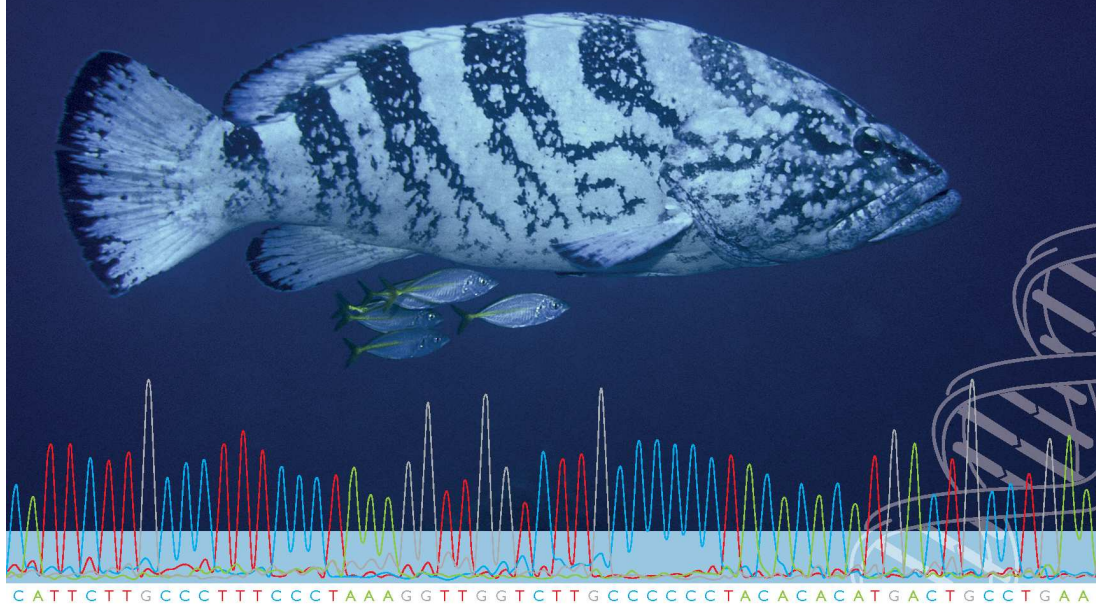


# Genetic connectedness between black cod (*E. daemeli*) collections

along the NSW coast and the Elizabeth & Middleton Reefs Reserve



Final report to the Department of Environment and Water Resources, June 2007

Investigators: CSIRO Marine and Atmospheric Research  
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### 3. EXECUTIVE SUMMARY

In this study, we investigated the population structure of black cod (*Epinephelus daemellii*) using samples from the Elizabeth and Middleton Reefs Marine National Nature Reserve (EMRMR) screened for variation in five regions of mitochondrial DNA and three nuclear microsatellite loci. Our initial aim was to investigate the genetic connectedness of black cod from the EMRMR and more coastal NSW regions but limited sampling prevented us from undertaking this comparison. Previous to this study, there was no information on the stock structure of *E. daemellii* in Australian waters nor on molecular markers in black cod. This study therefore reports the first use of mtDNA markers and microsatellite loci in *E. daemellii*.

Direct mtDNA sequencing of five gene fragments from 91 black cod individuals was successfully undertaken from DNA sourced from muscle biopsies, fin sections and dried scales. Up to 3192 base pairs were sequenced in black cod. Most gene regions were relatively easy to amplify, sequence and analyse except for the control region which was extremely difficult to amplify routinely. Two fragments (16S rRNA and cytochrome oxidase I) were suitable for species differentiation with only one haplotype observed in the 16S and two haplotypes in the *coxI* gene. The remaining three mtDNA gene fragments (cytochrome b, NADH dehydrogenase subunit 2 and the control region) demonstrated low to medium variation (% variable sites = 0.68, 0.78, 7.60 respectively) suitable for population analyses. Three of eight nuclear microsatellite loci that successfully amplified in *E. daemellii* were highly variable ( $H_o \approx 0.800$ ) and showed no evidence of null alleles (albeit in small sample sizes).

From a molecular genetic viewpoint, the evidence from this study suggests that the spatial and temporal collections of black cod from the EMRMR are similar. The null hypothesis of a single stock could not be rejected, but the small sample sizes reduce our power to critically evaluate this hypothesis. As other marine studies have shown that genetic heterogeneity among fish species is usually more pronounced across wider geographic scales, it is plausible that the observance of genetic homogeneity among these locations reflects pelagic egg or larval drift in these areas, coupled with adult movements, as only 45 km separates the two reefs.

Given the current data however, it is not possible to ascertain if these collections are genetically dissimilar to those from the eastern Australian coastal regions. Observations of genetic homogeneity in other *Epinephelus* species has been shown to be both species, marker and location specific. We found the cytochrome b fragment provided marginally significant differentiation (based on differences in haplotype frequencies) among samples from the eastern Australian coastal regions and those from the EMRMR and Lord Howe Island. The actual source of the variance was however difficult to determine given that only six cod were analysed from the coastal regions. Currently, the sample sizes from all areas are very low and need to be substantially increased to provide meaningful comparisons.

We therefore recommend the urgent acquisition of more *E. daemellii* samples across a broader range and examination of at least the markers outlined in this study, in order to determine the levels of gene flow between locations.

While this study provides only the initial inputs into management and conservation processes for *E. daemellii*, we firmly believe that the combining of genetic data with tagging and morphological data will enhance the power of future stock structure investigations for this protected species.

## 4. OUTCOMES ACHIEVED

This results of this research were aimed at addressing four major objectives. Three of these four objectives were fulfilled, the remaining objective could not be adequately addressed due to sampling limitations and difficulties our collaborators had in sourcing black cod tissues from the field. The objectives from the original contract were:-

1. using direct sequencing, develop and deploy mtDNA markers that are suitable for investigating stock structure in black cod collections from the NSW coast and EMRMR

The molecular markers developed in the current study were based on the polymerase chain reaction (PCR). PCR requires only small amounts of tissue; we used fin clips, scales, white muscle tissue and small biopsy samples. Using PCR techniques, tissues were obtained in a non-destructive manner from this protected species. Five mtDNA gene regions were sequenced and three demonstrated low to medium variation among the 91 black cod individuals screened in the current study.

2. analyse the genetic connectedness between black cod samples using the mtDNA sequences and analytical genetic software

Unfortunately, we did not undertake any cod sampling ourselves, and it was extremely difficult for our collaborators to acquire a comprehensive number of black cod samples from the field. The study also suffered significantly from a paucity of sampling locations; we only received six cod samples from the coast and near coast areas. These samples were insufficient for rigorous population analyses and subsequent tests of genetic subdivision between the EMRMR region and eastern areas were not undertaken. Therefore we were not able to fulfill this objective. However, good levels of genetic diversity were observed in the 80 black cod individuals that were sampled from the Elizabeth and Middleton Marine Reefs Marine National Nature Reserve; there was no temporal or spatial genetic heterogeneity detected in the EMRMR.

3. develop and optimise sequenced mtDNA markers for species identification. Link into the existing Bar Code of Life project by sequencing selected samples of *E. daemelii* for the *coxI* fragment and compare with already obtained *Epinephelus* sequences

Two of the five mtDNA gene regions (16S rRNA and cytochrome oxidase I) provided excellent species specific sequences which are suitable for accurate species detection. Sequences from these regions will further help to clarify the inter-relationships within the genus and will readily enable the molecular identification of this species in confiscated fish or fillet samples, in egg or larvae samples. In addition, as there was no molecular information on this species in publicly available databases, representative sequences from all five mtDNA gene regions, including the two regions more suited to species identifications have been submitted to both GenBank and the BarCode of Life database.

4. trial available microsatellite primers from other *Epinephelus* species on selected *E. daemelii* individuals

Nine nuclear microsatellites developed for *E. quernus* (see Rivera *et al.*, 2003) were trialed in *E. daemelii*. Three of these loci successfully amplified a PCR product. Medium to high levels of genetic diversity were observed at these three loci although no significant differences among the EMRMR samples were detected.

Overall, the current genetic findings are consistent with a management practice of considering black cod in the EMRMR as a single stock. The caveat on this statement is that the failure to reject the null hypothesis does not mean that stock structuring in this area does not exist; only that it was not detected in our current study. Our inability to reject the null hypothesis may be related to the power of the genetic markers employed (i.e. lower levels of variability observed in the mtDNA gene fragments) and the very small sample sizes examined (particularly for the hyper-variable microsatellite loci). While mtDNA is sometimes more sensitive to population bottlenecks and more subject to genetic drift and population differentiation than nuclear DNA (due to the maternal inheritance, haploidy, and hence reduced effective population size of mtDNA), in the current study the findings from both marker types were concordant.

This research represents the first preliminary study in black cod in any Australian waters, and while we do not have enough samples to comment on the population structure of black cod more widely, we would recommend the urgent acquisition of more *E. daemeli* samples across a broader range. Examination of at least cytochrome b, NADH dehydrogenase subunit 2 and the control region and the three microsatellite loci outlined in this study would help to determine the levels of gene flow between locations if more samples are acquired. Additionally, we would recommend that tagging studies are undertaken on this species so that adult movements can be monitored; larval studies should be established to give a better understanding of the movement and duration of the larval phase and the likelihood of local replenishment; and future genetic studies should be combined with more in depth morphological sampling via field photography studies.

## 5. BACKGROUND

### 5.1 *E. daemelia* population characteristics

*Epinephelus daemelia* (Günther, 1876) (black cod) is a large (up to 80 kg, 1.5 m in length) reef dwelling grouper species of the family Serranidae. It occurs in reef caves, gutters and beneath bommies (Gill and Reader, 1992) in warm temperate to subtropical waters of the southwestern Pacific. In Australia, *E. daemelia* is found in coastal and offshore reef habitats from southern Queensland to eastern Victoria (Pogonoski *et al.*, 2002). Black cod are a slow growing species and are considered sedentary yet territorial and aggressive (Heemstra and Randall, 1993). As with many other serranids (Rivera *et al.*, 2004), black cod are protogynous hermaphrodites (see Pears *et al.*, 2006 and references within), with smaller fish typically being female and becoming male at about 110 cm in length (Paulin and Roberts, 1992).

Apart from this, we know very little of the biology or ecology of black cod, although Hutchins and Swainston (1986) observed recently settled juveniles in coastal rock pools along the NSW coastline. It is unknown if adults form breeding groups and nothing is known of larval distribution, fecundity or life history. The species is listed as vulnerable under the *NSW Fisheries Management Act 1994* (since 1999), and has been protected in NSW since 1983 (see also Pogonoski *et al.*, 2002) following an apparent decline in numbers in the 1970's due to spear fishing (Leadbitter, 1992). Furthermore, *E. daemelia* is listed under section 15 of the *Commonwealth Fisheries Management Act 1991*. Under this Act, the taking of black cod in fishing operations is illegal unless covered by a scientific permit (Pogonoski *et al.*, 2002).

The general characteristics of slow growth, putative long life history, unequal sex ratios, territorial sedentary nature and apparent decline in population numbers have led to the need to obtain information regarding population structure for this species. Such information is extremely important for continued species management and protection - particularly within NSW state waters as this area provides the largest region of protection for the species.

There is currently nothing known regarding the stock structure of *E. daemelia*, but the successful management of any threatened species requires this assessment at an appropriate scale (Rivera *et al.*, 2003). This means implementing a variety of tools for the assessment. One such important tool for fin fish management is the analysis of genetic markers.

### 5.2 Genetic stock structure and population studies

As indicated above, knowledge of the genetic structure of populations forms the fundamental basis for management of all species, be they harvested, exploited or protected. Stock structure uncertainty restricts the ability of conservation managers to make assessments on local, regional or temporal scales. While the population structure for *E. daemelia* is currently unknown, we can expect the number of genetically distinct populations for *E. daemelia* to depend on reproductive and physiological factors, environmental factors, spawning grounds, thermo clines, and water currents. Additionally and very importantly, in marine fish such as the black cod, large population sizes or life-history stages such as pelagic larvae can play a role in determining genetic differentiation (Waples, 1988; Ward *et al.*, 1994). Marine species characterised by larval stages that are carried by the ocean currents across large distances, or marine populations that have few geographic barriers to dispersal, will probably show little stock structure across their wider geographic locations (Ward *et al.*, 1994; Bargelloni *et al.*, 2000).

The continued conservation of black cod therefore requires information on genetic connectedness among coastal populations and between these and populations within marine protected areas (i.e. areas of no-take). If genetic differences are not observed between marine protected and other areas, we would not be able to reject the null hypothesis of single genetic stock in the region. If genetic differences are observed, then gene flow across spatial areas must be limited. Any such deviations from panmixia in the black cod's range would necessitate more fine scale population management.

### 5.2.1 Molecular studies in other *Epinephelus* spp.

To date, there have been no population genetic studies on *E. daemeli* and available loci and markers are lacking. Research on other *Epinephelus* species include mitochondrial DNA (mtDNA) sequencing (for fillet identification from groupers, taxonomic studies in serranid species, phylogenetic research in Eastern Atlantic and Australian *Epinephelinae* species) (Craig *et al.*, 2001; Maggio *et al.*, 2005; Trotta *et al.*, 2005; Ward *et al.*, 2005), intraspecific phylogeography on *E. adscensionis* and *E. marginatus* using mtDNA surveys and Restriction Fragment Length Polymorphisms (RFLPs) (Gilles *et al.*, 2000; Carlin *et al.*, 2003; Maggio *et al.*, 2006), taxonomic relationships among species of the Genus *Epinephelus* based on Randomly Amplified Polymorphic DNA (RAPD) fingerprinting (Govindaraju and Jaysankar, 2004), population subdivision of Hawaiian grouper (*E. quernus*) using mtDNA sequences (Rivera *et al.*, 2004) and development and application of microsatellite loci, mtDNA RFLPs and allozymes more generally (*E. quernus*, *E. morio*, *E. coidides*, *E. polyphkadion*, *E. marginatus*, *E. malabaricus*, *E. fuscoguttatus*) (Richardson and Gold, 1993; Richardson and Gold, 1997; Nugroho *et al.*, 1998; De Innocentiis *et al.*, 2001; Rhodes *et al.*, 2003; Rivera *et al.*, 2003; Zatcoff *et al.*, 2004; Antoro *et al.*, 2005; Zhu *et al.*, 2005; Koedprang *et al.*, 2007).

The current research utilises molecular markers which employ PCR techniques. Such markers are relatively quick and easy to implement and do not require large tissue samples. Individuals can be sampled non-destructively; this is ideal for studying black cod as the species is protected and destructive sampling is not permitted.

Once obtained, molecular sequences are submitted to public databases (e.g. <http://www.ncbi.nlm.nih.gov>) for comparison against previously submitted data. However, this approach relies on the quality and quantity of submitted information to the databases; to date, there are a limited number of *Epinephelus* species sequences and no *E. daemeli* sequences in GenBank. For the current study, we therefore chose to use an integrated approach that employed both mtDNA and nuclear microsatellites to give a more informed indication of the stock structure as patterns of nuclear inheritance can be quite different from that of the mitochondrial genome (Awise, 1994; Appleyard *et al.*, 2002; Rubinoff, 2005).

### 5.3 Application of mitochondrial DNA

MtDNA is a haploid genome and is generally inherited through the maternal line. It has an effective population size one quarter that of nuclear DNA (making it highly sensitive to any bottleneck effects) and its evolutionary rate of change is estimated at five to ten times that of the nuclear genome; mtDNA therefore can show rapid rates of substitution (Brown *et al.*, 1979; Moore, 1995). MtDNA is considered a powerful molecular tool for investigating population differentiation, genetic diversity and species identification. Universal mtDNA primers are widely amplifiable across distantly related fish species and the sequences of most primers are published. In the current study, we have considered five different mtDNA gene fragments. Prior to this study, there was no mtDNA sequence information available for *E. daemeli* so we were

unsure of which (if any) gene fragment would provide enough sequence variation to undertake homogeneity studies.

From the literature, the 5' end of the control region (CR) of the mtDNA is usually highly variable and has the highest observed rate of base substitution and insertion/deletion events in vertebrates (Saccone *et al.*, 1987; see also Rivera *et al.*, 2004). Other mtDNA regions also suitable for population studies include the cytochrome b gene (*cytb*) and the NADH dehydrogenase subunit 2 gene (ND2) while the cytochrome oxidase I (*coxI*) gene and the 16S ribosomal RNA gene (16S) are usually more suited to phylogenetic comparisons. These five genes have traditionally been utilized for both inter and intra species molecular studies.

### 5.3.1 Background to mtDNA gene fragments

'Universal' primers enable access to the mitochondrial genomes of untested species (Palumbi, 1996). As *E. daemeli* is one such species, our strategy was to amplify several different mitochondrial gene fragments and test these for genetic diversity and variation.

The 16S ribosomal RNA (16S) is a large subunit RNA gene in the mtDNA. It is relatively conserved in sequence and structure and generally evolves more slowly than other mtDNA regions (Palumbi, 1996). The 16S rRNA gene may contain enough variation suitable for species level identification (phylogenetic analyses) and occasionally higher levels of variation in this fragment may be utilised in population comparisons. In *Epinephelus* species, 16S rRNA has been used for fillet identification from groupers and taxonomic studies in several serranid species (Craig *et al.*, 2001; Maggio *et al.*, 2005; Trotta *et al.*, 2005).

