Molecular phylogeography and climate change biology of the invasive green marine macroalgae Caulerpa taxifolia and Caulerpa cylindracea in Australia

Submitted by
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BSc (Hons)

Thesis submitted in total fulfillment of the requirements for the degree of Doctor of Philosophy

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Thesis Summary

Populations of the green marine macroalgae *Caulerpa taxifolia* and *Caulerpa cylindracea* have invaded and spread throughout Mediterranean after they were introduced from Australia. In Australia, these tropical to subtropical species have established invasive populations in New South Wales, South Australia, and most recently, for *C. cylindracea,* in Victoria.

Significant efforts have been made to elucidate the invasion history and geographic source locations of Mediterranean populations. The same effort has been lacking in Australia. Both species have provided challenges for molecular ecologists because of their predominantly clonal reproduction and low genetic variation within invasive populations in the case of *C. taxifolia*, and very high intra-individual genetic variation in *C. cylindracea*.

In chapter 2, I present a detailed review of the literature about the molecular ecology of *C. taxifolia* and *C. cylindracea*. I outline the phylogenetic and phylogeographic research on both of these species. The methodology and analysis of each study is critiqued. From this analysis I identify the knowledge gaps about invasive *Caulerpa* spp., and how to approach further research given advances in technology and knowledge of the organisms.

In chapter 3, I present a modified Ion Torrent next generation sequencing protocol that was used to identify novel single nucleotide polymorphisms (SNPs) in *C. taxifolia*. This approach resulted in the successful identification of a suite of SNPs. Primer development and SNP validation was performed for the Sequenom MassArray, and 184 specimens from 10 Australian populations from Queensland, New South Wales, South Australia, and Western Australia, were genotyped. The data build on existing phylogeographic data for *C. taxifolia* in Australia, and support a model of anthropogenic distribution rather than natural dispersal. Anthropogenically mediated primary introductions may have been caused by aquarium releases, while secondary spread has been facilitated by heavy boat traffic in affected areas. Finally I discuss the evidence of the so called “invasive strain” of *C. taxifolia*, and conclude that there is no such thing, and that

the assumption that an “invasive strain” exists has undermined the scientific objectivity of 30 years of study of invasive *Caulerpa* species.

Chapter 4 represents the first phylogeographic study of invasive and native *C. cylindracea* populations in Australia. In this chapter I used variation in the *rpl*16-*rps*3 region of the chloroplast to test hypotheses about the origins and dispersal mechanisms responsible for the establishment of invasive populations of *C. cylindracea* in South Australia. Molecular data does not support natural dispersal of *C. cylindracea*, a finding that is supported by a lack of species records of *C. cylindracea* for 2600 km between southern Western Australia and the SouthAustralia.

In chapter 5, I determined if RNA:DNA, Protein:DNA, and Protein:RNA ratios can be used to quantify the effect of ocean warming and acidification on *C. taxifolia* and *C. trifaria*. Protein profiles were also examined for effects of ocean warming and acidification. While no significant effects were observed on the ratios, concentrations of DNA were positively affected by temperature, while negative impacts on protein were associated with decreasing pH. The protein profiles also allowed me to determine if the invasive tropical and subtropical *C. taxifolia* is likely to be impacted differently by ocean warming and acidification than the native temperate *C. trifaria*. Protein profile data revealed that *C. trifaria* specimens experience greater levels of metabolic stress than *C. taxifolia* at lower pH, and that the invasive *C. taxifolia* will continue to thrive in warmer and more acidic ocean conditions while the native temperate species will be impacted negatively, possibly resulting in localized extinctions.

In chapter 6, I discuss how new techniques and their application aid in understanding the phylogeogaphy of invasive *Caulerpa* spp. in Australia, paving the way for continued phylogeographic analyses of these problematic species. I have also shown that the response of native and invasive *Caulerpa* spp. to climate change scenarios indicates that invasive populations of *Caulerpa* may become more abundant and continue to expand their invasive range in the

future. I also identify the limitations of this body of work and the issues encountered in the program of research, and discuss future research possibilities for invasive *Caulerpa* species.

Thesis Declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

I give consent to this copy of my thesis, when deposited in the University Library, being made available for loan and photocopying, subject to the provisions of the Copyright Act 1968.

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William Maxwell Grant

27/02/2015

Chapter 1

Introduction

1.1 Introduction

Invasive species are species that are able to establish a reproducing population or populations outside of their native range, and which cause negative ecological and economic impacts (Sakai et al. 2001, Richardson et al. 2011). Marine invasive species are a serious threat to biodiversity and ecosystem function, causing economic losses worldwide (Carlton 2000, Schaffelke and Hewitt 2007). The combined negative effects of widespread and cumulative local and global environmental stressors, including pollution and climate change, with the impact of species introductions, are expected to homogenize global biota, but also contribute to radical environmental transformations with severe loss of current ecosystem functions and services (Williams and Smith 2007, Hellman et al. 2008, Rahel and Olden 2008). Among the hundreds of known introduced marine organisms worldwide, macroalgae are of particular interest because they typically become abundant or dominant in invaded marine communities (Schaffelke et al. 2006, Williams and Smith 2007). Invasive macroalgae can alter ecosystem structure, dominance of foundation species, habitat heterogeneity, ecosystem function, the taxonomic and nutritional composition of food webs, and the prevailing source of primary productivity (Williams and Smith 2007). There are numerous vectors that transport marine invasive species. Williams & Smith (2007) reviewed the vectors associated with 277 algal invasions and reported that, from most to least common, they included hull fouling, aquaculture, ballast water, canal migrants, fishing equipment, the aquarium trade, and research.

Among the ~914 marine macroalgal species established outside their native range (Schaffelke et al. 2006, Schaffelke and Hewitt 2007), species in the genus *Caulerpa* J.V. Lamouroux are prominent, with two species reported as invasive due to rapid expansion, habitat competition, and exclusion of native flora and fauna. (Boudouresque et al. 1996, Chisholm et al. 1997, Wright et al. 2007, Žuljević et al. 2011). A further four have been identified outside their native range. The invasive species are; *Caulerpa taxifolia* (M. Vahl) C. Agardh (see Meinesz and

Hesse 1991), and *Caulerpa cylindracea* (Sonder) (see Belton et al. 2014), while the species outside their native range are *Caulerpa brachypus* forma *parvifolia* (Harvey) Cribb (Lapointe et al. 2006), *Caulerpa webbiana* Montagne (see Amat et al. 2008), *Caulerpa scalpelliformis* (R.Brown ex Turner) C.Agardh (see Falcao and Szechy 2005) and *Caulerpa filiformis* (Suhr) Hering (see Cummings and Williamson 2008).

Caulerpa spp. are coenocytic green macroalgae that attach to substrates by branched colourless rhizoids and possess creeping stolons that give rise to erect axes (assimilators or fronds) that are simple or branched (Price 2011). The morphology of *Caulerpa* spp. is a key contributor to their success as invaders, particularly the -

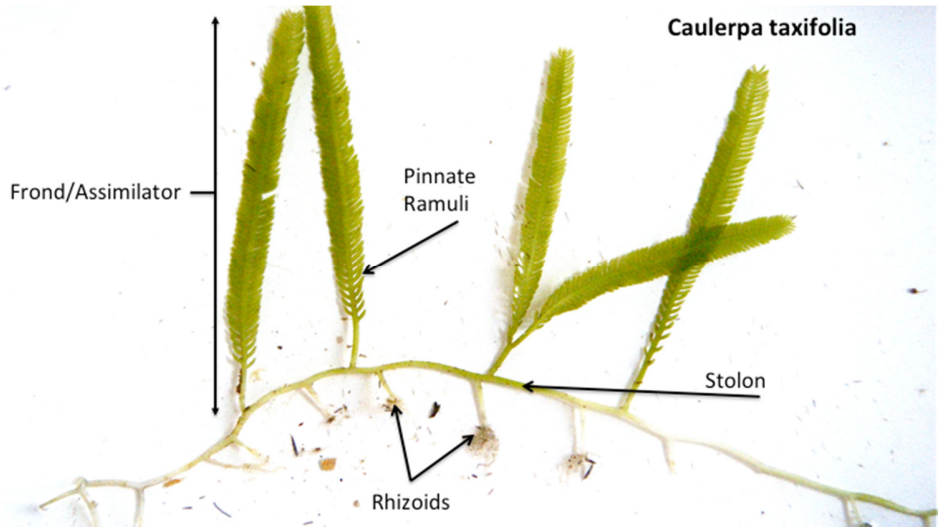

Fig 1.1 General morphology of the genus *Caulerpa*, displayed here is *Caulerpa taxifolia* from Sydney, New South Wales

rhizoids, which enable *Caulerpa* spp. to attach to a wide variety of substrates including mud, sand, and rock, and to inhabit these substrates in sheltered or exposed waters (Meinesz and Hesse 1991, Meinesz et al. 1993).

A unique characteristic of *Caulerpa* are the trabeculae, a dense network of anastomosing cylindrical cell wall ingrowths crossing the lumen of the cell, which provide the plant with structural support that helps resist the effects of compression and tension caused by changes in

cell turgidity (Domis et al. 2003). Trabeculae are hollow, communicate with the external environment, and are a major facilitator of diffusion between the environment and the internal contents of the coenocyte (Domis et al. 2003).

Molecular analyses indicate that *Caulerpa* is monophyletic (Famá et al. 2002b). The genus currently comprises 95 species, with the center of diversity in Australia, where 40 species are recorded and members of the genus are ubiquitous (Womersley 1984, Lam and Zechman 2006, Guiry and Guiry 2014). Of the 6 *Caulerpa* spp. that have been reported outside of their native range, 4 are native to northern Australia (*C. taxifolia, C. cylindracea, C. bracypus,* and *C. webbiana*), but also occur pan-tropically (Guiry and Guiry 2014). Of these, invasions by *C. taxifolia* and *C. cylindracea* have had the greatest impacts, particularly in the Mediterranean Sea and temperate Australia, to the extent that *C. taxifolia* is known as the "killer algae" (Meinesz 1999, Klein and Verlaque 2008), and is one of the world's 100 most invasive species (GISD 2014). These two species have consistently become invasive when introduced outside their native range, outcompeting native flora (Ceccherelli et al. 2002), and negatively impacting native fauna including fish (Gollan and Wright 2006, Felline et al. 2012), bivalves (Wright 2005), echinoderms (Bouderesque et al. 1996), sponges (Žuljević et al. 2011), and gorgonians (Cebrian et al. 2012). Both species can form ecosystem-changing monocultures (Meinesz et al. 1993, Katsanevakis et al. 2010). These *Caulerpa* spp. produce new individuals asexually by fragmentation, and fragments as small as 5 mm can grow into a new alga, making control difficult (Ceccherelli and Cinelli 1999).

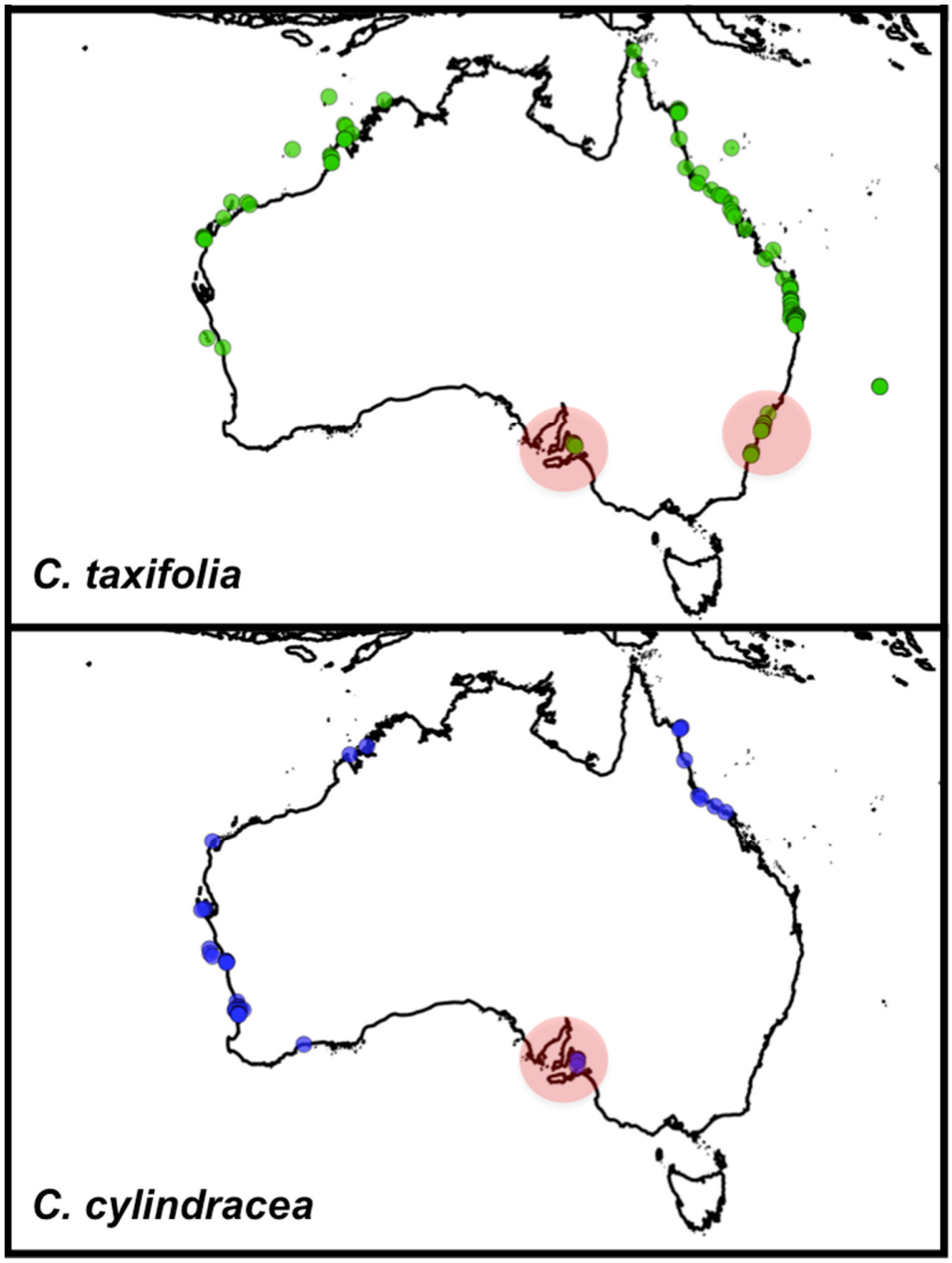

Fig 1.2: Distribution of all records of *Caulerpa taxifolia*, and *C. cylindracea* in Australia. Transparent red circles represent invasive populations. All data from Australia's Virtual Herbarium (2014).

Phyloegeography is the interpretation of the contemporary geographical distribution of populations of a species, with respect to an associated gene genealogy, allowing us to assess the evolutionary processes that have influenced the observed distribution (Avise et al. 1987, Avise

2000). Phylogeography is, in essence, a multidisciplinary activity incorporating biogeography, population genetics, and phylogenetics. Molecular studies of invasive species are crucial to develop an understanding of how invasive species disperse (Voisin et al. 2005, Andreakis et al. 2007, Munoz et al. 2013) and respond genetically to their new environments (Provan et al. 2008, Schlaepfer et al. 2008). Phylogeographic methods are particularly useful for identifying the source and likely vector of introduction of an invasive population, information that is often unknown when a new population is discovered (Benzie et al. 2000, Voisin et al. 2005, Provan et al. 2008, Zepeda-Paulo et al. 2010, Lillo et al. 2013).

Phylogeographic techniques are increasingly applied to enhance our understanding of marine invasions. Lionfish *Pterois volitans* (Linneaus), have spread throughout the Caribbean as a result of secondary founder events from an invasion epicenter in Florida (Toledo-Hernández et al. 2014). Invasive populations of the green crab, *Carcinus maenas* Linneaus, in North and South America were founded from European populations, and subsequent secondary invasions in the south Pacific originated in the Americas (Darling et al. 2008). Two cryptic species of the *Ciona intestinalis* (Linneaus) complex of tunicates are invasive with high gene-flow between populations in Europe and the Americas, indicating constant human mediated intercontinental dispersal followed by secondary spread with high regional population connectivity (Goldstein et al. 2010, Zhan et al. 2010). For macroalgae, a combination of historical herbarium samples and recent collections identified that *Codium fragile* ssp. *tomentosoides* (van Goor) P.C.Silva was widespread over 100 years before it was identified as invasive, and had been mistaken for other subspecies (Provan et al. 2008).

The phylogeography of populations of invasive *Caulerpa* spp. is poorly understood. This is particularly true for Australia, where genetic sampling effort has been smaller than in the Mediterranean Sea, and where there is a complex mix of native and invasive populations. Although some phylogenetic relationships among native and invasive species of *Caulerpa* are

understood (Piazzi et al. 2001a, Balata et al. 2004, Sauvage et al. 2012, Belton et al. 2014), genetic connectivity and structure for most populations remain unresolved. This is particularly important in Australia, which is often considered the origin of invasive populations in the Mediterranean Sea, California and Japan (Jousson et al. 1998, Jousson et al. 2000). It is important that the phylogeographic history of the Australian *C. taxifolia* and *C. cylindracea* populations is elucidated to aid in understanding the likely sources of invasive populations and their vectors.

The source of invasive populations of *C. taxifolia* is of particular interest because Jousson et al. (2000) asserted that there was an "invasive strain", based on the occurrence of a dominant genotype among invasive populations. Identification of invasive populations, and their relationships with native populations, is crucial for understanding past, present, and future invasions. If there are multiple invasive strains, then any population of an invasive *Caulerpa* species could be considered a potential source of invasions.

Anthropogenic climate change is driven by global CO_2 emissions and has accelerated over the past century (Harley et al. 2012). Elevated atmospheric CO_2 has caused an average temperature increase of 0.6°C since 1910 (Walther et al. 2002). Barnett et al. (2001) identified that anthropogenic climate change has warmed surface and deep waters of the earth's oceans. Australian coastal waters are predicted to warm 1-3°C by 2070 (Wernberg et al. 2011). Also associated with elevated atmospheric CO_2 is increased oceanic pCO_2, resulting in a decrease in pH and a reduction in carbonate ions in the world's oceans (Hoegh-Guldberg et al. 2007). The increase in temperature and pCO_2 modifies macroalgal community composition and structure (Porizo et al. 2011, Wernberg et al. 2011). A common prediction is that increased pCO_2 will cause declines and loss of calcified algae, which will be replaced by macrophytes (Porizo et al. 2011, Harley et al. 2012), including *Caulerpa* spp.

The effects of climate change on *Caulerpa* invasions are important because of the likely impacts of increased ocean temperature and increased pCO_2 on algal ecology and distribution.

Caulerpa spp. are restricted to waters where temperature minima exceed 10°C (Komatsu et al. 1997, Komatsu et al. 2003, Ivesa et al. 2006), and rising sea temperatures are likely to facilitate range extensions of *Caulerpa* spp. into areas from which they were previously excluded. The effects of dissolved pCO_2 on *Caulerpa* spp. are more difficult to predict, because changes in pCO_2 can affect protein synthesis and photosynthesis (Sarker et al. 2013, Tomanek 2014, Liu and Zou 2015). The effects of elevated environmental stress, including those related to elevated seawater temperature and pCO_2, have been studied in corals, fish, angiosperms and macroalgae using analysis of nucleic acid ratios, and the identification of stress response proteins (Buckley et al. 1999, Buckley and Szmant 2004, Ireland et al. 2004, Chicharo and Chicharo 2008, Reef et al. 2010, Cruces et al. 2012). This approach is also suitable for examining responses of *Caulerpa* spp., because large sample sizes can be analysed at relatively low cost, and these analyses inform improved understanding of health and function.

1.2 Study Aims.

Following a literature review on *C. taxifolia* and *C. cylindracea* in chapter 2, I present three data chapters that test a range of hypotheses. In chapter 3, I use next generation sequencing to develop a suite of SNPs to examine the hypothesis that *C. taxifolia* in SA originates from a single introduction, and furthermore, seek to identify the likely location of that origin and how the SA populations are related to other invasive populations in Australia. Currently, the origin of this population is unknown and previous studies have not utilised population genetic methods to assess relatedness, gene flow, and migration of invasive Australian populations.

In chapter 4, I test whether sequence data from the *rpl*16-*rps*3 region of the chloroplast can be used for phylogeographic and population genetic analysis of *C. cylindracea*. Using this data, I assess the hypothesis that the populations of *C. cylindracea* in SA are the result of natural

dispersal from southern WA. To date, no phylogeographic or population genetic studies on Australian populations of *C. cylindracea* exist; such studies are required to understand the invasive potential of this species in Australia.

In chapter 5, I use comparative proteomics and nucleic acid ratios of algae from a controlled experiment to predict how *C. taxifolia* and the native *C. trifaria* are likely to respond to climate change. I test if the RNA:DNA, PTN:DNA, and PTN:RNA ratios can be used to quantify the effects of ocean warming and acidification on *C. taxifolia* and *C. trifaria*. I also assess if the shape of extracted protein profiles can be used to identify if ocean warming and acidification negatively impact *these species*. Finally, I evaluate if invasive *Caulerpa* species are more resilient to the effects of ocean warming and acidification than native species. Studies using molecular tools to assess the effects of climate change on marine macroalgae are rare. Given the notoriety of invasive *Caulerpa* spp., a detailed understanding of how these species will respond to climate change at the molecular, as well as population level, is important.

