Molecular Systematics of Selected Australian Brown Algae

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Thesis Summary

This thesis focuses on the molecular systematics of four selected Australian brown algal genera (Phaeophyceae); *Acrocarpia*, *Caulocystis*, *Cystophora* (Fucales) and *Lobophora* (Dictyotales). The thesis consists of five chapters. **Chapter 1**: General introduction; **Chapter 2**: The phylogenetics, DNA barcoding and phylogeographic discrimination of *Caulocystis* and *Acrocarpia* species; **Chapter 3**: The molecular phylogenetics of *Cystophora*; **Chapter 4**: An assessment of species diversity within Australian *Lobophora* based on *rbc*L and *cox*1 DNA sequence analyses, and **Chapter 5**: General discussion.

The first chapter describes the marine floral biodiversity of Australia, the ecological importance of benthic brown algae, the development of taxonomic studies in macroalgae, and the aims and significance of this study.

The second chapter is the first molecular taxonomic study specifically targeting two endemic Australian Fucales genera, *Acrocarpia* and *Caulocystis*, based on *cox*1, *rbc*L and ITS2 DNA sequence analyses. Results from molecular and morphological data suggest that two known *Caulocystis* species are conspecific, and hence should be merged into a single taxon.

The third chapter aims to clarify taxonomic problems originating from the phenotypic plasticity of the Australian and New Zealand endemic genus *Cystophora*. Traditionally, *Cystophora* species identification has been based on morphological features alone. This study applies molecular phylogenetic analyses to reassess *Cystophora* taxonomy. Results demonstrate the competence of molecular phylogenetics for resolving species delineation problems and establishing evolutionary relationships within the genus.

The fourth chapter reports on the true levels of species richness in the brown algal genus *Lobophora* based on *rbc*L and *cox*1 DNA sequences. *Lobophora* is found all over the world, from tropical to warm temperate waters, and wherever it is found, species in this genus are common and conspicuous members of the benthic flora. In Australia there have been only four species recognized so far. However, the molecular data generated from approximately 300 new specimens collected widely around Australia suggest the existence of at least 22 species. Consequently, the species diversity within the genus has been highly underestimated, worldwide. The last chapter is a summary of the findings, including new taxonomic changes and the

significance of molecular phylogenetics in both, solving long standing phylogenetic questions and uncovering hidden brown algal species diversity in Australia.

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Chapter 1

Introduction

1.1 Background

Australia harbors one of the highest diversities of marine macroalgae (Phaeophyceae) in the world (Phillips 2001). This diversity is due to a range of historical and geological factors, particularly the varied number of habitats supported along the coast, from tropical to sub-tropical and temperate systems, including significant variation within some biomes. For example Bolton (1996) compared species diversity of marine brown algae from the coastlines of four warm temperate regions around the world: southern Australia, California, Africa and north-central Chile. Results from 100 km sections of coastline showed that southern Australia had the highest species diversity (over 140 species per section) and also high regional endemism. As a result of this high diversity, the Australian marine flora is a reference for understanding the ecological, taxonomic and evolution of marine plants globally.

The Phaeophyceae, or brown algae, is a group of almost exclusively marine autotrophic organisms that vary in morphology and size, from microscopic filaments to giant plants (Draisma *et al*., 2001; Phillips, 2008; Burrower *et al*., 2008). In temperate Australia they present one of the most conspicuous and ubiquitous elements of the marine flora, where most of the lower intertidal and subtidal rocky and reef habitats are typically dominated by brown algae. Currently, there are around 285 genera (Reviers *et al*., 2007) and 1,792 described species of brown algae worldwide (Guiry 2012). They are ecologically important in coastal marine ecosystems as primary producers, and help build the three-dimensional forest structure that other benthic plants and animals rely on for habitat and refuge. Many brown algae are also economically important in food, paint and chemical industries (Phillips, 2007).

Since brown algae are ecologically and economically important, the taxonomy and systematics of the group have been intensively studied. Traditionally, brown algal

taxonomy has been based primarily on morphological features, such as plant construction, branching patterns, mode of growth, anatomy of vegetative structures and life cycle (Draisma, 2001). Though morphological features usually provide information for classification and species identification, many brown algae are difficult to identify due to morphological simplicity, high levels of phenotypic plasticity, and the extensive presence of analogous or homoplasic characters.

Since the early 1990s, DNA sequencing technology and molecular-based phylogenetic analyses have been used to assist algal taxonomic studies (Draisma *et al*., 2001). Several DNA markers have been developed to aid in the study of phylogenetic relationships of brown algae; for example, the chloroplast-encoded subunit of the ribulose 1,5-biphosphate carboxylase oxygenase gene (*rbc*L), the nuclear encoded ribosomal DNA small and large subunit genes (18S and 26S respectively), and the mitochondrial encoded cytochrome oxidase 1 and 3genes (*cox*1, *cox*3). These DNA regions have been extensively used to reconstruct phylogenetic trees and resolve taxonomic problems at different taxonomic levels in several brown algae taxa (e.g. Rousseau and Reviers, 1999; Draisma *et al*., 2001; Yoon *et al*., 2001; Lee *et al*., 2002, Horiguchi *et al*., 2003; Payri *et al*., 2008; McDevit and Saunders, 2009; Lane *et al*., 2007; Sun *et al*., 2012).

Classification of Australian brown algae have also been mostly limited to morphological work (Womersley, 1987; Huisman, 2000). However, many studies have demonstrated that morphological features on their own are not sufficient to build natural classification systems and to describe the true diversity of brown algal taxa (Charrier and de Reviers, 2012). For example, a study of giant kelp, *Macrocystis*, showed that four morphological species, distinguished from each other based on holdfast and blade shape, are more appropriately classified as a single species based

on molecular analyses of ITS1 and ITS2 regions (Coyer *et al*., 2001). Recently these results were corroborated with more extensive ecological "common-garden" studies (Graham et al., 2007).

In this thesis, I apply molecular phylogenetic analyses to solve specific taxonomic questions in four Australian brown algal genera, *Acrocarpia, Caulocystis*, *Cystophora* and *Lobophora,* in order to generate a better understanding of their diversity and evolution.

1.2 Thesis structure and objectives

This thesis is separated into three data chapters.

Chapter 2: Phylogenetics, DNA barcoding and phylogeographic discrimination within the Australian endemic brown macroalgal genera, *Caulocystis* and *Acrocarpia* (Sargassaceae, Phaeophyceae), based on ITS, *cox*1 and *rbc*L DNA sequences.

Chapter 3: Molecular Phylogenetics of the Australian endemic brown algae genus *Cystophora*(Sargassaceae, Phaeophyceae) based on *cox*1, *rbc*L and ITS analyses.

Chapter 4: Unveiling species diversity of Australian *Lobophora* (Dictyotales) based on *rbc*L and *cox1* DNA sequence analyses.

Chapters 2 and 3 correspond to phylogenetic studies of three common and widespread temperate genera in southern Australia, *Acrocarpia*, *Caulocystis* and *Cystophora*. Species in these genera were previously grouped within a single genus, *Cystophora*, due to shared environmental and morphological similarities (Agardh, 1848, 1870, 1896; De Toni, 1895; Harvey, 1859, 1863; De Toni and Forti, 1923; Sonder, 1852; Womersley, 1950; Nizamuddin, 1960). However, Womersley (1964) separated them into three-genera based on the differences in the shape of the main axes and the distinct ramification patterns of lateral determined branches. The taxonomic status of those genera has only been established on comparative morphology data, and remains untested molecularly. As described above, considering the limitations of morphological characters to depict phylogenetic relationships in brown algae, an independent molecular assessment of the status of these taxa is called for.

In Chapter 2, I compare conventional morphological classification against newly generated molecular-based phylogenetic analyses of *Acrocarpia* and *Caulocystis*. The genus *Acrocarpia* was established by Areschoug (1854) with originally only one member, *A. paniculata*. *A. robusta* was later transferred from *Cystoseira* to *Acrocarpia* by Womersley (1964). These two species have disjunct distributions along the Australian temperate coast. *A. paniculata* occurs around South Australia State to the southeastern coast, while *A. robusta* is limited to southwestern Australia (Chapter 2, Fig. 3). Apart from the distributional differences, the two *Acrocarpia* species differ in their ramuli branching pattern, where the ramuli of *A. paniculata* are simple and those of *A. robusta,* bifurcating. The robustness of this taxonomic grouping has never been formally tested, and in this thesis, I use molecular phylogenetics to test whether *A. paniculata* and *A. robusta* are indeed different species.

Chapter 2 also reports on the molecular and morphological analyses of *Caulocystis*. *Caulocystis* was originally established by Areschoug (1854) at the same time as *Acrocarpia*. The genus consists of two species, *C. cephalornithos* and *C. uvifera*. Unlike *Acrocarpia*, the two species of *Caulocystis* share geographic distribution and also have the same appearance except for the morphology of pneumatocysts (air vesicles). *C. cephalornithos* bear fusiform-shaped vesicles while *C. uvifera* display round-shape vesicles. However, individuals with overlapping phenotypes are often observed (Areschoug, 1854; Womersley, 1964, 1987). In addition, some *Caulocystis* plants display no vesicles at all, making species identification impossible. In chapter 2, I apply molecular phylogenetic approaches to resolve pending taxonomic uncertainty between the two species of *Caulocystis*.

Chapter 3 corresponds to a molecular phylogenetic study of *Cystophora,* a genus endemic to temperate Australia and New Zealand. The genus consists of 25 species of which 23 are found in Australia, six in New Zealand, and only two being New Zealand endemics. *Cystophora* is a foundation species, very common in shallow to subtidal habitats across southern Australia, where it plays a major ecological role in coastal marine benthic communities. Species identification in *Cystophora* has been exclusively based on the morphological classification provided by Womersley (1964, 1987). Though Womersley's species descriptions are highly detailed, some taxa remain difficult to identify, particularly between some species pairs and triplets, for example, between *C. retorta* and *C. siliquosa, C. polycystidea* and *C. expansa*, and between *C. harveyi, C. tenuis* and *C. brownii.* In addition, some species exhibit a wide range of morphological variation, such as *C. subfarcinata* and *C. congesta*; while others still can't be distinguished from each other in the absence of receptacles (i.e. *C. cuspidata* and *C. subfarcinata*). Womersley (1964) was the first to propose a morphology-based reconstruction of the phylogenetic affinities between all known *Cystophora* species. The main characters recognized by Womersley were the degree of flattening or compression of the main axes, and the branching pattern of lateral branches. However, the tree diagram proposed by Womersley remains highly tentative and incomplete, with entirely missing internal branches and nodes. This study aimed to apply molecular phylogenetic approaches to reassess *Cystophora* species classification and identify evolutionary relationships in the genus.

Chapter 4 describes the application of molecular phylogenetic analyses and DNA barcoding approaches to uncover species diversity in *Lobophora*. *Lobophora* is a common brown algal genus in the order Dictyotales that occurs worldwide. Presently, in Australia, there are four recognized *Lobophora* species: *L. australis*, *L.*

nigrescens, *L. rickeri* and *L. variegata*. Previously, *Lobophora variegata* was the only known species for the entire continent, and occurred from tropical to temperate coasts (Womersley, 1977). Recently, Kraft (2009) described a new species, *L. rickeri*, collected from the southern Great Barrier Reef, and Sun *et al*. (2012) also reported two additional species from southern Australia, *L. nigrescens* and the newly described *L. australis*. The recent reports of Kraft (2007) and Sun et al. (2012) were obtained from only a few specific locations in Queensland and southern Australia. Apart from these locations, all Australian *Lobophora* specimens are still classified as the cosmopolitan, *Lobophora variegata*. In Chapter 4, I explore species diversity and uncover cryptic species of Australian *Lobophora* using DNA species delimitation approaches.

Overall, this thesis uses molecular phylogenetic analysis to (i) improve estimates of species diversity in selected brown algal genera in Australia, (ii) test the current taxonomy with newly generated morphology-independent data sets.

1.3 Molecular phylogenetic background

1.3.1 Phylogenetic Classification

Biological classifications are systems that show relationships among the organisms. Several kinds of classification have been applied to biological classification, for example, classification of natural kinds (members of the group possess the same properties), historical classifications or systematizations (members of the group have direct historical relationships). Phylogenetic classifications are biological classifications that systematize the names using phylogenetic trees as a reference for proposing and building classification schemes.

1.3.2 Phylogenetic Methods

Several methods and algorithms have been developed to construct phylogenetic relationships from both morphological and molecular data, each with their advantages and limitations. Most studies at present apply different methods for phylogenetic reconstruction to get more reliable results. If different methods and approaches produce the same or similar results, this is seen as a measure of accuracy and robustness of data.

Distance Methods

Distance methods are fast and simple methods for reconstructing phylogenetic trees. Algorithms calculate trees based on a matrix of pairwise distance values. The two most widely used methods for constructing distance trees are UPGMA (Sneath and Sokal, 1973) and Neighbor Joining (Saitou and Nei, 1987).

UPGMA

UPGMA method (Unweighted Pair-Group Method with Arithmetic Mean, Sneath and Sokal, 1973) is the simplest method for tree reconstruction. The algorithm searches for the pair of taxa with the least distance between them, and defines branching at a midway distance. Then the algorithm forms the two taxa into a cluster. The distance matrix of newly formed cluster and the remaining taxa are calculated again and the process is continued until all taxa are clustered. UPGMA method assumes that the evolutionary rate is the same in all branches that produce rooted ultrametric tree.

Neighbor Joining

Neighbor Joining or NJ (Saito and Nei, 1987) is similar to UPGMA in that it is distance matrix based, but differs in that NJ calculate internal node distances directly and it does not assume that all taxa are equidistant from the root. NJ algorithm starts with calculating the net divergence of each taxon from the sum of the individual distances from the taxon. The net divergence is then used to calculate an either corrected or uncorrected distance matrix. The NJ method searches for the pair of taxa with the minimum distance and creates a node. The new matrix is then created where the new node is substituted instead of those two taxa.

Though distance methods are fast and simple, the information about evolution of particular characters is lost because the process summarizes aligned DNA sequence data as a pairwise distance matrix. Also, several mathematical corrections have been developed to model DNA sequence evolution and account for excess mutations, which in itself leads to the reduction of information in the dataset.

Parsimony Methods

Parsimony methods are based on assumption that the best and most accurate phylogenetic tree is the tree that contains the fewest evolutionary changes. The total number of evolutionary changes, or tree length, are calculated by using the number of changes at all sites; invariant characters and characters that occur in one terminal taxon are ignored. Parsimony methods are reliable when homoplasy is rare but the methods may give misleading tree from long-branch attraction effect (Swofford et al., 1996; Bergsten 2005), where the terminal clades that are not closely related can be mistakenly clustered together.

Maximum Likelihood

Maximum Likelihood or ML algorithms (Felsenstein, 1981) search for the tree with the highest probability of the observing data given a defined model of evolution, the tree topology and the branch lengths between nodes. The model is used to calculate probability of observing data in a particular tree, and maximum likelihood methods search for the tree with the highest log-likelihood score. ML methods for DNA sequence data depend on an explicit model of molecular evolution and suitable model parameters. An appropriate model is necessary for obtaining the correct tree although the method has been proven very robust against deviations from optimum models. Furthermore, the algorithm is computational intensive.

Bayesian Inference

Bayesian analysis and ML are similar in that they both use likelihood calculation as the basis for inference, but Bayesian analysis calculates probability of

tree topology and model of evolution from the data, while ML computes the probability of the data given a defined model of evolution (Yang and Rannala, 1997). Bayesian analysis is based on the posterior probabilities, the probabilities estimated from the prior probability calculated from observed data and the likelihood derived from a probability model for the data to be observed. The algorithm searches for the tree topology that has the highest posterior probability and the favored method to explore posterior probability space is Markov Chain Monte Carlo (MCMC) integration. MCMC explores space of trees and parameters starting with randomly picking a model (tree topology), and then it randomly selects the second model and compares posterior probabilities, the higher probability is preferred. By running MCMC long enough, the area of highest posterior probability density is sampled. Bayesian analysis is faster than ML algorithm. Because Bayesian analysis infers the best tree from observed (not modeled) data. However the data set must be accurate to get an accurate tree.

For comprehensive accounts of each major analytical method, including step-by-steps descriptions on how the different methods of phylogenetic reconstructions work, the readers are referred to the excellent reviews by Swofford et al. (1996), Posada et al. (2001), Huelsenbeck et al. (2001), and Hall (2011)

1.3.3 Molecular Markers

Several techniques in molecular biology have been used to clarify macroalgal systematics; all of them have strengths and limitation.

Restriction fragment length polymorphism (RFLP)

RFLP is a technique that detects variation in homologous DNA sequences using restriction enzymes. After the enzyme digestion, the restriction DNA fragments are separated according to their length by electrophoresis, and followed by Southern blotting. DNA variation is visualized by radioactively or chromatically labelling DNA fragments or RFLP probes that hybridize with one or more fragments of the digested DNA on the blotting paper. Practical protocols for RFLP analysis are provided by Dowling et al. (1996) and Palmer (1986).

RFLP markers can be applied for estimation of gene diversity and population structure. The markers are also useful for phylogenetic and phylogeographic analyses but depend on the DNA sequence they derived. Though this method can detect large amounts of variation and are highly repeatable, it requires large amounts of DNA for the analyses and data from different laboratory are difficult to combine.

Microsatellites (SSRs)

Microsatellites are repetitive segments of two to five nucleotides that are assumed to be randomly distributed throughout the genome in non-coding regions. SSRs detect changes in number of repeat units using primers specific to the variable SSR and the polymorphism is detected using PCR and products length are identified using an agarose gel separation or DNA sequencing. Protocols and analyses for generating SSRs data can be found in Karp, Isaac, and Ingram (1998), Scribner and Pearce (2000).

SSRs are primarily applied to estimate gene diversity, population structure and analysis of gene flow because the gene regions targeted primarily identify differences

between individuals. Phylogenetic analysis of SSR data is limited because of the homoplasy.

Randomly amplified polymorphic DNA (RAPD)

RAPD markers are DNA fragments from PCR amplification using single arbitrary oligonucleotide primer. The annealing sites for the primer are scattered throughout the genome and phenotype patterns resulting from separation of PCR products are visualized on an agarose gel. The detailed RAPD protocols and analysis are provided by Weising at al. (1995) and William at al. (1993).

This method is cheap, requires small amount of DNA and is simple, but the PCR conditions and parameters can greatly affect the results. RAPD is useful for initial investigation, but the method has limited application and other methods are now preferred. RAPD technique has been used in phylogenetic and phylogeographic studies though the use is controversial since RAPD data cannot be ordered.

Amplified fragment length polymorphism (AFLP)

The AFLP technique (Mueller and Wolfenbarger, 1999; Savelkoul et al., 1999) is based on the selective PCR amplification of restriction fragment generated by the digestion of restriction enzymes. The amplicons are separated by electrophoresis to visualize band patterns.

AFLP is very reliable and highly polymorphic but the method requires large amounts of high quality DNA. The AFLP method is often applied to genome mapping and breeding studies. Phylogenetic and phylogeographic studies have applied AFLP analysis but it's not sensitive enough to detect single nucleotide changes and the reconstruction of phylogenies is prone to error.

DNA sequencing analysis

DNA sequencing techniques and PCR are applied to target specific DNA regions. The DNA sequence data make it possible to access changes in nucleotide composition, the ultimate genetic code. DNA sequencing analysis can be used in population genetic studies and the techniques are the information of choice for phylogenetic and phylogeographic analyses.

Gene markers of choice:

*rbc*L

The large subunit of ribulose-1,5-bisphosphate carboxylase oxygenase (*rbc*L) is one (the larger) of the two subunits of the key enzyme on CO_2 fixation during Calvin Cycle in photosynthesis. *Rbc*L is a single copy gene encoded in the chloroplast genome and is 1,467 bp in length in brown and red algae (Draisma et al., 2001). It was the first chloroplast gene ever sequenced (McIntosh et al. 1980). Many studies of molecular systematics on brown algae recommend *rbc*L s a good marker for revealing phylogenetic information across distinct levels of biological organization, from genus (Draisma et al., 2002; Phillips et al., 2005) to order (Bittner et al., 2008 (Dictyotales); Heesch et al., 2008(Sphacelariales)).

ITS region

Internal transcribed spacer (ITS) is a region of non-coding DNA located between the large and small subunits of the nuclear ribosomal DNA cistron. ITS consists of two regions; ITS1 and ITS2, with a short 5.8S gene located between the two regions. ITS regions have been applied with different levels of success to

reconstruct phylogenetic relationships at the family, genus and species levels in brown algae (e.g. Sasaki and Kawai, 2007 (Genus *Chorda*, Chordaceae, Laminarales); Stiger et al., 2003 (Genus *Sargassum*, Sargassaceae, Fucales)).

*Cox*1

Cytochrome oxidase subunit I (cox1) is the main subunit of a key enzyme in cellular respiration, it is encoded in the mitochondrial genome and is 1,529 bp in length. The mutation rate of *cox*1 is fast enough to distinguish species that are closely related and is primarily used for DNA barcoding in animals (Rubinoff, 2006) and red algae (Saunders 2005). McDevit and Saunders (2009) showed that *cox*1 is a competent marker for DNA barcoding and species differentiation in brown algae (29 species from five order).

1.3.4 Concept of species

A species concept is required for taxonomic classification. Several concepts have been proposed which can be broadly classified into pattern-based concepts and processed-based concepts.

Pattern-based concepts

Pattern-based concepts rely on identifying patterns of features (morphological or genetic variation) to define one group of species from another. Three widely applied pattern-based species concepts are taxonomic species concept, phylogenetic species concept and evolutionary species concept.

Taxonomic species concept

This concept identifies the members of the same species based on shared morphological features (Sokal and Crovello, 1970). The limitation of this concept is

when identifying cryptic species that posses little or no morphological differentiation and species with high phenotypic plasticity.

Phylogenetic species concept

This concept identifies species as the smallest cluster of individual organisms that descended from a common ancestor (Cracraft, 1983). The members of the same species share combinations of diagnosable characteristics (morphological, biochemical, physiological and behavioral) that other species lack. The limitation of this concept occurs when the morphologically different populations are connected by gene flow. Also, this concept fails to establish the level of differentiation to recognize different species.

Evolutionary species concept

Simpson (1961) defined evolutionary species as a series of ancestor descendent populations passing through time independently. Extinct species are also acknowledged and the lineage is recognized to be a transformation of several species. This concept is widely used in a paleontological context. However, the problems of this concept are that several criteria are used to define evolutionary species and can be arbitrary between species when gaps in the fossil record occur.

Process-based species concepts

Process-based species concepts define a group of species based on demonstrating the process of evolution and isolation. Many process-based species concepts were proposed but the most widely known is biological species concept.

Biological species concept

This concept was defined by Mayr (1942) as "groups of [actually or potentially] interbreeding natural populations which are reproductively isolated from other such groups". BSC is broadly used to identify species based on reproductive isolation; however, the concept cannot be applied to asexual organisms. Furthermore, BSC is not applied in the case of limited hybridization, where organisms occasionally form hybrids.

Recognition species concept

The concept recognizes member of species as a set of organisms that share common fertilization system (Patterson, 1985). Recognition species concept differs from BSC in that the hybrid producing from the members of species can be either fertile or sterile.

Ecological species concept

Van Valen (1976) defined ecological species as "a lineage or closely related set of lineages, which occupies an adaptive zone minimally different from that of any other lineage in its range and which evolves separately from all lineages outside its range". This concept recognizes species based on ecological niches and the development of genotypic selection. However, the concept cannot be applied when there are more than one species shared the same niche.

In this study I primarily apply the phylogenetic species concept, recognizing species based on patters of phylogenetic gene clustering.

1.4 Taxonomic description of the focal species and genera in this study.

Acrocarpia Areschoug, 1854

Plants dark brown to black. Holdfast lacerate or split basally, 0.5-2.5 cm in diameter, epilithic. Main axes tristichously to irregularly branched, bearing bushy upper parts and denuded with pointed scars on the lower parts. Vesicles absent.

Acrocarpia paniculata (Turner) Areschoug 1854

Plants 20-100 cm high. Ramuli simple, terete, 1-5 cm long and 0.4-0.7 mm thick. Receptacles develop panicles from the ends of ramuli, 1-4 mm long, 0.5-1 mm thick, terete to torulose. Conceptacles are numerous, bisexual, scattered on receptacles. Oogonia are sessile, ovoid, 80-120 μm in diameter. Antheridia are sessile, 20-25 μm long and 6-10 μm in diameter.

Acrocarpia robusta (J. Agardh) Womersley 1964

Plants 10-50 cm high with one to several primary axes arising from the holdfast. Laterals irregularly to spirally branched, 1-2 mm apart, 1-6 cm long. Ramuli furcate near the base, 1-4 mm long and 0.2-0.4 mm thick. Receptacles develop panicles from the ends of ramuli, 1-4 mm long, and 0.5-1 mm thick, terete and pedicellate. Conceptacles numerous, bisexual, scattered on receptacles. Oogonia sessile, ovoid, 80-150 μm in diameter. Antheridia sessile, 20-40 μm long and 7-10 μm in diameter.

Acrocarpia consists of only two species, *A. paniculata* and *A. robusta*. *Acrocarpia paniculata* has simple ramuli and distributed around South and Southeastern Australia, while *Acrocarpia robusta* has bifurcate ramuli and only found

in Western Australia. The type species of this genus is *A. paniculata.* *Acrocarpia* is closely related to *Caulocystis* in their morphology but the former genus differs in the lack of vesicles, lacerate holdfast, and the presence of a tristichous ramification pattern of the lateral branches of determined growth.

***Caulocystis* Areschoug, 1854**

Plants medium to dark brown, 10-100 cm high. Holdfast discoid-conical, 0.3-1 cm in diameter, epilithic. Primary branches terete, 2-4 mm in diameter, radially branched and denuded at the lower part with short residues. Laterals alternately to subdichotomously branched, 3-9 cm long. Receptacles develop from the ends of ramuli, 3-10 mm long, and 1-2 mm thick, terete to lanciform, smooth. Conceptacles numerous, bisexual, scattered on receptacles. Oogonia sessile, ovoid. Antheridia sessile.

***Caulocystis cephalornithos* (Labillardiere) Areschoug 1854**

Main axes branches near the base bearing 1-4 primary branches. Vesicles fusiform, ovoid to elongate, 3-10 mm long and 2-5 mm in diameter. Oogonia 60-130 μm in diameter. Antheridia 20-30 μm long and 6-10 μm in diameter.

***Caulocystis uvifera* (C. Agardh) Areschoug 1854**

Main axes branches near the base bearing 1-6 primary branches. Vesicles pedicellate, subspherical to ovoid, 3-9 mm in diameter and smooth. Oogonia 80-160 μm in diameter. Antheridia 20-30 μm long and 7-10 μm in diameter.

The genus *Caulocystis* also comprises only two species, *C. cephalornithos* and *C. uvifera*. These species are distinguished from each other by the shape of pneumatocysts (air vesicles), with *C. cephalornithos* exhibiting fusiform pneumatocysts and *C. uvifera* bearing spherical pneumatocysts.

***Cystophora* J.Agardh, 1841**

Cystophora as a genus can be easily recognized by the presence of a bilateral sympodial main axis ramification (or branching), and main axes bearing lateral branches of limited growth (i.e. determined growth). The type species of this genus is *C. retroflexa.* Key morphological features used in *Cystophora* species identification are listed on Table 1.1. Full species descriptions of *Cystophora* are listed in Appendix 5.

Table 1.1. Morphological features used in *Cystophora* species identification.

