Gulf of Carpentaria and the Arafura Sea. Given this study found two distinct populations of *H. scabra* it is recommended that this species be considered and managed for now as two separate, genetically distinct stocks.

We also found that a panel of nine of our microsatellite markers could be used to assign parentage confidently amongst the hatchery progeny. The partial dominance of some of the breeders in progeny of pooled matings should be taken into account, however, when establishing closed-cycle cultures of *H. scabra.* Effective genetic management will be necessary to maintain adequate effective population sizes in hatchery stocks to avoid inbreeding and to minimise the potential impact on genetic diversity of wild stocks in areas where aquaculture reared Sea Cucumbers are stocked for ranching. One way to mitigate the potential side effects of low diversity in progeny from large contributions from a small number of brood would be to separate brood into smaller numbers of brood lots for spawning and then mix the resultant progeny before release.

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Appendix A: Meeting summary: Final dissemination of results of the Sea Cucumber population genetics project (Seafood CRC Project No. 2008/733)

Venue: NT Fisheries' conference room, Goff Letts Building, Berrimah Farm, Darwin.

Attendees: Andrea Birch[1]; Will Bowman[2]; Alison Fitch[3]; Ann Fleming[1]; Mike Gardner[3]; Cameron Hartley[1]; Grant Leeworthy[2]; Graham Mair[4]; Steven Matthews[1]; Evan Needham[1]; Jim Prescott[5]; Thor Saunders[1]; Glenn Schipp[1]; Luke Turner[2]

[1]NT Department of Resources - Fisheries; [2]Tasmanian Seafoods; [3]Flinders University; [4]Seafood CRC; [5]Australian Fisheries Management Authority

Meeting Chair: Ann Fleming

Objectives of meeting identified before and at the start of the meeting:

1. Enhance understanding of the application of population genetic data and its role in fisheries management.
2. Report the findings of the population genetics study.
3. Use of working group session to develop and refine management recommendations for the *H. scabra* fisheries consistent with known stock delineations and dispersals.
4. Use working group sessions to define and develop genetic management strategies for propagated stock and guidelines for stocking of propagated juveniles based on known population structure.
5. Discuss implications of results from project for policy and industry practice.
6. Establish next steps to be taken/gaps in knowledge.

Outline of meeting:

Introduction of attendees and their expectations of what the meeting should achieve.

Mike Gardner delivered a PowerPoint presentation on results of the project with discussion taking place during the talk.

Steve Matthews provided further information on the delineation of the Sea Cucumber fishing zones.

Discussion on implications followed with all attendees participating.

After the meeting, several attendees (Alison Fitch, Mike Gardner, Graham Mair) were shown around the Darwin Aquaculture Centre by Tasmanian Seafoods employees.

Further discussion took place with Ann Fleming.

General points raised in meeting discussion:

Discussions surrounding population genetic structure identified:

The idea that more genetic populations may exist in areas not sampled was raised. Potentially the small local populations would then not experience new recruitment as would other stocks from localities with a larger genetic population.

The issue of understanding the abruptness of change from one genetic stock to the other was raised. It was noted that this could be clarified from more sampling and population genetic analysis in areas west of those sampled and around the Wessel Islands up to Millingimbi.

Additional sampling to identify the membership of Sea Cucumber stocks west of the sampling localities in this project was also raised as a worthwhile venture for future work to understand not only the full extent of the NT genetic populations but also for WA stocks.

Will recruitment occur in localities depleted in stock? Potentially yes, if they are areas under study but no comment can be made on areas not within the sampling regime of the project.

The need to ensure that stock enhancement activities would not lead to a homogenising of the wild stock – this ties back into two things, ensuring the effective population size of the progeny to be released is large and also understanding the relative proportion of the released animals compared with the size of the standing stock. If the proportion of released animals is very high compared to the standing stock then the potential effect of swamping the area with hatchery produced genotypes would be greater.

Coupled with this idea is the continued use of broodstock collected from the wild rather than from captive bred animals. Additionally, the idea that broodstock should be used on some sort of rotational basis to ensure the contribution from a few breeders doesn't dominate the progeny released.

The understanding of what drives the existence of the two genetic populations would be enhanced by understanding the localised currents in the regions under study. Potential contacts are David Williams and Dan Alongi from AIMS and also the CSIRO website. (Post meeting Jim Prescott forwarded an excerpt from an ATSEA biophysical report to Mike Gardner).

A genetic management plan for the Sea Cucumber enhancement should be produced by the industry partner in consultation with NT Fisheries officials and the Seafood CRC plus Flinders University. Under this management plan it was discussed that the ability to export product from fisheries enhancement activities needs to be established – there can be no industry if the exportation of product is not allowed by the Commonwealth.