The cytochrome oxidase I gene (*coxI*) is a subunit of the cytochrome oxidase complex which is part of the electron transport chain in the cell. It is a highly conserved fragment across phyla; as such it has been proposed as a global bio-identification system for animals and fish (see Ward *et al.*, 2005) and is the gene of choice for the Barcode of Life initiative (<http://www.barcodinglife.com/views/login.php>). While the utility of this gene fragment is based on its ability to differentiate between species (i.e. within species sequences are more similar to each other than to sequences in other species) there have been exceptions to this observation (Ward *et al.*, 2005).

The cytochrome b gene (*cytb*) also codes for a protein in the electron transport chain (Palumbi, 1996). It is a fully functional monomer (i.e. is not part of a larger subunit or complex) (Palumbi, 1996) and has a number of both conserved and variable regions which make it suitable for both species and population level analyses (depending on the species in question).

The NADH dehydrogenase subunit 2 gene (ND2) has been shown to be variable in other marine fish populations (Smith *et al.*, 2001; Appleyard *et al.*, 2002; Appleyard *et al.*, 2004; Maggio *et al.*, 2006) and so we chose to also investigate this fragment in black cod.

The control region (CR) of the mitochondrial genome contains the region for mtDNA replication and transcription (Palumbi, 1996). While there are a number of conserved blocks of sequence in the control region, there are also a number of flanking areas around these conserved regions that can vary and are often highly variable. These characteristics make this particular gene fragment highly suitable for population level analyses. This fragment was used for population study in *E. quernus* (Rivera *et al.*, 2003) and also been utilised extensively in other fin fish species (e.g. Ovenden *et al.*, 2004; Salini *et al.*, 2006; see others in this report)

## 5.4 Application of nuclear microsatellite loci

In contrast to the mtDNA gene fragments, microsatellites are co-dominant, bi-parental nuclear markers that display high levels of polymorphism. They are used widely in fisheries and aquaculture studies (O'Connell and Wright, 1997 and references within) but they may require primer sequences which are species-specific or developed from closely related species. PCR amplification conditions for primers may also vary among species.

There are currently no published *E. daemelli* microsatellite primers although there are a number microsatellites developed for other *Epinephelus* species (e.g. *E. quernus*, *E. morio*, *E. coidides*, *E. polyphkadion*, *E. marginatus*, *E. malabaricus*, *E. fuscoguttatus* (Richardson and Gold, 1993; Richardson and Gold, 1997; Nugroho *et al.*, 1998; De Innocentiis *et al.*, 2001; Rhodes *et al.*, 2003; Rivera *et al.*, 2003; Zatcoff *et al.*, 2004; Antoro *et al.*, 2005; Zhu *et al.*, 2005, and see evaluation of microsatellite loci in other *Epinephelus* spp. Koedprang *et al.*, 2007)). In the current study, we concentrated on the primers and loci developed by Rivera *et al.* (2003) for the closely related *E. quernus* in an effort to extend the available markers for *E. daemelli*.

## 6. NEED & OBJECTIVES

Currently, the Commonwealth Department of the Environment and Water Resources is focusing on the relationships between NSW coastal populations of black cod and those at the Elizabeth and Middleton Reefs Marine National Nature Reserve in the Tasman Sea. As outlined, the determination of genetic structure is fundamentally based on the relationship among alleles and genotypes in a population. In *E. daemellii*, there are no molecular markers in place with which to study population structure or describe allelic differences. Hence, the major objective of the current study:-

1. using direct sequencing, develop and deploy mtDNA markers that are suitable for investigating stock structure in black cod collections from the NSW coast and EMRMR

In addition, resolution of stock structure is better managed through a combination of approaches than by any single technique in isolation. Keeping this in mind, the other objectives of our research were:-

2. analyse the genetic connectedness between black cod samples using the mtDNA sequences and analytical genetic software
3. develop and optimise sequenced mtDNA markers for species identification. Link into the existing Bar Code of Life project by sequencing selected samples of *E. daemellii* for the *coxI* fragment and compare with already obtained *Epinephelus* sequences
4. trial available microsatellite primers from other *Epinephelus* species on selected *E. daemellii* individuals

## **7. METHODS**

### **7.1 *Sampling and DNA extraction***

Black cod samples were acquired from a number of sources and locations through our collaborators (CMAR did not undertake any sampling). These were provided as alcohol stored tissues or DNA extracted samples (see Table 1 and Figure 1) and consisted of white muscle tissue, dried fin sections, scales and biopsy samples. Sampling information was entered into a CMAR spreadsheet and saved on CMAR servers. Where possible, tissue samples were sent to CMAR and in instances where original tissue samples were limited, researchers at James Cook University (JCU) extracted DNA and sent the dried DNA to CMAR. Both JCU and DEWR undertook specific sampling field trips in the EMRMR region designed to acquire black cod samples although the acquisition of samples from the more coastal regions was opportunistic.



Table 1 Sampling locations, sample size and source details of *E. daemellii* used in the current study

Sampling location and abbreviation	Sample numbers	Approximate sample location		Date of sampling	Sample size	Source <sup>#</sup>	Sample type <sup>***</sup>
		Latitude	Longitude				
Bundaberg (Bundg)	AMS I. 39781 (62ED)	24 <sup>0</sup> 52'S	152 <sup>0</sup> 21'E	Sept 1999	1	Australian Museum via JCU	DNA
Saumarez Reef (Saumarez)	AMS I.40856-003 & AMS I.40856-005 (64ED & 65ED)	29 <sup>0</sup> 56'S	153 <sup>0</sup> 42'E	May 2001	2	Australian Museum via JCU	DNA
Port Stephens (PortS) <sup>*</sup>	1ED	-----	-----	Aug 2003	1	UTS	tissue
Bermagui (Berma)	AMS I. 43615 (63ED)	36 <sup>0</sup> 25'S	150 <sup>0</sup> 04'E	April 2005	1	Australian Museum via JCU	DNA
Rocky Point (RockyP) <sup>**</sup>	2ED	-----	-----	June 2005	1	NSW Fisheries & UTS	tissue, scales
Middleton Reef (Middle06)	3ED - 44ED	29 <sup>0</sup> 27'S	159 <sup>0</sup> 07'E	Feb 2006	42	JCU	23 tissues, 19 DNA
Elizabeth Reef (Eliza06)	45ED - 61ED	29 <sup>0</sup> 56'S	159 <sup>0</sup> 05'E	Feb 2006	16	JCU	3 tissues, 13 DNA
Lord Howe Island (LordH)	66ED	31 <sup>0</sup> 31.5'S	159 <sup>0</sup> 04'E	Aug 2006	1	LHMPA	tissue
Middleton Reef (Middle07)	67ED - 71ED	29 <sup>0</sup> 27'S	159 <sup>0</sup> 07'E	Feb 2007	5	DEWR	tissues
Elizabeth Reef (Eliza07)	72ED - 86ED	29 <sup>0</sup> 56'S	159 <sup>0</sup> 05'E	Feb 2007	15	DEWR	tissues

<sup>\*</sup>sample was taken from confiscated catch off a longline vessel that uses Port Stephens as its home base, fish was taken outside the state jurisdiction but no details on exact location are known

<sup>\*\*</sup>sample was taken from confiscated catch off Rocky Point, near the entrance of Port Stephens

<sup>#</sup>primary contact at source:- Australian Museum = R. Sweeney and P. Grumski; University of Technology Sydney = E. Buckle; NSW Fisheries = N. Otway; Lord Howe Marine Park Authority = G. Kelly; Department of Environment and Water Resources = P. Anderson

<sup>\*\*\*</sup>if tissue was provided, DNA extraction was as per Promega below, in all other instances JCU provided dried DNA



Figure 1 Sampling locations for *E. daemeli* (where positional information was available) off the eastern Australian coastline, Elizabeth and Middleton Reef regions and Lord Howe Island.

In addition to the samples outlined above, we also received five samples from juvenile black cod (small fin sections) from Emily Buckle (UTS) towards the end of April 07. These samples were not included in the overall analyses due to their late arrival. However DNA was extracted (individuals were from the Wollongong, Kiama, Lord Howe Island and Minnamurra River areas (i.e. cod numbers 87-91ED) and analysed for a component of the mtDNA gene fragments.

When tissues were provided, total genomic DNA (from approximately 25mg tissue or several scales) was extracted using Wizard SV Genomic DNA Purification Systems (Promega, USA) as per the manufacturer's instructions, except for elution volumes which were reduced to a total of 250  $\mu$ l. If any tissues remained these were stored, along with an aliquot of the extracted DNA, at  $-80^{\circ}\text{C}$ . Genomic DNA for each individual was then diluted to 10  $\text{ng}/\mu\text{l}$  where possible using a NanoDrop ND1000 Version 3.0 (NanoDrop Technologies Inc, USA). The DNA aliquots were stored at  $4^{\circ}\text{C}$  for working applications. Samples were used for both mtDNA and microsatellite amplification, although depending on observed variation, or lack thereof, not all samples were used for each application.

Initially, all samples were analysed independently to obtain diversity statistics (i.e. mtDNA haplotypes and microsatellite allele frequencies). As the sample sizes of several of the locations were extremely small and in several cases there was no detailed sampling location information (e.g. PortS), these samples were not used in subsequent detailed population analyses. The genetic differentiation results presented here are based on the two sets of temporal samples from the Elizabeth and Middleton Reefs (Eliza06 & 07 and Middle06 & 07) with limited comparisons with the samples from the other areas (e.g. Saumarez and LordH). Four of the five late arriving samples were used for ND2, *cytb* and CR amplification. However only the first two regions were amplified and sequenced successfully.

For the Elizabeth and Middleton Reef collections, if the molecular data demonstrated that the collections were not significantly different, these groups were pooled and further analyses undertaken.

In all multiple test comparisons, the significance levels of *P*-values were adjusted for multiple tests using the sequential Bonferroni correction (Rice, 1989).

## 7.2 MtDNA amplification and analyses

Table 2 outlines the primers used for mtDNA gene fragment amplification. The 16S, *coxI*, *cytb*, ND2 and CR fragments were separately analysed. PCR reactions were undertaken in a Perkin Elmer GeneAmp® System 9600 thermal cycler (Applied Biosystems, USA) in a total volume of 50  $\mu$ l. Reactions consisted of 1  $\mu$ l of 10 mM dNTP's (Promega), 3  $\mu$ l of 25 mM MgCl<sub>2</sub> (Applied Biosystems), 5  $\mu$ l of 10 $\times$  Amplitaq Gold Buffer (Applied Biosystems), 1  $\mu$ l of 10  $\mu$ M forward and reverse primer (Geneworks, South Australia), 0.25  $\mu$ l of Amplitaq Gold (Applied Biosystems), 20 - 300 ng of template DNA (depending on fragment), adjusted to a final volume of 50  $\mu$ l with ddH<sub>2</sub>O. The PCR cycling conditions were as follows: initial denaturation at 93°C for 10 min, 40 cycles of 93°C for 30 s, 53°C for 1 min 30 s, and 72°C for 2 min. A final extension cycle of 72°C for 10 min was followed by an indefinite 4°C cycle.

Table 2 mtDNA gene regions and primers used to screen variation in *E. daemellii* in the current study

mtDNA gene fragment	Primers and source	Annealing temperature	Length of fragment in <i>E. daemellii</i> (bp) <sup>#</sup>
16S ribosomal DNA	16SarL & 16SbrH (Palumbi <i>et al.</i> , 1991)	53°C	516
cytochrome oxidase subunit 1	FishF1 & FishR1 (Ward <i>et al.</i> , 2005)	53°C	609
cytochrome b	GLUDG-L & CB3-H (Palumbi <i>et al.</i> , 1991)	53°C	736
NADH dehydrogenase subunit 2	t-Met (Park <i>et al.</i> , 1993) & Mt76 (Smith <i>et al.</i> , 2001)	53°C	897
control region	A & E (Lee <i>et al.</i> , 1995)	50 - 53°C	434

<sup>#</sup>following trimming of sequences and removal of primer regions

PCR products were run on 2.5% TBE (Tris, boric acid, EDTA) buffer agarose gels containing ethidium bromide at 120V for 1 hour against a Hyperladder size standard (Bioline, USA). Fragments were visualized under UV light and photographed with a digital camera. Products from each gene fragment were purified using AMPure™ magnetic beads (Agencourt, USA) according to the manufacturer's instruction. We then used approximately 8 - 20 ng of purified PCR product (depending on fragment size) as template for bi-directional sequencing using ABI Big Dye® Terminator v. 3.1, Cycle Sequencing Kits (Applied Biosystems). Products were sequenced using the same primer sets that generated the initial PCR products.

Sequenced products were purified with CleanSEQ magnetic beads (Agencourt) according to the manufacturer's instructions. Fragments were sequenced on an Applied Biosystems 3100 DNA autoanalyser following ABI protocols. Reference sequences for each mtDNA gene fragment will be submitted to GenBank (<http://www.ncbi.nlm.nih.gov/Genbank/submit.html>). Five *coxI* sequences have also been submitted to the BOL database (<http://www.barcodinglife.com/>).

Forward and reverse sequences were analysed in SeqScape v2.1 (Applied Biosystems) (and additionally checked by eye) with consensus sequences extracted. Each mtDNA fragment was analysed separately. Removal of ambiguities in the beginning and end of the sequences was undertaken. Gaps were inserted to maintain alignment in the CR fragment. Subsets of sequences from each fragment were then compared with the NCBI databases (<http://www.ncbi.nlm.nih.gov/>, all GenBank+EMBL+DDBJ+PDB sequences) using the basic local alignment search tool (BLASTn) feature to ensure that the correct gene fragment had been amplified.

Aligned sequences for each gene fragment were analysed in MEGA v3.1 (Kumar *et al.*, 2004); this program was used to undertake exploratory data analysis and calculate molecular distances among sequences using the Kimura two parameter method (K2P) (Kimura, 1980). Neighbour-joining (NJ) trees of K2P distances were produced in MEGA which enabled visualisation of the distances and patterns for black cod samples analysed at each mtDNA fragment. Estimates of mean pair-wise sequence diversity were made using 2000 bootstraps. The NJ method does not assume that all lineages have diverged equal amounts although it does sequentially identify neighbour pairs that minimize the total length of the tree (Swofford *et al.*, 1996). Bootstrapping (Felsenstein, 1985) was used to estimate the reliability of the NJ trees. Tajima's test of neutrality which compares the number of segregating sites per site with nucleotide diversity, was also calculated on a per fragment basis in MEGA.

According to the geographic groupings in Table 2, haplotype diversity estimates were calculated based on the number of unique haplotypes and haplotype frequencies (unbiased haplotype diversity  $h$ , Nei, 1987) and nucleotide sequence diversity ( $\pi$ ) (i.e. average gene diversity over the sequence, the probability that randomly chosen homologous nucleotides are different, calculated from pair-wise sequence differences: Tajima, 1983; Nei, 1987) were calculated using ARLEQUIN vers 3.1 (Excoffier *et al.*, 2006). Deviation from equilibrium expectations were tested with the Tajima's D statistic (Tajima, 1989) for each collection based on an infinite-site model; significance was tested in ARLEQUIN based on 10000 randomisations. This test assesses the evidence for population expansions.

For the informative gene fragments, we also used pair-wise  $F_{ST}$  tests and analysis of molecular variance (AMOVA, based on Excoffier *et al.* (1992)), in ARLEQUIN to examine spatial differentiation within and between collection groupings. This test was used to partition the variance in genetic distances among sequences according to a pre-defined hierarchical structure – within collections, among collections within groups and among groups. Standard variance components for collection structure were calculated (resulting in  $\Phi$ -statistics). The  $\Phi$  statistics include the variance attributable to the molecular distances (based on K2P estimates) between each haplotype.

### **7.3 Microsatellite loci amplification and analyses**

In the current study, the nine microsatellite loci developed by Rivera *et al.* (2003) for *E. quernus* (Table 3) were trialled in black cod. In the screening phase, individual microsatellite loci were amplified separately in a sub-sample of individuals.

