Final report to the Department of the Environment, Water, Heritage and the Arts

Population genetic structure of Australian Galapagos reef sharks *Carcharhinus galapagensis* at Elizabeth and Middleton Reefs Marine National Nature Reserve and Lord Howe Island Marine Park

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1. Executive summary

The present study is the first to investigate genetic structure of the Galapagos reef shark *Carcharhinus galapagensis*. The only Australian populations of this apex predator occur at the Elizabeth and Middleton Reefs Marine National Nature Reserve and Lord Howe Island Marine Park, which includes Balls Pyramid. An insight into the Australian population genetic stock structure of this worldwide patchily distributed shark will inform management and conservation strategies, specifically at the Elizabeth and Middleton Reefs Marine National Nature Reserve and the Lord Howe Island Marine Park.

The aim of this study was to determine if *C. galapagensis* present at Middleton Reef (n=28), Elizabeth Reef (n=29) and Lord Howe Island (n=29) form a single or multiple stocks. The non-coding mitochondrial DNA (mtDNA) control region was sequenced for all individuals sampled and revealed 21 polymorphic sites and 36 haplotypes. Significant population structure was identified by an analysis of molecular variance (AMOVA), Fst = 0.5416, P<0.0001, confirming the presence of multiple Australian stocks. The Elizabeth and Middleton Reefs population forms a single stock, which is distinct from the Lord Howe Island population. Haplotype and nucleotide diversities differed substantially between these stocks (h= 0.186; π = 0.29% $\pm$ 0.002 and h= 0.933; π = 1.18% $\pm$ 0.006 respectively) and suggest that the Elizabeth-Middleton Reef population has low genetic resilience compared to the Lord Howe population. The overall diversity of Australian *C. galapagensis* mtDNA control region was h = 0.5717, which is relatively high compared to other sharks but, low when compared to marine teleosts. The low genetic diversity in sharks is attributed to various factors such as their role as apex predators with low effective population size, their slow life history, low fecundity and long generation time. Coalescence analyses suggested that the Elizabeth - Middleton Reefs population was sourced from the Lord Howe Island population during the late Pleistocene. This *C. galapagensis* genetic structure is based on female mediated gene flow (mtDNA) alone and may therefore indicate philopatric behaviour.

Whilst this report provides important management information, which implies that Elizabeth-Middleton Reef and Lord Howe Island populations be managed as separate

resources, which are unlikely to replenish each other in the event of the loss of either population from the area, it also identifies two additional studies that are required to ensure that management outcomes are optimal. These are:

1) It would be strategic to initiate a study that evaluates the impact of recreational and charter fishing activities in partially fished and no take reserves at Elizabeth and Middleton Reefs Marine National Nature Reserve and Lord Howe Island Marine Park, in order to test the hypothesis that partially protected areas open to recreational fishing will suffer reduced abundances and sizes of apex predators such as grouper (black cod) and Galapagos sharks, as was found in Hawaii, even if they are not target species.

2) Future genetic studies of *C. galapagensis* in the Tasman Sea will need to include additional samples from these (ideally at least fifty individuals) and other populations such as the Kermadecs, New Zealand, in order to identify what gene flow (if any) exists between this nearest neighbour to the Australian populations. They will also need to incorporate additional nuclear (microsatellite) markers to determine both the scale of gene flow and evidence for male-mediated gene flow, since this study had insufficient power to realise this.

2. Introduction:

Although sharks comprise more than 500 known species to date (Compagno, 1984), few shark population genetic studies have been published (Table1). These studies have been hindered by extremely low genetic differentiation at allozyme loci (Smith, 1982) and mtDNA (Nei, 1989). The low level of variation is considered to be due to relatively low mutation rates in sharks compared to other vertebrates (Martin, 1995). This in turn, may result from a number of shark properties, such as reproductive success, body size and habitat preference (Table 2).

The Galapagos shark (*Carcharhinus galapagensis*) is a large shark (maximum reported length = 370cm) that exhibits a patchy, circumglobal distribution in tropical and temperate waters (Compagno, 1984). In Australia, this species only occurs on coral reefs at the Elizabeth and Middleton Reefs Marine National Nature Reserve and Lord Howe Island, the southernmost coral reefs in the world. In general, *C. galapagensis* is characterized by a slow maturation time of approximately 10 years, a long gestation period of 12 months and the production of few offspring (6-16 pups/litter) (Wetherbee et al., 1997). Moreover, as for all sharks, it lacks a dispersal larval phase and parental care, therefore the use of nursery grounds is crucial to achieve successful recruitment (Keeney et al., 2003). Kato and Carvallo (1967) reported that *C. galapagensis* juveniles were limited to shallow water (<20m) at the Revillagigedo Islands and that adults seldom occur with juveniles. Moreover Wetherbee et al. (1997) noted a depth segregation based on sex and age displayed by *C. galapagensis*. It is noteworthy that most individuals appeared too small to be mature and it is therefore likely that larger Galapagos sharks occur in deeper unsurveyed waters at these reefs and at Lord Howe Island (Choat et al, 2006, Speare et al 2004). This suggests a capacity for philopatry, a common practice in most sharks (Springer, 1967). Molecular tools, specifically targeting the mtDNA, which is inherited exclusively from the mother, will therefore be useful to examine the evidence for philopatry in this species.

Densities of Galapagos sharks at Elizabeth and Middleton Reefs and at Lord Howe Island were almost an order of magnitude higher than on tropical coral reefs surveyed using the same techniques elsewhere (Great Barrier Reef Marine Park along the east Australian coast, oceanic atoll reefs of the Rowley Shoals Marine Park along the north

east Australian coast and in subtropical reefs of the Great Australian Bight in South Australian waters), indicating healthy populations at the times of survey (Oxley et al, 2004, Choat et al 2006, Speare et al 2004).

The current global distribution of *C. galapagensis* (Figure1) suggests a trans-oceanic dispersal ability. Tagging studies, however show that movements spanned small distances which ranged from 5 miles (Holzwarth et al., 2006) to 14 km (Kohler and Turner, 2001). Compagno (1984) suggested that these sharks are fairly localised around islands but are capable of travelling at least 50 km between islands. Genetic data will allow us to determine whether there is migration at this spatial scale (50 km) and further.

Figure 1. *C. galapagensis* geographic distribution.

Common name	Species name	Method	Population structure	Author(s)
Gummy shark	*Mustelus antarcticus*	A	No heterogeneity in southern and western Australia	MacDonald (1988)
spottail shark	*Carcharhinus sorrah*	A	No heterogeneity in northern Australia	Lavery and Shakel (1989)
Australian Blacktip shark	*Carcharhinus tilsoni*	A	No heterogeneity in northern Australia	Lavery and Shakel (1989)
Sandbar shark	*Carcharhinus plumbeus*	A,M	No heterogeneity between Gulf of mexico and mid Atlantic	Heist et al. (1995)
Shortfin mako	*Isurus oxyrinchus*	M	Significant heterogeniety between oceans	Heist et al. (1996a)
Atlantic sharpnose shark	*Rhizoprionodon terraenovae*	M	No heterogeniety btw mid Atlantic and Gulf of Mexico	Heist et al. (1996b)
Pacific angel shark	*Squatina californica*	A	Significant heterogeniety btw Channel Islands	Gaida (1997)
Gummy shark	*Mustelus antarcticus*	A,M	Significant allozyme heterogeniety btw east/south Australia	Ward and Garden (1997)
School shark	*Galeorhinus galeus*	A,M	No heterogeniety in Australia / heterogeniety among oceans	Ward and Garden (1997)
Great white shark	*Carcharodon carcharius*	M	heterogenety btw South Afric, Australia and New Zealand	Pardini et al. (2001)
Blacktip shark	*Carcharius limbatus*	M,Mt	heterogeneity btw w. Atlantic,Caribbean and Gulf of maxico	Keeney et al. (2005)
Hammerhead shark	*Sphyrna lewini*	M	heterogeneity btw Atlantic, Pacific and Indian Oceans.	Duncan et al. (2006)
Basking shark	*Cetorhinus maximus*	M	No heterogeneity btw ocean basins.	Hoelzel et al. (2006)
Whale shark	*Rhincodon typus*	M	heterogeneity btw Atlantic and Indo-Pacific.	Castro et al. (2007)

Table1. Summary of past shark population studies and their main outcome. (**A** = Allozymes, **M** = Mitochondrial DNA, **Mt** = Microsatellites).

Common name	Species name	Habitat	Reproductive mode	max.length	*H*	*No.loci*	Reference
Gummy shark	*Mustelus antarcticus*	Continental shelf	Aplacental viviparity	185cm TL	0.006	21	MacDonald (1988)
….	….	….	….	….	0.096	28	Ward& Gardner(1997)
spottail shark	*Carcharhinus sorrah*	Continental shelf	Placental viviparous	160cm TL	0.035	47	Lavery& Shakel (1989)
Australian Blacktip shark	*Carcharhinus tilsoni*	Continental shelf	Placental viviparous	200cm TL	0.037	47	Lavery& Shakel (1989)
Sandbar shark	*Carcharhinus plumbeus*	Oceanic banks	Placental viviparous	300cm TL	0.005	….	Heist et al. (1995)
Shortfin mako	*Isurus oxyrinchus*	Coastal and Oceanic	Ovoviviparous	400cm TL	….	….	Compagno (1984)
Atlantic sharpnose shark	*Rhizoprionodon terraenovae*	Continental shelf	Placental viviparous	67cm TL	….	….	Compagno (1984)
Pacific angel shark	*Squatina californica*	Continental shelf	Unknown	152cm TL	0.056	29	Gaida (1997)
School shark	*Galeorhinus galeus*	Continental shelf	Ovoviviparous	195cmTL	0.001	….	Ward and Garden (1997)
School shark	**	**	**	**	0.003	….	Smith (1986)
Great white shark	*Carcharodon carcharius*	Oceanic	Aplacental viviparity	600cm TL	0.694	38	Pardini et al. (2001)
Blacktip shark	*Carcharius limbatus*	Continental shelf	Placental viviparous	255cm TL	0.486	34	Keeney et al. (2005)
Scalloped hammerhead	*Sphyrna lewini*	Continental shelf	Viviparous	420cm TL	….	….	Compagno (1984)
Basking shark	*Cetorhinus maximus*	Continental shelf	Presumed oophagous	1000cm TL	….	….	Compagno (1984)
Whale shark	*Rhincodon typus*	Oceanic	Ovoviviparous	2100cm TL	….	….	Compagno (1984)

Table2. Summary of mean heterozygosity in correlation to habitat preference, reproductive strategy and body size between different shark species (***H*** Mean heterozygosity, **….** No data and ** repeated data).

