

# GREAT AUSTRALIAN BIGHT RESEARCH PROGRAM

## RESEARCH REPORT SERIES

### **Molecular assessment of benthic and pelagic biodiversity in the Great Australian Bight: Barcoding**

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[www.misa.net.au/GAB](http://www.misa.net.au/GAB)

## GREAT AUSTRALIAN BIGHT RESEARCH PROGRAM

The Great Australian Bight Research Program is a collaboration between BP, CSIRO, the South Australian Research and Development Institute (SARDI), the University of Adelaide, and Flinders University. The Program aims to provide a whole-of-system understanding of the environmental, economic and social values of the region; providing an information source for all to use.

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**Figure 3.** Neighbour-Joining tree for the genus *Ophiomusium* based on Kimura 2-parameter genetic distance with 1000 bootstraps. Samples with coloured circle are from the current study. Green circle indicates that the sample was identified at the species level ( $p\text{-dis} < 1.65\%$ ) using our proposed strategy, while red circle indicates that the sample was identified at the genus level ( $1.65\% < p\text{-dis} < 10.36\%$ ) using threshold values. Sequences from samples without coloured circles were obtained from databases (NCBI and BOLD) and their accession numbers and sample IDs are shown after the species names.

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<sup>1</sup> - Total number of samples received from two surveys, <sup>2</sup> - Total number of unique samples that were sequenced regardless of quality, <sup>3</sup> - Total number of DNA extractions carried out for a given phylum, <sup>4</sup> - Total number of PCRs carried out for a given phylum, <sup>5</sup> - The number of PCRs carried out for each primer combination, <sup>6</sup> - Total number of sequences obtained (including replicates) and <sup>7</sup> - Total number of sequences in the final dataset for a given phylum (after quality control).

## ACKNOWLEDGEMENTS

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## 1. EXECUTIVE SUMMARY

Species identification underpins any research in marine biology, being a fundamentally important process for assessments of biodiversity, community structure and ecosystem functioning. The aim of this sub-project was to use DNA barcoding to develop selective genetic datasets to complement and advance morphological taxonomy within informative benthic groups. Benthic samples from two field surveys (2013 and 2015) in the Great Australian Bight (GAB) were transferred to Flinders University's Molecular Ecology Laboratory, where all analyses and results reported here were carried out. The DNA extracted from these samples was used to amplify, via the polymerase chain reaction (PCR), the cytochrome c oxidase subunit 1 (COI) region of the mitochondrial genome that has been used as a DNA barcode for species identification. We optimized and developed several protocols for DNA extraction and amplification of the COI gene across a range of marine phyla from the GAB region. We also implemented analytical approaches to circumvent the lack of information about morphological identification, which allowed the proposal of thresholds of COI sequence similarity to identify samples at the species, genus and/or family levels. The identifications corresponded to 139 Arthropoda, 106 Echinodermata, 27 Mollusca, 14 Annelida, 11 Sipuncula and 6 Cnidaria. Identifications are provided for each individual sample as series' of barcode IDs and sequence data. We also found that species identification was substantially improved by using both the NCBI and BOLD sequence databases. Finally, according to the species distribution records from the Atlas of Living Australia and BOLD, we found three species that seem to be new to Australia: *Sperosoma biserialatum* (Echinodermata), *Granulifusus nopinicus* (Mollusca) and *Ebalia nux* (Arthropoda). We propose that the COI region should be considered as an appropriate and cost-effective tool for biodiversity assessment in future ecological monitoring in the GAB region, either as individual sequencing approaches, or using next-generation sequencing platforms. Information from morphological identification and species geographic ranges should be incorporated into future studies to improve the power of DNA barcoding for species identification in the GAB.

## 2. INTRODUCTION

The overall aim of the Benthic Biodiversity Theme is to provide the first knowledge of benthic biodiversity structure (composition, distribution and standing stock) in the deep waters of the Great Australian Bight (GAB). The sub-project 1 'Barcoding' is a molecular-based program that uses "DNA barcodes" to identify key taxonomic groups in the GAB region. It provides a contribution to the evaluation of leading-edge molecular techniques to identify informative and cost-effective biodiversity metrics relevant to future ecological monitoring.

Species identification underpins any research in marine biology, being a fundamentally important process for assessments of biodiversity, community structure and ecosystem functioning. However, marine environments impose numerous challenges for species identification including difficulties sampling key habitats and problems arising from the patchy distribution of many marine species. DNA barcoding has revolutionised the task of identification by providing reliable, inexpensive, and rapid ways to identify known species, discover new ones and discriminate between cryptic species (i.e. discrete species that are difficult or impossible to distinguish morphologically) (Hebert et al. 2003; Marshall 2005; Meusnier et al. 2008). In this method, identification is performed by using DNA sequences from a small fragment of the genome (the "DNA barcode"), with the aim of contributing to a wide range of studies in which traditional taxonomic identification is impractical.

A large number of researchers have embraced DNA barcoding as a practical tool to be used in conjunction with traditional morphology-based studies. For instance, [The International Barcode of Life project](#) (iBOL, [www.ibol.org](http://www.ibol.org)) is the largest biodiversity genomics project ever undertaken. It includes hundreds of scientists from 25 nations who collaborate to construct a DNA-based identification system for all multi-cellular life. DNA barcoding is now well established for animals and is based on sequences from the mitochondrial cytochrome c oxidase 1 (COI) gene (see [www.barcodeoflife.org](http://www.barcodeoflife.org)). The COI gene normally shows sequence variation that is high enough between species to discriminate one from another, and low enough within species to distinguish

intra- from inter-specific genetic variation (Herbert et al. 2003; Marshall 2005; Meusnier et al. 2008).

## **2.1 Aim**

The aim of this sub-project is to use DNA barcoding to develop selective genetic datasets to complement and advance morphological taxonomy within informative benthic groups.

## **2.2 Outputs and extensions**

The main output of this sub-project will be a series of barcodes for selected taxa of interest, which can then be used to form the basis of the molecular monitoring sub-project. In addition, we expect the major findings of the Barcoding Sub-project to be published in leading journals in marine science.



### 3. METHODS

#### 3.1 Sampling

All tissue samples required for this project were provided by projects 3.1 and 2.2 as part of two GAB surveys referred herein as 'Survey 2013' and 'Survey 2015'. Samples from 'Survey 2013' were divided into groups of 'infauna' and 'epifauna' organisms, whereas all samples from 'Survey 2015' were 'epifauna' organisms. Samples were frozen aboard and then preserved in ethanol (>95%) prior to being transferred to the Molecular Ecology Lab at Flinders University (MELFU) for DNA barcoding.

#### 3.2 Laboratory procedures

Most of the infauna samples were very small in size (~1-2 mm), whereas the majority of the epifauna samples were substantially larger (>5 mm). Samples were previously identified at the phylum level, and mostly included Arthropoda, Echinodermata, Annelida and Mollusca. Other phyla included Sipuncula, Porifera, Chordata (Ascidiacea), Cnidaria and Brachiopoda. Due to the high diversity of tissue types and sizes, we had to carry out optimisation of DNA extractions for each phylum using a variety of methods and conditions. We tested several protocols including DNeasy blood & tissue kit (Qiagen), CTAB, salting out protocol (Sunnucks & Hales 1996), Chelex 100 (Bio-Rad) and Vertebrate Lysis Buffer protocol (VLB) (Ivanova et al. 2006). The details about the methods and modifications of each DNA extraction protocol are presented in Appendix 1. DNA integrity was assessed by gel electrophoresis, and purity was measured using a NanoDrop 1000 spectrophotometer (Thermo Scientific).

The total DNA was then used to amplify, via the polymerase chain reaction (PCR), the COI region of the mitochondrial genome that has been used as DNA barcode for most species identification projects (Hebert et al. 2003; Ward et al. 2005; Bucklin et al. 2011). Several primer combinations and PCR conditions were trialled (Table 1). The 10  $\mu$ L PCRs consisted of 2  $\mu$ L of 5x PCR buffer, 0.25 mM of dNTPs, 1.5 mM of  $MgCl_2$ , 0.3  $\mu$ M of each primer, 0.2 mg/mL of BSA (NEB), 1 U of Mango Taq (Bioline), 1  $\mu$ L of template DNA and ddH<sub>2</sub>O. Resulting PCR products were visualised on 1% agarose gel using 1x TAE buffer (40 mM Tris (pH 7.6), 20 mM acetic acid and 1mM EDTA) using 1  $\mu$ L of each reaction. Successful amplifications were diluted in ddH<sub>2</sub>O to approximately 100 ng/ $\mu$ L if necessary and unidirectionally sequenced in an ABI3730XL (Applied Biosystems) automated sequencer at Macrogen, Korea. Primers used for sequencing correspond to those used for PCR and include LCO1490, LCOechi1aF1, LoboF1 and jgLCO.

**Table 1.** PCR cycles and primer combinations used to amplify the COI region for GAB samples.

LCO1490 x HCO2198			LCOechi1aF1 x HCO2198			jgLCO x jgHCO		
94C	1 min		94C	1 min	x1	94C	1min	x1
40-47C	1 min	x35-40	94C	30 sec		94C	30sec	
72C	1 min		45C	90 sec	x5	45C	90sec	x5
72C	10 min	x1	72C	1 min		72C	1min	
LoboF1 x LoboR1			94C	30 sec		94C	30sec	
94C	2 min	x1	54C	90 sec	x45	54C	90sec	x45
94C	30 sec		72C	1 min		72C	1min	
47C	90 sec	x45	72C	5 min	x1	72C	5min	x1
72C	1 min							
72C	5 min	x1						

### 3.3 Data analysis

Resultant sequences were visually inspected in GENEIOUS 10.0.5 (Kearse et al. 2012) and the data quality using Phred score was also checked in CODONCODE ALIGNER v.6.0.2 (CodonCode Aligner, Centerville, MA, USA). Any bases with Phred score of less than 20 were considered as missing data (i.e. N). The presence of a stop codon was examined with MESQUITE v.3.10 (Maddison & Maddison 2016) and any sequences with intermediate stop codons along the fragment were removed from the dataset. Sequences from the same phylum were aligned in MEGA 7.0.21 (Kumar et al. 2016) to check for the presence of indels and to remove the primer region. Clean and aligned sequences were then blasted using the NCBI BLAST (Altschul et al. 1990) based on default settings with expected threshold of 10 and match/mismatch score of 0.5 for possible contaminations. These sequences were also submitted to the animal identification system (IDS) implemented in BOLD (Ratnasingham & Hebert 2007) to obtain the identification of organisms that are the nearest match to our samples. Because of the lack of morphological ID for the barcoded samples, the highest similarity ID from either BLAST search or IDS from BOLD, regardless of percentage, was used to name our samples. Sequences with >500 bp length and with less than 1% of missing data were kept to compile a final data set.

To improve sample identification we used a direct sequence comparison approach known as the best close match (BCM), with support from phylogenetic trees. The BCM was proposed by Meier et al. (2006), who compared the success rate of species identification based on phylogenetic trees and direct sequence comparison. These authors found that direct sequence comparison had better identification and a relatively low misidentification. BCM involves calculating the frequency of intraspecific uncorrected distance (p-distance) to find the threshold value below 95% where all intraspecific distances can be found. If sequences do not have any barcode match even below the threshold value, they will remain unidentified. The remaining sequences are then compared to those of their closest match. Identification is considered successful if the taxon name was identical while

identification fails when the taxon differs. If there are more than two species that match below the threshold, identification is considered ambiguous. This method can be used when 1) you have replicates from species and genus, and 2) a reference sequence that is closely related to your samples.

Since we did not have enough replicates at species and genus levels, it was not feasible to use our dataset to calculate intraspecific distance. Instead, we used a different method and calculated intraspecific distance in TAXON DNA/SPECIES IDENTIFIER 1.8 (Meier et al. 2006). We obtained COI sequences from NCBI GenBank and BOLD representing both Echinodermata (genera *Amphiophiura*, *Benthopecten*, *Mellita* and *Meridiastra*) and Arthropoda (genera *Acanthephyra*, *Ebalia*, *Eumunida*, *Liocarcinus* and *Periclimenes*). These phyla were chosen since most of our samples were represented by Echinodermata and Arthropoda. Sequences accessed from GenBank and BOLD corresponded to the same genera as those in our dataset with the aim of obtaining genetic distances from similar organisms. The number of species per genus ranged from 4 to 6 and the number of replicates per species ranged from 2 to 10 (Table 2). Because intraspecific distance varies among taxa, we averaged distances below 95% from 4 Echinodermata genera and 5 Arthropoda genera so that thresholds could be applied to our dataset. This was also done for samples with only few available conspecific sequences as they cannot be used in Taxon DNA/Species Identifier. The lowest average interspecific distance and average congeneric distance calculated from Taxon DNA/Species Identifier were used as thresholds to identify genus and family, respectively. We then used 15% and 21% for classifying order and class, respectively, based on visual examinations of NCBI GenBank and BOLD results.

Uncorrected pairwise genetic distances (p-distance) were calculated between our samples and the closest match sequences in MEGA 7.0.21 and the threshold was applied to the pairwise p-distance to identify the taxonomic level. In order to confirm the results of identification using BCM, we also constructed Neighbour-Joining trees (NJ) based on Kimura-2-parameter with 1000 bootstraps. This was only done for species with available congeneric sequences. Finally, the known distributions of

species identified in this study was checked against the Atlas of Living Australia (Atlas 2015) and BOLD (Ratnasingham & Hebert 2007).

**Table 2.** Samples used to calculate intraspecific diversity. Bold G indicates that sequences were sourced from GenBank and bold B indicates that sequences were sourced from BOLD.