Table 3 Microsatellite loci and amplification conditions in *E. daemellii*, GenBank accession numbers for each locus are given. Loci were originally developed from *E. quernus* and described by Rivera *et al.* (2003)

Locus and accession number	Repeat motif	5' Forward primer sequence	5' Reverse primer sequence	Annealing temp
<i>CA-1</i> (AF539604)	(CA) <sub>n</sub> GA(CA) <sub>n</sub> (GA) <sub>3</sub> CA	TCTAGGTGCTGACA GCTACAAACA	CACAAAGATGTCAC TATTCCAGAAC	57 <sup>o</sup> C
<i>CA-2</i> (AF539606)	(CA) <sub>n</sub>	GACTTGATTCAGCA AAATAAAGATG	AGAGACGGTGCCAG TAAATGAA	45 <sup>o</sup> C
<i>CA-3</i> (AF539605)	(CA) <sub>n</sub>	ATGTGACACGTTG ACAGGCAAGT	GACCTTGATATTTTC ATTGCTTG	45 <sup>o</sup> C
<i>CA-4</i> (AF539607)	(CA) <sub>n</sub> CG(CA) <sub>n</sub>	GTGTGTTTATATAC ATATTAGTCA	AAATTATGAAAACA CAACAT	NP <sup>#</sup>
<i>CA-6</i> (AF539608)	(CA) <sub>n</sub>	GTGTTGCTGGGGTT ACTAATGAAG	TTAGACACATTGTCA CGATGGTCC	45 <sup>o</sup> C
<i>CA-7</i> (AF539609)	(CA) <sub>n</sub>	CACAGTCAAATAC TCATAAGTCATG	CAAGATGCCTGGGT ATTTTTGG	35 <sup>o</sup> C
<i>GA-1</i> (AF539610)	(GA) <sub>n</sub> GG(GA) <sub>n</sub>	GGCTGAGTTAGCA AGATGCAT	AAGAGCACAACCCC TGAAGA	55 <sup>o</sup> C
<i>CT-1</i> (AF539611)	(CT) <sub>n</sub> GT(CT) <sub>n</sub> GT (CT) <sub>n</sub>	AGCAAGAGCACAA TAGCCAGAA	CCATATGACAAAAT GAGACATAAG	55 <sup>o</sup> C
<i>GAA-1</i> (AF539612)	(GAA) <sub>n</sub>	GGAGTGTTAAATA TGCCACCA	CAGAAATCGAGGAC AAGAG	55 <sup>o</sup> C

<sup>#</sup>no product produced across a range of temperatures and MgCl<sub>2</sub> concentrations

Each locus was amplified separately in 25  $\mu$ l reactions in an ABI 9600 thermocycler (as above). Amplifications consisted of 1  $\mu$ l of 10mM dNTP's (Promega), 1.25 – 2.0  $\mu$ l of 25 mM MgCl<sub>2</sub> (Applied Biosystems) (depending on locus), 2.5  $\mu$ l of 10  $\times$  Amplitaq Gold Buffer (Applied Biosystems), 1.0  $\mu$ l each of 10  $\mu$ M forward (labeled primer with fluorescent dye, Applied Biosystems) and 10  $\mu$ M reverse primer (Applied Biosystems), 0.25  $\mu$ l of Amplitaq Gold (Applied Biosystems), 10 - 20 ng of template DNA, adjusted to a final volume of 25  $\mu$ l with ddH<sub>2</sub>O.

The PCR cycling conditions were as follows: initial denaturation at 93<sup>o</sup>C for 10 min, 35 cycles of 93<sup>o</sup>C for 30 s, 35 – 57.1<sup>o</sup>C for 1 min 30 s (depending on primer set, see Table 3), and 72<sup>o</sup>C for 2 min. A final extension cycle of 72<sup>o</sup>C for 10 min was followed by an indefinite 4<sup>o</sup>C cycle. 1.5 - 3.0  $\mu$ l of the amplified product was diluted in a mix of HiDi Formamide (Applied Biosystems) and water and denatured at 94<sup>o</sup>C for 2 min. Samples were run on an ABIPrism<sup>®</sup> 3100 Genetic Analyser against an internal GeneScan<sup>™</sup>-500 LIZ Size Standard (Applied Biosystems). We used GeneMapper<sup>™</sup> v.3.7 (Applied Biosystems) software to set various panels and bin ranges that enabled routine genotyping. For each collection, samples were scored as they were run; genotypes were checked again after all samples had been run for all loci.

In the first instance it was noted if amplifications produced a product in *E. daemellii* and secondly if the locus was variable. Subsequently, for each polymorphic locus, we then examined several diversity statistics, including allele frequencies, number of alleles ( $N_{alleles}$ ), observed heterozygosity ( $H_o$ ) and expected heterozygosity ( $H_e$ ) for each collection (as calculated in FSTAT v.2.9 (Goudet, 2001)). We used the rarefaction approach in FSTAT to calculate allelic richness ( $A$ ); this enables comparisons across the collections of differing sample sizes. Conformation to genotypic frequencies under Hardy-Weinberg Equilibrium (HWE) was assessed in ARLEQUIN, using a Markov chain approach but due to the very small sample sizes in some instances these results are not conclusive. The most robust HWE results are seen in the Middleton and Elizabeth collections.

Micro-checker v2.2.1 (van Oosterhout *et al.*, 2003) assessed the potential for large allele dropout, scoring errors due to stuttering and the potential of null alleles by comparing the observed and expected homozygote genotype frequencies and associated bin sizes. Linkage disequilibrium was tested by Fisher's exact tests for goodness of fit employed in GENEPOP vers 3.3 (Raymond and Rousset, 1995); but again due to the small sample sizes, the results should only be considered preliminary.

As in the mtDNA analysis, hierarchical AMOVA, global tests of genetic differentiation and calculation of traditional *F* statistics (Wright, 1951; Weir and Cockerham, 1984) (examined across all loci and collections on a pair-wise basis) were undertaken in ARLEQUIN. The same hierarchical divisions as outlined in the mtDNA section were used to analyse collection structure. Among collection differences (for nuclear microsatellite loci) were also examined in GENEPOP using pairwise comparisons (based on Fisher's exact test) across all loci.

## **8. RESULTS**

### **8.1 Nucleotide diversity in mtDNA gene fragments**

The five mtDNA gene regions were assessed for successful amplification, ease of sequencing, informativeness and utility for intra collection diversity studies. Two of the mtDNA fragments were more suited to *E. daemeli* species identification (16S and *coxI*) while the other three fragments were variable amongst the cod samples. Up to 3192 base pairs were sequenced from the mtDNA genome of *E. daemeli* (i.e. the combined size of the five gene fragments). Representative sequences from each of the mtDNA gene regions have been submitted to GenBank under Accession numbers EF644422-EF644424 (16S), EF644425-EF644431 (*cytb*), EF644432-644437 (ND2) and EF653810-EF653820 (CR). Five samples from the *coxI* gene have also been submitted to the BarCode of Life database under Accession numbers BW-A3708-BWA3712. All submitted sequences can be accessed in due course.

Three of the five gene fragments were easy to amplify and sequencing of the fragments was very repeatable and reliable from column prepared DNA templates. The ND2 fragment, the longest of the five fragments, was harder to amplify than 16S, *coxI* and *cytb* but eventually 81 individuals were screened for variation in this fragment. The CR fragment was often very difficult to amplify across a range of DNA templates. In some instances, cycling conditions and DNA template amounts had to be optimised for individual samples. It would appear that at least 150 - 300 ng of DNA is needed for successful amplification of the CR fragment in some samples; in the Middleton and Elizabeth collections, several samples were not concentrated enough to enable amplification. Of those templates that were amplified, sequencing was reliable and repeatable with the forward strand producing slightly better base pair resolution.

In the following analyses, we also included additional sequences from GenBank as outgroup representatives for each of the mtDNA regions. The outgroup accession numbers are listed in each section.

Table 4 Sequence diversity information for five mtDNA gene fragments amplified in *E. daemellii* samples from EMRMR and various eastern Australian coastal locations

mtDNA	Sample size	Average nucleotide composition %				Number sites <sup>#</sup>	Conserved sites	Variable sites <sup>##</sup>	Nucl. diversity	Tajima's $\theta$
		T	C	A	G					
16S	24	22.7	25.2	29.1	23.1	516 (504)	516	0 (0)	0.000	0.000
<i>cox I</i>	16	28.7	28.6	24.4	18.3	609 (598)	608	1 (1)	0.001	0.001
<i>cytb</i>	67	29.9	31.0	23.2	15.9	736 (708)	731	5 (3)	0.001	0.001
ND2	81	25.1	33.3	28.6	13.0	897 (701)	890	7 (6)	0.002	0.002
CR	52	32.7	16.1	39.2	12.0	434 (273)	401	33 (25)	0.016	0.017

<sup>#</sup>number of base pairs sequenced across the gene fragment, number of sites used in pair-wise distance calculations (based on complete deletion of gaps/missing data) shown in parenthesis

<sup>##</sup>number of parsimony informative sites given in parenthesis

### 8.1.1 16S

For the 16S fragment, 24 individuals were sequenced and aligned across 516 sites. This fragment was easily amplified across all DNA templates, with the sequences requiring little trimming other than that of the primer sequences. Neither gaps nor insertions were needed to achieve sequence alignment. All base pairs were conserved across this fragment; there were no transitions or transversions observed among the 24 black cod individuals.

Nucleotide composition of this fragment is shown in Table 4. There was no segregating sites amongst the 24 individuals and hence the mean K2P distance was  $d = 0.000 \pm 0.000$ . No further analysis on sample location groupings was undertaken.

Several of the 16S sequences were compared to the NCBI database; BLASTn search results gave a highest match of 97% to *E. moara* (Accession number DQ067303.2), *E. latifusciatus* (Accession number DQ088044.1) and *E. bruneus* (Accession number DQ067314.1). The 16S fragment therefore provides a robust 'species' identification fragment for *E. daemellii* but is not useful for intra species differentiation. A phylogenetic analysis based on the 24 samples and including *E. moara* (GenBank Accession no. DQ067303) as an outgroup demonstrated the single genetic grouping for *E. daemellii* (see Figure 2).

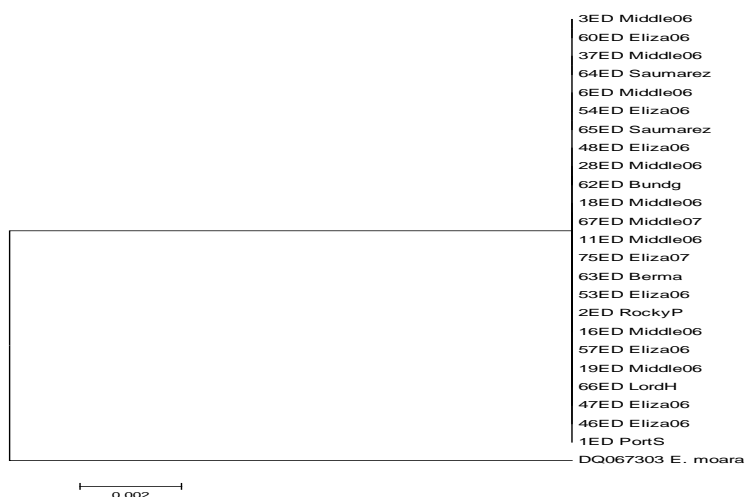


Figure 2 NJ tree based on 16S rRNA sequence data from 24 *E. daemellii* individuals. The distance scale bar represents 0.002 substitutions per site

### 8.1.2 *CoxI*

Sixteen *E. daemeli* individuals were sequenced and aligned across the *coxI* fragment. This fragment was also easily sequenced in both directions from the DNA templates with no insertions or deletions. The aligned sequence (following minimal trimming) consisted of part of the cytochrome oxidase I 5' region of the gene containing 609 base pairs. There were 608 identical pairs and one variable site which was parsimony informative among the 16 individuals. This variable site was a transition from G to A at base pair 353. The nucleotide composition of this fragment is shown in Table 4. With only one variable base among the 16 sequences, the overall genetic distance amongst all individuals was a very small  $0.001 \pm 0.001$ .

Sequences from five (I.40856-005 (65ED), I. 40856-003 (64ED), 18ED, 48ED, 1ED) of the 16 individuals were submitted for inclusion to the Barcode of Life Database (BOLD sample numbers BW-A3708, BW-A3709, BW-A3710, BW-A3711, BW-A3712 respectively). These five individuals were chosen based on the two haplotypes observed in the gene fragment, two of the individuals were voucher specimens from the Australian Museum (if possible voucher specimens are required for inclusion in the BOL database) and the other specimens were randomly chosen based on the number of sequences required for submission to BOLD ( $n = 5$ ). Figure 3 demonstrates the resulting NJ phenogram from comparisons of these five individuals with the BOL database. The branches are well supported with bootstrap values  $>75$ . The five *E. daemeli* individuals are clearly identified from other *Epinephelus* species.



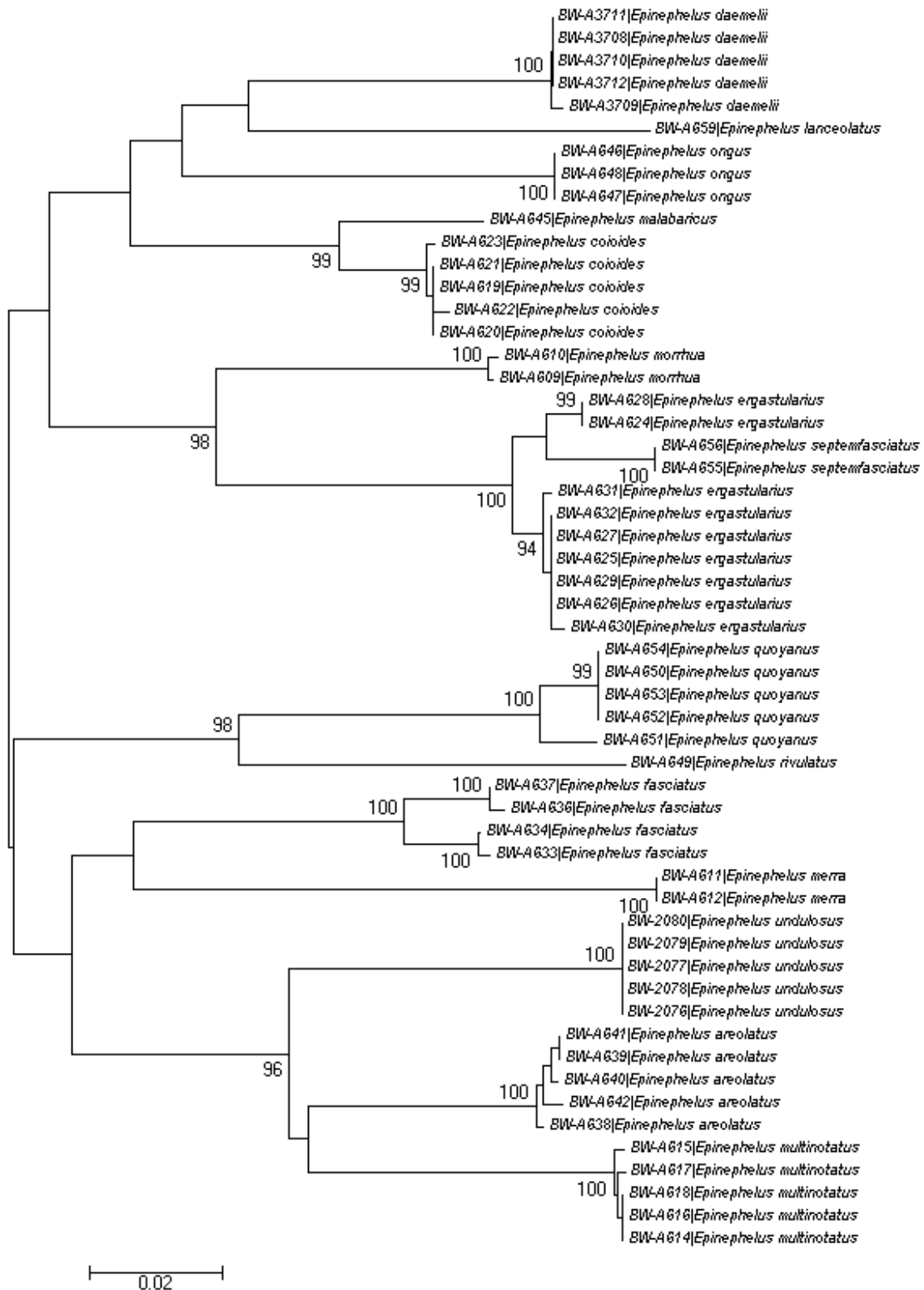


Figure 3 NJ tree based on cytochrome oxidase I sequence data from five *E. daemeli* individuals and other *Epinephelus* samples from the BarCode of Life database. Bootstrap values based on 2000 replicates are shown. The scale bar represents 0.02 nucleotide substitutions per site

The two *coxI* haplotypes are more clearly visible when just the 16 sequenced *E. daemelia* individuals are compared to a single outgroup (GenBank Accession Number DQ107891 (*E. coioides*)) (see Figure 4). As with the 16S fragment, due to the dearth of variation in this fragment, additional location testing was not undertaken.

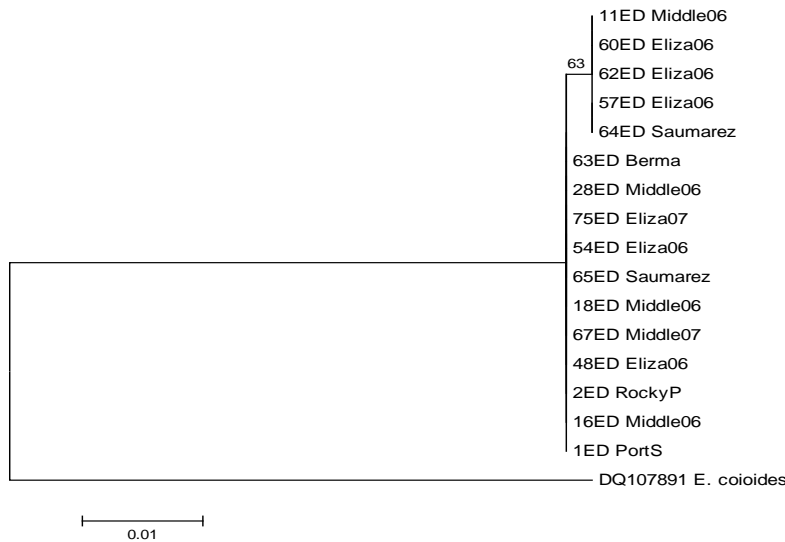


Figure 4 NJ tree based on cytochrome oxidase I sequence data from 16 *E. daemelia* samples. Bootstrap values based on 2000 replicates are shown. The scale bar represents 0.01 nucleotide substitutions per site

### 8.1.3 *Cytb*

A greater number of individuals were sequenced for the *cytb* fragment as initial testing demonstrated some variation in this fragment. Overall, 67 *E. daemelia* individuals were sequenced for 736 sites. This fragment was also easy to amplify and sequence with no insertions or deletions. The forward strand produced slightly better base pair resolution than the reverse.