Species	Primary axis	Laterals' branching patterns	Receptacles	Pneumatocysts (air vesicles)
C. botryocystis	Compressed to almost quadrangular with round edges, branched from the face	Alternately and distichously branched	Terete to torulose, conceptacles bisexual or unisexual	Spherical to slightly ovoid, densely clustered on lower part of laterals
C. brownii	Compressed, branched in one plane from the face of the main axes	Alternately distichous, branching with round axils, slender	Terete to strongly torulose	Absent
C. congesta	Compressed, fairy straight,	Tufty, irregularly branched	Slightly compressed,	Subspherical, occasionally

	main axes		awn	
C. cuspidata	Compressed, fairly straight, branced from the face of the main axes	Irregularly, alternately branched	Simple or branched with prominent swollen conceptacles	Absent
C. cymodoceae	Flattened, branched from the face of the main axes	Alternately, subdistichous to irregularly branched	Terete, simple or branched with sterile terminal awn	Usually numerous, elongate to subspherical replacing lower part of the ramuli
C. distenta	Compressed, strongly zig-zag, branched from the face of the main axes	Alternately distichously branched, with round axils	Compressed, torulose with rows of prominent conceptacles	Spherical to subspherical
C. expansa	Compressed, fairy straight, branched from face of the main axes	Tristichously branched	Terete to slightly compressed, slender	Small, elongate to ellipsoid, occasionally absent
C. gracilis	Ovoid to compressed, branched from the face of	Alternately to irregularly branched, in one plane	Terminating dichotoumous ramuli with sterile terminal	Absent

	the main axes		awn	
C. grevillei	Terete	Alternately distichous branched, with round axil	Subterete to ovoid in cross section	Usually present, spherical to slightly ovoid replacing basal ramuli
C. harveyi	Compressed with narrow edges, branched from the face only from the center of the main axes	Alternately distichously branched, in one plane	Terete to slightly compressed	Absent
C. intermedia	Oval in cross section, branched from the side of the axes	Alternately to subdichotomously branched, in one plane	Terete to torulose	Absent
C. monilifera	Compressed with narrow edges, branched from the face of the main axes	Tristichously branched from subterete rachis	Terete, slender, constricted between receptacles	Usually present, subspherical to ovoid replacing the basal ramuli of laterals
C. moniliformis	Flattened, fairly straight,	Alternately distichously	Moniliform to torulose	Absent

	branched from the edge of the main axes	branched		
C. pectinata	Terete to slightly ovoid or compressed,	Alternately distichous with simple, compressed ramuli	Transformed from ramuli, compressed	Absent
C. platylobium	Flattened, slightly flexous, branched from the edge of the main axes	Alternately distichously branched, complanate	Strongly flattened, lanceolate	Vesicles usually present, subspherical to ovoid
C. polycystidea	Compressed, quadrangular, branched from the face of main axes	Radially and spirally branched	Distantly moniliform	Small, numerous, elongate, tapering at both ends
C. racemosa	Compressed, branched from the face of the axes	Alternately distichous with simple, compressed lanceolate ramuli	Lanceolate to slightly torulose	Usually present, spherical to slightly ovoid replacing basal ramuli
C. retorta	Compressed, relatively straight, branched from the	Alternately distichously branched with round axils	Terete to slightly compressed	Usually absent, ellipsoid to ovoid replacing basal ramulus

	face of the main axes			
C. retroflexa	Compressed, branched from the face of the axes	Irregularly branched on all sides	Compressed to slightly torulose, apex attenuate with steril awn	Ovoid to spherical replacing basal ramuli of laterals
C. scalaris	Compressed, zig-zag, branched from the face of the main axes	Alternately distichously branched, complanate	Ovoid to compressed, smooth to torulose	Spherical to ovoid
C. siliquasa	Quadrangular to square, branched from the face of the main axes	Alternately distichously branched with round axils, complanate	Slightly to strongly compressed, conceptacles unisexual and thalli dioecious	Absent
C. subfarcinata	Compressed, slightly flexous, branched from the face of the main axes	Irregularly alternatly branched	Simple or branched with prominent swollen conceptacles separated by sterile tissue	Usually present, absent in rough water form, elongate ovoid to subspherical

C. tenuis	Compressed, branched from the face of the axes	Alternately distichously, branched in one plane	Terete to slightly compressed	Absent
C. torulosa	Compressed, branched from the face of the axes	Densely, irregularly to alternately subdistichously branched	Terete to three side swollen conceptacles	Subspherical to ovoid replacing basal ramuli
C. xiphocarpa	Flattened, branched from the face of the main axes	Unbranched, lanceolate, or branched in one plane	Strongly flattened, broadly lansiform	Absent

***Lobophora* J.Agardh 1894**

There are 11 species of *Lobophora* recognized worldwide (Guiry and Guiry 2013). *Lobophora* plants belong to the tribe Zonarieae based on the presence of a terminal row of apical cells along the margin of their blade-like thalli (Womersley 1987). Plants are medium to dark brown, but become dark brown to black when dried. Thalli are fan-shape, arising from rhizoidal holdfast and fronds can be erect, prostrate or encrusted on the substrate. Thalli are usually 7-12 cells thick with a distinctly larger central cell layer.

Currently, only four *Lobophora* species are recognized in Australia, *L. australis*, *L. nigrescens*, *L. rickeri* and *L. variegata*. Species distributions of the four known species in Australia are illustrated in Figure 1.

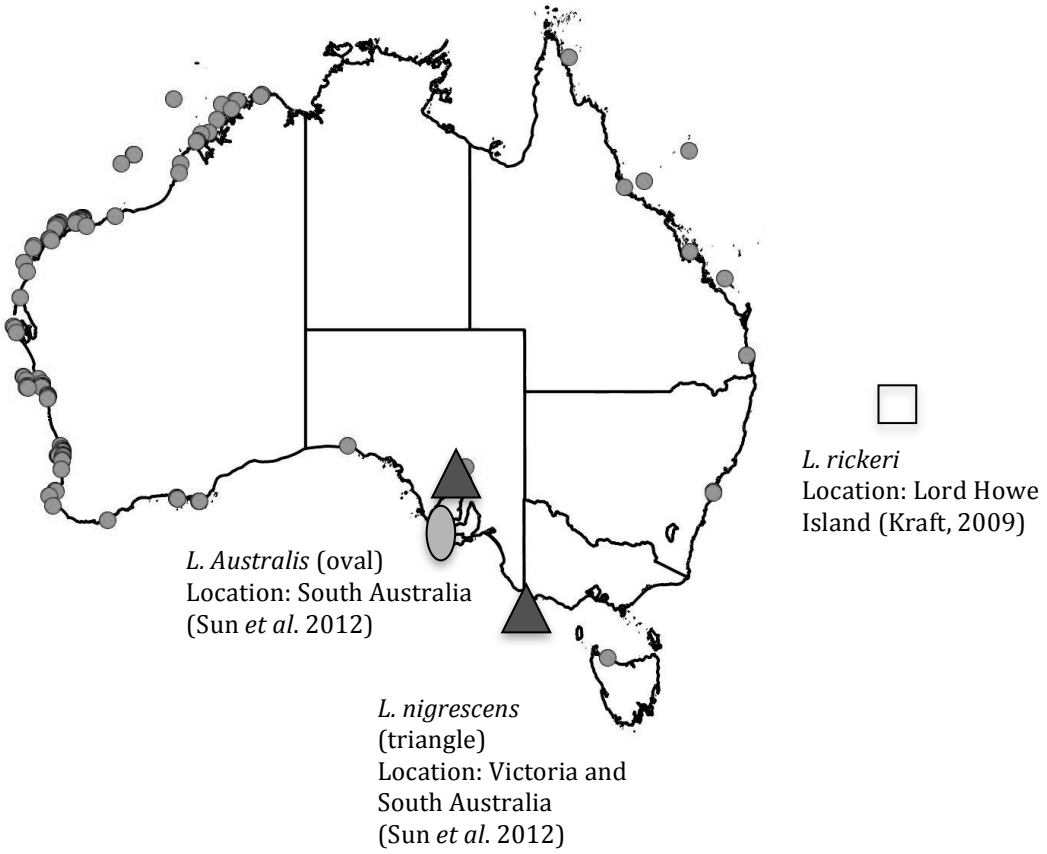

Fig 1. *Lobophora* species distribution in Australia based on Australia Virtual Herbarium database (2014), Kraft (2009) and Sun et.al. (2012). *L. veriegata* distributes around Australia (small dots) and other *Lobophora* species distributions are marked on the map.

Chapter 2: Phylogenetics, DNA barcoding and phylogeographic discrimination within the Australian endemic brown macroalgal genera *Caulocystis* and *Acrocarpia* (Sargassaceae, Phaeophyceae) based on ITS, *cox*1 and *rbc*L DNA sequences

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Abbreviations: Bayesian phylogenetic inference, BI; Bayesian posterior probabilities, PP; bootstrap proportion values, BP; *cox*1, cytochrome oxidase 1; ITS, internal transcribed spacer of the nuclear ribosome cistron; *rbc*L, large subunit of the Ribulose 1,5-bisphosphate carboxylase/oxygenase; Maximum likelihood, ML.

Statement of Authorship

Phylogenetics, DNA barcoding and phylogeographic discrimination within the Australian endemic brown macroalgal genera *Caulocystis* and *Acrocarpia* (Sargassaceae, Phaeophyceae) based on ITS, *cox*1 and *rbc*L DNA sequences

This chapter has been prepared as a submission for publication

Nuttanun Soisup
Executed fieldwork, laboratory work and data analysis, prepare manuscript as principal author.

Signed
Date: 26 July 2013

Andrew Lowe
Provided guidance with data analysis and contributed to revisions of the manuscript

Signed
Date: 26 July 2013

Fred Gurgel
Involved in developing the idea, executed fieldwork, provided guidance with laboratory work and data analysis, and contributed to revisions of the manuscript

Signed
Date: 26 July 2013

Abstract

Acrocarpia Areschoug and *Caulocystis* Areschoug are brown macroalgae genera endemic to the Australian temperate coast. The taxonomy to date of these species has been based on morphological characters alone, and their taxonomic distinctness has yet to be supported by molecular investigations, with all species previously being grouped within the genera *Cystophora*. The two species of *Acrocarpia* are morphologically distinct with discrete distributions, *A. paniculata* found in southeastern Australia and *A. robusta* restricted to southwestern Australia. In contrast, the two species of *Caulocystis* occur along the southern Australian coastline and both species share morphological features, except for the shape of pneumatocysts (air vesicles). *Caulocystis uvifera* exhibits round pneumatocysts while *C. cephalornithos* has fusiform pneumatocyst. However, species identification of *Caulocystis* remains problematic because of the wide variation in pneumatocyst shape. This study employed three molecular markers, encoded by three distinct genomic compartments (*cox*1 - mitochondrial, *rbc*L – chloroplast and ITS2- nuclear) to provide an independent test of the phylogenetic distinctness of these taxa. Results showed that the genera *Acrocarpia* and *Caulocystis* are phylogenetically distinct. In addition the two *Acrocarpia* species are phylogenetically distinct from each other and form a single clade with high support across all markers. However while the *Caulocystis* taxa formed a single clade with high phylogenetic support, there was extremely low genetic variation and no pneumatocyst morphological congruence. Results suggested that the two species of *Caulocystis* are conspecific, with *Caulocystis cephalornithos* bearing the name priority.

Cytoplasmic markers for *Caulocystis* exhibited phylogeographic structure between populations from South Australia and southeastern Australia (i.e. Victoria, NSW and Tasmania), a pattern that is highly concordant with those of other sympatric benthic marine species.

Introduction

Acrocarpia and *Caulocystis* are Australian endemic temperate brown algal genera in the order Fucales (Sargassaceae, Pheaophyceae). They are common, conspicuous benthic macroalgae ranging between 10 to 30 cm in length (although 150 cm long specimens have been recorded), found in most intertidal to subtidal habitats characterized by hard substrate and clear waters with some degree of wave exposure. These two genera are some of the most widely distributed canopy-forming macroalgae in temperate Australia. While *Acrocarpia* specimens tend to occur as isolated individuals or in small clumps, *Caulocystis* species can often be found forming medium sized intertidal meadows in the upper subtidal and lower intertidal regions.

In 1854, Areshoug established, the two genera, but not long before that they were referred to as synonyms of *Cystophora* by J. Agardh (Harvey 1859, 1863, J. Agardh 1870, 1896, Womersley 1950, Nizamuddin 1960). In 1964, Womersley monographed *Cystophora*, and related genera, and considered *Acrocarpia* and *Caulocystis* distinct from *Cystophora* based on overall habit morphology and distinct main axis branching pattern.

The genus *Acrocarpia* contains two species: *A. paniculata* and *A. robusta*. The genus is distinguished from closely related taxa by presenting an almost hapteroid holdfast, terete main axes which are tristichously to radially branched, lack of pneumatocysts (air vesicles responsible for improving plant upright flotation) and dense ramuli (equivalent to leaves in higher plants) which are thin (< 1.2 mm in diameter) and cylindrical (Fig. 1). Differences in ramuli thickness, branching pattern and density; density and distribution of secondary branches along main axes, and geographic distribution separate the

two species of *Acrocarpia*. *Acrocarpia paniculata* is distributed from South Australia to the eastern coast, has main axes more homogeneously covered with dense secondary branches, all of which are themselves covered with progressively slender laterals and thicker ramuli (> 400 μm in diameter). *Acrocarpia robusta's* geographic range is limited to the southwestern coast (Fig. 3), has secondary branches on the main axes more concentrated towards the apices of the thallus, and thinner ramuli (< 350 μm in diameter) that are bifurcated at the base. The genus was originally established with only one species, *A. paniculata* (Areschoug 1854), but Womersley (1964) later transferred *Cystoseira robusta* to the genus. Since 1964, species identification in *Acrocarpia* has been based on Womersley's morphology-based monograph and no molecular studies have so far focused on testing the phylogenetic position of these two species within the genus or between closely related genera as predicted on morphological grounds (as per Womersley 1964, 1987).

The genus *Caulocystis* consists of two species: *C. cephalornithos* and *C. uvifera*. *Caulocystis* as a genus is distinct from *Acrocarpia* and *Cystophora*, the two most morphologically similar taxa in the family, in displaying discoid-conical holdfasts, terete, radially branched and thinner main axes (compared to *Acrocarpia*), and the presence of pneumatocysts (Fig. 2). Only the shape of pneumatocysts separates the two *Caulocystis* species. *Caulocystis cephalornithos* is defined by possessing fusiform pneumatocysts with tapering ends, while *C. uvifera* is defined by presenting perfectly spherical pneumatocysts. Although a pneumatcyst is a prominent feature, some *Caulocystis* plants do not develop pneumatocyst at all. Furthermore, the two forms of pneumatocysts are not always distinct from each other, but

morphological intergrades can be found in the same habitat. Areschoug (1854) and Womersley (1964, 1987) mentioned this variation in pneumatocyst morphology, and Womersley (1964, 1987) suggested that the two taxa might in fact be ecological forms or subspecies of the same species, but without making any new taxonomic proposal. To this day, *Caulocystis* specimens without pneumatocysts are considered 'inadequate' samples and remain unidentified.

Specimens of *Caulocystis* have been used in molecular phylogenetic studies but none have directly tested the validity of these two species using characters other than morphological (Draisma et al. 2010, Silberfeld et al. 2010). A multi-locus time calibrated phylogeny of the entire brown algae showed that the two *Caulocystis* species present genetic differences but variation in pneumatocysts was not considered in that, or any other study to date (Silberfeld et al. 2010). Presently, species identification in *Caulocystis* remains problematic when non-typical pneumatocyst morphologies are found.

In this study, we tested whether species in these two genera are truly distinct from each other using a morphology-independent, multi-marker molecular phylogenetic approach.

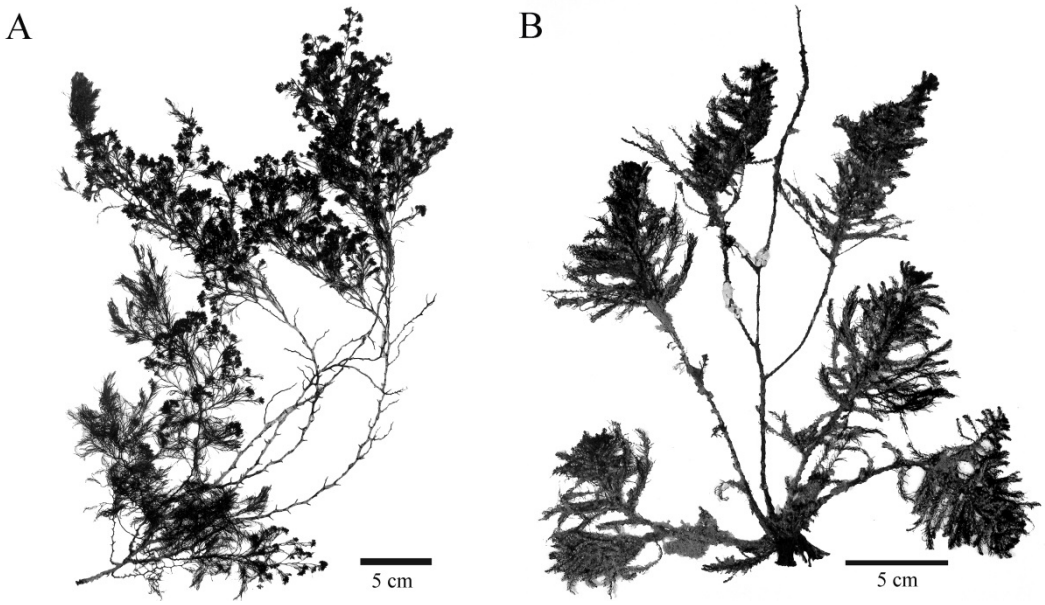

Figure 1. Photographs of the two species of *Acrocarpia*. (A) *Acrocarpia paniculata* (Turner) Areschoug (AD-A95297) and (B) *Acrocarpia robusta* (J. Agardh) Womersley (AD-A89241).

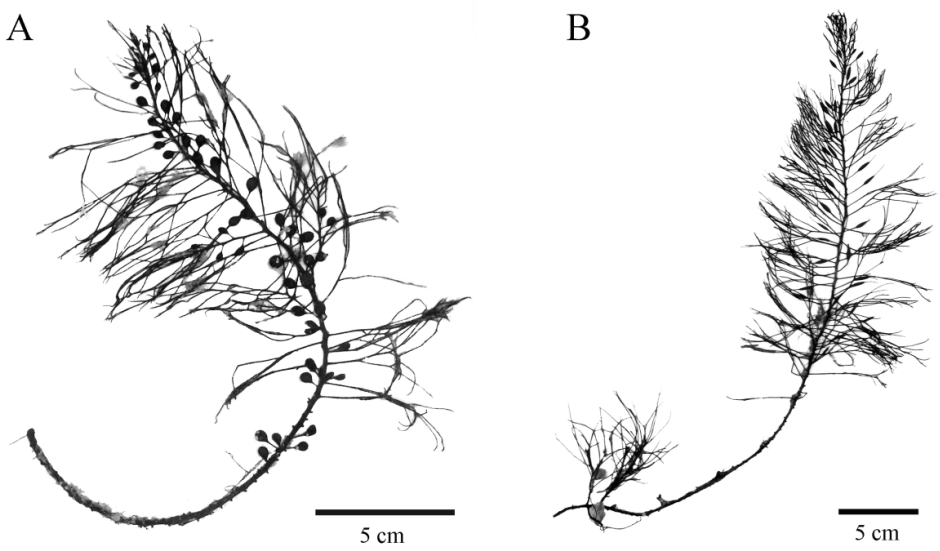

Figure 2. Photographs of the two species of *Caulocystis.* (2A) *Caulocystis uvifera* (C. Agardh) Areschoug (AD-A89188), (2B) *Caulocystis cephalornithos* (Labillardiere) Areschoug (AD-A89185).

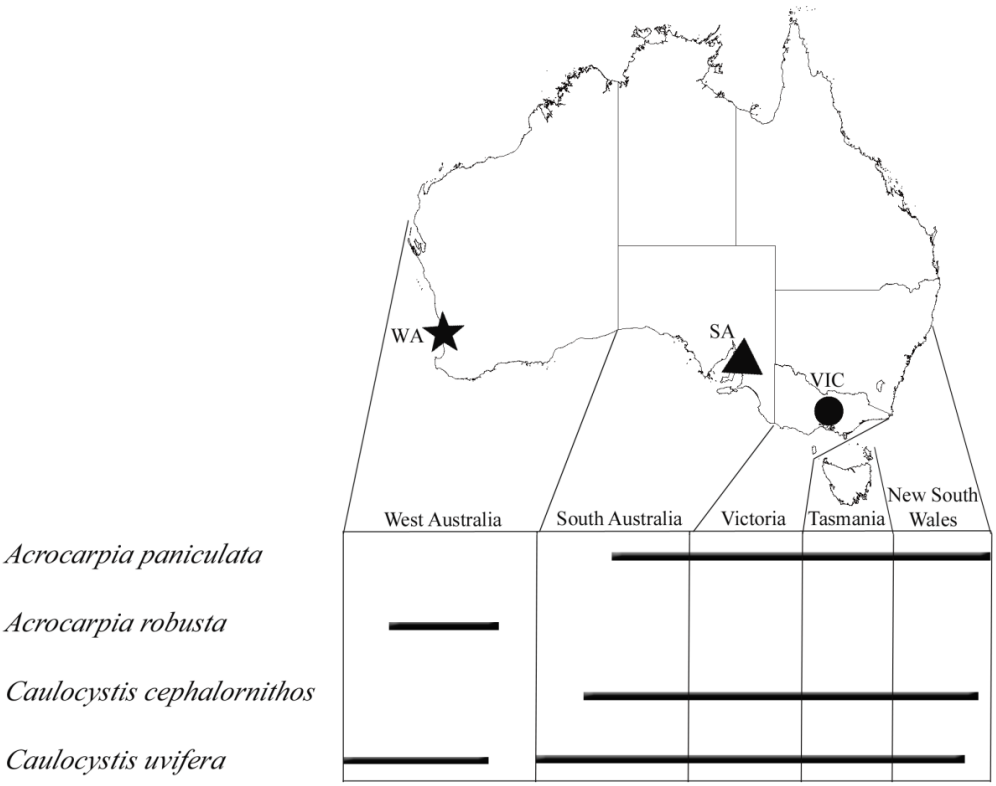

Figure 3. Distribution of the brown algal genera *Acrocarpia* and *Caulocystis* in temperate Australia based on herbarium records data based in the Australia Virtual Herbarium. General collection areas visited in this study were marked on the map (star = Fremantle, West Australia; triangle = Adelaide, South Australia; circle = Queenscliff, Victoria).

Materials and Methods

Collections and morphological analyses

Acrocarpia and *Caulocystis* were collected from West Australia, South Australia and Victoria coasts. A list of specimens used for DNA analyses with collection information and GenBank accessions are provided in Appendix 2. Specimens were morphologically examined according to Womersley (1964, 1987), pressed dry onto herbarium paper, and deposited at the South Australia State Herbarium (AD). Subsamples for DNA analysis were preserved in silica gel desiccant (Chase & Hillis, 1991). *Caulocystis* specimens with different pneumatocyst morphologies were classified into different morpho-groups (see results).

DNA extraction, amplification and sequencing

DNA was extracted from silica gel preserved materials using DNeasy Plant MiniKit (Qiagen, Doncaster, Australia) according to the manufacturer's specifications. Sequences of three well established and commonly used phylogenetic markers in brown algae, the chloroplast-encoded *rbc*L (partial, i.e. only the first ~700 bp from the 5' end were used), the nuclear-encoded ITS2 rDNA region, and the mitochondrium-encoded DNA barcode marker *cox*1 region (partial, i.e. only the first ~650 bp from the 5' end were used) were amplified using primers listed in Appendix 1 (see Chapter 1 as well). PCR amplifications were performed in 25 µl reactions composed of 1 X AmpliTaq Gold PCR buffer (Applied Biosystems, Foster City, CA, USA), 3 mM MgCl2, 0.2 µM of each primer, 0.2 µM of each dNTP, 1 M Betaine, 1.5 units of AmpliTaq Gold DNA polymerase, and 1 µl of DNA template diluted 1:10 or

1:100 in distilled water. The amplification was run on the Palm Cycler (Corbett Research, Australia) and parameters included an initial denaturation at 95˚C for 3 min followed by 40 cycles of 95˚C for 45 seconds, Tm (listed in Table2) for 1 min, and 72˚C for 1.30 min, terminated by 72˚C for 5 min. Products were cleaned using ExoSAP-IT (GE Healthcare, Buckinghamshire, UK) or MultiScreen PCR384 Filter Plate (Milipore, Billerica, MA, USA). Sequencing was performed using ABI BigDye Terminator v 3.1 (Applied Biosystems, Foster City, CA, USA) and sequencing products were cleaned using MultiScreen SEQ384 Filter Plate (Milipore, Billerica, MA, USA). All cleaned products were submitted to AGRF-Adelaide for capillary separation on an ABI 3730xl sequencing platforms.

Phylogenetic Analyses

Contig assembly and ITS2, *cox*1 and *rbc*L DNA sequence alignments were conducted in Geneious Pro v.5.5.2 (Biomatters, Aukland, New Zealand). DNA sequence alignments were performed using the Muscle algorithm with default parameters and then corrected manually. A concatenated dataset was produced with all three markers. Indels in the ITS2 alignments that contained more than 60% deletions across all sequences were removed from the analysis. *Sirophysalis trinodis* (Sargassaceae, Fucales), which is phylogenetically and morphologically distinct from *Caulocystis* and *Acrocarpia,* was designated as outgroup. Maximum-likelihood (ML) and Bayesian Inference (BI) trees were constructed on all alignments independently so results from each marker could be compared between each other and then all together in the concatenated analysis. The most suitable nucleotide substitution model for all alignments

was selected by jModeltest2 (Darriba et al. 2012) according to the Akaike Information Criterion (AIC). The concatenated alignment was partitioned in coding and non-coding regions (see below). The best fit models were HKY+I for ITS2, HKY+G for *cox*1, Trn (or K81)+G for *rbc*L, and GTR+G for both partitions in the concatenated dataset, where HKY refers to Hasegawa et al. (1985) model of nucleotide substitution, Trn = Kimura (1981) model with equal base frequencies, I = a proportion of nucleotide sites being invariable, and G = rate variation of variable sites following a gamma distribution with estimated alpha parameter. Maximum-likelihood analyses were for each independent marker was performed using PhyML 3.0 online execution version (Guindon and Gascuel 2003; Guidon *et al.*, 2005; http://www.atgc-montpellier.fr/phyml/) starting with BioNJ trees, using both options nearest-neighbor interchanges and subtree-prune-regraft moves (NNIs and SPRs), 10 starting random trees, and 1,000 bootstrap replicates. Bayesian Inference was performed in MrBayes v.3.1.2 (Ronquist and Hueselbeck 2003) plug-in on Geneious Pro and consisted of two parallel runs each of four incrementally heated chains, 3 million generations, sampled every 1,000 generations with 10% burn-in. Bayesian phylogenetic analysis on the *rbc*L dataset used the GTR+G model instead of Trn, as the latter is not available in MrBayes, and the GTR+G model was the second best fit model according to the AIC criterion. MCMC runs were monitored within Geneious, effective sample size were more than 500 in all runs, providing evidence that convergence had been reached.

The three-marker concatenated dataset was produced for two species of *Acrocarpia* and six specimens of *Caulocystis* representing five pneumatocyst morphotypes. The ML analysis of the concatenated dataset was run in RAxML

version 1.3 (Stamatakis, 2006, Silvestro and Michalak 2012) with independent rates for each partition, and model parameters estimated over the duration of specified runs. Bootstrap support was performed under the thorough bootstrap option, with 100 replicates. The Bayesian analysis of the concatenated dataset was run in MrBayes v.3.1.2 (Ronquist and Hueselbeck 2003) plug-in on Geneious Pro. The dataset was partitioned by codon and by markers using PartitionFinder V1.0.0 using Bayesian Information Criterion in an unlink analysis which allowed the rates to vary over the partitions. According to the results of PartitonFinder, the most appropriate model for the 1st and 2nd codon positions of *cox*1 and *rbc*L, and ITS2 markers is K80+G and the most appropriate model for 3rd codon position of *cox*1 and *rbc*L is GTR+G. MCMC consisted of two parallel runs each of four incrementally heated chains, 3 million generations, sampled every 1,000 generations with 10% burn-in. MCMC runs were monitored within Geneious, effective sample size were over 500 in all runs, providing evidence that convergence had been reached.