Chapter 2

Molecular Ecology and Phylogeography of the Invasive Macroalgae *Caulerpa taxifolia* and *Caulerpa cylindracea*: a review

Statement of Authorship

Molecular Ecology and Phylogeography of the Invasive Macroalgae *Caulerpa taxifolia* and *Caulerpa cylindracea*: a review

Grant, W. M. (Candidate)

Contributed to initial manuscript conceptualisation, performed all reviews of relevant literature, and acted as lead author in drafting of manuscript.

Signature…… Date…27/02/2015

Tanner, J. E. (Principal supervisor)

Contributed to initial manuscript conceptualisation, and commented on and edited manuscript drafts.

Signature …… Date…27/02/2015

Gurgel, C. F. (Associate supervisor)

Contributed to initial manuscript conceptualisation, and commented on and edited manuscript drafts.

Signature …….. Date…27/02/2015

Deveney, M. R. (Associate supervisor)

Contributed to initial manuscript conceptualisation, and commented on and edited manuscript drafts.

Signature …… Date…27/02/2015

2.1 Caulerpa taxifolia

Caulerpa taxifolia is a green macroalga found on sand, mud, or rock substrates in tropical and subtropical marine environments around the world (Benzie et al. 1997). There are records of *C. taxifolia* from at least 33 countries, displaying its pan-tropical distribution (Guiry and Guiry 2014). Members of *Caulerpa* are characterised by a long creeping thallus (stolon), with upright fronds (ramuli) and rhizoid like anchoring structures (Price 2011). The stolons of *C. taxifolia* grow in dense mats or as an open network, are 0.2 to 1.7mm in diameter and 0.1-3.0m long, with upright fronds 10 to 30cm tall, and 5-20mm wide, and pinnate ramuli as seen in Fig 1.1 (Price 2011).

Caulerpa taxifolia was discovered at Monaco in 1984, occupying approximately 1 m^2 of the sea floor (Meinesz and Hesse 1991). This species subsequently spread throughout the Mediterranean, covering over 130 km^2 of sea floor in six countries by 2007 (Meinesz and Hesse 1991, Komatsu et al. 1997, Ivesa et al. 2006). The rapid spread, perceived impacts and lack of coordinated attempts to control this invasion led to widespread media coverage and labeling of *C. taxifolia* as the "killer algae" (Meinesz 1999). In 2000, *C. taxifolia* was discovered in a lagoon in southern California (Jousson et al. 2000), but was eradicated using hypochlorite treatments (Anderson 2005). *Caulerpa taxifolia* has also been reported from the Sea of Japan, but seasonally low water temperature appears to have prevented its establishment (Komatsu et al. 2003). The species was also discovered in 11 waterways in New South Wales (NSW), Australia between 2000 and 2007 (Glasby et al. 2005, Glasby and Gibson 2007). The northern-most incursion in NSW is 850 km from the southern-most known native population of *C. taxifolia* in Moreton Bay, Queensland (QLD), which makes it unlikely that these populations represent natural range extensions (Schaffelke et al. 2002). In 2002, a population of *C. taxifolia* was discovered in the intra-coastal waterways of West Lakes and the Port River, Adelaide, South Australia (SA) (Cheshire et al. 2002). The West Lakes population was eradicated by enclosing the lake and

flooding it with freshwater, while the Port River population is considered ineradicable with current technology (Wiltshire 2010).

Caulerpa taxifolia outcompetes seagrasses in disturbed habitats by smothering the edges of seagrass beds and occupying cleared patches (Ceccherelli and Cinelli 1999). In nutrient enriched sediments, *C. taxifolia* stimulates nitrogen fixation by promoting microbial reactions in the substrate by releasing photosynthetic products from rhizoids (Ceccherelli and Cinelli 1997, Chisholm and Moulin 2003). This process also occurs in mats of dead *Posidonia oceanica* (Linneaus) Delile and *Cymodocea nodosa* (Ucria) Acherson invaded by *C. taxifolia*, accelerating their decomposition (Ceccherelli and Cinelli 1997, Chisholm and Moulin 2003). These mats act as a nursery for seagrass shoots by protecting juvenile seagrasses from wave damage; this protection is also afforded to the *C. taxifolia*, facilitating further invasion of seagrass beds (Ceccherelli and Cinelli 1997, Chisholm and Moulin 2003). The alga also has negative impacts on native fauna. Estuarine fish in NSW have poor survivorship and lower abundance in beds of *C. taxifolia* than in native algae and seagrass (Gollan and Wright 2006). The bivalve *Anadara trapezia* Deshayes has reduced population density and body mass where *C. taxifolia* occurs (Wright et al. 2007). *Caulerpa taxifolia* also contains toxic caulerpenynes. Boudouresque et al. (1996) found that the urchin *Paracentrotus lividis* Lamarck would either reject *C. taxifolia* and die of starvation, or eat the alga and show signs of toxicity. In vitro, caulerpenyne also has negative effects on cell division in developing *P. lividis* eggs (Boudouresque et al. 1996). McKinnon et al. (2009), Prado and Thibaut (2008), and Tanner (2011) however, reported greater diversity and biomass of epifauna and infauna in *C. taxifolia* patches than in nearby native seagrass beds. Chisholm and Moulin (2003) noted that nitrogen fixation by *C. taxifolia* facilitates the remediation of these disturbed environments. While *C. taxifolia* may have positive effects on previously bare substrate in disturbed habitats, Tanner (2011) acknowledged that *C. taxifolia*

could potentially out-compete native seagrass, leading to negative impacts on ecosystems caused by shifts in community structure driven by local food web destabilization.

In its native range, *C. taxifolia* can reproduce sexually (Meusnier et al. 2004). The life history of *Caulerpa* spp. is poorly understood, but *C. taxifolia* probably has a haplo-diplontic life cycle (Varela-Alvarez et al. 2012). Spawning has been observed in invasive populations of *C. taxifolia,* but only in water temperatures above 25°C, and only male gametes were found (Žuljević and Antolić 2000). Vegetative fragmentation, or clonal propagation, replaces sexual reproduction in Mediterranean populations, and supports the rapid spread of *C. taxifolia* (Ceccherelli and Cinelli 1999).

2.1.1 Molecular studies of Caulerpa taxifolia

Understanding the inter- and intra-population relationships and the geographical origin of invasive species is important in invasion biology (Booth et al. 2007). Molecular methods have improved since 1984, increasing the resolution with which the relationships within and between populations can be determined (Halkett et al. 2005).

There were no molecular taxonomic studies for any *Caulerpa* species before 1997. Benzie et al. (1997) used 6 allozyme markers to analyse *Caulerpa* to delineate species boundries in the genus.They examined seven morphologically defined species of *Caulerpa*, including *C. taxifolia*, from 6 inshore and offshore sites from Townsville, QLD, and 1 site from Lodestone Reef, in the central Great Barrier Reef, QLD (GBR). Benzie et al. (2000) used the same loci to compare new samples of *C. taxifolia* from Stradbroke Island, Moreton Bay, QLD and the Mediterranean Sea, with data from Benzie et al. (1997) and *Caulerpa mexicana* Sonder ex Kützing from Florida and the Mediterranean Sea. *Caulerpa mexicana* was included to test if *C. taxifolia* in the Mediterranean Sea is an ecomorphic variant (ecad) of *C. mexicana*, a Lessepsian migrant to the Mediterranean (Benzie et al. 2000). This hypothesis aimed to discredit evidence that populations

of *C. taxifolia* in the Mediterranean Sea were the result of an aquarium release and represented a biological invasion (Benzie et al. 2000).

Benzie et al. (1997) showed that allozymes can identify species, but could not identify phylogenetic relationships within the genus. The unweighted pair group method with arithmetic mean (UPGMA) analysis of genetic distance grouped the 6 populations from Townsville together. The Lodestone Reef sample grouped separately on a lone branch sister to the Townsville clade. When these data were reanalysed by Benzie et al. (2000) with new samples from Stradbroke Is. and the Mediterranean Sea, the new samples grouped together in a clade showing no genetic distance between samples, indicating a close relationship. Within the Townsville clade, however, genetic distance varied between populations.

All *C. mexicana* samples grouped together in a clade separated from the *C. taxifolia* clade by a long branch. Benzie et al. (2000) concluded that this deep separation between the species meant that it was unlikely that the Mediterranean *C. taxifolia* was an ecad of *C. mexicana.* This conclusion was further supported by a phylogeny based on ITS DNA sequence data (Olsen et al. 1998).

Allozymes are less informative than DNA sequence data for phylogenetic studies (Burton 1994), but Benzie et al. (1997, 2000) described similar genetic variation between *C. taxifolia* samples to phylogeographic studies using more variable ITS DNA markers (Olsen et al. 1998). The relationship between samples from Moreton Bay and the Mediterranean Sea was supported by allozyme data, and indicates that the likely origins of the Mediterranean invasion could have been identified when the invasion was discovered, rather than being first proposed 16 years later by Jousson et al. (2000).

Most phylogenetic and phylogeographic studies on *C. taxifolia* have used the nuclear encoded 18S, 5.8S rDNA internal transcribed spacers (ITS1 and ITS2). Olsen et al. (1998) used ITS sequence data to provide further support for the differentiation of Mediterranean *C. taxifolia*

and *C. mexicana*, identifying 12% sequence divergence between the species. Maximum parsimony and maximum likelihood methods produced identical trees with 100% bootstrap support (Olsen et al. 1998), indicating that ITS has sufficient resolving power to differentiate *Caulerpa* species. Olsen et al. (1998) found no variation in ITS among Mediterranean samples of *C. taxifolia,* but did not link this to clonality in *C. taxifolia* (see Meinesz et al. 1993) or to a founder effect of an invasion. Such conclusions, however, may have been premature given their small data set (n=3). Jousson et al. (1998) also used ITS sequence data to examine the origins of Mediterranean *C. taxifolia*. Based on phylogenies of samples from the Mediterranean Sea, the Pacific Ocean, the Caribbean Sea, 3 aquaria in Europe, 1 in Japan, and 1 in Hawaii, Jousson et al. (1998) concluded that Mediterranean populations of *C. taxifolia* were likely to be descended from an aquarium release of algae originally sourced from the Caribbean Sea. The Caribbean samples Jousson et al. (1998) examined were, however, the only non-Mediterranean/aquarium samples analysed, limiting their ability to make conclusions about geographic origin.

Jousson et al. (2000) employed ITS sequence data to establish relationships between Mediterranean and Californian *C. taxifolia*. Jousson et al. (2000) also included samples from the Adriatic Sea, the Red Sea, various public aquaria, the Caribbean Sea, Indonesia, New Caledonia, and Australia. The Australian populations comprised samples from Townsville, Fraser Island QLD, Moreton Bay, Port Hacking NSW, and Lake Conjola NSW. Their phylogeny identified that the populations in California were nearly identical to samples from the Mediterranean and European aquaria. A substantial proportion of Australian samples from Townsville, Moreton Bay, Port Hacking, and Lake Conjola also fell into this clade, but the number of individuals from each population that grouped in this clade was not described. Jousson et al. (2000) identified this clade as “invasive”. The Australian samples were the only native *C. taxifolia* samples in the invasive clade, and Jousson et al. (2000) hypothesized that eastern Australia was probably the source of *C. taxifolia* populations in the Mediterranean. Other samples from the Mediterranean,

the Adriatic, and Australia did not group in the invasive clade, implying that there had been multiple introductions into the Mediterranean and that a single "invasive strain" was unlikely to exist, although these data were not discussed further by Jousson et al. (2000).

Meusnier et al. (2001) generated ITS data for 14 new *C. taxifolia* samples from the Mediterranean, Polynesia, and Malaysia, and included data from Olsen et al. (1998) and Jousson et al. (1998). Their analyses showed that Mediterranean and Australian *C. taxifolia* populations shared four unique deletions, indicating a close phylogenetic relationship and also suggesting that the Mediterranean invasive populations originated from Australia. Meusnier et al. (2001) used only a single sample from Townsville, limiting the geographic coverage and depth of their findings, but provided further support for an eastern Australian origin for Mediterranean *C. taxifolia*.

Schaffelke et al. (2002) used ITS DNA sequences to confirm the identity of 3 invasive *C. taxifolia* populations from NSW. New samples from Port Hacking, Lake Conjola, Careel Bay NSW, Moreton Bay, Gladstone QLD, Arlington Reef QLD, Hastings Reef QLD, Michaelmas Reef QLD, Sudbury Reef QLD, Hicks Reef QLD, and Myrmidon Reef QLD were analysed with data from Olsen et al. (1998) and Jousson et al. (1998, 2000). The resulting phylogeny grouped Lake Conjola samples with samples from Moreton Bay. The Port Hacking population and one sample from Careel Bay grouped with populations from Gladstone, Townsville, and Fraser Is. The other two Careel Bay samples formed a distinct clade. Unlike other invasive populations, the 3 Careel Bay samples included five genotypes, indicating that the initial introduction had substantially more genetic variation than other invasions, or that multiple introductions occurred at this site. The source population for the Lake Conjola invasion is probably in Moreton Bay, and the Port Hacking invasion comprises algae that probably originated in central Queensland (Schaffelke et al. 2002). The clade in which the Lake Conjola and Moreton Bay *C. taxifolia* grouped also included samples from the Mediterranean, California, and European aquaria,

providing further evidence that Moreton Bay is likely to be the source of the Mediterranean and Californian invasive populations. The polyphyly of the Careel Bay samples prevented their source location from being identified, although one clade which included the majority of the genotypes was a sister clade to populations from the GBR. That population is distantly related to the clade in which the Moreton Bay, Mediterranean, Californian, and aquarium samples grouped, and represents an independent invasion from a coastal or reef population in QLD. Schaffelke et al. (2002) recognised that reductions in genetic diversity in Lake Conjola and Port Hacking populations reflected a founder effect associated with introduced populations. Schaffelke et al. (2002) recognised that the genotypes in Careel Bay samples being distinct from the Mediterranean/aquarium/Californian clade undermined the validity of the "invasive stain" and had implications for management of invasions.

Meusnier et al. (2004) re-analysed ITS sequence data from all previous studies. Five distinct indel patterns, dubbed 'indelotypes' were identified: I_3 (complete ITS1 sequence), I_2 (loss of sequence between positions 72-113), I_{1a} (loss of sequence between positions 72-113 and 127-129, I_{1b} (loss of sequence between 7-8 and 127-129 with partial sequence loss between 72-113), and I_0 (loss of sequence between 7-8, 72-113, and 127-129). Indelotypes I_{1a} and I_{1b} lost the 2bp region between positions 6-9 of ITS1 independently. These data indicate that there has been a stepwise loss of variation from tropical GBR populations (I_3), west to Townsville (I_{1a}, & $I_{0)}$, and south along the east coast of Australia to Moreton Bay (I_0). Most invasive populations have the I_0 indelotype, but Port Hacking populations have indelotype I_{1a}. Careel Bay populations have a unique combination of indelotypes I_{1b} and I_{1a}. All Mediterranean samples were characterised by indelotype I_0, except one sample from Tunisia, which had indelotype I_{1a}, providing further evidence that invasive populations in the Mediterranean Sea are derived from a native eastern Australian I_0 indelotype population. Meusnier et al. (2004) also identified a second indelotype, the I_{1a}indelotype of *C. taxifolia* from Tunisia, which grouped with samples from Careel Bay,

Townsville, Gladstone, Fraser Is., and Lord Howe Is., Australia. Meusnier et al. (2004) further suggested that indelotype I_0 should be regarded as an incipient species because of its derived status and persistent monophyly. To test if incipient speciation is occurring, further analysis with multiple neutrally evolving loci is needed. The use of multiple genetic markers increases the resolution with which species can be distinguished through the assessment of a consensus from across the genome (Famá et al. 2002b, Martel et al. 2004, Jongma et al. 2012). That incipient speciation is occurring in invasive *C. taxifolia* should therefore be regarded skeptically until data from multi-marker studies of conserved genes are available.

Famá et al. (2002a) and Meusnier et al. (2002) used the chloroplast RuBisCo (ribulose-1, 5-Bisphosphate carboxylase/oxygenase) large subunit (*rbc*L) introns to independently verify their ITS phylogenies.

Famá et al. (2002a) used ITS sequences from an additional 50 *C. taxifolia* samples from Lake Macquarie NSW, Lord Howe Is., Careel Bay, and the Mediterranean. These samples were compared to data from Olsen et al. (1998), Jousson et al. (1998, 2000) and Meusnier et al. (2001) to create a revised phylogeny including 228 samples to further assess the origin of invasive populations in the Mediterranean and NSW. Partial *rbc*L sequences were also obtained for the 50 new samples to test if it is a suitable marker for identification of invasive populations. The ITS phylogeny grouped aquarium, Mediterranean, Californian, and Moreton Bay populations together in a single clade which (Famá et al. 2002a) named ITS type 1, the “invasive strain”. All other Australian samples formed a separate clade, while samples from New Caledonia formed a distinct clade, samples from the Caribbean Sea, Red Sea, Indonesia, Japan and the Philippines group in a fourth clade and a fifth clade of samples from the Red Sea, Indonesia and the Philippines grouped at the base of the tree. The type 1 clade had homogenous ITS sequences, while the other clades had variable ITS sequences. Analysis of samples from the Caribbean Sea, Red Sea, and South-East Asia showed that the rbcL intron was ˜1000bp long, while the rbcL

intron of samples from aquaria, the Mediterranean Sea, Australia, and New Caledonia was ~250bp long. Famá et al. (2002a) concluded that all invasive populations, and the native populations of Australia and New Caledonia, are characterised by a 750 bp *rbc*L intron deletion (Meusnier et al. 2002).

To further assess the origin of Mediterranean *C. taxifolia* populations, and to evaluate if sexual reproduction occurred in native populations, Meusnier et al. (2002) used single stranded conformation polymorphism (SSCP) analysis of ITS2 and chloroplast 16S rDNA intron-2, on 16 new samples from Moreton Bay, Townsville, the GBR, the Philippines, Tahiti, and a sub-set of samples from Olsen et al. (1998) and Meusnier et al. (2001). ITS analyses identified 10 SSCP profiles, while 16S intron-2 results identified 5 SSCP profiles. All invasive populations belonged to ITS2 SSCP profile A. The other 8 ITS2 SSCP profiles were found in native populations from tropical and sub-tropical Australia, the Philippines and Polynesia. Phylogenetic analysis of the SSCP data grouped all samples into two clades displaying strong geographic structure: all samples from inshore Australian populations (Brisbane and Townsville) and invasive/aquarium sites (Monaco Aquarium, Mediterranean and California) formed clade one, and all samples from offshore Australian populations (Great Barrier Reef,) and other Pacific Islands (Tahiti and Philippines) formed clade two. Meusnier et al. (2002) suggested that this geographic grouping is evidence of incipient speciation between coastal and offshore populations because of consistent differences in the molecular data sets, habitat, and morphology, but acknowledged that this would be difficult to prove. The 16S intron-2 analyses produced five SSCP profiles. Only one 16S intron-2 profile was found in the invasive Mediterranean populations, which was shared with Australian native inshore populations. This profile displayed the same geographic structure as the ITS-2 DNA SSCP profile. In sexual reproduction, chloroplasts are inherited uniparentally so Meusnier et al. (2002) used pairwise comparison of ITS2 and 16S intron-2 SSCP profiles to identify if sexual reproduction was occurring. Invasive populations were characterised by a single

combination of ITS2 and 16S intron-2 SSCP profiles, providing strong evidence for nucleo-cytoplasmic linkage disequilibrium (Meusnier et al. 2002). This further supports that invasive populations are predominantly clonal (Meusnier et al. 2002). Pairwise comparisons of native populations showed that multiple nuclear ITS2 profiles were associated with 16S intron-2 profiles, providing the first direct genetic evidence of sexual reproduction in *C. taxifolia* (Meusnier et al. 2002). Pairwise comparisons of SSCP profiles indicate that invasive populations are the result of at least 2 founder events that limited genetic variation in invasive populations: one establishment of northern or central QLD *C. taxifolia* in Moreton Bay, and one from Moreton Bay to the Mediterranean invasive populations they sampled (Meusnier et al. 2002).

Cevik et al. (2007) aimed to identify 2 isolates of *C. taxifolia* from Turkey, 1 from the Gulf of Iskenderun, and 1 from an aquarium shop in Izmir, using ITS sequences. The Iskenderun sample grouped in a clade with inshore northern Queensland populations, but not the Mediterranean/Moreton Bay clade. The ITS1 indelotype of the Iskenderun sample is $I1_a$, the same as that of samples from Tunisia, but these samples fall into different clades in the ITS phylogeny, indicating that each population is derived from distinct populations in Australia. The aquarium sample from Izmir grouped in a clade with samples from the Philippines and offshore reef sites in Australia. These data suggest that the trade in *C. taxifolia* was (and probably is) ongoing, and poses continuing risk to vulnerable environments (Cevik et al. 2007). Cevik et al. (2007) regarded the presence of 2 new *C. taxifolia* genotypes in the Mediterranean as evidence that there have been multiple invasions.

Jongma et al. (2012) used ITS, the 16S rDNA intron-2 and *tuf*A markers to analyse a population of a slender morphotype of *C. taxifolia* from Sicily, which is distinct from the robust form observed in other Mediterranean invasive populations. Jongma et al. (2012) identified this isolate as *C. distichophylla*, native to southern Western Australia (WA). They found that *tuf*A and the chloroplast intron differed by only one nucleotide between *C. taxifolia* and *C. distichophylla,*

while the ITS sequence of the Sicilian sample and *C. distichophylla* from WA shared a 27bp deletion. Due to the lack of variation between *C. taxifolia* and *C. distichophylla*, Jongma et al. (2012) synonymised *C. distichophylla* with *C. taxifolia* as the variety *C. taxifolia* var. *distichophylla*.