<b>Echinodermata</b>			
<b>Genus</b>	<b>Species</b>	<b>Sample ID</b>	<b>Comments</b>
<i>Amphiophiura</i>	<i>Amphiophiura laudata</i>	EU869873.1 G	
		EU869874.1 G	
		EU869875.1 G	
		EU869876.1 G	
		KU894936.1 G	
	<i>Amphiophiura spatulifera</i>	KU894926.1 G	
		KU894927.1 G	
		HM400324.1 G	
	<i>Amphiophiura superba</i>	HM400325.1 G	
		HM400326.1 G	
		HM400327.1 G	
		EU869877.1 G	
	<i>Amphiophiura urbana</i>	EU869878.1 G	
		EU869879.1 G	
		EU869881.1 G	
		EU869880.1 G	
		HM542917.1 G	
<i>Benthopecten</i>	<i>Benthopecten acanthonotus</i>	HM542918.1 G	
		HM542919.1 G	
		HM542921.1 G	
		HM542920.1 G	
		HM542922.1 G	
	<i>Benthopecten claviger</i>	HM542923.1 G	
		HM542924.1 G	
		NZEC431-09 B	
	<i>Benthopecten munidae</i>	NZEC433-09 B	
		NZEC440-09 B	
		NZEC439-09 B	
	<i>Benthopecten pikei</i>	NZEC441-09 B	
		NZEC442-09 B	
<i>Mellita</i>	<i>Mellita_grantii</i>	KF204793.1 G	
		KF204794.1 G	
		KF204795.1 G	
		KF204796.1 G	

		KF204797.1 G	
	<i>Mellita longifissa</i>	KF204798.1 G	
		KF204799.1 G	
		KF204800.1 G	
		KF204807.1 G	
		KF204830.1 G	
	<i>Mellita notabilis</i>	KF204749.1 G	They are grouped together for analysis as their p-dis ranged from 0 - 0.72%. <i>M. kanakoffi</i> was suggested to be junior synonym of <i>M. notabilis</i> (Coppard et al. 2013).
		KF204756.1 G	
		KF204757.1 G	
		KF204758.1 G	
		KF204759.1 G	
	<i>Mellita kanakoffi</i>	KF204773.1 G	
		KF204774.1 G	
		KF204775.1 G	
		KF204776.1 G	
		KF204777.1 G	
	<i>Mellita quinquiesperforata</i>	KF204724.1 G	
		KF204725.1 G	
		KF204726.1 G	
		KF204727.1 G	
		KF204728.1 G	
		KF204729.1 G	
	<i>Mellita tenuis</i>	KF204857.1 G	They are grouped together for analysis as their p-dis ranged from 0 - 0.72%. <i>M. isometra</i> was suggested to be synonymise to <i>M. tenuis</i> (Coppard et al. 2013).
		KF204859.1 G	
		KF204842.1 G	
		KF204858.1 G	
		KF204860.1 G	
	<i>Mellita isometra</i>	KF204848.1 G	
		KF204849.1 G	
		KF204850.1 G	
		KF204851.1 G	
		KF204852.1 G	
<i>Meridiastra</i>	<i>Meridiastra calcar</i>	EU869944.1 G	
		EU869945.1 G	
		EU869946.1 G	
		EU869947.1 G	
		EU869948.1 G	
	<i>Meridiastra gunnii</i>	AY458432.1 G	
		AY458434.1 G	
		AY458436.1 G	
		AY458437.1 G	
		AY458438.1 G	
	<i>Meridiastra medius</i>	AY458477.1 G	
		AY458473.1 G	
		AY458474.1 G	
		AY458476.1 G	
	<i>Meridiastra occidens</i>	AY458501.1 G	
		AY458505.1 G	

		AY458502.1 G	
		AY458503.1 G	
		AY458504.1 G	
	<i>Meridiastra oriens</i>	AY458450.1 G	
		AY458451.1 G	
		AY458452.1 G	
		AY458453.1 G	
		AY458455.1 G	
<b>Arthropoda</b>			
<b>Genus</b>	<b>Species</b>	<b>Sample ID</b>	<b>Comment</b>
<i>AcanthePHYra</i>	<i>AcanthePHYra eximia</i>	KP759360.1 G	
		KP759359.1 G	
		KP759358.1 G	
		KP759357.1 G	
		KP759356.1 G	
	<i>AcanthePHYra curtirostris</i>	KP076164.1 G	
		KP076163.1 G	
		KP076162.1 G	
		KP076161.1 G	
		GU183785.1 G	
	<i>AcanthePHYra pelagica</i>	KP759361.1 G	
		KP076182.1 G	
		KP076179.1 G	
		KF930998.1 G	
		JQ305961.1 G	
	<i>AcanthePHYra purpurea</i>	KP076173.1 G	
		KP076171.1 G	
		KP076170.1 G	
		GU183787.1 G	
	<i>AcanthePHYra quadrispinosa</i>	KP759362.1 G	
		KP759363.1 G	
		KP076178.1 G	
<i>Ebalia</i>	<i>Ebalia cranchii</i>	KT209387.1 G	
		KT209435.1 G	
		KT209457.1 G	
		KT209478.1 G	
		KT209497.1 G	
		KT209523.1 G	
	<i>Ebalia nux</i>	JQ348856.1 G	
		JQ348859.1 G	
		JQ348860.1 G	
	<i>Ebalia tuberosa</i>	KT208846.1 G	
		KT209089.1 G	
		KT209151.1 G	
		KT209162.1 G	
		KT209479.1 G	
	<i>Ebalia tumefacta</i>	KT208981.1 G	
		KT208779.1 G	

		KT209285.1 G
		KT209393.1 G
		KT209463.1 G
<i>Eumunida</i>	<i>Eumunida annulosa</i>	EU243354.1 G
		EU243403.1 G
		EU243408.1 G
		EU243461.1 G
	<i>Eumunida capillata</i>	EU243341.1 G
		EU243342.1 G
		EU243343.1 G
		EU243344.1 G
	<i>Eumunida keijii</i>	EU243337.1 G
		EU243338.1 G
		EU243340.1 G
		EU243514.1 G
	<i>Eumunida minor</i>	EU243502.1 G
		EU243548.1 G
		EU243550.1 G
		EU243551.1 G
	<i>Eumunida parva</i>	EU243345.1 G
		EU243346.1 G
		EU243518.1 G
		EU243521.1 G
		EU243522.1 G
	<i>Eumunida spinosa</i>	EU243500.1 G
		EU243501.1 G
		EU243513.1 G
		EU243533.1 G
<i>Liocarcinus</i>	<i>Liocarcinus pusillus</i>	GQ268534.1 G
		KT209364.1 G
		KT208625.1 G
	<i>Liocarcinus depurator</i>	GQ268531.1 G
		KT209206.1 G
		KT209341.1 G
		KT209461.1 G
		KT209530.1 G
	<i>Liocarcinus holsatus</i>	GQ268538.1 G
		KT208798.1 G
		KT208821.1 G
		KT209279.1 G
		KT209350.1 G
	<i>Liocarcinus marmoreus</i>	GQ268535.1 G
		KT208606.1 G
		KT209205.1 G
		KT209214.1 G
		KT209329.1 G
		KT209516.1 G



	<i>Liocarcinus navigator</i>	cGQ268537.1 <b>G</b> KT208849.1 <b>G</b> KT208868.1 <b>G</b> KT209223.1 <b>G</b> KT209424.1 <b>G</b> KT209515.1 <b>G</b>
<i>Periclimenes</i>	<i>Periclimenes imperator</i>	GQ415634.1 <b>G</b> GQ415635.1 <b>G</b> GQ415636.1 <b>G</b> GQ415637.1 <b>G</b>
	<i>Periclimenes rathbunae</i>	KX090123.1 <b>G</b> KX090120.1 <b>G</b> KX090121.1 <b>G</b> KX090122.1 <b>G</b> KX090124.1 <b>G</b> KX090125.1 <b>G</b>
	<i>Periclimenes soror</i>	GQ415629.1 <b>G</b> GQ415630.1 <b>G</b> GQ415631.1 <b>G</b> GQ415632.1 <b>G</b> GQ415633.1 <b>G</b>
	<i>Periclimenes thermohydrophilus</i>	AB298102.2 <b>G</b>  AB298103.2 <b>G</b> AB298104.2 <b>G</b> AB298105.2 <b>G</b> AB298106.2 <b>G</b>
	<i>Periclimenes yucatanicus</i>	KU065010.1 <b>G</b> KX858822.1 <b>G</b>

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## 4. RESULTS AND DISCUSSION

### 4.1.1 Optimisation of laboratory procedures

Although different protocols yielded varying levels and qualities of DNA extractions for different phyla, the salting out protocol yielded the highest concentration of cleaner DNA (260/280 and 230/280 NanoDrop measurements) for all major phyla such as Arthropoda, Annelida, Echinodermata and Mollusca (Table 3). These results were more apparent for Echinodermata, except for the class Holothuroidea of which mucous slime appeared during the DNA precipitation step hindering the success of extraction and thus affecting downstream application. We found no substantial differences in DNA extraction based on the way tissues were preserved (frozen or ethanol). The optimisation of DNA extraction protocols was achieved for all phyla, except for Porifera (DNA yield was much better for denser morphological species than coarser ones) and Foraminifera.

**Table 3.** Results of DNA extractions and corresponding protocol for each phylum. O: satisfactory result, X: poor result, Δ: inconsistent result (protocol works for some samples but not all), NA: protocol was not used for the phylum, D<sup>1</sup>: indicates denser morphotype Porifera and C<sup>2</sup>: indicates coarser morphotype Porifera.

DNA protocol	Amphipoda	Echinodermata	Polychaeta	Mollusca	Porifera
DNeasy	O	Δ	O	O	D <sup>1</sup> : Δ / C <sup>2</sup> :X
CTAB	NA	Δ	NA	NA	X
Salting out	O	O	O	O	NA
Chelex	X	X	X	NA	X
VLB	NA	X	NA	NA	X

The combination of primers LCO1490 and HCO2198 (Folmer et al. 1994) was the most effective across the taxonomic diversity of our sample. For Echinodermata, a different forward primer, LCOech1aF1, together with HCO2198 was used for most samples, whereas a combination of LoboF1 and LoboR1 (Lobo et al. 2013) was used for several Arthropoda and Cnidaria, and a combination of jgLCO and jgHCO (Geller et al. 2013) was used for Porifera. We also tested combinations of polyLCO and polyHCO (Carr et al. 2011), MinibarF1 and MinibarR1 (Meusnier et al. 2008) and COLceF and COLceR (Hoareau & Boissin 2010) during optimisation but results were inconsistent and they were not used to produce a final dataset (Table 4).

The PCR amplifications were also improved as different primer sets were tested beside the better performing Folmer primers. For example, we observed signs of amplification for Echinodermata with Folmer primers but resultant sequences were mostly contaminated with teleost (i.e. fish) DNA.

Examples of source of teleost contamination included *Cetonus globiceps* and *Coelorinchus acanthiger*. These species are found along the Great Australian Bight and were sampled in the same round of bottom trawling as the samples used for this project. We propose that echinoderms absorbed the mucoprotein coating of these teleosts before they were individually processed, which accounts for the high concentration of foreign DNA in the tissue of our target samples. A range of dissecting methods and tissue sources were attempted from the echinoderm samples with inconsistent results. In the best cases we needed 3-4 rounds of processing (i.e. dissecting, DNA extraction, PCR and sequencing) for each echinoderm sample, as opposed to one round as initially planned, in order to obtain a non-teleost ID for the echinoderm. We also tested whether DNA quantity or a combination of primers was more important for amplification of these targeted samples. The LCOech1aF1 x HCO2198 combination successfully amplified Echinodermata sequences while the Folmer primers often continued to amplify fish DNA when using the same DNA template. None of the sequences obtained from Brachiopoda and Chordata (Ascidiacea) were from target species, suggesting that none of the primers were suitable for these phyla. Another recurrent issue we experienced relates to a low rate of amplification success for the 2013 infauna samples. For

instance, the PCR protocol optimised for the 2015 epifauna Arthropoda failed to amplify many of the infauna Arthropoda (Table. 4), an issue attributed to the very small size of these samples.

**Table 4.** Summary of efforts on DNA extraction, PCR and sequencing.

<sup>1</sup> - Total number of samples received from two surveys, <sup>2</sup> - Total number of unique samples that were sequenced regardless of quality, <sup>3</sup> - Total number of DNA extractions carried out for a given phylum, <sup>4</sup> - Total number of PCRs carried out for a given phylum, <sup>5</sup> - The number of PCRs carried out for each primer combination, <sup>6</sup> - Total number of sequences obtained (including replicates) and <sup>7</sup> - Total number of sequences in the final dataset for a given phylum (after quality control).