Table 4 shows the average nucleotide composition within this fragment. Three of the variable sites were parsimony informative and all were transitions (T-C at base pair 397, G-A at base pair 508, C-T at base pair 518). Average nucleotide composition consisted of approximately 30% T, 31% C, 23% A and 16% G.

The overall K2P distance among the 67 individuals was  $d = 0.001 \pm 0.001$ . Figure 5 demonstrates the very close association of the black cod individuals at this fragment. Three main groups (1, 2 & 3) are shown in the phenogram, with several individual outliers, although the distance between these individuals and the major groups (and indeed between the three groups) is still very small. This is emphasised once the outgroup sequence is used in the analysis.

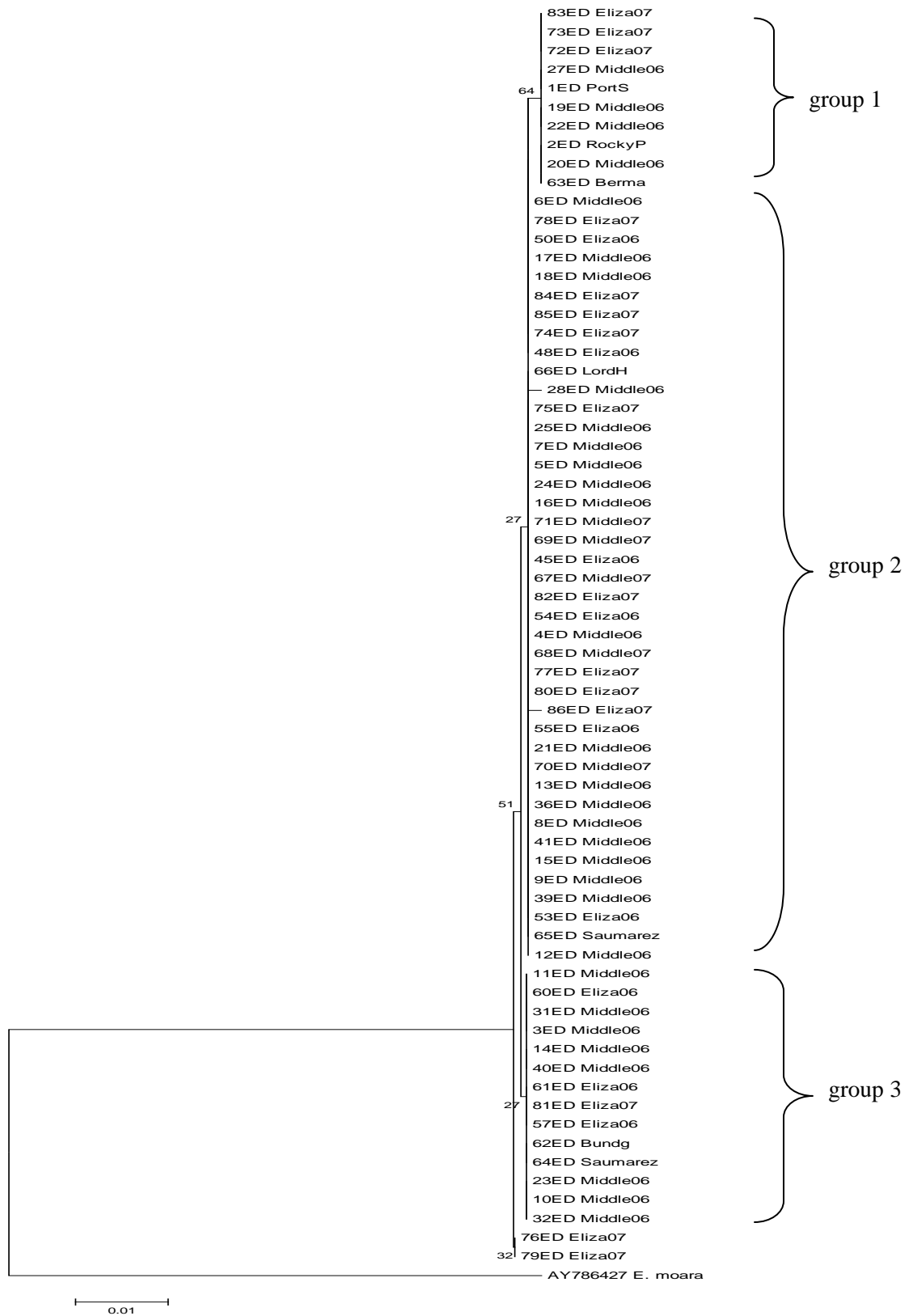


Figure 5 NJ tree based on cytochrome b sequence data from 67 *E. daemellii* samples. Bootstrap values based on 2000 replicates and the scale bar representing 0.01 nucleotide substitutions per site are shown

#### 8.1.4 ND2

This fragment was generally easy to amplify and sequence, although some DNA templates did not amplify successfully (despite standardisation of genomic quantity used in the PCR reactions). Of those templates that did amplify, sequencing was reliable and repeatable with the forward strand producing slightly better base pair resolution than the reverse primer.

Alignment of this fragment consisted of part of the ND2 region across 897 base pairs with no insertions or deletions. However a number of base pairs in the beginning and end of this fragment produced sequencing ambiguities and as such, the number of base pairs used (after pair-wise deletion of missing data) for the distance calculations was approximately 700 bp. As Table 4 shows, 890 base pairs were conserved and six of the seven variable sites were parsimony informative. All informative sites were transitions (C-T at base pair 212; A-G at base pair 266; G-A at base pair 296; C-T at base pair 470; A-G at base pair 734; T-C at base pair 867). Average nucleotide composition was 25.1% T, 33.3% C, 28.6% A and 13.0% G (see Table 4). The overall K2P genetic distance among the 81 individuals was 0.002. The resulting phenogram using *E. tukula* as an outgroup shows four major groups, although again the distance between the cod individuals is small but well supported (Figure 6).

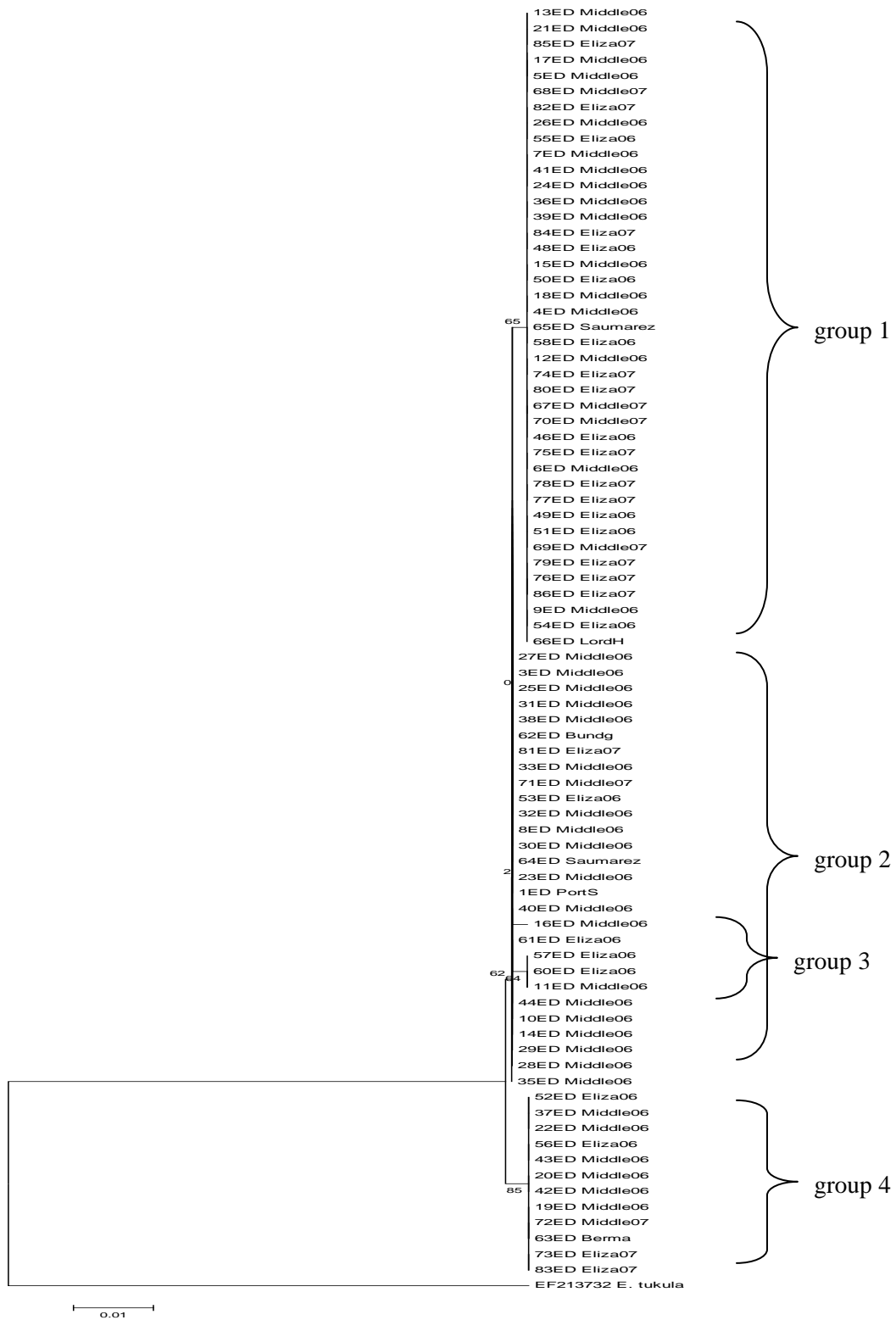


Figure 6 NJ tree based on NADH dehydrogenase subunit 2 sequence data from 81 *E. daemeli* samples. Bootstrap values based on 2000 replicates are shown. The scale bar represents 0.01 nucleotide substitutions

### 8.1.5 CR

Alignment of this fragment included part of the left hand side of the D-loop or control region from the tRNA-proline gene to the central conserved region across 434 base pairs. A number of base pairs were trimmed from the beginning and end of the sequences due to sequencing ambiguities. This fragment was often difficult to amplify and sequence on a routine basis; as such only 52 individuals across all the sampling locations are represented in the analysis. Of these, not all sequences contained the full length read, hence the reduced number of sites used in the distance calculations (see Table 4, cf. sites used for 16S and *cytb* distance calculations).

In black cod, the CR fragment was highly variable with 33 variable sites (across the 434 base pairs) and 75% of these are parsimony informative (see Table 4). While too numerous to list here (see Appendix E for the aligned sequences), the variable sites were all transitional pairs. Nucleotide composition across the 52 fish was; T = 32.7%, C = 16.1%, A = 39.2% and G = 12.0%; due to a tandem TA repeat section in this fragment, the average composition of these two bases was the highest of all five fragments.

The tandemly repeated TA sequence started at the 184 base pair. Variation amongst the samples was due both to the 33 variable sites and the insertion/deletions (indels) events in the tandem repeat area. The largest number of repeats was 14 and the smallest was five. In the K2P distance analysis, gaps were inserted to adjust for the internal length changes when aligning the sequences (see also Swofford *et al.*, 1996). Sequence positions with gaps were then omitted from the overall analyses according to the 'complete deletion' method which deletes a site from all pair-wise comparisons if any of the sequences in the 52 individuals have a gap at that site. As Swofford *et al.* (1996) point out, this method discarded more information but is considered more appropriate for the CR fragment as its regions are more prone to indel events than other mtDNA fragments. Due to the variable number of indels in black cod, each sequence was manually aligned based on the number of TA repeats observed in the forward and reverse sequences of the traces. The length of the control region has previously been reported to be highly variable even among closely related species (and individuals) due to the presence of tandem repeat sequences and large insertions (Lee *et al.*, 1995).

Additionally, due to the high variability observed among species with this region, BLASTn comparisons with the database at NCBI demonstrated very low level matches (across a very limited number of base pairs) to the hypervariable control and D loop regions in tuna - *Thunnus alalunga* (Accession number AF390333) and *T. thynnus* (Accession number DQ087592) (90 - 91% match across only 49 base pairs, with very low  $E = 7e-06$ ), and a low match to the D loop region in *Chromis triptoralis* (Accession number DQ212281) ( $E = 1e-04$ ), a coral reef fish from the Great Barrier Reef. It was also difficult to obtain an outgroup CR sequence from GenBank. This fragment does not lend itself to phylogenetic analyses due to its highly variable internal and length sequence differences. However, as Figure 7 shows, the NJ tree for the 52 black cod samples using *E. quernus* as the outgroup demonstrates at least three very closely related groups within *E. daemeli*.

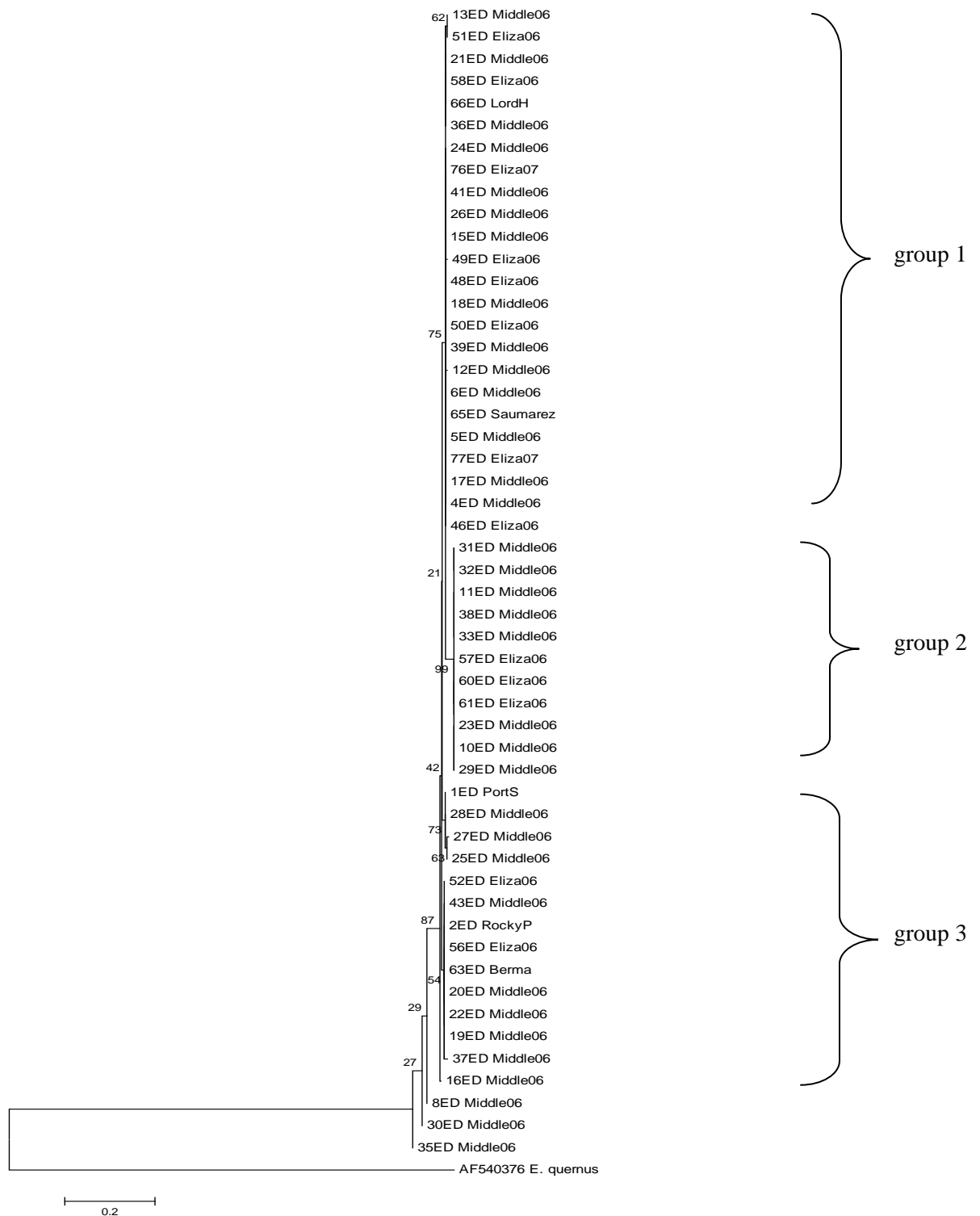


Figure 7 NJ tree based on control region sequence data from 52 *E. daemellii* samples. Bootstrap values based on 2000 replicates are shown. The scale bar represents 0.2 nucleotide substitutions per site

## 8.2 Variation in mtDNA gene fragments – testing for genetic homogeneity

As indicated above, only three of the five mtDNA fragments were variable enough to undertake sample location analyses and due to the very small sample sizes in six of the ten locations, the more robust genetic homogeneity tests are based only on the four collections from the Elizabeth and Middleton Reef marine reserve for *cytb*, ND2 and CR (although some preliminary eastern Australian coast v EMRMR & LordH area comparisons are presented). An inspection of the phenograms produced from each of the fragments indicates that for all of the fragments, the groupings of the individuals were not location specific. Furthermore, sequencing for *cytb* and ND2 from the samples that arrived late in April 07 (88 - 91ED) demonstrated that these haplotypes were the same as those observed from the other cod collections; there were no apparent genetic differences or indeed location specific differences with these samples.

