



Australian Government

**A survey of the terrestrial reptiles of Norfolk Island
March 2005:**

**Report 3. The levels of intraspecific genetic differentiation among island
populations of the gekkonid lizard *Christinus guentheri* in the Norfolk Island
complex**

prepared for

the Department of the Environment and Heritage

by

D. Colgan, G. Muir, G. Shea and H. Cogger



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A survey of the terrestrial reptiles of Norfolk Island March 2005:

Report 3: the levels of intraspecific genetic differentiation among island populations of the gekkonid lizard *Christinus guentheri* in the Norfolk Island Complex

by

Don Colgan¹, Glenn Muir², Glenn Shea³ and Hal Cogger⁴

Introduction

The gekkonid lizard *Christinus guentheri* is gazetted as a Vulnerable species under the Commonwealth of Australia's *Environment Protection and Biodiversity Conservation Act 1999* (EPBC Act). It is listed as Vulnerable in the World Conservation Union (IUCN) Red Data Book (2004). It occurs only on two oceanic island complexes in the western Pacific off the eastern coast of Australia: Lord Howe Island and Norfolk Island.

Gecko populations on both islands are morphologically similar but their genetic relationships to each other are currently unknown. Both island complexes consist of a large main island and a series of smaller offshore rocks and islets, which together represent the eroded remnants of once larger and continuous Gondwanan land masses sitting atop seamounts on oceanic ridges. Their continuous existence as islands probably dates from the late Eocene. These islands and their smaller satellite islands are eroded remnants of once larger islands, and doubtless their offshore islands were connected to the present main islands on many occasions since that time through the combined effects of orogenic uplift and fluctuating sea levels. At least some of them would have been connected to the main island as recently as the last 8000–20,000 years.

The broader study reported here incorporates the results of two separate field surveys. The first was conducted by the Australian Museum for ANCA (a predecessor of the Department of the Environment and Heritage (DEH) in 1978 (Cogger *et al.*, 1979, 1983). However in 2004, while preparing a draft recovery plan (Cogger, 2004) for *Christinus guentheri* on Norfolk Island, it became clear that the plan would have to rely primarily on data collected in 1979. Subsequently DEH contracted the authors, through Australian Museum Business Services, to undertake a new survey of the Norfolk Island complex to determine whether the conservation status of this species (and of a native scincid lizard confined to Phillip Island, *Oligosoma lichenigera*) had changed significantly in the intervening 25 years.

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In the 1978 survey of the reptiles of Norfolk Island it was confirmed that the gecko did not then occur on Norfolk's main island, while historic records indicated that the species was also absent from the main island at the time of European colonisation in 1788. However subfossil records associated with archaeological excavations in the Kingston area of Norfolk Island have revealed the presence of the species (including eggs) at around 800 A.D. It was from about this time that the Polynesian Rat (*Rattus exulans*) is first recorded from Norfolk Island, with clear association with a Polynesian presence. Anthropologists (Matisoo-Smith, Horsburgh, Robins and Anderson, 2001; Smith, Clark and White, 2001) have concluded that these two events are related, and it has been further postulated that, in the absence of any earlier fossil record, the introduction of *Rattus exulans* by Polynesian visitors in ca. 800 A.D. brought about the extinction, on Norfolk's main island, of the native gecko *Christinus guentheri*. The Pacific Rat has now been largely displaced by the post-settlement introduction of the larger and more aggressive Black Rat (*Rattus rattus*).

Whether *Rattus exulans* dates from an earlier introduction has yet to be determined, but the impact of the introduction of *R. exulans* to New Zealand and many of its off-shore islands by Maori colonisers certainly resulted in the decline and extinction of many organisms, including lizards. Similarly, the great decline of this gecko (and a native scincid lizard) on Lord Howe's main island following accidental introduction of the Black Rat (*Rattus rattus*) from a shipwreck in 1927 is well documented, and resulted in the native lizards being confined to small pockets of microhabitats in which rat-proof oviposition sites were abundant.

It has also been postulated that the high impact of rodents on island lizard populations has been largely confined to oviparous species, via predation on eggs and the consequent recruitment failure.

In 1978 (Cogger *et al.*, 1983) *Christinus guentheri* was recorded from Phillip Island and Nepean Island, both lying to the south of the main island, and from three small rocky islets – Moo'oo Stone, Green Pool Stone and Bird Island - all lying within 100 metres or so of the northern coast of the main island. Given the habitat utilised by the gecko on these islets (grassy rock slopes and stunted shrubs and trees) it is likely that the species also occurs on a number of other small rocky islets, extremely difficult to access, off the northern coast. We were unable to gain access to these latter islets, and with the exception of Cathedral Rock (where Mr Owen Evans advises that the geckos do occur) the occurrence on them of geckos is yet to be determined.