Murphy and Schaffelke (2003) undertook amplified fragment length polymorphism (AFLP) analysis on samples of *C. taxifolia* from Lake Conjola, Moreton Bay, and the Mediterranean Sea. The Lake Conjola isolate grouped with the Moreton Bay samples in a sister clade to those from the Mediterranean. Murphy and Schaffelke (2003) regarded this as a good indicator that the Lake Conjola population was not introduced from the Mediterranean Sea, but most likely was a separate introduction from a source population in Moreton Bay.

Grewe et al. (2008) used microsatellites to identify population structure and connectivity between native Australian: Moreton Bay, Fraser Island, the GBR Townsville, Torres Straight Northern Territory, Ningaloo Reef WA and invasive Australian: West Lakes SA, Port River SA, North Haven Harbour SA, Batemans Bay NSW, St Georges Bay NSW, Lake Conjola, Pittwater Sydney, Gunnamatta Sydney, Botany Bay Sydney populations in Australia, and identify differences between Australian and Mediterranean populations. Twenty-seven loci were selected, but only 9 primers were successfully developed and used to genotype 518 samples, from which 96 genotypes were identified. Invasive populations were characterised by multiple genotypes, but with lower diversity than native populations. In invasive populations, one genotype typically dominated at frequencies of 50-90%. Genotypes differed between invasive populations in SA and around Sydney, while SA and southern NSW populations shared a genotype, which occurred in 84% of SA samples and 83% of southern NSW samples. No shared genotypes were found between native and invasive populations, preventing the development of supported hypotheses about the geographic origins of invasive populations. Excess heterozygotes were found, and in South Australian populations, one allele showed 100% heterozygosity. Grewe et al. (2008)

differentiated *C. taxifolia* from Australian invasive sites into 3 clades. The South Australian, Batemans Bay and Lake Conjola populations grouped together and were a sister group to the second invasive clade, which consisted of Pittwater NSW. These invasive Australian samples were sister clades to the samples from the Mediterranean Sea, and samples from the mainland side of Fraser Is, GBR in QLD. The third invasive clade included samples from Port Hacking and Botany Bay and was a sister clade to a population from Townsville. The Mediterranean samples showed no geographic variation. All loci tested show that none of the samples from Australia have the microsatellite profile of the Mediterranean samples, indicating that the invasive Australian populations are not the result of a re-introduction from Europe. The native population at Victoria Point, Moreton Bay QLD was sampled in 2002 and 2007 and tested for temporal variation. No genotypes were shared between sampling years, but UPGMA analysis showed that the time-series samples were closely related.

Grewe et al. (2008) showed that more variation can be detected in *C. taxifolia* than could be using traditional sequence based markers. Further marker development may allow discrimination between NSW and SA populations that grouped together in the UPGMA phylogeny. Grewe et al. (2008) cited inadequate sample numbers for not performing population genetic analyses, because they treated each sampling site as an individual population. Had sites been pooled, population genetic analyses would have been feasible, and more informative analyses could have been performed.

Meusnier et al. (2001) used 16S rDNA sequence data from endosymbiotic bacteria in *Caulerpa* spp. to infer relationships between and within invasive and native populations. Analysis of these sequences supported a close relationship between Australian and Mediterranean Sea populations of *C. taxifolia,* similar to the relationships inferred from analyses of ITS rDNA sequences. Bacterial endosymbionts from families that are uncommon in the Mediterranean Sea but common in Australia were found in Mediterranean and Australian *C. taxifolia*. How *C.*

taxifolia obtains its endosymbionts, and if these are transmitted to offspring or if they are only shared among clones, and if they are shared consistently, is unknown. This approach furthermore, cannot provide population genetic data on *C. taxifolia*, additionally limiting its utility.

Varela-Alvarez et al. (2012) collected native *C. prolifera* and invasive *C. cylindracea* and *C. taxifolia* from the Mediterranean and measured ploidy and genome size using nuclear fluorescence microspectophotometry. Only *C. prolifera* thalli were reproductive, which further supports that invasive *Caulerpa* populations are clonal. Methodological issues prevented quantification of the total mass of *Caulerpa* nuclei, and instead, Cx-values were calculated based on the gametes of *C. prolifera*. The Cx value refers to the DNA content (C) of an organism in a monoploid (x) life stage (haplophase in *Caulerpa*) and can be prefixed by a number to indicate ploidy (Greilhuber et al. 2005). *Caulerpa cylindracea* and *C. taxifolia* had smaller genomes than *C. prolifera*. Varela-Alvarez et al. (2012) also found that *C. taxifolia* has a DNA content of 2Cx, and populations in the Mediterranean are in the haplophasic stage of a haplo-diplontic life history. If Mediterranean *C. taxifolia* populations entered diplophase, they would be tetraploid (Varela-Alvarez et al. 2012). Smaller genome size has been correlated with invasiveness and rapid growth in over 150 angiosperm weed species (Bennett et al. 1998), and Varela-Alvarez (2012) proposed that clonal reproduction of haplophasic polyploids in invasive populations of *C. taxifolia* contributes to its success as an invasive species. Clonal propagation and a reduction in genome size in *C. taxifolia* may therefore be an adapted trait, and is reflected in the deletion pattern observed in the ITS indelotypes of Meusnier et al. (2004). A reduction in genome size might allow for faster DNA replication and/or transcription potentially facilitating higher growth rates in invasive *C. taxifolia.*

2.2 Caulerpa cylindracea

Caulerpa racemosa was shown to be a polyphyletic species-complex composed of at least seven taxa by Belton et al. (2014), who raised *C. racemosa* var. *cylindracea* from a variety to a species,

C. cylindracea, based on *tuf*A analyses. Like *C. taxifolia, C. cylindracea* occurs on sand, mud or rock substrates from the intertidal zone to depths of at least 45m (Price 2011).

Caulerpa cylindracea is native to temperate southern WA, tropical Australia, Papua New Guinea, and New Caledonia (Sauvage et al. 2012) and has invaded the Mediterranean Sea (Verlaque et al. 2000) and South Australia (Collings et al. 2004). Although its morphology is variable, *C. cylindracea* is characterized by slender stolons ~2 mm thick, fixed to the substrate by rhizoids up to 15 mm in length. The stolon bears upright fronds up to 10 cm in height with clavate to cylindrical ramuli 5mm long and ~2 mm wide (Fig 2.1)(Verlaque et al. 2003).

The appearance of invasive *C. racemosa* in the Mediterranean Sea during the early 1990's prompted Verlaque et al. (2000) to study contemporary and historical Mediterranean collections attributed to this species complex. Verlaque et al. (2000) identified three varieties of *C. racemosa*: var. *turbinata-uvifera* (the earliest record of *C. racemosa* in the Mediterranean from Tunisia, 1926), *C. racemosa* var. *lamourouxii* f. *requienii* (identified from Israel in 1951), and *C. racemosa* aff. var. *occidentalis* (the invasive variety first recorded in Libya in 1990, referred to hereafter as *C. cylindracea*). Verlaque et al. (2000) proposed that *C. cylindracea* represented -

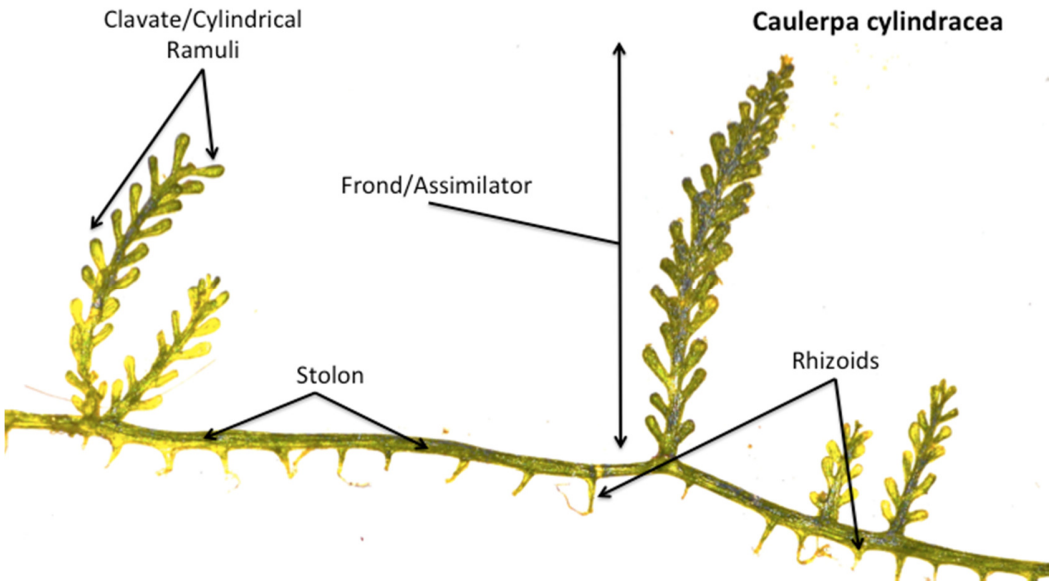

Fig 2.1 Labeled herbarium pressing of *C. cylindracae* from South Australia

a recent introduction into the Mediterranean, and refuted that it was a Lessepsian migrant as proposed by Giaccone and Di Martino (1995, 1997 cited in Verlaque et al 2000) because its biogeography did not conform to a westward pattern of migration. Verlaque et al. (2000) also refuted the possibility that *C. cylindracae* populations represented range expansions of native Mediterranean *C. racemosa* varieties in response to warming sea temperatures, because the increase in sea temperature at invaded sites was inadequate to support the warmer water varieties.

Caulerpa cylindracea spread rapidly throughout the Mediterranean Sea after 1990 (Verlaque et al. 2003) including the Adriatic Sea (Nuber et al. 2007) and the Ionian Sea (Katsanevakis et al. 2010). *Caulerpa cylindracea* is also recorded at two localities on the metropolitan coast of Adelaide (Collings et al. 2004), and at Portland, Victoria (VIC), Australia.

Caulerpa cylindracea thrives on dead or dying seagrass mats and can invade already impacted, low-density *Posidonia oceanica* beds (Klein and Verlaque 2008). In these disturbed habitats, *C. cylindracea* can out-compete seagrass, but unlike *C. taxifolia*, it has little effect on large patches of healthy seagrass (Ruitton et al. 2005). *Caulerpa cylindracea* decreased alpha diversity (within-habitat) and beta diversity (between-habitats) by up to 45% in Tuscany, leading to biotic homogenization of invaded areas (Piazzi and Balata 2008). Homogenization can decrease the capacity of a system to respond to further disturbance (Bulleri et al. 2010). After removal of *C. cylindracea* from invaded areas, communities did not recover to a pre-invaded state, displaying modified habitat structure and richness for both flora and fauna (Piazzi and Ceccherelli 2006).

2.2.1 Molecular studies of Caulerpa cylindracea

Molecular studies on *C. cylindracea* have used nuclear DNA ribosomal regions 18S, ITS1, 5.8S and ITS2, or combinations thereof, and the chloroplast gene *tuf*A. Famá et al. (2000) examined ITS-1 of samples from 8 sites in the Mediterranean Sea, one from Panama, and one from WA.

The proportion of polymorphic loci was high at ~23%, and intra-individual polymorphisms were sometimes greater than inter-individual polymorphisms. This polymorphism meant that phylogenetic trees were not informative, but the intercalation of WA and Mediterranean samples indicated that they are almost certainly related. Famá et al. (2000) suggested that polyploidy could account for the high levels of intra-individual variation found in *C. cylindracea* populations. Varela-Alvarez et al. (2012) identified that *C. cylindracea* in the Mediterranean Sea, like *C. taxifolia*, is a haplophasic polyploid with a DNA content of 3Cx. Unlike *C. taxifolia*, *C. cylindracea* is an endopolyploid, that is it had experienced chromosome duplication without nuclear division. Endopolyploidy increases vegetative growth (Kapraun 1994), and is probably responsible in part for the success of *C. cylindracea* as an invader (Varela-Alvarez et al. 2012).

Durand et al. (2002) examined ITS1 + ITS2 and 18S rDNA sequences of *C. racemosa* from the Mediterranean Sea, and found that the three morphologically distinct varieties identied by Verlaque et al. (2000): *C. racemosa* var. *turbinata*, *C. racemosa* var. *lamourouxii*, and *C. cylindracea* are also genetically distinct. ITS analyses grouped *C. cylindracea* into a monophyletic clade, while the 16S intron grouped *C. cylindracea* into 2 separate clades (Durand et al. 2002). Durand et al. (2002) proposed that *C. cylindracea* may have arisen as a hybrid of *C. racemosa* var. *turbinata-uvifera*, and an unknown introduced tropical *C. racemosa* taxon. Durand et al. (2002) asserted that differential rates of concerted evolution accounted for the discrepancies between the ITS and 16S intron datasets providing support for their hybridization hypothesis.

Verlaque et al. (2003) used morphological data and ITS sequence data to clarify the identity of invasive Mediterranean *C. cylindracea* populations. Morphology did not differentiate samples from invasive Mediterranean populations and WA, but could distinguish *C. cylindracea* from *C. racemosa* var. *occidentalis*, which Verlaque et al. (2000) had proposed as the possible identity of the invasive Mediterranean populations. Maximum likelihood, neighbour joining, and

maximum parsimony phylogenies all had the same topology and carried high bootstrap values, supporting strongly the relationships identified (Verlaque et al. 2003). Invasive and WA samples showed no sequence variation, and clustered in a clade, leading Verlaque et al. (2003) to conclude that WA was the likely source of the Mediterranean populations, and showing that the Mediterranean invasive variety was not a unique hybrid. Verlaque et al. (2003) found that morphological and molecular evidence distinguished the invasive variety from *C. racemosa* var. *laetevirens,* leading them to propose a new variety, *C. racemosa* var. *cylindracea*, nomenclature that was widely used in *C. cylindracea* studies from 2003-2014. Nuber et al. (2007) also used ITS1 and ITS2 markers to analyze relationships between samples of *C. racemosa* from the Adriatic Sea and the Mediterranean Sea. Their results unequivocally confirmed the presence of *C. cylindracea* in the Adriatic Sea.

2.3 Conclusion

Considerable effort has been expended to elucidate the source populations for *C. taxifolia* and *C. cylindracea* invasions in the Mediterranean Sea and Australia. Much of this work has been based on analyses of ITS rDNA, sequenced from numerous samples over more than ten years, providing a comprehensive dataset that facilitates examination of temporal genetic variation and which may be used to identify patterns of invasion (Olsen et al. 1998, Jousson et al. 1998, 2000, Meusnier et al. 2001, 2002, 2004, Famá et al. 2002, Cevik et al. 2007, Jongma et al. 2012). These studies combine to make a strong argument for the release and spread of *C. taxifolia* in the Mediterranean Sea from aquarium samples that originated from Moreton Bay in the 1970s or early 80s.

Reduced genetic diversity in introduced populations is a reflection of the stochastic processes inherent in founder events (Nei 1987), and subsequent genetic drift (Wright 1931). These were observed in most studies on invasive *Caulerpa* spp. but were discussed and framed in the context of a biological invasion only by Meusnier et al. (2002, 2004) and Schaffelke et al.

(2002), and then only briefly. The ongoing fixation with the existence of an "invasive strain" has led to some results not being adequately examined. Jousson et al. (2000), in particular, did not discuss Mediterranean genotypes that did not fall in the "invasive strain" clade. It is now clear that the assumption that the *C. taxifolia* that established in the Mediterranean Sea is an invasive strain is spurious. The detection of distinct genotypes in Monaco (Meusnier et al. 2002), Tunisia (Cevik et al. 2007) and the identification of invasive C. *taxifolia* var. *distichophylla* in Turkey (Jongma et al. 2012), all with distinct ITS genotypes and 16S intron-2 haplotypes, means that there were at least 3 different geographical sources of Mediterranean *C. taxifolia*. The first *C. taxifolia* population identified in the Mediterranean is related to populations from Moreton Bay (Meusnier et al. 2002), the Tunisian population is related to populations from Fraser Is. (Cevik et al. 2002), and *C. taxifolia* var. *distichphylla* in Turkey is related to populations in southern WA (Jongma et al. 2012). *Caulerpa taxifolia* from any biogeographic region, when introduced to a suitable locality, is therefore likely to display invasive characteristics. At least 6 species of *Caulerpa* are reported outside of their native range, with *C. taxifolia* and *C. cylindracea* being reported as invasive (Meinesz and Hesse 1991, Verlaque et al. 2003, Falcao and Szechy 2005, Lapointe et al. 2006, Amat et al. 2008, Cummings and Williamson 2008) indicating that many members of the genus have traits that make them suited to invading new environments, including asexual reproduction (Ceccherelli and Cinelli 1999), rapid growth and colonization of varied habitats (Amat et al. 2008), toxic secondary metabolites that deter herbivores (Boudouresque et al. 1996, Gollan and Wright 2006), and the ability to modify substrates to facilitate further invasion (Chisholm and Moulin 2003).

The *C. taxifolia* and *C. cylindracea* populations in the Mediterranean are not the result of natural range extensions. Anthropogenic dispersal produces populations that are geographically isolated from native populations with shared genotypes (Lambrinos 2004), and there are no proximal *C. taxifolia* and *C. cylindracea* populations to the Mediterranean with similar

genotypes. It is likely that all invasive populations in the Mediterranean were introduced through the aquarium trade (Jousson et al. 1998, Cevik et al. 2007).

It is unclear if native and invasive populations of *C. taxifolia* and *C. cylindracea* in Australia are connected. Fewer studies have analysed invasive Australian populations than Mediterranean populations, and most examined few samples. The lack of genetic variation in invasive *C. taxifolia* and *C. cylindracea* populations (Meusnier et al. 2004, Verlaque et al. 2003), indicates that founding populations were genetically limited or comprised few individuals (Allendorf and Lundquist 2003). This is likely to occur in propagated aquarium stock, or for algae transported inadvertently on vessels. The geographic origins of the primary invasions in Australia are unknown, but most Australian invasive populations occur in areas with heavy vessel traffic, or at heavily used public boat ramps. West et al. (2007) demonstrated that fragments of *C. taxifolia* are readily caught on anchors and survive emersion for up to 10 hours. This indicates that small vessels probably act as a secondary vector and may be responsible for local spread following invasion (Hewitt et al. 2007).

Although there is a substantial body of work on *C. cylindracea,* the origins of the Mediterranean populations remain obscure because samples from other parts of the world have not been included in the phylogeographic analyses, and sample sizes have been small ($n \leq 7$). This is particularly relevant now that the native range of *C. cylindracea* is known to include tropical Australia, Papua New Guinea, and New Caledonia (Sauvage et al. 2013).

One of the shortfalls of all studies on *C. taxifolia* and *C. cylindracea* is that they have been based only on phylogenies. Phylogenies identify samples that are related, but cannot provide important population genetic measures such as fixation indices, heterozygosity or number of migrants. These data are invaluable to study invasive species because they can be combined with occurrence and geographic data to test for natural dispersal. Large data sets are also needed to facilitate population genetic analysis with robust statistical testing.

The future of the molecular ecology, and in this case specifically population genetics and phylogeography, of invasive *Caulerpa* species lies in next-generation sequencing technologies. These platforms can process a large number of samples rapidly, produce exponentially larger data sets than previous methodologies, and at a fraction of the cost (Glenn 2011). This reduction in cost has allowed routine de-novo sequencing of non-model organisms (Helyar et al. 2011). Larger datasets facilitate the development of numerous molecular markers, such as single nucleotide polymorphisms. New markers are crucial for population level studies of *C. taxifolia* because of the low genetic variation found using conventional markers.

There are other obstacles preventing the collection of quality data from *Caulerpa* species, including the presence of intra- and extra-cellular bacteria, which is co-extracted with *Caulerpa* DNA (Meusnier et al. 2001, Delbridge et al. 2004). Bioinformatics can be used to identify and remove prokaryotic DNA sequences from eukaryotic DNA sequence datasets. To assess the patterns of invasion and genetic connectivity of invasive *Caulerpa* populations, new highly variable genetic markers must be developed which allow the application of population genetic methodologies. Next-generation sequencing datasets can be mined for markers that can be used to genotype the large sample sets that are required to elucidate the history of *Caulerpa* invasions in Australia. Another approach to assessing if invasive and native *Caulerpa* can be differentiated would be to examine the transcriptomes of invasive and native populations of *C. taxifolia* and *C. cylindracea* in situ and under controlled conditions, to identify if particular genes are expressed differently, and if those differences are environmentally determined. Transcriptome analysis of the invasive plant *Scenecio madagascariensis* (Poir. (1817)), showed that genes for fungal and microbial defense were up-regulated in native populations and down-regulated in an invasive population, indicating that the invasive population was using fewer resources for immune defense, contributing to its invasiveness (Prentis et al. 2010). Similar studies of invasive *Caulerpa* may be valuable for understanding what makes species in this genus invasive. Such

analyses would determine if a biotic or abiotic factor triggers a change in transcription that aids invasiveness. Such studies would further contribute to determining if all *Caulerpa* spp. are potentially invasive, and if among invasive species, some populations or genotypes are more successful invaders. If a particular transcriptome profile is characteristic of invasive populations, this technique could be used as a surveillance tool in native populations that may start to display invasive characteristics due to global or local environmental change.