Two past population structure studies of worldwide distributed sharks showed heterogeneity based on maternally inherited DNA. Ward and Garner (1997) studied the school shark from the Indian Ocean, Bass Strait, North Atlantic, South Atlantic and New Zealand and reported a significant variation in the haplotype frequencies between samples, suggesting genetic partitioning within and among Ocean basins. Likewise, Heist et al. (1996a) reported similar genetic heterogeneity in the shortfin mako shark (*Isurus oxyrinchus*) from North and South Pacific and North and South Atlantic populations.

Insight into the genetic structure of *C .galapagensis* at a relatively small geographical scale (in the order of 50 km between Middleton and Elizabeth Reefs and 175 km between Elizabeth Reef and Lord Howe Island) will inform us about connectivity at these spatial scales and identify conservation strategies that are needed to manage these important members of the marine ecosystem in a sustainable way.

In this study we test the null hypothesis that there is a single Australian population that shares a common gene pool. The main aim of the study was therefore to determine the population genetic structure of *C. galapagensis* from Middleton Reef, Elizabeth Reef and Lord Howe Island by obtaining and comparing genetic data from the non coding region of the mtDNA (M) with nuclear markers (Mt) in order to:

1. Confirm the identity of Australian *C. galapagensis* by comparison to Hawaiian *C. galapagensis* and other sharks, using mtDNA sequence data and phylogenetic analysis.

2. Determine population structure of *C. galapagensis* in Australia, using both mtDNA and nuclear (microsatellite) markers in order to detect philopatry and/or connectivity between sites.

3. Assess the level of genetic diversity of *C. galapagensis* and compare it to other broadly distributed sharks, marine reptiles, marine mammals and teleosts. (M)

4. Identify challenges associated with the limited reproductive output and dispersal ability of *C. galapagensis*.

3. Materials and methods

3.1 Collection of tissues and study sites

Eighty six samples of *C. galapagensis* were collected randomly from Middleton and Elizabeth Reefs Marine National Nature Reserve and Lord Howe Island Marine Park (Figure 2).

Eighty samples were collected as dorsal fin clips from sharks caught by line fishing and immediately released, while the remaining six were taken as skin and white muscle tissues by *in situ* underwater biopsy probe following Robbins (2006). All tissue samples were immediately placed in 80% EtOH, and stored at room temperature.

Additional samples were included in the study through external collaboration (Table 3). From Hawaii two *C. galapagensis* were used as a comparative control to confirm the Australian *C. galapagensis* species identity. Four sand bar shark *(Carcharhinus plumbeus)*, two from Hawaii and two from Australia; two bull sharks (*Carcharhinus leucus)* and two whitetip reef sharks *(Triaenodon obesus*) were included as out groups to root the phylogenetic tree.

Common name	Scientific name	Location	Date of collection	Sample size	Source
Galapagos shark	*C.galapagensis*	Middleton Reef	Feb.2006	28	JCU-DEWR survey
	C.galapagensis	Elizabeth Reef	Feb.2006	29	JCU-DEWR survey
	C.galapagensis	Lord Howe Islad	Jan.2007	29	Fisher men*
	C.galapagensis	Hawaii	Aug.2006	2	Ms. Toby Day Engel
Sandbar shark	*C.plumbeus*	Australia	Feb.2006	2	Will Robbins
	C.plumbeus	Hawaii	Feb.2006	2	Ms. Toby Day Engel
Bull shark	*C.leucus*	Australia	Feb.2006	2	Will Robbins
Whitetip reef shark	*T.obesus*	Australia	Feb.2006	2	Will Robbins

Table 3: Summary of sampling sites, date of collection, sample size and source.
* Fishermen, S. Wilson, G. Johnson, K. Galloway and J. Shick

3.2 DNA extraction

Total genomic DNA was extracted from approximately 15mg of tissue. Extractions were performed using phenol/chloroform and precipitated using ethanol following protocols of Sambrook et al (1989). DNA pellets were resuspended in 50 µl of TE buffer. The quality and quantity of extracted DNA was checked against a size standard (New England Biolabs, www.neb.com) using a 0.8% agarose gel. Electrophoresis was performed for 20 minutes at 100 Volts (V). Elizabeth and Middleton Reefs populations were screened using 1µl of neat DNA while the Lord Howe population was screened using 1 µl of 1/4 diluted DNA for both mtDNA and microsatellite PCR amplification reactions.

3.3 mt-DNA amplification and sequencing

The non-coding region of the mitochondrial DNA was selectively amplified using Light strand ProL2 (5'-CTG CCC TTG GCT CCC AAA GC-3') (Pardini et al. 2001) and heavy strand 282H (5'-AAG GCT AGG ACC AAA CCT-3') (Keeney et al. 2003) primers by polymerase chain reaction (PCR) using an MJ Research PTC-200 thermal cycler as follows:

Elizabeth and Middleton Reefs samples required different reaction conditions than Lord Howe Island samples. In the former populations, reactions were carried out in a total volume of 20 µl which consisted of, 2.0µl PCR buffer (10X), 1.3 µl of 2mM deoxynucleotide triphosphates (DNTPs), 1µl of Forward primer (10 pmol/µl), 1µl of Reverse primer (10 pmol/µl), 0.3 µl of *Taq* DNA polymerase (Qiagen kit) and 1 µl of non- diluted DNA.

The Lord Howe Island samples had to be processed differently: reactions took place in 30 µl aliquots and contained 3.0µl PCR buffer (10X), 2.0 µl deoxynucleotide triphosphates (DNTPs) (2mM each), 1.5µl of each Forward and Reverse primers (10 pmol/µl), 0.5µl of *Taq* DNA polymerase (Qiagen kit) and 1 µl of 1/4 diluted DNA.

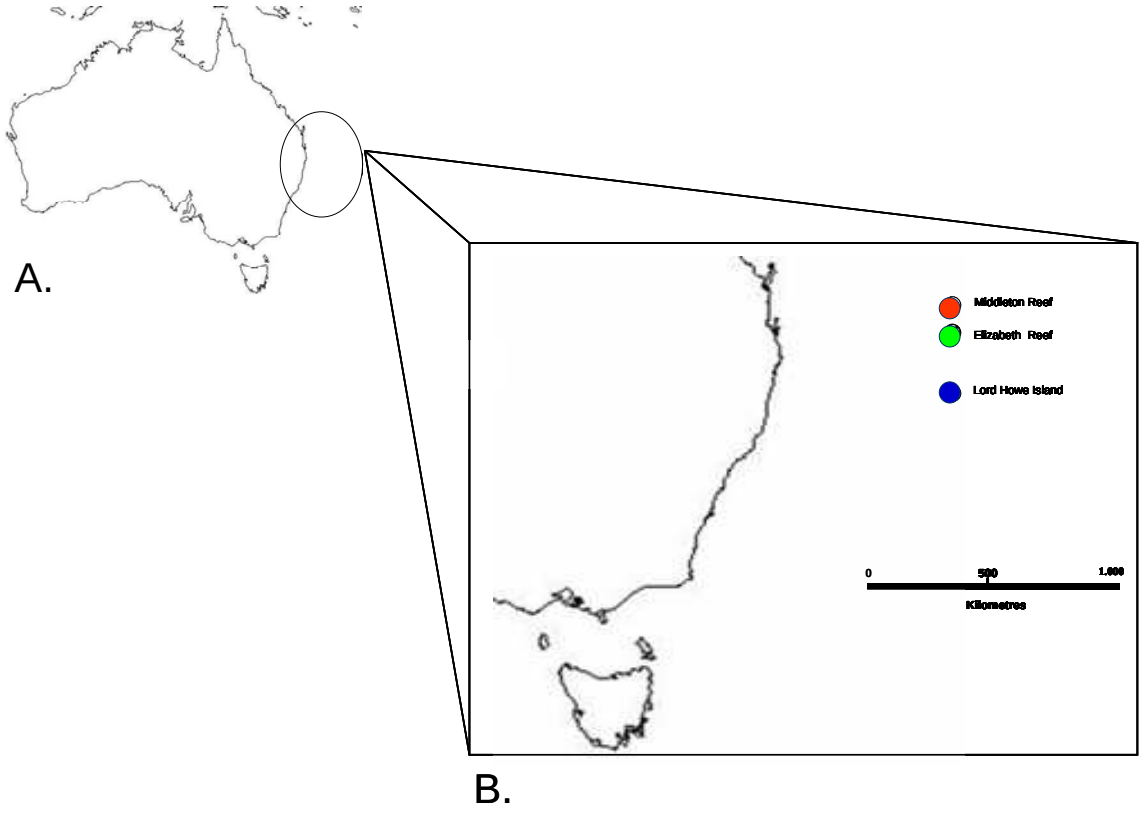


Figure 2: Study site, **A.** South East Australia. **B**. Middleton Reef (Red) (*latitude 29°27'S and longitude 159°07'E*); Elizabeth Reef (Green) (*latitude 29°56'S and longitude 159°05'E*) and Lord Howe Island (Blue) (*31°30'S latitude, some 700 km north-east of Sydney*).

The PCR cycling conditions for all samples were an initial denaturation at 94 ºC for 2.0 minutes, 30 cycles of 94 ºC for 30 seconds, 53 ºC for 30 seconds, and 72 ºC for 1:30 minutes. A final extension of 72 ºC for 10 minutes was done.

PCR product was evaluated using 5µl of product, which was run on 1.5 % agarose gel containing ethidium bromide. Products, which showed strong single bands on the check gels, were cleaned up with 70% isopropanol, vacuumed and quantified before being sent for sequencing. Sequencing was done by Macrogen (www.macrogen.com) using the forward primer after confirming that Forward and Reverse sequences are compatible.