The gecko-inhabited islets of the north coast, occurring as they do mostly within 50–100 metres of the main island, were likely to have exchanged occasional individual geckos with mainland populations when the latter existed. However the probability of direct genetic exchange between these northern islet populations and those on the larger islands to the south of the main island is very low. Prevailing winds and sea movements would be unlikely to carry large, gecko-carrying flotsam to the northern side of Norfolk Island, involving a distance of more than 20 km across open ocean.

The gecko populations of the northern islets appear to be small, probably fewer than 100 individuals. Individuals are generally hard to locate during diurnal searches,

although nocturnal surveys are likely to be more productive. Assuming that these populations have effectively been small, closed populations since *Christinus guentheri* became extinct on the main island (ca. 1200 ybp), one might expect considerable in-breeding and loss of genetic variability compared with populations from the large southern islands, where population size is measured in the tens – and possibly hundreds – of thousands.

Yet the large population on Phillip Island is probably the most vulnerable because this island is extensively used as a base for fishing, tourism and other forms of passive recreation, making it extremely vulnerable to unwanted introductions (such as rodents and invasive plant species) that could devastate the gecko population (and other native species).

For this reason the conservation of the small populations on small rocky islets may prove to be critical to the long-term survival of the species on Norfolk Island. Certainly, from the perspective of managing such a threatened species, the vulnerability of small oceanic islands to exotic predators and other invasive species makes the population on any given island not only vulnerable to rapid decline or extinction, but conversely also a potential source for recolonising, following remediation, any islands that might suffer a decline from any cause.

On Norfolk Island's main island, a wide range of actions (e.g. rodent and weed control programmes) are being undertaken by the relevant land managers to ameliorate the impacts of invasive weeds and other introduced exotic species to promote the natural recovery of many native species. In considering the reintroduction of a species to an area in which it has significantly declined or disappeared, the genetic relationships of both the original population and the population to be sourced for reintroduction can be of considerable import in conservation biology terms. In the case of *Christinus guentheri* within the Norfolk Island complex this is not merely a theoretical consideration; there is considerable local interest in reintroducing this species to Norfolk's main island if suitable sites, managed for rodent control and the minimisation of other threatening processes, can be identified. This issue is the subject of a separate report by the present authors to DEH.

It is important to assess the patterns of intraspecific genetic variation in threatened species, as this can reveal:

- a. unsuspected geographic or taxonomic subdivision in a species *or*
- b. that a particular population is depauperate in variability and thereby exposed to an increased risk of extinction (Spielman *et al.*, 2004).

This investigation was conducted to assess whether there is significant genetic variation between the populations of *C. guentheri* in the Norfolk Island complex, by examining:

- a. whether there are divergent haplotypes restricted to a particular locality *or*
- b. whether there are haplotype frequency differences between populations.

The investigation was also intended to identify whether there was a restriction on genetic variability in any population that would threaten its viability in the immediate future. Two gene segments from the mitochondrial DNA were chosen for investigation. These have been shown to be quite variable in previous studies of

reptiles, and primers are available for both that are suitable for amplifying most species of vertebrates (Mousseline *et al.*, 2005; Sadlier *et al.*, 2005). The segments were cytochrome *b* (abbreviated as Cytb, herein) and cytochrome *c* oxidase subunit I (COI).

Materials and methods

Autotomy and regrowth of partially or completely lost tails is characteristic of many lizard species, including most gekkonid lizards. The frequency of reproduced tails in adult populations of *Christinus guentheri* in the Norfolk Island complex is very high (89% - see *Background Document*), indicating that single or multiple tail loss events from intraspecific interactions and/or gecko-predator interactions is a normal occurrence in the lives of these lizards, and clearly does not constitute a life-threatening event. Under Australian Museum Animal Care and Ethics Committee's Approval 02–03 a short section of the tail tip (<10mm) of selected hand-captured geckoes was removed, either with clean forceps or by slicing cleanly with a fresh scalpel blade, and placed in 0.5 ml of RNA later (Ambion) for storage. The tissue was stored in this solution until processed on 2/5/5. For DNA extraction the specimen was removed from the RNA later and drained on tissue paper. Approximately half of each sample was dissected for use. DNA was extracted by the CTAB method of Saghai-Marroof *et al.* (1984).

The polymerase chain reaction (PCR) was performed to amplify specific short sequences of DNA. The reactions were set up using 1.0 Units of BioTaq DNA polymerase, in the manufacturer's buffer made to 1X concentration, 0.05 mM dNTPs, 3.5 mM MgCl₂ and 12.5 pmol of each primer in a total reaction volume of 50 µL. 1 µL of DNA was used. Samples with more concentrated DNA were diluted before use (see Table 1). Negative controls were included in each reaction array. The cycling profile was as follows; (95°C for 5 min, 50°C for 1 min, 72°C for 1 min) for one cycle, (95°C for 30 sec, 50°C for 1 min, 72°C for 1 min) for 32 cycles and 72°C for 3 min for the final cycle.