Results

Morphological analyses.

Morphological features of all *Acrocarpia paniculata* and *Acrocarpia robusta* specimens agreed with descriptions in the literature. All *Caulocystis* specimens were classified into six morphological categories, M0 to M5, according to their air vesicle shape (pneumatocysts) as depicted in Fig. 4. Pneumatocyst morphology in *Caulocystis*, a diagnostic feature between the two species, shows the occurrence of intergrades between fully spherical (M1) to fully fusiform (M4), including some newly described, unreported shapes, M3 and M5 (Fig. 4).

Taxonomy and phylogeny

cox1

The *cox*1 alignment contained 11 newly generated DNA sequences and two *Caulocystis* sequences downloaded from GenBank, was 660 bp long, and consisted of 122 variable sites. The ML and Bayesian analyses produced identical tree topologies (only ML tree is shown, Fig. 5). All *Caulocystis* specimens formed a single clade with high support values (BP/PP = 90/0.99, respectively). The uncorrected *p*-distances within the *Caulocystis* clade ranged between 0-1.4% while the distance between the *Caulocystis* and *Acrocarpia clades* ranged between 12.2-13.9%. Within the *Caulocystis* clade, samples from NSW and Victoria, and samples from South Australia formed two distinct sub-clades with 1.2-1.4% sequence divergence between them. Two species of *Acrocarpia* formed a single highly supported clade (BP/PP = 96/1, respectively). The *p*-distant between the two species of *Acrocarpia* was 7.4%

and no genetic difference was observed between the *A. robusta cox*1 sequences.

*rbc*L

A total of 20 newly generated *rbc*L DNA sequences were produced and two *Caulocystis* sequences from GenBank were added to the dataset, producing a 1,322 bp long alignment with 135 variable sites. The ML and Bayesian analyses produced similar tree topologies (only ML tree is shown, Fig. 6). *Caulocystis* displaying different air vesicle morphologies (pneumatocyst morphotypes M0 to M5, Fig. 4) formed a single clade with moderate support (BP/PP = 70/0.69, respectively) and no genetic distinctiveness between specimens with distinct pneumatocyst morphotypes. The uncorrected *p*-distances within the *Caulocystis* clade ranged between 0-0.6% while the distances between the *Caulocystis* clade and its sister *Acrocarpia* clade ranged between 3.7-5.4%. The ML analysis reveals two sub-clades of *Caulocystis* associated with geographic locations, one sub-clade composed of Victoria and Tasmania samples and a second sub-clade composed solely of South Australian samples. The p-distance between the two *Caulocystis* sub-clades was 0.6%. The two species of *Acrocarpia* are phylogenetically distinct from each other and formed a single clade with high support (BP/PP = 99.8/1.0, respectively). The genetic variation between the two species of *Acrocarpia* was 1.6-2.3% and within *Acrocarpia paniculata* was 0.6% while no genetic differences were observed between the two *rbc*L sequences of *A. robusta.*

ITS2

ITS2 alignment contained 20 newly generated DNA sequences, 14 *Caulocystis*, four *Acrocarpia*, and two *Sirophysalis trinodis* as outgroup. The original global sequence alignment provided by MUSCLE was 709 bp long (including outgroup sequences), displayed 241 invariable nucleotide sites (= 34%) and an overall pairwise percentage identity of 79%. When *Caulocystis* ITS2 sequences were aligned independently, the alignment showed no substantial indels (i.e. 5 sites with 3-5 bp long indels) and all sequences could be easily aligned. However the introduction of *Acrocarpia* and *Sirophysalis* sequences caused the appearance of 18 sites that show more than 4 bp long indels, in contrast with other two protein-coding markers which had neither indels nor difficulties in alignment because they are coding DNA regions. After all indels were removed the alignment comprised 554 sites and an overall pairwise percentage identity of 85.5%. Appendix E shows the original alignment with excluded indels marked. The ML and Bayesian analyses showed all *Caulocystis* samples forming a single clade with high support (BP/PP = 98/1 respectively). The uncorrected p-distances within *Caulocystis* ITS2 clade ranged between 0-1.6% while the distant between the two species of *Acrocarpia* clades was 26.7%. All *Caulocystis* specimens formed a single clade, but there was no phylogeographic signal between South Australia and Victoria+Tasmania samples as observed for the two cytoplasmic markers. All 14 specimens in this sub-clade displayed pneumatocysts with different morphologies. The two *Acrocarpia* species formed a clade with high support (BP/PP = 97/1.0 respectively).

Concatenated dataset

The concatenated dataset was 2,485 bp in length, contained six *Caulocystis* sequences representing distinct five morphotypes, two *Acrocarpia* sequences representing the two different species known in the genus and two *Sirophysalis trinodis* sequences as outgroup. *Caulocystis* samples formed a clade with high support (BP/PP =89/0.94). Within the *Caulocystis* clade, the two sub-clades with strong geographic associations were revealed, with samples from Victoria formed a sub-clade with low bootstrap support and relatively high posterior probability (BP/PP =63/0.92) and samples from South Australia formed a sub-clade with high support (BP/PP =90/0.98). Two species of *Acrocarpia* formed a clade with full support (BP/PP =100/1.0). Overall, the three distinct markers from three distinct genomes when analyzed independently and as the concatenated dataset were highly congruent in recognizing the phylogenetic distinctiveness between the two *Acrocarpia* species and the lack of any clear phylogenetic distinction between the two *Caulocystis* species.

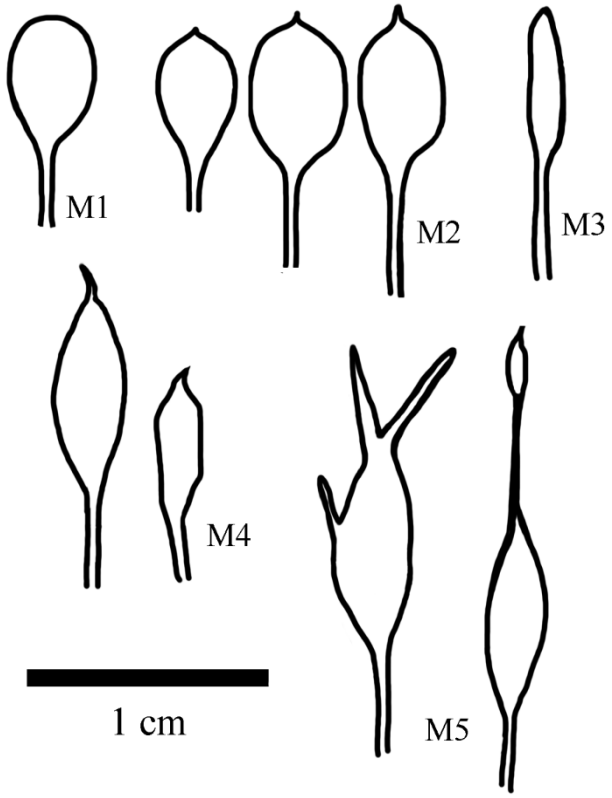

Figure 4. Variations of *Caulocystis* pneumatocyst morphology. Pneumatocyst morphologies were categorized into 6 morphotypes: M0 for absent (not illustrated), M1 for round shape (stereotypical *Caulocystis uvifera*), M2 for round shape with tapering end (often recognized as *Caulocystis uvifera*), M3 for a fusiforme shape (stereotypical *Caulocystis cephalornithos*), M4 for elliptical to fusiform in shapes with short and small awns; and M5 for more unusual forms such as fusiform shapes with long awns or strings of pneumatocysts.

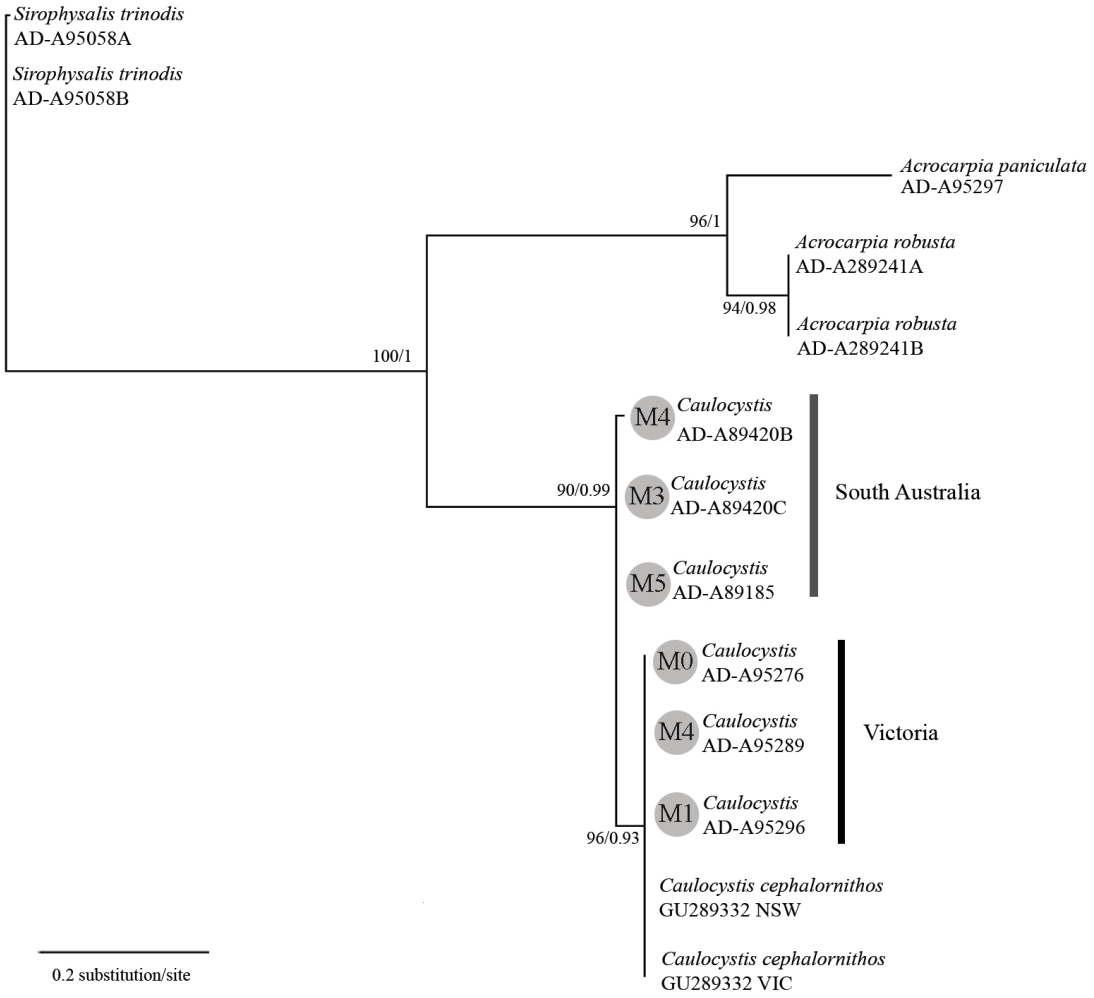

Figure 5. Maximum likelihood tree based on *cox*1 DNA sequences of *Acrocarpia* and *Caulocystis* with *Sirophysalis trinodis* as outgroup. The numbers associated with each branch represent bootstrap proportions based on 1000 replications and Bayesian posterior probabilities, respectively. *Caulocystis* pneumatocyst morphotypes were identified as M0 (absent) to M5 according to morphological classification depicted in Figure 4. *Sirophysalis trinodis* were used as outgroup.

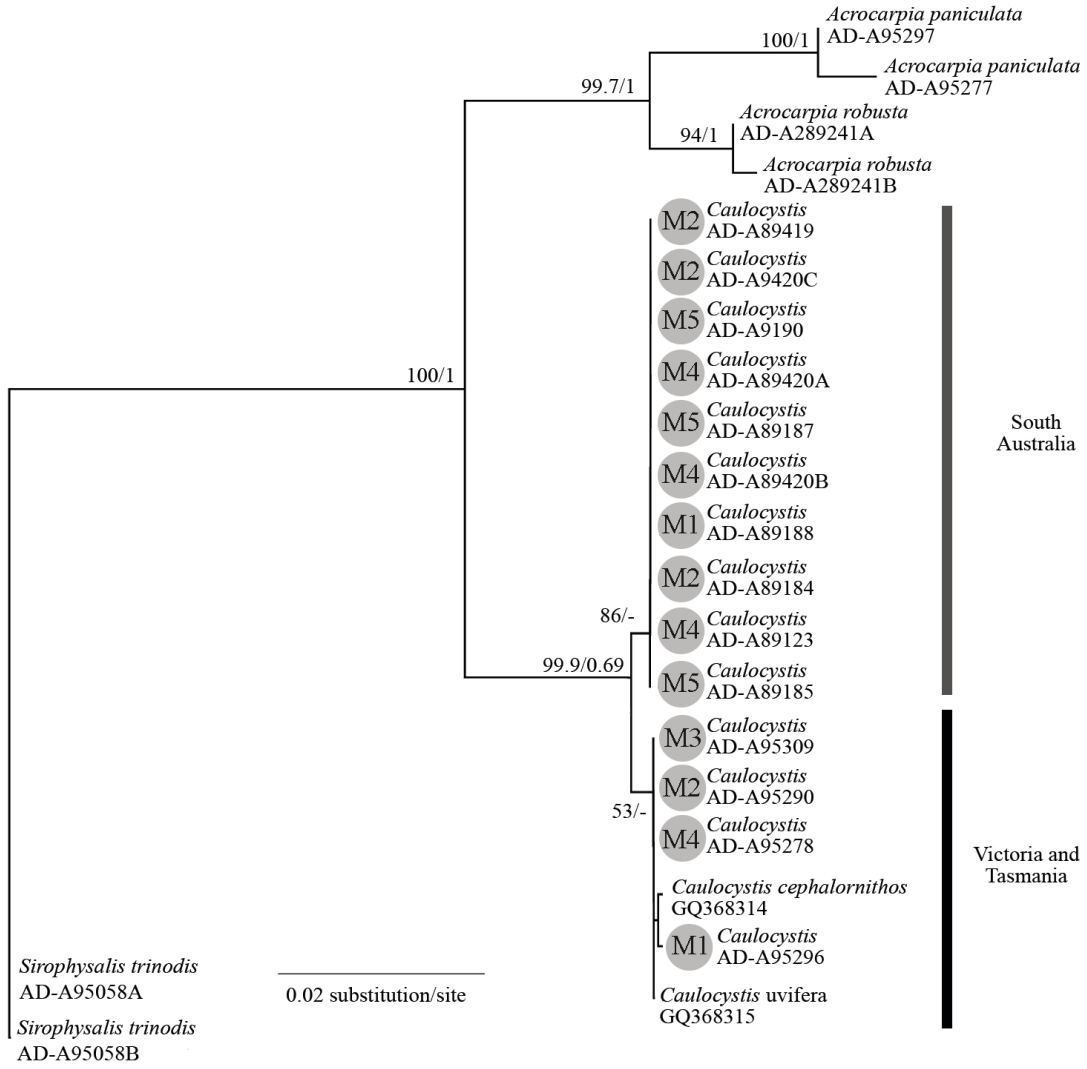

Figure 6. Maximum likelihood tree based on *rbc*L DNA sequences of *Acrocarpia* and *Caulocystis* with *Sirophysalis trinodis* as outgroup. The numbers associated with each branch represent bootstrap proportions based on 1000 replications and Bayesian posterior probabilities, respectively. *Caulocystis* pneumatocyst morphotypes were identified as M0 (absent) to M5 according to morphological classification depicted in Figure 4. *Sirophysalis trinodis* were used as outgroup.

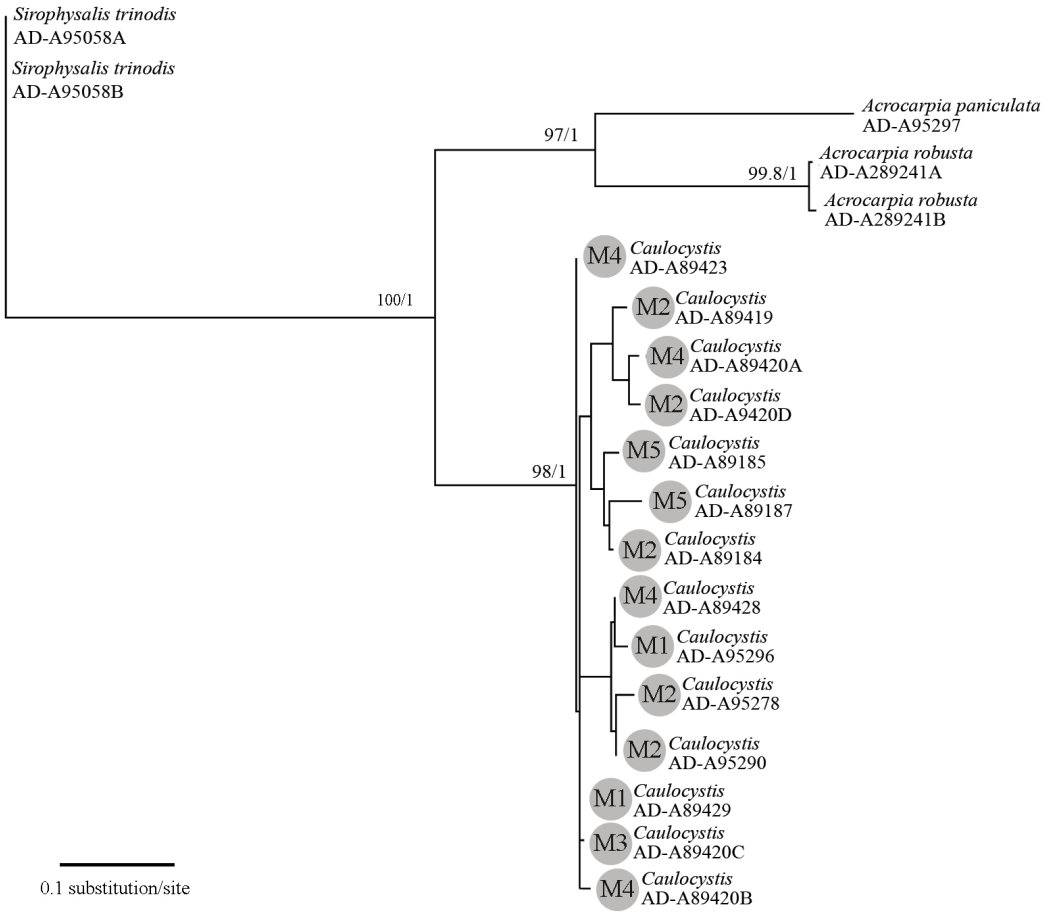

Figure 7. Maximum likelihood tree based on ITS2 DNA sequences of *Acrocarpia* and *Caulocystis* with *Sirophysalis trinodis* as outgroup. The numbers associated with each branch represent bootstrap proportions based on 1000 replications and Bayesian posterior probabilities, respectively. *Caulocystis* pneumatocyst morphotypes were identified as M0 (absent) to M5 according to morphological classification depicted in Figure 4. *Sirophysalis trinodis* were used as outgroup.

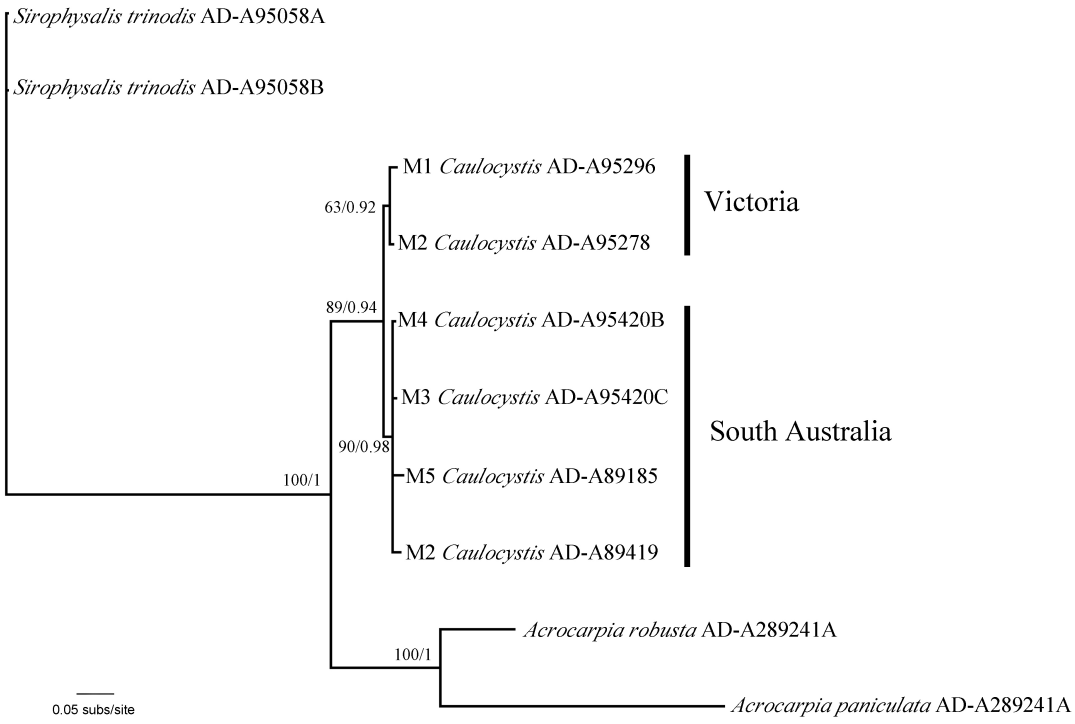

Figure 8. Phylogenetic analysis of combined *cox*1, *rbc*L and ITS2 dataset inferred with the Bayesian analysis method. Numbers at internal nodes indicate support values (Maximum likelihood and Bayesian Inference respectively). *Caulocystis* pneumatocyst morphotypes were identified as M0 (absent) to M5 according to morphological classification depicted in Figure 4. *Sirophysalis trinodis* were used as outgroup.

Discussion

Molecular phylogenetic analyses of the genus *Acrocarpia* using ITS2, *cox*1 and *rbc*L reveal that *A. paniculata* and *A. robusta* are closely related but distinct taxa, and hence these two species should be maintained as originally proposed based on morphological data (Womersley 1965, 1987). However, the results of ITS2, *cox*1, *rbc*L, and the concatenated dataset for *Caulocystis* revealed that *C. cephalornithos* and *C. uvifera* correspond to a single species, which contrasts with the original morphological classification and the current taxonomy of the genus. All *Caulocystis* with different pneumatocyst morphologies clustered together with low to no genetic differences and high phylogenetic support, suggesting that these two *Caulocystis* species should be merged. No genetic association of pneumatocyst morphology was observed across all three distinct genetic markers and genomic regions (i.e. chloroplast, mitochondria and nucleus). The name priority is attributed to *C. cephalornithos* as this species was described first, in 1806, by Labillardiere; compared to *C. uvifera* described in 1824 by C. Agardh. The lectotype for the genus is therefore changed from *C. uvifera* (De Toni 1891) to *C. cephalornithos*. A revised genus and species description are included at the end of this discussion.

Apart from resolving taxonomic uncertainties within *Caulocystis*, results from *rbc*L, *cox*1 and the concatenated dataset also supported the existence of phylogeographic structure between populations from South Australia and those from southeastern regions (i.e. Victoria and Tasmania). Ocean currents, thermal and climate gradients, habitat and substrate discontinuities, and shifts in land bridges associated to changes in sea-level across geological time are recognized as some of the most potent abiotic

factors driving marine phylogeographic and biogeographic structuring (e.g. Miller et al. 2013, Fraser et al. 2010). It is likely that several of these factors, including biotic factors (i.e. life history traits and ecology) are responsible for the genetic structuring observed within *Caulocystis*. Similar phylogeographic patterns have also been detected in other marine benthic organisms across the same geographic range. Genetic discontinuity for continuously distributed marine species in this region have been extensively documented in recent years for a range of animal and plant taxa and using a range of molecular methods. When phylogeographic patterns are found in the region they are attributed to what is known as the Bassian Isthmus Hypothesis or the East-West Genetic Disjunction of the southeastern coast of Australia (Waters 2005, Waters et al. 2008, York et al. 2008, Miller et al. 2013). This hypothesis predicts that during the last glacial maxima (i.e. the Pleistocene) a dry land-bridge between continental Australia and Tasmania, combined with colder temperatures in southern Tasmania, promoted genetic isolation between marine populations to the east and west of this biogeographic barrier. However whether any phylogeographic structure subsequently dissipated or is maintained to the present day remains an open question for most species. Interestingly, along the same region, paleo and extant oceanographic processes are also attributed to the formation of marine biogeographic disjunctions above the species level, in what is known as the shift between the Peronian and the Flindersian marine provinces along southeastern Australia (Bennett and Pope 1953, Waters et al. 2010). Similar phylogeographic structuring has been reported for the barnacle *Catomerus polymerus* (York et al. 2008), the polychete *Galeolaria caespitosa* (Styan et al. 2006), the starfish *Coscinasterias muricata* (Waters and Roy

2003), the chiton *Plaxiphora albida* (Ayre et al. 2009) and pulmonate limpets of the genus *Siphonaria* (Colgan and da Costa 2013). Closer to the specific case of this study, this pattern has also been observed for the *Caloglossa leprieurii* red algal species complex where South Australian (Adelaide) populations are genetically and reproductively distinct from Victoria and NSW populations (Kamiya 2004)

During field collections, different morphotypes of *Caulocystis* were found growing together in the same habitat. The pneumatocyst shapes varied between plants but never within plant, except for morphotype M5, which exhibited pneumatocysts with awns and no awns. Our results also showed random association between pneumatocyst type, haplotype, genotype and any geographic region or habitat, but further studies are needed to identify the processes determining their presence, absence and morphology.

The *cox*1 DNA barcode marker has been used for species identification in a diverse number of macroalgal taxa (brown algae: Kucera and Saunders, 2008, McDevit and Saunders 2009, 2010; red algae: Saunders *et al* 2005). In this study, the number of *cox*1 sequences is limited, because retrieving *cox*1 sequences from both *Caulocystis* and *Acrocarpia* was far more challenging compared to ITS2 and *rbc*L markers (although all markers were sequenced using the same DNA samples). DNA extractions from *Caulocystis* and *Acrocarpia* were noted to often have low overall success (low yield and high degeneration) when compare with other recently co-extracted taxa such as *Sirophysalis trinodis*, *Cystophora* and *Lobophora.* Brown algae are known to be rich in polysaccharides, tannins and polyphenols which when co-extracted with DNA and known to compromise both final DNA yield and downstream

sequencing. DNA analysis of *Caulocystis* and *Acrocarpia* samples will benefit from the development of more optimal automated extraction protocols.