Carcharhinus galapagensis stocks: Elizabeth-Middleton Reefs and Lord Howe Island

3.4 Microsatellite loci amplification, genotyping and analyses

3.4.1 Microsatellite amplification and genotyping

Six microsatellites were developed and used for the blacktip shark *(Carcharhinus limbatus)* by Keeney and Heist (2003) (Table 4). These loci were tested for the Galapagos shark (*Carcharhinus galapagensis*) by Daly-Engel *et al.* (2006). The PCR conditions were as was followed in the mtDNA amplification with some modifications (depending on locus) (Table 4).

Each microsatellite locus was amplified separately in 20 µl (Elizabeth and Middleton Reefs) or 30µl (Lord Howe Island) reactions using the MJ Research PTC-200 Peltier thermal cycle device and an optimized PCR recipe. PCR cycling conditions were as follows: initial denaturation at 94 ºC for 2.0 min, 30-40 cycles of 94 ºC for 30 sec., 52-60 ºC for 30 sec. (depending on primer set, see (Table 4), and 72 ºC for 1:30 min. A final extension cycle of 72 ºC for 10 min was followed by an indefinite 11 ºC cycle.

Three loci (one of each fluorescent dye were combined together at a different ratio depending on the strength of the PCR product, to a total volume of 30 µl of the PCR fragment and treated as one set. *Set.1* consisted of (Cli 12, Cli 108 and Cli 107) and *set.2* consisted of (Cli102, Cli103 and Cli106). The combined products of each set for each individual was cleaned with Ethanol precipitation by adding 3 µl of ammonium acetate and 90µl of cold 100% Ethanol and spin it for 20 min at full speed (4000 rpm). After that samples were inverted to tip out the solution and were spun upside down on paper towel for one min. at (500rpm). After that 150µl of 70% cold Ethanol from the freezer was added to the samples and inverted immediately on paper towel to remove as much ethanol as possible and spin up side down on paper towel at a speed of (500rpm) for one min., then samples were allowed to air dry for few minutes and sent for genotyping at the Genetic Analysis Facility of James Cook University (GAF).

Despite extensive optimisations, only three of the six markers provided satisfactory results (cli12, cli 107 and cli 108). As a result these three markers were the only microsatellite markers included in population genetic analyses here.

Locus	5' to 3' primer sequence	Range (bp)	Ta (ºC)	Mg Con.	Dye label	Cycle#
Cli 12	F: TCCCAGTCACATTTACACATGC R:GGAAGACCATTGAACCCAATC	184-194	58	1.5	TET	35
Cli 102	F:GACTGGCTGACCTAACTAAGC R:ATCCTGTGGTCCTTCTATC	128-130	54	2.5	HEX	40
Cli 103	F:GCTTCATTCCATGAGAG R:TTTCTCTGTCCTGGTGTTTC	118-124	52	1.5	FAM	40
Cli 106	F:GATTCTACAACCGCAACATTCG R:GCTCCCCTAACTATTCCACGC	180-200	60	2.5	TET	40
Cli 107	F:GGATTCACAACACAGGGAAC R:CTCATTCTTAGTTGCTCTCG	105-113	56	1.5	FAM	40
Cli 108	F:TCACTGGGTTAGACACTTCC R;CCACAGTCAGAAAACAAATTG	126-142	56-54	1.5	HEX	30

Table 4: Summarizes the microsatellite loci obtained from Keeney and Heist (2003), their sequences, annealing temperature, Mg^{+2} concentration, fluorescent dye (Gene work) label of forward primer and number of thermal cycles in each PCR reaction.

3.5 mtDNA data analysis:

Forward sequences were automatically aligned (CLUSTALW) then manually aligned and edited using BioEdit (Hall, 1999). This was performed after the removal of ambiguities in the beginning and the end of each sequence. Sequence alignment was maintained by inserting gaps where required.

3.5.1 Phylogenetic analysis

Sequences were analysed using MEGA ver 3.0 (Kumar *et al.,* 2004) and PAUP* (Swofford, 2001) to construct phylogenetic trees. On the other hand Arlequin ver 3.1 (Excoffier *et al.,* 2005) was used for population genetic analysis. All forward sequences (822 bp) were included in phylogenetic analyses to identify genetically distinct lineages and relationships between samples within lineages. The best substitution model to fit the data was selected using Modeltest (Bohonak, 2002).

Neighbour Joining (NJ) and Maximum parsimony (MP) trees using MEGA ver 3.0 (Kumar *et al.,* 2004) and Maximum likelihood trees (ML) were constructed using PAUP* version 4.10b (Swofford, 2001). Bootstrapping (Felsenstein, 1985) was used to estimate the reliability of the trees by constructing consensus trees following 1000 replicates. The differences between individual sequences were determined for five characters (A, G, T, C and gaps). The NJ and ML trees were rooted using the outgroup shark species. These were, sand bar shark *(Carcharhinus plumbeus*), bull shark (*Carcharhinus leucus)* and white tip reef shark *(Triaenodon obesus*).

3.5.2 Population genetic analysis

3.5.2.1 Minimum spanning tree (MST)

A Minimum Spanning Tree was constructed from Arlequin ver 3.1 (Excoffier *et al.,* 2005) to explore the amount of shared haplotypes between the three different locations and the haplotype and nucleotide diversities of the data.

Nucleotide diversity (π) was obtained directly from the population genetic analysis performed in Arlequin. This index is used to determine whether the population exhibits a deep (many substitutions between haplotypes) or shallow (few substitutions between haplotypes) population history. On the other hand, haplotype diversity (*h*) was calculated using the following equation:

$$h = n (1- \sum xi2)/ (n - 1) \qquad \text{(Nei, 1989)}$$

Where:

n = number of sampled individuals.

xi = frequency of the ith haplotype in the population.

This index (*h*) presents information about the number of shared haplotypes (Avise, 2004). In summary, the MST indicates the number of shared haplotypes and the number of substitutions between haplotypes. Haplotype and nucleotide diversities were then interpreted for both populations based on Grant and Bowen (1998) (Table 5).

h		
π	Small	Large
Small	1. Recent population bottleneck or founder event by single or a few mtDNA lineages.	2. Population bottleneck followed by rapid population growth and accumulation of mutations.
Large	3. Divergence between geographically subdivided populations.	4. Large stable population with long evolutionary history or secondary contact between differentiated lineages.

Table 5: The four categories stated by Grant and Bowen (1998) to interpret haplotype and nucleotide diversities of marine fish.

3.5.2.2 Pairwise Fst and isolation by distance test (IBD)

The pairwise *F-statistics* fixation indices (F_{st}, F_{ct} and F_{sc}) were measured to determine evidence for gene flow between populations (Scheinder et al., 2000). The *P*-values of these indices were also measured directly from Arlequin ver 3.0 following 10000 resampling of the data. Comparing the pairwise *Fst* among populations of different locations should reveal if there is population subdivision, where this subdivision occurs and whether it is likely that isolation by distance affects the genetic structure (Bohonak 2002). Isolation by distance was tested formally using the Mantel test (Bohonak, 2002). The program for isolation by distance, IBD ver.1.4, can be obtained freely at http:// www.bio.sdsu.edu/pub/andy/IBD.html. This test plots genetic distance (pairwise F_{st}) against geographic distance (km) (Bohonak, 2002). The IBD program establishes if the two measures are significantly correlated or not. This is done using both non-transformed and log-transformed analyses of both axes in order to determine confidence intervals and statistical significance.

3.5.2.3 Hierarchical analyses

AMOVA (Analysis of Molecular Variance) (Weir and Cockerham 1984; Excoffier et al. 1992) was performed to confirm if there is population genetic partitioning between locations sampled. The partitioned data was initially identified using pairwise *Fst* measured in Arlequin (Excoffier *et al.*, 1992). The hierarchical AMOVA addressed variation at two different levels. First approach, individuals were grouped into three

populations (division by site: Middleton Reef vs. Elizabeth Reef vs. Lord Howe Island) and second approach individuals were combined into two groups (a combined Elizabeth and Middleton Reefs group and a Lord Howe Island group) to accommodate the signal obtained from pairwise Fst values. This test is useful to investigate the spatial differentiation within and between the structured groups.

3.5.3 Coalescence analyses

Coalescence is a powerful modelling approach that performs a backward tracing of the genealogical relationships among gene copies (allele) at a locus from the offspring to the parents until it reaches a common ancestor (Avise, 2004). Therefore, it provides information about the age of the population and its demographic history (expanding or stable population). Coalescence was measured by obtaining the raggedness indexes and *Tau* values from Arlequin ver. 3.0. Then coalescence aging was estimated using parameters obtained from Arlequin ver 3 and the following equations:

$u = 2 \mu k$ (1) (Roger and Harpending, 1992)

Where:

u= mutation rate for the whole sequence.

μ= mutation rate per nucleotide.

k= total sequence length.

$t = tau/2u$ (2) (Roger and Harpending, 1992)

Where:

t= generation divergence time.

tau= unit of mutational time.

u= mutation rate for the whole sequence.

After that the number of generations since divergence was calculated for the mean Tau value as well as the upper and lower Tau limits by the following equation:

$t = Nt \times T$ (3) (Pianka, 1978)

Where:

Nt = Number of generations since divergence.

t = divergence time (in years).

T= generation time (in years per generation).

The generation time was calculated from the following equation:

Generation time (T) = ($\alpha + \omega/ 2$) (4) (Adams et al., 2000)

Where:

α = age at maturation.

ω = longevity.

C. galapagensis was estimated to reach maturity at the age of ten years (Wetherbee et al.,1997). On the other hand De Crosta et al. (1984) has reported different ages of maturity (Females = 6.5-9 and Males= 6.5-8). In our calculation we considered Wetherbee's findings, since mtDNA is maternally inherited and Wetherbee's study was applied only to females.

The mutation rate of *C. galapagensis* was calculated for the conserved and variable (slow and fast rate) sites along the sequence, these calculations were performed as indicated below:

1. Mutation rate of the variable sites in sharks was obtained from (Martin et al., 1992), and was calculated as follows:

tv = 0.072% Myr^{-1}

ti = 2.3% Myr^{-1}…… calculated for (*Sphyrna tiburo*)

Where:

tv = Transversion sites (slow rate).

ti = Transition sites (fast rate).