Details of primer pairs are as follows:

Cytochrome oxidase subunit 1 (Folmer *et al.* 1994):

COIL1490F 5'-GGTCAACAAATCATAAAGATATTGG-3'

COIR 2198R 5'-TAAACTTCAGGGTGACCAAAAAATCA-3'

These primers amplify a fragment of approximately 700 base pairs (including the primer sequences themselves).

Cytochrome *b* (Kocher *et al.*, 1989):

CytBF: 5'-AAAAGCTTCCATCCAACATCTCAGCATGATGAAA-3' and

CytBR: 5'-AAACTGCAGCCCCTCAGAATGATATTTGTCCTCA-3'

These primers amplify a fragment of approximately 400 base pairs (including the primers).

Reaction products were separated by electrophoresis through 2% agarose gels containing ethidium bromide. Single band products were purified using AMPURE magnetic beads (Agencourt). Products were sequenced (generally in both

directions) with a General Electric MEGABACE automatic capillary sequencer using the ET (General Electric) or Big Dye (ABI), version 3.1 sequencing chemistries according to the manufacturer's protocols with the following modifications. Sequencing buffer (4 μ l) was used in all reactions. The amount of ET used was 4 μ l. Big Dye was generally reduced to 2 μ l. Sequencing primers (1 μ L) were used at a concentration of 3.2 pM/ μ L for Big Dye and 5 pM for ET. Reactions were purified by ethanol precipitation.

Sequences were edited using Sequencher (Anon., Gene Codes Corporation). Sequences determined in only one direction are identified by asterisks in the figures and tables. Sequence alignment was performed using the default parameters in CLUSTALX (Thompson et al., 1997). Nexus files were edited using MacClade (Maddison and Maddison, 1992).

Analyses using maximum parsimony were carried out using PAUP*4.0 (Swofford, 2001) with default settings assumed except that transformations were assumed to be delayed. Heuristic searches were conducted with 200 replicates of random taxon addition sequence.

Mega v.3.0 (Kumar et al., 2004) was used to determine average pairwise genetic distances. The calculations are based on Tamura-Nei distances, assuming equal substitution rates per alignment position, with pairwise deletion of missing data.

Results

Sequences were collected from all individuals for the cytochrome b dataset. For one sample, it was possible to collect data for sequencing in one direction only. All other specimens were sequenced in both directions. In the alignment of the cytochrome b sequences, there were 313 invariant sites and 21 variable sites.

Sequences were collected from 11 specimens for the COI dataset. Eight of these were sequenced in both directions but three could only be sequenced in one direction. In the alignment of these sequences, there were 617 invariant sites and 11 variable sites.

Average pairwise genetic distances both within (results not shown) and between populations (Table 2) were low for both gene segments indicating that all specimens clearly belonged to the same species. Nevertheless, some notable differences between populations were apparent in the distribution of haplotypes.

There were four Cyt b haplotypes from Phillip Island in six scored individuals. Two of the haplotypes are shared with Nepean I and two were found nowhere else. Five of six scored specimens from Nepean Island had identical haplotypes, with the only variant being EBU37018. There are two haplotypes in the three specimens from Moo-oo Stone. The sample from Bird Rock has the same sequence as one of these haplotypes. The other is not found in any other population in the sample.

Eighty-nine trees were found in maximum parsimony analyses of the cytochrome b data (Figures 1 and 2). These had 21 steps and returned the maximum possible consistency index of 1.000.

The four scored geckos from Nepean Island included one haplotype found in three individuals and a variant found in EBU37022. Each of the other seven specimens has a distinct haplotype that is found nowhere else. Four of these are from Phillip Is., two are from Moo-oo Stone and the seventh is the Bird Rock specimen.

Seventy six trees were discovered in maximum parsimony analyses of the COI data (Figure 3). These were 12 steps long and had a consistency index of 1.00.

In combination of the two gene segments, there were 11 haplotypes. Each of the individuals had a unique haplotype except for (i) EBU37003 and EBU37005, (ii) EBU37016, EBU37017, EBU37019 and EBU37020 (iii) EBU370018 and EBU37026. In total Phillip Is. had five haplotypes, Nepean Is. three, Moo-oo Stone three and Bird Rock one.

Discussion

Low genetic distances between individuals indicate that all are conspecific. These distances and the general sharing of haplotypes suggests that the species is not structured into recognised sub-species or, as far as determinable with the present data, into genetically isolated regions. Surveys using the much more labour intensive technique of microsatellite analysis would be required to determine whether such regionalisation is completely absent.