Other potential projects were discussed including the engagement of the Sea Cucumber research ongoing in Indonesia – also to interact with Didi and Jim Fox from ANU who are working on Roti Island with ranching of *H. scabra*.

Discussion relating specifically to Broodstock:

What is the effective population size of the progeny to be released into the wild areas? – the idea that an effective population size of 200 has been cited in the literature.

The need for understanding of the standing stock of Sea Cucumbers in areas where the enhancement of stock was discussed as critical. The proportion of hatchery reared stock to be released compared with the standing stock will impact upon any potential swamping of local genetic diversity.

It became evident that the spawning event used for the broodstock/progeny analysis in the genetics study was atypical due to cyclonic events prior to the spawning attempt causing broodstock to be in poor condition.

Two possibilities were raised: 1) redo the broodstock/progeny genotyping and analysis with more typical spawning events to more accurately represent the normal situation; 2) take the current information as a worst case scenario.

It was pointed out that the reduced contribution of some broodstock to the spawning event may also be due to poor survival of some progeny. The potential for assessing the broodstock contribution at several different life stages, particularly before known high mortality events during the hatchery rearing phase was discussed.

There is a need for the genetic diversity of the progeny released to match the wild stock – such that progeny should be from the same genetic population identified within the study and also that the genetic diversity of progeny needs to be kept at levels which match those found in the wild. Additionally, the areas of release should not be near the boundaries of the two stocks such that unwanted mixing of stocks occurs.

Discussions surrounding policy:

Glenn Schipp identified that the release of a policy on fisheries enhancement, focusing on broad scale (not specific to species) ranching, stock enhancement and restocking was imminent. The type of information that NT Fisheries would require to make a decision would include economic and social benefit and environmental impact. A genetic management plan would be part of the requirement of a permit application. This plan would need to show that the genetic integrity of wild populations would be maintained. Some level of genetic monitoring would be required. A smaller scale pilot study would be required as the initial step.

Next steps:

1. Further genotyping of progeny arrays to more accurately represent typical spawning should be carried out.
2. Clarification of target effective population size of broodstock.
3. Areas for intended ranching activities should be identified.
4. Standing stocks should be estimated in potential ranching areas.

5. Tasmanian Seafoods to develop genetic management plan in consultation with NT Fisheries, Seafood CRC and Flinders University representatives.
6. Information on local currents should be sourced to identify potential drivers of population genetic structure.

Appendix B: Primer Note Draft for submission to Australian Journal of Zoology.

Development of eighteen microsatellite markers for the commercially valuable sea cucumber, *Holothuria scabra* (Echinodermata: Holothuriidae).

We isolated 18 new polymorphic microsatellite markers from *Holothuria scabra*, a commercially important species of Sea Cucumber found throughout the Asia-Pacific region. Next generation sequencing was used to identify 206 unique loci for which primers could be designed. Of these unique loci we trialled 65 in the target species, 48 (74%) amplified a product of the expected size. Eighteen loci were found to be polymorphic and reliable, and were screened for variation in 50 individuals, from a single population from Croker Island, Northern Territory, Australia. Observed heterozygosity ranged from 0.00 to 0.96 (mean 0.46) and the number of alleles per locus from 2 to 28 (average of 9.61). These loci should be useful for investigation of population structure and mating systems in *H. scabra* and may also be of use in other holothurian species.

Keywords: *Holothuria scabra*; sea cucumber; microsatellites; GS-FLX; next generation sequencing.

The Sea Cucumber *Holothuria scabra*, commonly called the sandfish, is a commercially important species found throughout the Asia-Pacific region and marketed under the name bêche-de-mer, trepang or haisom. With the economic development of the People's Republic of China and improved living standards over the last 20 years the demand for bêche-de-mer has increased both in China and in Chinese communities around the world (Chen 2004, Friedman *et al*. 2011). *Holothuria scabra* is easily harvested from inshore habitats and is vulnerable to over-exploitation, as has been shown for commercially important species of sea cucumbers around the world (for examples see: Battaglene and Bell 2004, D'Silva 2001, Gamboa *et al*. 2004, Hamilton and Lokani 2011, Uthicke *et al*. 2004). Background information on the fisheries biology and recruitment patterns of *H. scabra* are scarce and thus current regulations governing Sea Cucumber fisheries tend not to be well informed. At present management is based on assuming one stock along the Northern Territory coastline and is by limited access, area restrictions based on an arbitrary line on the water and trigger limits based on historical data. Basic research including knowledge of population structure is required to enable informed decisions on the sustainable management of existing fisheries.