Due to a lack of studies reporting transition mutation rates in sharks in general and *C. galapagensis* in particular, we used the fast mutation rate of the bonnethead shark, *(Sphyrna tiburo)* to represent the fast mutation rate of the variable sites in *C. galapagensis*.

The total mutation rate was then calculated as follows:

Rate of mutation (total) = Proportion of **ti** to total variable sites x fast rate%

\+

Proportion of **tv** to total variable sites x slow rate %

2. Mutation rate of the conserved sites (slow mutating rate) was calculated as follows:

Total sequence length – variable sites = **X**

Since we obtained the variable sites ratio we can calculate the conserved site ratio as follows:

X / total sequence length= ratio of conserved sites in the sequence.

Mutation of shark mtDNA is 7 to 8 fold slower than other vertebrate mtDNA (Martin et al., 1992). Therefore we represented *C. galapagensis* mtDNA mutation rate to be 7.5 times slower than vertebrates (7 + 8/ 2), and calculated the mutation rate as follows:

$(1.1 / 7.5)B + (12.5/7.5)\ D = 0.1467B + 1.6667D\ \%\ /Myr^{-1}$

where 1.1% and 12.5% are the mtDNA mutation rates of conserved and variable sites in vertebrates, respectively, per Million years (Alvarado et al., 1995).

Therefore the total mutation rate= (A x B) + (C x D).

A = mutation rate of conserved sites.
B = ratio of conserved sites in the whole sequence.
C= mutation rate of variable sites.
D= ratio of variable sites in the whole sequence.

3.6 Microsatellite data analyses:

Heterozygosity was scored using Fragment profiler (Amersham Biosciences). Scored data were transferred to Arlequin to be tested for adherence to assumptions for population genetic structure analyses using microsatellite data. Specifically, do the markers used conform to the expectation that they are not linked and are the markers in Hardy Weinberg equilibrium or not. If any of the markers used does not conform to these assumptions, it should be eliminated from population genetic structure analyses. The probability that any of the loci were in Hardy-Weinberg equilibrium was tested using Arlequin ver.3 (Excoffier *et al.,* 2005). The test was performed using Chi-squared test, which calculates the observed genotype frequencies obtained from the data and the expected genotype frequencies obtained using Hardy-Weinberg principle. The tested null hypothesis is that the population is in Hardy-Weinberg principle (Excoffier *et al.,* 2005). Hardy-Weinberg principle suggests that the genotype frequencies in a population is in equilibrium when passed from generation to another unless it was disturbed by introduced influences, such as non-random mating, new mutations, random genetic drift, gene flow and selection. This principle is considered as a basic concept of population genetics that should be maintained. Therefore any data that does not meet this concept should be removed.

4. Results

4.1. Mitochondrial data

4.1.1 Phylogenetic analyses

The non coding region of the mt-DNA (822bp) was sequenced for 86 *Carcharhinus galapagensis* individuals representing three samples. The nucleotide composition showed an A-T base pair bias AT:GC of 3:1 (***T*= 36.02%**, *C*= 19.77%, ***A*=30.27%,** and *G*= 13.95%). Such bias is typical for marine fish mitochondrial DNA including sharks (McMillan and Plumbi, 1995). Out of the 822 bp (including 50 gaps), twenty-one variable nucleotide positions were detected consisting of ten parsimony informative sites. This variability resulted in 36 different haplotypes among the Australian *C. galapagensis* (Table 6.). Most of these changes were transitions (ti) (C-

T or A-G), only one site at position 693bp was a transversion (tv) (A-C). The ti:tv ratio was high, 7.5.

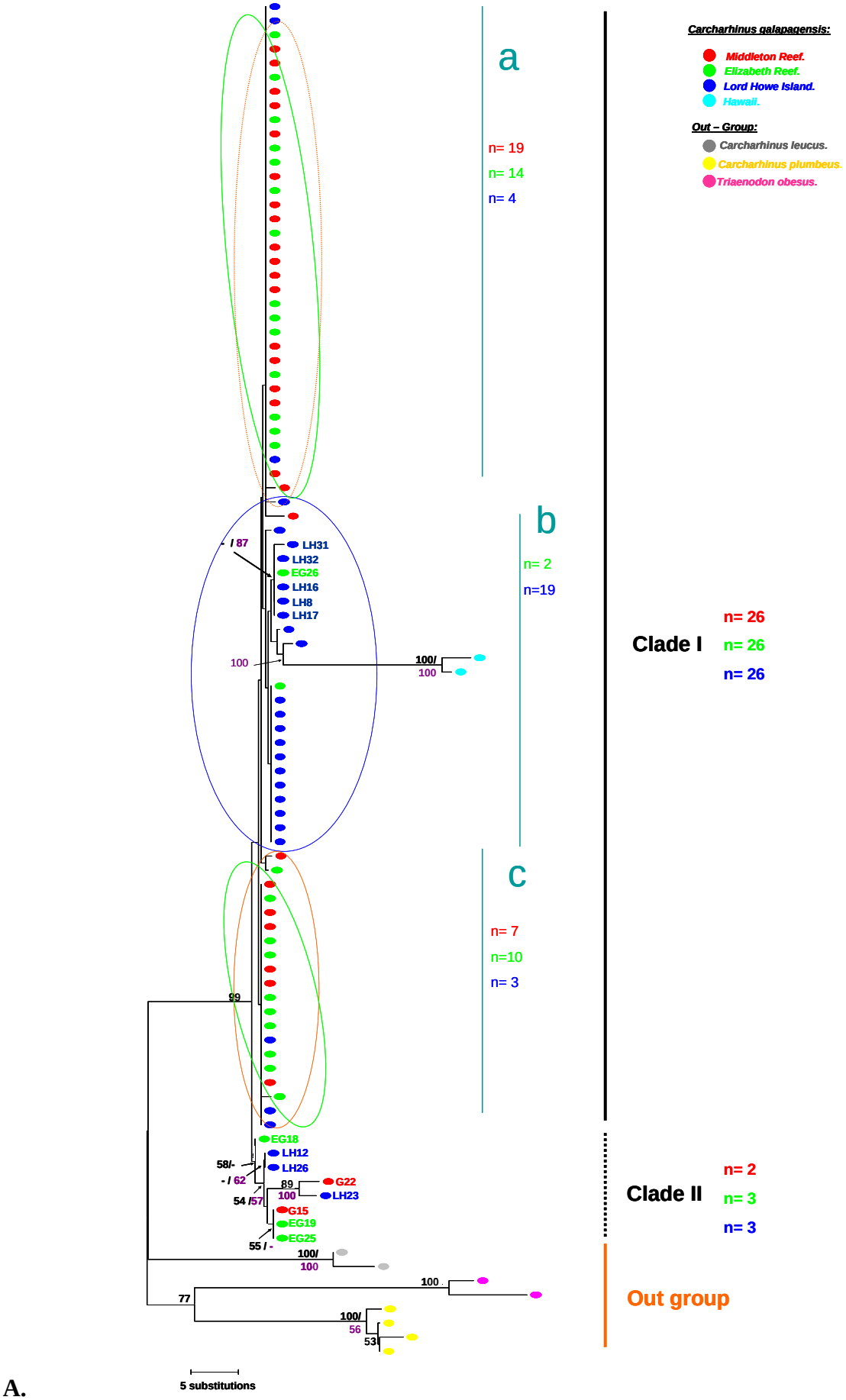
Both NJ and ML trees showed the same topology with the exception that the ML tree had grouped *C. leucus* (outgroup) within the *C. galapagensis* ingroup. Therefore we chose the NJ tree to display our first estimates of *C. galapagensis* population structure. Importantly, the Hawaiian *C. galapagensis* was placed within the ingroup in the NJ tree. Thus, confirming the identity of the sampled sharks as *C. galapagensis* (aim 1). Although the NJ tree displayed a geographic subdivision between individuals from Elizabeth and Middleton Reefs and the Lord Howe Island individuals (Figure 2, explained by the shaded areas), few clusters were supported with high bootstrap support, which produced only two strongly supported clades (**I and II**). **Clade II** suggests that individuals (**G: 22and 15**; **EG**: 18;**19and 25** and **LH:** 12;26 and **23**) shared more genetic similarities when compared to the other individuals from the three different locations (Elizabeth/Middleton Reefs and Lord Howe Island). Whereas **clade I** included the rest of the individuals obtained from these three locations and displayed them as an intermixing single population. Individuals highlighted in bold were also supported by the parsimony informative tree (Figure 3) which displayed a close relation between the following individuals (**G:22&15**; **EG: 19&25** and **LH:** 13, **23**&26) as well as a sharing genetic similarity between (EG: 26 and LH: 8, 16, 17, 25, 31&32) which were not supported by the NJ tree. However, these data need to be examined using population structure analyses, which were performed using Arlequin ver 3.1 (Excoffier *et al.*, 2005).