Despite the high level of within species variation typically found for ITS2 (Hillis et al. 1996), this nuclear region has been commonly used in phylogenetic studies of brown algae, including the order Fucales (Dixon *et al*. 2012, Serrão *et al*. 1999, Stiger *et al*. 2000) and other phylogeographic studies of marine organisms in Australia (e.g. Miller et al. 2003). Within the Fucales, various studies showed that ITS2 is phylogenetically informative at the species level (Dixon *et al*. 2012, Serrão *et al*. 1999). However, the ITS2 alignment in the present study showed multiple indels. The global alignments performed in either Muscle or ClustalW methods differed in terms of alignment length, homology assignment and indel number and size, but not in final overall pairwise percentage identity (= 81% in both). Hand made final adjustments in both alignments were minimal and more extensive attempts to improve homology quickly became futile as improvements on one side of the alignment would immediately result in the decrease homology in another location. Most indels larger than 4 bps were attributed to differences between genera and species, and the Muscle software produced a visually better albeit longer alignment (707 bp instead of 610 bp). In the literature, the effect of sequence alignments and nucleotide homology on final phylogenetic topology vary widely between studies (Kumar and Filipski 2007). A growing amount of evidence also suggests that alignment accuracy obtained using common default values of alignment parameters is not much worse than that obtained from supposedly true penalties for inserting gaps and allowing base substitutions (Landan 2005). However recent computer simulations demonstrated that if

60% or more of the sites are accurately aligned, further improvements make little difference in the final results and even highly accurate alignments can produce trees with substantial variation in quality (Kumar and Filipski 2007). In this study, treating all gaps as missing data considerably reduced most of the differences between ITS2 alignments making the remaining differences irrelevant to the final ML and Bayesian results. Despite the morphological similarities between *Caulocysits* and *Acrocarpia* and their close phylogenetic proximity, their ITS2 DNA sequences are only marginally alignable.

Absence of phylogeographic structure in the ITS2 nuclear DNA dataset compared to our cytoplasmic markers is highly concordant with phylogeographic studies in general (Avise 2000). The reasons for this pattern have been well established in the literature: cytoplasmic markers are haploid, have smaller population sizes, inherited by only one gender in macroalgae, are generally free from recombination, experience faster coalescence times and hence tend to be fixed faster in the population compared to nuclear markers (Moore 1995). On the other hand, nuclear markers are more likely to suffer from incomplete lineage sorting, introgression and gene duplication/extinction events (Maddison 1997). More specific discordances between nuclear and cytoplasmic markers in regards to presence or absence of genetic structuring in marine phylogeographic studies have been reported before in the literature such as *Gracilaria tikvahiae* (Gurgel et al. 2004), and the clam *Lasaea australis* (Li et al. 2013)

In conclusion, this study used molecular approaches as an independent test of the veracity of morphology-based taxonomy and classification of endemic species in the genus *Acrocarpia* and *Caulocystis*. In addition,

phylogeographic structure observed between geographically separated *Caulocystis* may offer an evolutionary pathway towards parapatry or allopatry, and the formation of distinct species as seen in *Acrocarpia.* Further studies that increase the number of specimens and collection sites may expand the knowledge of biogeographic patterning and evolutionary history for these two groups of macroalgae, particularly C. *cephalornithos*. Phylogeographic concordance between distinct organisms has the power to uncover shared evolutionary histories, identify historical and extant processes promoting genetic isolation and hence, help us better understand speciation in the marine environment and identify genetically distinct populations and communities which in themselves might require distinct conservation strategies.

Acknowledgements:

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Taxonomic changes.

***Caulocystis cephalornithos* (Labillardiere) Areschoug 1854:334.**

Basionym: *Fucus cephalornithos* 1806:114, pl. 261

Heterotypic synonym: *Caulocystis uvifera* (C. Agardh) Areschoug 1854:335.

Sargassum uviferum C. Agardh 1824: 306; 1826: 165. Sonder 1846:165.

Cystophora uvifera (C. Agardh) J. Agardh 1848: 246. De Toni 1895: 317.

Cystoseira cephalornithos (Labillardiere) C. Agardh 1824: 291. Greville 1830, synop.: 33.

Cystophora uvifera (Labillardiere) J. Agardh 1848: 246. De Toni 1895: 138.

Description: Thallus 10-30 (-100) cm long, with a discoid-conical holdfast. Primary branches terete, straight and denuded below, bearing dense and radially branched laterals. Lateral branches terete, simple or laterally branched one to several times. Vesicles either absence or present, when present they borne on primary branches or sometimes on lower parts of old laterals that develop further, vesicles pedicellate, spherical, subspherical to ovoid or fusiform, sometimes apiculate with awns at the ends, or occasionally developed string of pneumatocysts. Ramuli simple to much branched, terete, usually 5-15 cm long, 0.5-1.5 mm in diameter.

Thallus monoecious. Receptacles developed on upper ends of ramuli, usually simple, terete and smooth, 2-15 mm long and 1-2 mm in diameter, with scattered ostioles. Conceptacles numerous, bisexual with some phaeophycean hairs and paraphyses. Oogonia sessile, ovoid, 60-130 μm long and 40-80 μm in

diameter, antheridia sessile or on branched paraphyses, elongate-ovoid, 20-30 μm long and 6-10 μm in diameter.

Distribution: from Shark Bay, West Australia, to Sydney, New South Wales, around Tasmania. One record from Norfolk Island (NSW 397998, A.J.K. Millar 11 May 1996).

Specimen examined: (information is listed as follows: locality, voucher number): South Australia, Glenelg Beach: AD-A89187, AD-A89184, AD-A89185, AD-A89190, AD-A89188, South Australia, Yorke Peninsular: AD-A89423, AD-A89425, AD-A89429, AD-A89428, AD-A89424; Victoria, Point Lonsdale Reef: AD-A95309, AD-A95276, AD-A95315, AD-A89419, AD-A89420A, AD-A89420B, AD-A89420C, AD-A89420D

Chapter 3: Molecular Phylogenetics of the Australian endemic brown algae genus *Cystophora* (Sargassaceae, Phaeophyceae) based on cox1, rbcL and ITS analyses.

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Abbreviations: Abbreviations: Bayesian phylogenetic inference, BI; Bayesian posterior probabilities, PP; bootstrap proportion values, BP; CSR, character state reconstruction; *cox*1, cytochrome oxidase 1; ITS, internal transcribed spacer of the nuclear ribosome cistron; *rbc*L, large subunit of the Ribulose 1,5-bisphosphate carboxylase/oxygenase; Maximum likelihood, ML.

Statement of Authorship

Molecular Phylogenetics of the Australian endemic brown algae genus *Cystophora*(Sargassaceae, Phaeophyceae) based on cox1, rbcL and ITS analyses.

This chapter has been prepared as a submission for publication

Nuttanun Soisup
Executed fieldwork, complete laboratory work and data analysis, and prepare manuscript as principal author.

Signed
Date: 26 July 2013

Andrew Lowe
Provided guidance with data analysis and contributed to revisions of the manuscript

Signed
Date: 26 July 2013

Fred Gurgel
Involved in developing idea, executed fieldwork, provided guidance with laboratory work and data analysis and contributed to revisions of the manuscript

Signed
Date: 26 July 2013

Abstract

Cystophora is an Australasian endemic brown algal genus commonly found in temperate marine benthic environment. The genus consists of 25 species; 23 of which occur in Australia, and six in New Zealand, where only two species are endemic. The genus has been considered the most species rich in the order Fucales in temperate Australia, and to date species identification and any attempts at phylogenetic reconstruction have been based solely on morphological analyses. However, many species display high levels of phenotypic plasticity, and occasionally, key diagnostic characters are absent, making species discrimination and phylogenetic reconstruction challenging. This study applied three molecular markers (*cox*1, *rbc*L and ITS rDNA) to reconstruct phylogenetic relationships among 19 of the most abundant *Cystophora* species in Australia to test species delineations. Results suggest that *C. cuspidata* and *C. subfarcinata* should be merged into a single morphologically plastic species. Four major evolutionary lineages where found in *Cystophora*. Character evolution of main diagnostic features was compared against novel hypotheses based on newly generated phylogenetic trees. In general, with the exception of a few species, molecular and morphological results were highly congruent.

Introduction

The marine brown algal genus *Cystophora* J. Agardh (Sargassaceae, Fucales) is endemic in New Zealand and the temperate southern Australia. There are 25 species of *Cystophora,* 23 occurring in southern Australia and six in New Zealand (two of which are endemic to New Zealand). Some species are limited to deep waters and most are dominant, canopy-forming species in intertidal areas, shallow waters and rock pools. *Cystophora* are common and conspicuous members of subtidal habitats, and can be easily recognized at the genus level by the presence of a bilateral sympodial main axis containing several lateral branches of determined growth. However, identification at the species level is often problematic due to the high levels of phenotypic plasticity, the absence of key diagnostic characters (e.g. reproductive structures) and unclear diagnostic characters, particularly when dealing with immature or atypical specimens.

Most *Cystophora* species were first described under the genera *Cystoseira* or *Fucus* (Womersley, 1964). Decaisne (1840, 1841) established the genus *Blossevillea* based on *Cystoseira* species characterized by secondary axes branched off from the flat face of the main axis, and receptacles with two rows of the conceptacles but no species were listed under *Blossevillea*. Later, Decaisne (1842) included five species in the genus: *B. paniculata, B. torulosa, B. spartioides, B. dumosa,* and *B. platylobium*. At the same time, Agardh (1841) established the genus *Cystophora* for the Australian species that were previously recognized as *Cystoseira.* Agardh (1841) characterized *Cystophora* based on the presence of retroflex main axes and the presence of air vesicles (pneumatocysts) but again, no species were listed under the genus. Later,

Agardh (1848) included 20 species in *Cystophora*, 12 of which are currently accepted in the genus (Womersley, 1964; Guiry and Guiry 2013). *Cystophora* and *Blossevillea* were acknowledged as synonymous but the former name had been commonly used (Agardh, 1848, 1870, 1896, Lucas 1936, Womersley 1964). Cotton (1935) recognized the merit of conserving *Cystophora* against *Blossevillea* when the genus was placed on the list of *Nomina Generica Conservanda Proposita* in the Botanical Rules for 1935. In 1964, Womersley recognized *Acrocarpia* and *Caulocystis* as distinct genera from *Cystophora* due to the lack of key *Cystophora* morphological features in those two taxa and at present, the classification and *Cystophora* species identification are based on Womersley's monographs (1987, 1964). At higher taxonomic ranks, *Cystophora* was previously placed under the family Cystoseiraceae with *Acrocarpia* and *Caulocystis* as the closely related genera (Womersley 1964, 1987). However, several recent molecular studies have recommended the merger of Cystoseiraceae into Sargassaceae (Rousseau and De Reviers, 1999, Cho et al., 2006 Saunders and Kraft, 1995; Rousseau et al., 1997).

Previous molecular phylogenetic studies based on 23S mtDNA DNA sequences using specimens of *Cystoseira* and other Sargassaceae genera revealed that *Caulocystis* and *Acrocarpia* were not closely related to *Cystophora*, but were the earlier diverging clade of Sagassaceae, while *Cystophora* formed a distinct clade together with *Landsburgia quercifolia* and *Halidrys siliquosa* (Draisma et al., 2010). In the same year, a multi-locus time calibrated phylogeny of the brown algae placed *Caulocystis* as the earlier diverging taxon of a clade consisted of *Cystophora*, *Sargassum, Halidrys, Cystoseira* and *Bifurcia* (Silberfeld et al., 2010). These molecular phylogenetic

studies suggested that, despite their morphological similarities, *Caulocystis* and *Acrocarpia* are not the most closely related genera to *Cystophora*; as previously hypothesized by Womersley (1964, 1987).

According to Womersley's descriptions (1978, 1964), 12 species of *Cystophora* are morphologically distinct, however the remaining species display a wide range of overlapping lateral branching patterns and receptacle shapes, for example, between *C. subfarcinata* and *C. cuspidata,* and between *C. retorta* and *C. siliquosa*. Womersley (1964) proposed the possible phylogenetic relationships of the species attempted the first evolutionary reconstruction among the 23 *Cystophora* species using the shape of main axes and pattern of lateral branching. However, the diagram lacks of internal nodes and relationships between several species of *Cystophora* remains unclear.

Several chemotaxonomy studies have attempted to solve phylogenetic relationships within *Cystophora* (Valls and Piovetti 1995, Amico 1995, Laird and van Altena 2006, Laird et al. 2010). Laird and van Altena (2006) isolated tetracyclic meroditerpenoids from *Cystophora fibrosa* Simons (1970) from South Africa and confirmed Womersley's (1987) morphological diagnosis that that species should be designated as *Cystoseira*. More recently, Laird et al. (2010) analyzed the secondary metabolite profile of further 13 *Cystophora* species. From their results, *C. monilifera* and *C. expansa* were considered the most advanced (derived) species and phylogenetic relationships were detected between some species pairs showing similar chemistry such as, between C. *scalaris* and *C. monilifera,* between *C. harveyi* and *C. torulosa,* and between *C. intermedia* and *C. moniliformis*. In addition, the differences in the isolated compounds indicated that *C. harveyi* and *C. brownii* might not be as closely

related as previously suggested by Womersley (Bian and van Altena, 1998). To date, the chemistry data could not establish a robust phylogenetic tree within the genus and Laird *et al*. (2010) suggested that the combination of morphology, chemistry and genetic data is needed to propose better phylogenies.

Recently, Buchanan (2011) generated *cox*1 and ITS DNA sequences for 12 *Cystophora* species from Australia and New Zealand. Despite the low levels of phylogenetic support in his trees, Buchanan proposed the merger of two species pairs, *C. distenta* and *C. scalaris* (both NZ endemic species) and *C. retroflexa* and *C. congesta*, supported by both morphological and genetic similarities.

Because only half of all *Cystophora* species have been analyzed using molecular approaches thus far, the classification and phylogenetic relationships within the genus, including the taxonomic position of certain species, remain uncertain. This study aimed to apply a molecular phylogenetic approach to a wider range of *Cystophora* species in order to investigate the phylogenetic relationships within the genus, using three molecular markers from three distinct genomic compartments: *cox*1 (mitochondrial), *rbc*L (chloroplast) and ITS 1 and 2 (nuclear rDNA cistron). In addition, we compared our new molecular results with the character evolution of key morphological characters proposed by Womersley (1964, 1987).

Materials and Methods

Collections and morphological analyses

Specimens were collected and identified according to Womersley (1964, 1987). Entire specimens were pressed onto herbarium sheets without being previously fixed in formalin solution. Subsamples for DNA analysis were preserved in silica gel desiccant in the field right after collection (Chase & Hillis, 1991). A list of specimens with collection information and sequence accessions is provided in Appendix 3. Multiple specimens belonging to the same species were numbered sequentially. All specimens were deposited in the State Herbarium of South Australia (AD).

DNA extraction, amplification and sequencing

DNA was extracted from silica gel preserved materials using DNeasy Plant MiniKit (Qiagen, Doncaster, Australia) according to the manufacturer's specifications. Sequences of *rbc*L, ITS 1 and 2 and *cox*1 region were amplified using primers listed in Appendix 1. PCR amplifications were performed in 25 µl reactions as described in chapter 2. The amplification was run on the Palm Cycler (Corbett Research, Australia) and parameters included an initial denaturation at 95˚C for 3 min followed by 40 cycles of 95˚C for 45 seconds, Tm (listed in Table 1) for 1 min, and 72˚C for 1.5 min, terminated by 72˚C for 5 min. Products were cleaned using ExoSAP-IT (GE Healthcare, Buckinghamshire, UK) or MultiScreen PCR_{384} Filter Plate (Milipore, Billerica, MA, USA). Capillary separation of sequencing products was performed by the Australian Genome Research Facility Ltd (AGRF) on the ABI 3730*xl* sequencing platforms (Applied Biosystems, Foster City, CA, USA) using ABI

BigDye Terminator V.3.1 (Applied Biosystems, Foster City, CA, USA).

Phylogenetic Analyses

ITS rDNA, cox1 and rbcL DNA sequences were aligned in Geneious Pro v5.5.8 (Biomatters, available from http://www.geneious.com) using the MUSCLE algorithm (Edgar 2004) with default parameters and then corrected manually. Outgroups were chosen based on data availability and closest phylogenetic proximity to the ingroup. The work of Draisma et al. (2010, figs. 1 and 2) was used as guidance of between genus relationship for outgroup selection. Maximum-likelihood (ML) and Bayesian Inference (BI) trees were constructed for ITS, *cox*1 and *rbc*L sequence alignments independently using nucleotide substitution model selected by jModelTest2 (Darriba et al. 2012) with AIC criterion. The best fit models were GTR + G for ITS, HKY + I + G for *cox*1, and Trn + I + G for *rbc*L. In the case of BI analysis for *rbc*L, GTR+G was used instead of Trn because the latter model is not available in MrBayes, and GTR+G was the second best fit model selected for that dataset according to the AIC criterion. ML analyses were performed using PhyML plug-in on Geneious Pro 5.5.8 software with 1,000 bootstrap replicates (Guidon et al. 2010). Bayesian Inference was performed in MrBayes 3.2 (Ronquist et al. 2012) plug-in on Geneious Pro consisted of two parallel runs, each of three incrementally heated chains, ran for 5.5 million generations, sampled every 1,000 generations with 500,000 generations burn-in. MCMC runs were monitored within Geneious, the effective sample size (ESS) were over 500 in all runs, providing evidence that convergence had been reached.

A three-marker concatenated dataset was produced from sequences representing each species but not always coming from the same specimen because it was not always possible to amplify all three markers from the same sample. Reassignment of sequences into their respective species was confirmed based on the results obtained in the single-marker datasets. The ML analysis of the concatenated dataset was run in RAxML version 1.3 (Silvestro and Michalak 2012) using the GTR+G model of evolution with independent rates for each partition, and model parameters estimated over the duration of specified runs. Bootstrap support was performed under the thorough bootstrap option, with 100 replicates. The Bayesian analysis of the concatenated dataset was run in MrBayes v.3.1.2 (Ronquist and Hueselbeck 2003) plug-in on Geneious Pro. The dataset was partitioned by codon and by markers using PartitionFinder V1.0.0 using Bayesian Information Criterion in an unlink analysis which allowed the rates to vary over the partitions. According to the results of PartitonFinder, the most appropriate model for the 1st and 2nd codon positions of *cox*1 and *rbc*L, and ITS markers is HKY+G and the most appropriate model for 3rd codon position of *cox*1 and *rbc*L is GTR+G. MCMC consisted of two parallel runs each of four incrementally heated chains, 3 million generations, sampled every 1,000 generations with 10% burn-in. MCMC runs were monitored within Geneious, effective sample size were over 500 in all runs, providing evidence that convergence had been reached.

Ancestral state reconstruction

Ancestral state reconstructions were performed in Mesquite V.2.75 (Maddison and Maddison, 2011) onto the phylogenetic hypothesis inferred

from Bayesian analysis of the concatenated dataset. As the phylogenetic tree contains soft polytomies, maximum parsimony was preferred over other methods because maximum parsimony does not require knowledge of branch lengths and can operate with polytomies as observed in our phylogenetic tree. Four characters were applied to the analyses: axis branching, lateral branching, simplified interpretation of lateral branching, and presence or absence of pneumatocysts. Axis branching characters were categorized into three states based on the shape of primary axes in cross section and modes of lateral branch insertion: flat axis with laterals arising from the edges, flat axis with laterals arising from the face, and terete axes. Lateral branching pattern character consisted of six states: unbranched, pinnate, bipinnate, distichous, tristichous, and irregular or radial. The first two characters correspond to the two main features used by Womersley (1964) to create his proposed *Cystophora* phylogeny. These features also formed the basis for the creation of *Cystophora* identification keys published in his two monographs (i.e. Womersley 1964, 1987). A simplified interpretation of lateral branching pattern categorized branching pattern into three states: branch in one plane or complanate (flatten), branch in three plane or tristichous, and radial to irregular branching. The simplified interpretation mode included unbranched, pinnate, bipinnate, distichous states from Womersley's interpretation into complanate state. Pneumatocyst character was separated into presence and absences of pneumatocysts or air vesicles.

The robustness of our results was examined against alternative tree topologies following Asplen et al. (2009). Randomly resolve polytomies command was used to generate 100 polytomy resolved trees and ancestral state

reconstructions were performed on the generated trees. The results were then compared with the most parsimonious reconstructions that contain polytomies.

Results

Molecular phylogenetic analyses.

*Cox*1 dataset

The *cox*1 dataset contained 37 new *Cystophora* sequences and two outgroup taxa, *Caulocystis cephalornithos* (from this study) and *Carpoglossum confluens* (downloaded from Genbank). The alignment was 688 base pairs (bp) long and comprised of 132 polymorphic sites of which 72 were parsimony informative. The ML and BI analyses produced very similar tree topologies (only ML tree is shown, Fig. 1). Most specimens clustered according to their morphological species delineation (i.e. specimen identification based on morphological analysis) however only four lineages received high phylogenetic support from both analytical methods: the *C. platylobium* clade (BP=89, PP=1.0), the *C. torulosa* clade (BP=99, PP=1.0), *C. polycystidea* (BP=99, PP=1.0), and *C. moniliformis* (BP=92, PP=1.0). *Cystophora intermedia* was the most divergent species in the cox1 datset forming a long branch positioned at the base of the ingroup clade. The largest clade comprised of *C. torulosa, C. polycistidea, C. retroflexa*, and *C. subfarcinata* received high BP support (90%) but no PP support (< 0.5) and hence showed a support disagreement between the two phylogenetic methods herein adopted. Despite resolving specimens according to their previous morpho-species assignments, the remaining lineages showed very low to no phylogenetic support (Fig. 1).

*rbc*L dataset.

The *rbc*L dataset consisted of 32 newly generated *Cystophora* DNA sequences, and was 1,316 bp long, comprising 14 distinct species. The alignment contained 141 polymorphic sites, 71 parsimony informative sites and no indels. The ML and Bayesian analyses produced very similar tree topologies and only ML tree is shown in Figure 2. *Cystophora* samples formed lineages and clades according to their prior morphological identifications with the exception of the clade composed of *C. intermedia* and C. *moniliformis*, and the clade composed of *C. cuspidata* and *C. subfarcinata*. *Cystophora intermedia* sequence was placed in a clade with one of the longest long branch lengths in the ingroup, in a position basal to the remaining species in the genus, and accompanied by all *C. moniliformis* specimens. The single *C. intermedia* specimen presented a *rbc*L sequence identical to *C. moniliformis* #1 and #2 but differed from *C. moniliformis* #3 by only 1 bp. These two species and four sequences formed a monophyletic group with maximum phylogenetic support (Fig. 2). In addition, *C. cuspidata* clustered with all *C. subfarcinata* sequences, excepting *C. subfarcinata #1,* with moderate to high support (BP=87, PP=1.0). *Cystophora subfarcinata #1* differed from all other *C. subfarcinata* sequences by only 1-2 bp, however that was enough to considerably lower the support of the assignment of this sequence to the *C. subfarcinata* clade. Based on morphological observations, *C. subfarcinata* #1 was exactly the same as *C. subfarcinata* but the plant lacked fertile organs and air vesicles (air vesicles are not always present in *C. subfarcinata*). Major lineages observed in the *cox*1 trees were also observed in the *rbc*L trees (Fig. 2): the *C. pectinata* - *C. grevillei* - *C. expansa* clade (BP=68, PP <0.5), the *C. platylobium* – *C.*

siliquosa – C. retorta association (BP=70, PP <0.93), and the large *C. polycistidea – C. congesta – C. torulosa – C. retroflexa – C. cuspidata – C. subfarcinata* clade (BP=87, PP <0.97).

ITS dataset.

The ITS alignment (ITS1, partial 5.8S rDNA, and ITS2) was comprised of 36 newly generated *Cystophora* sequences from 13 morpho-species, was 1,177 bp in length, contained 334 variable sites, 250 parsimony informative sites and 32 remaining indels. The ML and Bayesian analyses produced very similar tree topologies and hence only the ML tree is shown in Figure 3. Sequences were clustered according to their prior morphology-based identification except for the lineage formed by *C. retroflexa*, *C. subfarcinata*, *C. torulosa, C. botryocystis,* and *C. cuspidata* where overall low levels of genetic divergence among species was observed across all species. This clade received low support in the ML analysis (BP = 33%) but high support in the BI result (PP=0.99). Despite their close phylogenetic relationship, small and consistent genetic differences between these species occurred (not clear in the Fig. 3) with the exception of *C. cuspidata* which presented ITS sequences 100% similar to *C. subfarcinata* #6 and #29 (Fig. 3). The only *C. botryocystis* sequence generated in this study was for the ITS marker, was relatively short (527 bp) and exhibited a distinct long branch from other members in the clade with low BP but high PP support (43% and 0.98, respectively). Until data from the other two or more markers are generated for this species, its phylogenetic position in lineage should be considered with care.

Concatenated dataset

The concatenated dataset was 3,078 bp in length, contained 19 sequences representing distinct *Cystophora* species, and two outgroup taxa: *Sirophysalis trinodis* and *Landsburgia quercifolia*. The BI tree is shown in Figure 4. Phylogenetic trees inferred from the separate (*cox*1, *rbc*L and ITS) and concatenated datasets highly were congruent. Lineage comprised *Cystophora moniliformis* and *C. intermedia* is the basal group in the *Cystophora* tree with full support in Bayesian Inference while the ML tree shows the polytomy of these two species (Fig. 4). The lineage comprised *C. brownii, C. racemosa, C. pectinata, C. expansa* and *C. grevillei* branch off as the second group with relatively low support in ML tree (BP=70) and relatively high support in BI tree (PP=0.94). *Cystophora xiphocarpa* formed a sister branch to two lineages: the lineage comprised *C. platylobium, C. retorta* and *C. siliquosa*; and lineage that composed of *C. congesta, C. torulosa, C. retroflexa, C. botryocystis, C. subfarcinata* and *C. cuspidata* (Fig. 4). The BI analysis of concatenated dataset shows higher support values than the ML analysis. Similarly to the ITS results, *C. botryocystis* exhibits a long branch and formed a polytomy with *subfarcinata* and *C. cuspidata* (BP=50/PP=100). Another polytomy is also observed between *C. expansa*, *C. grevillei* and a clade contained *C. racemosa* and *C. pectinata.*

Morphological evolution

The ancestral state reconstruction of main axis branching pattern (Fig 5) suggests that the ancestral state for main axis branching pattern in *Cystophora* cannot be determined. Regardless of *C. moniliformis* and *C. intermedia*, a

transition from flat axis/branched from faces state to flat axis/ branched from edges state occurred once, and a transition from flat axis/branched from faces state to terete axis occurred three times. For the lateral branching pattern (Fig 6), distichous lateral branching pattern is the ancestral state in *Cystophora* and has lost multiple times through the evolution to other states. Lateral tristichously branched in *C. expansa*, lateral unbranched in *C. xiphocarpa* and lateral bipinnately branched in *C. brownii* occurred once within *Cystophora* and are considered autapomorphy. Pinnate and radial lateral branching patterns exhibited multiple independent origins.

For the simplified interpretation of lateral branching pattern character (Fig 7), results suggest that complanate branching pattern is the ancestral state, whereas radially to irregularly and tristichous branching patterns are derived. Tristichous branching pattern occurred once and was considered autapomorphy, while complanate branching pattern occurred twice, at the origin and *C. distenda*.

Regarding air vesicles evolution shown in figure 8, the absent of vesicles is more likely to be ancestral state. The phylogram suggests that the transition between the absent to the presence of vesicles occurred once in the outgroup and at least twice within *Cystophora*.