The population on Phillip Island appears to retain a good level of genetic variability but there is no indication that it contains representatives of all of the haplotypes in *Christinus guentheri*. Therefore the other populations have conservation value if the goal is to preserve as much as possible of the remaining genetic diversity in this species. The low nucleotide divergence between the haplotypes on Phillip Is. and those found only in other populations suggests however, that the majority of the evolutionary divergence of the species in the Norfolk complex is at least represented in this population. Specimen number EBU37013, in particular, is quite distinct for both gene segments, so Phillip Island may include representatives of the most divergent lineages within *C. guentheri*.

More than one haplotype variant was observed only in the Moo-oo Stone sample, emphasising the importance of this population and indicating that it has maintained at least two distinct lineages despite possibly being isolated for many hundreds of years. Although only one specimen was available from Bird Rock, this had a unique haplotype for the COI segment.

Two haplotypes observed in each of the gene segments scored in individuals from Nepean Islands. Neither of the Cytb haplotypes was unique to this population. Both COI haplotypes were unique to the island but differed by only one or two base changes from a haplotype found on Phillip Island. The low numbers of haplotypes compared to the number of specimens sequenced might indicate a reduction in genetic variability in this population but this may also be due to small sample effects.

Summary

Sequencing of specimens of *Christinus guentheri* for two gene segments clearly indicates that all are conspecific and does not suggest that the species is regionally genetically structured in the Norfolk Island group. Specimens were available from four populations, two larger at Phillip and Nepean Islands and two from smaller islands at Moo-oo and Bird Rocks. Unique haplotypes were found in all of these populations suggesting that each has some significance for the preservation of the genetic variation in the species.

References cited

Anonymous, (1994). *Sequencher*. Gene Codes Corporation.

Folmer, O., Black, M., Hoeh, W., Lutz, R., and Vrijenhoek, R. (1994) DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Molecular Marine Biology and Biotechnology*, 3, 294–299.

Kocher, T. D., Thomas, W. K., Meyer, A., Edwards, S.V., Pääbo, S., Villablanca, F. X. and Wilson, A. C. (1989). Dynamics of mitochondrial DNA evolution in animals: Amplification and sequencing with conserved primers. *Proceedings of the National Academy of Sciences of the U.S.A.*, 86, 6196-6200.

Kumar, S., Tamura, K., and Nei, M. (2004) MEGA3: Integrated software for Molecular Evolutionary Genetics Analysis and sequence alignment. *Briefings in Bioinformatics*, 5, 150–163.

Maddison, W. P., and Maddison, D. R. (1992). *McCLADE, version 3.0*. Sunderland, Massachusetts, Sinauer Associates.

Moussalli, A., Hugall, A.F. and Moritz, C. (2005). A mitochondrial phylogeny of the rainforest skink genus *Saproscincus*, Wells and Wellington (1984). *Molecular Phylogenetics and Evolution*, 34, 190–202.

Matisoo-Smith, E., Horsburgh, K.A., Robins, J.H. and Anderson, A. (2001). *Genetic variation in archaeological *Rattus exulans* remains from the Emily Bay settlement site, Norfolk Island.* pp. 81–84 in Anderson, A. and White, P. (eds) *The prehistoric archaeology of Norfolk Island, Southwest Pacific. Records of the Australian Museum, Supplement 27*, i–vi, 1–142

Sadler, R. A., Couper, P., Colgan, D. J., Vanderhuys, E. and Rickard, E. *Saproscincus eungellensis, a new species of scincid lizard from Mid-eastern Queensland.* *Records of the Queensland Museum*, 51, 559–571.

Saghai-Marooof, M.A., Soliman, K.M, Jorgensen, R.A. and Allard, R. W. (1984). *Ribosomal DNA spacer length variation in barley: Mendelian inheritance, chromosomal location and population dynamics.* *Proceedings of the National Academy of Science of the U.S.A.* 81, 8018–8021.

- Smith, I., Clark, G. and White, P. (2001). *Mammalian and reptilian fauna from Emily and Cemetery Bays, Norfolk Island*. pp. 75–79 in Anderson, A. and White, P. (eds) *The prehistoric archaeology of Norfolk Island, Southwest Pacific*. Records of the Australian Museum, Supplement 27, i–vi, 1–142
- Spielman, D., Brook, B.W. and Frankham, R. (2004). *Most species are not driven to extinction before genetic factors can impact?* PNAS 101, 15261–15264.
- Swofford, D.L. (2001) *PAUP* Phylogenetic Analysis Using Parsimony (* and other methods)*. Version 4b10. Sinauer Associates, Sunderland Massachusetts.
- Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F. and Higgins, D.G. (1997) *The CLUSTAL X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools*. Nucl. Acids Res., 25, 4876–4882.