'Survey 2013' infauna samples							
Phylum	Total # <sup>1</sup>	Seq # <sup>2</sup>	All DNA # <sup>3</sup>	All PCR # <sup>4</sup>	# of PCR for each primer combination <sup>5</sup> 140 (Folmer), 4 (Minibar), 7 (jgLCO), 14 (Lobo), 67 (polyLCO)	All seq # <sup>6</sup>	Final seq # <sup>7</sup>
Annelida	109	6	82	232		15	0
Arthropoda	59	4	52	255	190 (Folmer), 59 (Lobo), 3 (jgLCO), 3 (Minibar)	20	0
Echinodermata	13	0	11	38	12 (Folmer), 24 (LCOechi1aF1), 2 (Lobo)	0	0
Miscellaneous	17	0	10	15	15 (Folmer)	0	0
<b>TOTAL</b>	<b>198</b>	<b>10</b>	<b>155</b>	<b>540</b>		<b>35</b>	<b>0</b>
'Survey 2013' epifauna samples							
Phylum	Total # <sup>1</sup>	Seq # <sup>2</sup>	All DNA # <sup>3</sup>	All PCR # <sup>4</sup>	# of PCR for each primer combination <sup>5</sup>	All seq # <sup>6</sup>	Final seq # <sup>7</sup>
Annelida	5	2	8	17	17 (Folmer)	3	1
Arthropoda	12	12	19	24	24 (Lobo)	15	10
Cnidaria	1	1	2	1	1 (jgLCO)	1	0
Echinodermata	34	16	85	187	32 (COIceF), 12 (LCOechi1aF1), 141 (Folmer), 2 (Lobo)	39	12
Mollusca	6	5	7	12	12 (Folmer)	4	4
Porifera	19	4	42	163	94 (jgLCO), 63 (Folmer), 6 (Lobo)	7	0
Sipuncula	2	1	3	4	4 (Folmer)	3	1
<b>TOTAL</b>	<b>79</b>	<b>41</b>	<b>166</b>	<b>408</b>		<b>72</b>	<b>28</b>
'Survey 2015'							
Phylum	Total # <sup>1</sup>	Seq # <sup>2</sup>	All DNA # <sup>3</sup>	All PCR # <sup>4</sup>	# of PCR for each primer combination <sup>5</sup>	All seq # <sup>6</sup>	Final seq # <sup>7</sup>
Annelida	19	13	28	33	29 (Folmer), 4 (Lobo)	15	13

Arthropoda	153	145	156	196	168 (Folmer), 28 (Lobo)	155	129
Brachiopoda	14	12	28	28	28 (Folmer)	15	0
Chordata	10	8	20	44	34 (Folmer), 10 (Lobo)	14	0
Cnidaria	12	11	13	43	17 (Folmer), 3 (jgLCO), 33 (Lobo)	12	6
Echinodermata	275	100	299	593	85 (Folmer), 2 (COlceF), 506 (LCOechi1aF1)	154	93
Mollusca	45	32	43	65	65 (Folmer)	33	23
Sipuncula	16	12	16	35	35 (Folmer)	25	11
Dendrogramma	3	2	3	3	3 (Folmer)	2	0
<b>TOTAL</b>	<b>360</b>	<b>335</b>	<b>606</b>	<b>1040</b>		<b>425</b>	<b>275</b>

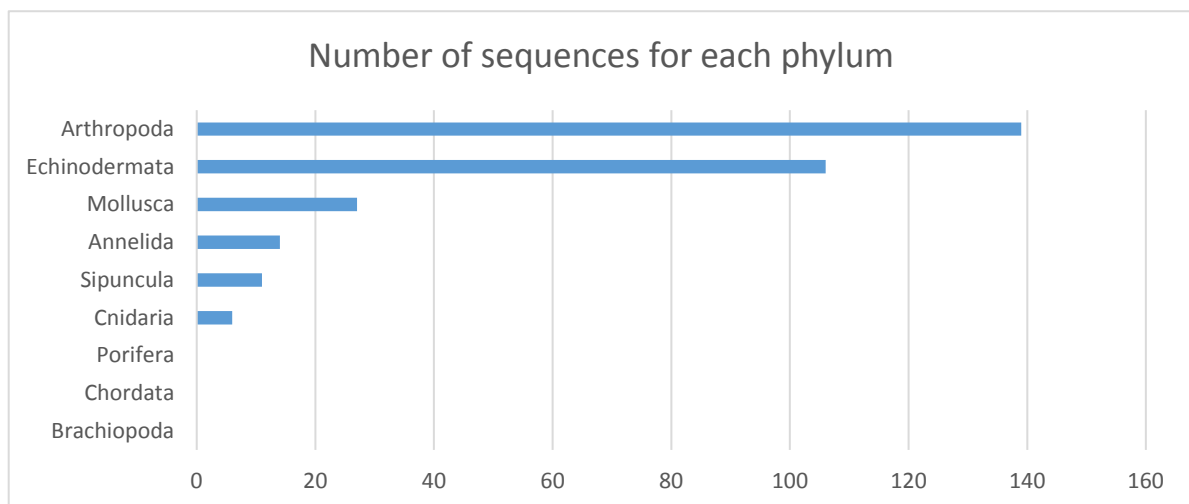
#### SUMMARY

		All	All				
	Total	Seq	DNA	PCR		All seq	Final
Phylum	# <sup>1</sup>	# <sup>2</sup>	# <sup>3</sup>	# <sup>4</sup>	# of PCR for each primer combination <sup>5</sup>	# <sup>6</sup>	seq # <sup>7</sup>
Annelida	133	21	118	282		33	14
Arthropoda	224	161	227	475		190	139
Brachiopoda	14	12	28	28		15	0
Chordata	10	8	20	44		14	0
Cnidaria	13	11	13	43		12	6
Dendrogramma	3	2	3	3		2	0
Echinodermata	322	116	395	818		193	105
Miscellaneous	17	0	10	15		0	0
Mollusca	51	37	50	77		37	27
Porifera	19	4	42	163		7	0
Sipuncula	18	13	19	39		28	12
TOTAL	824	385	925	1987		531	303

#### 4.1.2 Summary statistics

From the total number of samples received ( $n=824$ ), we carried out 925 DNA extractions, 1,987 rounds of PCRs and obtained 531 sequences. These numbers include replication due to issues with teleost contamination and minute size of tissue samples (described above). After quality control, we obtained a total of 303 high quality sequences, which correspond to 139 Arthropoda, 106 Echinodermata, 27 Mollusca, 14 Annelida, 11 Sipuncula and 6 Cnidaria (Figure 1). The aligned sequences from each phylum were trimmed to 565 bp (all Arthropoda), 563 bp (all Cnidaria), 535 bp

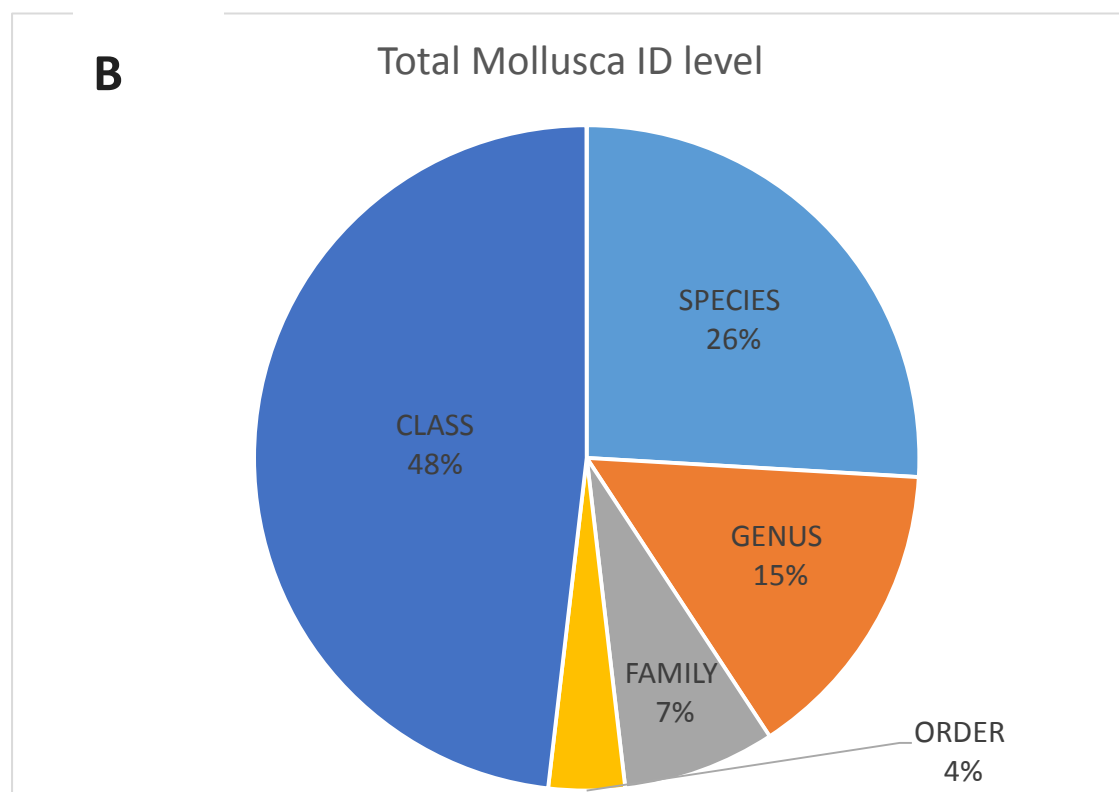
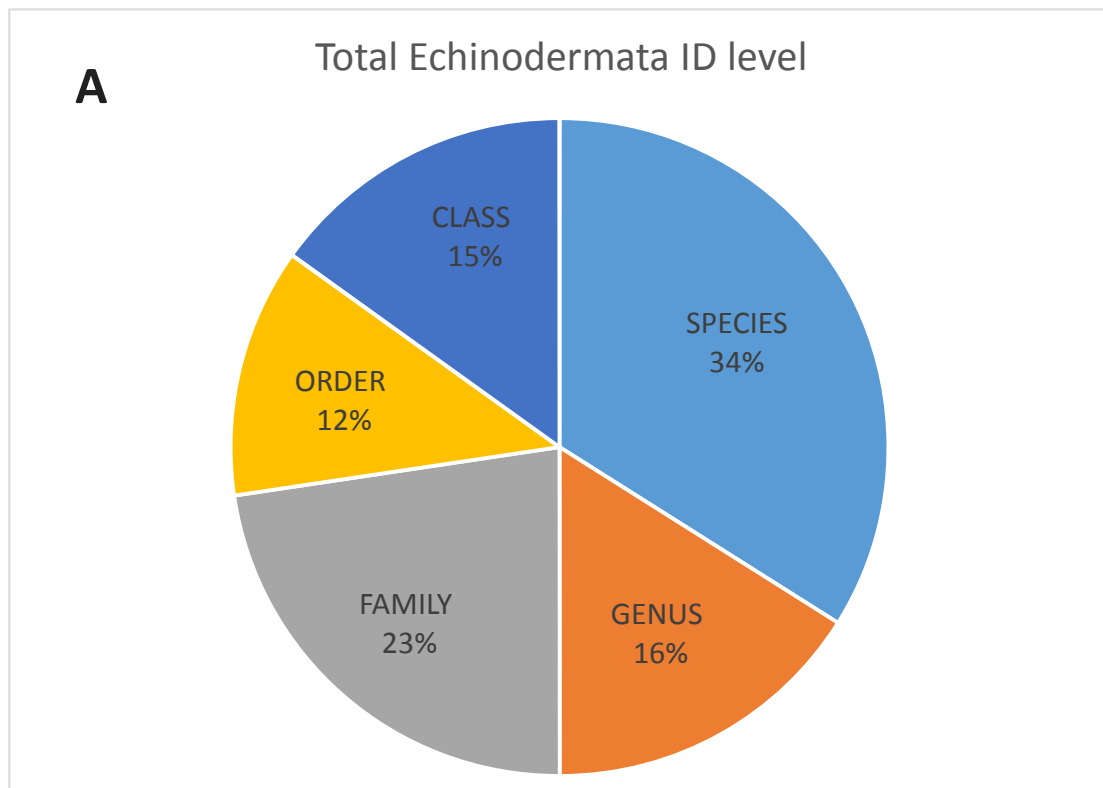
(three classes of Echinodermata), 556 bp (all Mollusca), 587 bp (all Annelida) and 533 bp (all Sipuncula). The aligned sequences from the Echinodermata class Holothuroidea were trimmed separately from other Echinodermata because many of the available Holothuroidea sequences were approximately 200 bp different in length compared to the standard barcode region. Sequence lengths for Holothuroidea samples ranged from 434 to 605 bp. Data from the following phyla were not available for identification due to low annealing during PCR (Brachiopoda and Chordata), due to the presence of a stop codon approximately in the middle of the fragment (Porifera) or due to an excess of missing data.



**Figure 1.** The number of sequences in the final data set for each phylum.

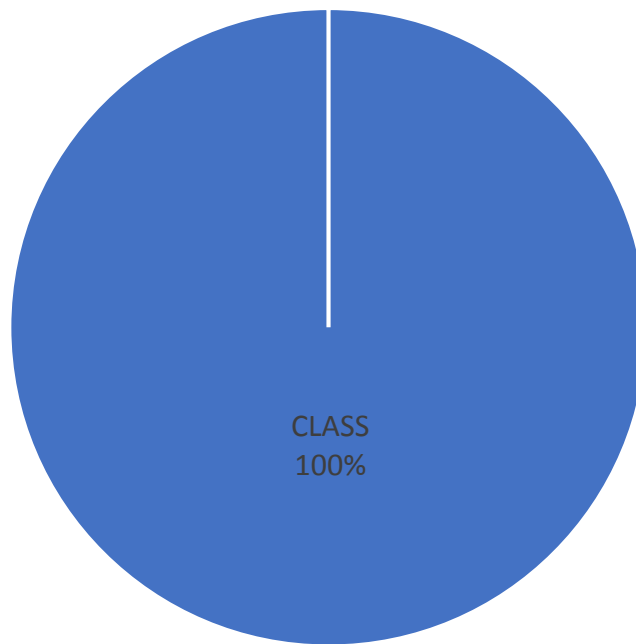
Searches conducted in Blast returned species ID with similarity percentages ranging from 79 to 100% (see Appendix 2 for IDs for each sample). Uncorrelated pairwise distances between our sample sequences and GenBank sequences ranged from 0 to 20.54%. Our estimates of average sequence distance based on five Arthropoda and four Echinodermata genera indicated that intraspecific distances ranged from 0 to 1.65%, interspecific distance were up to 10.36% and intrageneric genetic distances were up to 12.9%. These thresholds, based on average sequence distances, were then used to identify species, genus, and family, respectively. They successfully identified a number of

species, genera and families for Echinodermata, Mollusca, Arthropoda and Cnidaria. All Annelida and Sipuncula samples were only identified at the class level (Figure 2).