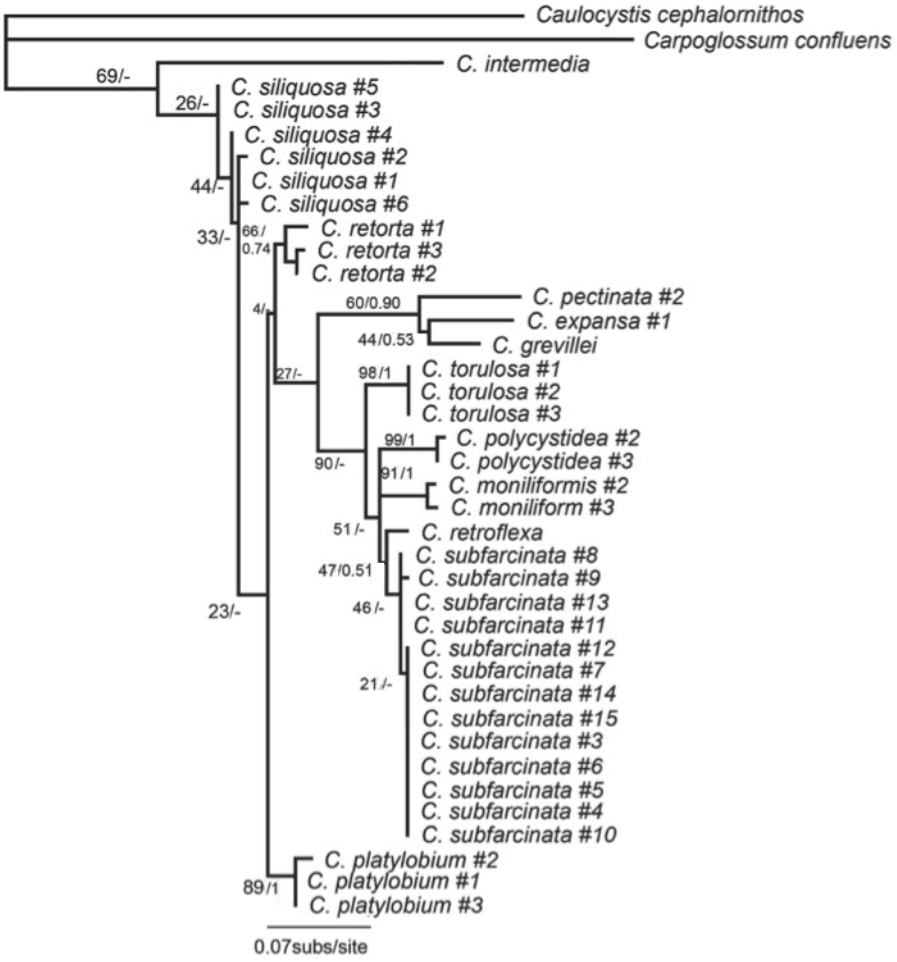

Figure 1. Maximum likelihood tree based on *cox*1 DNA sequences of *Cystophora* species with *Caulocystis cephalornithos* and *Carpoglossum confluens* as outgroups. Numbers at branches represent phylogenetic support values, i.e. bootstrap proportions based on 1000 replications and Bayesian posterior probabilities, respectively.

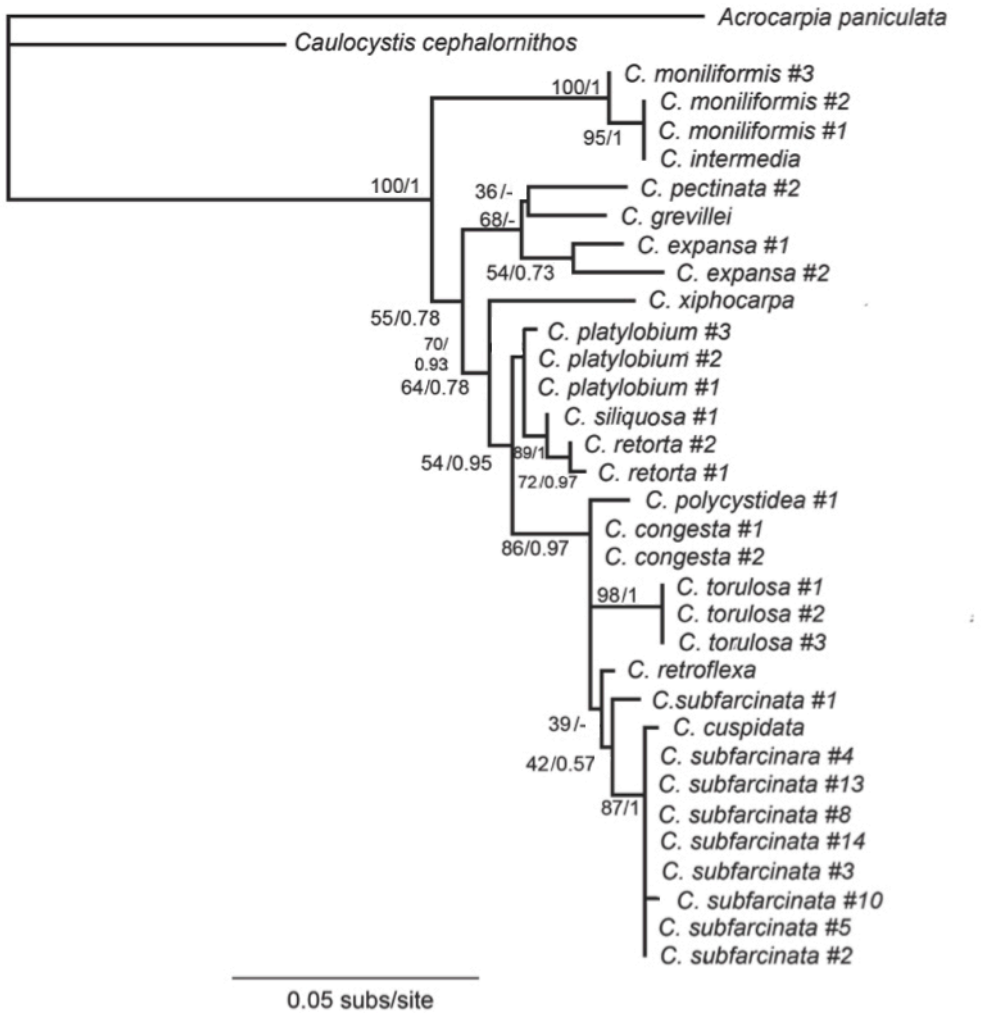

Figure 2. Maximum likelihood tree based on *rbc*L sequences of *Cystophora* species with *Caulocystis cephalornithos* and *Acrocarpia paniculata* as outgroups. Numbers at branches represent phylogenetic support values, i.e. bootstrap proportions based on 1000 replications and Bayesian posterior probabilities, respectively.

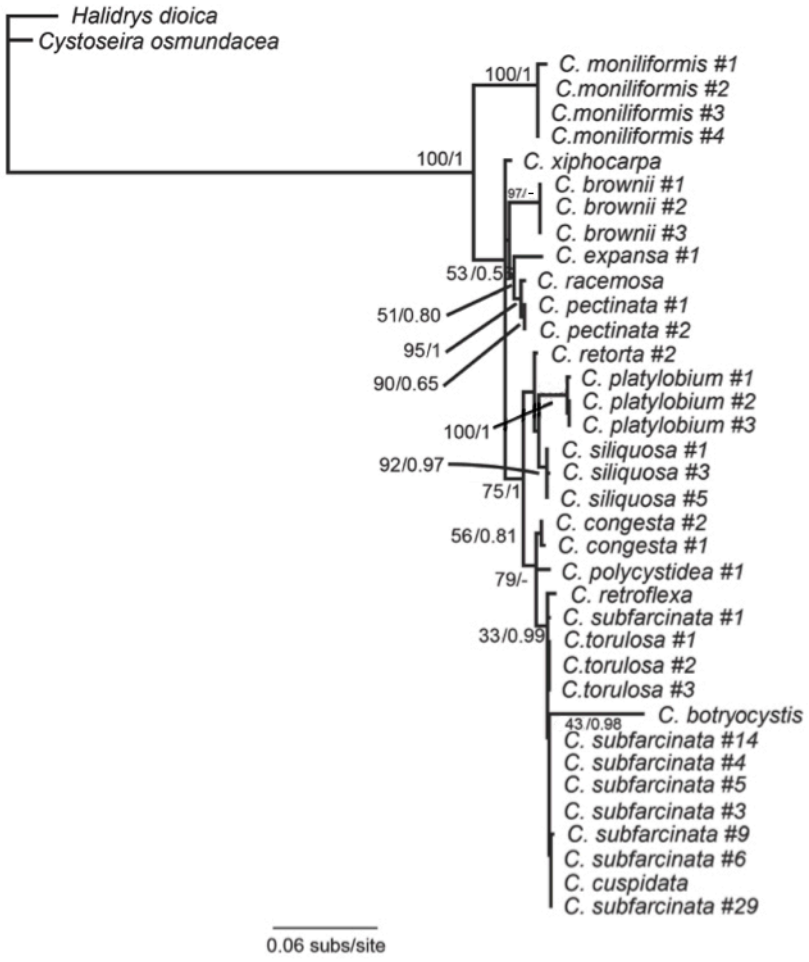

Figure 3. Maximum likelihood tree based on ITS1 and 2 rDNA sequences of *Cystophora* species with *Caulocystis cephalornithos* and *Acrocarpia paniculata* as outgroups. Numbers at branches represent phylogenetic support values, i.e. bootstrap proportions based on 1000 replications and Bayesian posterior probabilities, respectively.

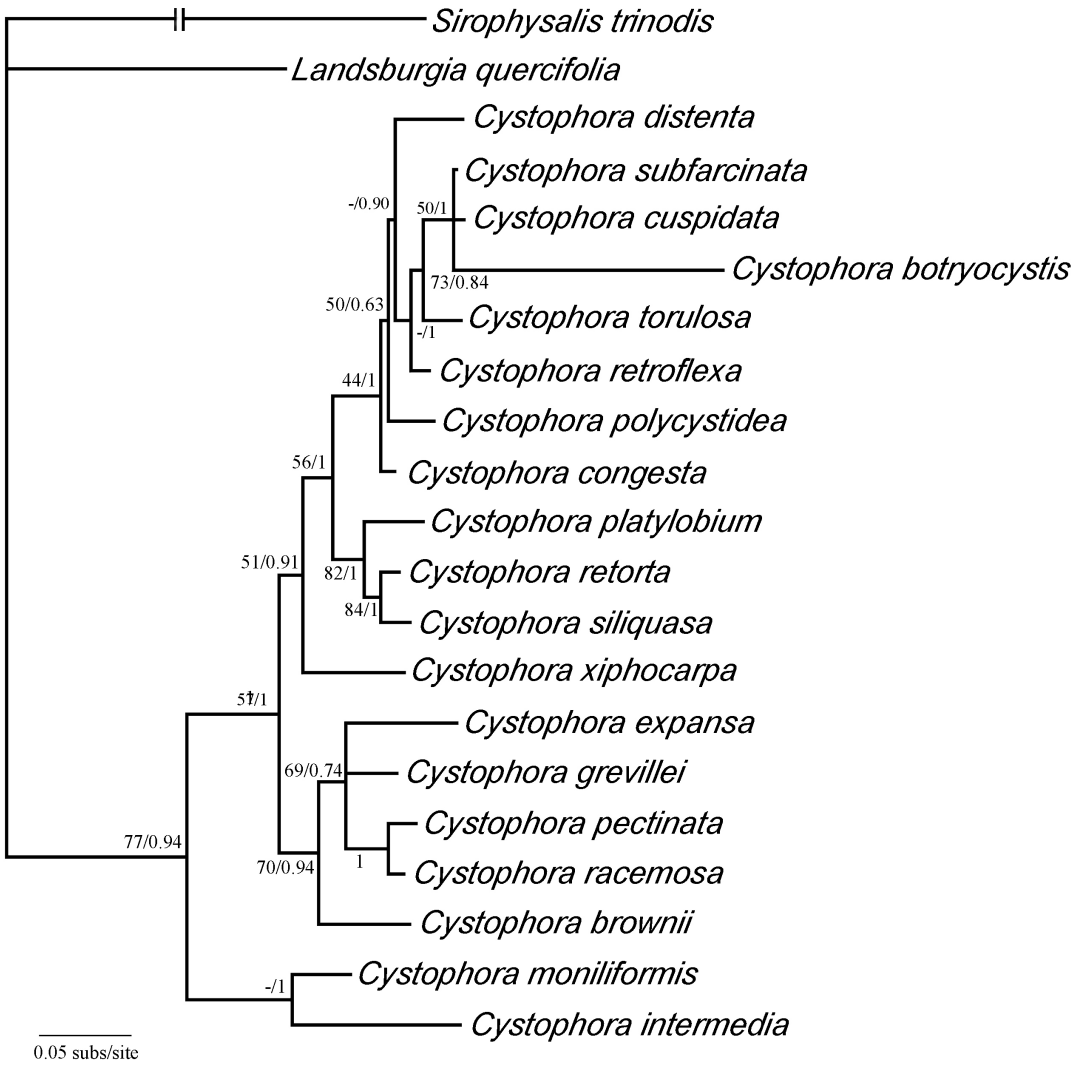

Figure 4. Phylogenetic analysis of combined cox1, rbcL and ITS dataset of *Cystophora* inferred with the Bayesian analysis method. Numbers at internal nodes indicate support values (Maximum likelihood and Bayesian Inference respectively). *Sirophysalis trinodis* and *Landsburgia quercifolia* were used as outgroup.

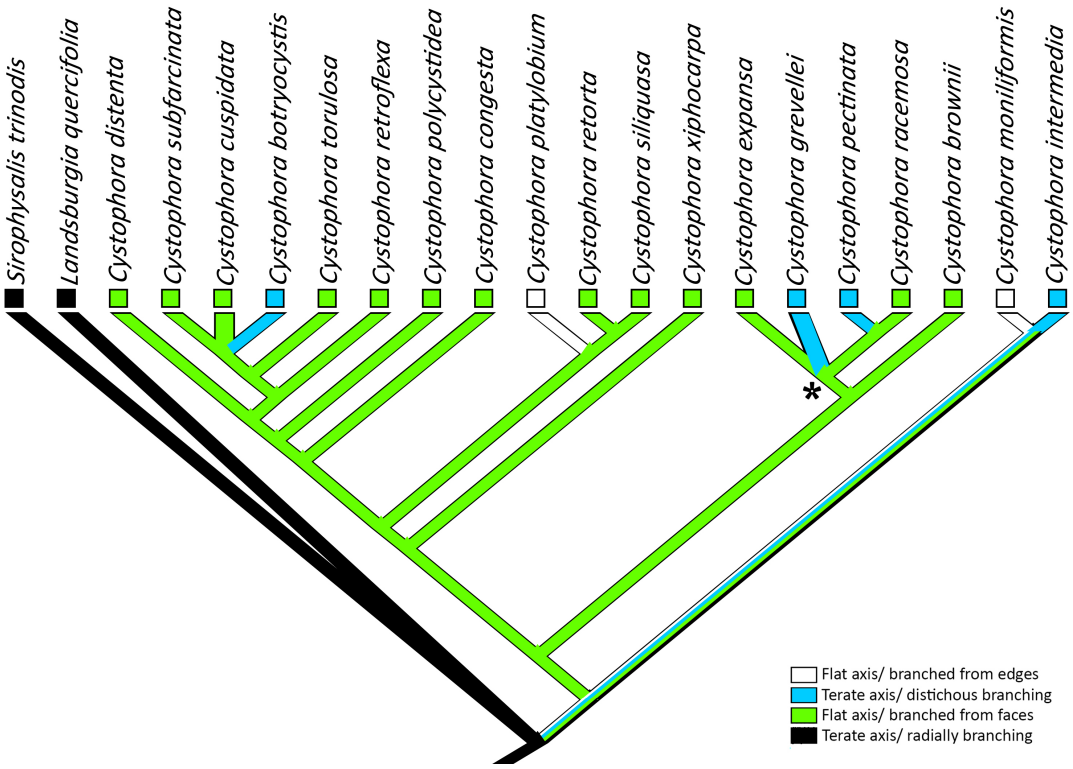

Figure 5. Ancestral state reconstruction of main axis branching pattern based on Maximum parsimony approach using Mesquite V2.7.5 (Maddison and Maddison, 2011). Colored branches indicate the most parsimonious hypotheses given the character state distribution of *Cystophora* species. Asterisks indicate clades where relationships change from the most parsimonious reconstruction after polytomies were randomly resolved.

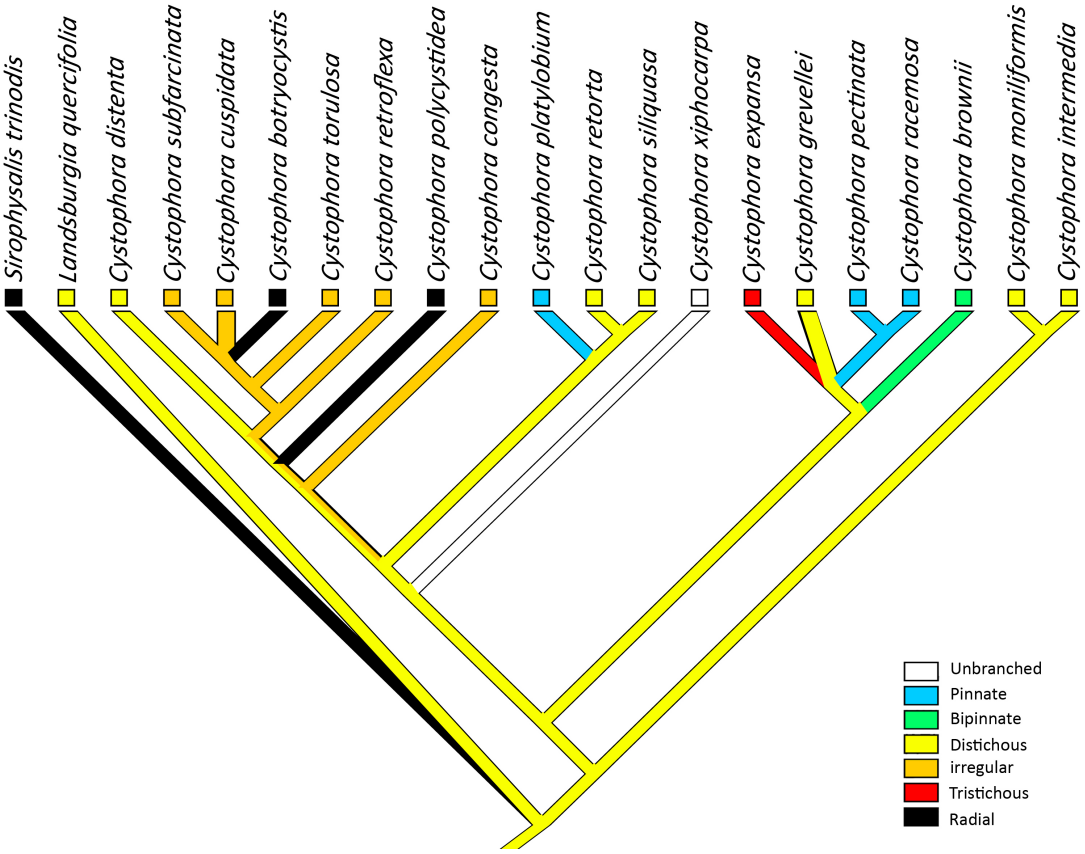

Figure 6. Ancestral state reconstruction of lateral branching pattern based on Maximum parsimony approach using Mesquite V2.7.5 (Maddison and Maddison, 2011). Colored branches indicate the most parsimonious hypotheses given the character state distribution of Cystophora species.

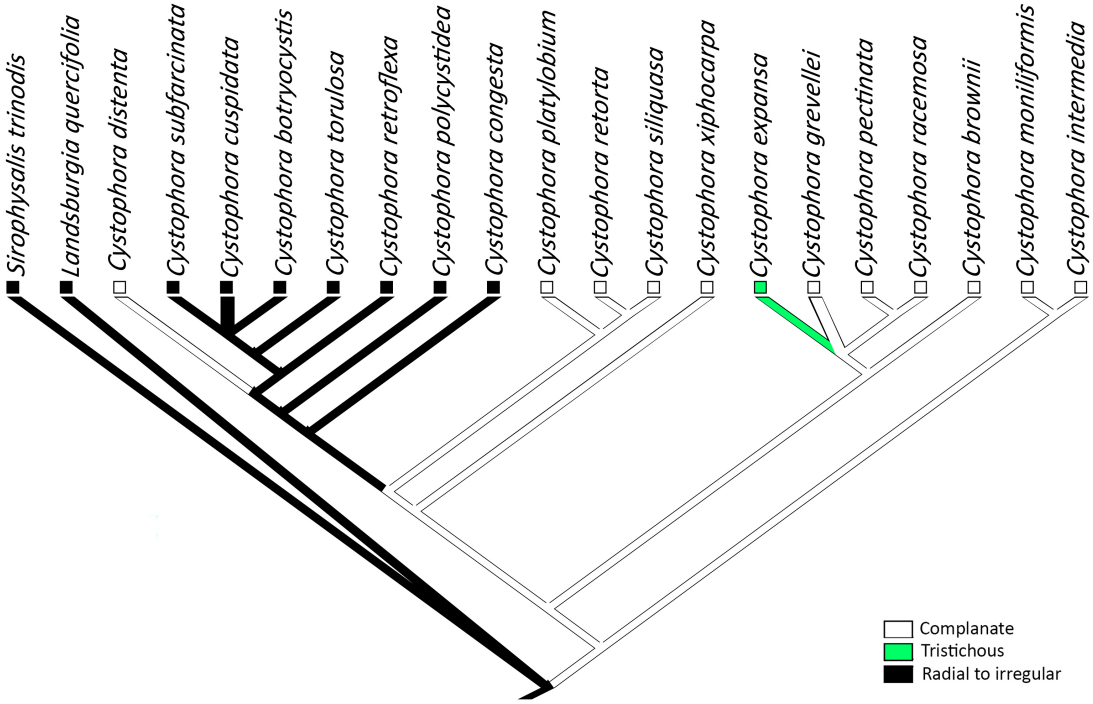

Figure 7. Ancestral state reconstruction of simplified main axis branching pattern based on Maximum parsimony approach using Mesquite V2.7.5 (Maddison and Maddison, 2011). Colored branches indicate the most parsimonious hypotheses given the character state distribution of *Cystophora* species.

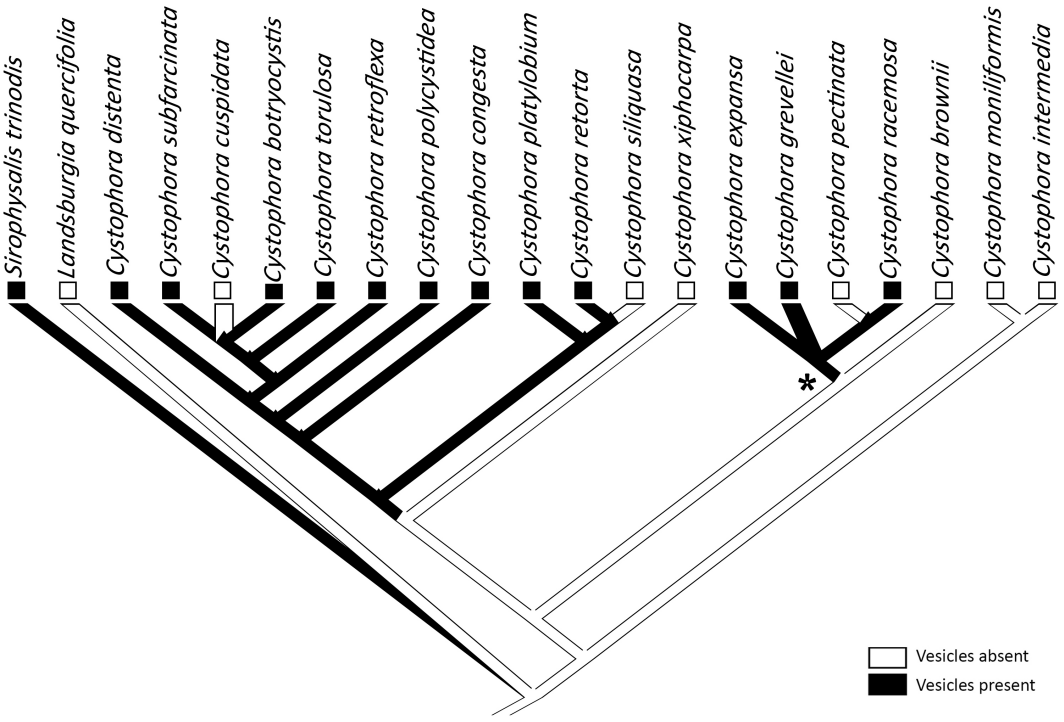

Figure 8. Ancestral state reconstruction of the presence of vesicles based on Maximum parsimony approach using Mesquite V2.7.5 (Maddison and Maddison, 2011). Colored branches indicate the most parsimonious hypotheses given the character state distribution of *Cystophora* species. Asterisks indicate clades where relationships change from the most parsimonious reconstruction after polytomies were randomly resolved.

Discussion

Results from the *rbc*L, ITS and concatenated datasets showed that *C. subfarcinata* and *C. cuspidata* cannot be separated on molecular grounds, suggesting that they are conspecifics. Furthermore, *C. cuspidata* and *C. subfarcinata* are morphologically similar to each other in their overall habit, except the size of receptacles where for *C. cuspidata* they are distinctly larger. Womersley (1964) also noted the presence of intermediate specimens between *C. subfarcinata* and *C. cuspidata* that he could not identify. *Cystophora subfarcinata,* receptacles also exhibit wide morphological variation. They can be simple or branched, their conceptacles can be irregularly placed or can be found forming two rows along the receptacles. Conceptacles in *C. subfarcinata* are mostly monoecious but unisexual plants were occasionally observed (Womersley 1964). Additionally, air vesicles in *C. subfarcinata* are absent in rough water forms, while *C. cuspidata* exhibits no air vesicles and occurs mainly on exposed sites. From my morphological observations and molecular results, *C. cuspidata* is one of the *C. subfarcinata* forms, occurring in exposed sites. I propose the merger of these two species, with *C. subfacinata* bearing the name priority (*C. subfarcinata* was originally reported as *Fucus subfarcinatus* by Mertens in 1819 while *C. cuspidate* was first reported by J. Agardh in 1896). Unfortunately, I was unable to generate *cox*1 sequences from *C. cuspidata,* which would have helped provide further support for the conspecificity of this species with *C. subfarcinata*.

Womersley (1964, 1987) suggested many pairs of closely related species in *Cystophora.* The molecular phylogenetic results obtained in this study also agree with his perception, and many of those morphologically

similar species pairs turned out to be sister clades, for example, the *C. pectinata* and *C. racemosa* clade (Figs. 3, 4), and the *C. retorta* and *C. siliquosa* clade (Figs. 2, 4). However, some pairs of morphologically closely related species were not in agreement with the molecular results. For example Womersley identified *C. platylobium* and *C. xiphocarpa* as one such pair and *C. platylobium* is similar to *C. xiphocarpa* in having flattened, lanceolate determined laterals, and receptacles displaying two marginal rows of conceptacles. However, the phylogenetic trees suggest that *C. platylobium* is sister to the clade composed of *C. siliquosa* and *C. retorta* in the *rbc*L, ITS, and concatenated dataset analyses (Figs. 2, 3, 4). Within *C. platylobium* - *C. siliquosa* - *C. retorta* clade, all members exhibit laterals that branch in one plane (i.e.complanate) but they differ in main axis shape: *C. platylobium* has flat main axis and the secondary axes are branched from the edges, *C. siliquosa* has main axis with quadrangular to almost square in cross section, while *C. retorta* has flat main axis with secondary axes branching off from the face.

The ancestral state reconstruction in Figures 5-8 suggests that the ancestor of *Cystophora* possibly had no vesicles and possessed distichous lateral branching pattern. However, the ancestral state for the shape of the main axis cannot be determined in the analysis. Womersley (1964) proposed that *C. xyphocarpa* was the most primitive and interpreted its morphology as the simplest or least complex among all *Cystophora* species (e.g. complanate branching patterns across all branching orders, from primary axes to ramulli, and hence absence of a three dimensional habit). Whereas the ancestral state reconstruction suggested that the 'simpler' features of *C. xiphocarpa* (Figs. 5-

8; see also Chapter 1, Table 1.1) can be interpreted as derived rather than primitive (e.g. secondary loss, character reversal).