Table 5 lists the within collection diversity statistics for each of the larger collections from the EMRMR: the majority of genetic homogeneity tests were based on this data. As highlighted previously, the CR fragment was the most variable of the three mtDNA gene fragments with up to 12 haplotypes (and  $h = 0.941$ ) observed in the Middle06 collection. It should be remembered that this estimate is calculated only on the bases used in the distance calculations; this is a conservative estimate as complete base pair deletions were used amongst cod individuals in the K2P analyses. The tandem repeat sequences themselves provide a source of variation due to the differing number of repeats among black cod.

The sequences revealed generally low nucleotide diversity in each of the fragments (*cytb* average  $\pi = 0.0007$ ; ND2 average  $\pi = 0.0016$ ; CR average  $\pi = 0.0100$ ) and moderate to high haplotype diversity depending on fragment type (Table 5). Overall the CR fragment was the most variable with gene diversity ranging from 0.927 to 1.000 while the *cytb* fragment displayed the lowest amount of gene diversity across the collections (0.000 – 0.781). In five of the eleven comparisons, Tajima's D test was negative although none was significant.



Table 5 Within collection diversity statistics (across the various sequences) in *E. daemellii* for the three variable mtDNA fragments screened in the Elizabeth and Middleton Reef collections; N = sample size,  $S_p$  = number of polymorphic sites,  $h$  = gene diversity (mean  $\pm$  s.d) ,  $\pi$  = nucleotide diversity averaged over loci (mean  $\pm$  s.d), D = Tajima's test of selective neutrality ( $P$  value shown in parenthesis)

mtDNA region	Middle06	Eliza06	Middle07	Eliza07
<i>cytb</i>				
N	31	9	5	15
$S_p^{\#}$	3	1	0	4
$h$	0.604 $\pm$ 0.076	0.500 $\pm$ 0.128	0.000 $\pm$ 0.000	0.781 $\pm$ 0.102
$\pi$	0.0009 $\pm$ 0.0008	0.0007 $\pm$ 0.0007	0.0000 $\pm$ 0.0000	0.0012 $\pm$ 0.0010
D	-0.182 (0.439)	0.986 (0.901)	1.000 (1.000)	-0.972 (0.202)
ND2				
N	40	15	5	15
$S_p^{\#}$	7	6	1	5
$h$	0.824 $\pm$ 0.036	0.695 $\pm$ 0.109	0.900 $\pm$ 0.161	1.000 $\pm$ 0.0243
$\pi$	0.0019 $\pm$ 0.0010	0.0024 $\pm$ 0.0016	0.0005 $\pm$ 0.0006	0.0017 $\pm$ 0.0012
D	0.027 (0.583)	0.583 (0.736)	-0.817 (0.297)	-0.511 (0.356)
CR				
N	34	11	----	2
$S_p^{\#}$	31	16	----	0
$h$	0.941 $\pm$ 0.023	0.927 $\pm$ 0.067	----	1.000 $\pm$ 0.000
$\pi$	0.0177 $\pm$ 0.0094	0.0134 $\pm$ 0.0078	----	0.0000 $\pm$ 0.000
D	-0.189 (0.484)	0.053 (0.564)	----	0.000 (1.000)

<sup>#</sup>all variable sites were transitions

Across all locations (see Table 1), the global tests of differentiation (based on haplotype frequencies) at each of the three mtDNA fragments (across all cod that were successfully sequenced) were non-significant in all instances ( $P > 0.999$ ). There was no evidence of differentiation amongst the overall sampling locations.

Furthermore, when all locations were considered together (the actual number of locations included in each AMOVA analysis is dependent on the fragment type, see Table 6), a non-hierarchical analysis of variance demonstrated relatively low and non-significant variance estimates for all fragment types. Between 91- 100% of the variance was attributed to 'within' collection differences (i.e. differences in individual sequences from each location).

Table 6 *E. daemellii* non-hierarchical AMOVA based on mtDNA sequencing across the three most variable gene fragments

mtDNA fragment	$n^*$	% of variation	$\Phi_{ST}$	$P$ value
<i>cytb</i>	10	90.7	0.093	0.077
ND2	9	97.0	0.030	0.254
CR	8	100.0	-0.071	0.823

\*  $n$  = number of sampling locations used in the AMOVA comparison, see Table 1 for details

On a pair-wise basis, relatively large (but non-significant)  $F_{ST}$  values were obtained between sampling locations that consisted of only one or two haplotypes and the locations with larger sample sizes (i.e. *cytb*  $F_{ST}$  Middle06 & Bunda = 0.403,  $P = 0.999$ ). This same trend was observed in all three mtDNA fragments and this is more of a sample size issue than demonstrating biological meaningful differences between the locations.

As Table 6 demonstrates, the largest value of the non-hierarchical fixation index was observed for *cytb*. There did appear to be differences in the haplotype frequencies observed in the samples from the eastern Australian coastal regions (Bunda, Berma, PortS, RockyP and

Saumarez) in comparison to those from the Elizabeth and Middleton Reef and Lord Howe Island areas (Middle06, Middle07, Eliza06, Eliza07, LordH) (i.e.  $\Phi_{CT}$  in hierarchical AMOVA for *cytb* = 0.117,  $P = 0.016$ ;  $\Phi_{ST} = 0.175$ ,  $P = 0.072$ ), however the actual source of the variance is difficult to determine given that only six cod were analysed from these coastal regions. When the four additional samples from the eastern Australian coastal area were included in the *cytb* analysis (the samples from E. Buckle April 07, 88 – 91ED), the genetic variance among the two groups was reduced to  $\Phi_{CT} = 0.101$  ( $P = 0.011$ ) and the overall variance among all samples was only slightly above that from the non-hierarchical AMOVA shown in Table 6 ( $\Phi_{ST} = 0.140$ ,  $P = 0.103$  ca.  $\Phi_{ST} = 0.093$ ,  $P = 0.077$ ). The *cytb* fragment may provide a low level of genetic diversity but importantly no significant differences were seen across the black cod individuals in the marine reserve area. Furthermore, while the data from the *cytb* fragment suggests some level of genetic differentiation among the areas, the differences are marginally significant.

In contrast to the *cytb*, the corresponding hierarchical values from the other two mtDNA fragments (based on the same eastern coastal regions v EMRMR and LHI as above) were lower and not significant (ND2  $\Phi_{CT} = -0.019$ ,  $P = 0.504$ ;  $\Phi_{ST} = 0.015$ ,  $P = 0.245$ ; CR  $\Phi_{CT} = 0.108$ ,  $P = 0.083$ ;  $\Phi_{ST} = 0.014$ ,  $P = 0.820$ ). Many more samples from multiple ‘coastal’ areas would need to be analysed before the *cytb* finding could be corroborated.

Concentrating on just the EMRMR collections, non-hierarchical AMOVA analysis of Middle06, Middle07, Eliza06 and Eliza07 also indicated no significant variance arising from among collection differences for any of the three mtDNA fragments (Table 7). All variance was accounted for by within collection differences when these collections were compared in a group. Likewise, the pair-wise  $F_{ST}$  comparisons among these four collections were all low and non-significant (*cytb*  $F_{ST}$  ranged from -0.037 to 0.151; ND2  $F_{ST}$  ranged from -0.0389 to 0.082; CR  $F_{ST}$  ranged from -0.026 to -0.153). As there was no significant differences between the two temporal collections at each sampling location, the data were combined and used in a hierarchical AMOVA analysis between Elizabeth and Middleton sampling locations.

Table 7 *E. daemellii* non-hierarchical AMOVA based on mtDNA sequencing in the Elizabeth and Middleton Reef locations (across the three most variable gene fragments)

mtDNA fragment	$n^*$	% of variation	$\Phi_{ST}$	$P$ value
<i>cytb</i>	4	97.6	0.024	0.218
ND2	4	97.2	0.028	0.174
CR	3	100.0	-0.044	0.807

\*  $n$  = number of sampling locations used in the AMOVA comparison, see Table 1 for details

Not unexpectedly, the AMOVA analysis on the combined temporal locations demonstrated that there was no significant evidence to consider the samples from the two reef areas as belonging to separate populations. While a sizeable component of the variance from the CR fragment was attributed to differences among the two combined collections ( $\Phi_{CT} = 0.143$ ), this was not significant ( $P = 0.334$ ) (Table 8).

Table 8 *E. daemellii* hierarchical AMOVA from the EMRMR region based on mtDNA sequencing across variable gene fragments – *cytb*, ND2 & CR

Source of variation	Gene fragment	Total variance	Fixation indices	<i>P</i> value
Among groups ( $\Phi_{CT}$ )	<i>cytb</i>	-0.020	-0.060*	1.000
	ND2	0.005	0.006*	0.663
	CR	0.481	0.143**	0.334
Among collections within group ( $\Phi_{SC}$ )	<i>cytb</i>	0.023	0.065*	0.141
	ND2	0.020	0.023*	0.229
	CR	-0.606	-0.210**	0.900
Within collections ( $\Phi_{ST}$ )	<i>cytb</i>	0.330	0.009*	0.228
	ND2	0.839	0.029*	0.171
	CR	3.493	-0.037**	0.801

\*these groups consisted of Middle06 &amp; Middle07 v Eliza06 &amp; Eliza07

\*\*these groups consisted of Middle06 v Eliza06 &amp; Eliza07

### 8.3 Microsatellite loci in *E. daemellii* – qualitative results for the Rivera *et al.* (2003) markers

A range of PCR annealing and cycling conditions for a subset of *E. daemellii* individuals were trialled for all loci from Rivera *et al.* (2003). In attempts to produce single, discrete PCR banding patterns, tested annealing temperatures ranged from 35°C to 60°C and 1.25 mM – 2.5 mM MgCl<sub>2</sub> concentrations were used. Table 2 shows the annealing temperatures at which a product was produced for each of the primer combinations.

In *E. daemellii*, CA-2 was amplified at 45°C. A large 323 base pair banding artefact was also produced, but the scored allelic pattern was consistent. This locus was amplified in all individuals. CA-3 was also amplified at 45°C with the resultant banding pattern highly repeatable and alleles easy to score (several artefacts were produced up and down stream of the alleles but these non-variable bands were not considered). Similarly, CA-6 was also amplified at 45°C with the banding pattern displaying the typical ‘cock’s comb’ appearance of a dinucleotide repeat but the alleles were repeatable and easy to score. Resultant genotypes at these three loci depended on the quality and quantity of genomic DNA left after the mtDNA gene fragment amplifications. The resulting statistical information for these loci is shown in Table 9.

For the remaining loci, CA-1 was difficult to amplify and produced a very weak product with multiple bands. Analysis on the DNA autosequencer showed a very messy multiple peak profile and hence this locus was not used further. CA-4 could not be amplified despite trialling across a wide range of annealing temperatures and MgCl<sub>2</sub>. PCR amplification at CA-7 was only observed at 35°C and due to the very low annealing temperature, a multiple banding pattern was observed. The locus however did appear non-variable and as such was not screened. GA-1 and GAA-1 were amplified at 55°C. These loci were easy to score but both were monomorphic. Finally, CT-1 amplified successfully at 55°C and while the banding pattern of ‘alleles’ was repeatable and relatively easy to score, three putative alleles in each genotype were observed. This locus was not used for any further screening.

### 8.4 Genetic diversity at microsatellite loci

Table 9 lists the various genetic and diversity estimates. However, given the relatively small sample sizes in most of the collections, the majority of genetic homogeneity comparisons were based on the Middle06, Middle07, Eliza06 and Eliza07 collections. Furthermore, none of the alleles in the samples from the coastal or near coast locations were unique to these areas. Tests

for conformity to HWE and linkage disequilibrium were only undertaken in these four collections - it is not possible to estimate these statistics in 'collections' where the sample size is very small.

For the three microsatellite loci, between 71 and 85 black cod were genotyped, depending on the locus (see Appendix F for allele frequencies). Tests for linkage disequilibrium in the four Middleton and Elizabeth Reef collections (following Bonferroni correction) supported the independent assortment of alleles at different loci.

Generally, the three loci displayed moderate to high levels of polymorphism. The number of alleles at each locus in the larger collections ranged from 4 (at CA-6, in Middle07) to 22 (at CA-3, in Middle06) and average numbers of alleles per locus ranged from 11 (CA-2) to 13 (CA-6) (Table 9). In the four reef collections and on a per collection basis, average  $H_o$  ranged from 0.786 at CA-2 in Eliza07 to 1.000 at CA-3 in Eliza07. Locus CA-3 displayed the highest average  $H_o$  and locus CA-6 the lowest (Table 9). Mean allelic richness was consistent amongst the collections (and has been adjusted for a minimum sample size of 1, ranging from 1.644 to 1.944 in the four larger collections). There was low-level evidence of private alleles (i.e. alleles observed in a single collection) but of the 16 private alleles observed over the three loci, 69% of these were observed in the largest collection, Middle06 and each was present at a frequency of less than 0.060.  $F_{IS}$  estimates across the four EMRMR collections on a per locus basis were all small which indicated that mating can be considered essentially random in these cod collections ( $F_{IS}$  CA-2 = 0.037;  $F_{IS}$  CA-3 = 0.052;  $F_{IS}$  CA-6 = 0.068)

Average  $H_e$  across the three loci in the four larger collections was at least 89%. On a per locus scale, in all instances,  $H_e$  was very high ranging from 0.644 at CA-6 in Middle07 to 0.944 at CA-6 in Eliza07; genotypic tests demonstrated that all loci in these collections conformed to HWE (following Bonferroni correction) (Table 9). Similarly, results from MICROCHECKER indicated that none of the loci were characterised by null alleles, problematic scoring due to stutter effects or large allelic dropout.

Table 9 Summary statistics for three microsatellite loci screened in *E. daemellii* collections from the EMRMR and various eastern Australian regions; mean sample size per locus ( $N$ ), number of alleles ( $N_{alleles}$ ), allelic richness ( $A$ ), heterozygosity observed ( $H_o$ ), and heterozygosity expected under equilibrium conditions ( $H_e$ ). All loci in the larger collections conformed to HWE

Collection	CA-2	CA-3	CA-6
<b>Bunda</b>			
$N$	1	1	1
$N_{alleles}$	2	1	2
$A^*$	2.000	1.000	2.000
$H_o$	1.000	NA	1.000
$H_e$	1.000	NA	1.000
<b>Saumarez</b>			
$N$	1	1	2
$N_{alleles}$	2	2	3
$A^*$	2.000	2.000	1.833
$H_o$	1.000	1.000	0.500
$H_e$	1.000	1.000	0.833
<b>PortS</b>			
$N$	1	1	1
$N_{alleles}$	2	2	2
$A^*$	2.000	2.000	2.000
$H_o$	1.000	1.000	1.000
$H_e$	1.000	1.000	1.000
<b>Berma</b>			
$N$	1	1	1
$N_{alleles}$	2	2	1
$A^*$	2.000	2.000	1.000
$H_o$	1.000	1.000	NA
$H_e$	1.000	1.000	NA
<b>RockyP</b>			
$N$	1	1	1
$N_{alleles}$	1	2	2
$A^*$	1.000	2.000	2.000
$H_o$	NA	1.000	1.000
$H_e$	NA	1.000	1.000
<b>Middle06</b>			
$N$	36	34	42
$N_{alleles}$	15	22	17
$A^*$	1.903	1.933	1.910

$H_o$	0.944	0.853	0.833
$H_e$	0.903	0.933	0.910
Eliza06			
$N$	10	8	16
$N_{alleles}$	10	10	13
$A^*$	1.905	1.933	1.887
$H_o$	0.800	0.875	0.813
$H_e$	0.905	0.933	0.887
LordH			
$N$	1	1	1
$N_{alleles}$	2	2	2
$A^*$	2.000	2.000	2.000
$H_o$	1.000	1.000	1.000
$H_e$	1.000	1.000	1.000
Middle07			
$N$	5	5	5
$N_{alleles}$	7	6	4
$A^*$	1.911	1.889	1.644
$H_o$	0.800	0.800	0.800
$H_e$	0.911	0.889	0.644
Eliza07			
$N$	14	14	15
$N_{alleles}$	12	15	12
$A^*$	1.931	1.944	1.924
$H_o$	0.786	1.000	0.867
$H_e$	0.931	0.944	0.924
<i>Average</i> **			
$N$	16.25	15.25	19.5
$N_{alleles}$	11	13	11.5
$H_o$	0.833	0.882	0.828
$H_e$	0.913	0.925	0.841

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<sup>†</sup>based on a minimum sample size of 1 diploid individual

<sup>\*\*</sup>based on samples from Middle06, Middle07, Eliza06, Eliza07

## 8.5 Variation in microsatellite loci – testing for genetic homogeneity

Significant allele frequency variation was not observed in the three microsatellite loci across the all sampling locations ( $P = 1.000$ ). Exact tests of allelic frequencies showed no significant differences among the ten sampling locations when analysed on a per locus basis (CA-2  $P = 0.109$ ; CA-3  $P = 0.277$ ; CA-6  $P = 0.095$ ). Likewise there were no significant (following Bonferroni correction) pair-wise comparisons among the collections across all loci (Fisher's  $P$  value  $> 0.030$ ). Indeed when Middle06 & Middle07 and Eliza06 & Eliza07 were analysed, no significant differences in allelic frequencies were generally observed (CA2  $P = 0.209, 0.083$ ; CA3  $P = 0.593, 0.222$ ; CA6  $P = 0.545, 0.042$  respectively).