Hapl.	n.	Polymorphic nucleotide sites:																						
		14	**43**	**81**	**90**	**168**	**186**	**223**	**241**	**262**	263	**298**	**316**	**323**	**356**	**418**	**646**	**656**	**684**	**693**	696	700	709	719
1	1	A	C	~	A	G	C	G	A	C	~	C	G	T	G	A	~	T	T	A	~	~	~	~
2	31	.	.	.	.	A	.	C	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.
3	1	.	.	.	.	.	.	C	.	.	.	.	.	.	T	C	.	.	.	.	.	.	.	.
4	14	.	.	.	.	.	.	C	.	T	.	.	.	.	T	.	.	.	.	.	.	.	.	.
5	3	.	.	.	.	A	T	C	.	T	.	.	.	.	T	.	.	.	.	.	.	.	.	.
6	1	.	.	.	.	.	.	C	.	T	.	.	.	.	T	.	.	.	.	.	.	.	.	.
7	1	.	T	.	.	.	T	C	G	T	.	T	A	C	T	.	.	.	.	.	.	.	.	.
8	1	.	.	.	.	.	.	C	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.
9	1	.	.	.	.	A	.	C	.	T	.	.	.	.	T	.	.	.	.	.	.	.	.	.
10	3	.	.	.	.	.	.	C	.	T	.	.	.	.	T	.	.	.	G	.	.	.	.	.
11	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G
12	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
13	1	.	.	G	.	.	.	C	.	T	.	.	.	.	T	.	.	.	.	.	C	.	.	.
14	4	G	.	.	.	.	.	C	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.
15	1	.	.	.	.	.	.	C	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.
16	1	.	.	.	.	.	.	C	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.
17	1	.	.	.	.	.	.	C	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.
18	1	.	.	.	.	.	.	C	.	T	.	.	.	.	T	.	A	.	.	.	.	A	A	.
19	1	.	.	.	.	A	T	C	.	T	.	.	.	.	T	.	.	.	.	.	.	.	.	.
20	1	R	.	.	.	.	.	C	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.
21	1	.	.	.	.	.	.	C	.	.	.	.	.	.	T	.	.	.	.	C	.	.	.	.
22	1	G	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.
23	1	G	.	.	.	.	.	C	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.
24	1	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
25	1	G	.	.	.	.	.	C	.	.	.	.	.	.	T	.	.	.	.	.	C	.	A	G
26	1	G	.	.	.	A	T	C	G	T	.	T	A	C	T	.	.	C	.	.	.	.	.	.
27	1	.	.	.	.	A	T	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.
28	1	G	.	.	C	.	.	C	.	T	.	.	.	.	T	.	.	.	.	C	.	.	.	.
29	1	.	.	.	.	.	.	C	.	T	.	.	.	.	T	.	.	.	.	.	.	.	.	G
30	1	.	.	.	.	.	.	C	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	G
31	1	G	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G
32	1	G	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G
33	1	G	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
34	1	G	.	.	.	.	.	C	.	T	.	.	.	.	.	.	.	.	.	.	.	A	.	G
35	1	G	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
36	1	G	.	.	.	.	.	C	.	.	.	.	.	.	T	.	.	.	.	.	.	A	.	G

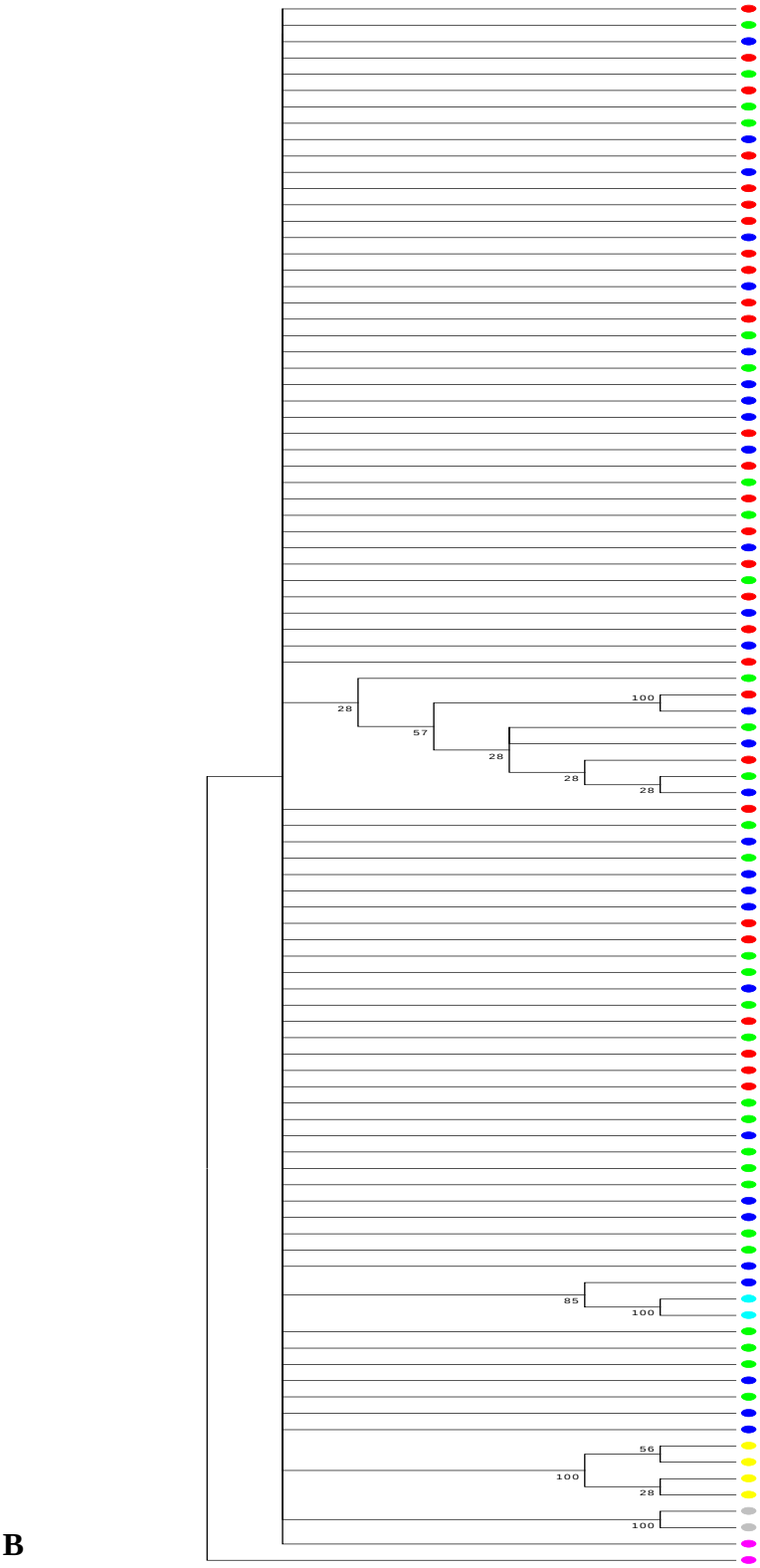
Table 6. Polymorphic nucleotide positions for the Australian *C. galapagensis* (822bp); Haplo = haplotype ID and n. = number of individuals sharing a particular haplotype. The nucleotide at each position is given for haplotype 1, only nucleotides different from haplotype1 are listed for all other haplotypes. Identical nucleotides between haplotypes are indicated by (.); Middleton Reef (Red), Elizabeth Reef (Green) and Lord Howe Island (Blue). Parsimony informative sites highlighted in blue and Variable sites are in bold.

Hapl.	Polymorphic nucleotide sites:																		
	736	**737**	**746**	750	753	759	768	769	771	778	781	785	**789**	795	**805**	806	807	**811**	818
1	~	A	~	~	~	~	~	~	~	~	~	~	A	~	A	~	~	A	~
2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
3	.	.	.	.	.	.	.	.	.	.		.	.	.	.	.	.	.	.
4	.		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
5	.	.	.	.	.	.	.	.	.	.	.	.		.	G	.	.	.	.
6	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.
7	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.
8		.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.
9	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
10	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
11	.	.	.	C	.	G	T	.	G	.	.	G	.	.	.	.	.	.	.
12	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
13	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		.
14	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
15	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
16	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
17	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
18	.	.	.	.	.	.	.	.	.	.	G	G	.	G	.	A	A		G
19	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
20	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.
21	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
22	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
23	.	.	C	.	C	.	.	.	.	.	.	.	.	.	.	A	A		G
24	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
25	.	.	.	C	C	G	T	T	.	.	G	G	A	.	.	.	A	.	G
26	.	.	A	.	.	.	.	.		.	.	.	.	.	.	A	A	.	G
27	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
28	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
29	C	.	.	.	.	G	.	.	.	.	.	C	.	.	.	A	.	.	G
30	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	A	.		G
31	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	G
32	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.
33	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.
34	.	.	.	C	.	G	T	.	.	.	.	.	.	G	.	A	.	C	.
35	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.
36	.	.	.	C	C	G	.	.	G	A	.	.	.	.	.	A	.	.	.

Continue table 6.


Carcharhinus galapagensis:
Middleton Reef.
Elizabeth Reef.
Lord Howe Island.
Hawaii.
Out – Group:
Carcharhinus leucus.
Carcharhinus plumbeus.
Triaenodon obesus.
a
n= 19
n= 14
n= 4
b
n= 2
n=19
c
n= 7
n=10
n= 3
Clade I
n= 26
n= 26
n= 26
Clade II
n= 2
n= 3
n= 3
Out group
LH31
LH32
EG26
LH16
LH8
LH17
EG18
LH12
LH26
G22
LH23
G15
EG19
EG25
5 substitutions

A.



B

Figure 3. mtDNA data analysis of the *Carcharhinus galapagensis* including all locations in Australia; Elizabeth and Middleton Reefs and Lord Howe Island. **A.** Rooted NJ bootstrapped Phylogenetic tree of *C. galapagensis.* NJ bootstrap support values (of 1000 bootstrap replicates) are indicated in black and majority rule support values for the Maximum Parsimony tree are indicated in purple, adjacent to relevant nodes that are supported by these respective analyses. Colour dots are used to identify the location and species identity of all samples, as indicated in accompanying key. Numbers of individuals in each clade are indicated by the values given for n. the blue clades indicate weakly supported clades which display a defined structure. **B.** Majority rule consensus tree of Maximum Parsimony analysis of same data presented in A above, refer to key in A above for location by colour codes.

4.1.2 Population genetic analyses

4.1.2.1 Minimum spanning trees (MST)

The MST shows a total of 36 unique haplotypes, where 31 of these were not shared by any others. The MST identifies the two most common haplotypes, both of which are shared between Elizabeth and Middleton Reefs exclusively. The first haplotype was shared by 31 individuals (17 from Middleton Reef and 14 from Elizabeth Reef), while the second haplotype was shared between 14 individuals (six Middleton Reef and eight Elizabeth Reef). Individuals from Lord Howe Island had unique haplotypes with the exception of two shared haplotypes with Elizabeth and Middleton Reefs (Figure 4). The first of these was shared between two individuals from Lord Howe and one from Elizabeth Reef, whereas the second haplotype was shared between four individuals from Lord Howe exclusively.