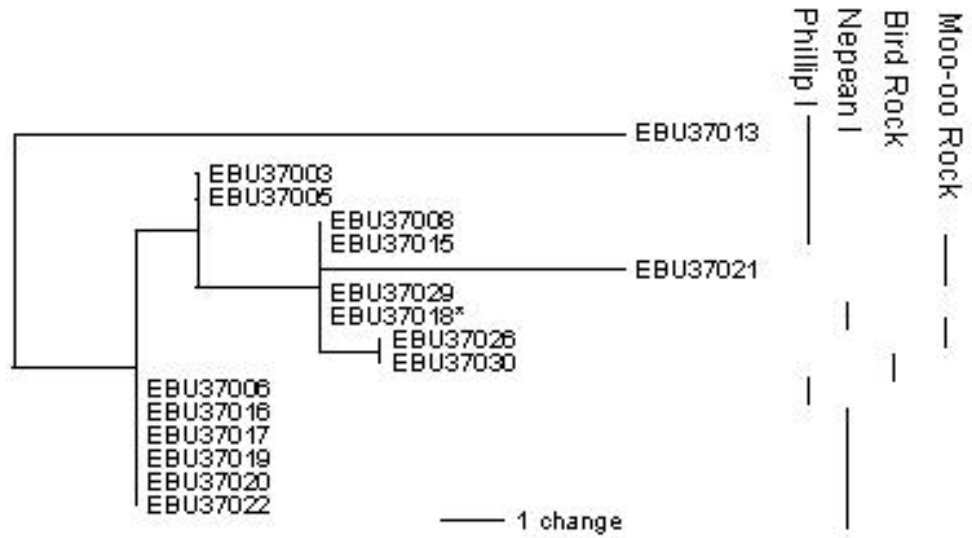
Table 1: Locality and specimen data for the sequenced specimens of *Christinus guentheri*. Specimens are identified by Evolutionary Biology Unit (EBU) tissue collection number. Localities are identified by the waypoint number. Haplotypes are separately designated within the individual gene segments. Upper and lower case letters are used to indicate that no relation between the schemes is intended. The designations were made in order of the first appearance of the haplotype in the numerical listing of the EBU numbers.

Locality	Waypoint	Specimen Number	DNA dilution	Cyt b	COI	Combined	Remarks
Phillip Island	12	EBU37003	1 in 8	a		1	
	22	EBU37005	1 in 8	a	A	1	Evan's Camp
	5	EBU37006	1 in 20	b	B	2	
	15	EBU37008	1 in 20	c	A	3	Long Valley pit trap line
	19	EBU37013	1 in 20	d	C	4	
	12	EBU37015	1 in 20	c		5	
Nepean Island	45	EBU37016	1 in 20	b	D	6	
	45	EBU37017	1 in 20	b	D	6	
	45	EBU37018	undiluted	c		3	
	45	EBU37019	undiluted	b		6	
	45	EBU37020	undiluted	b	D	6	
	45	EBU37022	1 in 20	b	E	8	
Moo-oo Stone		EBU37021	undiluted	e		7	
		EBU37026	1 in 20	f	F	9	
		EBU37029	1 in 20	f	G	10	
Bird Rock		EBU37030	1 in 20	f	H	11	

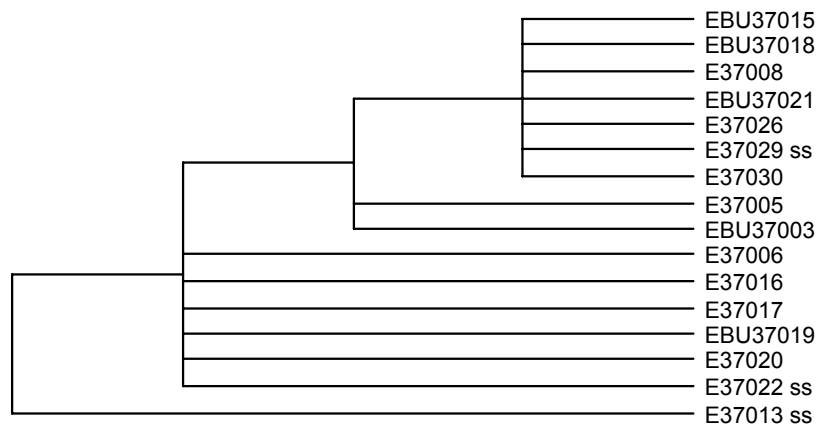
Table 2: Average pairwise genetic distances between four samples of *Christinus guentheri*. The figures in the lower triangular half of the table are for cytochrome *b* and the figures for the upper half are for cytochrome *c* oxidase. Two figures are given for each cell. The first is the average genetic distance between pairs of individuals comprised of one member each of the indicated populations. The second is the standard deviation of these averages.