Chapter 3

Phylogeography of the invasive marine green macroalga *Caulerpa taxifolia* (M. Vahl) C.Agardh in Australian waters: a next generation sequencing approach to marker discovery

Statement of Authorship

Phylogeography of the invasive marine alga *Caulerpa taxifolia* (M. Vahl) C.Agardh in Australian waters: a next generation sequencing approach to marker discovery

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Contributed to initial manuscript conceptualisation, collection of samples, carried out all labwork (unless specified otherwise), carried out all data analysis, produced all figures and tables, and acted as lead author in writing of manuscript.

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Abstract:

Caulerpa taxifolia is one of the world's most invasive marine macroalgae. Since 1984, incursions have been observed in at least six Mediterranean countries, as well as Japan and California. Low diversity among these populations and apparently divergent biology led to the hypothesis that an "invasive strain" exists. In Australia, *C. taxifolia* is native in tropical latitudes but invasive across 16 temperate sites. Molecular studies suggest that worldwide, all studied invasive populations are related to northeast tropical and subtropical Australian populations. To date, no detailed phylogeographic study has been conducted on *C. taxifolia* in Australia. I developed new SNP markers from *de novo* next-generation genomic sequence data to assess patterns of genetic diversity and structure among Australian native and invasive populations, including populations of the recently synonymized *C. taxifolia* var. *distichophylla*. Native populations showed more genetic diversity than invasive populations (H= 0.30 ± 0.20 s.d. vs. H= 0.15 ± 0.05 s.d., respectively). Moderate (r = 0.65) but significant ($p \leq 0.001$) isolation by distance was also detected. Invasive populations were characterized by multiple genotypes, which is not consistent with the invasive strain hypothesis.

3.1 Introduction:

The marine benthic green macroalga *Caulerpa taxifolia* is considered one of the world's worst marine invasive species (Williams and Smith 2007). Since the discovery in 1984 of a population in the Mediterranean Sea at Monaco (Meinesz and Hesse 1991), *C. taxifolia* has spread along the coast of six Mediterranean nations, covering at least 130km^2 of sea floor (Meinesz and Hesse 1991, Komatsu et al. 1997, Ivesa et al. 2006). This alga has been found subsequently in the Adriatic Sea in 1994 (Ivesa et al. 2006), Southern California in 2000 (Anderson 2005), in at least 16 sites in temperate eastern and southern Australia between 2000-2007 (Cheshire et al. 2002, Glasby et al. 2005, Glasby and Gibson 2007), and in the Sea of Japan from 1992-1993, although it failed to establish there due to low water temperature (Komatsu et al. 2003). Extensive shifts in community structure have been observed at invaded sites, including loss of native seagrass beds (Chisholm and Moulin 2003), exclusion of native herbivorous fishes because of unpalatability and toxicity (Gollan and Wright 2006), a decrease in native bivalve health and population size (Wright et al. 2007), and increased mortality and exclusion of echinoderms due to toxicity (Boudouresque et al. 1996).

Determining the source or origin of invasive *C. taxifolia* populations is of particular scientific, political and environmental interest. Information on where invasive species come from can help identify vectors, which in turn assists in taking the appropriate measures to prevent further invasions. Studies addressing the origin of the *C. taxifolia* invasions in Europe have used a range of molecular methods, which are listed in Table 3.1 and reviewed in chapter 2. ITS 1+2 nuclear rDNA studies have so far provided the majority of data on the subject, and the strongest evidence that the *C. taxifolia* populations in the Mediterranean Sea were derived from eastern Australian populations, more specifically those from Moreton Bay, Queensland (QLD) (Meusnier et al. 2001, Famá et al. 2002, Meusnier et al. 2002, Schaffelke et al. 2002, Meusnier et al. 2004, Cevik et al. 2007, Jongma et al. 2012).

Paper	Marker
Benzie et al. (1997)	Allozyme
Jousson et al. (1998)	ITS 1 + 2 *r*DNA
Olsen et al. (1998)	ITS 1 + 2 *r*DNA
Benzie et al. (2000)	Allozyme
Jousson et al. (2000)	ITS 1 + 2 *r*DNA
Meusnier et al. (2001)	ITS 1 + 2 *r*DNA
Weidmann et al. (2001)	Restriction Digests
Fama et al. (2002)	ITS 1 + 2 *r*DNA
Meusnier et al. (2002)	ITS 1 + 2 *r*DNA and *rbc*L
Schaffelke et al. (2002)	ITS 1 + 2 *r*DNA
Murphy & Schaffelke (2003)	AFLP
Meusnier et al. (2004)	ITS 1 + 2 *r*DNA
Grewe et al. (2008)	Microsatellites
Jongma et al, (2012)	ITS 1 + 2 *r*DNA and *rbc*L

Table 3.1 List of studies by Author/Date and the molecular markers used for phylogeographic analysis.

Further evidence from ITS 1+2 suggested that there have been at least three separate introductions of *C. taxifolia* into the Mediterranean Sea, with populations found at Monaco (Jousson et al. 1998), Tunisia (Jousson et al. 2000), and Turkey (*C. taxifolia* var. *distichophylla*) (Cevik et al. 2007) being most closely related to samples form Moreton Bay QLD, central QLD, and Perth, Western Australia (WA) respectively. It is likely that the introduction of *C. taxifolia* into the Mediterranean Sea has occurred multiple times by release of aquarium specimens (Jousson et al. 1998, Cevik et al. 2007), with each introduction being followed by population expansion facilitated by fragmentation and marine currents, together with local human assisted dispersal caused by commercial and recreational activities including fishing, boating, and the aquarium trade (Walters et al. 2006, Cevik et al. 2007, West et al. 2007, Abdulla and Linden 2008, Jongma et al. 2012). Despite evidence for multiple introductions of *C. taxifolia* into the Mediterranean, a hypothesis describing an invasive strain has been proposed and maintained for the last 2 decades.

Invasive populations of *C. taxifolia* in Australia were first reported in Port Hacking, Sydney, New South Wales (NSW) in 2000 (Phillips and Price 2002). Invasive populations were subsequently reported in five other water bodies in the greater Sydney area between 2000-2006, a further 8 estuaries south of Sydney between 2000-2007, and one north of Sydney in 2001 (Creese et al. 2004, Glasby et al. 2005, Glasby and Gibson 2007). Two of these invaded locations, Lake Macquarie, north of Sydney, and Wallagoot Lake, the southern-most population in NSW, were declared *C. taxifolia* free in 2009 and 2013 respectively (NSW 2014). *Caulerpa taxifolia* from Port Hacking and Lake Conjola share the same ITS1 rDNA genotype as isolates from the Mediterranean Sea, while the Careel Bay, Pittwater population presented a unique ITS1 genotype (Schaffelke et al. 2002, Meusnier et al. 2004). A second ITS1 genotype identified from Pittwater and Port Hacking also occurs in Tunisia (Meusnier et al. 2004).

In 2002, *C. taxifolia* was identified in West Lakes, Adelaide, South Australia (SA), a manmade estuary, and subsequently in the Port River estuary (Cheshire et al. 2002). It was eradicated from West Lakes, but established and is ineradicable in the Port River (Wiltshire 2010). In comparison to the Mediterranean Sea and some NSW populations, limited phylogeographic data exist for these populations. Using a microsatellite approach, Grewe et al. (2008) identified three distinct genetic clades: 2 composed of samples from the greater metropolitan Sydney area, and 1 of all SA specimens and samples from NSW populations south of Sydney.

It is assumed that *C. taxifolia* can reproduce sexually, but sexual reproduction is not documented in invasive populations (Žuljević and Antolić 2000). Meusnier et al. (2002) inferred from ITS genotypes in native QLD populations that sexual reproduction probably occurs only infrequently in native populations. An excess of heterozygotes in *C. taxifolia* also supports a predominantly clonal habit (Benzie et al. 1997, Grewe et al. 2008). Clonality has a significant effect on genetic diversity, and must be considered when undertaking population genetic studies

due to it directly impacting the choice of molecular marker that is used. This is because the reduction of genetic variation between individuals and populations associated with clonality requires a hyper-variable marker to provide adequate differentiation (Rozenfeld et al. 2007).

Studies on the invasion history of *C. taxifolia* in Australia have been hindered by low genetic variation. Low genetic variation limits phylogeographic resolution, leading to a failure to identify clear relationships among native and invasive populations. Detecting more variation is key to understanding how populations are related. A greater understanding of the relatedness of invasive and native populations will clarify if there is a single invasive strain of *C. taxifolia,* or if invasive populations are descended from a variety of genotypes and that likelihood of invasion success is linked to ecological factors rather than the genotype.

The origin of populations in eastern and southern Australia has not been examined with the same effort as those from the Mediterranean Sea. In the few studies of the phylogeography of invasive *C. taxifolia* in Australia, a limited number of samples from the invasive populations have been analyzed (~10 samples on average), and the majority of these data are from the NSW populations. Data on the SA populations have only been published in grey literature (Grewe et al. 2008), and *C. taxifolia* var. *distichophylla* from Western Australia (WA) has not been studied. In this study, a *de novo* next generation reduced representation sequencing approach was used to develop new single nucleotide polymorphism (SNP) markers for *C. taxifolia*. These markers were used to genotype a large sample set from QLD, NSW, SA, and WA populations to determine their phylogeographic relationships. This study aimed to determine how invasive Australian populations of *C. taxifolia* are related to one another and to native populations. I also aimed to assess if populations had experienced recent gene flow to assess if older invasive populations provided the propagules for more recent invasions. By combining the data from these investigations on population structure and relatedness, I aimed to determine if there is a single invasive strain of *C. taxifolia* in Australia.

3.2 Materials and Methods

3.2.1 Collection

All samples were collected by wading, snorkeling, or SCUBA between 2011 and 2013 at sites in QLD, NSW, SA, and WA (Table 3.2). Specimens were collected at intervals of > 3m to reduce the risk of sampling the same individual multiple times. Fifty samples per population were collected, except where this was not possible due to low population densities or small population size. Samples for nucleic acid extraction were preserved in the field by removing any epiphytic material, wrapping in a Kimwipe and storing in silica gel desiccant within a ziplock bag (Chase and Hills 1991). One pressed voucher was taken per population and housed at the State Herbarium of South Australia.

3.2.2 DNA Extraction for library construction

Total DNA extractions were carried out from specimens from three distinct populations (Moreton Bay, Port Jackson, and Port River, Garden Island - see Fig. 3.3 for locations). Six samples from each population were randomly chosen and combined into a single pooled homogenate, as library construction only requires a representative sample from the entire population being studied and does not require assignation to individual samples. These populations were chosen as they represent one native population, the most central invasive population collected in NSW, and one SA population respectively. For each pooled sample, approximately 500 mg of silica-dried tissue was homogenized in a mortar and pestle with sterile sand prior to extraction. Total DNA extractions were performed using the DNeasy Plant Maxi Kit (Qiagen) following the manufacturer's protocol. The final spin columns were eluted twice with 750µL of elution buffer and combined. All elutions were quantified using a Qubit 2.0 fluorometer (Life Technologies). DNA yields ranged from 0.2 to 146 µg/mL. Elutions were visualized following electrophoresis

Populations	Site Name	Status / Climate	No. Collected/ No. Genotyped	Latitude	Longitude	Collection Date
SA1-PRGI	Port River Garden Island SA	Inv/Temp	20/14	-34.8036	138.5467	08/12/2011
SA2-PRQS	Port River Quarantine Station SA	Inv/Temp	20/12	-34.7852	138.5173	15/08/2012
NSW1-PWSI	Pittwater Scotland Island NSW	Inv/Temp	52/36	-33.6386	151.2963	16/02/2012
NSW2-PJ	Port Jackson NSW	Inv/Temp	52/40	-33.8403	151.2251	17/02/2012
NSW3-BBSB	Botany Bay Silver Beach NSW	Inv/Temp	20/20	-34.0078	151.2035	15/02/2012
NSW4-GBSC	Gunnamatta Bay Sailing Club NSW	Inv/Temp	51/46	-34.0551	151.1491	18/02/2012
NSW5-BL	Burrill Lake NSW	Inv/Temp	4/1	-35.3821	150.4365	12/02/2012
QLD1-MB1	Moreton Bay QLD	Nat/Trop	40/12	-27.4914	153.3981	23/11/2011
QLD2-MB2	Moreton Bay QLD	Nat/Trop	10/5	-27.6595	153.3898	16/08/2012
WA1 (COTSTH)	Cottesloe South WA	Nat/Temp	7/2	-32.0045	115.7508	26/02/2013
WA1 (COTNTH)	Cottesloe North WA	Nat/Temp	7/2	-31.9848	115.7512	26/02/2013
WA1 (MARAN)	Marmion Anglers club WA	Nat/Temp	9/6	-31.8385	115.7493	26/02/2013
WA1 (WTMN)	Watermans Beach WA	Nat/Temp	8/1	-31.8579	115.7518	28/02/2013

Table 3.2 *Caulerpa taxifolia* population and specimen sampling information. Inv = Invasive population, Nat = Native population, Temp = Temperate population, Trop = Tropical population.

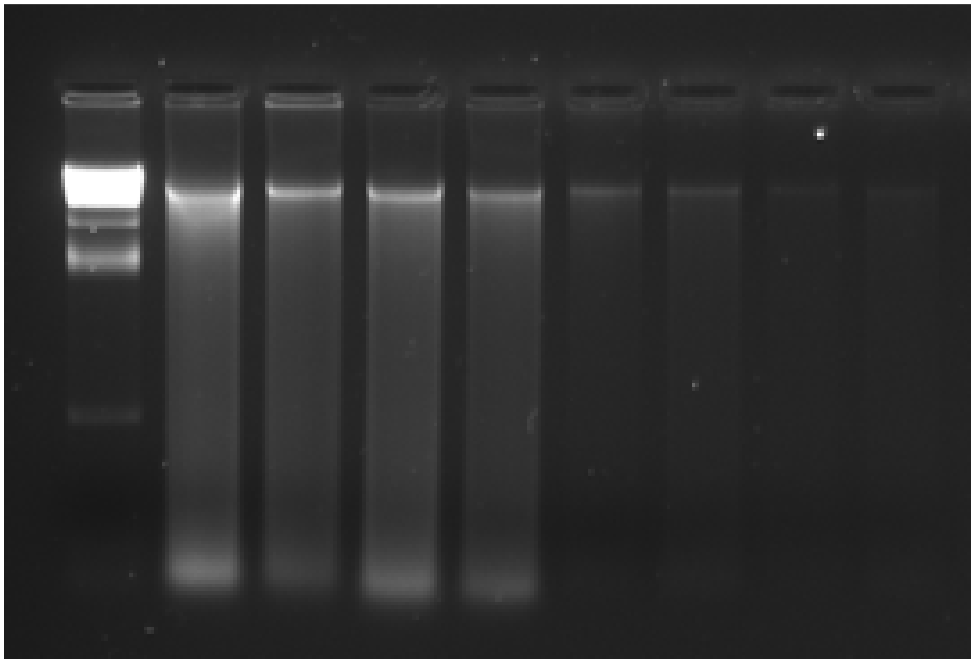

Figure 3.1 1% agarose gel of Genomic DNA and AluI restriction digests. Lane 1 = Lambda hindi III 2 = PRGI genomic DNA, 3 = MB genomic DNA, 4 = PJ genomic DNA, 5 *C. cylindracea* genomic DNA (not used in this study), 6-9 = AluI digested DNA for PRGI, MB, PJ, and *C. cylindracea* respectively

on a 1% agarose gel with 1x TAE (tris-acetate-ethylenediaminetraacetic acid) buffer to assess DNA integrity. High molecular weight genomic DNA was visible as a bright band at the top of the gel (Figure 3.1).

3.2.3 DNA cleanup

Extracted genomic DNA was further cleaned using a lithium chloride extraction. An equal volume of 4.28 M LiCl was added to each sample, mixed by pipetting and incubated at -20ºC for 30 min. Samples were then centrifuged at maximum speed (18,416g) for 15 min at 4ºC and the supernatant was removed into a new microcentrifuge tube. Cold, -20°C, 100% ethanol was added to the supernatant to precipitate DNA, which was followed by centrifugation at 4°C at maximum

(18,416g) speed for 60 min. The supernatant was discarded and the DNA pellet was air-dried for 10 min, and then resuspended in 30 µL of molecular grade H_2O.

3.2.4 Reduced representation library preparation

Reduced representation Ion Torrent sequencing libraries were prepared following a modification of the standard Ion Fragment Library Kit protocol (Life Technologies). Two 500 ng aliquots of genomic DNA from each pooled sample were incubated with 10 U of AluI restriction enzyme in a 50µL reaction overnight. The two digested aliquots were combined and the DNA purified using a MinElute Cleanup Kit (Qiagen), after which the purified DNA was eluted in 25 µL of molecular grade H_2O. The Ion Torrent protocol for 100 ng library preparation was followed, using MinElute Cleanup columns (Qiagen), and the DNA was eluted from the MinElute columns using molecular grade H_2O. The unamplified library was size selected using a 2% E-Gel Size Select Agarose Gel (Life Technologies), from which a 330-bp target peak was collected for the preparation of 200-bp sequencing libraries according to the manufacturer's instructions. Library quality control was performed in a MCE®-202 MultiNA microchip electrophoresis system for DNA/RNA analysis (Shimadzu) to determine the size distribution. Library concentration was determined using a Qubit 2.0 fluorometer (Life Technologies). Both size and concentration were used to determine the library dilution factor required to achieve a working concentration in a defined volume, using the following equation:

$$= (x \text{ pM} * 10^{-15}) * (y \text{ µl} * 10^{-6}) * (z * 660 \text{ Da}) * (1*10^{12} \text{ ng})$$

where x = desired library concentration (20 pM in this case), y = final diluted library volume (minimum 20 µl required for Ion OneTouch), and z = library mean fragment size calculated by the MultiNA. Emulsion PCR and enrichment of template positive ion sphere particles were performed using the Ion OneTouch 200 Template Kit v2, Ion OneTouch system and Ion OneTouch ES (Life Technologies) according to the manufacturer's instructions. Sequencing on

the Ion Torrent PGM was performed using an Ion PGM 200 Sequencing Kit, and 314 and 316 chips with 500 flows per run.

3.2.5 Polymorphism Discovery

Ion Torrent raw sequencing results were imported into CLC Bio Genomics Workbench v6.0 (Qiagen). Sequences were trimmed based on 100- or 200-bp long fragment sizes expected from the library preparation. Sequences with a PHRED score below 13 (p=0.05) were removed (Figure 3.1). After the trimming step, 135,639 sequences (66.3 %) were recovered with an average length of 133-bp. Sequences were pooled and assembled *de novo* using assembly settings as follows: map reads back to contigs, update contigs, automatic word size, bubble size = 267, 100bp minimum contig length, perform scaffolding, auto-detect paired distances, mismatch cost of 2, insertion cost of 3, deletion cost of 3, length fraction of 0.9, and similarity fraction of 0.9. Contig consensus sequences were used to map trimmed sequences (this is required for variant detection). Mapping settings were as follows: no masking, mismatch cost of 2, insertion cost of 3, deletion cost of 3, length fraction of 0.9, similarity fraction of 0.9, global alignment, map randomly. Using the mapped contigs, probabilistic variant detection was carried out with the following settings: ignoring non-specific matches and broken pairs, minimum coverage of 10, variant probability of 90, variant required in both forward and reverse reads, filter 454/Ion homopolymer indels, maximum of 2 expected variants, standard genetic code, and quality scores not ignored. *Caulerpa* species are known to harbor extensive endogenous and exogenous bacterial communities; therefore the consensus sequences for all mapping contigs containing polymorphisms were extracted and analysed by BLAST searches. Any contig with a BLAST result that matched a prokaryotic sequence was removed to ensure any downstream genotyping applications would only target polymorphisms in *C. taxifolia.* All SNPs were genotyped using the Sequenom MassARRAY platform (Sequenom Inc., USA). Sequenom assay design criteria required that the

SNP of interest be flanked by at least 50-bp of sequence that did not contain any additional variants.

3.2.6 DNA Extraction for population genetics analyses.

DNA from 291 samples from 13 distinct populations (Table 3.2) was extracted using automated NucleoSpin® 96 Plant II kits (MACHEREY-NAGEL, Germany) following the manufacturer's protocol at the Australian Genome Research Facility (AGRF), Adelaide node. After the DNA was extracted, the DNA concentration was determined using the Quantifluor dsDNA System (Promega, USA).