Results from independent analyses across all three genetic markers were in agreement not only to each other but also, and to a certain extent, to Womersley's phylogenetic proposal (Figs. 1-3). Between the molecular phylogenies, incongruent results occurred for the position of *C. moniliformis*. In the *rbc*L tree, *C. moniliformis* displayed low to no genetic differences with *C. intermedia* a forming clade with very high support, while the *cox*1 trees placed *C. moniliformis* is sister to *C. polycystidea*. The use of a third marker (ITS) and the total evidence (i.e. concatenated data) analysis were paramount to solve this disparity and recognize *C. moniliformis* forming as a basal lineage in the *Cystophora* phylogeny, together with *C. intermedia*. Furthermore C. *moniliformis* and *C. intermedia* share key morphological features and were recognized by Womersley (1964, 1987) as closely related species (Fig. 1). Therefore two out of three markers plus the concatenated results and the morphological data are in agreement and support the basal positioning of *C. moniliformis* and *C. intermedia* possibly forming a single lineage in the *Cystophora* phylogeny.

Also, the relationship of *C. torulosa* and *C. subfarcinata* in the ITS tree showed relatively low genetic differences between these two species whereas the *rbc*L and *cox*1 trees suggested that they are distinct from each other. In this case, cytoplasmic and nuclear molecular markers have clear different evolutionary rates, leading to different evolutionary relationships, although all results, including morphological data agree that these two species are closely related and belong to the same evolutionary linage. Alternatively, an example

of an evolutionary process that can potentially cause marker disagreement includes ancient hybridization. To test for the present of hybridization in the evolutionary history of some *Cystophora* species and clades would require explicit and targeted research, including specimen morphometric analysis to see whether putative hybrids also display mixed morphological characters. In this study we used only stereotypical specimens in order to avoid species identification problems and ensure we were dealing with and discussing about the right taxa.

Recently, the DNA barcode marker *cox*1 known in animals and red algae for its high levels of interspecific genetic variability, and low intraspecific variation (Herbert et al. 2003, Saunders 2005, respectively) was developed for brown algae (Lane et al. 2007). As such, it was applied in this study as a way to aid in *Cystophora* species identification. However, it is important to point out that the primary application of this marker has not been to build phylogenies. While cox1 turned out to be very good in discerning apart species, phylogenetic reconstructions, particularly those between deeper nodes, should be considered with great care. Marker and topology incongruence in species-level studies can be a result of several factors, such as different rates of molecular evolution, saturation, misalignment, introgression, unrecognized paralogy, interlineage inhomogeneity of models (Sanderson and Shafer 2002), and incomplete lineage sorting (the latter a result of recent speciation and the absence of strong selective sweeps, Maddison and Knowles 2006). Simulations show that larger number samples per species rather than more markers sequenced is the best approach to solve problems associated with incomplete lineage sorting (Maddison and Knowles 2006). Consequently, the next steps in

Cystophora systematic research should aim not only in generating similar data for the species not included in this study but also in obtaining data for other specimens with limited sampling in this study, such as *C. expansa, C. pectinata, C. grevillei, C. retroflexa, C. intermedia.*

The phylogenetic trees of combined dataset of three markers exhibited moderate to low support for several clades in ML analysis (BP = 44-84%), whereas the Bayesian Inference tree showed better support values but two clades contained polytomy were present; *C. subfarcinata – C. cuspidata – C. botryocystis*, and *C. expansa – C. grevillei –* and a clade contained *C. racemosa* and *C. pectinata*. These polytomies in this analysis are soft (i.e. lack of phylogenetic information) (Walsh et al, 1999) rather than 'hard' polytomy (i.e. rapid speciation) (Walsh et al, 1999). The results of combined dataset from ML and BI analyses indicated that more phylogenetic information (number of markers and samples per species) are needed to resolve the polytomy and obtain better phylogenetic support values.

Buchanan (2011) reconstructed phylogenetic relationships in *Cystophora* using both *cox*1 and combined ITS and *cox*1 DNA sequence datasets. His results suggested the merger of the New Zealand endemic species *C. distenda* and *C. scalaris*, two species not included in this study, that display high morphological resemblances, although are not recognized in the same evolutionary lineage as seen in Womersley's phylogenetic diagram (Fig. 1). From Buchanan's (2011) *cox*1 analysis, *C. distenda* and *C. scalaris* specimens formed one clade with high bootstrap support, and within that clade, sequences were grouped into two sub-groups with moderate support. When the location of these specimens was considered, a strong phylogeographic signal could be

observed between northern and southern New Zealand biogeographic provinces. Buchanan (2011) also suggested the synonym of *C. retroflexa* and *C. congesta* based on both molecular and morphological analyses. However, according to our results, *C. congesta* and *C. retroflexa* were not conspecific and were not found in the same clade as Buchanan's trees. *Cystophora retroflexa* specimen used in this study were all compared to Womersley's collections at State Herbarium of South Australia and their morphologies were in agreement. This discrepancy between the results from Buchanan (2011) and my results requires further analyses. The results of this study are in agreement with Buchanan's (2011) *cox*1 results, showing signs of the absence of lineage sorting in *C. retroflexa, C. subfarcinata* and *C. congesta* (data not shown). I decided to not include Buchanan's data in this study, as I was not able to confirm sample identification used by that author.

In summary, this study revealed the phylogenetic relationships in *Cystophora*, and recognized *C. cuspidata* as an ecological form of *C. subfarcinata*. Ancestral state reconstruction results revealed that morphological characters in *Cystophora* have limited power for inferring phylogenetic relationships and identifying monophyly due to a high incidence of homoplasies. This study also advanced our knowledge on how three of the most commonly used genetic markers in brown algal systematics contribute to this information. Overall genetic divergence among *Cystophora* species is small across all markers used suggestive of recent radiation. Due to low levels of genetic divergence among ITS sequences and the presence of incomplete lineage sorting in *cox*1, single marker species identification should be, whenever possible, avoided. *Rbc*L phylogenies produced the most consistent

results with overall higher phylogenetic support values; consequently, this marker should be prioritized in *Cystophora* systematic studies. A multi-marker approach together with a better within and between species taxon sampling, including the addition of rare, difficult to find uncommon species such as *C. harvey*. *C. gracilis, C. tenuis* and *C. cymodoceae*, the latter only known from the type locality, will undoubtedly solve once and for all, all phylogenetic relationships among *Cystophora* extant species.

Acknowledgements:

We thank Carolyn Ricci and Bob Baldock (State Herbarium of South Australia) for providing access to specimens and advice, Gareth Belton, Rainbo Dixon and Maria Marklund for providing *C. cuspidata, C. racemosa, C. pectinata* specimens and Gerald Kraft for providing *C. xiphocarpa* specimen.

Taxonomic changes.

Cystophora subfarcinata (Mertens) J. Agardh 1848:240.

Basionym: *Fucus subfarcinata* 1819:184.

Heterotypic synonym:

Cystophora cuspidata J. Agardh 1896: 48.

Cystoseira subfarcinata (Mertens) C. Agardh 1821:83; 1824: 289. Sonder 1846: 160

Blossevillea subfarcinata (Mertens) Kuetzing 1849: 628; 1860: 26

Sargassum subfarcinata (Mertens) Kuntze 1880: 228.

Description: Thallus is 20-80 cm long, medium to dark brown. Holdfast is discoid-conical, 3-12 mm in diameter, epilithic. Primary axes are fairly straight, compressed to strongly flatten, 2-7 mm broad, 1-2 mm thick, distichously branched from the faces. Secondary axes are slightly retroflex and distichously branched. Laterals are 1-3 cm long, simple to irregularly branched with simple or branched terete ramuli. Vesicles absent or present; are numerous replacing ramuli at the base of laterals. Vesicles absent in rough waters, when present, vesivles are elongate-ovoid to subspherical, pedicellate, 1-3 cm long and 2-3 mm thick. Receptacles are simple or often branched, 1-3 cm long and 1-2 mm thick but in rough water form, receptacles are distinctly larger, up to 6 cm long with very prominent conceptacles closely arranged in two to three rows.

Distribution: From Fremantle (Perth), W.A., around South Australia, Victoria and Tasmania.

Specimens examined (information is listed as follows: taxon name, locality, voucher number): As *C. cuspidata* - South Australia, Eyre Peninsula region, Elliston: AD-A13446, AD-A13600, AD-A19407, AD-A13445, AD-A15125B; St Vincent Gulf, Adelaide: AD-A18655, AD-A15842; Encounter Bay, Victor Harbour: AD-A15516, AD-A15517, AD-A01848; Port Eliot: AD-A09411, AD-A21084, AD-A18669; Robe: AD-A29284B; Great Australian Bight: AD-A94920. As *C. subfarcinata* - South Australia, Eyre Peninsula region: AD-A94918, AD-A95051; South Australia, Kangaroo Island: AD-A74674, AD-A98021, AD-A98022, AD-A74652, AD-A74675, AD-A74667, AD-A74663, AD-A74656, AD-A98024, AD-A98026, AD-A74640, AD-A74647, AD-A79084

Chapter 4: Unveiling species diversity of *Lobophora* (Dictyotales) in Australia with *rbc*L and *cox*1 DNA sequence analyses.

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Statement of Authorship

Unveiling species diversity of *Lobophora* (Dictyotales) in Australia with *rbc*L and *cox*1 DNA sequence analyses

This chapter has been prepared as a submission for publication

Nuttanun Soisup
Executed fieldwork, complete laboratory work and data analysis, and prepare manuscript as principal author.

Signed
Date: 26 July 2013

Andrew Lowe
Provided guidance with data analysis and contributed to revisions of the manuscript

Signed
Date: 26 July 2013

Fred Gurgel
Involved in developing idea, executed fieldwork, provided guidance with laboratory work and data analysis and contributed to revisions of the manuscript

Signed
Date: 26 July 2013

Abstract

Lobophora is a common brown algal genus widely distributed in both temperate and tropical zones around the world. Currently the entire genus consists of nine species, four of which are found in Australia. In this study, we explored the diversity of *Lobophora spp*. in Australia based on *rbc*L and *cox*1 DNA sequences based on ~200 samples collected across the country, including some samples from other parts of the world. Primary species hypotheses (PSHs) based on consensus results of Generalized Mixed Yule Coalescent (GMYC) models and Automatic Barcode Gap Discovery (ABGD) methods from both markers were used to identify species. Results suggest the existence of at least 33 species worldwide. Six species (PSH) agree with published taxa, however, *L. pachyventera* samples were separated into two PSHs. Of all 33 PSHs, 22 PSHs occurred in Australia and eight PSHs were widely distributed from sub-tropical eastern Asia to tropical Australia. *L. variegata* was also the only species known from Atlantic Ocean but our results suggested at least four PSHs occurred in that area.

Introduction

Lobophora J. Agardh (1894) is a common benthic marine brown alga distributed in tropical to temperate oceans, worldwide. It occurs intertidally to depths of more than 120 m (Littler and Littler 2000). In tropical areas, *Lobophora* is often abundant and found in association with coral reefs (Huisman 2000). *Lobophora* thalli are flattened, fan-shaped and not calcified. The genus is distinct from other Dictyotales genera in that its central row of medullary cells is prominently larger.

There are nine *Lobophora* species currently recognized worldwide (Guiry and Guiry 2013, Sun et al. 2012). *Lobophora variegata* is the most common and widely distributed species in the genus. This species has been reported from all tropical and temperate oceans, while the rest are restricted in their distribution. For example, *Lobophora papenfussii* is known from the Marshall Islands, Micronesia, Fiji and Madagascar. *Lobophora dichotoma* is known only from eastern South Africa, and *Lobophora rickeri* is known only from southern Great Barrier Reef, Australia. Until recently, all *Lobophora* species were described and identified based on morphological characters, however, Sun *et al*. (2012) recently combined morphological and molecular analyses to describe four new species from the western Pacific Ocean: *Lobophora pachyventera, Lobophora asiatica, Lobophora crassa,* and one new species from southern Australia, *Lobophora australis*. Sun *et al*. (2012) also resurrected an old name previously known as a *L. variegata* synonym, *Lobophora nigrescens*, to identify specimens from temperate Australia.

Australia is one of the top five temperate marine macroalgal biodiversity and endemism hot spots in the world (Bolton 1996) and consists of

different marine habitats from tropical to temperate environments. In Australia, *Lobophora* is widely distributed around the country's entire coastline except for Tasmania (Womersley 1987, Kraft 2009). Four *Lobophora* species are known in Australia: *L. rickeri* from Queensland, *L. nigrescens* from Victoria and South Australia, *L. australis* from South Australia, and *L. variegata*, which is Australia-wide. In Australia, *L. variegata* occurs in various habitats from shallow water rock pools, coral reefs to deep-water environment (36 m) (Womesley 1987).

Morphological plasticity is very common in *L. variegata*. Littler and Littler (2000) placed *L. variegata* from Caribbean Sea into three morphotypes, according to their habitats: a decumbent form in shaded area or the deep waters, a crust form in shallow subtidal waters and a ruffled form in calm and shallow areas. However, many other authors have described two morphological forms in *L. variegata*: an encrusting (prostrate) form and an erect form (Womersley 1987, Taylor 1960, Earle 1969, Lawson and John 1982, Kraft 2009, Huisman 2000). The encrusting form is common in shallow water habitats and in the presence of high herbivory pressure (such as pristine offshore coral reefs) or wave-exposed habitats. The erect form tends to be more common in protected, low-intertidal to subtidal and deep-water habitats, usually with low herbivory pressure. Additionally, other studies have also identified that the different morphotypes of *L. variegata* also display variation in their rate of dispersal and susceptibility to herbivory (Coen and Tanner 1989, de Ruyter van Steveninck and Breeman 1987, de Ruyter van Steveninck et al. 1988). *L. variegata* morphological and physiological variation is possibly caused by genetic divergence and the existence of multiple cryptic and locally

adapted species rather than a single genetically connected (panmictic) population, or taxon, with worldwide distribution. From our field observations, *L. variegata* in Australia also shows wide morphological and habitat variation. However, no studies to date have tested whether *L. variegata* in Australia corresponds to a single taxon or multiple genetically distinct entities.

The application of DNA sequence techniques to delimit species and support biodiversity surveys are currently widely used (Castelin *et al*. 2012, Hebert *et al*. 2003, Puillandre *et al*. 2012a). Several approaches have been developed to define species boundaries, especially when taxa under examination exhibit phenotypic plasticity or cryptic and subtle morphological differences between species. Since species identification, particularly in Dictyotalean brown algae, has been problematic due to morphological variation, many studies have applied molecular techniques to identify species and refine their geographic distributions (Silberfeld *et al*. 2013, Tronholm *et al*. 2012). In this study, I aimed to unveil cryptic diversity of Australian *Lobophora* based on DNA sequence data.

Materials and Methods

Collections

Lobophora samples were collected across five major regions in Australia via snorkeling or SCUBA diving (Fig. 1). All samples were preserved for DNA analysis in silica gel desiccant (Chase & Hillis 1991) and whole plants were either herbarium pressed as morphological vouchers or preserved in 4% formalin/seawater solution and deposited at the South Australia State Herbarium (AD). The list of specimens with collection information and GenBank accession numbers were provided in Appendix 4.

DNA extraction, sequencing and *alignment*

DNA was extracted from hand-cleaned tissue samples using NucleoSpin® 96 Plant II kits (Macherey-Nagel, Düeren Germany) following the manufacturer's protocol. Sequences of *rbc*L and *cox*1 region were amplified using the previously published primers *rbc*L-68F/*rbc*L-608R (Draisma *et al*. 2001) and GAZF2/GAXR2 (Lane *et al*. 2007), respectively. PCR amplifications were performed in 25 µl reactions composed of 1X AmpliTaq Gold PCR buffer (Applied Biosystems, Foster City, CA, USA), 3 mM $MgCl_2$, 0.2 µM of each primer, 0.2 µM of each dNTP, 1 M Betaine, 1.5 units of AmpliTaq Gold DNA polymerase, and 1 µl of DNA template diluted 1:10 in distilled water. The amplification was run on the Palm Cycler (Corbett Research, Australia) and parameters included an initial denaturation at 95˚C for 3 min followed by 40 cycles of 95˚C for 45 seconds, 52˚C for*cox*1 and 50˚C for *rbc*L regions for 1 min, and 72˚C for 1.30 min, terminated by 72˚C for 5 min. Products were cleaned using ExoSAP-IT (GE Healthcare,

Buckinghamshire, UK) or MultiScreen PCR_{384} Filter Plate (Milipore, Billerica, MA, USA). Sequencing was performed using ABI BigDye Terminator v 3.1 (Applied Biosystems, Foster City, CA, USA) and sequence products were cleaned using MultiScreen SEQ_{384} Filter Plates following the manufacturer's protocol (Milipore, Billerica, MA, USA). All cleaned products were submitted to the Australian Genome Research facility (AGRF-Adelaide)Ltd. Pty. for capillary separation on an ABI 3730*xl* sequencing platforms. Sequence contig assembly was conducted in Geneious Pro v.5.5.8 (Biomatters, Aukland, New Zealand) and *rbc*L and *cox*1 sequences were aligned using the MUSCLE algorithm implemented in Geneious.

Molecular species delineation

To identify candidate species, we applied two molecular-based species delineation approaches, the General Mixed Yule-Coalescent (GMYC) model and the Automatic Barcode Gap Discovery (ABGD) to both markers independently. General Mixed Yule-Coalescent (GMYC) model (Monaghan et al. 2005, Pons et al. 2006) uses maximum likelihood approach to detect transition signals from speciation to coalescent branching patterns in an ultrametric tree. We constructed the ultrametric tree using Bayesian analysis implemented in BEAST version 1.7.4 (Drummond et al., 2012). Identical sequences in *rbc*L and *cox*1 alignments were reduced to unique haplotypes using MetaPIGA 2.1.3 (Helaers and Milinkovitch, 2010). The tree was constructed under the coalescent model of constant population prior with GTR+I+G model and an uncorrected lognormal relaxed molecular clock. Two independent analyses were run for 10 million generations and sampled every

1,000 steps. The log files were checked with Tracer 1.5.0 (Rambaut and Drummond, 2009), 10% burn-in was discarded from each run and a combined log from two independent runs had effective sample size (ESS) more than 1000 after burn-in. The maximum clade credibility tree was extracted from the combined MCMC runs using TreeAnnotator 1.7.4 (Drummond et al., 2012) with a posterior probability limit of 0.5. GMYC analyses were run with single and multiple threshold models from Splits package (Ezard et al., 2009) for R (available from http://r-forge.r-project.org/projects/splits).

The Automatic Barcode Gap Discovery (ABGD) software (Puillandre et al., 2012) was also used to delimit species boundaries. The ABGD method is based on detection of the barcode gap in the distribution of pairwise genetic distance values. The uncorrected p-distances of *rbc*L and *cox*1 dataset were calculated using MEGA5 (Tamura et al., 2011) and the ABGD analysis was performed using online version of the program (available from http://wwwabi.snv.jussieu.fr/public/abgd/abgdweb.html).

Proposing primary species hypotheses

Primary species hypotheses (PSHs) were proposed based on un-contradicted (i.e. agreement between methods GMYC and ABGD methods) positive species identification described above.

Phylogenetic Analyses

*Rbc*L and *cox*1 DNA sequences representing unique haplotypes were selected and aligned as described above. *rbc*L and *cox*1 dataset were independently used to construct ML and Bayesian Inference tree. The model

GTR+I+G was selected as the best fitting model for both markers using jModelTest2 (Darriba et al. 2012). The maximum likelihood tree was performed using PhyML online execution (available from http:// www.atgc-montpellier.fr/phyml/) with NNI & SPR option, 10 random starting trees and 1,000 bootstrap replicates. Bayesian Inferences were analysed by MrBayes (Huelsenbeck and Ronsquist 2001) plug-in implemented in Geneious Pro 5.5.8. Markov chains were run for 5 million generations, sampled every 1,000th generation with a specified 500,000 generations burn-in. MCMC runs were monitored within Geneious, effective sample size were more than 1000 in all runs after the burn-in, providing evidence that convergence had been reached.

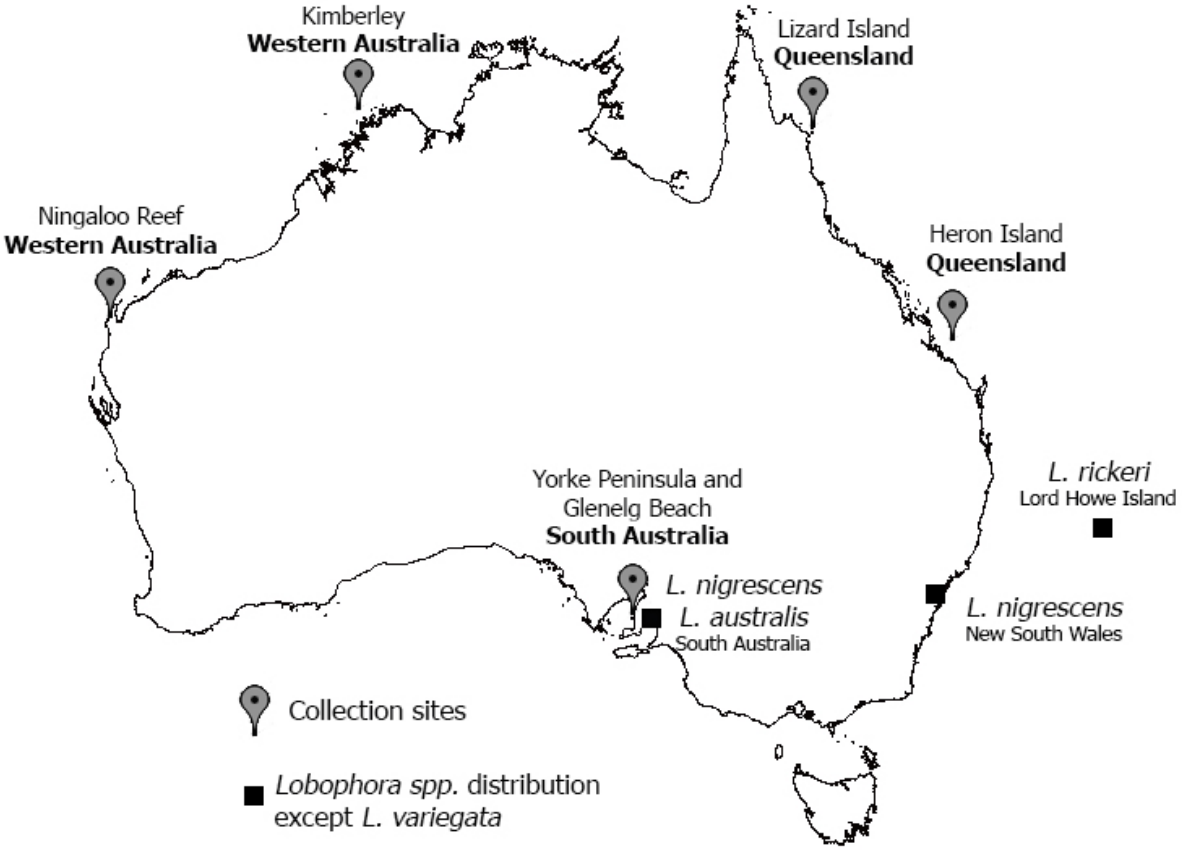


Figure 1. Collection sites and distribution of *Lobophora* spp. in Australia except *L. variegata* that occurs across Australia except in Tasmania.

Results

Species delimitation

*rbc*L dataset

The *rbc*L dataset contained 312 *Lobophora* DNA sequences (624 bp long) comprised of 273 newly generated and 30 published sequences (Appendix 4). We found 80 distinct *rbc*L haplotypes displaying 11 parsimony informative sites.

GMYC models were applied to the Bayesian ultrametric tree to find the maximum likelihood for the transition between intra and interspecific branching patterns. The likelihood of single and multiple models increased from the null model (Table 1). The single-threshold GMYC model defined 32 entities from 80 haplotypes with confidence interval of 28 – 41, while the multiple-threshold model defined 42 entities with confidence interval of 28-42.

Applying the ABGD method to the 312 *rbc*L sequence dataset using standard settings with relative width gap (x-value) = 10, no barcode gap was detected. When lowered the x-value to 1.5, 9 priori thresholds were detected with the prior of intraspecific divergence ranging from 0.001 to 0.059 (Fig.2). At the intraspecific divergence range between 2-3% where the numbers of group reach stationary state before there is no barcode gap gap detected, ABGD method estimated 29 groups.

*cox*1 dataset

The *cox*1 dataset consisted of 52 newly generated sequences (634 bp long) displaying 203 polymorphic sites and 182 parsimony informative sites (Appendix 4). Forty-two distinct cox1 haplotypes were recognized.

GMYC models were applied to the Bayesian ultrametric tree constructed from 42 sequences representing different haplotypes. The likelihood of single and multiple models were significantly different from the null model (Table 1). The single-threshold GMYC model defined 18 entities with confidence interval of 17-19 while multiple-threshold model defined 20 entities with confidence interval of 19-20.

Applying the ABGD method to 52 *Lobophora* cox1 sequences using standard setting with relative width gap (x-value) = 10, no barcode gap was detected. When lowered the x-value to 1.5, 10 priori thresholds were detected with the prior of intraspecific divergence ranged from 0.001 to 0.12 (Fig.2). At the intraspecific divergence up to 3.5%, ABGD method estimated 15 species groups.

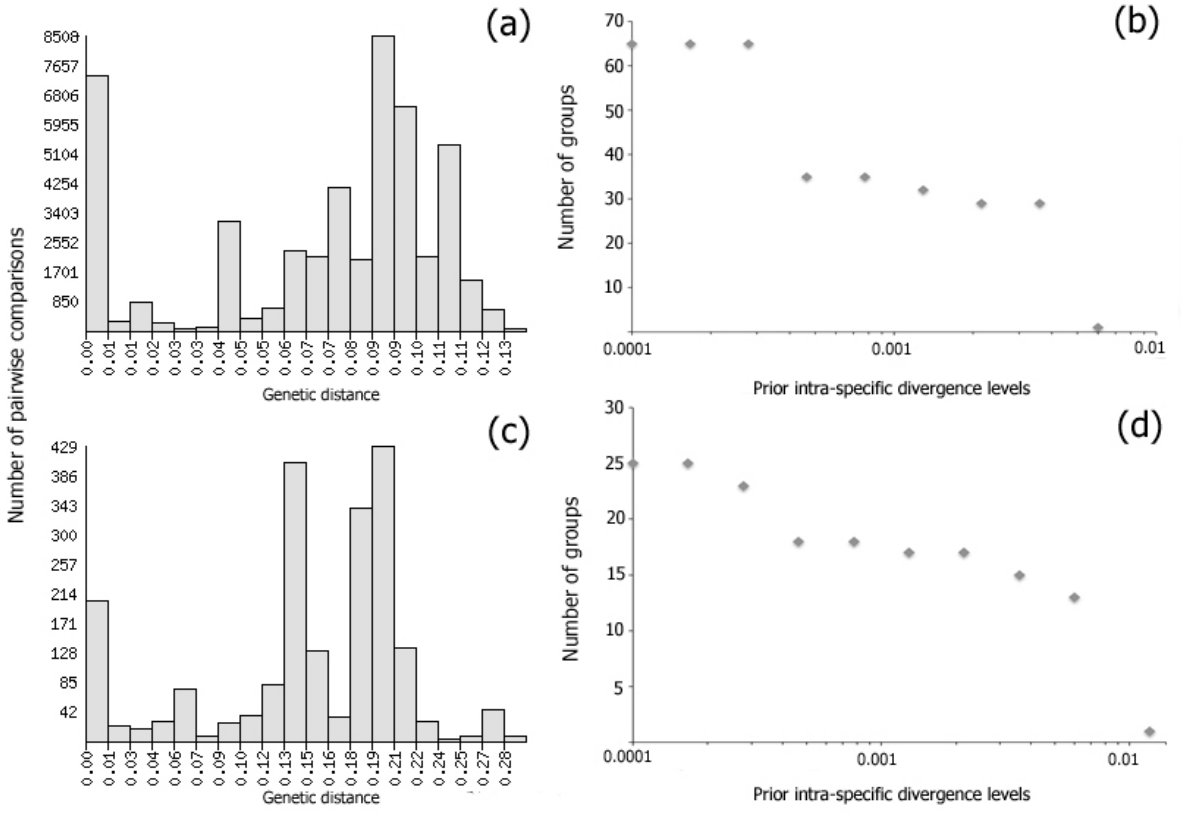


Figure 2. (a) Frequency distribution of pairwise sequence comparison based on p-distance of *rbc*L dataset; (b) ABGD results indicating number of groups given each prior intraspecific divergence value of *rbc*L dataset; (c) frequency distribution of pairwise sequence comparison based on p-distance of *cox*1 dataset; (d) ABGD results indicating number of groups given each prior intraspecific divergence value of *cox*1 dataset.