Pair-wise  $F_{ST}$  comparisons among the 10 sampling locations (data not shown) demonstrated no significant differences in heterogeneity; all pair-wise estimates were not significant following Bonferroni correction for multiple tests. In many instances, particularly between the larger collections, the  $F_{ST}$  values were essentially zero (e.g. Middle06 and Eliza06  $F_{ST} = -0.030$ ). The  $F_{ST}$  values between the 'more' coastal and reef samples (e.g. Bunda and Eliza06  $F_{ST} = 0.200$ ) were often higher but it must be remembered that the sample size of these collections was at most just two individuals; these samples did not provide robust or meaningful  $F_{ST}$  pair-wise comparisons. Turning to the four larger collections, Table 10 shows the small and non-significant  $F_{ST}$  values among the EMRMR locations. These indicate that there is much greater genetic diversity within the four collections than between collections.

Table 10 Pair-wise  $F_{ST}$  comparisons among the four *E. daemellii* Middleton and Elizabeth collections, based on three nuclear microsatellite loci (below the diagonal).  $P$  values of the  $F_{ST}$  estimates are given above the diagonal, significant values following Bonferroni correction are shown in bold. Values are given to three decimal places; negative values are equal to zero

Collection	Middle06	Eliza06	Middle07	Eliza07
Middle06	-----	0.972	0.026	0.181
Eliza06	-0.030	-----	0.781	0.968
Middle07	0.036	-0.034	-----	0.052
Eliza07	0.006	-0.044	0.037	-----

As with the mtDNA gene fragment, AMOVA analyses (using the microsatellite genotypes) on all ten sampling locations demonstrated very low  $\Phi_{ST}$  results ( $\Phi_{ST} = 0.007, P = 0.266$ ). There was no evidence to suggest sub-structuring of cod at the various sampling locations.

A hierarchical AMOVA across each of two geographic groups (i.e. between Lord Howe Island and EMRMR collections ( $n = 5$ ) and the east coast Australian collections ( $n = 5$ )) demonstrated that the great majority of observed variation in the two groups was due to within collection differences ( $\Phi_{ST} = 0.025, P = 0.260$ ), but a low and significant amount of the variance (2.3%) was due to among group differences ( $\Phi_{CT} = 0.023, P = 0.008$ ). However, there were only six individuals in this combined 'east coast group' and hence this AMOVA finding should be viewed with caution until a much greater number of coastal samples are genotyped for these three microsatellite loci and included in the comparisons.

A non-hierarchical AMOVA on Middle06, Middle07, Eliza06 and Eliza07 demonstrated no evidence of genetic sub structuring ( $\Phi_{ST} = -0.008, P = 0.785$ ). Following the non-significant pair-wise allelic comparisons between the two Middleton and two Elizabeth reef collections, the data were combined to form a single Middleton and a single Elizabeth reef collection. AMOVA analysis on these two combined collections again demonstrated there was no significant genetic structuring among the two groups ( $\Phi_{ST} = -0.006, P = 0.784$ ). AMOVA analysis on a locus by

locus basis demonstrated very small and non-significant differences were attributable to among collection variation at two of the three loci (Table 11). The small but significant spatial differentiation at *CA-6* still only accounted for 2% of the variation among the four collections. The overall sample size of the four collections is also still considered minimal for a study of this type.

Table 11 Locus by locus AMOVA results for *E. daemeli* from the EMRMR

Microsatellite locus	Sample size	Among collection		
		% variation	$\Phi_{ST}$	<i>P</i> value
<i>CA-2</i>	65	0.82	0.008	0.215
<i>CA-3</i>	61	1.04	0.010	0.171
<i>CA-6</i>	78	2.01	0.020	0.030

## 8.6 Additional molecular trials undertaken in *E. daemeli*

In addition to the mtDNA sequencing from the black cod individuals which were generally undertaken on either muscle or biopsy samples, we also used dried scales and alcohol stored fin tissue for DNA extraction and subsequent molecular screening. Both ‘tissue’ types provided good DNA although the overall quantity of DNA was directly proportional to the sample amount. Caudal, pectoral, anal, pelvic and dorsal fins plus several scales from one individual (1ED) were used to amplify the five mtDNA fragments. All sequences from the same fragment showed concordance to each other and that obtained from white muscle DNA.

During the current research and with an aim developing other variable molecular markers, several exon primed, intron crossing (EPIC) primers from Jarman *et al.* (2002) were trialled in two black cod samples. Although PCR and sequencing was successful, no sequence variation was detected in the ATP Synthetase Subunit  $\alpha$  and  $\beta$  (ATPS $\alpha$  and ATPS $\beta$ ) loci. EPIC screening in the Adenine Nucleotide Transporter/ADP-ATP Translocase (ANT) and Signal Recognition Particle 54-kDA subunit (SRP54) loci produced multiple banding patterns which could not be optimised (to produce a clean single product) and hence we did not sequence these loci. The EPIC loci screening to date has not provided useful molecular markers.

## 9. DISCUSSION

In the current study, we used genetic variation in three mtDNA gene fragments (*cytb*, ND2 and CR) and three microsatellite loci (*CA-2*, *CA-3*, *CA-6*) to investigate the population structure of black cod primarily from the Elizabeth and Middleton Marine Reef Reserve. As part of this research, two temporal collections of cod from these areas were examined but no temporal heterogeneity was observed.

The use of PCR based markers enabled black cod to be sampled non-destructively from various tissue types and molecular analyses undertaken. However, in some instances, the very small tissue samples obtained from the biopsy gun and the need to subsequently divide tissue or DNA samples among collaborators resulted in a number of individuals with limited amounts of DNA available particularly for the mtDNA amplifications. Despite this, the use of fin clips and scales not just white muscle tissue or biopsy samples is extremely important for population assessment of *E. daemeli* as it is a protected species and destructive sampling is not permitted.

The mtDNA gene regions were generally easy to amplify and sequence except for difficulties encountered with the control region. Difficulty in amplifying this region has been observed in



other fish species (either directly or by comparing numbers of fish amplified for the control region and other molecular markers in each study) (Bérubé *et al.*, 1998; Ovenden *et al.*, 2002; Ovenden and Street, 2003; Chen *et al.*, 2004; Ovenden *et al.*, 2004; Salini *et al.*, 2006). Furthermore, scoring of genotypes at the microsatellite loci was relatively routine despite the use of cross species developed loci.

Unfortunately, CMAR did not undertake any tissue sampling, and it was extremely difficult for our collaborators to acquire a substantial number of black cod samples from the field. The study also suffered significantly from a paucity of sampling locations. While the original objective was to develop and deploy mtDNA markers that were suitable for investigating stock structure in black cod collections from the NSW coast and EMRMR region, we only received six cod samples from the coast and near coast areas. These samples were insufficient for rigorous population analyses and subsequent tests of genetic subdivision between the EMRMR region and eastern areas. Therefore we were not able to fulfill this objective.

## 9.1 Genetic variability within collections

As will be discussed, the utility and application of the five mtDNA fragments varies among species, sampling locations, regions and fragment type. It is for this very reason that more than one fragment was screened in the current study. In contrast, microsatellite loci are usually implemented for population diversity and intra species studies due to their high variation and high allelic content.

As several previous studies have shown (Craig *et al.*, 2001; Maggio *et al.*, 2005; Trotta *et al.*, 2005), the functional value of the 16Sr RNA fragment and the cytochrome oxidase I fragment was in excellent species identification of *Epinephelus* species and other fin fish more widely. The new *E. daemellii* specific sequences obtained in this study will add to the available tools and sequences used for *Epinephelus* molecular taxonomy - this will further help to elucidate the inter-relationships within the genus (see also Craig *et al.*, 2001). It will also readily enable the identification of this species in any confiscated fish or fillet samples (or indeed in egg and larvae samples).

In contrast to the almost complete lack of intra *E. daemellii* variation in the above two fragments, we observed relatively low levels of variation in the *cytb* and ND2 fragment among all cod individuals ( $d = 0.001, 0.002$  based on 708 and 701 base pairs respectively) while the CR fragment was more variable ( $d = 0.016$ , based on 273 base pairs).

The *cytb* fragment has previously been used for both intra and inter species differentiation studies. A case in point is Maggio *et al.* (2005) investigation of phylogenetic relationships among eastern Atlantic *Epinephelus* species. However, while Maggio *et al.* (2005) did not detect any *cytb* differences among individuals of the same *Epinephelus* species, we observed several *cytb* haplotypes in *E. daemellii*. Maggio *et al.* (2005) only analysed 397 bases of this fragment while we sequenced up to 736 bases in *E. daemellii*.

In comparison to our study, Carlin *et al.* (2003) also detected variation in a 593 bp fragment of the *cytb* fragment in *E. adscensionis* (across a sample size of 109 individuals from nine locations) which resulted in slight but significant population genetic differentiation across the tropical Atlantic Ocean ( $\Phi_{ST} = 0.056, P < 0.001$ ). As with our study, Carlin *et al.* (2003) also reported low nucleotide diversity in this fragment ( $\pi = 0.008$ ) yet moderate haplotype differences (cf. with the current study  $\pi = 0.001$  and five variable sites). While we did not detect

any strong source of collection differentiation based on this fragment in black cod, there were some low level differences between the EMRMR and more coastal Australian locations – a definitive conclusion is not possible however due to the inadequate sample sizes from the coastal locations. Unlike our study, the samples in the Carlin *et al.*, (2003) study were obtained from a much wider geographic area across multiple locations including Florida, Bahamas, Barbados and Brazil. Carlin *et al.* (2003) reported a single major bifurcation in *E. adscensionis* with one branch restricted to Florida while the other was widespread across the geographical range of the fish. Gilles *et al.* (2000) also detected intraspecific sequence variation in a 353 base pair region of the *cytb* fragment in 29 *E. marginatus* individuals from the western Mediterranean Sea. Similarly, this study also detected a major bifurcation that separated the Mediterranean *cytb* sequences although the authors could not rule out the presence of a cryptic species. While we observed variation in this same fragment, we did not detect such a major bifurcation; several small branches (or ‘twigs’ see Carlin *et al.*, 2003) were observed in the NJ phenogram of *E. daemeli* but no major division or inter locality divergence.

With regards to the ND2 fragment, only one other *Epinephelus* study has utilised this fragment for population differentiation and that was based on RFLP analyses (Maggio *et al.*, 2006). Our direct sequencing of 897 base pairs across this fragment in black cod demonstrated a low level of nucleotide diversity and haplotype diversity which was ten fold less than the variation observed in *E. marginatus*. Maggio *et al.* (2006) detected strong genetic differentiation among Atlantic and Mediterranean samples of *E. marginatus* with ND2 RFLP haplotypes but not with *cytb* sequencing, yet both marker types provided evidence of genetic differentiation in the Mediterranean regions. We did not detect strong ND2 differences in the black cod.

As in many recent marine fin fish studies (e.g. Arnaud *et al.*, 1999; Ovenden *et al.*, 2004; Correia *et al.*, 2006; van Herwerden *et al.*, 2006; Zhang *et al.*, 2006; Klanten *et al.*, 2007 and other references within this report), we too observed the CR to be best suited to analysis of collection differentiation due to its variability and high haplotype diversity. In the current study, we detected up to 12 haplotypes in 52 fish, and in the EMRMR cod, average haplotype diversity was high ( $h = 0.956$ ). The observed base transitions and the numerous insertion/deletion events in the 5' end of the control region in *E. daemeli* appear to behave in a similar manner to that observed for this region in vertebrates (Saccone *et al.*, 1987) and indeed for other fin fish. We also observed a high AT bias (A = 39.2% , T = 32.7%) but this seems common in many marine fish species (Dudgeon *et al.*, 2000; Ovenden *et al.*, 2004; Correia *et al.*, 2006; Salini *et al.*, 2006; van Herwerden *et al.*, 2006; Klanten *et al.*, 2007). The high gene diversity in *E. daemeli* the CR fragment observed in the larger cod collections was similar to that observed for other finfish species and comparable to *E. quernus* at 0.870 - 0.990 (Rivera *et al.*, 2004) although we detected fewer variable sites (i.e. 33 across 434 base pairs) than Rivera *et al.* (2004) in *E. quernus* (76 variable sites across 398 bases)

Mirroring the high levels of haplotype diversity in at least the CR, good levels of microsatellite variation were also detected in the cod collections with up to 22 alleles at CA-6. We have no evidence of deviations from HWE and hence assume that the fish at least in the EMRMR areas are randomly mixing; we also did not detect the presence of null alleles at any locus although the sample sizes used for the microsatellite analyses are very much on the lower end of the acceptable scale for population comparisons.

As is often the case, microsatellite loci screened in this study (and indeed in many other *Epinephelus* studies) have been isolated from non-target *Epinephelus* species and utilised for cross species amplification. We used *E. quernus* microsatellites to obtain *E. daemeli* genotypes. Despite this, the observed and expected heterozygosities (average  $H_o = 0.848$ ; average  $H_e =$

0.893) observed in the black cod collections were comparable to those in other *Epinephelus* species and marine fish more widely (although levels of polymorphism do vary considerably depending on species and locus). In *E. polyphkadion* average  $H_o$  was 0.808 from three loci (Rhodes *et al.*, 2003); average  $H_o$  in *E. coioides* from Thailand and Indonesia was 0.360 - 0.550 (Antoro *et al.*, 2005); *E. morio* from southeastern Atlantic was slightly more variable at  $H_o = 0.681$  (Zatcoff *et al.*, 2004); average  $H_o$  from other *Epinephelus* species ranged from 0.370 - 0.660 (Nugroho *et al.*, 1998; Koedprang *et al.*, 2007)), and level of heterozygotes for the *E. quernus* loci screened in 24 *E. quernus* individuals ranged from 0 - 92% (Rivera *et al.*, 2003). More generally, De Woody and Avise (2000) reported average expected heterozygosity in 12 marine fish species for microsatellites was 0.770 (De Woody and Avise, 2000).

## 9.2 Lack of differentiation and population structure

Marine studies often report genetic heterogeneity among fish species but usually differentiation is more pronounced across wider geographic scales or in estuarine or near shore habitats (Shaklee and Bentzen, 1998; Ovenden *et al.*, 2004 and references within). With this in mind, varying population conclusions in *Epinephelus* species from both a mitochondrial and nuclear perspective are present in the literature (i.e. ongoing gene flow in *E. morio* - Richardson and Gold, 1997; genetic heterogeneity in *E. marginatus* - De Innocentiis *et al.*, 2001; regional homogeneity in *E. polyphkadion* - Rhodes *et al.*, 2003; genetic differentiation maintained by oceanic currents in *E. quernus* - Rivera *et al.*, 2004; minimal genetic differences in *E. morio* - Zatcoff *et al.*, 2004; conflicting mtDNA heterogeneity in *E. marginatus* - Maggio *et al.*, 2006). This suggests that observation of genetic structure is entirely marker and species specific; there are no generalisations that fit all *Epinephelus* species across their broader geographic range. This observation was pivotal to our study in *E. daemellii* as we had no preconceived ideas about suitable markers or probable outcomes in this protected species.

The mtDNA analyses in the current study indicated no significant collection differentiation on a local or broader scale in black cod. There is some indication that the more coastal samples may be slightly differentiated from individuals at EMRMR at the *cytb* fragment, although given the very small numbers of fish from the coastal region and the ambiguity around some sampling locations, it is difficult to be conclusive. In direct comparison with our other mtDNA data, many coral reef and marine fin fish studies based on the CR have also reported significant differences in fish populations across wide geographic scales but not within Australian waters (*Lutjanus malabaricus* and *L. erythropterus* grouped into broad stocks with boundaries between Kupang and the Timor Sea - Salini *et al.*, 2006; *Plectropomus maculatus* and *P. leopardus* form single mtDNA lines in eastern Australia but display more regional differences elsewhere - van Herwerden *et al.*, 2006; *Pristipomoides multidens* display significant stock structure among the Indonesian and Australian national boundaries yet homogeneity among Australian collections - Ovenden *et al.*, 2002; *Scarus frenatus* and *Chlorurus sordidus* show high levels of gene exchange along the Great Barrier Reef - Dudgeon *et al.*, 2000).

In support of the mtDNA results, the three microsatellite loci also displayed the same trend - a lack of strong heterogeneity across the sampling locations. Locus by locus analyses indicated a low level heterogeneity among the EMRMR collections but this was only the result of differences at one locus and the sample size for this locus was considerably smaller than we would consider useful for robust population comparisons. Overall, our findings are in contrast to that in *E. polyphkadion* populations in five sites across the western central Pacific which displayed significant allele frequency differences throughout a 5 000 km study area (Rhodes *et al.*, 2003). However our study concentrated on samples from across a much smaller area and

even considering the more eastern coastal samples, this distance is well less than 1 000km. More directly comparable, Rhodes *et al.* (2003) found that *E. polyphkadion* sampled in the Great Barrier Reef clustered with those from New Caledonia.

We do however caution that this apparent lack of genetic heterogeneity across the wider geographic locations should be viewed in light of the sampling limitations. We were not able to obtain samples from several known *E. daemeli* locations and indeed the samples we did screen from the eastern Australian area were far too small to be considered robust. In contrast, across a wider sampling area for *E. marginatus* (which is also listed as an endangered species in the Mediterranean Sea), 227 individuals across 15 populations were not considered part of a larger panmictic population (De Innocentiis *et al.*, 2001).