The Elizabeth and Middleton Reefs population was dominated by haplotype 1 and 2, resulting in a much lower haplotype diversity (h=0.185). The low level of mutations between haplotypes resulted in a low nucleotide diversity of this population as well (π = 0.3% +/- 0.2%). Whereas in case of the Lord Howe population, haplotype diversity was very high (h= 0.983), as was nucleotide diversity (π = 1.2% +/- 0.6%) (Table 6). This suggests that the Lord Howe population has a different history from the Elizabeth and Middleton Reefs population. Moreover, the total genetic (haplotype and nucleotide) diversity of *C. galapagensis* showed a relatively high genetic variability when compared to other sharks. However, this genetic variability is still considered low in relation to teleosts (table 8).

The substitution rate between most haplotypes was quite low, 86% of the haplotypes were separated by $\leq$6 substitutions (base change) and only 14% of the haplotypes were separated by >6 substitutions. The Lord Howe individuals had more substitutions between haplotypes than the Elizabeth and Middleton Reefs population.

Haplotype diversity	%Nucleotide diversity	n^a	n_h^b	Geographic region
0.1858	0.29%±0.00178	29	11	Elizabeth & Middleton
0.9833	1.18%±0.0062	29	24	Lord Howe Island
0.5717	0.57% ± 0.0031	58	35	*C. galapagensis* (Total)

Table 7: *Carcharhinus galapagensis* haplotype and nucleotide diversities per region

[a] number of samples per region; [b] number of haplotypes per region

h: Haplotype diversity (Nei 1989);

π: Nucleotide diversity as per Arlequin Ver.2.01

Scientific name	Common name	nucleotide diversity π	haplotype diversity *h*	Reference
Cetorhinua maximus	Basking shark	0.13% +/- 0.0009	0.72 +/- 0.028	Hoelzel *et al.* (2006).
Carcharhinus limbatus	Blacktip shark	0.21% +/- 0.0013	0.805 +/- 0.018	Keeney *et al.* (2005).
Carcharhinus taurus	Sand tiger shark	0.3% +/- 0.0001	0.717+/- 0.01	Stow *et al.* (2006).
Carchardon carcharias	Great White shark	2.03%	…..	Pardini *et al* .(2001).
Sphyrna lewini	Scalloped Hammerhead	1.3% +/- 0.0068	0.8 +/- 0.02	Duncan *et al* .(2006).
Caretta caretta	loggerhead turtle	2.36% +/- 0.0121	0.579+/- 0.028	Bowen *et al.* (2004).
Delphinus delphis	Common dolphin	1.2% +/- 0.021	0.853 -1.0	Natoli *et al.* (2006).
Orcinus orca	Killer whale	0.53% +/- 0.0031	0.874+/- 0.013	Hoelzel *et al.* (2002).
Physeter macrocephalus	Sperm whale	0.2% +/- 0.0003	0.86	Lyrholm *et al.* (1996).
Thunnus obesus	Bigeye tuna	5.40%	0.98 - 1.0	Martinez *et al.* (2006).
Xiphias gladius	Swordfish	1.48% +/- 0.0005	0.997	Lu *et al* . (2006).
Acanthocybium solandri	Kingfish	5.30%	0.999	Garber *et al.* (2005).

Table 8. Summary of haplotype and nucleotide diversities reported from past studies of sharks, turtles, marine mammals and teleosts.

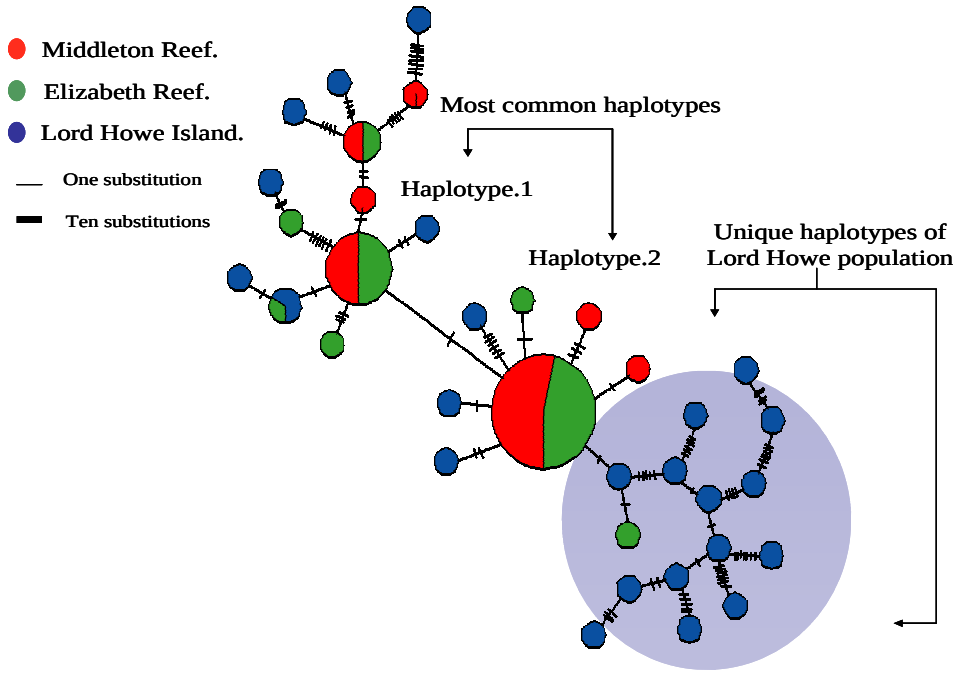


Figure 4 Minimum spanning tree showing the relationships between haplotypes of *C. galapagensis* from Middleton, Elizabeth Reefs and Lord Howe Island. Sizes of the circles are proportional to the number of individuals sharing a particular haplotype and crosses on the connecting lines reflect numbers of substitutions separating different haplotypes. Each colour represents different location as indicated in the figure key.

4.1.2.2 *Pairwise F_{st} and geographic distance*

Assessment of the pairwise F_{st} values indicated that the haplotype differences between Elizabeth and Middleton Reefs regions were quite low (F_{st}= 0.002) where as the Lord Howe Island haplotypes were significantly different from those at Elizabeth and Middleton Reefs (F_{st}= 0.52 and 0.45 respectively with $P \leq 0.05$) (Table 9). These results confirm the suggested structure obtained from the MST, which suggests high female mediated gene flow within *C. galapagensis* at Elizabeth and Middleton Reefs with limited gene flow to and from Lord Howe Island. This identifies Lord Howe as a genetically distinct population from a single Elizabeth and Middleton Reefs population. The Mantel test was then used to investigate the correlation between genetic similarity and geographic distance (Manly, 1994). The two dimension test compared the two variables which were the pairwise F_{st} matrix (genetic distance) and the log transformed pairwise geographic distance (km) (Figure 5). The matrix produced a non significant p-value (P> 1.000) with (r =0.9986, z=2.2454) which indicates non significant correlations found in the Mantel analyses (Bohonak, 2002), therefore an isolation by distance effect (increase in haplotype variability with

increased distance) was not evident in the *C. galapagensis* populations, but this may be limited by the limited number of locations sampled in this study.

	M	**Elz**	**LH**
M	**-**		
Elz	0.00185	-	
LH	***0.5180****	***0.44702****	-

* *P*< 0.05, ** *P*<0.005, *** *P*<0.0001

Table 9 Pairwise F_{st} comparisons between *C. galapagensis* populations among three regions supported with the P-values significant level which is indicated by asterisks as indicated in the key.

4.1.2.3 Hierarchical analyses

The *AMOVA* analysis is used to measure the proportion of genetic variation among subdivided populations (Avise, 2004). These analyses have detected a strong structure within the Australian population (*Fst*= 0.5416, *P*=0.000) when Elizabeth and Middleton Reefs are grouped together and compared to the Lord Howe population, as suggested by the pairwise Fsts (Table 9). There is confirmation of a statistically significant variation among Australian *C. galapagensis* haplotypes from two populations (Elizabeth and Middleton Reefs vs. Lord Howe Island). The Elizabeth and Middleton Reefs individuals were genetically similar to each other, but distinct from the Lord Howe Island population. This confirms that there are two, but not three discrete populations. While 55% of the genetic variation was among locations (Middleton Reef vs. Elizabeth Reef vs. Lord Howe Island), this was not significant (*Fct*= 0.55, *P*= 0.34). Likewise, genetic variation among populations within regions was not significant (*Fsc*= - 0.02, *P*= 0.38) and it accounted for none of the genetic variation (negative value). Whereas genetic variation within populations relative to the total sample was the only highly significant measure (*Fst*= 0.54, *P*<0.0001) and accounted for 45% of the total observed genetic variation.

Source of variance	**d.f**	**S.S.M**	**Variance component**	**%of variance**	**Fixation indices(P)**
Among groups	1	109.102	2.80760Va	54.95	***Fct*** 0.549 (0.343)
Among populations with in groups	1	1.187	-0.04054Vb	-0.79	***Fsc*** -0.0176 (0.385)
With in populations	83	194.385	2.34199Vc	45.84	***Fst*** 0.5416 (***)

* P< 0.05, ** P<0.005 and *** P<0.0001

Table 10 Hierarchical Analysis of Molecular Variance (AMOVA) of *C. galapagensis*.

4.1.3 Coalescence analyses

Coalescence analyses were used to examine the population demographic history (e.g. population expansion and bottlenecks) from the mtDNA. The mitochondrial control region substitution rate for the variable and conserved sites was estimated as (3% of the sites mutating at a rate of 2.07% MY^{-1}, and 97% of the sites mutating at a rate of 0.15% MY^{-1} respectively). Therefore the total mutation rate was calculated as 0.196% MY^{-1} bp^{-1}. The mismatch distribution histograms (Figure 6) were calculated by comparing observed and expected sequence differences among all possible pairs of sequences in the sample to determine the population demographic state (Roger and Harpending, 1992).