Table 1. Summary of the *rbc*L and *cox*1 dataset analyses for the single and multiple threshold variants of GMYC method

	Model	T	N_{gmyc}	CI	Shared entities	rbcL only	L_0	L_{gmyc}	Likelihood ratio
*rbc*L	Single	-0.0083	32	28-41	14	18	539.2873	548.062	17.5494*
	Multiple	-0.0102	42	28-42	21	22	539.2873	549.202	19.9504*
		-0.006							
		-0.0033							
		-0.0015							
*cox*1	Single	-0.0055	18	17-19	15	-	224.1778	236.567	24.7799**
	Multiple	-0.0055	20	19-20	19	-	224.1778	236.858	25.3621**
		-0.0006							

T, threshold genetic distance from the branch tips where transition occurred

Ngmyc, number of entities delineated by GMYC models

CI, confidence intervals

Shared entities, entities that were delimited based on rbcL and cox1 sequences

rbcL only, entities that were delimited based on only rbcL sequences

L0, likelihood for null model

Lgmyc, likelihood for GMYC model

LR, significance of the likelihood ratio

*$p<0.005$

**$p<0.001$

Proposing primary species hypotheses

Thirty-three PSHs were delineated based on consensus results of the analyses described above (Fig. 3). 16 PSHs were identified based on both *rbc*L and *cox*1 analyses while the 17 PSHs were delineated based on *rbc*L results only. Average genetic distance between PSHs ranged between 1.2-10 % in *rbc*L and 3-18 % in *cox*1.

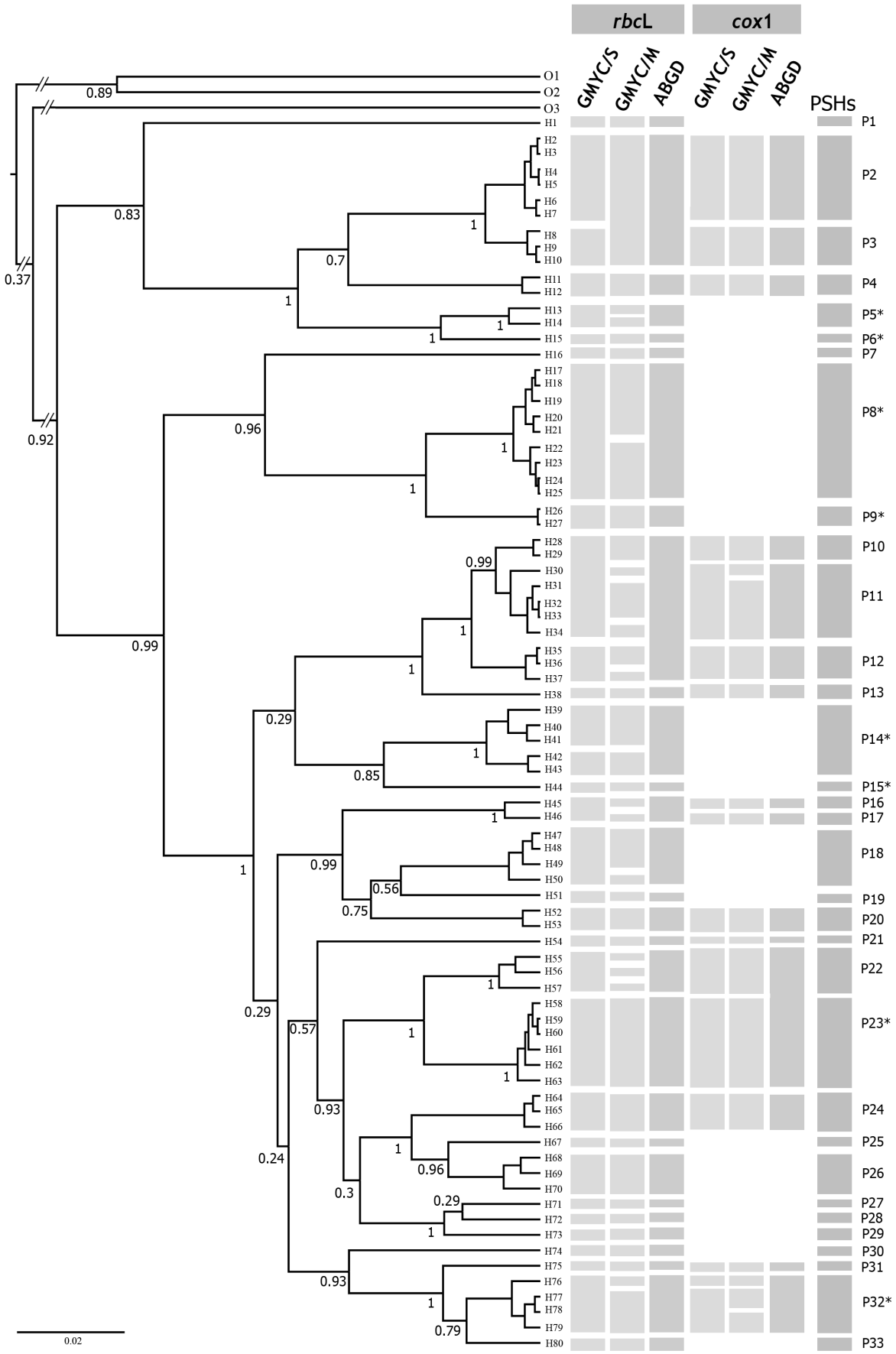
Of all 36 published *rbc*L sequences from GenBank, 21 sequences contained 7 described species while the remaining 15 sequences have yet to be named. *"L. variegata*" sequences from New Caledonia from GeneBank (EU579956, EU579957) corresponded to PSH5, and the only *L. papenfussi* sequence in GenBank (EU579953) was recognized as our PSH 6. *L. nigrescens* sequences from South Australia and New South Wales (AB665257, AB646541) are grouped with samples from Heron Island, Ningaloo Reef and South Australia as PSH 8. *L. australis* sequence (AB665258) was grouped with South Australia samples in PSH 9. *L. crassa* sequences (AB665260, AB665261, AB665262, AB665264) were clustered with samples from Kimberley and Heron Island in PSH 32. However, *P. pachyventera* composed of five published sequences grouped into 2 distinct PSHs, 4 samples from China and Japan (AB665265, AB665266, AB665267, AB665268) and 4 samples from Lizard Island formed PSH14 while a *P. pachyventera* sample from Malaysia (AB665269) was proposed as a distinct species (PSH15).

Phylogenetic analyses

We analysed 80 *rbc*L sequences representing unique *Lobophora* haplotypes, and three phylogenetically closely related outgroup sequences:

Zonaria angustata, *Padina australis* and *Newhousia inbricata*. Maximum likelihood and Bayesian Inference trees displayed the same topology (only ML tree shown, Fig. 4). Sequences belonging to the same PSHs clustered together with high support values. The *cox*1 dataset of 42 sequences from different haplotypes also clustered according to PSHs with high support in both ML and BI trees (Fig. 4). *Cox*1 tree also showed congruence with *rbc*L results.

Figure 3 (on the next page). On the left: Ultrametric tree generated with BEAST software from rbcL dataset containing 80 unique haplotypes (H1-H80) and three outgroup: *Padina australis* (O1), *Newhousia imbricata* (O2), and *Zonaria angustata* (O3). Numbers at the nodes indicate posterior probabilities shown only on nodes that separated PSHs. In the middle: summary of entities delineated by GMYC models and ABGD methods of *rbc*L and *cox*1 dataset. On the right: Primary species hypotheses (PSHs) proposed from un-contradicted results of analyses. Unique PSHs were labeled as P1-P33 and PSHs that contained described species sequences were marked with an asterisk. Species identifications were cited in Appendix 4.


rbcL
cox1
GMYC/S
GMYC/M
ABGD
GMYC/S
GMYC/M
ABGD
PSHs
O1
O2
O3
H1
H2
H3
H4
H5
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P23*
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P26
P27
P28
P29
P30
P31
P32*
P33
0.89
0.37
0.83
0.7
1
1
1
0.92
0.96
1
1
0.99
0.99
1
1
0.29
1
0.85
1
1
0.99
0.56
0.75
1
0.29
0.57
1
1
0.93
0.24
1
0.96
0.3
0.29
1
0.93
1
0.79
0.02

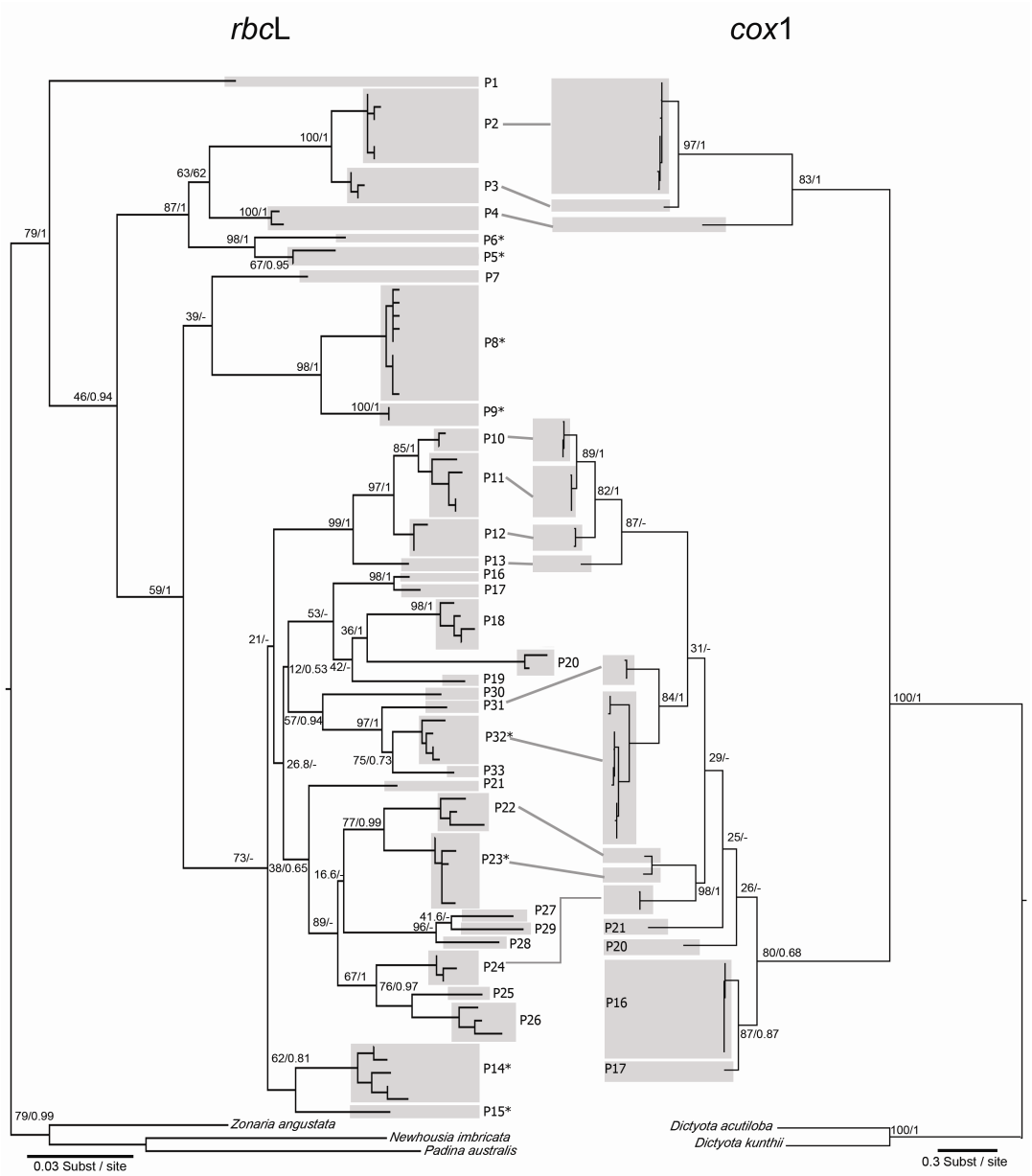

Figure 4. On the left: *Lobophora* maximum likelihood tree based on 80 unique *rbc*L haplotypes. All haplotypes were grouped according to primary species hypotheses, PSH (P1-P33). On the right: Maximum likelihood tree based on 42 unique *cox*1 haplotypes. *Cox*1 haplotypes were separated into 15 groups according to PSHs results. Lines between two trees indicate the congruence between *rbc*L and *cox*1 trees. The support values are shown only at the node identifying distinct PSHs (bootstrap values from ML analyses / posterior probability from Bayesian inferences).

Discussion

This study proposed 33 *Lobophora* primary species hypotheses (PSHs) based on *rbc*L and *cox*1 datasets. Of the nine formally described *Lobophora* species present in the literature, seven were confirmed using molecular classification. The exceptions include *L. dichotoma* and *L. rickeri*. Four published species agree with our PSHs delineation: *L. papenfussi* as PSH 6, *L. nigrescens* as PSH 8, *L. australis* as PSH 9, and *L. crassa* as PSH 32. However, our results support the splitting of *L. pachyventera* into two distinct entities: PSH14 and PSH15 (Fig. 3). PSH14 clade was composed of four published sequences from China and Japan (AB665265, AB665266, AB665267, AB665268) and four samples from Lizard Island with intra-specific genetic distances up to 1.6% in *rbc*L. However, the inter-specific genetic distance between PSH14 and PSH15 was up to 4.6%. Sun *et al*. (2012) suggested that for *Lobophora*, within species divergence should not exceed 5% in *rbc*L and 7% in *cox*1, with *L. pachyventera* holding the highest intra-specific divergence observed in their study, up to 4.6% in *rbc*L and 6.3% in *cox*3. Comparing with other published species, intra-specific genetic divergences are up to 3.1% in PSH5, and approximately 1-2% in other PSH. In addition, PSH15 (AB665269) contains only one specimen collected from Malaysia, and the inclusion of more specimens from this clade in future analyses will certainly clarify the genetic and morphological differences between PSH14 and PSH15.

The phylogenetic trees constructed by Sun *et al*. (2012) based on *rbc*L and *cox*3 sequences suggested 12 clades of *Lobophora*, named A to L, but only seven species were reported. Clades B, C, D, G, and L were not formally described as new species due to limited number of specimens or the lack of

distinctive morphological characters between them. Our analyses incorporated those sequences and delineated them into different PSHs. Clade C was separated into three PSHs: PSH25, PSH 26, and PSH24. Clade D was separated into two PSHs: PSH28 and PSH29. Clade G is PSH 30, Clade L is PSH 1 and Clade B is PSH 22. The inclusion and analysis of more samples in this study showed that there is likely more than one species contained in Clades C and D, demonstrating the effect of taxa sampling in DNA barcoding studies that can lead to biodiversity underestimations.

Due to their morphological simplicity and often paucity of morphological characters to distinguish hidden species diversity, molecular species delimitation and DNA barcoding have currently been widely used in marine macroalgal systematics. Indeed DNA barcode surveys have revealed a plethora of highly diverse species complexes (Payo et al. 2013, Puillandre et al. 2012). Several methods have been developed to delimit species from DNA barcode datasets, for example Hebert et al. (2004) suggested that inter-specific threshold distances should be 10 times greater than intraspecific distances. For large datasets, two recent methods were developed for delimiting species, the General Mixed Yule Coalescent model analysis (GMYC) and Automatic Barcode Gap Discovery (ABGD). Though those two approaches are convenient they still have limitations, especially when distinct species are represented in the datasets with only a few specimens (Loshe 2009, Puillandre 2011). From the 33 PSHs we proposed, there were 10 PSHs that were represented by only one specimen and six PSHs by two to three specimens. Collecting more samples belonging to those PSHs would refine GMYC and ABGD species delimitation results in *Lobophora*.

Several molecular phylogenetic studies have incorporated *Lobophora* specimens in their analyses (Bittner et al. 2008, Phillips et al. 2008, Sun et al. 2012), though Sun et al. (2012) were the first to target *Lobophora* phylogenetic relationships at the species level. Bittner et al. (2008) analyzed 26S rDNA, *psa*A and *rbc*L regions of four *Lobophora* sequences identified as *L. variegata*, *L. papenfussi* and *Lobophora spp*. The study simply suggested that *Lobophora* was closely related to *Newhousia* and *Zonaria*. Sun *et al*. (2012), applied *rbc*L and *cox*3 regions to reassess *Lobophora* diversity and showed that both markers were applicable for species level phylogenetic reconstruction. In our study, we used partial *rbc*L and *cox*1 regions to evaluate species diversity of *Lobophora*. The *cox*1 region contained more variable sites comparing with *rbc*L region. Moreover, ABGD and GMYC analyses of samples for which there were both *cox*1 and *rbc*L sequences showed that *cox*1 region is able to delineate more entities than *rbc*L (Fig. 3 and Table 2). However, partial *rbc*L region contained sufficient information for reconstructing species-level phylogenies with high bootstrap support especially at the terminal nodes (Fig. 4).

My results showed that several *Lobophora* species reported from eastern Asia also occurred in Australia. For example, *L. pachyventera* (PSH14) reported from Japan and China is also present in Lizard Island, Queensland. *L. asiatica* (PSH23) known from China, Japan and Malaysia is also present in Ningaloo Reef, Western Australia. *L. crassa* (PSH32) known from Hawaii, China and Japan was also found in Heron Island, Queensland and offshore reefs in the Kimberley region, Western Australia. These three coral reef species show a wide geographic distribution in the tropical western Pacific. Species

that have yet to be named also clustered with Australian specimens. Clade C (PSH26) consisted of specimens from Curaçao Island, West Indies and Shioya, Minamidatojima Island, Japan, clustered with samples from Ningaloo Reef, Western Australia. A sample from Malaysia belonging to Clade C (PSH24) clustered with samples from Lizard Island, Queensland. Samples from Clade B (PSH22) from China and Japan clustered with samples from Lizard Island, Queensland and Ningaloo Reef, Western Australia. In addition, *Lobophora* sequence from Palau (AB096899) was clustered with samples from Lizard Island in PSH4. In PSH11, one sample from Palau clustered with samples from Ningaloo Reef, Western Australia and Lizard Island, Queensland. In summary, there are eight PSHs that co-occurred between Asia and tropical Australia. Those PSHs showed that they have a wide spatial distribution from the subtropical Islands of Japan to tropical Australia but more collections from Southeast Asia and Pacific Islands will improve the understanding of their biogeography.

From our results, 22 PSHs present in Australia instead of only four as previously known. Eight PSHs were shared between tropical Australia and Asia as described above. In Kimberley, Western Australia, there are at least two PSHs presented: PSH2 consisted of samples from Kimberley and Ningaloo Reef, Western Australia and PSH32 (*L. crassa*). There are nine PSHs presented in Ningaloo Reef (PSHs 2, 8, 11, 20, 21, 22, 23, 26, and 31), with only PSH 21 and 31 restricted to that site. There are ten PSHs presented in Lizard Island, Queensland (PSHs 4, 11, 12, 13, 14, 18, 20, 22, 24, and 27) and only two PSHs (13 and 27) were unique to that region. Heron Island also contained nine widespread and four unique PSHs (widespread: 8, 10, 12, 13, 16, 17, 18, 19,

32; unique: 10, 16, 17, and 19). Based on our recent sampling, which are the most robust collections ever conducted in Australia, Heron Island presented the highest level of *Lobophora* species endemism. However, these putative species might be found elsewhere as more collections are made.

In South Australia, Womersley (1987) proposed the existence of only one species, *L. variegata*. However, Sun *et al*. (2012) suggested two species occurred in South Australia: *L. nigrescens* and *L. australis*. *L. nigrescens* was first reported from Dromana Bay, Victoria (type locality, J. Agardh 1984), but *L. nigrescens* was later merged into *L. variegata* by Womersley (1987). Sun *et al*. (2012) analyzed recent collections made in South Australia and New South Wales and proposed the resurrection of *L. nigrescens* as distinct species according to new morphological and molecular evidence. From our results, *L. nigrescens* sequences were grouped into PSH8 and clustered with specimens from Heron Island and Ningaloo Reef. However, another species from South Australia, *L. australis* (PSH9) formed a clade with specimens collected only in South Australia. *L. australis* is possibly a true endemic species with restricted distribution in southern temperate Australia.

Previously, *Lobophora variegata* was the only species known from Atlantic Ocean, however, our analyses revealed the presence of at least 4 PSHs in that area: PSH3 confirmed from Panama and deep-water Gulf of Mexico (i.e. dredge specimens), PSH7 composed of sample from Belize, PSH1 from Guadeloupe, West Indies; and PSH26 composed of specimens from Curaçao Island, West Indies, Shioya (Japan) and Ningaloo Reef (Western Australia). In addition, specimens from New Caledonia in PSH5 identified as *L. variegata* by Bittner et al. (2008) should be re-identified since the type locality of *L.*

variegata is Antilles, West Indies, and our results showed that no Caribbean specimens belonged to PSH5. From our analyses, there were 2 PSHs: PSH1 and PSH26, that contained samples from West Indies, the type location of *L. variegata*.

Results from our study suggested that *Lobophora* species diversity has been greatly underestimated due to its subtle morphological structures and high phenotypic plasticity. *Rbc*L and *cox*1 markers showed the utility of molecular species delimitation in resolving taxa with poor and ambiguous morphological characters. Although 33 PSHs are proposed from *rbc*L and cox1 dataset in this study, there are some missing data from cox1 marker and some PSHs posses limited samples which possibly misleading number of species proposed. Furthermore, additional morphological study in those groups of PSHs specimens is necessary to subjectively define putative *Lobophora* species and to provide key morphological features for field identification markers.

Acknowledgements:

We thank Carolyn Ricci and Bob Baldock (State Herbarium of South Australia) for providing access to specimens and advice.

Chapter 5

General Discussion

This thesis applied molecular phylogenetic approaches to study several Australian brown algal genera in order to cast light on their evolutionary relationships, to improve their taxonomic classification, and reveal novel patterns of generic and species diversity. This chapter is divided into 5 sections. Sections 1 to 4 refer to all scientific findings of this dissertation and the utility of molecular phylogenetics; and lastly, Section 5 describes the significance of this work.

5.1 Taxonomic revision and phylogenetic relationships of *Acrocarpia*, *Caulocystis* and *Cystophora*

5.1.1 Phylogenetic relationship within *Acrocarpia*

Acrocarpia is an Australian endemic brown algae genus common in temperate marine shallow water habitats. Traditionally, the genus is identified via morphological examinations based on Womersley (1964, 1987). No studies have reassessed the relationships between the two species in the genus. Molecular analyses of *A. paniculata* and *A. robusta* in Chapter 2 confirmed that those two species are distinct from each other and belong to the same genus. In this case, the molecular analyses agree with the historical morphological diagnoses.

5.1.2 Phylogenetic relationship within *Caulocystis*

Areschoug (1854) and Womersley (1964, 1987) proposed two species of *Caulocystis*: *C. cephalornithos* and *C. uvifera*, which differed by the shape of their pneumatocysts (air vesicles). However, both authors observed specimens with intermediate morphology. Womersley (1964, 1987)

hypothesized that the two *Caulocystis* taxa could possibly be morphological variants of the same species, but he could not confirm the statement using only morphological evidence. Previous work has included *Caulocystis* species in their molecular analyses (Draisma *et al*., 2010; Silberfeld *et al*., 2010), but no studies to date have compared genetic differences and phylogenetic relationships among different *Caulocystis* morphotypes. From the molecular analyses in Chapter 2, *Caulocystis* specimens from various sites with different morphotypes showed low to no genetic differences in all three molecular markers and indicate that the two species should be considered conspecific.

5.1.3 Phylogenetic relationship within *Cystophora*

Cystophora is one of the most common and conspicuous brown algal genera in temperate Australia and New Zealand. Identification at generic level is based on the presence of bilateral and sympodial main axes, which is easy to recognize. However, identification at species level is problematic due to high levels of phenotypic plasticity. In addition, Womersley's monographs of the genus (1964, 1987) remains unclear in many parts. For example, Womersley (1964) described the differences between *C. congesta* and *C. retroflexa* on page 87 as " *C. congesta is most closely related to C. retroflexa, differing in the much denser laterals and secondary axes, shorter receptacles, and thicker, more rigid, primary axis. Occasional intergrades between these species occur*." According to this statement, it is relatively difficult to identify these two species without comparing a wide range of specimens and looking for overall patters rather than clear diagnostic features. It also makes the identification of atypical and juvenile specimens often impossible. In addition,

Womersley did not describe the characteristics of the intergrade cases in a way to solve this conundrum. Apart from ambiguous morphological delineations, molecular-based phylogenetic relationships in *Cystophora* as seen across all three markers herein tested were also unclear. Womersley was the first person to propose evolutionary relationships for *Cystophora* species based on the main axis and lateral branching patterns, however his diagram and tree remains incomplete. In this study, I focused on constructing phylogenetic trees solely based on specimens that agreed with the stereotypical descriptions provided by Womersley. The intent was to use only DNA sequences from specimens with unequivocal stereotypical morphotypes and hence, of unquestionable taxonomic identification, to identify standard molecular data with which other atypical morphologies could be compared to and identified against. Because of the lack of clear cut molecular and phylogenetic differences found across all taxa, this quest remains open for further investigations, or perhaps many species other then those here merged will have to have their delineations revised. Moreover, newly generated phylogenetic trees were compared with Womersley's (1964) morphological character-evolution hypotheses. The analysis in Chapter 3 showed that morphological characters used by Womersley to define evolutionary relationships are not free from homoplasy, when compared to hypothesis derived from the molecular data, suggesting a more complex evolutionary history exists within the genus than previously considered.

5.2 Species recognition and taxonomic changes

5.2.1 Morphological and molecular phylogenetic analyses and species recognition

In this thesis, I used molecular phylogenetic analyses to test morphological based classification. Morphological classification has been used to classify members of species based on shared phenotypic similarities, but phylogenetic classification defines members of species based on the phylogenetic tree. Morphological classification is widely used and key morphological features of the species are useful especially when identifying species in the field. However, morphological features can have limited information and some characters are homoplasic. Molecular phylogenetic analyses can produce reliable classification because the numbers of DNA characters are abundant when compared with morphological characters, but species recognition using DNA analyses is not as simple as using morphological characters. The combination of two approaches, morphological and molecular analyses, is advantageous for species recognition.

In addition, the results of Chapter 2 and 3 suggest that some morphological characters do not reflect evolutionary relationships, for example, pneumatocysts (air vesicles). In chapter 2, the phylogenetic relationships of *Caulocystis* suggest that air vesicle shapes are not relevant to genetic differentiation. *Caulocystis* sequences tend to cluster according to the geographic location, not the shape of pneumatocysts. In addition, the ancestral state reconstruction of the genus *Cystophora* (chapter 3) reveals that the presence or absence of pneumatocysts in *Cystophora* is homoplastic.

5.2.2 *Caulocystis cephalornithos* and *C. uvifera*.

Based on molecular phylogenetic analyses and morphological examination in chapter 2, I propose to merge of *C. cephalornithos* and *C. uvifera*. The priority is given to *C. cephalornithos* as the species was described prior to *C. uvifera*.