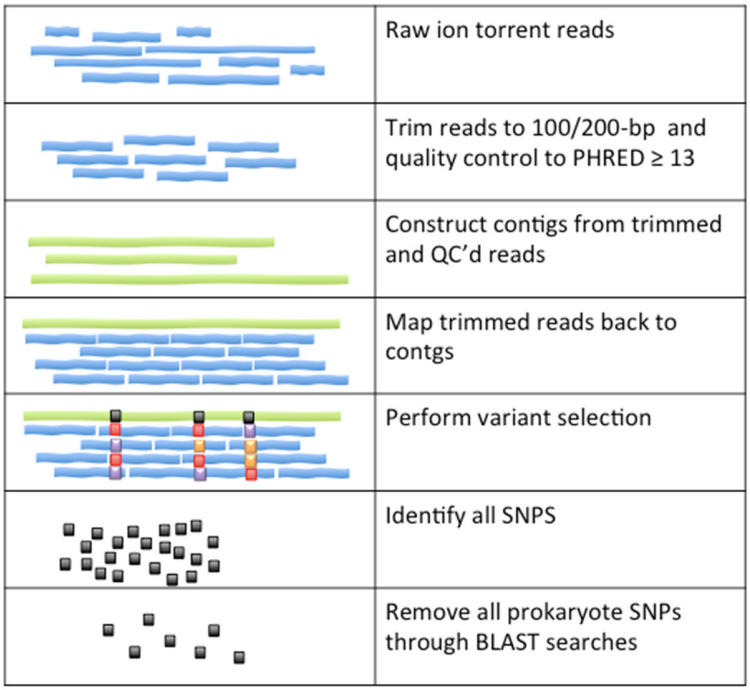

Figure 3.2 Workflow for SNP detection in CLC Bio

3.2.7 Sequenom genotyping

The concentration of each DNA sample was standardized to 10 ng/mL. Samples for which the total DNA extraction concentration fell below this value were dried to completion using a

SpeedVac and subsequently resuspended to the desired concentration using molecular grade H_2O. Primer and multiplexing design for the 31 SNP regions suitable for Sequenom analysis was performed using MassARRAY Typer version 4.0 (Sequnom Inc., USA). Of the 31 contigs containing SNPs suitable for Sequenom, only 16 passed validation by way of attempting to genotype a subset (96) of samples. Failed regions either presented chemistry incompatibility or inability to be incorporated into a single multiplex due to primer conflict.

3.2.8 Population genetic analyses

Due to low success rates during genotyping, samples from all WA populations were pooled into a combined population of 11 individuals for the purpose of statistical analyses (Table 3.2). The population from Burrill Lake NSW was represented by only 1 sample, and was therefore omitted from the majority of population genetic analyses, but it remains in the distribution of genotypes (Figure 3.3), the statistical parsimony network (Figure 3.4), and the UPGMA tree (Figure 3.5).

Tests of Hardy Weinberg equilibrium (HWE) were carried out in GenAlEx according to the procedure of (Guo and Thompson 1992). Measures of heterozygosity were calculated in GenAlEx (Peakall and Smouse 2006, 2012).

Locus-by-locus analyses of molecular variance (AMOVA) followed by permutation tests for significance were performed in Arlequin following the methods of Excoffier et al. (2005). Multiple AMOVAs were carried out to separately test for the presence of significant genetic variation between invasive and native populations, climatic zones (temperate vs tropical), and geographical groups (QLD vs NSW vs SA vs WA). Values for F_{CT}, the proportion of the total variation within groups, and F_{SC}, the proportion of total variation among subpopulations within groups, were calculated.

Values for F_{ST} (Slatkin 1995) and M, where M=2Nm (Nm = number of migrants) (Slatkin and Voelm 1991) were calculated using Arlequin.

A statistical parsimony network was constructed using TCS v.1.2.1 (Clement et al. 2000) with a connection limit set to 5 using an artificial DNA sequence generated by combing all SNP loci for each individual sample.

Analysis of isolation by distance (IBD) was calculated using the isolation by distance web service (Jensen et al. 2005), and by implementing a partial Mantel test with F_{ST} values and uncorrected shoreline distances in km, using 30,000 randomisations. The indicator matrix took into account the biogeographic break at Wilsons Promontory (39°S 146°E), the base of what was the Bassian Isthmus during the last glacial maximum (Ayre et al. 2009).

A phylogenetic tree was calculated using Nei's Genetic Distance (Nei 1972) and the unweighted pair group method with arithmetic mean algorithm (UPGMA) implemented in the BioINFO Project web site. Nei's genetic distance was calculated in GenAlEx v.6.5.

STRUCTURE (Pritchard et al. 2000) analysis was carried out to estimate the number of genetically distinct populations (K) within the dataset. An initial run with parameters set to a 10,000 burn-in length, 10,000 MCMC replications post-burn-in, K = 1-20, and 5 iterations was performed. Results of the first run were extracted and analysed in STRUCTURE Harvester v.6.94 (Dent and vonHoldt 2012) to determine an optimum estimate of K values to be analysed further. A second run was performed with parameters set to 100,000 burn-in length, 1,000,000 MCMC replications post burn-in, K = 1-10, and 10 iterations. Results were analysed with STRUCTURE Harvester to identify the final value of K.

3.3 Results:

3.3.1 Polymorphism discovery

Four Ion Torrent sequencing runs (3x 314 and 1x 316), each corresponding to the sequencing of one of the four populations, yielded 204,536 raw sequencing reads. After trimming, 135,639 reads remained, of which 89,511 were assembled into 10,636 contigs by *de novo* assembly. Re-

mapping of the trimmed sequences to the *de novo* assembled contigs resulted in a total of 92,473 mapped reads, from which a total of 329 polymorphisms were detected composed of 17 deletions, 7 insertions, and 305 SNPs. After BLAST searches were performed to remove prokaryotic sequences, 60 eukaryotic contigs containing 179 polymorphisms remained. One hundred and seventy eight of these polymorphisms had a PHRED quality score above 15 (p=0.05), of which 31 were appropriate for SEQUENOM analysis.

3.3.2 Sequenom genotyping

Of the 16 SNP loci that were successfully genotyped on the Sequenom MassARRAY, 3 were removed from the study, one due to the large number of failed samples, and two due to loci being monomorphic. From the 291 samples analysed, 206 were genotyped successfully; the other 85 failed due to poor DNA concentration and/or quality. From the 206 that were successfully genotyped, 184 remained after the removal of samples that failed to genotype for all SNPs.

3.3.3 Population genetic analyses

In total, 14 genotypes were identified among all samples analysed. Only two genotypes, A and B, were present in multiple populations (Table 3.3, Figure 3.3). Furthermore, populations represented by genotypes A and B were invasive. Fifty eight percent, or 107/206 samples, belonged to genotype A with genotype B representing the second highest membership at 13%. The third most common genotype (J) accounted for 12.5% of the samples, and was geographically restricted to Pittwater Bay (NSW). The rest of the genotypes are also exclusive to individual populations, but were found in relatively low frequency (Table 3.3). Higher genotype diversity was found in the native populations, with the MB populations collectively having 6 genotypes, and the WA population having 4 (Figure 3.3). Collectively the NSW populations have 3 genotypes, however individual populations were monotypic. SA populations collectively have -

Genotype	SNP													Hetero	N
	1	8	11	14	16	17	20	22	23	25	26	28	31		
A	CC	GA	CC	TT	AA	AA	CC	GG	TA	GG	CC	GA	CC	3/13	107
B	CC	GA	TC	TT	AA	GA	GG	GC	TT	GG	CA	GG	CC	5/13	24
C	TC	GA	TC	TT	CA	GA	GG	GC	TT	GG	CA	GA	TC	9/13	3
D	CC	GA	TC	TT	CA	GA	GG	GC	TA	GG	CA	GA	TC	9/13	6
E	CC	GA	TC	CT	AA	GG	GC	GC	TA	GG	CC	GA	TC	8/13	1
F	CC	GA	TC	CT	CA	GA	GC	GC	TA	GG	CA	GA	TC	11/13	2
G	CC	GA	TC	CT	AA	GA	GC	GC	TA	GG	CC	GA	TC	9/13	3
H	CC	GA	TC	TT	AA	GA	GC	GC	TA	GG	CC	GA	CC	7/13	2
I	CC	GA	TC	TT	AA	GA	GG	GC	TT	AG	CA	GG	CC	6/13	2
J	CC	GA	CC	CT	CA	AA	CC	GG	TT	GG	CC	GA	TC	5/13	23
K	CC	GG	CC	TT	AA	AA	GG	GG	AA	GG	CC	AA	TC	1/13	5
L	CC	GG	CC	TT	AA	AA	GG	GG	AA	GG	CC	GA	TC	2/13	2
M	CC	GG	CC	TT	AA	AA	GG	GG	AA	GG	CC	GG	TT	0/13	1
N	CC	GG	CC	TT	AA	AA	GG	GG	AA	GG	CC	GG	TC	1/13	3

Table 3.3 Genotypes detected in 184 Australian *Caulerpa taxifolia* samples from 13 SNPs, Red samples indicate haplotypes present in invasive populations.

3 genotypes, with 2 being unique to individual populations.

The statistical parsimony network produced a sprawling, mostly linear network displaying a strong geographic structure in the distribution of genotypes across populations (Figure 3.4). An unresolved topology (network loop) involved genotypes B, C and H. The genetically most distant genotypes (which are 5 to 7 mutation steps away from the nearest genotype, i.e. genotypes K-N from WA) are also the geographically most distant to any other population. Invasive populations were the only ones to share genotypes A and B. The SA and MB genotypes are closely linked to one another with only one ancestral genotype between genotype B and H.

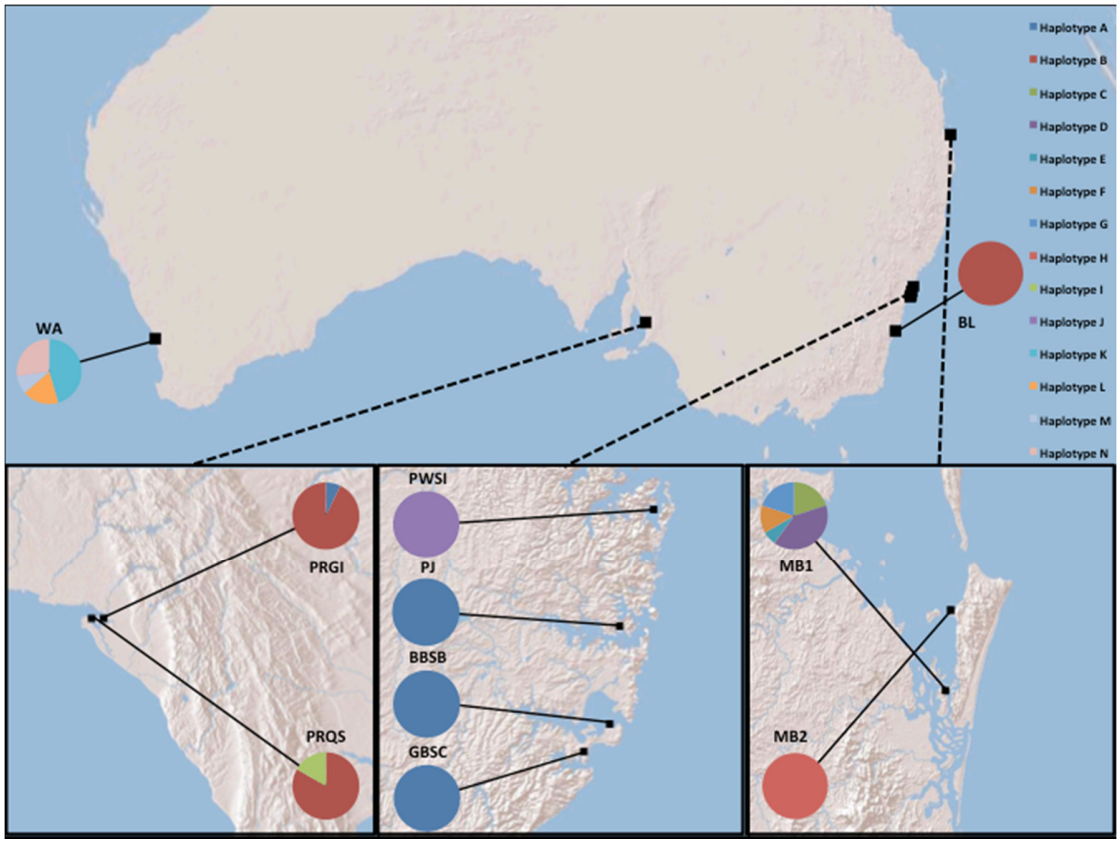

Figure 3.3 Map showing the genotype frequency of 10 *Caulerpa taxifolia* populations in Australia based on SNP data. % of genotype contribution is represented by pie graphs.

Most loci in all populations were not in HWE. The MB populations have the lowest levels of monomorphic homozygote loci (7.6 - 30%) and the highest frequencies of excess

heterozygotes (54 - 70%) (Table 3.4, Figure 3.4). Invasive populations, however, presented an opposite trend with higher frequencies of monomorphic homozygote loci (38 - 77%) and a low frequency of excess heterozygotes (23-46 %). The native Western Australian population on the other hand had the highest proportion of monomorphic loci (85%) and the lowest levels of excess heterozygotes found in this study (7.7%). The majority of deviations from HWE were highly significant (Table 3.4).

Minimum F_{ST} and maximum M values between MB populations indicate panmixia (Table 3.5). F_{ST} and M pair-wise values between MB and WA suggest the presence of moderate to strong genetic structure, but with continued migration between them (M = 0.4 - 1.3 migrants per generation). Moderate levels of genetic structuring were observed between MB and NSW populations, with low levels of migration (F_{ST} = 0.321-0.442, M = 0.630-1.162). There was a low level of genetic structure between MB and SA populations, which had the highest levels of migration (F_{ST} = 0.116-0.160, M = 2.614-3.808). Population PWSI showed a moderate level of genetic structuring and migration with the other three NSW populations (F_{ST} = 313-0.342, M = 0.959-1.094), which were panmictic with high levels of migration (F_{ST} = <0, M = ∞). All NSW populations showed a high level of genetic structuring with both SA and WA populations, with low levels of migration (F_{ST} = 0.494-0.561, M = 0.359-0.510). South Australian populations were panmictic with high levels of migration (F_{ST} = <0, M = ∞), and both display a high level of genetic structuring with WA (F_{ST} =0.549-0.587, M = 0.351-0.409).

The topology of the UPGMA tree reflects the topology of the SPN in Figure 3.4 and the F_{ST} values in Table 3.5 and Figure 3.5. Both Moreton Bay populations clustered in a sister clade to that containing both SA populations and the single sample from Burrill Lake NSW. The three genetically identical southern Sydney populations of PJ, BBSB, and GBSC formed a sister clade to the Northern Sydney population of PWSI. The Western Australian population occupied a lone branch sister to the cluster of Sydney clades.

	MB1	MB2	PWSI	PJ	BBSB	GBSC	PRGI	PRQS	WA
SNP-1	0.667	-	-	-	-	-	-	-	-
SNP-8	<0.001	0.157	<0.001	<0.001	<0.001	<0.001	<0.001	0.001	-
SNP-11	<0.001	0.157	-	-	-	-	0.001	0.001	-
SNP-14	0.333	-	<0.001	-	-	-	-	-	-
SNP-16	0.025	-	<0.001	-	-	-	-	-	-
SNP-17	0.001	0.157	-	-	-	-	0.001	0.001	-
SNP-20	0.333	0.157	-	-	-	-	<0.001		-
SNP-22	<0.001	0.157	-	-	-	-	0.001	0.001	-
SNP-23	0.010	0.157	-	<0.001	<0.001	<0.001	0.890		-
SNP-25	-	-	-	-	-	-	-	0.753	-
SNP-26	0.025	-	-	-	-	-	0.001	0.001	-
SNP-28	<0.001	0.157	<0.001	<0.001	<0.001	<0.001	0.890	-	0.036
SNP-31	<0.001	-	<0.001	-	-	-	-	-	0.006

Table 3.4. Outcomes (p-values) of Hardy Weinberg Equilibrium test from single nucleotide polymorphism data from nine Australian populations of the green marine benthic macroalga *Caulerpa taxifolia*. **-** = Monomorphic homozygous loci, blue cells = excess heterozygotes, red cells = excess homozygotes, black cells = not significant

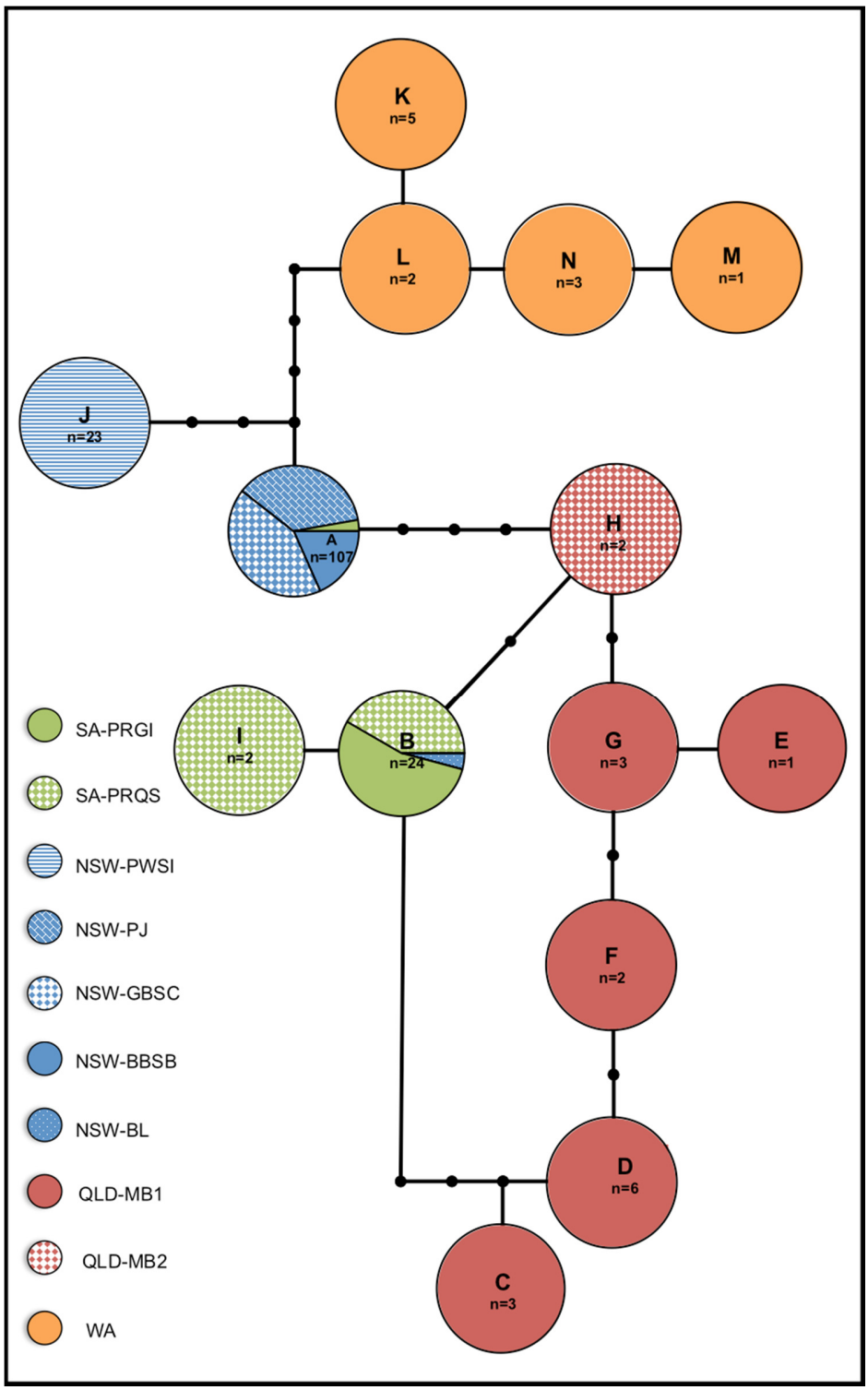

Figure 3.4. Statistical Parsimony Network showing the evolutionary relationships among 14 genotypes of *Caulerpa taxifolia* based on 13 SNPs from184 specimens across 10 populations. Connection limit used =5. Circles represent genotypes; lines represent a single mutation step, with nodes representing a hypothetical unobserved genotype.

	MB1	MB2	PWSI	PJ	BBSB	GBSC	PRGI	PRQS	WA
MB1	0	inf	1.058	0.659	0.834	0.630	3.111	2.821	1.104
MB2	-0.016	0	0.821	1.040	1.162	1.024	3.808	2.614	0.472
PWSI	0.320	0.378	0	0.979	1.094	0.959	0.510	0.449	0.396
PJ	0.431	0.324	0.337	0	inf	inf	0.440	0.3662	0.403
BBSB	0.374	0.300	0.313	-0.019	0	inf	0.497	0.408	0.389
GBSC	0.442	0.327	0.342	-0.011	-0.0182	0	0.431	0.359	0.405
PRGI	0.138	0.116	0.494	0.531	0.501	0.536	0	inf	0.409
PRQS	0.150	0.160	0.526	0.577	0.550	0.581	-0.0327	0	0.351
WA	0.311	0.514	0.564	0.553	0.562	0.552	0.549	0.587	0

Table 3.5 Pairwise table of linearised F_{ST} (Slatkin 1995) values below the diagonal, and M values where M=2Nm (Slatkin 1991) above the diagonal for *Caulerpa taxifolia*. Green cells indicate a significant result ($p<0.05$), whilst yellow indicate a non-significant result.

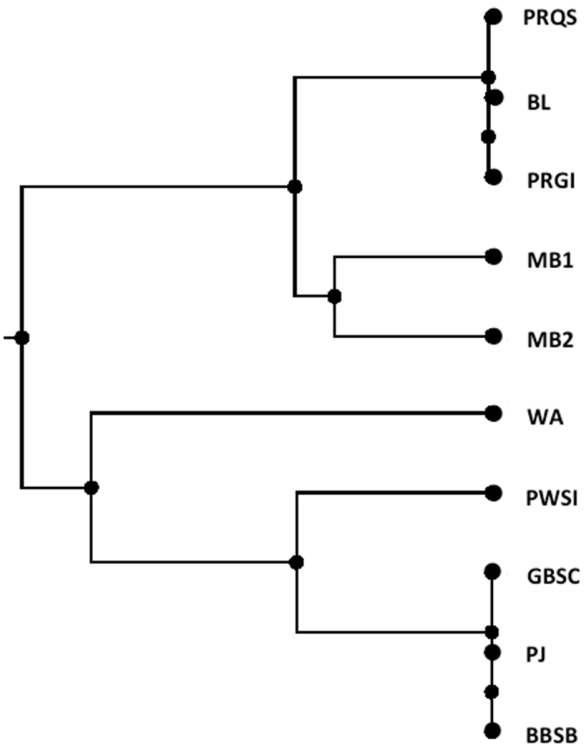

Figure 3.5 UPGMA Tree of Nei Genetic Distance values.