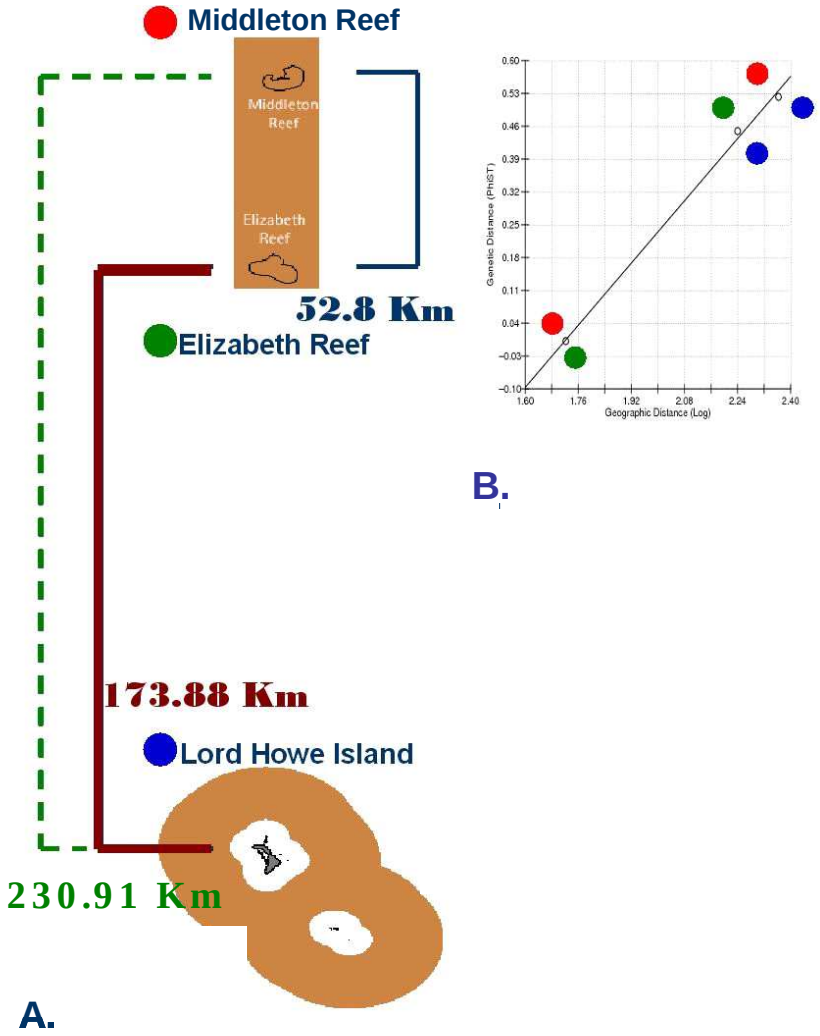
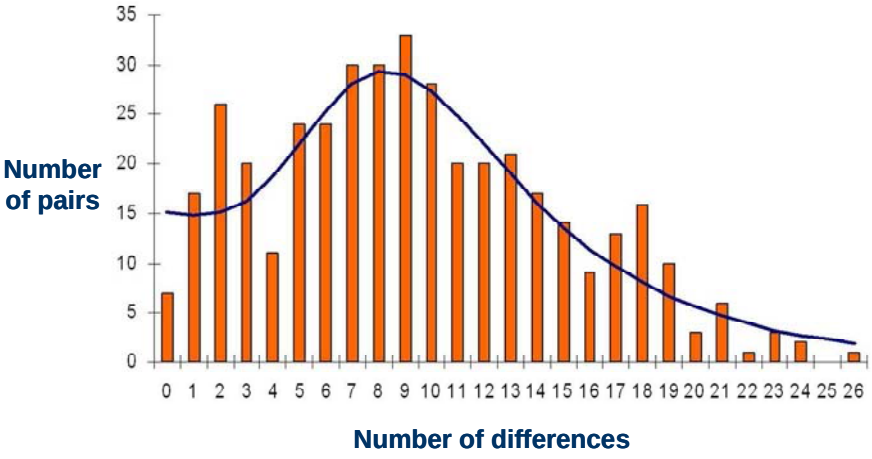


Figure 5 Pairwise comparison of **A)** geographic distance (km) against **B)** F_{st} (genetic distance). Sample locations are: (M: Middleton reef, Elz: Elizabeth reef and LH: Lord Howe Island.

The mismatch distribution histogram of the Lord Howe population, displayed a smooth unimodal graph, which is a characteristic of a long-term equilibrium population expansion. The same expanding pattern is displayed by the Elizabeth and Middleton Reefs populations, yet expansion in this population is likely more recent as it represents fewer haplotypes with fewer mutations.

By calculating years since population expansion of the two populations, we concluded that the Lord Howe population is older than the Elizabeth and Middleton Reefs population. High haplotype and nucleotide diversity result when more time has passed since expansion started, allowing more mutations to accumulate. On the other hand, the Elizabeth and Middleton Reefs population showed reduced haplotype and nucleotide diversities, which indicates a more recent expansion with less time since the expansion.

A.



B.

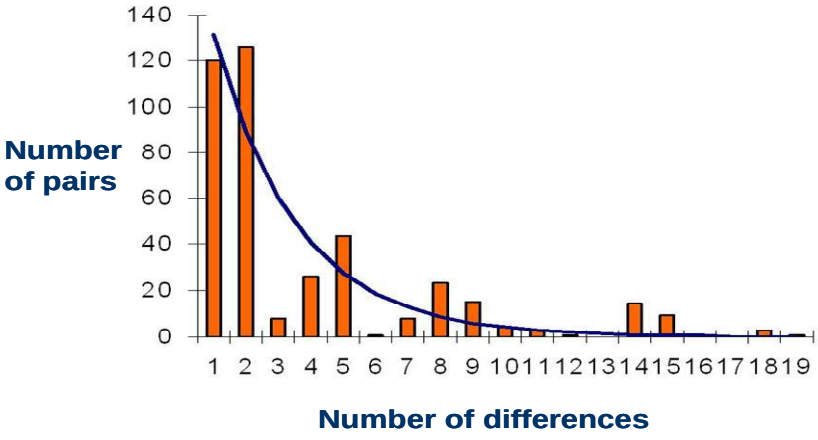


Figure 6 Mismatch distribution histogram for Australian *C. galapagensis* populations; A. Lord Howe Island and B. Elizabeth and Middleton Reefs population. The observed frequency of pairwise base pair differences are indicated by histogram bars and the expected values by line. The null hypothesis for the expected values is that the populations are expanding.

Estimates of the mtDNA expansion times were then calculated using the total mutation rate and the *C. galapagensis* female generation time which was assumed based on two longevity estimates (20 years or 15 years) (De Crosta et al., 1984), therefore the female generation time is calculated as (15 years or 12.5 years respectively).

Coalescence analyses suggest that the Lord Howe Island population started expanding 30577.9 years ago, with a range between **(**11 417.274 and 58 512.773 years ago), whereas the Elizabeth and Middleton Reefs population started expanding 6 599.8 years ago ranging between (580.9 and 16 630.17 years ago) (Table 11). Therefore the coalescence analyses and mismatch distribution both suggest that the Lord Howe population appears to be older than the Middleton and Elizabeth Reefs population when minimum, mean and maximum ages are considered.

Tau **(95%CI)**	**Theta $_0$**	**Theta$_1$**	**SSD**	**Raggedness index**	**t divergence (K year)**		**Mean nr of diff.**
2.17 (0.191-5.468)	**M**=463.596	0.32	0.022***ns***	0.0898***ns***	**6599.8** (580.9-16630.2)	**Eliz & Mid**	1.562
10.054 (3.754-19.239)	1.9	50.046	0.003***ns***	0.0049***ns***	**30577.9** (11417.3-58512.8)	**LHI**	9.587

Table 11 Coalescence analysis parameters for *Carcharhinus galapagensis* calculated from Arlequin vr.3

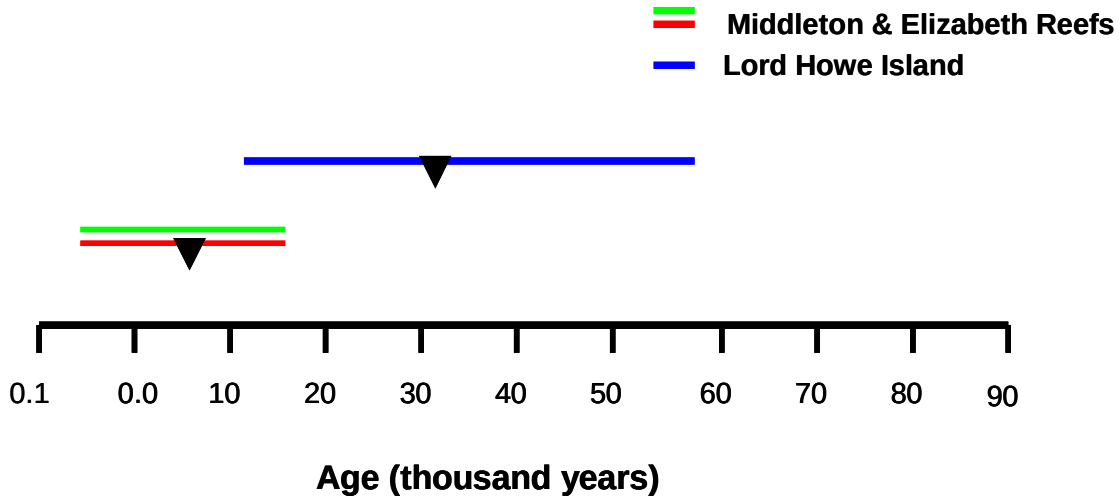


Figure 7 Coalescence age range of Australian C .galapagensis populations obtained from Elizabeth - Middleton Reefs and Lord Howe Island populations as indicated in the key. The mean age is specified by an arrow.

4.*2 Nuclear data:*

The Hardy-Weinberg equilibrium test revealed significant *P*-values for the expected and observed data for cli108 and cli107 among Lord Howe individuals. Therefore, the null hypothesis that the population is in Hardy-Weinberg principle is rejected for these two loci. Consequently the nuclear data are not presented due to insufficient data, as a single microsatellite locus can not determine population genetic structure, particularly for such limited sample sizes, as were available for this study.

5. Discussion

The four specified aims of this study (listed below) were achieved and we consider the relevance of these findings here.

5.1. Confirm the identity of Australian C. galapagensis

All phylogenetic analyses confirmed that Australian *C. galapagensis* are of the same species as the Hawaiian *C. galapagensis*. However, these analyses did not partition *C. galapagensis* populations into identifiable genetic clades. Population genetic analyses were required to detect stock structure.