Genomic DNA was extracted using the Gentra Puregene Tissue Kit (Qiagen, Australia), following the manufacturer's instructions, from the tube-feet of two *H. scabra* individuals collected from waters off the Northern Territory of Australia. The DNA was sent to the Australian Genomic Research Facility (AGRF, Brisbane, Australia) for next generation sequencing on a Titanium GS-FLX (454 Life Sciences/Roche FLX) following Gardner *et al*. (2011). The sample occupied 12.5% of a plate and

produced 184,954 individual sequences, with an average fragment size of 369 bp, 3.3% of which were found to contain microsatellites. Sequences were screened for di-hexanucleotide repeat classes with ≥ 8 tandem repeats using MSATCOMMANDER v0.8.2 (Faircloth 2008) and primers were designed using Primer3 (as automated within MSATCOMMANDER) (Rozen and Skaletsky 2000). To identify and exclude loci with flanking region similarities, which may represent loci which are not necessarily unique, sequences for which primers had been designed were then analysed in the program MICROFAMILY v1.2 (Meglécz 2007).

Following the procedure outlined in Gardner *et al.* (2011) 65 loci were chosen for further development. Initially the loci were trialed for amplification in three individuals of *H. scabra* using the multiplex-ready PCR (MRT) method as described in Hayden *et al*. (2008). Forty-eight (74%) loci amplified an unambiguous product of the expected size. These 48 loci were then tested for polymorphism in six individuals selected from across the sampled range on a 5% polyacrylamide gel. Of the 48 loci, 22 (46%) loci were polymorphic, 1 (2%) was monomorphic, 9 (19%) did not amplify, and alleles in 16 (33%) loci were unclear.

Eighteen loci were selected for genotyping from the 22 polymorphic loci, each forward primer was labelled with a fluorescent tag at the 5′ end: FAM (GeneWorks), NED, PET or VIC (Applied Biosystems) and these 18 loci then screened for variation in 50 individuals from a single population from Croker Island, Northern Territory, Australia using the Qiagen® Multiplex PCR Kit (Qiagen, Australia). DNA from these individuals was extracted from either body wall or longitudinal muscle tissue using a modified cetyltrimethylammonium bromide (CTAB) method (Doyle and Doyle 1987). Multiplex PCR amplifications (between 3-4 loci per reaction, Table 1) were performed in 10 µL volumes containing template DNA, 1X Qiagen Mulitplex PCR Master Mix and 0.15-0.35 µM of each primer (Table 1). Amplifications were performed using the following cycling protocol: 95 ^{0}C for 15 min; 35 cycles of 94 ^{0}C for 30 s, 57 ^{0}C for 90 s, 72 ^{0}C for 1 min; 60 ^{0}C for 30 min. PCR products from each individual were first pooled and then separated on an AB3730 DNA Analyser (Applied Biosystems). Microsatellite alleles were sized according to an internal standard, GeneScan 500LIZ in the program GENEMAPPER v4 (Applied Biosystems).

For each locus we calculated the number and range of alleles, observed and expected heterozygosity, polymorphic information content and estimated null allele frequencies using CERVUS v3.0.3 (Kalinowski 2007) and deviation from Hardy-Weinberg Equilibrium (HWE) using GENEPOP v4.1.10 (Raymond and Rousset 1995) (Table 1). *P* values from HWE tests were adjusted for multiple tests of significance using the sequential Bonferroni method (Rice 1989). We used MICRO-CHECKER v2.2.3 (Van Oosterhout *et al.* 2004) to check each locus for further evidence of null alleles, scoring error due

to stuttering and large allele drop out. Twelve loci showed significant null allele frequencies possibly due to heterozygote excess and three showed the possibility of scoring error due to stuttering due to a lack of heterozygotes. None of the loci showed evidence for large allele drop out. We checked all pairs of loci for linkage disequilibrium in GENEPOP v4.1.10 and none were significant after sequential Bonferroni adjustment. These molecular markers will be useful for investigation of population structure, levels of gene flow and mating systems which will provide valuable information for fisheries management and development of a sustainable fishery.

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Table 1. Characterisation of polymorphic loci. Primer sequences, PCR multiplex, PCR primer concentration of each locus primer, GenBank accession number, repeat motif, and diversity characteristics of 19 microsatellite loci from *Holothuria scabra*. N indicates the sample size; Na indicates number of alleles; *Ho* and *He* indicate observed and expected heterozygosities respectively; PIC indicates polymorphic information content; HWE indicates Hardy-Weinberg equilibrium. * indicates significance after Bonferroni correction. Superscripts F, N, V, and P indicate primers were labelled with the dyes 6-FAM, NED, VIC and PET respectively.