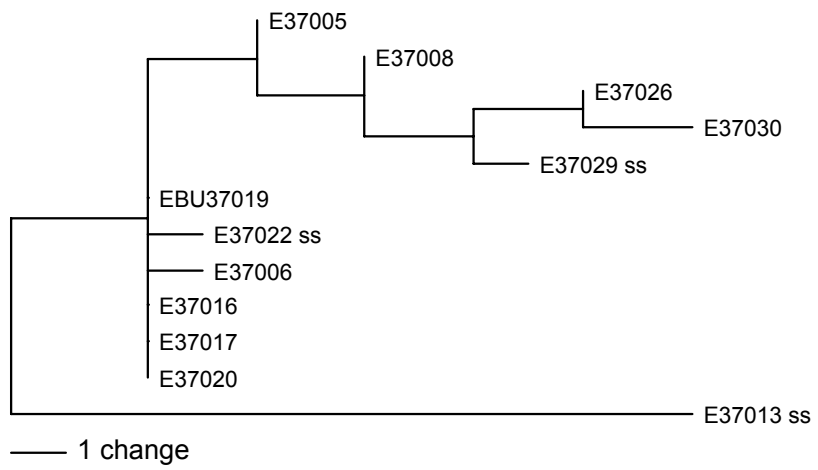
Population	Phillip Island	Nepean Island	Moo-oo Stone	Bird Rock
Phillip Island		0.003 ± 0.001	0.006 ± 0.003	0.010 ± 0.004
Nepean Island	0.020 ± 0.005		0.007 ± 0.003	0.011 ± 0.004
Moo-oo Stone	0.021 ± 0.005	0.008 ± 0.003		0.004 ± 0.002
Bird Rock	0.023 ± 0.006	0.012 ± 0.005	0.006 ± 0.006	

The figure below are (in order) (1) one of the trees for the Cyt b data, (2) the consensus of the MP trees from this analysis; (3) one of the trees for the COI data.



Cytochrome b contree
Strict





Appendix 1: Cytochrome *b* sequence data collected for the report. The specimens are identified by EBU number (see Table 1). The dots indicate that the base at that position in the specified sequence is the same as the base in the sequence written at the top of the compilation. The data are presented in NEXUS file format so that they can be edited and saved as a text formatted file for analysis in bioinformatics programmes such as PAUP, etc. Dashes indicate missing data. Letters other than A,C, G or T indicate uncertainties according to standard codes (M = A or C, K = G or T, R = A or G, S = C or G, W = A or T, Y = C or T).

```
#NEXUS

BEGIN DATA;
DIMENSIONS  NTAX=16  NCHAR=334;
FORMAT MISSING=- GAP=-  MATCHCHAR=.  INTERLEAVE  DATATYPE=DNA ;

MATRIX

[           10           20           30           40           50]
[           .           .           .           .           .]

EBU37003  CATCCAACAT CTCAGCATGA TGAAATTTTCG GCTCACTATT AGGACTATGC
[ 50]
EBU37005  .....
[ 50]
EBU37006  .....
[ 50]
EBU37008  .....
[ 32]
EBU37013  -----
[ 0]
EBU37015  ----- .....
[ 40]
EBU37016  ----- --- .....
[ 37]
EBU37017  ---- ..... - .....
[ 46]
EBU37018* .....
[ 50]
EBU37019  .....
[ 50]
EBU37020  ---- .....
[ 45]
EBU37021  ..... ..... A .....
[ 50]
EBU37022  .....
[ 50]
EBU37026  .....
[ 50]
EBU37029  ----- -- .....
[ 38]
EBU37030  .....
[ 50]
```

```

[           60           70           80           90
100]
[           .           .           .           .           .]

```

```

EBU37003   CTAATTCTAC AAATCCTAAC AGGTCTATTC CTAGCAATAC ACTACTCAGC
[100]
EBU37005   .....
[100]
EBU37006   .....-.....
[99]
EBU37008   .....C.....
[82]
EBU37013   -----
[0]
EBU37015   .....-.....C.....
[89]
EBU37016   .....-.....
[86]
EBU37017   .....M.....
[96]
EBU37018*  .....C.....
[100]
EBU37019   .....M.....M.....
[100]
EBU37020   .....
[95]
EBU37021   .....C.....C.....
[100]
EBU37022   .....
[100]
EBU37026   .....Y.....
[100]
EBU37029   .....C.....
[88]
EBU37030   .....C.....
[100]

```

```

[          110          120          130          140
150]
[           .           .           .           .           .]