5.2.3 *Cystophora cuspidata* and *C. subfarcinata*

Caulocystis subfarcinata and *C. cuspidata* are morphologically similar except that the receptacles in *C. cuspidata* are prominently larger. Based on molecular analyses of *rbc*L, ITS 1and 2, and concatenated datasets in chapter 3, *C. cuspidata* clustered in a clade with *C. subfarcinata* in all analyses, and with low to no genetic differences between these two species. In addition, my observations revealed receptacles in *C. subfarcinata* specimens showed wide morphological variation, overlapping with those of *C. cuspidata*. Based on molecular and morphological evidence, I propose the recognition of *C. cuspidata* as a synonym of *C. subfarcinata*.

5.3 *Lobophora species diversity*

In chapter 4, I applied DNA approaches to reveal cryptic species in the genus *Lobophora*. *Lobophora variegata* was the only species in the genus with a worldwide distribution while all other known species in the genus have a more restricted distribution, although several different morphotypes of *L. variegata* have been recognized. Since phenotypic plasticity is common in brown algae and differences between *L. variegata* morphotypes have been attributed to ecological factors, the true number of species passing under the name *L. variegata* could not be assessed by traditional methods. From the analyses in chapter 4, application of DNA sequence methods in species delimitation unveiled a large number of cryptic diversity in *Lobophora*. Contrary to *Cystophora*, the results for *Lobophora* showed that, in cases where morphological characters are limited or lacking, the power of molecular analyses can reveal clear taxonomic differences between groups of species. The next steps in this research will be to use the clear species delimitations based on molecular analyses as a guide to recognize morphological differences and patterns between taxa. New species can be described based on the congruence of both, molecular and morphological data.

5.4 Species distribution and phylogeographic inferences in temperate southern Australia

5.4.1 Species distributions in *Acrocarpia, Caulocystis* and *Cystophora*

The two species of *Acrocarpia* are genetically very distinct and do not share the same geographic distribution. One species is confined to the west coast, while the other to the mid-east coast of southern Australia. Strangely, there are no historical collections of *Acrocarpia* recorded from Saint Francis Isles, Southern Australia to Israelite Bay, Western Australia. Whether this gap in distribution is due to poor sampling or historical and/or oceanographic processes remains unknown. Temperate rocky reefs occur across the entire extension of the Australian southern coastline so lack of substrate for new *Acrocarpia* settlements is not a reason for this disjunction. It is possible that the historical biogeography of *Acrocarpia* may have reflected that of *Caulocystsis*, where once a single common ancestor of both *Acrocarpia* species occurred across the entire southern Australian coast, which then followed a process of population isolation by distance and differentiation, allopatry, and ultimately speciation. Signs of genetic structuring were detected for *Caulocystis* across this biogeographic divide and may be a sign of incipient speciation.

5.4.2 Phylogeographic relationships in *Caulocystis*

In chapter 2, *rbc*L and *cox*1 markers reveal phylogenetic relationships in *Caulocystis* clade. There is a clear phylogeographic structure between *Caulocystis* populations from South Australia and populations from southern Australia regions (i.e. Victoria and NSW). This pattern is in agreement with two biogeographic provinces: the Flindersian Province (western and south

Australia) and Peronian Province (eastern Australia) (Whitley, 1932; Poore and O'Hara, 2007). A recent study of the characteristics of subtidal habitat along the Australian coastline suggests that the species composition of the two provinces is different: Flindersia possesses the most extensive canopy-forming algae, whereas in Peronia, the algae represent a patchwork (Connell and Irving, 2009). Connell and Irving (2009) also suggest that canopy-forming algae patch in Peronia Province is created by the strong herbivory of black urchins, *Centrostephanus rodgersii*, that removes all erect algae at their base. However, the black urchins do not recruit in Flindersia because the East-Australian Current does not flow to the south and west coast of mainland Australia. Habitat characteristics and ocean current possibly cause the disjunction between two populations of *Caulocystis* in this study.

5.4.2 Phylogeographic patterns and species distribution of *Lobophora*

According to the analyses in chapter 4, there are 22 species (as PSHs) of *Lobophora* in Australia, eight of which have a wide spatial distribution from the subtropical Islands of Japan to tropical Australia. There are only two species currently known from South Australia: *L. nigrescens* and *L. australis*. *Lobophora nigrescens* occurs widely across tropical and temperate coasts while *L. australis* is geographically restricted to South Australia and probably endemic to that region. More collections from across southwestern and southeastern Australia when compared with the baseline data herein, will certainly improve our understanding about *Lobophora* distribution and species diversity in other temperate Australian coastlines.

5.5 Significance of the thesis

Brown algae identification in Australia has been predominantly morphology-based, and many taxa still have unreliable taxonomic descriptions due to high levels of intergrading morphologies between several species. This thesis demonstrated the utility, power and limitations of DNA taxonomy and phylogenetic reconstruction to clarify taxonomic uncertainties in several Australian taxa. Furthermore, the results also produced evidence for the presence of phylogeographic structure of brown algae in southern Australia coasts. Apart from clarifying long-standing taxonomic uncertainties, molecular taxonomy tools also unveiled the presence of unknown and unexpected cryptic and pseudo-cryptic species in groups that have limited morphological characters.

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Appendices

Appendix 1: Lists of primers used in this study

Primers		Sequences(5'-3')	Region	Tm (˚c)	References
*rbc*L-68F	F	GCNAAAATGGGNWAYTGGGATGC	*rbc*L	52	Draisma *et al.*, 2001
*rbc*L-708R	R	TTAAGNTAWGAACCYTTAACTTC	*rbc*L	52	Bittner *et al*., 2008
*rbc*L-543F	F	CCWAAATTAGGTCTTTCWGGWAAAAA	*rbc*L	52	Bittner *et al*., 2008
*rbc*L-1381R	R	ATATCTTTCCATARRTCTAAWGC	*rbc*L	52	Burrowes *et al*., 2003
*gaz*F2	F	CCAACCAYAAAGATATWGGTAC	*cox*1	50	Lane *et al.*, 2007
*gaz*R2	R	GGATGACCAAARAACCAAAA	*cox*1	50	Lane *et al.*, 2008
ITS1-BF	F	GGCGGAGCTATCTTGCTTCGTCC	*ITS*-1	55	This study
5.8-BR	R	ATCGCATTTCGCTGCGTTCTTCATCG	*ITS*-1	55	This study
5.8S-BF	F	CGATGAAGAACGCAGCGAAATGCGAT	*ITS*-2	55	Yoshida *et al*. 2000
25BR-2R	R	TCCTCCGCTTAGTATATGCTTAA	*ITS*-2	55	Yoshida *et al*. 2000

Appendix 2: Lists of specimens used in Chapter 2 with their collection information.
Table. 1 Lists of specimens with their collection information

Taxon	Herbarium ID	Locality	Date of collection	collectors	*rbc*L	*cox*1	ITS2
A. robusta	AD-A89241a	South Coast, WA	18-Jan-09	RMD	KF285935	KF285945	*
A. robusta	AD-A89241b	South Coast, WA	18-Jan-09	RMD	KF285936	KF285946	
A. paniculata	AD-A95297	Queenscliff Beach, VIC	10-Dec-09	NS	KF285937	KF285937	*
A. paniculata	AD-A95277	Queenscliff Beach, VIC	10-Dec-09	NS	KF285938	-	-
C. cephalornithos	AD-A89187	Glenelg, SA	7-Jun-10	NS	*	KF285943	*
C. cephalornithos	AD-A89184	Glenelg, SA	7-Jun-10	NS	*	-	*
C. cephalornithos	AD-A89185	Glenelg, SA	7-Jun-10	NS	*	*	*
C. cephalornithos	AD-A89190	Glenelg, SA	7-Jun-10	NS	*	-	-
C. cephalornithos	AD-A89188	Glenelg, SA	7-Jun-10	NS	*	-	-

C. cephalornithos	AD-A89423	Yorke Peninsular, SA	9-Oct-09	NS + CFG	*	-	*
C. cephalornithos	AD-A89425	Yorke Peninsular, SA	9-Oct-09	NS + CFG	-	-	*
C. cephalornithos	AD-A89429	Yorke Peninsular, SA	9-Oct-09	NS + CFG	-	-	*
C. cephalornithos	AD-A89428	Yorke Peninsular, SA	9-Oct-09	NS + CFG	-	-	*
C. cephalornithos	AD-A89424	Yorke Peninsular, SA	9-Oct-09	NS + CFG	-	-	*
C. cephalornithos	AD-A95309	Point Lonsdale Reef, VIC	8-Dec-09	NS	*	-	-
C. cephalornithos	AD-A95276	Point Lonsdale Reef,	8-Dec-09	NS	-	KF285944	-

		VIC					
C. cephalornithos	AD-A95278	Point Lonsdale Reef, VIC	8-Dec-09	NS	*	-	*
C. cephalornithos	AD-A95290	Point Lonsdale Reef, VIC	8-Dec-09	NS	*	-	*
C. cephalornithos	AD-A95296	Point Lonsdale Reef, VIC	8-Dec-09	NS	*	KF285939	*
C. cephalornithos	AD-A95315	Queenscliff Beach, VIC	10-Dec-09	NS	-	*	-
C. cephalornithos	AD-A89419	Yorke Peninsular, SA	17-Jan-10	CFG+NS	*	-	*
C. cephalornithos	AD-A89420A	Lowly point, Whyalla, SA	20-Jan-10	CFG+NS	*	-	*
C. cephalornithos	AD-A89420B	Lowly point, Whyalla, SA	20-Jan-10	CFG+NS	*	KF285941	*

C. cephalornithos	AD-A89420C	Lowly point, Whyalla, SA	20-Jan-10	CFG+NS	*	KF285942	*
C. cephalornithos	AD-A89420D	Lowly point, Whyalla, SA	20-Jan-10	CFG+NS	*	-	*
Sirophysalis trinodis	AD-A95058A	Glenelg, SA	10-Jun-10	NS	KF285934	KF285949	*
Sirophysalis trinodis	AD-A95058B	Glenelg, SA	10-Jun-10	NS	KF285933	KF285948	*

*accession numbers can be searched via Herbarium ID

Appendix 3: Lists of specimens used in Chapter 3 with their collection information.

Species name	#	*cox*1	*rbc*L	ITS	Herbarium ID
C.botrycystis	-	-	-	*	AD-A76499
C.brownii	1	-	-	*	AD-A94920
C.brownii	2	-	-	*	AD-A94922
C.brownii	3	-	-	*	AD-A94921
C.congesta	1	-	*	*	AD-A95286
C.congesta	2	-	*	*	AD-A95287
C.cuspidata	-	-	*	*	AD-A94920
C.expansa	1	*	*	*	AD-A74642
C.expansa	2	-	*	-	AD-A94922
C.grevillei	-	*	*	*	AD-A95302
C.intermedia	-	*	*	-	AD-A74643
C.moniliformis	1	-	*	*	AD-A74658
C.moniliformis	2	*	*	*	AD-A98019
C.moniliformis	3	*	*	-	AD-A79104
C.moniliformis	4	-	-	*	AD-A98028
C.moniliformis	5	-	-	*	AD-A95280
C.pectinata	1	-	-	*	AD-A91427
C.pectinata	2	*	*	*	AD-A91426
C.platylobium	1	*	*	*	AD-A74651
C.platylobium	2	*	*	*	AD-A95303
C.platylobium	3	*	*	*	AD-A95304
C.polycystidea	1	*	*	*	AD-A98013
C.polycystidea	2	*	-	-	AD-A98012
C.polycystidea	3	-	-	-	AD-A98015
C.racemosa	-	-	-	*	AD-A91428
C.retorta	1	-	*	-	AD-A98027
C.retorta	2	-	*	*	AD-A98009
C.retorta	3	-	-	-	AD-A98016
C.retroflexa	-	-	-	-	AD-A95314
C.siliquasa	1	*	*	*	AD-A74653
C.siliquasa	2	*	-	-	AD-A74669
C.siliquasa	3	*	-	*	AD-A74649
C.siliquasa	4	*	-	-	AD-A94928
C.siliquasa	5	*	-	*	AD-A74654
C.siliquasa	6	*	-	-	AD-A47641
C.subfarcinata	1	-	*	*	AD-A94918
C.subfarcinata	2	*	*	*	AD-A74674
C.subfarcinata	3	*	*	*	AD-A98021
C.subfarcinata	4	*	*	*	AD-A98022
C.subfarcinata	5	*	*	*	AD-A74652
C.subfarcinata	6	*	-	*	AD-A74675

C.subfarcinata	7	*	-	-	AD-A74667
C.subfarcinata	8	*	*	-	AD-A74663
C.subfarcinata	9	*	-	*	AD-A74656
C.subfarcinata	10	*	*	-	AD-A98024
C.subfarcinata	11	*	-	-	AD-A98026
C.subfarcinata	12	*	-	-	AD-A74640
C.subfarcinata	13	*	*	-	AD-A74647
C.subfarcinata	14	*	*	*	AD-A95051
C.subfarcinata	15	*	-	-	AD-A79084
C.torulosa	1	*	*	*	AD-A95315
C.torulosa	2	*	*	*	AD-A95316
C.torulosa	3	*	*	*	AD-A95273
C.xiphocarpa	1	-	*	*	GW5016580**

*accession numbers can be searched via Herbarium ID

** collection ID instead of AD-A accession number.

Appendix 4: Lists of *Lobophora* specimens used in Chapter 4 with their collection information.

	AD-A number	Species/Clade name	rbcL	cox1	Location
PSH1		Clade L	EU579955	-	
PSH2	87699		*	-	Ningaloo Reef, WA
	89501		*	-	Ningaloo Reef, WA
	87697		*	-	Ningaloo Reef, WA
	89896		*	-	Ningaloo Reef, WA
	87697		*	-	Ningaloo Reef, WA
	88279		*	-	Ningaloo Reef, WA
	91944		*	*	Ningaloo Reef, WA
	98043		*	*	Ningaloo Reef, WA
	98046		*	*	Ningaloo Reef, WA
			*	*	Ningaloo Reef, WA
	98042A		*	*	Kimberly, WA
	98042B		*	*	Kimberly, WA
	98042C		*	*	Kimberly, WA
	98042D		*	-	Kimberly, WA
	98042E		-	*	Kimberly, WA
	98042F		-	*	Kimberly, WA
	98042G		*	-	Kimberly, WA
	98042H		*	-	Kimberly, WA
	98042I		*	*	Kimberly, WA
	98042J		*	*	Kimberly, WA
	98042K		*	-	Kimberly, WA
	98043		*	*	Kimberly, WA
	98044		*	*	Kimberly, WA
PSH3	98047		*	*	Panama
	1234**		*	-	Panama
	1220**		*	-	Panama
	98047		*	-	Gulf of Mexico

	98047		*	-	Gulf of Mexico
PSH4			AB096899	-	
	92355A		*	*	Lizard Is., Queensland
	92355B		*	-	Lizard Is., Queensland
	92355C		*	-	Lizard Is., Queensland
PSH5		*L.variegata*	EU579956	-	
		L.variegata	EU579957	-	
PSH6		*L.papenfussi*	EU579953	-	
PSH7	98052		*	-	Belize
PSH8	90507D		*	-	Heron Is., Queensland
	90507D		*	-	Heron Is., Queensland
	88467B		*	-	Heron Is., Queensland
	90507B		*	-	Heron Is., Queensland
	88467E		*	-	Heron Is., Queensland
	88467D		*	-	Heron Is., Queensland
			EF990239	-	Australia
	88467C		*	-	Heron Is., Queensland
	90507B		*	-	Heron Is., Queensland
		L.nigrescens	AB646541	-	NSW
	98091		*	-	Lizard Is., Queensland
	91632		*	-	Ningaloo Reef, WA
	98086A		*	-	Ningaloo Reef, WA
	91804B		*	-	Ningaloo Reef, WA
	90894B		*	-	Ningaloo Reef, WA
	91895		*	-	Ningaloo Reef, WA
	98090A		*	-	Ningaloo Reef, WA
	91633		*	-	Ningaloo Reef, WA
	91675E		*	-	Ningaloo Reef,

			WA
91522A	*	-	Ningaloo Reef, WA
87697	*	-	Ningaloo Reef, WA
18.5.10.2.5**	*	-	Ningaloo Reef, WA
91852B	*	-	Ningaloo Reef, WA
91522B	*	-	Ningaloo Reef, WA
91978	*	-	Ningaloo Reef, WA
91679A	*	-	Ningaloo Reef, WA
91679C	*	-	Ningaloo Reef, WA
98089B	*	-	Ningaloo Reef, WA
91997G	*	-	Ningaloo Reef, WA
98086G	*	-	Ningaloo Reef, WA
91775A	*	-	Ningaloo Reef, WA
16.6.8.2.8E**	*	-	Ningaloo Reef, WA
87675A	*	-	Ningaloo Reef, WA
91852A	*	-	Ningaloo Reef, WA
98086B	*	-	Ningaloo Reef, WA
98089A	*	-	Ningaloo Reef, WA
91779D	*	-	Ningaloo Reef, WA
98093	*	-	Ningaloo Reef, WA
91775B	*	-	Ningaloo Reef, WA
91829	*	-	Ningaloo Reef, WA
15.5.9.1.52**	*	-	Ningaloo Reef, WA
87675E	*	-	Ningaloo Reef, WA

91679F		*	-	Ningaloo Reef, WA
91679B		*	-	Ningaloo Reef, WA
	L.nigrescens	AB665257	-	
98088		*	-	Edithburgh, SA
98029		*	-	Glenelg Beach, SA
98030AZ		*	-	Moorowie Port, SA
98030AG		*	-	Moorowie Port, SA
98030H		*	-	Moorowie Port, SA
98030		*	-	Moorowie Port, SA
98030AK		*	-	Moorowie Port, SA
98030AS		*	-	Moorowie Port, SA
98030O		*	-	Moorowie Port, SA
98030A		*	-	Moorowie Port, SA
98030AM		*	-	Moorowie Port, SA
98030L		*	-	Moorowie Port, SA
98030AP		*	-	Moorowie Port, SA
98030AW		*	-	Moorowie Port, SA
98030G		*	-	Moorowie Port, SA
98030AE		*	-	Moorowie Port, SA
98030AX		*	-	Moorowie Port, SA
98030AD		*	-	Moorowie Port, SA
98084A		*	-	Rickbly, SA
98084B		*	-	Rickbly, SA
98084C		*	-	Rickbly, SA
98084D		*	-	Rickbly, SA
98084E		*	-	Rickbly, SA
98084F		*	-	Rickbly, SA
98084G		*	-	Rickbly, SA
98084H		*	-	Rickbly, SA

98084I	*	-	Rickbly, SA
98084J	*	-	Rickbly, SA
98084K	*	-	Rickbly, SA
98085BC	*	-	Whyalla, SA
98072	*	-	Whyalla, SA
98073	*	-	Whyalla, SA
98074	*	-	Whyalla, SA
98075	*	-	Whyalla, SA
98076	*	-	Whyalla, SA
98077	*	-	Whyalla, SA
98078	*	-	Whyalla, SA
98079	*	-	Whyalla, SA
98080	*	-	Whyalla, SA
98081	*	-	Whyalla, SA
98082	*	-	Whyalla, SA
98083	*	-	Whyalla, SA
98087	*	-	Whyalla, SA
98085	*	-	Whyalla, SA
98071AG	*	-	Innes National Park, SA
98071N	*	-	Innes National Park, SA
98071S	*	-	Innes National Park, SA
98071O	*	-	Innes National Park, SA
98071Y	*	-	Innes National Park, SA
98071C	*	-	Innes National Park, SA
98071AH	*	-	Innes National Park, SA
98071AS	*	-	Innes National Park, SA
98071Q	*	-	Innes National Park, SA
98071E	*	-	Innes National Park, SA
98071AK	*	-	Innes National Park, SA
98071U	*	-	Innes National Park, SA
98071AQ	*	-	Innes National Park, SA
98071B	*	-	Innes National Park, SA
98071AM	*	-	Innes National

					Park, SA
PSH9	98031AB		*	-	Edithburgh, SA
	98056		*	-	Glenelg Beach, SA
	98055		*	-	Whyalla, SA
	98057		*	-	Glenelg Beach, SA
	98058		*	-	Glenelg Beach, SA
	98053		*	-	Whyalla, SA
	98065		*	-	Edithburgh, SA
	19.1.10.1.9**		*	-	Rickaby, SA
	98031AN		*	-	Edithburgh, SA
	98031H		*	-	Edithburgh, SA
	98066		*	-	Edithburgh, SA
	98070		*	-	Edithburgh, SA
	98059		*	-	Glenelg Beach, SA
	98060		*	-	Glenelg Beach, SA
		L.australis	AB665258	-	
	98061		*	-	Glenelg Beach, SA
	98068		*	-	Edithburgh, SA
	98031AG		*	-	Edithburgh, SA
	98062		*	-	Glenelg Beach, SA
	98063		*	-	Glenelg Beach, SA
	98031U		*	-	Edithburgh, SA
	98031AH		*	-	Edithburgh, SA
	98054		*	-	Whyalla, SA
	98064		*	-	Glenelg Beach, SA
	98069		*	-	Edithburgh, SA
	98067		*	-	Edithburgh, SA
PSH10	90791		*	-	Heron Is., Queensland
	88305		*	-	Heron Is., Queensland
	88679		*	-	Heron Is., Queensland
	21.11.9.1.4J**		*	-	Heron Is., Queensland
	90791G		*	-	Heron Is., Queensland
	90791M		*	-	Heron Is., Queensland
	98099		*	-	Heron Is., Queensland
	98098		*	-	Heron Is., Queensland
	21.11.9.1.3**		*	*	Heron Is., Queensland
	21.11.9.1.3A**		*	*	Heron Is.,

					Queensland
	90791L		-	*	
					Heron Is.,
	90791E		*	-	Queensland
					Heron Is.,
	21.11.9.1.4I**		*	-	Queensland
					Heron Is.,
	18.11.9.1.3A**		*	-	Queensland
					Heron Is.,
	90705C		*	-	Queensland
					Heron Is.,
	21.11.9.1.3B**		*	-	Queensland
					Heron Is.,
	21.11.9.1.3G**		*	-	Queensland
					Heron Is.,
	90791K		*	-	Queensland
					Heron Is.,
	98098		*	-	Queensland
					Heron Is.,
	90705A		*	-	Queensland
					Ningaloo Reef,
PSH11	98097		*	-	WA
					Ningaloo Reef,
	87664		*	-	WA
					Ningaloo Reef,
	88281		*	-	WA
					Ningaloo Reef,
	87664		*	-	WA
					Ningaloo Reef,
	98100		*	-	WA
					Ningaloo Reef,
	89604		*	-	WA
					Ningaloo Reef,
	87664		*	-	WA
		AB096896	*	-	
					Lizard Is.,
	88164D		*	*	Queensland
					Lizard Is.,
	88164E		*	-	Queensland
					Lizard Is.,
	88214B		*	-	Queensland
					Lizard Is.,
	88214A		*	-	Queensland
					Lizard Is.,
	88214C		*	-	Queensland
					Lizard Is.,
	88164A		*	*	Queensland

	88164B		*	*	Lizard Is., Queensland
	88164C		*	*	Lizard Is., Queensland
PSH12	98103		*	-	Lizard Is., Queensland
	9.2.9.1.1E**		*	-	Lizard Is., Queensland
	92496		*	*	Lizard Is., Queensland
	98105I		*	*	Lizard Is., Queensland
	98104J		*	-	Lizard Is., Queensland
	92496B		*	-	Lizard Is., Queensland
	91339		*	-	Lizard Is., Queensland
	98106		*	-	Lizard Is., Queensland
	98104I		*	-	Lizard Is., Queensland
	88146		*	-	Lizard Is., Queensland
	88144		*	-	Lizard Is., Queensland
	98106		*	-	Lizard Is., Queensland
	88212A		*	-	Lizard Is., Queensland
	88212B		*	-	Lizard Is., Queensland
	91254		*	-	Lizard Is., Queensland
	92496		*	*	Lizard Is., Queensland
	98106F		*	-	Lizard Is., Queensland
	88169		*	-	Lizard Is., Queensland
	90748H		*	-	Heron Is., Queensland
	90791H		*	-	Heron Is., Queensland
PSH13	92414		*	*	Lizard Is., Queensland
PSH14		*L.pachyventera*	*	-	

		L.pachyventera	*	-	
		L.pachyventera	*	-	
		L.pachyventera	*	-	
	87985		*	-	Lizard Is., Queensland
	88214D		*	-	Lizard Is., Queensland
	88214E		*	-	Lizard Is., Queensland
	87985A		*	-	Lizard Is., Queensland
PSH15		*L.pachyventera*	*	-	
PSH16	90990A		*	*	Heron Is., Queensland
	90990F		-	*	
	90990B		*	*	Heron Is., Queensland
	90990G		*	-	Heron Is., Queensland
	90556A		*	-	Heron Is., Queensland
	90556E		*	-	Heron Is., Queensland
	90556G		*	-	Heron Is., Queensland
	90819A		*	*	Heron Is., Queensland
	90819B		*	*	Heron Is., Queensland
	90819C		*	*	Heron Is., Queensland
PSH17	90990C		*	*	Heron Is., Queensland
PSH18	90990D		*	-	Heron Is., Queensland
	90705E		*	-	Heron Is., Queensland
	90705B		*	-	Heron Is., Queensland
	90705D		*	-	Heron Is., Queensland
	92412		*	-	Lizard Is., Queensland
	92421		*	-	Lizard Is., Queensland
PSH19	90714A		-	*	
	90714B		*	-	Heron Is.,

	90714C		*	-	Queensland Heron Is., Queensland
PSH20	91724		*	*	Ningaloo Reef, WA
	88052		*	-	Lizard Is., Queensland
	98096		*	-	Lizard Is., Queensland
PSH21	98101C		*	*	Ningaloo Reef, WA
	98102B		*	-	Ningaloo Reef, WA
PSH22	92601		*	-	Lizard Is., Queensland
	91617		*	*	Ningaloo Reef, WA
		Clade B	AB665276	-	
		Clade B	AB665274	-	
		Clade B	AB665278	-	
PSH23	98094		*	*	Ningaloo Reef, WA
		L.asiatica	AB665270	-	
		L.asiatica	AB096897	-	
		L.asiatica	AB548390	-	
		L.asiatica	AB665271	-	
		L.asiatica	AB665273	-	
		L.asiatica	AB665272	-	
PSH24	88212		*	-	Lizard
	88085		*	-	Lizard
	92305		*	-	Lizard
		Clade C	AB665280	-	
	92605		*	*	Lizard
	92606		*	-	Lizard
	89217		*	*	Lizard
	89215		*	-	Lizard
PSH25		Clade C	AB665277	-	
PSH26	98095		*	-	Ningaloo Reef, WA
		Clade C	AB665281	-	
		Clade C	AB665279	-	
PSH27	92368		*	-	Lizard Is., Queensland
PSH28		Clade D	AB665275	-	
PSH29		Clade D	AB096898	-	
PSH30		Clade G	AB665263	-	

PSH31	98041		*	-	Ningaloo Reef, WA
	98040		*	*	Ningaloo Reef, WA
	98039		*	*	Ningaloo Reef, WA
PSH32	98032		*	*	
	98033		*	*	
	98034		*	*	
	98035		*	*	
	98036		*	*	
	98037		*	*	
	98038		*	*	
		L.crassa	AB665261	-	
		L.crassa	AB665262	-	
		L.crassa	AB665260	-	
		L.crassa	AB665264	-	
	90828		*	*	Heron Is., Queensland
	90803A		*	-	Heron Is., Queensland
	90680		*	*	Heron Is., Queensland
	90803B		*	-	Heron Is., Queensland
	90803C		*	-	Heron Is., Queensland
	90803D		*	-	Heron Is., Queensland
	90803E		*	-	Heron Is., Queensland
PSH33			EU579954	-	

*accession numbers can be searched via Herbarium ID

** Field and sample unique identifiers instead of herbarium's accession numbers. All information available in AD-A.