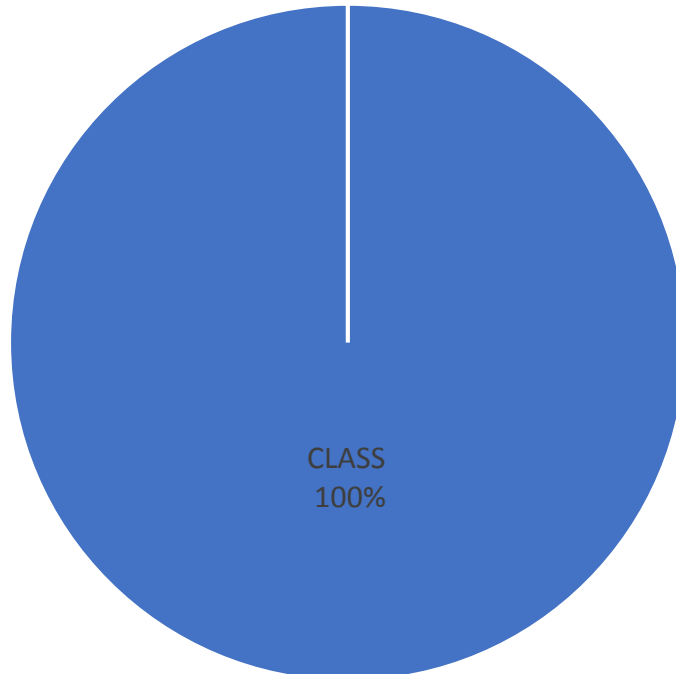
**C**

Total Annelida ID level

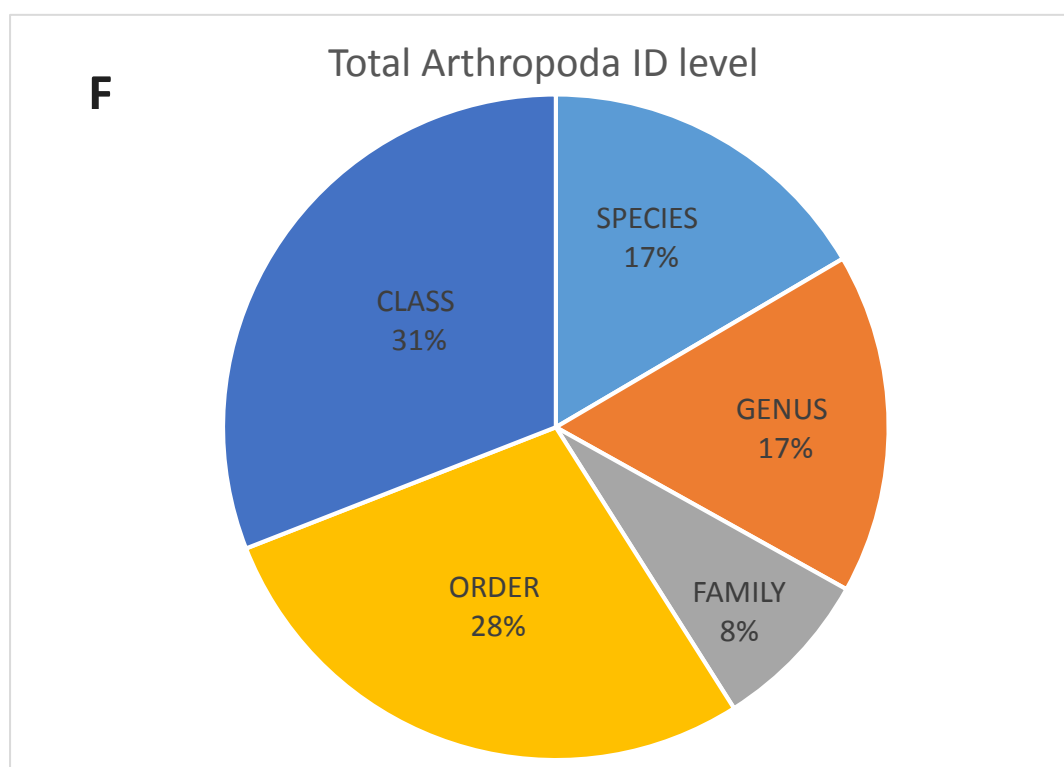
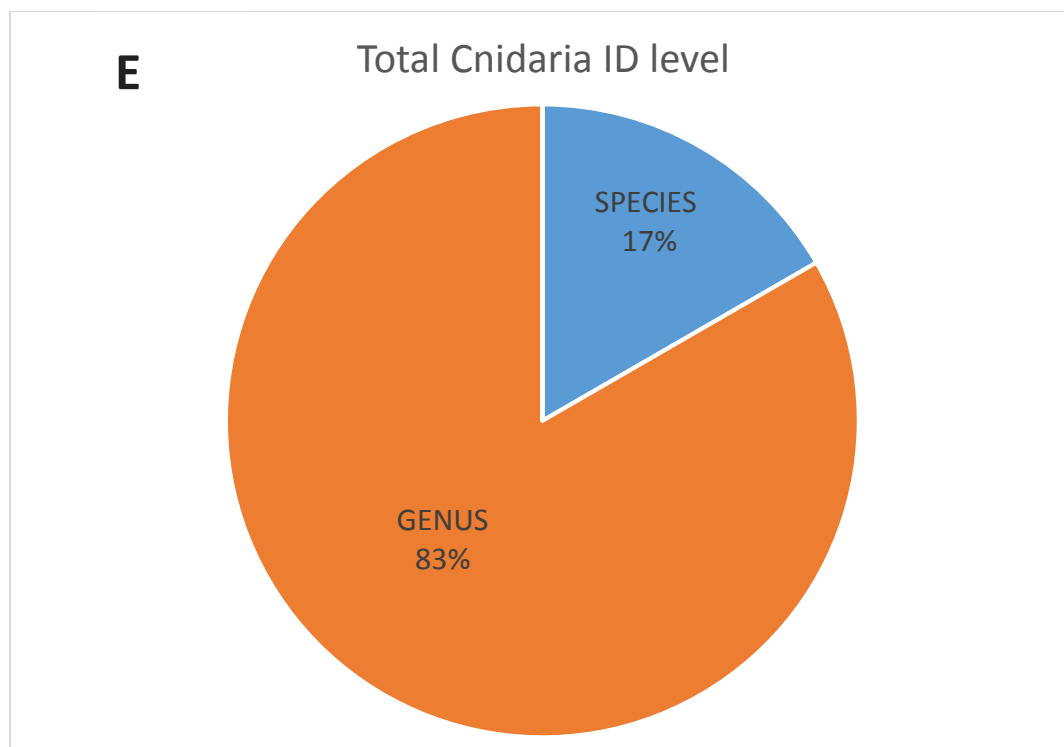


**D**

Total Sipuncula ID level



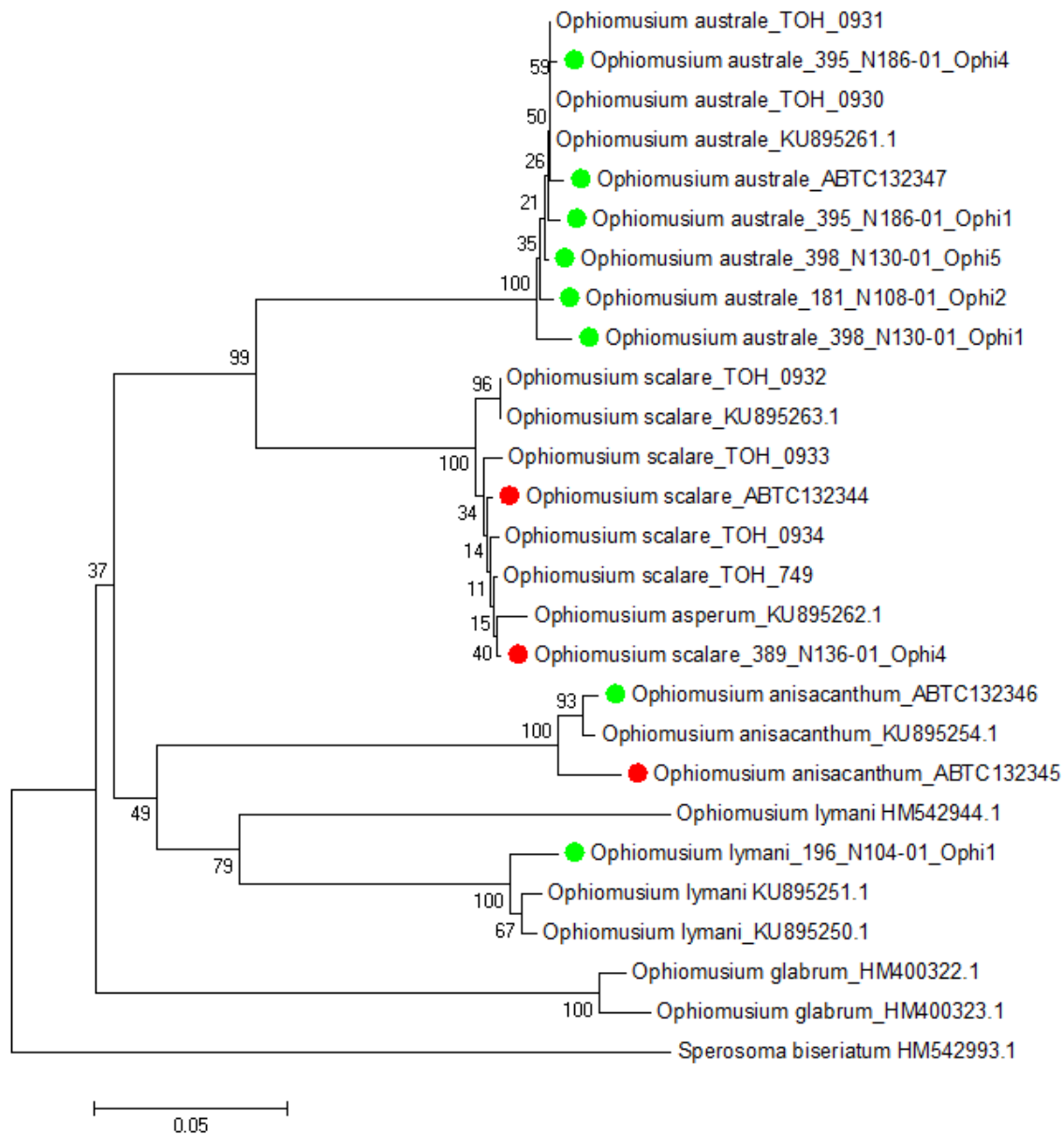




**Figure 2.** The proportion of sample identification for a given taxonomic level using p-distance thresholds (species: 0.00 – 1.65%, genus: 1.66 – 10.36%, family: 10.37 – 12.90%, order: 12.91 – 15.00% and class: 15.01 – 21.00%). (A) Echinodermata, (B) Mollusca, (C) Annelida, (D) Sipuncula, (E) Cnidaria and (F) Arthropoda.

#### 4.1.3 Proof of concept of identification strategy

The proof of concept for our identification strategy and resulting thresholds is exemplified below using a Neighbour-Joining (NJ) tree that we constructed for the Echinodermata genus *Ophiomusium* (Figure 3). Results from additional NJ trees for the other genera are shown in Appendix 3. The tree shows that most of our samples with species level identification clustered within the same clade with samples from the database (NBCI and BOLD), whereas the samples with genus level identification were clustered with two different species (*O. scalare* and *O. asperum*). Interestingly, pairwise p-distance between *O. asperum* and *O. scalare* ranged from 0.92 to 1.48%, which probably means that these samples were obtained from the same species under this BCM threshold. Using additional loci and coalescent-based analytical approaches, such as species delimitation methods (e.g. Yang 2015), is recommended to test for species boundaries and to provide stronger delimitation support for such situations. Although our *O. anisacanthum* sample (ABTC132345, red circle) was identified only to the genus level using BCM threshold (p-dis = 2.71%), it did form a single and discrete clade that only contained other samples identified as *O. anisacanthum* (Figure 3).



**Figure 3.** Neighbour-Joining tree for the genus *Ophiomusium* based on Kimura 2-parameter genetic distance with 1000 bootstraps. Samples with coloured circles are from the current study. Green circles indicate that the sample was identified at the species level ( $p\text{-dis} < 1.65\%$ ) using our proposed strategy, while red circles indicate that the sample was identified at the genus level ( $1.65\% < p\text{-dis} < 10.36\%$ ) using threshold values. Sequences from samples without coloured circles were obtained from databases (NCBI and BOLD) and their accession numbers and sample IDs are shown after the species names.

## 5. CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE STUDIES

Our results have shown that the approach of DNA barcoding using sequences from the mitochondrial DNA COI gene successfully identified key marine taxonomic groups in the GAB. We optimized and developed a number of protocols for the extraction of DNA and for the amplification and sequencing of the barcoding COI gene across a range of marine phyla from the GAB region. These developments enabled the extraction of 925 samples, 1,987 rounds of PCRs and the generation of 531 raw and 303 high quality sequences. We also implemented analytical approaches to circumvent the lack of information about morphological identification, which enabled us to propose thresholds of COI sequence similarity to identify samples at the species, genus and/or family levels. Identifications based on the data generated and analysed as part of this report correspond to 139 Arthropoda, 106 Echinodermata, 27 Mollusca, 14 Annelida, 11 Sipuncula and 6 Cnidaria. Identifications are provided for each individual sample as series' of barcode IDs and sequence data. The sequences obtained are available via the eResearch SA repository database; the corresponding author should be contacted to obtain access to this database.