The partial Mantel test (IBD) resulted in a significant (p = 0.0003) yet moderate partial correlation (r = 0.65) between genetic and geographic distance after correcting for the presence of the biogeographic divide at Wilsons Promontory in southern Victoria (Ayre et al 2008).

Locus by Locus AMOVA Results					
Invasive vs Native		**Temperate vs Sub-Tropical**		**QLD vs NSW vs SA vs WA**	
% Va	16.5	% Va	17	% Va	42
p-value Va and FCT	0.021	p-value Va and FCT	0.040 5	p-value Va and FCT	≤0.00 1
%Vb	29.76	%Vb	30.79 6	%Vb	8.872
p-value Vb and FSC	≤0.001	p-value Vb and FSC	≤0.00 1	p-value Vb and FSC	≤0.00 1
FSC	0.356	FSC	0.369	FSC	0.151
FCT	0.165	FCT	0.166	FCT	0.416

Table 3.6 Results of Locus-by-locus AMOVA (Excoffier et al 1992)

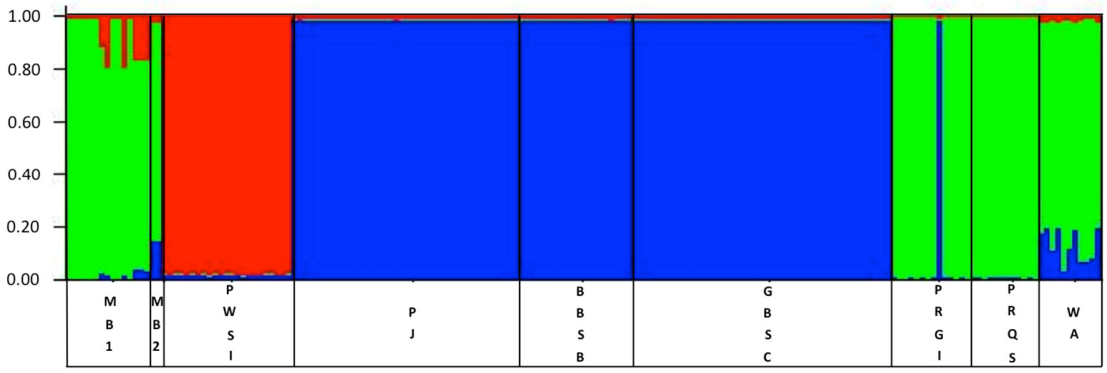

Figure 3.6 STRUCTURE (Pritchard et al. 2009) bar plot representing graphically the posterior probabilities (vertical axis) of a sample (horizontal axis) belonging to one of three hypothetical genetic populations or K in red, green, and blue. Geographical populations are identified below the horizontal axis.

AMOVA results showed that 16.5% of the observed molecular variation was between native and invasive populations, and 29.7% was among populations within either group (Table 3.6). Similarity between climatic regions accounted for 16.6% of the variation. Membership to one of four demes accounted for 41.6% of observed variation, while only 8% of variation was found among populations within each deme. All AMOVA results were significant (Table 3.6). Within population variation was not significant, with negative values indicating a lack of variation amongst populations (Table 3.7).

STRUCTURE analysis identified three distinct genetic populations (Figure 3.6). All South Australian samples were assigned to the green cluster, except for one from PRGI that was assigned to the blue cluster. The Sydney populations were assigned to 1 of 2 ancestral populations, PWSI in red, PJ, BBSB, and GBSC in blue. These populations are fixed for their designated genotype. The native populations were assigned among the three above-mentioned clusters, with the majority in the green cluster and only low-level assignment to red and blue.

Population Specific FIS	
MB1	-0.79295
MB2	-0.86364
PWSI	-1
PJ	-1
BBSB	-1
GBSC	-1
PRGI	-0.14359
PRQS	-0.94302
WA	-0.05263

Table 3.7 Populations specific FIS from AMOVA analysis

3.4 Discussion

Genetic variability in native populations of *C. taxifolia* was double, on average, that of invasive populations. Clonality was prevalent among the invasive populations. Populations PJ, BBSB, and GBSC (Fig. 3.3) are represented by a single clone (genotype A); PWSI is also a single clone (Genotype J), while genotype B accounts for 83% and 93% of genetic variation in PRQS and PRGI, respectively. In contrast, the native populations from Moreton Bay and WA had 6 genotypes amongst 15 individuals, and 4 genotypes amongst 11 individuals, respectively. Similar patterns of genotypes were found using microsatellites by Grewe et al. (2008). PJ, BBSB, and GBSC populations were characterized by a dominant genotype and 2 minor genotypes, while PWSI was characterized by 1 dominant genotype and 2 minor genotypes. The minor genotypes identified by Grewe et al. (2008) are redundant as they are unique to each population and provide no phylogeographic resolution. This comparison of SNP and microsatellite data indicates that the SNP markers generated here are more sensitive than the microsatellites of Grewe et al. (2008).

Analysis of molecular variance also shows that variation is high between invasive and native populations (F_{SC} =0.356 p = ≤0.001). The non-conformation to Hardy-Weinberg Equilibrium, specifically the high excess heterozygotes (Table 3.4) is an indication of predominantly asexual reproduction in these invasive *C. taxifolia* populations, as found using other markers by Meusnier et al. (2002) and Grewe et al. (2008).

Low genetic variability in invasive populations compared to native populations is indicative of a single origin, a small founding population, and the absence of subsequent additional introductions. A single clone or a small number of clones is likely to represent the total genetic variation of recently founded populations of asexual or clonal organisms (Amsellem et al. 2000, Zepeda-Paulo et al. 2010). This pattern is a characteristic of the founder effect (Nei 1987, Allendorf and Lundquist 2003), which is well documented in invasive species (Provan et al. 2004, Alexander et al. 2009, Henry et al. 2009, Toledo-Hernández et al. 2014, Williams and Fishman 2014). If persistent, the effects of drift and/or natural selection may cause a further reduction in genetic variation, with possible genotypic fixation, where a single clone comprises the entire genetic diversity of the invasive population (Zepeda-Pauo et al. 2010).

Temperate invasive populations of *C. taxifolia* in Australia are descended from native sub-tropical populations, specifically from the Moreton Bay area (Fama et al 2002, Meusnier et al 2004, Grewe et al. 2008). Moreton Bay is also recognized as the likely origin of the *C. taxifolia* found in the Mediterranean Sea at Monaco (Wiedenmann et al. 2001). I observed no shared genotypes in invasive and native populations, making it difficult to establish a direct recent link between populations or assess gene flow. Two population histories can account for the variation observed in the invasive populations. The NSW populations are older than the SA populations and have had more time for isolation and differentiation to act on them after establishment. Temperate *C. taxifolia* stands die back over winter due to low water temperatures and grow back in spring (Ivesa et al. 2006). This seasonal variation in biomass may include loss of individuals,

which could cause genetic drift and further reduce genetic variation until the population comprises only one genotype (clone) or one genotype becomes dominant, as we observed in the NSW invasive populations of *C. taxifolia*. The SA populations are a more recent invasion and the effects of seasons on the populations and the accompanying genetic drift have had less time to manifest. Alternatively, a single clone may have founded each of the NSW populations while the SA populations may have been founded by a more variable founding population, or multiple founding populations. My data cannot identify which scenario is more likely, but both are feasible.

Evidence for genetic connectivity between invasive populations from SA and NSW is low. Only one specimen from PRGI shared genotype A with PJ, BBSB, and GBSC. High F_{ST} and low M values between SA and NSW populations also indicate that the invasive populations are isolated from each other, and thus it is unlikely that the invasive populations in NSW are the origin of the populations in SA, or *vice versa*. M values indicate high local migration between groups of sites around Sydney and Adelaide. MB1 and MB2 also show a high number of migrants. Many of the invasive sites in NSW and SA are near boat ramps and marinas; the high regional genotypic homogeneity implies that local vessel traffic is involved in the translocation of *C. taxifolia*. West et al. (2007) showed that *C. taxifolia* fragments from NSW populations can survive out of water for at least 10 hours if kept on wet anchor rope, which could facilitate fragments collected at one anchoring site being translocated long distances on vessels or trailered recreational boats. This mechanism of local translocation is likely to have caused the patchy but broad distribution of *C. taxifolia* in the Sydney metropolitan region, which experiences substantial boat traffic.

Estimations of membership coefficients from Structure, the results from the statistical parsimony network and the UPGMA phylogeny provide strong evidence for the Moreton Bay area as the origin of the NSW PJ, BBSB, and GBSC populations. These analyses also support the

Moreton Bay area as the origin of the PWSI populations, but the PWSI population probably represents an independent invasion. This is further supported by the removed position of genotype J in the statistical parsimony network, and the single branch occupied by PWSI in the UPGMA phylogeny. F_{ST} values between the MB and NSW populations are also low, supporting a close relationship between these populations. F_{ST} values between the MB populations and the SA populations are very low, also indicating a close relationship. The UPGMA tree, statistical parsimony network, and Structure analysis, including the low F_{ST} and high M values, also indicate that individuals from the Moreton Bay area founded the SA populations.

High F_{ST} and low M values indicate that the WA population is distantly related to all other populations examined. The statistical parsimony network showed an isolated position for the WA population, and the UPGMA analysis places it on a lone long branch. Reducing the connection limit of the statistical parsimony network from 5 to 4 results in the WA population separating from the main network into a separate WA only network. These findings support the hypothesis that the WA population is not related to invasive populations in Australia. Jongma et al (2012) provided evidence for an invasion of WA *C. taxifolia* in the Mediterranean, so the populations in WA remain a potential source of future invasions. WA populations were once considered *Caulerpa distichophylla,* a distinct species recently synonymized with *C. taxifolia* based on *tuf*A cpDNA, ITS-1 rDNA, and 16S intron-2 rDNA sequenced data (Jongma et al 2012). My results show, however, that the morphologically slender and geographically isolated *Caulerpa taxifolia* var. *distichophylla* populations from WA are genetically distinct from *C. taxifolia* var. *taxifolia*, but SNP markers are not appropriate for delineating species because they are highly variable within species, and do not reflect the taxonomic status of these populations. My results indicate that more comprehensive sampling of native *C. taxifolia* of both varieties is required to resolve their taxonomy.

The broad geographic range and genetic relationships between the native and invasive populations sampled facilitated IBD analysis, to test the hypothesis that the genetic structure of *C. taxifolia* populations is correlated with their geographic distances. Correlation can be produced by natural dispersal following a stepping-stone model along the coast, or successive anthropogenic introductions in one direction. A significant correlation was detected between geographic and genetic distance, indicating that geographically proximal populations were also genetically similar and that natural long distance dispersal is either absent or very rare. If a stepping stone model is assumed, populations of *C. taxifolia* should occur between the native source populations and the invasive populations. *Caulerpa taxifolia* collections stored at Australia's Virtual Herbarium (AVH 2014) show no record between Perth and Adelaide (3600km), Moreton Bay and Sydney (750km), and Batemans Bay and Adelaide (1700km). The absence of records of *C. taxifolia* between tropical and introduced temperate populations is strong evidence against natural dispersal, and for anthropogenic translocation. The analysis of molecular variance revealed that variation among groups was greatest between geographic locations (F_{CT} = 0.416 p = ≤0.001), implying that populations are isolated between regions (QLD, NSW, SA, WA), further supporting anthropogenic translocation as the source of these populations. Sites where the Australian invasive populations occur(ed) are popular for recreational boating and fishing, and most are estuaries or estuarine lakes, often either land-locked (West Lakes, South Australia) or which receive low inward flow from the sea (e.g. NSW estuaries). These conditions support anthropogenic translocation as the main source of introductions (Lambrinos 2004). The Sydney populations (PWSI, PJ, BBSB, GBSC), however, occur in embayments with near oceanic conditions, but which are heavily anthropogenically modified and have heavy vessel traffic.

Although comparisons of SNP discovery via reduced representation sequencing with other algal species cannot be made, because there are no similar studies in the literature, comparisons can be made with other studies of non-model organisms. Table 3.8 shows the

number of raw base pairs per putative SNP discovered for 3 non-model species. My SNP mining experiment shows that there were far fewer SNPs discovered in *C. taxifolia* per bp than the other studies, additionally the total number of bp sequenced by these 3 studies was much higher than this study, resulting in higher numbers of SNPs discovered.

Species	1 SNP per *n*bp	Minimum coverage (*n*x)
Atlantic Cod (Carlsson et al. 2013)	1 SNP per 3500bp	4x
Rainbow Trout (Sanchez et al. 2009)	1 SNP per 11000bp	6x
Bornean Elephant (Sharma et al. 2012)	1 SNP per 81500bp	12x
C. taxifolia (this study)	1 SNP per 93000bp	10x

Table 3.8 A comparison of the number of sequenced base pairs per SNP discovered in three studies on non-model organisms.

It is important to recognize that *C. taxifolia*, including the native Moreton Bay population used in the SNP discovery, reproduces asexually, and I expected genetic variation to be low. The genotyping technology used in this study also reduced the number of SNPs I was able to assay, so although 305 SNPs were identified, I was only able to use 13. Had the sequencing experiments performed to specification, conservative estimates suggest I would have produced 2.2Gbp, which may have produced ~23,500 putative SNPs instead of 305. Although the number of SNPs in this study were low, the number of genotypes discovered was comparable to Grewe et al. (2008), indicating that allele for allele, the 13 SNPs reported here have more resolving power than the 9 microsatellite markers used by Grewe et al. (2008).

The four invasive genotypes identified here demonstrate that the hypothesis of a single invasive strain should be rejected, and that any non-native population of *C. taxifolia* should be treated as invasive regardless of its origin and genotype. The fixation on the identification of an "invasive strain" of *C. taxifolia* impacted the reporting of some of the previous molecular studies,

particularly Jousson et al. (2000). Discussion of stochastic processes, founder effects and genetic drift that occur during introductions and the impact on established populations has been rare. That Mediterranean *C. taxifolia* has originated from at least 3 separate introductions (Jousson et al. 1998, Cevik et al. 2007, Jongma et al. 2012) further supports the rejection of an invasive strain. In Australia, invasive *C. taxifolia* populations are descended from source populations in the Moreton Bay area of southern Queensland. These invasions are almost certainly anthropogenically mediated, either through recreational boating (West et al. 2007, West et al. 2009) or through the aquarium trade (Stam et al. 2006, Smith et al. 2010). Due to the distance between Moreton Bay and Sydney, NSW populations could have been introduced via boat traffic from Brisbane, while SA populations are too distant from native populations for this to be likely (West et al 2007). Both NSW and SA invasive populations could have been introduced by the release of unwanted aquarium specimens (Hewitt et al. 2007, Williams and Smith 2007). *Caulerpa* species are readily available for purchase either online or at aquarium stores (Stam et al. 2006, Smith et al. 2010), and are undoubtedly present in private aquaria. These primary releases have been followed by localized secondary invasions, probably facilitated by recreational boating activities and local spread associated with vegetative growth and clonal production of new algae. The success of these populations probably stems from clonality, and their ability to persist with limited genetic diversity. The success of multiple genotypes in broadly separated localities implies that *C. taxifolia* from any native population, when introduced into a suitable temperate site, may establish and become invasive.

Chapter 4

Phylogeography of the invasive marine green macroalga *Caulerpa cylindracea* Sonder in Australia.

Statement of Authorship

Phylogeography of the invasive marine green macroalga *Caulerpa cylindracea* Sonder in Australia

Grant, W. M. (Candidate)
Contributed to initial manuscript conceptualisation, collection of samples, carried out all labwork (unless specified otherwise), carried out all data analysis, produced all figures and tables, and acted as lead author in writing of manuscript.

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Supervised the study, secured funding, contributed to initial manuscript conceptualisation, and commented on and edited manuscript drafts.

Signature ……Date…27/02/2015

Abstract

Caulerpa cylindracea is a benthic green marine macroalga that is invasive in the Mediterranean Sea and Canary Islands, usually in association with its notorious invasive congener *C. taxifolia*. In Australia, *C. cylindracea* is native in tropical regions and sub-tropical to temperate coastal reefs of Western Australia. In South Australia, a population of this species was discovered in 2001. I assessed if application of the universal chloroplast primers of the *rpl*16-*rps*3 region described by Provan et al. (2004) and subsequent sequence analysis show enough variation in *Caulerpa cylindracea* to differentiate populations, and if the variation in *C. cylindracea* UCP6 data can identify if natural or anthropogenic dispersal was responsible for the establishment of *C. cylindracea* in SA. Strong genetic differentiation associated with haplotype fixation between tropical and temperate native populations was detected (F_{st} = 0.8, G_{st} = 0.21). Native temperate populations displayed the highest genetic diversity (Hd = 0.508, $\Theta = 1.43 \times 10^{-3}$), followed by the South Australian population (Hd = 0.107, $\Theta = 2.8 \times 10^{-4}$) and the tropical populations (Hd = 0.0, Θ = 0.0). South Australian populations comprise algae with temperate or tropical haplotypes, but none were detected with both haplotypes, suggesting a single introduction of *C. cylindracea* with a mixed geographic origin, or multiple introductions from more than one source region. Lack of support for natural dispersal indicates that SA populations of *C. cylindracea* were introduced by anthropogenic translocation and should be considered invasive.

4.1 Introduction

Caulerpa cylindracea Sonder (formerly *Caulerpa racemosa* var. *cylindracea* (Sonder) Verlaque, Huisman, and Boudouresque) is a marine benthic green macroalga native to temperate southwestern Australia, tropical Australia, Papua New Guinea and New Caledonia (Sauvage et al. 2012). *Caulerpa cylindracea* is an invasive species in temperate marine systems, usually co-occurring with another high profile invasive macroalga, *Caulerpa taxifolia* (M.Vahl) C. Agardh, known as the 'killer algae' (Meinesz 1999, Ceccherelli et al. 2002). Invasive populations of *C. cylindracea* were discovered in the Mediterranean Sea off Libya in 1991 (Nizamuddin 1991). This alga subsequently spread to the coasts of Italy, Greece, Albania, Cyprus, France, Turkey, Malta, Spain, Tunisia, Croatia, Algeria, and all major islands in the Mediterranean Sea, covering more than 164 km^2 of seafloor (Verlaque et al. 2003, Klein and Verlaque 2008). Discovery of *C. cylindracea* in the Canary Islands in 1997-1998 may be the result of secondary invasion from the Mediterranean Sea (Verlaque et al. 2004). In 2001, *C. cylindracea* was reported in temperate Australia in the Port River estuary, Adelaide, South Australia (SA) (Womersley 2003), where it has proliferated. It was subsequently found at O'Sullivan Beach Marina, a man-made coastal mooring 25 km south of the Port River (Collings et al. 2004, Belton et al. 2014). The most recent detection is at Portland, Victoria, Australia, ~575 km southeast of Adelaide (Belton unpublished data). The identity of these recent southern Australian populations has been confirmed by *tuf*A/*rbc*L DNA sequence analysis (Belton et al. 2014).

The negative effects of this species on marine ecosystems are well described. *Caulerpa cylindracea* invades *Posidonia oceanica* seagrass meadows (Ruitton et al. 2005), smothers and outcompetes other benthic organisms (Piazzi et al. 2001a, Piazzi et al. 2001b), and decreases alpha and beta diversity leading to biotic homogenization, which can decrease the capacity of the ecosystem to respond to further disturbance (Piazzi and Balata 2008). Removal of *C. cylindracea* from an invaded site only facilitates partial community recovery, with a long term decrease in

species composition and richness (Piazzi and Ceccherelli 2006). Presence of the alga decreases growth, and causes tissue necrosis and death, of the sponge *Sacrotragus spinosulus* in the Adriatic Sea (Žuljević et al. 2011), and of the gorgonian *Paramuricea clavata* in the northwestern Mediterranean (Cebrian et al. 2012). Caulerpin, a toxin found in *C. cylindracea*, occurs in the *Caulerpa* eating fish *Diplodus sargus* at concentrations that lead to deleterious changes in enzyme pathway activity (Felline et al. 2012). These modifications lead to detrimental effects on health, changes in behavior and overall loss of reproductive output (Felline et al. 2012). Thus, the impact of *C. cylindracea* on costal marine benthic ecosystems is potentially substantial, and its ongoing spread highlights the need to record when and where, and understand how, this species invades, to inform strategies to prevent further spread.

Caulerpa cylindracea has a dynamic taxonomic history in the Mediterranean Sea, possibly dating back to 1926 when the first '*Caulerpa racemosa sensu lato'*, a taxon typical of tropical and sub-tropical distribution, was recorded in Tunisia (Verlaque et al. 2003). It was not until 2000 that distinct varieties of '*C. racemosa'* were recognized (Verlaque et al. 2000). The invasive strain was identified as closely related to *C. racemosa* var. *laetevirens* f. *cylindracea* (Famá et al. 2000), and later elevated to *C. racemosa* var. *cylindracea* Verlaque, Huisman & Boudouresque (2003). Verlaque et al. (2003) analyzed DNA sequence data from nuclear 18S, ITS1, 5.8S and ITS2 to identify the invasive variety in the Mediterranean Sea. Mediterranean samples phylogenetically grouped with *C. racemosa* var. *cylindracea* from Australia, identifying the Mediterranean alga as conspecific with Australian populations, and recognizing Australia as its probable origin (Verlaque et al. 2003). How *C. cylindracea* was introduced to the Mediterranean is unknown, but it is likely that it arrived through the aquarium trade; a vector that has been associated with multiple introductions of *C. taxifolia* into the Mediterranean Sea (Jongma et al. 2012).