```

```

EBU37003   AGACATCTCC CTGGCCTTTT CATCCATCTC CCACATCTGT CGAGACGTAC
[150]
EBU37005   .....
[150]
EBU37006   .....
[149]
EBU37008   .....
[132]
EBU37013   -----
[21]
EBU37015   .....
[139]
EBU37016   .....
[136]

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EBU37017 .S.....
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 EBU37018*
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 EBU37021R.....M.....
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 EBU37026
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 EBU37029
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 EBU37030
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[160 170 180 190
 200]
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EBU37003 AGTACGGCTG ATTAATCCGA AATATTCACG CCAACGGCGC ATCTTTATTC
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 EBU37005
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 EBU37006
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 EBU37008
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 EBU37013G.. -..... T.AT..... -.....A.....
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 EBU37016
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 EBU37017
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 EBU37020
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 EBU37021 .R.....M.....Y.....M.....W.....
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 EBU37029
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 EBU37030
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[210 220 230 240
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 EBU37006
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 EBU37022
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 EBU37026
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 EBU37029
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 EBU37030
 [250]

[260 270 280 290
 300]
 [. . . .]

EBU37003 CACAGCCACA AAAACATGAA ACATCGGAAT CCTGCTATTA TTTTGTAGTAA
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 EBU37006 ..T.....
 [299]
 EBU37008A.....
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 EBU37013 ..TGTA..A. G.....Y.....Y-.....
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 EBU37015-.....A.....
 [288]
 EBU37016 ..T.....
 [286]

```

EBU37017 ..T.....
[296]
EBU37018* .....- - - - -
[259]
EBU37019 ..T.....
[300]
EBU37020 ..T.....
[295]
EBU37021 .....G .....A.....-C...
[299]
EBU37022 ..T.....
[300]
EBU37026 .....G. ...A.....
[300]
EBU37029 .....- - - - -
[261]
EBU37030 .....G. ...A.....
[300]

```

```

[           310           320           330   ]
[           .           .           .       ]

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```

EBU37003 TAGCAACTGC CTTTGTTGGA TATGTCCTAC CCTG [334]
EBU37005 ..... [334]
EBU37006 ..... [333]
EBU37008 ..... [316]
EBU37013 .....- - - - - [188]
EBU37015 .....W.. - - - - - [299]
EBU37016 .....-... [320]
EBU37017 ..... [330]
EBU37018* - - - - - [259]
EBU37019 .....-- [332]
EBU37020 .....- - - - - [309]
EBU37021 ..-.....C ..... [332]
EBU37022 .....-...Y... [333]
EBU37026 ..... [334]
EBU37029 - - - - - [261]
EBU37030 .....- - - - - [316]

```

```

;
END;

```

Appendix 2: Cytochrome c oxidase subunit I sequence data collected for the report.

The specimens are identified by EBU number (see Table 1). The dots indicate that the base at that position in the specified sequence is the same as the base in the sequence written at the top of the compilation. The data are presented in NEXUS file format so that they can be edited and saved as a text formatted file for analysis in bioinformatics programmes such as PAUP, etc. Dashes indicate missing data. Letters other than A,C, G or T indicate uncertainties according to standard codes (M = A or C, K = G or T, R = A or G, S = C or G, W = A or T, Y = C or T). 'ss' indicates that the specimen was sequenced in one direction only.

#NEXUS

BEGIN DATA;

DIMENSIONS NTAX=11 NCHAR=628;

FORMAT MISSING=- GAP= MATCHCHAR=. INTERLEAVE DATATYPE=DNA ;

MATRIX

```
[           10           20           30           40
50]
[           .           .           .           .           .]
```

E37005 CTGAGCGGGA ATAGTGGGAA CTGCCCTAAG TCTATTAATC CGCGCTGAAT

[50]

E37006

[50]

E37008

[50]

E37013* ----- ----- T.....

[36]

E37016S.....

[50]

E37017

[50]

E37020

[50]

E37022* ----- -----

[27]

E37026

[50]

E37029* ----- -----

[50]

E37030

[50]