Overall, more taxonomic units (i.e. species, genus and family levels) were obtained for Echinodermata and Arthropoda due to the larger number of sequences obtained in the final data set for these phyla. We also found that species identification was substantially improved by using both the NCBI and BOLD databases. Finally, according to the species distribution records from the Atlas of Living Australia and BOLD, we found three species that are new to Australia. These are *Sperosoma biseriatum* (Echinodermata), *Granulifusus nopinicus* (Mollusca) and *Ebalia nux* (Arthropoda).

The COI region should be considered as an appropriate and cost-effective tool for biodiversity assessment in future ecological monitoring in the GAB region. That could be achieved using

individual sequencing approaches (such as the one implemented here based on direct Sanger sequencing), or by using next-generation sequencing platforms for parallel acquisition of DNA barcode sequences from hundreds of specimens simultaneously (e.g. Shokralla et al. 2014). Undoubtedly, the power of DNA barcoding for species identification can be improved substantially with the availability of extensive DNA sequence data from the regional biota being studied, and with information from morphological data (including identification) and from records about the ranges of the targeted taxonomic units. This information was not available to our team for the samples targeted in this GAB sub-project. Finally, resolution of some technical issues identified in this project would enable more efficient application of DNA barcoding to the benthic fauna of the GAB. Improvements include development of specific primers for Echinodermata and sampling methods to target larger infauna samples during fieldwork.

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## APPENDIX 1: DNA EXTRACTION PROTOCOLS

**Appendix 1.** Summary of DNA extraction protocols tested for the different phyla and tissues.

Protocol	DNA Extraction Procedure
<b>DNeasy Blood &amp; Tissue kit (Qiagen)</b>	<ol style="list-style-type: none"> <li>1. Cut up to 25 mg tissue (up to 10 mg spleen) into small pieces, and place in a 1.5 mL microcentrifuge tube. For rodent tails, place one (rat) or two (mouse) 0.4–0.6 cm lengths of tail into a 1.5 mL microcentrifuge tube. Add 180 µL Buffer ATL. Earmark the animal appropriately.</li> <li>2. Add 20 µL proteinase K. Mix thoroughly by vortexing, and incubate at 56°C until the tissue is completely lysed. Vortex occasionally during incubation to disperse the sample, or place in a thermomixer, shaking water bath, or on a rocking platform.</li> <li>3. Vortex for 15 s. Add 200 µL Buffer AL to the sample, and mix thoroughly by vortexing. Then add 200 µL ethanol (96–100%), and mix again thoroughly by vortexing.</li> <li>4. Pipet the mixture from step 3 (including any precipitate) into the DNeasy Mini spin column placed in a 2 mL collection tube (provided). Centrifuge at &gt;6,000 x g (8,000 rpm) for 1 min. Discard flow-through and collection tube.</li> <li>5. Place the DNeasy Mini spin column in a new 2 mL collection tube (provided), add 500 µL Buffer AW1, and centrifuge for 1 min at &gt;6,000 x g (8,000 rpm). Discard flow-through and collection tube.</li> <li>6. Place the DNeasy Mini spin column in a new 2 mL collection tube (provided), add 500 µL Buffer AW2, and centrifuge for 3 min at 20,000 x g (14,000 rpm) to dry the DNeasy membrane. Discard flow-through and collection tube.</li> <li>7. Place the DNeasy Mini spin column in a clean 1.5 mL or 2 mL microcentrifuge tube (not provided), and pipet 30 µL ddH<sub>2</sub>O directly onto the DNeasy membrane. Incubate at room temperature for 1 min, and then centrifuge for 1 min at &gt;6,000x g (8,000 rpm) to elute.</li> </ol>
<b>CTAB</b>	<ol style="list-style-type: none"> <li>1. For mollusks and echinoderms mix 5 mL of 2×CTAB and 0.5 mL of Proteinase K, 20 mg/mL in a sterile container. Add 50 µL of Lysis Mix to each Eppendorf tubes containing small pieces of tissue (1–3 mm<sup>3</sup>). Cover with fresh strip caps. Incubate at 56°C for a minimum of 6 hours or overnight to allow digestion. Centrifuge at 1,500 g for 1 min to remove any condensate from the cap strips.</li> <li>2. Add 100 µL of Plant Binding Buffer (PBB) to each sample using multichannel pipette. Incubate for 5 min at RT.</li> <li>3. Mix lysate 5–10 times by pipetting, transfer the lysate (about 150 µL) from the Eppendorf tube into the silica membrane spin column placed on collection tube using pipette. Close the tubes. Centrifuge at 5,000 g for 5 min to bind DNA to the silica membrane.</li> <li>4. First wash step: Add 180 µL of Protein Wash Buffer (PWB) to each spin column. Close the lids and centrifuge at 5,000 for 2 min.</li> </ol>



	<ol style="list-style-type: none"> <li>5. Second wash step: Add 750 µL of Wash Buffer (WB) to each spin column. Close the lids and centrifuge at 5,000 for 5 min.</li> <li>6. Open the lids and place spin columns on the rack. Incubate at 56°C for 30 min to evaporate residual ethanol.</li> <li>7. Position new Eppendorf tubes and place the spin columns (without collection tubes) on top to collect DNA. Dispense 20 µL of ddH<sub>2</sub>O (prewarmed to 56°C) directly onto the membrane in each well of GF plate and incubate at room temperature for 1 min.</li> <li>8. Centrifuge spin columns in the Eppendorf tubes at 5,000 g for 5 min to collect the DNA eluate. Remove the spin column and discard it.</li> </ol> <p>(Ivanova et al. 2016)</p>
<b>Salting out</b>	<ol style="list-style-type: none"> <li>1. 1-3 mg of tissue were crushed in a 1.5mL microfuge tube and incubated at 55°C in 600 µL TNES (50 mM Tris, pH 7.5, 400 mM NaCl, 20 mM EDTA, 0.5% SDS) with 100 µg/ml Proteinase K. After overnight incubation, proteins were precipitated with 170 µL of 5M NaCl and hard shaking for 15 sec.</li> <li>2. Proteins were pelleted in a microfuge at 14,000 rpm for 5 min. then DNA was precipitated from the decanted supernatant with 1 volume 100% ethanol. DNA was pelleted, washed in 70% ethanol twice, air-dried, and dissolved in 10-15 µL of sterile water.</li> </ol> <p>(Sunnucks &amp; Hales 1996)</p>
<b>Chelex (Bio-rad)</b>	<ol style="list-style-type: none"> <li>1. Add small sample (1-2 mm) in the Eppendorf tube with 80 µL of 10% Chelex 100 (Bio-rad) and incubate sample at 99°C for 20 min.</li> </ol>
<b>VLB</b>	<ol style="list-style-type: none"> <li>1. Mix 45 µL of VLB (100 mM NaCl, 50 mM Tris-HCl pH 8.0, 10 mM EDTA pH 8.0 and 0.5% SDS) and 5 µL of proteinase K (10 mg/ mL) in the Eppendorf tube and add 1-2 mm<sup>3</sup> of tissue. Close the lid and incubate overnight at 56°C and then centrifuge at 1,000 g for 1 min.</li> <li>2. Add 100 µL of Binding Mix (1 part of 96% ethanol and 1 part of Binding Buffer, BB, (6 M GuSCN, 20 mM EDTA pH 8.0, 10 mM Tris-HCL pH 6.4 and 4% Triton X-100)) to each tube, mix and centrifuge at 1,000 g for 20 s.</li> <li>3. Open the lid and transfer 125 µL of each lysate into a silica membrane spin column with collection tube. Close the lid of spin column and centrifuge at 5,000 g for 5 min to bind DNA to the silica membrane.</li> <li>4. For the first wash step, add 180 µL of Protein Wash Buffer (73% of 96% ethanol and 27% of BB) to each spin column and centrifuge at 5,000 g for 2 min.</li> <li>5. For the second wash step, add 750 µL of Wash Buffer (60% ethanol, 50 mM NaCl, 10 mM Tris-HCL pH 7.4 and 0.5 mM EDTA pH 8.0) to each spin column and centrifuge at 5,000 g for 5 min.</li> </ol>

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- |  |   |
|--|---|
|  | <ol style="list-style-type: none"><li>6. Open the lid, place spin column in new Eppendorf tube and incubate at 56°C for 30 min to evaporate residual ethanol. Add 30 µL of ddH<sub>2</sub>O to each spin column, close the lid and incubate at room temperature for 1 min.</li><li>7. Centrifuge spin column in the Eppendorf tube at 5,000 g for 5 min to collect eh DNA elute. Remove the spin column and discard it.</li></ol> |
|--|---|
- 

(Ivanova et al. 2006)

## APPENDIX 2: SPECIES IDENTIFICATIONS

### Appendix 2. Species identification for each sample.

ECHINODERMATA: Species (<1.65%), Genus (<10.36%), Family (<12.9%), Order (<15%), Class (<21%)				
ID	Most similar species ID	p-dis	Accession	ID level
181_N111-01_Ophi1	<i>Amphiophiura distincta</i>	0%	KU894937.1	Species
181_N111-01_Ophi5	<i>Amphiophiura distincta</i>	0%	KU894937.1	Species
389_N146-01_Ophi1	<i>Amphiophiura urbana</i>	0.54%	EU869880.1	Species
389_N146-01_Ophi2	<i>Amphiophiura urbana</i>	0.91%	EU869880.1	Species
202_N114-01_Aste	<i>Benthopecten pikei</i>	0.37%	33398	Species
202_N114-01_Aste2	<i>Benthopecten pikei</i>	0%	33398	Species
281_N106-01_Aste3	<i>Benthopecten pikei</i>	0.37%	33398	Species
ABTC132393_Holo	<i>Holothuria austrinabassa</i>	0%	EU220818.1	Species
ABTC132346	<i>Ophiomusium anisacanthum</i>	0.72%	KU895254.1	Species
181_N108-01_Ophi2	<i>Ophiomusium australe</i>	0.54%	KU895261.1	Species
395_N186-01_Ophi1	<i>Ophiomusium australe</i>	0.36%	KU895261.1	Species
395_N186-01_Ophi4	<i>Ophiomusium australe</i>	0.18%	KU895261.1	Species
398_N130-01_Ophi1	<i>Ophiomusium australe</i>	1.26%	KU895261.1	Species
398_N130-01_Ophi5	<i>Ophiomusium australe</i>	0.18%	KU895261.1	Species
ABTC132347	<i>Ophiomusium australe</i>	0.36%	KU895261.1	Species
196_N104-01_Ophi1	<i>Ophiomusium lymani</i>	1.63%	KU895250.1	Species
330_N120-01_Ophi	<i>Ophiomyxa crinita</i>	0.56%	KU895172.1	Species
389_N126-01_Ophi1	<i>Ophiomyxa crinita</i>	0.56%	KU895172.1	Species
389_N126-01_Ophi2	<i>Ophiomyxa crinita</i>	0.00%	KU895172.1	Species
389_N126-01_Ophi3	<i>Ophiomyxa crinita</i>	0.56%	KU895172.1	Species

389_N145-01_Ophi1	<i>Ophiothrix aristulata</i>	0.36%	KF663151.1	Species
389_N145-01_Ophi2	<i>Ophiothrix aristulata</i>	0.18%	KF663151.1	Species
389_N145-01_Ophi3	<i>Ophiothrix aristulata</i>	0.90%	KF663103.1	Species
389_N145-01_Ophi4	<i>Ophiothrix aristulata</i>	0.54%	KF663138.1	Species
389_N145-01_Ophi5	<i>Ophiothrix aristulata</i>	0.18%	KF663151.1	Species
159_N101-01_echi	<i>Phormosoma bursarium</i>	0%	33522	Species
382_N132-01_echi2	<i>Phormosoma bursarium</i>	0%	33643	Species
382_N132-01_echi4	<i>Phormosoma bursarium</i>	0%	33643	Species
382_N132-01_echi5	<i>Phormosoma bursarium</i>	0%	33643	Species
435_N109-01_Aste4	<i>Plutonaster complexus</i>	0.40%	33403	Species
435_N109-01_Aste1	<i>Plutonaster complexus</i>	0.40%	33403	Species
202_N106-01_echi	<i>Sperosoma biserialatum</i>	1.28%	HM542993.1	Species
202_N106-01_echi2	<i>Sperosoma biserialatum</i>	1.10%	HM542993.1	Species
202_N106-01_echi4	<i>Sperosoma biserialatum</i>	1.28%	HM542993.1	Species
202_N106-01_echi5	<i>Sperosoma biserialatum</i>	1.28%	HM542993.1	Species
281_N102-01	<i>Sperosoma biserialatum</i>	1.10%	HM542993.1	Species
395_N124-01_echi5	<i>Clypeaster japonicus</i>	10.20%	JQ341144.1	Genus
ABTC132360	<i>Ophiernus vallincola</i>	4.15%	KU895179.1	Genus
276_N112-01_Ophi2	<i>Ophiocten hastatum</i>	3.97%	KJ620610.1	Genus
449_N112-01_Ophi2	<i>Ophiocten hastatum</i>	3.97%	KJ620610.1	Genus
ABTC132345	<i>Ophiomusium anisacanthum</i>	2.71%	KU895254.1	Genus
389_N136-01_Ophi4	<i>Ophiomusium asperum/Ophiomusium scalare</i>	0.9%/1.26%	KU895262.1/KU895263.1	Genus
ABTC132344	<i>Ophiomusium scalare/Ophiomusium asperum</i>	1.09%	KU895263.1/KU895262.1	Genus