Although the identity and origins of Mediterranean invasive populations of *C. cylindracea* are now better resolved (Verlaque et al. 2003), comparisons of population parameters between endemic and invasive Australian populations are unexplored. This is particularly important for Australian populations in South Australia and Victoria. Understanding genetic relationships is key to aid identification of invasion sources, number of invasions, patterns of subsequent local spread, and to help determine if populations in southern Australia are invasions or unreported natural occurrences. Models predict further spread of *C. cylindracea* along the coasts of western Europe, west Africa and southern Australia as a result of climate change (Verbruggen et al. 2013). The use of phylogeographic methods would contribute greatly to testing if populations in southern Australia are early manifestations of this spread.

Traditional genetic markers are ineffective at measuring fine-scale population genetic differences in *C. cylindracea*. Nuclear ITS sequences used extensively in *C. taxifolia* studies (Meusnier et al. 2001, Famá et al. 2002a, Meusnier et al. 2004) show sufficient intra-individual variation in *C. cylindracea* the same as, or higher than, intra- and inter-population differences (Famá et al. 2000) making phylogeographic inference and the detection of geographic genetic structure impossible. Cloning is required to characterise intra-genomic variation, which is costly and slow. Chloroplast RuBisCo large and small subunits, including their spacer regions, also often fail to provide resolution for finer scale population genetics in green algae (Provan et al. 2004). The chloroplast *tuf*A gene has also been used extensively in phylogenetic studies, but it is highly conserved, which makes it more suitable for species and higher taxonomic level phylogenetics (Famá et al. 2002b, Zuccarello et al. 2009, Sauvage et al. 2012, Belton et al. 2014). To see if these challenges could be overcome, I evaluate if the universal chloroplast primers for the *rpl*16-*rps*3 region described by Provan et al. (2004) would amplify successfully in *Caulerpa cylindracea*, and if the resulting sequences contain enough genetic variation to differentiate

populations. I then assess if the genetic variation in *C. cylindracea* UCP6 data can identify if natural or anthropogenic dispersal was responsible for the establishment of *C. cylindracea* in SA. UCP6 has been used successfully in phylogeographic studies in the Chlorophyta; it has greater intraspecific variation than markers commonly used for phylogenetic analysis (Provan et al. 2008). Provan et al (2008) used the *rpl*16-*rps*3 region to robustly differentiate cosmopolitan *Codium fragile* populations. Variation was low, with only 5 variable sites giving rise to 11 haplotypes, but this indicates that UPC6 primers for *rpl*16-*rps*3 are suitable for assessing genetic variation within a species, confirming taxonomic identifications, and identifying cryptic taxa among historic and extant specimens.

4.2 Materials and Methods

4.2.1 Collection

All samples were collected by wading, snorkeling, or SCUBA diving between 2008 and 2013 at two sites at Port Adelaide in South Australia (populations SA1 and SA2), and six sites in Western Australia (here treated as 3 populations: WA1, WA2 and WA3) (Table 4.1). Garden Island (SA1) and Torrens Island (SA2) are 6 km apart. WA1 is at Point Peron, 50 km south of WA2. Algae from three reefs located along the Perth metropolitan region (Cottesloe, Marmion, and Watermans Beach), with an average distance of 8 km between each other, were considered a single genetic population (WA2) for phylogeograhic analysis. WA3 is composed of two northwestern reef systems, Ningaloo Reef and offshore Montgomery Reef, which are 1,200 km apart but are considered a single tropical population for phylogeographic analysis. Between temperate (WA1, WA2) and tropical WA populations (WA3), there is an approximate shoreline distance of 1,200 km. Between the SA and temperate WA populations there is an approximate shoreline distance of 2,900 km (Fig. 4.3).

4.2.2 DNA sequencing.

DNA was extracted from *C. cylindracea* fronds with automated NucleoSpin® 96 Plant II kits (MACHEREY-NAGEL, Germany) using a 50:50 mix of lysis buffer PL1 and PL2, and then following the manufacturer's protocol, at the Australian Genome Research Facility (AGRF), Adelaide node. Polymerase chain reaction (PCR) of the *rpl*16-*rps*3 was performed at the Australian Center for Evolutionary Biology and Biodiversity at the University of Adelaide using UCP6 primers and following a modified protocol of Provan et al. (2004).

Population (site)	n samples	Latitude	Longitude	Date of collection
SA1 (Garden Island)	19	-34.7852	138.5173	08/12/2011
SA2 (Quarantine Jetty)	18	-34.7852	138.5173	15/08/2012
WA1 (Point Perron)	25	-32.2716	115.6868	25/02/2013
WA2 (Cottesloe)	6	-31.9848	115.7512	26/02/2013
WA2 (Marmion)	9	-31.8385	115.7493	26/02/2013
WA2 (Watermans Beach)	1	-31.8579	115.7519	28/02/2013
WA3 (Ningaloo Reef)	1	-22.6666	113.65	18/06/2008
WA3 (Montgomery Reef)	2	-15.9933	124.2397	21 & 24/10/2009

Table 4.1. Collection details of *Caulerpa cylindracea* specimens analyzed in this study.

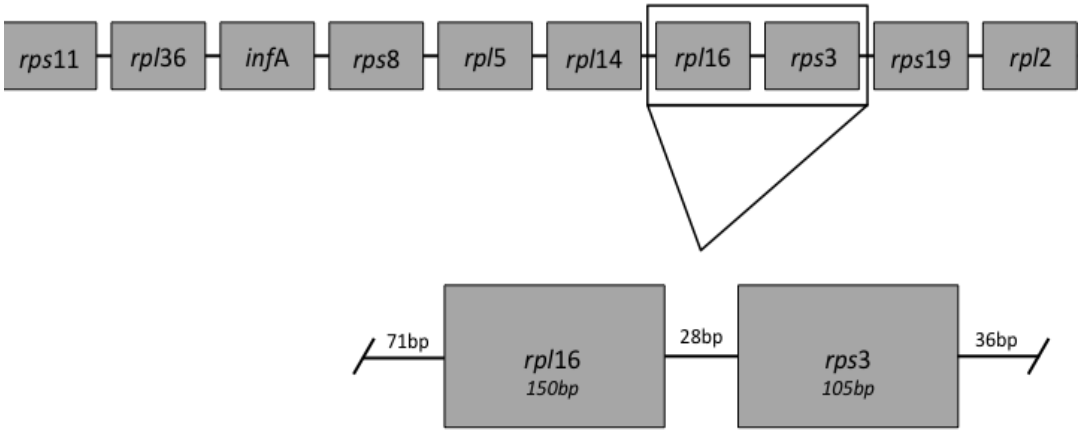

Figure 4.1. Graphical representation of the *rps*11-*rpl*12 gene cluster in the chloroplast genome of green macroalgae. Blown up section is representative of the UPC6 region amplified in this study.

Final PCR reaction concentrations were: 5 pmol of forward and reverse primers, 1x PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% (w/v) gelatin (Applied Biosystems, Carlsbad, CA, USA), 3 mM $MgCl_2$ (Applied Biosystems), 15g μl^{-1} BSA, 0.1 mM dNTPs, 0.2 U *Taq*-polymerase (Applied Biosystems). PCR clean-up was performed using an Agencourt AMPure PCR Purification system (Beckman Coulter Inc., Massachusetts, USA), sequencing reactions were performed using BigDye Terminator v4.1 chemistry (Thermo Fisher Scientific, Massachusetts, USA), and capillary separations were performed using an AB37030xl automated DNA analyser with a 96 capillary 50 cm array (Thermo Fisher Scientific). DNA sequence data were assembled and edited by eye using Sequencher v. 4.9 (Gene Codes Corp., Ann Arbor, MI, USA). DNA sequences were aligned in Geneious v. 5.4.6 (Biomatters, Auckland, New Zealand) using the Geneious Alignment option with 93% similarity, gap open penalty of 12, a gap extension penalty of 3, global alignment, no use of a guide tree, and 2 refinement iterations. Three UCP6 sequences from *C. cylindracea* (JN831125, JN831124, JN831122) were downloaded from GenBank and aligned to the new sequences; these downloaded sequences represent the individuals that make up WA3.

4.2.3 Statistical analyses.

Indices of intra-population genetic diversity (proportion of segregating sites (*S*), haplotype diversity (*h*), and nucleotide diversity (Pi) (Nei 1987)), as well as population genetic differentiation parameters (F_{st}, G_{st}), and gene flow estimates (Nm) (Hudson et al. 1992), were quantified using DNAsp v.5.10.01 (Librado and Rozas 2009). Tajima's D (Tajima 1989) and Fu & Li's D (Fu and Li 1993) neutrality tests were performed to assess the presence of a mutation/drift equilibrium under an infinite sites model (null hypothesis).

Statistical parsimony networks were constructed in TCS v.1.2.1 (Clement et al. 2000) using a 95% connection limit. Isolation by distance (IBD) analysis was performed using a partial

Mantel test as implemented in IBDWS v.4.23 (Jensen et al. 2005), with corrected PhiST genetic distances (Jukes and Cantor 1969) tested against untransformed geographic distances, 30,000 randomizations and a one-dimensional stepping stone model along the Australian coast following Rousset (1997). The indicator matrix considered the split between the regions north and south of latitude 27°S on the Australian west coast, which marks a shift in coastal currents (Woo et al. 2006), and the climatic shift between tropical and temperate zones, which is also the boundary between the Flindersian and the Damperian biogeographic provinces (Womersley 1981). The partial Mantel test dissociates variation due to spatial autocorrelation from that putatively related to other factors responsible for genetic spatial structuring such as physical barriers to gene flow, local adaptations to distinct climatic zones and selection (Jensen et al. 2005). Geographical distances were estimated manually along the coastline.

Cluster analysis using the Unweighted Pair Group Method with Arithmetic mean (UPGMA) algorithm was undertaken using DendroUPGMA (Garcia-Vallve et al. 1999) to infer distances among populations derived from a haplotype frequency matrix. The cophenetic correlation coefficient was calculated using 100 bootstrap replicates.

4.3 Results

A data set of 81 UPC6 sequences with a length of 390 bp was constructed. Three non-coding regions were present, a spacer region in between *rpl*16 and *rps*3, a region before *rpl*16, and one after *rps*3 (Fig. 4.1). Non-coding data accounted for 34.6% of the total sequence length, no indels were present, and all the polymorphism occurred in three of the 390 segregating sites (Table 4.2).

Six haplotypes were identified (A-F), with an estimated total haplotype diversity of h = 0.409 ± 0.062 (Table 4.3). Except for the tropical WA population (WA3), which was fixed for haplotype B, haplotype diversity varied between populations from 0.105 (SA1) to 0.542 (WA2).

Overall nucleotide diversity was low within both the species (Θ = 0.00156, ± 0.00095 s.d.) and the individual populations ($\Theta_{(\pi)}$ = 0.00117, ± 0.00020 s.d.; Table 4.3). Native temperate WA populations had the highest genetic diversity (Hd= 0.509, K= 0.556), five times higher than that observed for the SA populations (Hd= 0.107, K= 0.108) (Table 4.3).

The statistical parsimony network produced an unresolved star-shaped topology (Figure 4.2), which is concordant with the consistent negative, albeit insignificant, values obtained for Tajima's D, and Fu and Li's D (Table 4.3). The central haplotype A was the most common, and was found in four of the five populations studied (Table 4.2). Three of the five populations had a haplotype unique to their location, but SA1 and WA3 did not (Table 4.4, Figure 4.3).

There was no genetic differentiation within each of the four temperate populations (F_{st} and G_{st} = 0.00). F_{st} and G_{st} values between individual WA and SA temperate populations ranged from 0.159 to 0.265 (F_{st}) and 0.110 to 0.116 (G_{st}). The highest measures of genetic differentiation were observed between tropical and temperate populations (Table 4.3). F_{st} values were the highest between the tropical WA3 and all other populations (>0.78), and approached fixation between WA3 and SA populations ($F_{st} \approx 0.95$, Table 4.4). G_{st} values ranged from 0.27 to 0.32 between tropical and temperate WA populations, and from 0.70 to 0.71 between tropical WA and SA, better reflecting the spatial relationship between these populations. The partial Mantel test identified a significant, near perfect partial correlation between genetic and geographic distance after correcting for the presence of the shift in climatic zones (indicator matrix), r = 0.98, p = 0.0074 (Fig 4.4). A strong significant partial correlation between genetic distance and climatic zone was detected only after controlling for the effect of geographic distance (r= 0.996, p = 0.0074).

UPGMA analysis demonstrates the absence of monophyly between SA and any single WA population, and showed that SA1 has a genetic structure more similar to temperate WA populations, while SA2 is sister to both temperate and tropical WA populations (Fig 4.5).

Haplotype name	Nucleotide Position			N
	1	4	309	
A	T	A	A	61
B	A	A	A	4
C	T	G	A	1
D	T	T	A	13
E	T	T	G	1
F	T	A	G	1

Table 4.2. Haplotypes detected in 81 Australian UPC6 sequences of *C. cylindracea*. N= number of sequences (specimens sequenced).

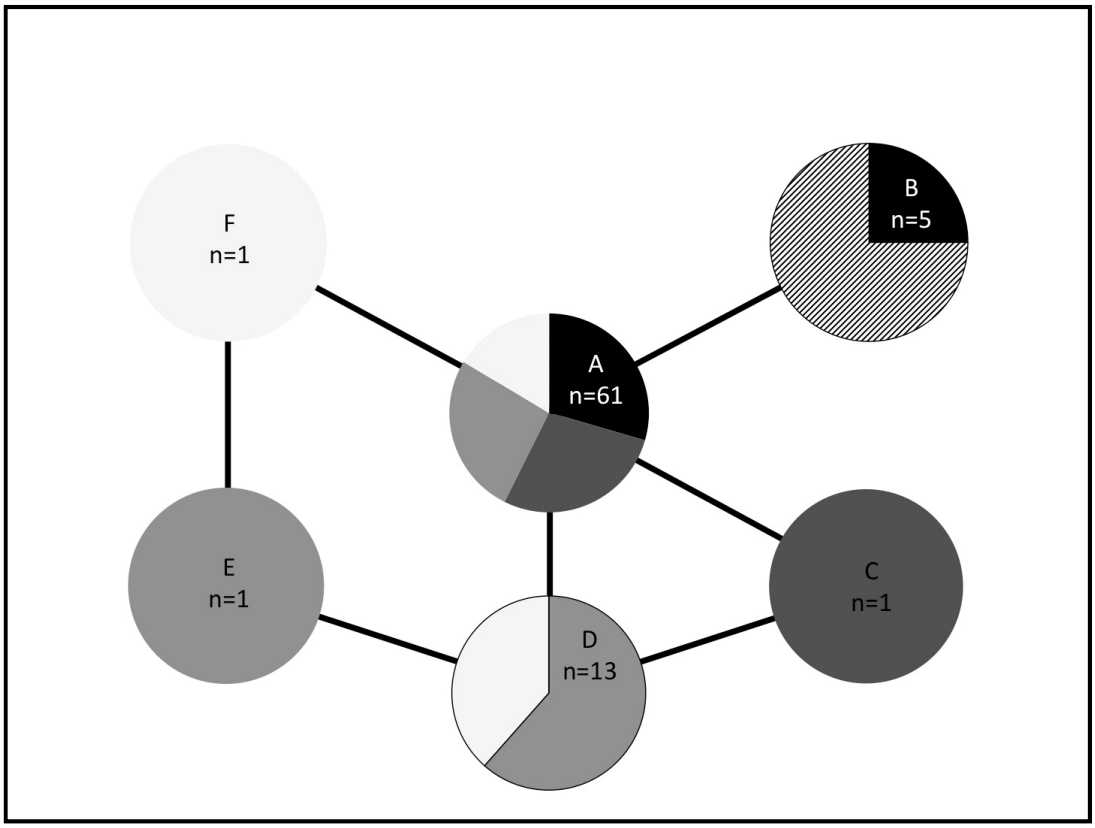

Figure 4.2. Statistical parsimony network showing the evolutionary relationships among six UPC6 haplotypes of *Caulerpa cylindracea* based on 82 DNA sequences sampled across 5 populations in Australia (black = SA1, dark gray = SA2, light gray = WA1, white = WA2, and cross-hatch = WA3). Connectivity threshold used = 95%. Circles represent haplotypes and lines between haplotypes correspond to a single mutation step.

Population name	Location	Ecological status	Climate zone	N	H	Hd	S	Pi (x 10^{-4})	K	Θ (from Eta)	Fu & Li's D test	Tajima's D test
SA1	Port Adelaide, Garden Island	invasive	temperate	19	2	0.105	1	2.7	0.105	0.00073 ± 0.00073	-1.520 (p>0.10)	-1.164 (p>0.10)
SA2	Port Adelaide, Torrens Island	invasive	temperate	18	2	0.111	1	2.8	0.111	0.00075 ± 0.00075	-1.499 (p>0.10)	-1.165 (p>0.10)
WA1	Point Peron	native	temperate	25	3	0.507	2	14.4	0.56	0.00136 ± 0.00101	-0.675 (p>0.10)	-0.124 (p>0.10)
WA2	Perth Metropolitan reefs	native	temperate	16	3	0.542	2	15.6	0.583	0.00155 ± 0.00116	-0.479 (p>0.10)	-0.082 (p>0.10)
WA3	Ningaloo and Kimberly Group	native	tropical	3	1	0	0	0	0	na	na	na
Total				81	6	0.409	3	11.7	0.454	0.00207	-0.21683 (p>0.10)	-0.89940 (p>0.10)

Table 4.3. Summary statistics of intra-population molecular variation and neutrality tests. Total number of samples (N), number of haplotypes per population (H), haplotype diversity (Hd, Nei 1987), number of segregating sites (S), nucleotide diversity (Pi = Θ from π), average number of nucleotide differences (K, Tajima 1983), Θ using the finite sites model estimated from the minimum number of mutations (Fu and Li 1993). na = not applicable when only 1 haplotype is detected.

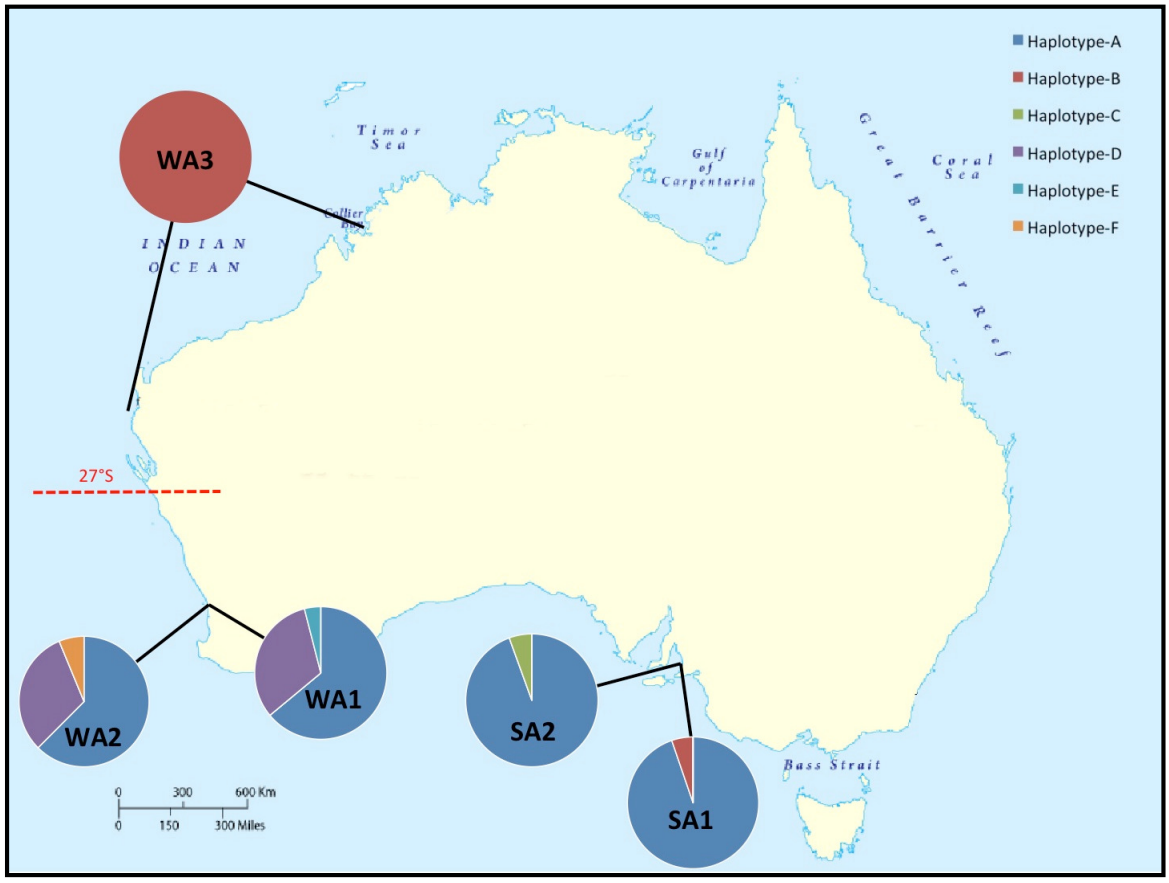

Figure 4.3. Map showing the Haplotype frequencies for the 5 populations of *C. cylindracea* in South and Western Australia with the oceanographic break at 27°S.