Locus		Primer sequence (5'-3')	PCR multiplex	Primer conc^{n} (µM)	GenBank accession no.	Repeat motif	N	Allele size range	Na	*Ho*	*He*	PIC	Null allele freq	HWE *P*
Hsc1	F:	^{N}CTTAGTCTGGTACGGTTGTCC	2	0.15		$(GT)_8$	24	79-105	5	0.250	0.682	0.607	+0.452	0.000*
	R:	GTTTACTAAGCAAGTGTCACAAAC												
Hsc4	F:	^{F}AGAGCATGTATGTATCATCGAAACC	4	0.20		$(GT)_{11}$	38	111-127	8	0.263	0.676	0.624	+0.448	0.000*
	R:	AAACGGAACGGAACAAGCC												
Hsc11	F:	^{F}TGTTCATAGAGGGAATGTGAGG	1	0.15		$(GT)_{12}$	50	150-168	6	0.400	0.396	0.374	+0.007	0.408
	R:	CGTTGAGTTAGAGCGTACCG												
Hsc12	F:	^{V}TGAGGTCATCTGTTCGCCC	1	0.15		$(GT)_{14}$	46	158-164	3	0.043	0.085	0.082	+0.288	0.022
	R:	CCTACGCATTTCGATCCGTC												
Hsc14	F:	^{N}GGGCATGTAGGCAAACTCTTC	1	0.20		$(AC)_{13}$	18	154-164	2	0.000	0.108	0.099	+0.765	0.029
	R:	TATTGCTCCAGTTCGCCCC												
Hsc17	F:	^{P}AGATTCATTTGGGAACTTTGGC	4	0.20		$(AC)_{11}$	38	185-199	7	0.211	0.729	0.674	+0.547	0.000*
	R:	AGGGTTGATGTAAGCTGCG												
Hsc20	F:	^{F}TGCGTGTGGTGATTTTGAC	2	0.15		$(AC)_{12}$	47	188-228	17	0.872	0.930	0.914	+0.025	0.139
	R:	ACCATTCTACAGCTCGTCCC												
Hsc24	F:	^{V}TCCTTCGTCGCAGCATGAC	4	0.20		$(AC)_{18}$	43	193-219	11	0.628	0.849	0.821	+0.142	0.056
	R:	TTCTTGTATTCCTTTGCAGGC												
Hsc28	F:	^{P}TTCTGGTCTCGACTGGCAC	5	0.30		$(GAT)_{15}$	41	217-484	28	0.317	0.952	0.937	+0.499	0.000*
	R:	TCAGTATCGGCTCCACAGG												
Hsc31	F:	^{V}TGTAGGTAGGTAGGTAGGTATGTATG	5	0.30		$(ATGT)_{11}$	40	170-176	3	0.925	0.515	0.391	-0.295	0.000*
	R:	CAGCAGTGGGTTTGGACAC												
Hsc40	F:	^{P}GCATTGATCATGTGGAATTTGCG	3	0.35		$(ATGT)_{13}$	49	233-345	26	0.959	0.954	0.941	-0.008	0.723
	R:	CACCATAGACCTGGCTTGC												
Hsc42	F:	^{N}TCCTTCGTCGCAGCATGAC	5	0.25		$(AC)_{18}$	47	248-254	4	0.191	0.251	0.236	+0.118	0.107
	R:	TTCTTGTATTCCTTTGCAGGC												
Hsc44	F:	^{F}GACGGTACGTCACCAGAGG	5	0.30		$(AAAC)_9$	50	170-198	8	0.760	0.797	0.760	+0.019	0.648
	R:	TTCTTCGTCTTTTGGCGGG												
Hsc48	F:	^{V}ACAATGCGGACGACAATGG	4	0.15		$(GT)_8$	48	151-163	5	0.375	0.619	0.579	+0.226	0.000*
	R:	ATCGTGTTTACAAGCGGGC												
Hsc49	F:	^{P}TGAGCACGGTGTATTGTCC	2	0.20		$(ACAG)_8$	46	157-237	18	0.674	0.930	0.914	+0.152	0.000*
	R:	TGATGTGAGCCACTGCG												
Hsc54	F:	^{F}AGACAGTTGTGGGAAGGGC	3	0.20		$(CTGT)_8$	48	190-206	3	0.125	0.192	0.178	+0.202	0.039
	R:	TGGATGGAATAACAATAGGTGTCC												
Hsc59	F:	^{F}AGAGCACACGTATCCCCAC	3	0.25		$(AC)_8$	49	234-264	13	0.816	0.837	0.812	+0.010	0.720
	R:	GGGGCAGGATAGAGCACATAG												
Hsc62	F:	^{V}AGCTAGCAGGGAAAAGAAGAAAG	2	0.20		$(AT)_8$	31	245-255	6	0.484	0.739	0.687	+0.188	0.003*
	R:	AGAGGCGGATGCTCTTACC												