So what factors could be working to maintain the apparent genetic homogeneity in black cod? The two reef systems that the majority of our samples originated from are separated by just 45 km of deep oceanic waters. While black cod may prefer to inhabit caves and gutters on these reefs (to depths of approximately 50 m (Heemstra and Randall, 1993), if the larvae are pelagic, it could be that the water currents across the 45 km distance are sufficient to provide mixing in this region. Without tagging information, we can not know if cod make contemporary movements across these areas.

Conceivably, adult movements could be occurring as *E. daemeli* displays similar husbandry habits to those of *E. morio* from US Atlantic and Mexico. Field observations on this *Epinephelus* species show that both juveniles and adult red grouper are fairly sedentary and prefer to hide in crevices and ledges (Richardson and Gold, 1997), but maturing adults do migrate from shallow water to depths greater than 36 m at approximately five years of age (see in Richardson and Gold, 1997). Indeed, reproductive output may not occur in some *Epinephelus* species until females are at least nine year old (Pears *et al.*, 2006). Tagging studies on *E. morio* and larval information from other *Epinephelus* species suggest adults can move up to 80 km with a pelagic larval stage of up to 40 days (see references in Richardson and Gold, 1997) so perhaps similar events are taking place in *E. daemeli* but without tagging information or basic larval studies, we can not be certain. Despite previous evidence that *E. daemeli* is an aggressive and territorial species that may live in one particular cave for its entire life (Heemstra and Randall, 1993), our genetic results from the EMRMR suggest that the fish sampled from both reef regions would be considered panmictic; it is therefore reasonable to assume that biological or water based factors that help maintain good gene flow among locations must be operating.

It is more difficult to ascertain the factors that may contribute to the lack of strong genetic heterogeneity, if there truly is no such heterogeneity, between the EMRMR region and the more eastern Australian coastal region given that the marine reserve is 600 km to the east of the NSW coastline. We also have such a paucity of samples on which to base a robust conclusion.

Despite this, the homogeneity in both the mtDNA haplotypes and nuclear microsatellites coupled with a lack of strong geographic structuring makes us unable to reject the null hypothesis that black cod particularly from the EMRMR represent a single breeding stock. However, there are several caveats around this statement. The failure to disprove the null hypothesis does not mean that stock structuring does not exist; only that we did not detect it in the current study. This might well reflect the small sample sizes available to us rather than any true lack of stock structure. In addition, the observation of genetic homogeneity does not necessarily reflect a single unit stock. While homogeneity is consistent with the null hypothesis, this does not give an indication of the level of relative mixing as only small amounts of gene

flow via larval drift or adult migration are required to keep collections homogeneous. Molecular theory states that essentially just one migrant per generation can homogenise populations (Nei, 1987) (as detected by molecular markers). A lack of genetic heterogeneity could also reflect historical gene flow rather than present levels. Furthermore, our inability to detect population sub-structuring could also be a reflection of the marker types. However, we surveyed both slower and faster evolving mtDNA and fast evolving nuclear markers – all with the same outcome. It should also be considered that given the available evidence and small sample sizes, it is difficult to determine whether the various collections have been separated long enough for significant genetic differences to occur.

### 9.3 Concordant results from mtDNA and microsatellites

The majority of black cod were assayed with both mitochondrial and nuclear markers. These marker types produced relatively concordant estimates of population differentiation with *cytb* sequences and one microsatellite locus revealing weak differentiation among the collections; although sample size limitations prevent us from making conclusive statements about samples from the more eastern Australian coastline regions. Mitochondrial  $\Phi_{ST}$  estimates (considered as equivalent  $F_{ST}$  estimates in this study) for the EMRMR collections ranged from -0.044 to 0.024 (depending on fragment type) and in microsatellites ranged from 0.008 – 0.020 (again depending on locus). Both marker types reflected the same lack of sub-structuring in the EMRMR collections on both a spatial and temporal basis (between year 2006 and 2007), albeit the analyses were based on small sample sizes.

While mtDNA may provide greater power to detect population structure due to its reduced effective population size, this was not observed in the current study. It has been proposed that differentiation observed in (maternally inherited) mtDNA haplotypes but not in (biparentally inherited) nuclear DNA markers such as microsatellites reflects females returning to their place of origin for reproduction (e.g. whales and black tip sharks - Baker *et al.*, 1999; Keeney *et al.*, 2005; Dalebout *et al.*, 2006). However, we did not observe this inconsistency between the molecular markers and have no information on natal spawning areas or tag movements of mature adults to suggest female philopatry in black cod.

### 9.4 Future developments

The primary focus of this research was molecular tool development and preliminary investigation into genetic differences of black cod in the EMRMR region. In this regard and due to a paucity of samples, we have only looked briefly at the possible structure of cod in the marine reserve – the main purpose of this study should be viewed as the initial step towards progressing tools for stock assessment in black cod, and not the examination in detail of population genetic structure of the western Pacific population of *E. daemellii*. As there is little scientific information on any other traits, further research regarding life history, juvenile and adult movements and larval distribution is required in order to understand on a wider level how gene flow might occur throughout the broader range of this fish.

While we have highlighted three mtDNA gene regions that were useful for population comparisons and trialled nine microsatellites developed from *E. quernus*, the search for molecular markers in black cod is by no means complete. Additional mtDNA regions including the ATPase genes could be screened as could a number of other nuclear markers or SNPs (single nucleotide polymorphisms) although our preliminary screening of several EPIC markers did not provide any variable fragments.

As we screened non *E. daemeli* microsatellites in the current study, perhaps a next step forward is to develop species specific repeat loci. Nonetheless this process is expensive and time demanding and does not always result in useful loci – but such an investigation might provide additional *Epinephelus* species markers to add to the relatively limited array of polymorphic microsatellites already in the literature. Furthermore, we were not able to completely optimise the microsatellite genotyping due to the relatively limited number of samples that were screened (n = 78). This only results from screening a greater number of fish across a wider geographic range so that we can determine if we are typing the alleles correctly (especially important when non target species loci are used). As sample sizes were limited, we were also not able to sufficiently assess HWE, the presence of null alleles and linkage disequilibrium in black cod. These attributes could be tested further if additional samples are obtained. Likewise, although we did not progress this line of investigation due to the lack of larger numbers of samples, future research could be undertaken into the co-plexing or multiplexing of the microsatellite loci. Co-plexing and or multiplexing of loci during the visualisation and PCR amplification stages enables the loci to be analysed together; reducing costs and time needed for analysis.

Furthermore, whilst the molecular markers are just one source of data, we firmly believe that tagging studies (of juveniles and adults) would greatly help to understand individual movements, migration patterns and possible discernment of spawning areas (if they exist) for *E. daemeli*. Tagging studies highlight adult movements and give an indication of sex ratios in the population. Skewed sex ratios, if present, can lead to reductions in effective population sizes and flow on effects for inbreeding levels and overall genetic health of the population. Moreover, whilst molecular markers provide an historical or generational perspective on population structure, tagging studies provide a more contemporary estimate of the population on an ecological relevant time scale (Slatkin, 1994). A downside of tagging although is that it gives no reliable identification of interbreeding or gene flow - the presence of a migrant fish at a distant location does not necessarily mean that that migrant will contribute to the gene pool at that location.

Originally, we wanted to target 50 - 100 individuals per population as this level of sampling gives greater insight into population structure given that, particularly for microsatellites, a larger sample size (>100) may be needed to confirm or identify any small but significant levels of genetic differentiation. Unfortunately we were not able to acquire this number of samples. Supplementary samples from the EMRMR region would also be beneficial, particularly if both juvenile, sub-adult and adult fish could be sampled.

Consequently, an important focus of any future black cod studies should be the sampling of many more individuals (and possibly eggs and larvae) from across its known range. Nothing is known of larval ecology and hence dispersal potential of *E. daemeli*. Larval dispersal in most marine species has long been assumed to be widespread which results in little genetic differentiation among populations (Ward *et al.*, 1994; Bay *et al.*, 2006). Without larval studies and egg dispersal information, and given the lack of genetic heterogeneity among the EMRMR, we have no way of identifying if this is the mechanism which is maintaining homogeneity.

It would of course be very interesting to examine black cod from the Kermadec Islands in New Zealand and other marine reserves (Great Barrier Reef Marine Park in Queensland and the Julian Rocks Aquatic Reserve off Byron Bay, NSW) in order to determine if the observed lack of genetic differentiation in the EMRMR sites is more widespread. Leading on from this, it will also be important for future management of black cod to determine the effect of marine reserves and their implicit protection for the species as compared to other locations – further research is required into the genetic structure of cod populations in the marine reserves and more widely, particularly within Queensland waters where *E. daemeli* is not a protected species. Managers need to consider the importance of connectivity processes between coastal areas and other

marine reserves (see Pérez-Ruzafa *et al.*, 2006). Currently we do not have the data to progress this line of study but it clearly needs addressing in future cod surveys.

## 9.5 Overall findings

The results of this work support the intuitively reasonable belief, given that only 45 km separates the two sampling locations, that stocks of cod at the Elizabeth and Middleton Reef in the EMRMR region are genetically similar. The current findings based on our limited dataset suggest that there are no significant genetic differences indicating that we may be dealing with a single genetic population in this region.

While we can not reject the null hypothesis of genetic homogeneity in the EMRMR region, we would strongly suggest gathering additional information from other sources and using this, not just the genetic data, to refine management decisions. We have no data to suggest that the stock at EMRMR are not a single unit and there appears to be good gene flow among black cod in this region.

Given the lack of comprehensive sampling, we can not comment on the possible level of gene flow (or lack thereof) in black cod between the EMRMR and more coastal areas. It may be that the 600 km that separates the two regions and a lack of suitable habitat between established populations in the EMRMR and eastern Australian coast area prevents mixing of individuals. However, we do not know anything of the larval duration in black cod nor if the eggs are pelagic for some period of time. Interestingly, recent isotope tagging data suggest that some reef fish show substantial local replenishment (Almany *et al.*, 2007), so a precautionary approach to management is certainly advisable.

The observation of non-significant Tajima D statistics in the EMRMR samples suggests that these cod collections have not been fished down or experienced a population bottleneck – perhaps the establishment of the marine reserve and the total ban on cod fishing in these areas has ensured the species has not been adversely affected by human pressure. We can not know if the historical decline in cod numbers in NSW waters (due to spearfishing in the 1970's) have affected the population characteristics of the more coastal regions as our sampling was not extensive enough to enable any population data to be obtained. If indeed this fish is a relatively long lived species (as documented in other *Epinephelus* species), it is foreseeable that historical fishing pressure could have detrimentally impacted on the coastal populations by skewing the sex ratio of the populations and removing maturing sub-adults and mature adults.

On a broader perspective, our successful mtDNA sequencing demonstrated the utility of different gene fragments for both inter or intra species differentiation studies. The deployment of microsatellites for screening of nuclear variation was also successful. These data can now be used to refine conservation and management plans for *E. daemellii* and future sampling events for this species. Uncertainty regarding black cod population structure has seriously restricted the ability of conservation managers to make confident statements about ongoing protection levels. While this current study provides only the initial inputs into this process, we firmly believe that the combining of genetic data with tagging data and morphological measurements should enhance the power of future stock structure investigations for this protected species.

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#76ED.	...	[ 546 ]
#9ED.	...	[ 546 ]
#75ED.	...	[ 546 ]
#84ED.	...	[ 546 ]
#86ED.	...	[ 546 ]
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#6ED.	...	[ 546 ]
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#4ED.	...	[ 546 ]
#54ED.	...	[ 546 ]
#5ED.	...	[ 546 ]
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#50ED.	...	[ 546 ]
#55ED.	...	[ 546 ]
#7ED.	...	[ 546 ]
#51ED.	...	[ 546 ]
#49ED.	...	[ 546 ]
#21ED.	...	[ 546 ]
#17ED.	...	[ 546 ]
#12ED.	...	[ 546 ]
#13ED.	...	[ 546 ]
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#26ED.	...	[ 546 ]
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#46ED.	...	[ 546 ]
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#27ED.	...	[ 546 ]
#28ED.	...	[ 546 ]
#30ED.	...	[ 546 ]
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#56ED.	...	[ 546 ]
#42ED.	...	[ 546 ]
#63ED.	...	[ 546 ]
#19ED.	...	[ 546 ]
#72ED.	...	[ 546 ]
#20ED.	...	[ 546 ]
#37ED.	...	[ 546 ]
#43ED.	...	[ 546 ]
#22ED.	...	[ 546 ]
#52ED.	...	[ 546 ]
#73ED.	...	[ 546 ]
#35ED.	...	[ 546 ]
#44ED.	...	[ 546 ]
#8ED.	...	[ 546 ]
#57ED.	...	[ 546 ]
#60ED.	...	[ 546 ]
#11ED.	...	[ 546 ]
#81ED.	...	[ 546 ]
#61ED.	...	[ 546 ]
#32ED.	...	[ 546 ]

#10ED. .... [546]  
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#74ED. .... [546]

#69ED. GAA TAA TTT TAA TCC TCC AAT TCT CAC CCT CCC TGA CCC [585]  
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#30ED. .... [585]  
#83ED. .... [585]  
#56ED. .... [585]







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#84ED.	...	[663]
#86ED.	...	[663]
#80ED.	...	[663]
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#67ED.	...	[663]
#41ED.	...	[663]
#66ED.	...	[663]
#68ED.	...	[663]
#4ED.	...	[663]
#54ED.	...	[663]
#5ED.	...	[663]
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#7ED.	...	[663]
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#49ED.	...	[663]
#21ED.	...	[663]
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#12ED.	...	[663]
#13ED.	...	[663]
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#65ED.	...	[663]
#46ED.	...	[663]
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#24ED.	...	[663]
#18ED.	...	[663]
#53ED.	...	[663]
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#28ED.	...	[663]
#30ED.	...	[663]
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#42ED.	...	[663]
#63ED.	...	[663]
#19ED.	...	[663]
#72ED.	...	[663]
#20ED.	...	[663]
#37ED.	...	[663]
#43ED.	...	[663]
#22ED.	...	[663]
#52ED.	...	[663]
#73ED.	...	[663]
#35ED.	...	[663]
#44ED.	...	[663]
#8ED.	...	[663]
#57ED.	...	[663]
#60ED.	...	[663]
#11ED.	...	[663]
#81ED.	...	[663]
#61ED.	...	[663]
#32ED.	...	[663]
#10ED.	...	[663]
#23ED.	...	[663]
#29ED.	...	[663]
#31ED.	...	[663]
#33ED.	...	[663]
#38ED.	...	[663]

#3ED. .... [663]  
#40ED. .... [663]  
#62ED. .... [663]  
#64ED. .... [663]  
#25ED. .... [663]  
#1ED. .... [663]  
#71ED. .... [663]  
#14ED. .... [663]  
#16ED. .... [663]  
#74ED. .... [663]

#69ED. ACA TAC TCG CCA CCT CTT GAA CAA AAG CCC CTG CAC TAA [702]  
#77ED. .... [702]  
#78ED. .... [702]  
#79ED. .... [702]  
#85ED. .... [702]  
#82ED. .... [702]  
#76ED. .... [702]  
#9ED. .... [702]  
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#86ED. .... [702]  
#80ED. .... [702]  
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#67ED. .... [702]  
#41ED. .... [702]  
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#68ED. .... [702]  
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#42ED. .... [702]  
#63ED. .... [702]  
#19ED. .... [702]  
#72ED. .... [702]  
#20ED. .... [702]  
#37ED. .... [702]





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#67ED.	...	[780]
#41ED.	...	[780]
#66ED.	...	[780]
#68ED.	...	[780]
#4ED.	...	[780]
#54ED.	...	[780]
#5ED.	...	[780]
#48ED.	...	[780]
#50ED.	...	[780]
#55ED.	...	[780]
#7ED.	...	[780]
#51ED.	...	[780]
#49ED.	...	[780]
#21ED.	...	[780]
#17ED.	...	[780]
#12ED.	...	[780]
#13ED.	...	[780]
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#26ED.	...	[780]
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#46ED.	...	[780]
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#18ED.	...	[780]
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#28ED.	...	[780]
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#43ED.	...	[780]
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#23ED.	...	[780]
#29ED.	...	[780]
#31ED.	...	[780]
#33ED.	...	[780]
#38ED.	...	[780]
#3ED.	...	[780]
#40ED.	...	[780]
#62ED.	...	[780]
#64ED.	...	[780]
#25ED.	...	[780]
#1ED.	...	[780]