5.2 Population structure of C. galapagensis in Australia

Although genetic variability was extremely low in *C. galapagensis* sequences, mtDNA analyses provided evidence to reject the null hypothesis that the samples share a single gene pool. Significant structure, which grouped Elizabeth and Middleton Reefs as a single population that is genetically distinct from the Lord Howe Island population was detected. Specifically, the population structure was supported by significant pairwise *Fst* measures, which were low for Elizabeth and Middleton Reefs, but high for both these sites and Lord Howe. This was confirmed by more statistically rigorous AMOVA analyses, which displayed a highly significant F_{ST} value between the Elizabeth - Middleton Reefs population and the Lord Howe population, with 55% of the total molecular variance attributable to variation among the three regions.

The mantel test suggested a direct correlation between genetic and geographic distances in all matrices (both standard and log transformed matrices), but was not significant, probably due to a lack of power. Additional sample locations across a wider geographical scale are needed to test this hypothesis more rigorously.

The observed geographic partitioning of *C. galapagensis* is not surprising, especially in the absence of a pelagic dispersal phase. Yet, it is unexpected from a species that is distributed worldwide. Similar findings were displayed in other widely distributed

shark species such as the blacktip shark (*Carcharhinus limbatus*) (Keeney and Heist, 2006) and the Pacific angelshark (*Squatina califorrnica*) with limited gene flow in the latter species being due to the restricted trans oceanic migration of adults (Gaida, 1997).

Although evidence for a strong structure of *C. galapagensis* in Australia is significantly supported, this study could not fully resolve the population structure question, as it did not account for male gene flow. Microsatellites were eliminated from the study due to insufficient data, time and money. However, further analysis is essential to determine if the currently identified genetic structure in *C. galapagensis* is derived from restricted female mediated gene flow (philopatry) alone or if it is present for the entire population. Similar degrees of population separation have been recorded in female carcharhinids returning to nursery areas to give birth (Feldheim et al. 2002, Keeney et al. 2003, Keeney et al. 2005). We suggest that the degree of population separation between the study sites may represent nursery ground site fidelity in *C. galapagensis*. In coral reef habitats, the high level of site fidelity has a great advantage in increasing both foraging success and resource awareness of the organism (Bradshaw et al. 2004). However, additional data from more nuclear markers and ideally more individuals than were available for this study, are required to evaluate this hypothesis further.

5.3 Level of genetic diversity in C. galapagensis

Grant and Bowen (1998) divided marine fishes into four groups depending on their genetic (nucleotide and haplotype) diversity measures obtained from mtDNA sequence analysis. Based on these criteria, the Elizabeth and Middleton Reefs population fitted into the first category with low haplotype and nucleotide measures, which suggests that the population represents a recent colonization and shallow history or that this population experienced a recent bottleneck which produced the observed low levels of genetic diversity. In contrast, the Lord Howe population, with high haplotype and nucleotide diversities, fits the fourth category. This suggests that high genetic diversity of this population is attributed to i) secondary contact between previously differentiated allopatric lineages (e.g. receiving migrants from other distinct populations such as Elizabeth - Middleton Reefs and/or the Kermadecs, New

Zealand) or ii) a large stable population. Additional material from neighbouring populations, such as the Kermadecs, is required to evaluate this.

Although little is known about the evolutionary history of *C. galapagensis* from – Elizabeth - Middleton Reefs, their low genetic diversity indicates periods of low effective population size within their recent past. This low genetic diversity suggests that the Elizabeth - Middleton Reefs population has lower resilience and is more susceptible to being adversely affected by anthropogenic and/or environmental stresses, such as global warming and disease outbreaks, than is the Lord Howe population. Comparing the genetic diversity of *C. galapagensis* to diversity indices obtained for other globally distributed marine organisms, including other shark species, other marine organisms including fishes, marine mammals and reptiles, indicated that the overall level of diversity in *C. galapagensis* is relatively high compared to other sharks, but low when compared to teleosts and is remarkably similar to the genetic diversity of the killer whale (*Orcinus orca*), a cetacean which exhibits a viviparous reproductive mode and a nektonic dispersal the same as *C. galapagensis*.

Another possible reason for low genetic diversity in sharks is their life history. This was investigated by Mitton and Lewis (1989), who stated a significant relationship between genetic variability and generation time, maturation age and maximum size (high genetic variability = short generation time, quick maturation and small maximum size). As most sharks (including *C. galapagensis)* do not display any of these features it may be that life history contributes to the low genetic diversities seen.

5.4 Challenges associated with limited reproductive output (fecundity) and dispersal ability of C. galapagensis

Sharks in general display a slow life history pattern, which is suggested to be one reason for their low genetic variability and slow mutation rate (Martine et al., 1992). Since *C. galapagensis* reproduce at most once every two years, with the production of relatively few offspring (6-16 pups per litter), they have a long generation time (Compagno, 1984). In addition to this low fecundity, gene flow is limited to migration by adults and/or juveniles. This is in stark contrast to high fecundities and dispersal

abilities of most teleosts, which release orders of magnitude more offspring more frequently, in the form of pelagic larvae, which are capable of extensive dispersal (Hedgecock, 1986). Thus, the *C. galapagensis* life history is thought to constrain their dispersal ability and consequently their ability to recover from population depletion, whatever the cause.

Sexual segregation in *C. galapagensis* (Wetherbee et al., 1994) is also thought to affect population structure, since it may result in sex-biased gene flow (Pardini et al. 2001) or philopatry. Such behaviour has been reported in other large shark species, such as the scalloped hammerhead, *Sphyrna lewini* (Duncan et al., 2006) and sand tiger sharks (grey nurse), *Carcharias taurus* (Stow et al., 2006). The presence of sexually mediated gene flow in *C. galapagensis* was investigated by the use of microsatellites to compare with the female mediated gene flow structure, which is inferred from mtDNA. However, only three of the 6 microsatellites applied in this study generated data and when analysed, there was insufficient statistical power to address the question. This shortcoming was exacerbated by the small numbers of samples available in this initial study and should be revisited given additional resources (time and money) to increase the number of microsatellite loci, the number of locations sampled and the number of individuals per sampled location.

6. Implications for management and conservation

1) The presence of two *C. galapagensis* stocks in Australia (Elizabeth - Middleton Reefs and Lord Howe Island) indicates that both populations require individual management. This is particularly important to maximize the conservation of genetic diversity. Although this study found that the Lord Howe Island population apparently has received migrants from the Elizabeth and Middleton Reefs population, this appears to be one-way and is likely insufficient to sustain either population. The Lord Howe Island population may be self-replenishing and/or be replenished from elsewhere (e.g. the Kermadecs in New Zealand). Further research is required to discriminate between these two very different possibilities, which will impact on the management strategies required.

2) The low genetic diversity of the Elizabeth and Middleton Reefs population, which was only established recently by a small founder population, is of particular concern and suggests rare migration to this population from elsewhere. This results in a lower genetic resilience of the Elizabeth and Middleton Reefs population than in the Lord Howe Island population. The implication of these results for the long-term conservation of *C. galapagensis* at the Elizabeth and Middleton Reef Marine National Nature Reserve is that this population should be carefully managed to eliminate or minimize impacts of fishing in the area, since recreational fishing is permitted at Elizabeth Reef under the current Management Plan (Oxley 2006). This is particularly important in light of the plight of shark populations world-wide (Myers et al 2007, Ward and Myers 2005, Myers and Worm 2005, Shepard and Myers 2005), including Australia (Robbins et al 2006), where shark abundances have plummeted, even if they have not been targeted by fishermen. Given this, it is most likely that the healthy populations of Galapagos sharks at Elizabeth and Middleton Reefs and at Lord Howe Island will suffer reductions if fishing is permitted in these areas, since the sharks (and black cod, *Epinephelus daemelii*) at these locations are not accustomed to human activities and are very curious towards both divers and baited hooks, even if the sharks or black cod are not themselves the target. Although this is an untested hypothesis at Elizabeth and Middleton Reefs and at Lord Howe Island, it has been tested in Hawaii, where there is striking evidence that fishing pressure (including recreational and subsistence fishing) in the main Hawaiian Islands has led to drastic reductions in number and size of a number of fish species, mainly apex predators, including sharks and the Hawaiian grouper, *Epinephelus quernus*. In contrast, these species are still common at the lightly fished remote North Hawaiian Islands (Friedlander and DeMartini 2002). Importantly, the study by Friedlander and DeMartini (2002) found that fully protected no take reserves in the main Hawaiian Islands had higher standing stocks of apex predators and other reef fishes than areas with partial or no protection from fishing, even if these areas were only fished recreationally.

3) The genetic partitioning between these Australian populations must be taken into account if any restocking between locations is to be considered.

4) Finally, it is fundamental to realise that limited fecundity and dispersal abilities of this species makes it highly susceptible to extirpation (the loss of the population from an area), especially if exploited. As reproduction is the main recovery strategy for *C. galapagensis*, this would be a very slow recovery at best. In general, the worldwide decline in shark species (Myers et al 2007, Ward and Myers 2005, Myers and Worm 2005, Shepard and Myers 2005, Castro et al., 1984) highlights the increasing importance of conserving these ecologically important apex predators.

7. Future research directions

1) Initiate a study to evaluate the impact of recreational and charter fishing activities in partially fished and no take reserves at Elizabeth and Middleton Reefs Marine National Nature Reserve and Lord Howe Island Marine Park. Partially protected areas open to recreational fishing in both Hawaii and the GBR have suffered substantial declines in shark numbers compared to areas with complete protection. Fishing at Elizabeth Reef could deplete the abundances and sizes of apex predators such as grouper (black cod) and (Galapagos) sharks even if they are not target species.

2) Future genetic studies of *C. galapagensis* in the Tasman Sea will need to include additional samples and estimates of abundance from these and other populations such as the Kermadecs, New Zealand, in order to identify what gene flow (if any) exists between this nearest neighbour to the Australian populations. They will also need to incorporate additional nuclear (microsatellite) markers to determine both the scale of gene flow and evidence for male-mediated gene flow, since this study had insufficient power to realise this. Lastly, the number of individuals per sampled location will need to be increased to 50 if possible in order to improve the statistical power of analyses.

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