```
[           60           70           80           90
100]
[           .           .           .           .           .]
```

E37005 TAAGCCAACC TGGCGCGCTT CTGGGGGACG ATCAAATTTA CAACGTCATT

[100]

E37006

[100]

E37008

[100]

E37013* .-.....T.....-.-.....
 [86]
 E37016
 [100]
 E37017
 [100]
 E37020
 [100]
 E37022*-.....R.....-.....
 [77]
 E37026G.....
 [100]
 E37029*-.....G.....
 [100]
 E37030G.....
 [100]

[110 120 130 140
 150]
 [.]

E37005 GTCACAGCAC ACGCATTTCAT TATAATCTTT TTTATAGTCA TACMTGTGAT
 [150]
 E37006S.....C.....
 [150]
 E37008C.....
 [150]
 E37013*S.....C.....
 [136]
 E37016C.....
 [150]
 E37017C.....
 [150]
 E37020C.....
 [150]
 E37022*C.....
 [127]
 E37026C.....
 [150]
 E37029*-.....C.....
 [150]
 E37030C.....
 [150]

[160 170 180 190
 200]
 [.]

E37005 AATCGGCGGC TTTGGAAACT GGCTCATCCC ACTAATAATT GCGCCCCAG
 [200]
 E37006
 [200]
 E37008
 [200]
 E37013*
 [186]

E37016
 [200]
 E37017
 [200]
 E37020
 [200]
 E37022*
 [177]
 E37026
 [200]
 E37029*
 [200]
 E37030T.....
 [200]

[210 220 230 240
 250]
 [.]

E37005 ATATGGCCTT CCCACGAATA AATAATATAA GCTTTTGATT ACTTCCACCA
 [250]
 E37006
 [250]
 E37008
 [250]
 E37013*
 [236]
 E37016
 [250]
 E37017
 [250]
 E37020
 [250]
 E37022*-.....-.....-.....
 [227]
 E37026
 [250]
 E37029*
 [250]
 E37030
 [250]

[260 270 280 290
 300]
 [.]

E37005 TCATTATTAC TATTGCTTGC CTCCTCAGGA GTCGAAGCCG GTGCTGGCAC
 [300]
 E37006
 [300]
 E37008
 [300]
 E37013*R.....
 [286]
 E37016
 [300]

E37017
 [300]
 E37020
 [300]
 E37022*
 [277]
 E37026
 [300]
 E37029*
 [300]
 E37030
 [300]

[310 320 330 340
 350]
 [.]

E37005 CGGCTGAACA GTTTATCCCC CACTAGCTGC CAACCTCGCC CACTCTGGGG
 [350]
 E37006
 [350]
 E37008
 [350]
 E37013*
 [336]
 E37016
 [350]
 E37017
 [350]
 E37020
 [350]
 E37022*
 [327]
 E37026
 [350]
 E37029*
 [350]
 E37030
 [350]

[360 370 380 390
 400]
 [.]

E37005 CATCTGTAGA TMTAGTAATC TTCTCCCTAC ATTTGGCTGG GGTATCGTCT
 [400]
 E37006C.....
 [400]
 E37008C.....
 [400]
 E37013*C.....
 [386]
 E37016C.....
 [400]
 E37017C.....
 [400]

E37020C.....
 [400]
 E37022*
 [377]
 E37026C.....
 [400]
 E37029*C.....
 [400]
 E37030
 [400]

[410 420 430 440
 450]
 [.]

E37005 ATTCTGGGTG CAATCAACTT TATCACCACC TGCATCAACA TAAAATCCCC
 [450]
 E37006
 [450]
 E37008
 [450]
 E37013*
 [436]
 E37016
 [450]
 E37017
 [450]
 E37020
 [450]
 E37022*
 [427]
 E37026
 [450]
 E37029*
 [450]
 E37030
 [450]

[460 470 480 490
 500]
 [.]

E37005 CTCACTATCA CAATATAATA CCCCCCTTTT TGTATGATCT GTATTAATCA
 [500]
 E37006
 [500]
 E37008K.....
 [500]
 E37013*
 [486]
 E37016
 [500]
 E37017
 [500]
 E37020
 [500]

E37022*
 [477]
 E37026
 [500]
 E37029*
 [500]
 E37030 A.....
 [500]

[510 520 530 540
 550]
 [.]

E37005 CAGCAGTTCT TTTATTATTA GCACTTCCGG TCTTGGCAGC AGGCATTACC
 [550]
 E37006
 [550]
 E37008
 [550]
 E37013*
 [536]
 E37016
 [550]
 E37017
 [550]
 E37020
 [550]
 E37022*
 [527]
 E37026 A.....
 [550]
 E37029* A.....
 [550]
 E37030 A..... M.....
 [550]

[560 570 580 590
 600]
 [.]

E37005 ATGCTACTCA CCGATCGTAA CCTTAATACC ACCTTCTTTG ACCCAGCCGG
 [600]
 E37006 -..... M..... T..G.....
 [599]
 E37008
 [600]
 E37013* -----
 [580]
 E37016 -..... -..... -G.....
 [597]
 E37017 -..... G.....
 [599]
 E37020 G.....
 [600]
 E37022* C..... -----
 [571]

```

E37026      .....C.....
[600]
E37029*    .....C.....-----
[600]
E37030     .....M..... M-.C.....
[600]

```

```

[           610           620           ]
[           .           .           ]

```

```

E37005      CGGGGGCGAC CCAGTATTGT ATCAACAC [628]
E37006      .....-.....-... [625]
E37008      .....M..... [628]
E37013*     ----- [580]
E37016      ..... [625]
E37017      .....R..... [627]
E37020      .....Y..... [628]
E37022*     ----- [571]
E37026      .....-..... [627]
E37029*     ----- [628]
E37030      .....-..... [627]
;
END;

```