Appendix E CR aligned sequence data for *E. daemelia*

#10ED.	ACC	GCC	TAT	AAC	GCA	TAT	TGA	GTA	ACC	AAA	TAT	AGG	ACC	[ 39]
#11ED.	...	...	...	...	...	...	t..	...	...	...	...	...	...	[ 39]
#12ED.	..c	...	...	...	...	...	...	...	...	...	..t	agg	...	[ 39]
#13ED.	...	...	...	...	...	...	...	...	...	...	...	...	...	[ 39]
#15ED.	...	...	...	...	...	...	...	...	...	...	...	...	...	[ 39]
#16ED.	...	...	...	...	...	...	...	...	...	...	...	...	...	[ 39]
#17ED.	...	...	...	...	...	...	...	...	...	...	...	...	...	[ 39]
#18ED.	...	...	...	...	...	...	...	...	...	...	...	...	...	[ 39]
#19ED.	...	...	...	...	...	...	...	...	...	...	...	...	...	[ 39]
#1ED.	...	...	...	...	...	...	t..	...	...	...	...	...	.T.	[ 39]
#20ED.	...	...	...	...	...	...	...	...	...	...	...	...	...	[ 39]
#21ED.	...	...	...	...	...	...	...	...	...	...	...	...	...	[ 39]
#22ED.	...	...	...	...	...	...	...	...	...	...	...	...	...	[ 39]
#23ED.	...	...	...	...	...	...	t..	...	...	...	...	...	...	[ 39]
#24ED.	...	...	...	...	...	...	...	...	...	...	...	...	...	[ 39]
#26ED.	..c	...	...	...	...	...	...	...	...	...	...	...	...	[ 39]
#27ED.	...	...	...	...	...	...	...	...	...	...	...	...	.T.	[ 39]
#28ED.	...	...	...	...	...	...	...	...	...	...	...	...	.T.	[ 39]
#29ED.	...	...	...	...	...	...	...	...	...	...	...	...	...	[ 39]
#2ED.	...	...	...	...	...	...	...	...	...	...	...	...	...	[ 39]
#30ED.	...	...	...	...	...	...	...	...	...	...	...	...	...	[ 39]
#31ED.	...	...	...	...	...	...	...	...	...	...	...	...	...	[ 39]
#32ED.	...	...	...	...	...	...	...	...	...	...	...	...	...	[ 39]
#33ED.	...	...	...	...	...	...	...	...	...	...	...	...	...	[ 39]
#35ED.	...	...	...	...	...	...	...	...	...	...	...	...	...	[ 39]
#36ED.	...	...	...	...	...	...	...	...	...	...	...	...	...	[ 39]
#37ED.	...	...	...	...	...	...	...	...	...	...	...	...	...	[ 39]
#38ED.	...	...	...	...	...	...	...	...	...	...	...	...	...	[ 39]
#39ED.	...	...	...	...	...	...	...	...	...	...	...	...	...	[ 39]
#41ED.	...	...	...	...	...	...	...	...	...	...	...	...	...	[ 39]
#43ED.	...	...	...	...	...	...	...	...	...	...	...	...	...	[ 39]
#46ED.	...	...	...	...	...	...	...	...	...	...	...	...	...	[ 39]
#48ED.	...	...	...	...	...	...	...	...	...	...	...	...	...	[ 39]
#49ED.	...	...	...	...	...	...	...	...	...	...	...	...	...	[ 39]
#4ED.	...	...	...	...	...	...	...	...	...	...	...	...	...	[ 39]
#50ED.	...	...	...	...	...	...	...	...	...	...	...	...	...	[ 39]
#51ED.	...	...	...	...	...	...	...	...	...	...	...	...	...	[ 39]
#52ED.	...	...	...	...	...	...	...	...	...	...	...	...	...	[ 39]
#56ED.	...	...	...	...	...	...	...	...	...	...	...	...	...	[ 39]
#57ED.	...	...	...	...	...	...	...	...	...	...	...	...	...	[ 39]
#58ED.	...	...	...	...	...	...	...	...	...	...	...	...	...	[ 39]
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#66ED.	...	...	...	.A.	...	...	...	...	...	...	...	...	...	[273]
#76ED.	...	...	...	.A.	...	...	...	...	...	...	...	...	...	[273]
#77ED.	...	...	...	.A.	...	...	...	...	...	...	...	...	...	[273]
#10ED.	TTG	ATT	TAC	AAT	AAT	TAG	TTG	GTT	ATG	GAG	TGT	ACA	ATG	[312]
#11ED.	...	...	...	...	...	...	...	...	...	...	...	...	...	[312]
#12ED.	...	...	.a.	...	...	...	...	.A.	...	...	.A.	...	...	[312]
#13ED.	...	...	...	...	...	...	...	.A.	...	...	.A.	...	...	[312]
#15ED.	...	...	...	...	...	...	...	.A.	...	...	.A.	...	...	[312]





#35ED. ....T ..... [390]  
 #36ED. ....T .....t ..... [390]  
 #37ED. ....T .....T. .... [390]  
 #38ED. ....T ..... [390]  
 #39ED. ....T ..... [390]  
 #41ED. ....T ..... [390]  
 #43ED. ....T ..... [390]  
 #46ED. ....T ..... [390]  
 #48ED. ....T ..... [390]  
 #49ED. ....T ..... [390]  
 #4ED. ....T ..... [390]  
 #50ED. ....T ..... [390]  
 #51ED. ....T ..... [390]  
 #52ED. ....T ..... [390]  
 #56ED. ....T ..... [390]  
 #57ED. ....T ..... [390]  
 #58ED. ....T ..... [390]  
 #5ED. ....T ..... [390]  
 #60ED. ....g. .... [390]  
 #61ED. .... [390]  
 #63ED. --- --- --- --- --- --- --- --- --- --- --- [390]  
 #65ED. ....T ..... [390]  
 #8ED. ....T ...ac ...ag. .a. .... [390]  
 #6ED. ....T ..... [390]  
 #25ED. ....c .....c. .... [390]  
 #66ED. ....T ..... [390]  
 #76ED. ....T ..... [390]  
 #77ED. ....T ...a. .... [390]

#10ED. ATA CAG CGA TCA CTT AAT GCA TAC GGT TAT TGA TAA TGA [429]  
 #11ED. .... [429]  
 #12ED. .... [429]  
 #13ED. ....T. .... [429]  
 #15ED. ....T. .... [429]  
 #16ED. ....T. .... [429]  
 #17ED. ....T. .... [429]  
 #18ED. ....T. .... [429]  
 #19ED. ....T. .... [429]  
 #1ED. ....T. .... [429]  
 #20ED. ....T. .... [429]  
 #21ED. ....T. .... [429]  
 #22ED. ....T. ....C. .... [429]  
 #23ED. .... [429]  
 #24ED. ....T. .... [429]  
 #26ED. ....T. .... [429]  
 #27ED. ....T. .... [429]  
 #28ED. ....T. ....t. .... [429]  
 #29ED. .... [429]  
 #2ED. ....T. .... [429]  
 #30ED. ....T. .... [429]  
 #31ED. .... [429]  
 #32ED. .... [429]  
 #33ED. .... [429]  
 #35ED. ....T. .... [429]  
 #36ED. ....TG. .... [429]  
 #37ED. ....T. .... [429]  
 #38ED. .... [429]  
 #39ED. ....T. .... [429]  
 #41ED. ....T. .... [429]  
 #43ED. ....T. .... [429]  
 #46ED. ....T. .... [429]  
 #48ED. ....T. .... [429]  
 #49ED. ....T. .... [429]

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#4ED.      ... .. .T. ..t ... .. [ 429]
#50ED.     ... .. .T. ... .. [ 429]
#51ED.     ... .. .T. ... .. [ 429]
#52ED.     ... .. .T. ... .. [ 429]
#56ED.     ... .. .T. ... ..C ... .. [ 429]
#57ED.     ... .. .T. ... .. [ 429]
#58ED.     ... .. .T. ... .. [ 429]
#5ED.      ... .. .T. ... .. [ 429]
#60ED.     ... .. .T. ... .. [ 429]
#61ED.     ... .. .T. ... .. t.. [ 429]
#63ED.     --- --- --- --- --- --- [ 429]
#65ED.     ... .. .T. ... .. [ 429]
#8ED.      ... .. .T. ... .. [ 429]
#6ED.      ... .. .T. ... .. t.. [ 429]
#25ED.     ... .. .t. ... .. [ 429]
#66ED.     ... .. .T. ... .. [ 429]
#76ED.     ... .. .T. ... .. [ 429]
#77ED.     a.. .. .T. ... .. [ 429]

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#10ED.     AAG AT [ 434]
#11ED.     ... .. [ 434]
#12ED.     --- -- [ 434]
#13ED.     ... .. [ 434]
#15ED.     ... .. [ 434]
#16ED.     ... .. [ 434]
#17ED.     ... .. [ 434]
#18ED.     ... .. [ 434]
#19ED.     ... .. [ 434]
#1ED.      ... .. [ 434]
#20ED.     ... .. [ 434]
#21ED.     ... .. [ 434]
#22ED.     ... .. [ 434]
#23ED.     ... .. [ 434]
#24ED.     ... .. [ 434]
#26ED.     ... .. [ 434]
#27ED.     ... .. [ 434]
#28ED.     ..g .. [ 434]
#29ED.     ... .. [ 434]
#2ED.      ... .. [ 434]
#30ED.     ... .. [ 434]
#31ED.     ... .. [ 434]
#32ED.     ... .. [ 434]
#33ED.     ... .. [ 434]
#35ED.     ... .. [ 434]
#36ED.     --- -- [ 434]
#37ED.     ... .. [ 434]
#38ED.     ... .. [ 434]
#39ED.     ... .. [ 434]
#41ED.     ... .. [ 434]
#43ED.     ... .. [ 434]
#46ED.     ... .. [ 434]
#48ED.     ... .. [ 434]
#49ED.     ... .. [ 434]
#4ED.      ... .. [ 434]
#50ED.     ... .. [ 434]
#51ED.     ... .. [ 434]
#52ED.     ... .. [ 434]
#56ED.     ... .. [ 434]
#57ED.     ... .. [ 434]
#58ED.     ... .. [ 434]
#5ED.      ... .. [ 434]
#60ED.     ... .. [ 434]
#61ED.     ..g .. [ 434]

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#63ED. --- -- [434]  
#65ED. ... .. [434]  
#8ED. ... .. [434]  
#6ED. ... .. [434]  
#25ED. ..g at [434]  
#66ED. ... .. [434]  
#76ED. ..g at [434]  
#77ED. ... .. [434]

*Appendix F Microsatellite allele frequencies for E. daemellii across the various sampling locations (n = sample size, sampling location abbreviations as per Table 1)*

CA-2											
Allele	Bunda	Saumar.	PortS	Berma	RockyP	Middle 06	Eliza 06	LordH	Middle 07	Eliza 07	Overall
255	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.036	0.007
257	0.000	0.000	0.000	0.000	0.000	0.014	0.000	0.500	0.100	0.107	0.042
261	0.000	0.000	0.000	0.000	1.000	0.028	0.100	0.000	0.000	0.000	0.042
267	0.500	0.000	0.000	0.000	0.000	0.028	0.000	0.000	0.000	0.071	0.035
269	0.500	0.000	0.000	0.500	0.000	0.167	0.050	0.000	0.100	0.071	0.127
271	0.000	0.000	0.000	0.000	0.000	0.000	0.150	0.000	0.200	0.107	0.056
273	0.000	0.500	0.500	0.500	0.000	0.181	0.250	0.500	0.300	0.071	0.190
277	0.000	0.000	0.000	0.000	0.000	0.056	0.050	0.000	0.000	0.071	0.049
281	0.000	0.500	0.500	0.000	0.000	0.056	0.000	0.000	0.100	0.107	0.070
287	0.000	0.000	0.000	0.000	0.000	0.028	0.000	0.000	0.000	0.000	0.014
289	0.000	0.000	0.000	0.000	0.000	0.014	0.000	0.000	0.000	0.000	0.007
291	0.000	0.000	0.000	0.000	0.000	0.069	0.000	0.000	0.100	0.179	0.078
293	0.000	0.000	0.000	0.000	0.000	0.125	0.100	0.000	0.000	0.000	0.078
295	0.000	0.000	0.000	0.000	0.000	0.097	0.050	0.000	0.000	0.036	0.063
297	0.000	0.000	0.000	0.000	0.000	0.097	0.150	0.000	0.100	0.107	0.099
299	0.000	0.000	0.000	0.000	0.000	0.028	0.050	0.000	0.000	0.036	0.028
301	0.000	0.000	0.000	0.000	0.000	0.014	0.000	0.000	0.000	0.000	0.007
303	0.000	0.000	0.000	0.000	0.000	0.000	0.050	0.000	0.000	0.000	0.007
<i>n</i>	1	1	1	1	1	36	10	1	5	14	71
CA-3											
Allele	Bunda	Saumar.	PortS	Berma	RockyP	Middle 06	Eliza 06	LordH	Middle 07	Eliza 07	Overall
329	0.000	0.000	0.500	0.000	0.000	0.059	0.188	0.000	0.000	0.071	0.075
333	1.000	0.000	0.000	0.500	0.000	0.132	0.000	0.000	0.200	0.179	0.142
335	0.000	0.000	0.000	0.000	0.000	0.103	0.063	0.500	0.000	0.107	0.090
337	0.000	0.000	0.000	0.000	0.000	0.015	0.000	0.000	0.000	0.000	0.008
339	0.000	0.000	0.000	0.000	0.000	0.059	0.125	0.000	0.000	0.000	0.045
341	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.036	0.008
345	0.000	0.000	0.000	0.000	0.000	0.015	0.000	0.000	0.000	0.000	0.008
347	0.000	0.000	0.000	0.000	0.000	0.000	0.063	0.000	0.000	0.036	0.015
349	0.000	0.000	0.000	0.000	0.000	0.074	0.000	0.000	0.300	0.071	0.075
351	0.000	0.000	0.000	0.000	0.000	0.000	0.063	0.000	0.000	0.071	0.022
353	0.000	0.000	0.000	0.000	0.000	0.162	0.063	0.000	0.100	0.036	0.105
355	0.000	0.500	0.000	0.000	0.500	0.029	0.188	0.000	0.200	0.071	0.082
357	0.000	0.000	0.000	0.000	0.000	0.015	0.000	0.500	0.100	0.071	0.037
359	0.000	0.000	0.000	0.500	0.000	0.044	0.000	0.000	0.000	0.036	0.037
361	0.000	0.000	0.000	0.000	0.500	0.044	0.125	0.000	0.000	0.036	0.052
363	0.000	0.500	0.000	0.000	0.000	0.044	0.063	0.000	0.000	0.000	0.037
365	0.000	0.000	0.500	0.000	0.000	0.015	0.000	0.000	0.000	0.036	0.022
367	0.000	0.000	0.000	0.000	0.000	0.029	0.000	0.000	0.100	0.000	0.022
369	0.000	0.000	0.000	0.000	0.000	0.015	0.000	0.000	0.000	0.036	0.015
371	0.000	0.000	0.000	0.000	0.000	0.059	0.000	0.000	0.000	0.000	0.030
373	0.000	0.000	0.000	0.000	0.000	0.015	0.000	0.000	0.000	0.107	0.030
375	0.000	0.000	0.000	0.000	0.000	0.015	0.000	0.000	0.000	0.000	0.008
377	0.000	0.000	0.000	0.000	0.000	0.015	0.063	0.000	0.000	0.000	0.015
379	0.000	0.000	0.000	0.000	0.000	0.015	0.000	0.000	0.000	0.000	0.008
399	0.000	0.000	0.000	0.000	0.000	0.029	0.000	0.000	0.000	0.000	0.015

*n*      1      1      1      1      1      34      8      1      5      14      67

CA-4

Allele	Bunda	Saumar.	PortS	Berma	RockyP	Middle 06	Eliza 06	LordH	Middle 07	Eliza 07	Overall
311	0.000	0.000	0.000	0.000	0.000	0.024	0.031	0.000	0.000	0.000	0.018
313	0.000	0.250	0.000	0.000	0.000	0.024	0.063	0.000	0.000	0.000	0.029
315	0.000	0.000	0.000	0.000	0.000	0.024	0.000	0.000	0.000	0.033	0.018
317	0.000	0.000	0.500	0.000	0.000	0.000	0.031	0.000	0.000	0.000	0.012
319	0.000	0.000	0.000	0.000	0.000	0.119	0.219	0.000	0.000	0.033	0.106
321	0.500	0.000	0.000	0.000	0.500	0.179	0.250	0.500	0.600	0.133	0.212
323	0.000	0.000	0.000	0.000	0.000	0.083	0.063	0.000	0.000	0.100	0.071
325	0.000	0.000	0.000	0.000	0.000	0.024	0.031	0.000	0.000	0.000	0.018
327	0.000	0.000	0.500	0.000	0.000	0.000	0.000	0.000	0.000	0.033	0.012
329	0.000	0.000	0.000	0.000	0.000	0.012	0.000	0.000	0.000	0.000	0.006
331	0.000	0.000	0.000	0.000	0.000	0.012	0.000	0.000	0.000	0.000	0.006
335	0.000	0.250	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.006
339	0.000	0.000	0.000	0.000	0.000	0.024	0.000	0.000	0.100	0.000	0.018
341	0.000	0.000	0.000	0.000	0.000	0.071	0.031	0.000	0.000	0.167	0.071
343	0.500	0.000	0.000	1.000	0.500	0.048	0.031	0.000	0.000	0.133	0.077
345	0.000	0.000	0.000	0.000	0.000	0.119	0.063	0.000	0.200	0.033	0.088
347	0.000	0.500	0.000	0.000	0.000	0.131	0.063	0.500	0.000	0.100	0.112
349	0.000	0.000	0.000	0.000	0.000	0.024	0.094	0.000	0.000	0.067	0.041
351	0.000	0.000	0.000	0.000	0.000	0.012	0.000	0.000	0.000	0.067	0.018
353	0.000	0.000	0.000	0.000	0.000	0.071	0.000	0.000	0.100	0.100	0.059
359	0.000	0.000	0.000	0.000	0.000	0.000	0.031	0.000	0.000	0.000	0.006
<i>n</i>	1	2	1	1	1	42	16	1	5	15	85