151_N114-01_Ophi4	<i>Ophiosphalma fimbriatum</i>	3.88%	KU895360.1	Genus
151_N114-01_Ophi5	<i>Ophiosphalma fimbriatum</i>	3.88%	KU895360.1	Genus
216_N115-01_Ophi1	<i>Ophiosphalma fimbriatum</i>	4.43%	KU895360.1	Genus
202_N103-01_Aste	<i>Plutonaster complexus</i>	5.79%	33403	Genus
435_N109-01_Aste3	<i>Plutonaster complexus</i>	5.40%	33403	Genus
ABTC132366_echi	<i>Plutonaster complexus</i>	5.79%	33403	Genus
435_N109-01_Aste5	<i>Plutonaster knoxi/ Plutonaster complexus</i>	0.40%	15388/33403	Genus
207_N122-01_echi	<i>Salenocidaris profundus</i>	3.99%	KF642994.1	Genus
207_N122-01_echi3	<i>Salenocidaris profundus</i>	3.26%	KF642994.1	Genus
207_N122-01_echi5	<i>Salenocidaris profundus</i>	3.62%	KF642994.1	Genus
395_N124-01_echi	<i>Clypeaster japonicus</i> (Family: Clypeasteridae)	10.70%	JQ341144.1	Family
395_N124-01_echi2	<i>Clypeaster japonicus</i> (Family: Clypeasteridae)	12.30%	JQ341144.1	Family
395_N124-01_echi3	<i>Clypeaster japonicus</i> (Family: Clypeasteridae)	11.80%	JQ341144.1	Family
398_N133-01_echi	<i>Clypeaster japonicus</i> (Family: Clypeasteridae)	10.60%	JQ341144.1	Family
141_N117-01_Aste2	<i>Hymenaster pellucidus</i> (Family: Pterasteridae)	11.50%	KU495861.1	Family
207_N124-01_Aste1	<i>Hymenaster pellucidus</i> (Family: Pterasteridae)	11.50%	KU495861.1	Family
207_N124-01_Aste2	<i>Hymenaster pellucidus</i> (Family: Pterasteridae)	11.70%	KU495861.1	Family
207_N124-01_Aste4	<i>Hymenaster pellucidus</i> (Family: Pterasteridae)	11.50%	KU495861.1	Family
207_N124-01_Aste5	<i>Hymenaster pellucidus</i> (Family: Pterasteridae)	11.50%	KU495861.1	Family
216_N106-01_Aste2	<i>Hymenaster pellucidus</i> (Family: Pterasteridae)	11.50%	KU495861.1	Family
216_N106-01_Aste4	<i>Hymenaster pellucidus</i> (Family: Pterasteridae)	11.50%	KU495861.1	Family
216_N106-01_Aste5	<i>Hymenaster pellucidus</i> (Family: Pterasteridae)	11.50%	KU495861.1	Family
141_N131-01_Holo2	<i>Peniagone vignoni</i> (Family: Elpididae)	11.78%	HM196385.1	Family
ABTC132350_echi	<i>Phormosoma bursarium</i> (Family: Echinothuriidae)	11.78%	46086	Family

207_N123-01_Aste4	<i>Psilaster charcoti</i> (Family: Astropectinidae)	12.48%	38450.1	Family
216_N128.01_Aste	<i>Psilaster charcoti</i> (Family: Astropectinidae)	12.85%	38450.1	Family
276_N108-01_Aste2	<i>Psilaster charcoti</i> (Family: Astropectinidae)	12.85%	38450.1	Family
276_N108-01_Aste3	<i>Psilaster charcoti</i> (Family: Astropectinidae)	12.85%	38450.1	Family
276_N108-01_Aste4	<i>Psilaster charcoti</i> (Family: Astropectinidae)	12.85%	38450.1	Family
281_N101-01_Aste2	<i>Psilaster charcoti</i> (Family: Astropectinidae)	12.48%	38450.1	Family
281_N101-01_Aste3	<i>Psilaster charcoti</i> (Family: Astropectinidae)	12.48%	38450.1	Family
281_N101-01_Aste5	<i>Psilaster charcoti</i> (Family: Astropectinidae)	12.66%	38450.1	Family
281_N108-01_Aste2	<i>Psilaster charcoti</i> (Family: Astropectinidae)	12.48%	38450.1	Family
435_N109-01_Aste2	<i>Psilaster charcoti</i> (Family: Astropectinidae)	12.66%	38450.1	Family
ABTC132353_echi	<i>Ctenodiscus crispatus</i> (Order: Paxillosida)	14.21%	HLC-23907	Order
207_N123-01_Aste1	<i>Psilaster charcoti</i> (Order: Paxillosida)	13.22%	38450.1	Order
207_N123-01_Aste5	<i>Psilaster charcoti</i> (Order: Paxillosida)	13.22%	38450.1	Order
276_N108-01_Aste5	<i>Psilaster charcoti</i> (Order: Paxillosida)	13.22%	38450.1	Order
281_N101-01_Aste4	<i>Psilaster charcoti</i> (Order: Paxillosida)	13.41%	38450.1	Order
330_N132-01_Aste	<i>Thrissacanthias penicillatus</i> (Order: Paxillosida)	13.65%	RBCM EC00045	Order
389_N120-01_Aste	<i>Thrissacanthias penicillatus</i> (Order: Paxillosida)	13.90%	RBCM EC00045	Order
181_N105-01_Aste2	<i>Uniophora granifera</i> (Order: Forcipulatida)	13.54%	Ech031	Order
395_N154-01_Aste2	<i>Uniophora granifera</i> (Order: Forcipulatida)	13.54%	Ech031	Order
395_N154-01_Aste3	<i>Uniophora granifera</i> (Order: Forcipulatida)	13.72%	Ech031	Order
398_N138-01_Aste2	<i>Uniophora granifera</i> (Order: Forcipulatida)	13.72%	Ech031	Order
398_N138-01_Aste3	<i>Uniophora granifera</i> (Order: Forcipulatida)	13.54%	Ech031	Order
ABTC132342	<i>Uniophora granifera</i> (Order: Forcipulatida)	13.36%	Ech031	Order

202_N105-01_Aste1	<i>Hymenaster pellucidus</i> (Class: Asteroidea)	15.21%	KU495861.1	Class
202_N105-01_Aste2	<i>Hymenaster pellucidus</i> (Class: Asteroidea)	15.21%	KU495861.1	Class
202_N105-01_Aste3	<i>Hymenaster pellucidus</i> (Class: Asteroidea)	15.21%	KU495861.1	Class
202_N105-01_Aste5	<i>Hymenaster pellucidus</i> (Class: Asteroidea)	15.01%	KU495861.1	Class
276_N104-01_Aste	<i>Hymenaster pellucidus</i> (Class: Asteroidea)	19.49%	KU495861.1	Class
281_N104-01_Aste1	<i>Hymenaster pellucidus</i> (Class: Asteroidea)	15.21%	KU495861.1	Class
281_N104-01_Aste2	<i>Hymenaster pellucidus</i> (Class: Asteroidea)	15.21%	KU495861.1	Class
281_N104-01_Aste3	<i>Hymenaster pellucidus</i> (Class: Asteroidea)	15.01%	KU495861.1	Class
435_N111-01_Aste1	<i>Hymenaster pellucidus</i> (Class: Asteroidea)	15.21%	KU495861.1	Class
ABTC132355_ophi_aste	<i>Marthasterias glacialis</i> (Class: Asteroidea)	16.79%	HM107750.1	Class
155_N110-01_Holo	<i>Protelpidia murrayi</i> (Class: Holothuroidea)	17.24%	KF713386.1	Class
207_N127-01_Holo	<i>Protelpidia murrayi</i> (Class: Holothuroidea)	16.88%	KF713386.1	Class
276_N106-01_Holo	<i>Protelpidia murrayi</i> (Class: Holothuroidea)	16.88%	KF713386.1	Class
281_N103-01_Holo	<i>Protelpidia murrayi</i> (Class: Holothuroidea)	17.24%	KF713386.1	Class
ABCT132332_Holo	<i>Protelpidia murrayi</i> (Class: Holothuroidea)	17.06%	KF713386.1	Class
ABTC132365	<i>Psilaster charcoti</i> (Class: Asteroidea)	19.31%	GU227097.1	Class

**MOLLUSCA: Species (<1.65%), Genus (<10.36%), Family (<12.9%), Order (<15%), Class (<21%)**

ID	Closest Species ID	p-dis	Accession	ID level
186_N106-01_mol	<i>Granulifusus niponicus</i>	0.54%	KT753935.1	Species
186_N106-01_Mol3	<i>Granulifusus niponicus</i>	0.54%	KT753935.1	Species
186_N106-01_Mol4	<i>Granulifusus niponicus</i>	0.18%	KT753935.1	Species
186_N106-01_Mol5	<i>Granulifusus niponicus</i>	0.54%	KT753935.1	Species
389_N108-01_mol	<i>Granulifusus niponicus</i>	0.36%	KT753935.1	Species

389_N108-01_Mol2	<i>Granulifusus niponicus</i>	0.54%	KT753935.1	Species
389_N108-01_Mol3	<i>Granulifusus niponicus</i>	0.54%	KT753935.1	Species
141_N124-01_mol	<i>Aforia serranoi</i>	3.96%	KT448836.1	Genus
167_N102-01_mol	<i>Muusoctopus oregonensis</i>	3.98%	HM572180.1	Genus
196_N101-01_mol	<i>Muusoctopus oregonensis</i>	3.98%	HM572180.1	Genus
ABTC132319_mol	<i>Stoloteuthis japonica</i>	9.17%	AB591072.1	Genus
ABTC132348_mol	<i>Amoria hunteri</i> (Family: Volutidae)	12.48%	JN182226.1	Family
389_N107-01_mol	<i>Cranopsis cucullata</i> (Family: Fissurellidae)	10.67%	GQ160755.1	Family
ABTC132340	<i>Dentalium majorinum</i> (Order: Dentaliida)	14.29%	AY260823.1	Order
281_N125-01_Mol2	<i>Echinolittorina paytensis</i> (Class: Gastropoda)	18.10%	AJ623027.1	Class
155_N105-01_mol	<i>Lorabela pelseneeri</i> (Class: Gastropoda)	17.88%	KT448824.1	Class
276_N122-01_Mol3	<i>Lorabela pelseneeri</i> (Class: Gastropoda)	17.88%	KT448824.1	Class
276_N122-01_Mol4	<i>Lorabela pelseneeri</i> (Class: Gastropoda)	17.88%	KT448824.1	Class
281_N125-01_Mol3	<i>Lorabela pelseneeri</i> (Class: Gastropoda)	18.07%	KT448824.1	Class
281_N125-01_Mol4	<i>Lorabela pelseneeri</i> (Class: Gastropoda)	17.88%	KT448824.1	Class
281_N125-01_Mol5	<i>Lorabela pelseneeri</i> (Class: Gastropoda)	18.07%	KT448824.1	Class
449_N118-01_mol	<i>Lorabela pelseneeri</i> (Class: Gastropoda)	18.07%	KT448824.1	Class
449_N118-01_Mol2	<i>Lorabela pelseneeri</i> (Class: Gastropoda)	17.70%	KT448824.1	Class
449_N118-01_Mol3	<i>Lorabela pelseneeri</i> (Class: Gastropoda)	17.88%	KT448824.1	Class
449_N118-01_Mol4	<i>Lorabela pelseneeri</i> (Class: Gastropoda)	17.70%	KT448824.1	Class
449_N118-01_Mol5	<i>Lorabela pelseneeri</i> (Class: Gastropoda)	17.70%	KT448824.1	Class
ABTC132318_mol	<i>Sepia hirunda</i> (Class: Cephalopoda)	15.29%	AY530213.1	Class

**ANNELIDA: Species (<1.65%), Genus (<10.36%), Family (<12.9%), Order (<15%), Class (<21%)**

ID	Closest Species ID	p-dis	Accession	ID level
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186_N108-01_poly	<i>Laetmonice filicornis</i> (Class: Polychaeta)	17.72%	JN852919	Class
186_N108-01_poly2	<i>Laetmonice filicornis</i> (Class: Polychaeta)	16.87%	JN852919	Class
186_N108-01_poly3	<i>Laetmonice filicornis</i> (Class: Polychaeta)	17.21%	JN852919	Class
186_N108-01_poly5	<i>Laetmonice filicornis</i> (Class: Polychaeta)	17.21%	JN852919	Class
191_N104-01_poly	<i>Laetmonice filicornis</i> (Class: Polychaeta)	17.55%	JN852919	Class
395_N146-01_poly	<i>Laetmonice filicornis</i> (Class: Polychaeta)	17.72%	JN852919	Class
395_N146-01_poly2	<i>Laetmonice filicornis</i> (Class: Polychaeta)	17.21%	JN852919	Class
395_N146-01_poly3	<i>Laetmonice filicornis</i> (Class: Polychaeta)	17.21%	JN852919	Class
395_N146-01_poly4	<i>Laetmonice filicornis</i> (Class: Polychaeta)	17.21%	JN852919	Class
395_N148-01_poly1	<i>Laetmonice filicornis</i> (Class: Polychaeta)	17.89%	JN852919	Class
395_N148-01_poly2	<i>Laetmonice filicornis</i> (Class: Polychaeta)	17.89%	JN852919	Class
395_N148-01_poly3	<i>Laetmonice filicornis</i> (Class: Polychaeta)	17.89%	JN852919	Class
395_N148-01_poly4	<i>Laetmonice filicornis</i> (Class: Polychaeta)	17.89%	JN852919	Class
ABTC132333_poly	<i>Laetmonice filicornis</i> (Class: Polychaeta)	17.21%	JN852919	Class

**SIPUNCULA: Species (<1.65%), Genus (<10.36%), Family (<12.9%), Order (<15%), Class (<21%)**

ID	Closest Species ID	p-dis	Accession	ID level
382_N150-01_sip	<i>Nephasoma diaphanes</i> (Class: Sipunculida)	18.64%	JN182658.1	Class
382_N150-01_Sip3	<i>Nephasoma diaphanes</i> (Class: Sipunculida)	18.64%	JN182658.1	Class
382_N150-01_Sip4	<i>Nephasoma diaphanes</i> (Class: Sipunculida)	18.64%	AY533769.1	Class
382_N150-01_Sip5	<i>Nephasoma diaphanes</i> (Class: Sipunculida)	18.64%	JN182658.1	Class
ABTC132337_sip	<i>Nephasoma diaphanes</i> (Class: Sipunculida)	19.02%	DQ300128.1	Class
151_N111-01_sip	<i>Sipunculus nudus</i> (Class: Sipunculida)	20.35%	GU014047.1	Class
151_N111-01_Sip4	<i>Sipunculus nudus</i> (Class: Sipunculida)	20.35%	GU014047.1	Class
435_N114-01_sip	<i>Sipunculus nudus</i> (Class: Sipunculida)	20.35%	AB542575.1	Class

435_N114-01_Sip2	<i>Sipunculus nudus</i> (Class: Sipunculida)	20.54%	AB542575.1	Class
435_N114-01_Sip3	<i>Sipunculus nudus</i> (Class: Sipunculida)	20.15%	AB542575.1	Class
435_N114-01_Sip4	<i>Sipunculus nudus</i> (Class: Sipunculida)	20.15%	KC404840.1	Class

**CNIDARIA: Species (<1.65%), Genus (<10.36%), Family (<12.9%), Order (<15%), Class (<21%)**

ID	Closest Species ID	p-dis	Accession	ID level
330_N133.01	<i>Corallimorphus profundus</i>	0.18%	KP938440.1	Species
151_N147.01_1	<i>Halipteris californica</i> / <i>Halipteris willemoesi</i>	0.89%	KF874202.1/KF874192.1	Genus
151_N147.01_3	<i>Halipteris californica</i> / <i>Halipteris willemoesi</i>	0.89%	KF874202.1/KF874192.1	Genus
151_N147.01_4	<i>Halipteris californica</i> / <i>Halipteris willemoesi</i>	0.89%	KF874202.1/KF874192.1	Genus
151_N123.01	<i>Stomphia didemon</i>	4.09%	KHBC-S13-0033-02	Genus
155_N123.01	<i>Stomphia didemon</i>	4.09%	KHBC-S13-0033-02	Genus

**ARTHROPODA: Species (<1.65%), Genus (<10.36%), Family (<12.9%), Order (<15%), Class (<21%)**

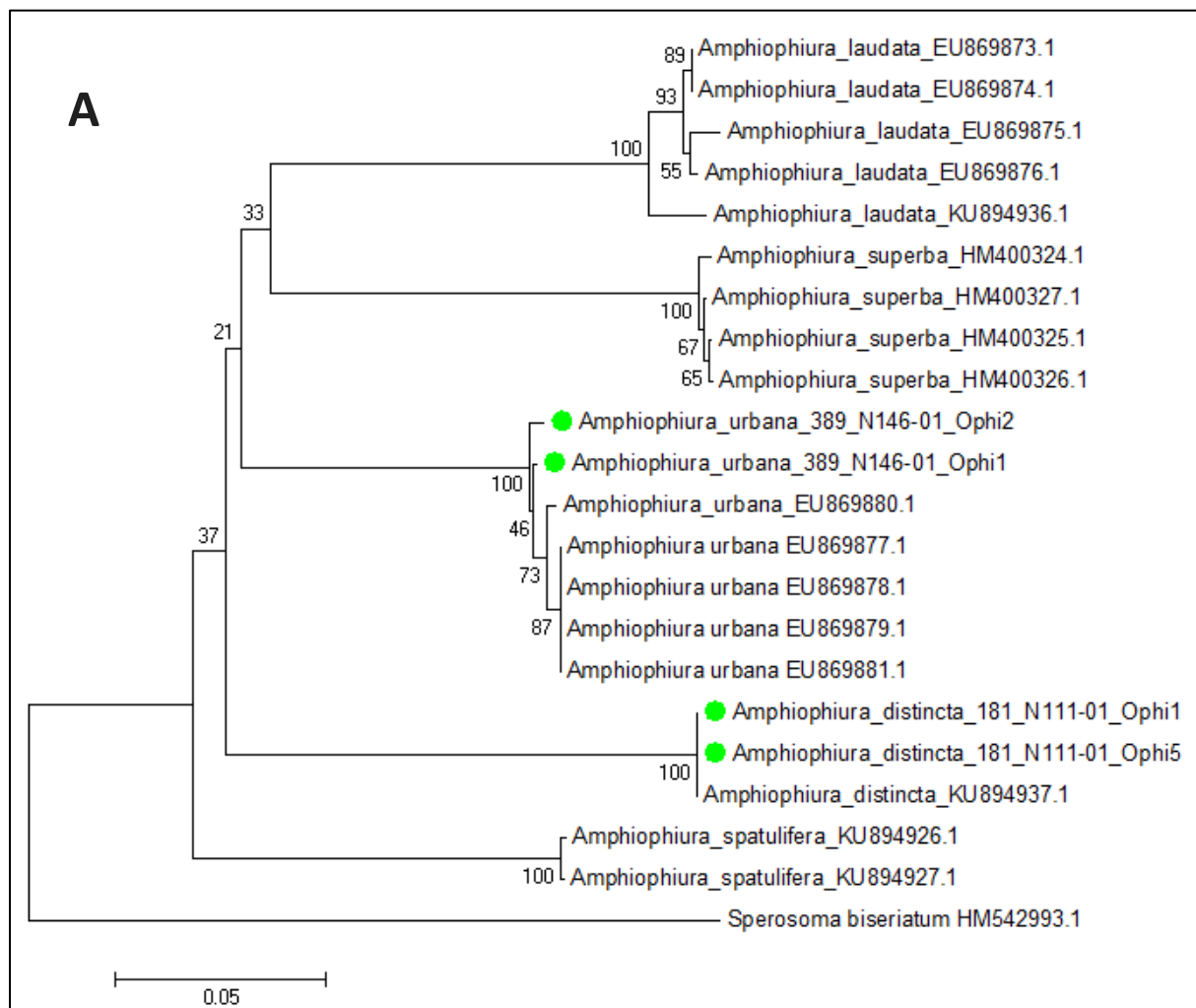
ID	Closest Species ID	p-dis	Accession	ID level
ABTC132373_arth	<i>Aegla alacalufi</i> (Class: Malacostraca)	15.75%	FJ471763.1	Class
292_N114-01_arth	<i>Aristeus antennatus</i> (Class: Malacostraca)	15.40%	JQ305889.1	Class
292_N115-01_arth	<i>Aristeus antennatus</i> (Class: Malacostraca)	15.40%	JQ305889.1	Class
292_N116-01_Arth	<i>Aristeus antennatus</i> (Class: Malacostraca)	15.40%	JQ305889.1	Class
292_N117-01_Arth	<i>Aristeus antennatus</i> (Class: Malacostraca)	15.75%	JQ305889.1	Class
292_N118-01_arth	<i>Aristeus antennatus</i> (Class: Malacostraca)	15.40%	JQ305889.1	Class
435_N105-01_Arth4	<i>Aristeus antennatus</i> (Class: Malacostraca)	15.40%	JQ305889.1	Class
ABTC132322_arth	<i>Aristeus antennatus</i> (Class: Malacostraca)	15.58%	JQ305889.1	Class

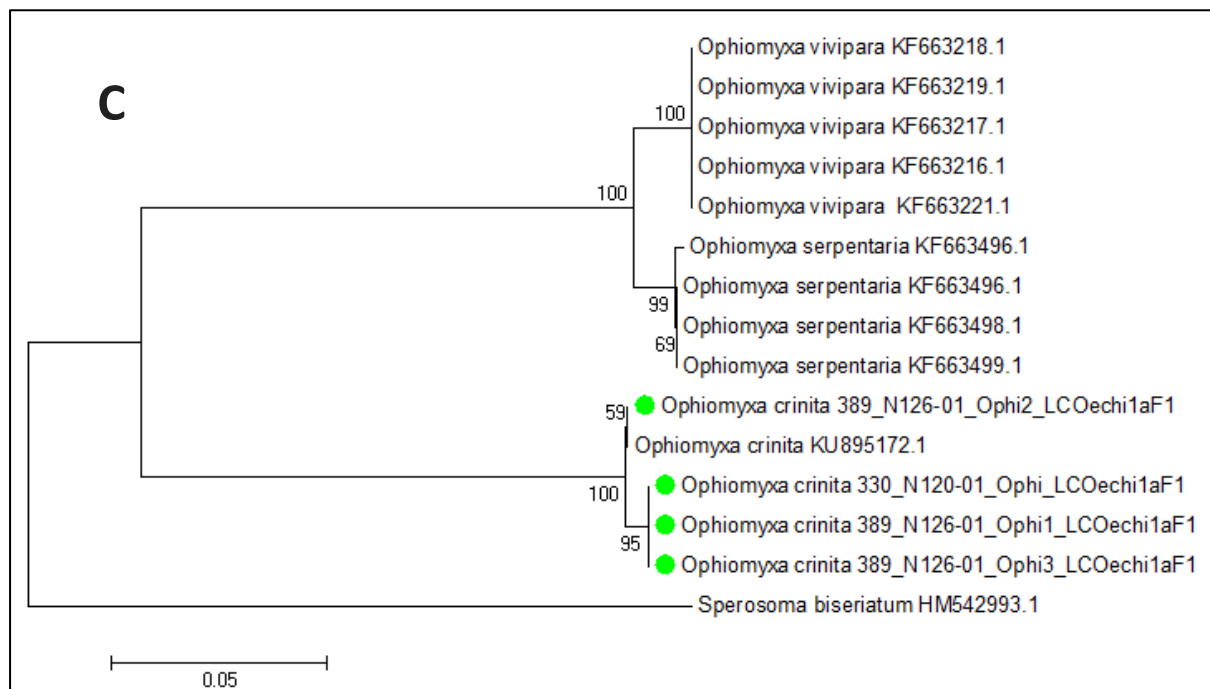
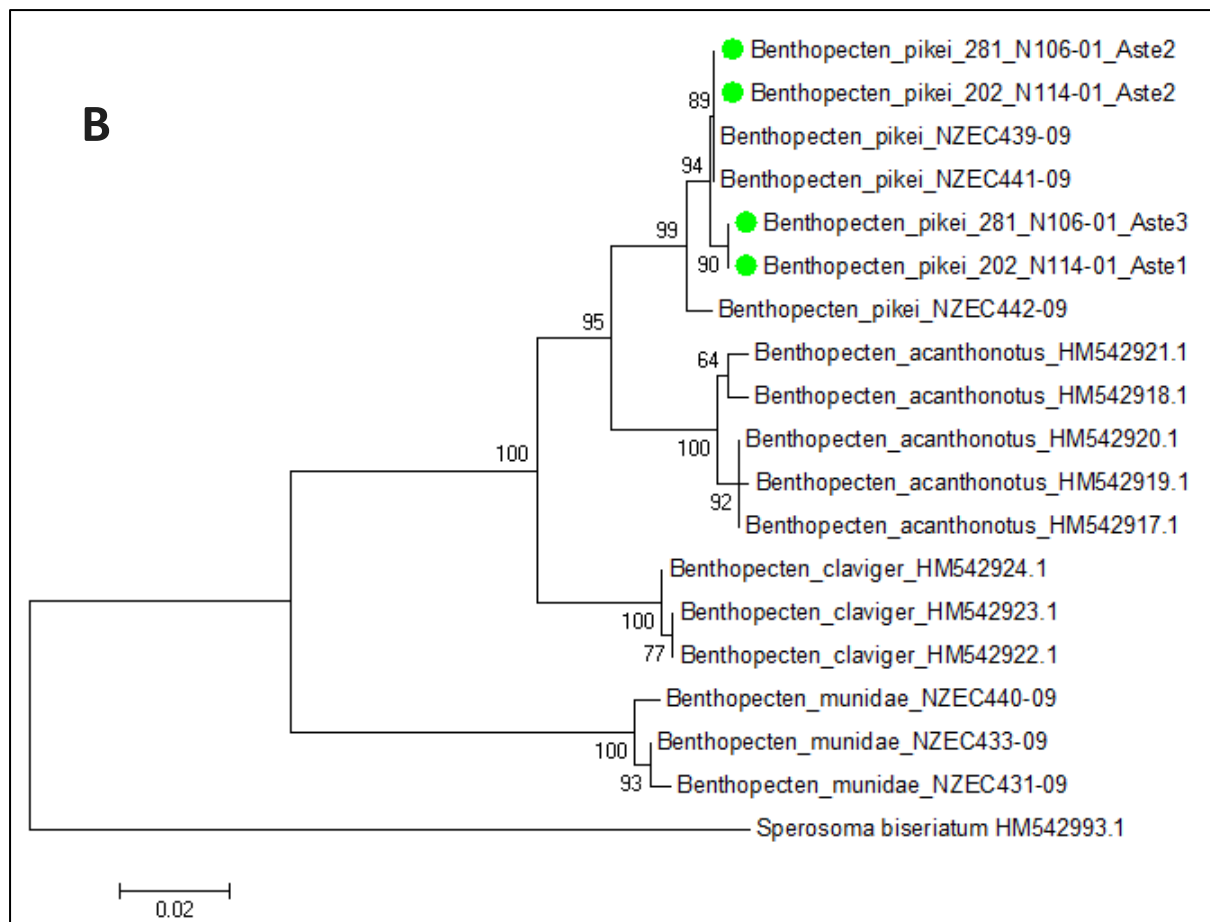
207_N106-01_arth	<i>Austromegabalanus psittacus</i> (Class: Maxillopoda)	18.58%	KJ756056.1	Class
207_N106-01_Arth2	<i>Austromegabalanus psittacus</i> (Class: Maxillopoda)	18.23%	KJ756056.1	Class
395_N140-01_arth	<i>Ceylonthelphusa cavatrix</i> (Class: Malacostraca)	16.64%	GQ982586.1	Class
395_N140-01_Arth2	<i>Ceylonthelphusa cavatrix</i> (Class: Malacostraca)	16.46%	GQ982586.1	Class
395_N140-01_Arth3	<i>Ceylonthelphusa cavatrix</i> (Class: Malacostraca)	16.46%	GQ982586.1	Class
398_N115-01_arth	<i>Chionoecetes tanneri</i> (Class: Malacostraca)	19.57%	JQ407478.1	Class
398_N115-01_Arth2	<i>Chionoecetes tanneri</i> (Class: Malacostraca)	19.57%	JQ407478.1	Class
398_N115-01_Arth4	<i>Chionoecetes tanneri</i> (Class: Malacostraca)	19.57%	JQ407478.1	Class
174_N142-01_arth	<i>Chionoecetes tanneri</i> (Class: Malacostraca)	19.57%	JQ407478.1	Class
330_N121-01_arth	<i>Chionoecetes tanneri</i> (Class: Malacostraca)	19.57%	JQ407478.1	Class
398_N155-01_Arth2	<i>Clinothelphusa kakoota</i> (Class: Malacostraca)	15.75%	GQ289664.1	Class
398_N155-01_Arth4	<i>Dromia personata</i> (Class: Malacostraca)	15.22%	JQ306068.1	Class
151_N121-01_Arth3	<i>Eumunida annulosa</i> (Class: Malacostraca)	15.01%	EU243493.1	Class
141_N134-01_arth3	<i>Eumunida annulosa</i> (Class: Malacostraca)	15.19%	EU243493.1	Class
216_N110-01_arth	<i>Eumunida annulosa</i> (Class: Malacostraca)	15.19%	EU243493.1	Class
216_N112-01_arth	<i>Eumunida annulosa</i> (Class: Malacostraca)	15.19%	EU243493.1	Class
274_N117-01_arth	<i>Eumunida annulosa</i> (Class: Malacostraca)	15.19%	EU243493.1	Class
274_N118-01_arth	<i>Eumunida annulosa</i> (Class: Malacostraca)	15.01%	EU243493.1	Class
274_N120-01_arth	<i>Eumunida annulosa</i> (Class: Malacostraca)	15.01%	EU243493.1	Class
382_N138-01_arth	<i>Eumunida minor</i> (Class: Malacostraca)	15.40%	EU243550.1	Class
ABTC132374_arth	<i>Eumunida sternomaculata</i> (Class: Malacostraca)	15.22%	EU243484.1	Class

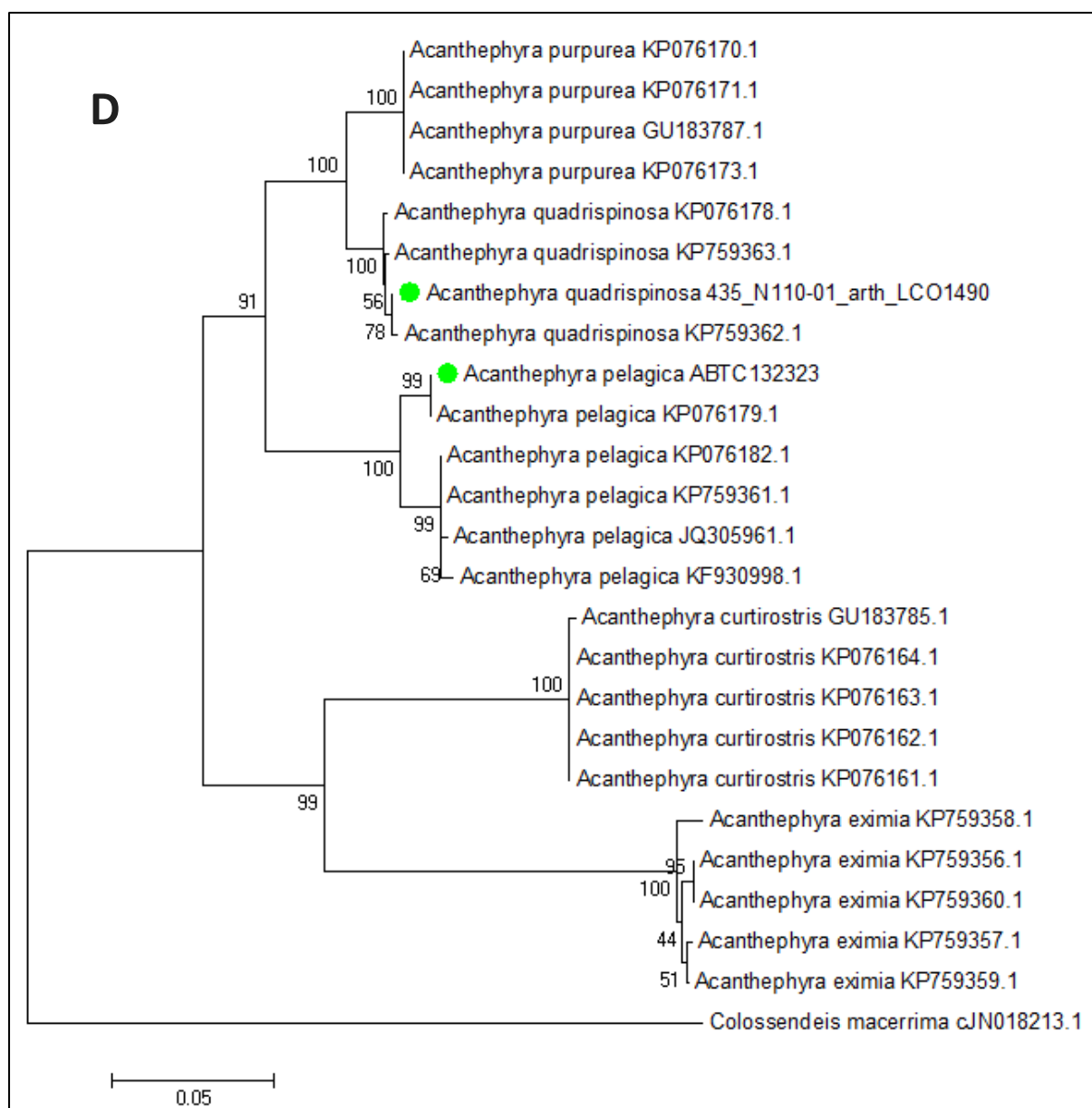
395_N104-01_Arth3	<i>Haptosquilla hamifera</i> (Class: Malacostraca)	15.04%	KM074037.1	Class
395_N104-01_Arth4	<i>Haptosquilla hamifera</i> (Class: Malacostraca)	15.22%	KM074037.1	Class
398_N114-01_Arth2	<i>Haptosquilla hamifera</i> (Class: Malacostraca)	15.04%	KM074037.1	Class
398_N114-01_Arth5	<i>Haptosquilla hamifera</i> (Class: Malacostraca)	15.04%	KM074037.1	Class
186_N103-01_Arth2	<i>Neonrosella vitiata</i> (Class: Maxillopoda)	15.07%	KM974424.1	Class
382_N120-01_arth	<i>Nephropsis atlantica</i> (Class: Malacostraca)	17.62%	JQ305974.1	Class
449_N142-01_arth	<i>Pagurus prideaux/Pagurus venturensis</i> (Class: Malacostraca)	16.07%	JQ306249.1/GU442192	Class
449_N144-01_arth	<i>Pagurus prideaux/Pagurus venturensis</i> (Class: Malacostraca)	16.07%	JQ306249.1/GU442192	Class
435_N105-01_Arth2	<i>Periclimenes wirtzi</i> (Class: Malacostraca)	16.46%	KU065009.1	Class
435_N105-01_Arth5	<i>Periclimenes wirtzi</i> (Class: Malacostraca)	16.28%	KU065009.1	Class
ABTC132329_arth	<i>Pugettia gracilis</i> (Class: Malacostraca)	18.65%	KX039768.1	Class
395_N140-01_Arth4	<i>Pugettia gracilis</i> (Class: Malacostraca)	15.75%	KX039768.1	Class
ABTC132324	<i>Synalpheus fritzmuelleri</i> (Class: Malacostraca)	17.70%	KJ595081.1	Class
ABTC132327_arth	<i>Synalpheus fritzmuelleri</i> (Class: Malacostraca)	17.52%	KJ595081.1	Class

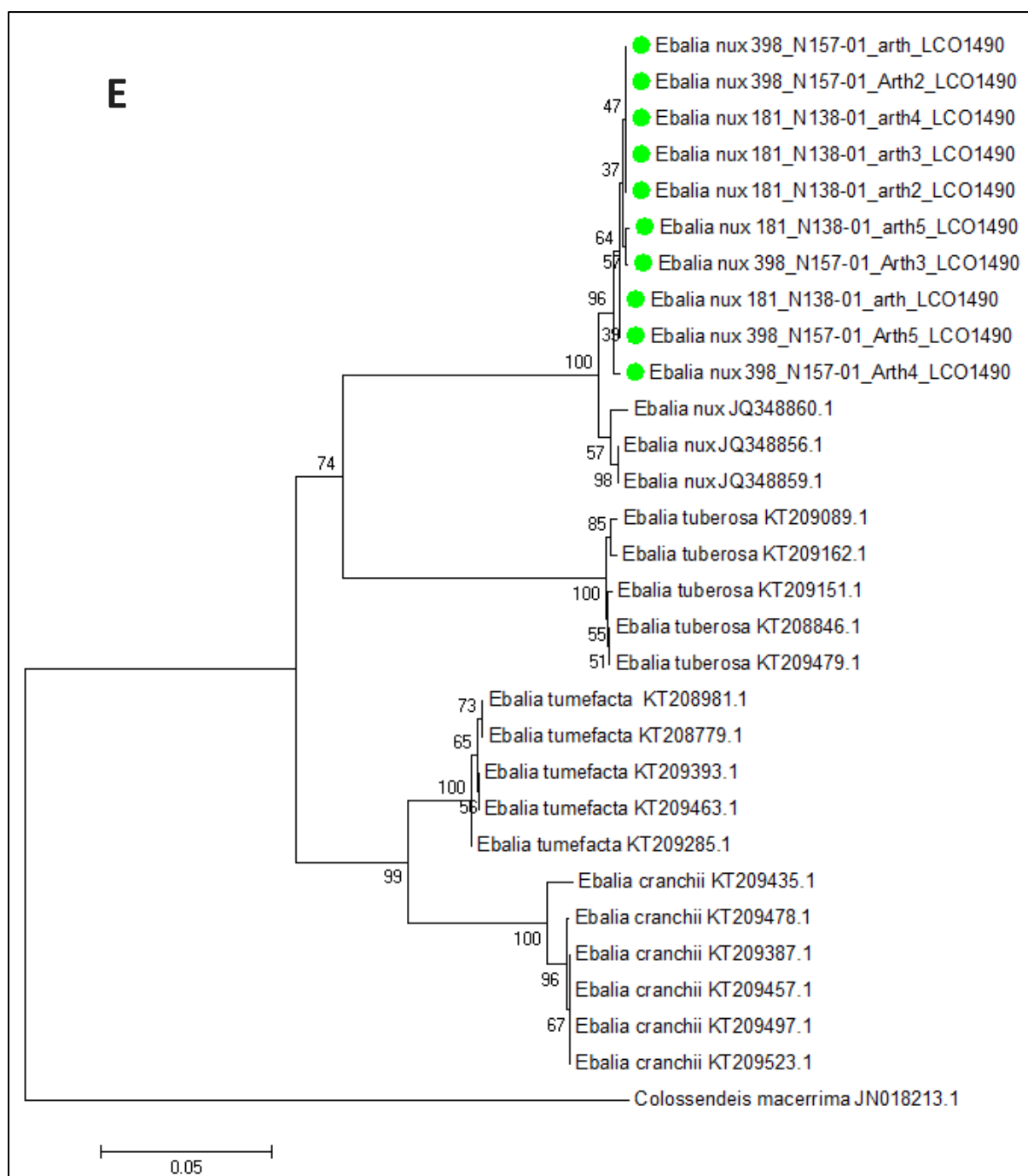
## APPENDIX 3: NEIGHBOUR-JOINING TREES

**Appendix 3.** Neighbour-Joining trees based on Kimura-2-paramter with 1000 bootstraps for genera used to calculate intra- and interspecific diversity. A: *Amphiophiura*, B: *Benthopecten*, C: *Ophiomyxa*, D: *Acanthephyra*, and E: *Ebalia*. Samples with coloured circles are from the current study. Green indicates that the sample was identified at species level using p-dis threshold.













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