	SA1	SA2	WA1	WA2	WA3
SA1		0.000	0.114	0.121	0.703
SA2	0.000		0.110	0.116	0.712
WA1	0.265	0.229		0.000	0.271
WA2	0.194	0.159	0.000		0.320
WA3	0.944	0.947	0.800	0.787	

Table 4.4. Population genetic structure for *Caulerpa cylindracea*. Inter-population pairwise matrix of F_{st} values (lower diagonal, Lynch & Creese 1990), and G_{st} values (upper diagonal, Nei 1973) for selected South Australian (invasive) and Western Australian (native) populations based on 82 UPC6 DNA sequences. Negative values were treated as 0.

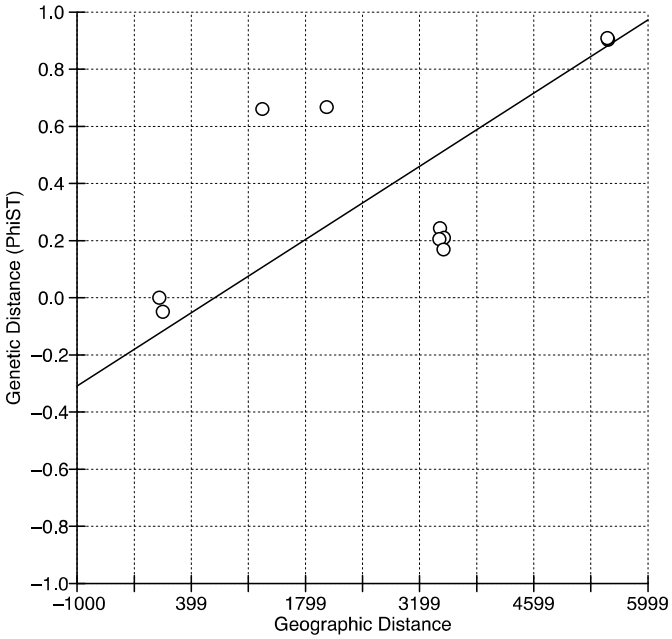

Figure 4.4. Reduced major axis (RMA) regression analysis showing relationship between geographic and genetic distances for *C. cylindracea* populations.

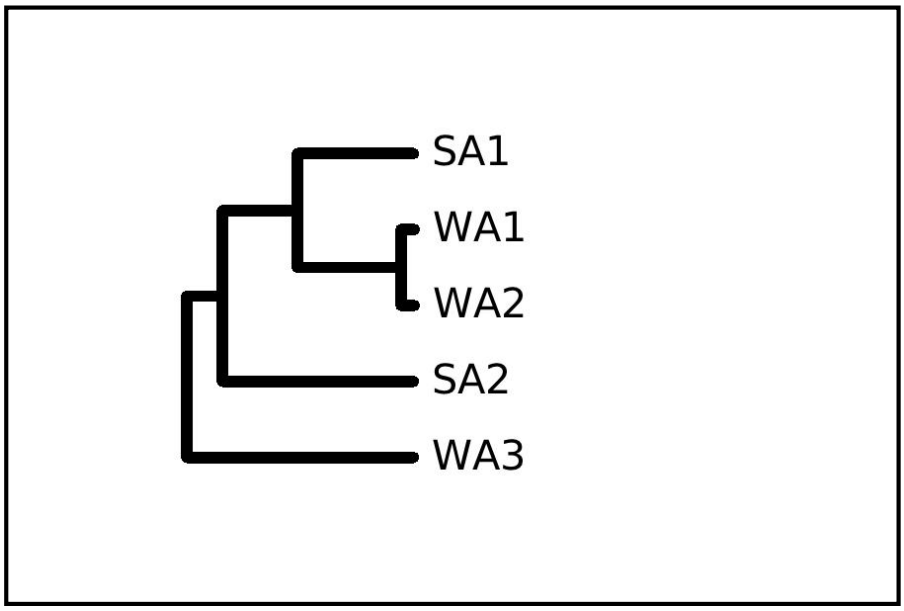

Figure 4.5. UPGMA dendrogram describing relationships among *Caulerpa cylindracea* populations (cophenetic correlation coefficient =0.96).

Discussion

No genetic structure was detected between the SA populations (F_{st} and G_{st} = 0.0), indicating panmixia, but F_{st} and G_{st} cannot distinguish between continuous gene flow and recent ancestry. Such genetic homogeneity is a strong indicator of a founder effect (Alberto et al. 2001). The recent discovery of abundant *C. cylindracea* in SA in a well surveyed area (Womersley 2003), suggests that both populations are likely to have been founded by a recent introduction, including individuals with haplotypes A, B and C. Further sampling may identify individuals with haplotypes B or C in both populations. Flow in the Port River Estuary is likely to distribute fragments. No individuals were found with mixed haplotypes; sexual reproduction is necessary for emergence of hybrid individuals with mixed haplotypes. Sexual reproduction has not been observed in invasive populations of *C. cylindracea*, although spawning that produced only female gametes has been observed in the Adriatic Sea (Žuljević et al. 2012). No sexual reproduction has been observed in SA (unpublished data).

No genetic structure was detected between the WA1 and WA2 populations (F_{st} and G_{st} = 0.0), probably due to continuous gene flow between them facilitated by the Leeuwin Current (Woo et al. 2006). Dispersal of zygotes or propagules could be facilitated by this current, as described for the kelp *Ecklonia radiata* (Coleman et al. 2013). The greater genetic diversity observed in WA populations than in SA populations could be explained by WA populations being sexually reproductive while SA populations are asexual.

Isolation by distance explains the differences between tropical and southern WA populations (F_{st} = ˜0.8, and G_{st}= ˜0.3). Consecutive founding populations colonising the WA coastline would manifest as low F_{st} values between geographically adjacent populations and high F_{st} values between distant populations. The results of the IBD analysis support this correlation between genetic and geographic distance (r = 0.98, p = 0.0074). The Australian Virtual Herbarium (AVH 2014) shows records of *C. cylindracea* along the WA coast from as far north as the Kimberly Region, south to Hopetoun, 2600 km west of Adelaide. It is likely that there are intermediate haplotypes along the coast between the tropical WA population

and the temperate populations sampled here. These intermediate haplotypes may resemble the SA1 population with a combination of tropical and temperate haplotypes.

SA1 comprises individuals with temperate or tropical WA haplotypes. It is likely, therefore that the populations in SA originated from the central WA coast or an intermediate location where both southern and northern haplotypes are present. The pattern of invasive populations with multiple haplotypes from non-overlapping distributions of the native range is indicative, however, of a mixed population as the likely source (Geller et al. 2012).

The occurrence of the unique tropical WA haplotype in the SA populations, and its absence from the temperate WA populations, makes a scenario involving natural dispersal of *C. cylindracea* to SA unlikely. Although the IBD analysis indicates that the SA populations of *C. cylindracea* may have been the result of natural dispersal, there is strong evidence to indicate that this is not the case. The F_{st}, G_{st} and Nm values indicate limited gene flow between SA and WA, but shared recent ancestry more plausibly explains the observed relationship between SA and WA. Collings et al (2004) sampled systematically from Fowlers Bay (1500 km west of Adelaide) to Adelaide and found no *C. cylindracea.* There are, furthermore, no records of this alga between Hopetoun and Adelaide (AVH 2014). Anthropogenic introductions may create artificial correlations, and thus by chance, translocation of particular genotypes may result in a significant correlation. Anthropogenic dispersal remains the most likely source of the SA populations. *Caulerpa* spp. survive emersion poorly (West et al. 2007), which makes translocation with vessels or equipment over long distances unlikely. *Caulerpa* spp. are available through the aquarium trade in Europe, America, New Zealand (Stam et al. 2006, Smith et al. 2010, Jongma et al. 2012) and southern Australia (unpublished data). Live rock (rock or dead coral with a living fouling community), and algae for marine aquaria are traded commercially and by hobbyists from WA. Live rock purchased in Adelaide sometimes includes attached *Caulerpa* spp. (unpublished data). If *Caulerpa* is discarded from aquaria into the environment, it can

colonise its disposal site and become invasive (Schaffelke et al. 2006, Hewitt et al. 2007), making this a plausible scenario for the introduction of *C. cylindracea* into SA.

Analysis of the UCP6 sequence identified population level differences in *C. cylindracea*. SA populations of *C. cylindracea* have lower genetic variability than WA populations and are very unlikely to have arrived in SA by natural dispersal, but *Caulerpa cylindracea* could have been translocated in the aquarium trade, either as algae or with live rock. The populations of *C. cylindracea* in SA are, therefore, probably the result of an introduction of algae from a mixed population containing individuals that originated in northern and southern WA. It is likely that it was introduced by the disposal of aquarium algae into the Port River estuary. The populations of *C. cylindracea* in the Port River should therefore be regarded as invasive, like its congener *C. taxifolia*.

Chapter 5

The effect of climate change experiments on DNA, RNA, and protein concentrations, and protein profiles of native and invasive *Caulerpa* spp.

Statement of Authorship

The effect of climate change experiments on DNA, RNA, and protein concentrations, and protein profiles of native and invasive *Caulerpa* spp.

Grant, W. M. (Candidate)
Contributed to initial manuscript conceptualisation, collection of samples, carried out all labwork (unless specified otherwise), carried out all data analysis, produced all figures and tables, and acted as lead author in writing of manuscript.

Signature…… Date…27/02/2015

Tanner, J. E. (Principal supervisor)
Supervised the study, secured funding, contributed to initial manuscript conceptualisation, provided guidance on statistical analyses, and commented on and edited manuscript drafts.

Signature ……Date…27/02/2015

Gurgel, C. F. (Associate supervisor)
Supervised the study, secured funding, contributed to initial manuscript conceptualisation, and commented on and edited manuscript drafts.

Signature ……Date…27/02/2015

Deveney, M. R. (Associate supervisor)
Supervised the study, secured funding, contributed to initial manuscript conceptualisation, commented on and edited manuscript drafts and kicked the candidate's ass when required.

Signature ……Date…27/02/2015

Abstract

Caulerpa spp. are invasive green marine macroalgae; *C. taxifolia* is invasive in the northern and southern hemisphere. Biological invasions will intensify with ongoing effects of climate change. Established populations of invasive species are likely to extend their ranges poleward, contributing to biotic homogenization. I tested the effects of increased temperature and pCO_2 on invasive *C. taxifolia* and native *C. trifaria* by analyzing DNA, RNA, and protein concentrations, and changes in protein profiles. DNA concentrations increased with elevated temperature, and there were overall negative impacts on proteins with decreasing pH. The results suggest that elevated temperature and pCO_2 will not impact *C. taxifolia*, while *C. trifaria* will decline due to decreased growth rates and increased mortality.

5.1 Introduction

Caulerpa taxifolia (Vahl) C. Agardh is a coenocytic, marine green macroalga with a circumtropical distribution (Meinesz 2002). In 1984, a population of *C. taxifolia* was discovered at Monaco (Meinesz and Hesse 1991) and subsequently spread throughout the Mediterranean Sea (Meinesz et al. 2001). Invasive populations of *C. taxifolia* were subsequently reported from the Agua Hedionda Lagoon in California, United States of America (Anderson 2005), at least 12 sites in New South Wales (NSW) (Glasby et al. 2005, Glasby and Gibson 2007), Australia, and 2 sites in South Australia (SA)(Cheshire et al. 2002).

Seagrass beds are heavily impacted in the Mediterranean Sea by poor water and substrate quality caused by coastal development and effluent discharge (Meinesz et al. 1991, Chisholm et al. 1997). *Caulerpa taxifolia* has accelerated this decline by occupying areas that were inhabited by seagrass and preventing re-establishment of seagrass (Ceccherelli and Cinelli 1997). In Australia, Glasby (2013) reported that *C. taxifolia* outcompetes native *Posidonia australis* Hook.f. and *Zostera capricorni* Irmisch ex Asch, but that it is an opportunistic coloniser of degraded environments rather than an active invader. *Caulerpa taxifolia* also stimulates the decomposition of seagrass mats, the dense carpet of dead seagrass material that is an important habitat for invertebrates (Chisholm and Moulin 2003). Herbivores avoid grazing on *C. taxifolia*, and this alga is linked to declines in native bivalve assemblages in Australia (Boudouresque et al. 1996, Gollan and Wright 2006, Wright et al. 2007). Epiphytic and epifaunal biodiversity, however, can be higher in *C. taxifolia* beds than in seagrass meadows (Prado and Thibaut 2008, McKinnon et al. 2009, Tanner 2011).

The range of *C. taxifolia* in temperate regions is restricted by seasonal temperature minima, which limit growth and kills the alga if below 10 °C (Komatsu et al. 1997, Komatsu et al. 2003). Populations of *C. taxifolia* have repeatedly established in the Sea of Japan and the northern Adriatic Sea, but were killed by low winter water temperatures (Komatsu et al. 2003, Ivesa et al. 2006). This temperature and latitudinal restriction is unlikely to continue if

the predicted 1.8-3.4°C increase in average global ocean temperature by 2050 is realized (IPCC 2007). Invasive species are extending their ranges in response to shifting climatic conditions (reviewed by Carlton (2000)) and as ocean temperatures rise, habitats suitable for *C. taxifolia* invasion will expand poleward (Hellman et al. 2008).

Another process associated with marine climate change is ocean acidification (OA). OA is a result of the increased atmospheric CO_2 concentrations observed since the industrial revolution. Approximately 25% of all atmospheric CO_2 ends up in the oceans (Wolf-Gladrow and Rost 2014). CO_2 dissociates in water producing carbonic acid, which decreases the pH. Oceanic pH has already declined from 8.2 to 8.1 since the end of the 19th century (Wolf-Gladrow and Rost 2014). The effects of OA on macroalgae are varied; some are negatively affected, some can tolerate the changes, while others increase their growth rate (Kubler et al. 1999, Porzio et al. 2011). Most calcified rhodophytes are particularly negatively affected or killed (Gao et al. 1993, Israel et al. 1999, Porzio et al. 2011). Increased pCO_2 can alter abundance of important habitat forming species, leading to shifts in community structure (Connell and Russell 2010). The effects of increased oceanic pCO_2, temperature and their interaction on *C. taxifolia* are unknown. Considering the importance of invasive populations and the detrimental effects they have on invaded ecosystems, predictions about how *C. taxifolia* will respond to climate change are needed to understand the potential for further impacts.

Caulerpa emerged and radiated from temperate Australia (Calvert et al. 1976). Southern Australia is the center of *Caulerpa* diversity, with 40 recorded species (Womersley 1984, Lam and Zechman 2006, Guiry and Guiry 2014). No native *Caulerpa* species with a southern distribution, however, are invasive (Price 2011). Unlike invasive species and populations, the ecology of native *Caulerpa* species that are not invasive is poorly understood (Crockett and Keough 2014), and data to predict the responses of all *Caulerpa* spp. to climate change are lacking.

To understand how *Caulerpa* spp. will respond to climate change, an experiment was conducted to examine the effects of two temperatures and four CO_2 concentrations on *Caulerpa* spp. responses to climate change scenarios. I examined how the RNA:DNA, RNA:protein, and DNA:protein ratios of invasive *C. taxifolia* and the native *Caulerpa trifaria* Harvey, one of the most common and abundant endemic *Caulerpa* species in southern Australia, varied in response to temperature and CO_2 manipulations. I also analyzed the total protein profile and assessed its response to different CO_2 concentrations and temperatures.

The DNA concentration in most somatic cells is stable, while RNA concentration varies with the rate of protein synthesis (Rooker and Holt 1996). In coenocytic organisms such as *Caulerpa*, however, DNA concentration varies in association with changes in nuclei number per total mass of tissue (Liddle et al. 1998, Varela-Alvarez et al. 2012).

Organisms require protein synthesis for growth and cellular function, so the RNA:DNA ratio serves as an indicator of organism health (Reef et al. 2010). This ratio has been used by Rooker and Holt (1996), to quantify growth and effects of starvation in larvae of the fish *Sciaenops ocellatus* Linneaus, by Wagner et al. (1998) to assess the nutritional condition in the copepod *Calanus finmarchicus* Gunnerus, by Zhou et al. (2001) to measure the effects of hypoxia in the fish *Cyprinus carpio* Linneaus, by Buckley and Szmant (2004) to assess the effects of depth and light attenuation on the anemone *Aiptasia pallida* Agassiz, and seasonal effects on the reef building corals in *Montastraea* Ellis and Solander, and by Reef et al. (2010) to quantify growth rates in the mangrove trees *Avicennia marina* (Forsk.) Vierh and *Ceriops australia* (C.T.White) Ballment, T.J.Sm. & J.A.Stoddart. While the RNA:DNA ratio of an organism represents the potential level of protein synthesis, the RNA:protein ratio, and total protein concentration, measure realized protein synthesis. Comparisons of protein profiles of stressed and unstressed macroalgae have been used to identify if stress proteins (such as *heat shock proteins*, HSPs) are induced by a range of abiotic factors, including heavy metals, ultraviolet light and elevated temperature (Lewis et al. 1998, Cruces et al. 2012).

5.2 Materials and Methods

5.2.1 Climate Change Experiment

As part of a research program on the effects of climate change on *C. taxifolia*, an experiment to compare the responses of indigenous and invasive species of *Caulerpa* to forecasted summer average temperatures (current + 4°C) and pCO_2/pH levels predicted for 2100 by IPCC (2007) was carried out (Gurgel, Tanner, Wiltshire, Papantoniou and Deveney, unpublished data). Forty 19L aquaria were installed in a controlled environment room and were used in a replicated mesocosm experiment. A multifactorial, randomized block design was used to randomly assign the experimental treatments to the tanks to control for any microclimatic differences in the controlled environment room. *Caulerpa trifaria* and invasive *C. taxifolia* were used in the experiment. Automated submersible heaters controlled the two temperatures tested, 20.9°C ± 1.3°C sd (control), and 24.1°C ± 1.3°C (future). Four pH treatments were crossed with both temperatures, through the addition of CO_2 (via bubbling) or a 10% HCl solution (via peristaltic pumps) to decrease pH to target values. The pH treatments were pH 8.21 ± 0.06 (control); pH8.10 ± 0.09 (future1-CO_2); pH7.85 ± 0.07 (future2-CO_2); pH7.98 ± 0.21 (future2-HCL). A light:dark cycle of 14:10 was applied to the tanks, with average light intensity of 109μE ± 27.38. Seawater was supplied to each tank at $8Lh^{-1}$. This system and variances meet the IUCN marine climate change research guidelines (Herr and Galland 2009) and the guidelines from the European Commission (Riebesell et al. 2010). Algae were collected from the Port River, Adelaide, South Australia, and individual specimens were randomly assigned to tanks, with 5 replicates per species per treatment. After 28 days of exposure to the experimental treatments, 3 plants from each treatment were selected randomly and frozen at -80°C and protein, RNA and DNA concentration were quantified.

5.2.2 DNA, RNA, and Protein Extraction

All handling was optimised to prevent loss of cytoplasm. Frozen samples were kept frozen on dry ice during processing, and cut to approximately the same size and weighed. Following weighing, samples were transferred to a chilled DNAse away (Sigma Aldrich, St Louis, Missouri) treated mortar and pestle on dry ice and homogenized under liquid nitrogen. Homogenised plant tissue was decanted into a test tube and excess liquid nitrogen was evaporated. DNA, RNA, and protein were then extracted from the homogenised tissue using the Qiagen AllPrep DNA/RNA/Protein Mini Kit (Qiagen, Germany). Extracted nucleic acids and proteins were stored at -80°C until further analysis.

5.2.3 DNA, RNA, Protein Quantification

Total DNA, RNA, and protein concentrations were calculated using a Qubit Fluorometer V. 2.0 and Qubit DNA-BR, RNA, and Protein assay reagent kits (ThermoFisher Scientific, Waltham, Massachusetts). Each sample was assayed three times and the average result was used as the final concentration. Final concentrations were divided by the wet weight of each sample to obtain a standardised nucleic acid or protein concentration for each sample.

5.2.4 Protein Profile Analysis

Protein analysis was carried out at the National Collaborative Research Infrastructure Strategy (NCRIS) photobioreactor facility, West Beach, South Australia. Proteins were quantified using a Bradford assay (Thermo Scientific), and the protein profiles were generated on a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, California), with an Agilent High Sensitivity Protein 250 Assay kit.

5.2.5 Statistical Analysis

The effects of temperature and pH on total DNA, RNA, and protein concentration were analysed in Primer6 with the PERMANOVA+ add-on, using the permutational analysis of

variance (PERMANOVA) function. Four factors were considered in the PERMANOVA design; species, temperature, pH, and tank. Tank was nested within temperature and pH, with the remaining 3 factors being orthogonal. Similarities among samples were calculated using Euclidean distances, and tests conducted with 999 permutations of residuals under a reduced model. The RNA:DNA, protein:RNA, and protein:RNA ratios were calculated and analysed using PERMANOVA in the same way as the total DNA, RNA, and protein concentrations. Pairwise tests were conducted where required to determine which treatment combinations differed.

Four outliers were identified in the DNA concentrations of *C. trifaria* (<0.001µg/mL). Exclusion of these values would provide insufficient replicates to perform the PERMANOVA; these data were therefore included.

Protein size (kDa) data from the bioanalyzer was sorted into 48 equal-sized bins from 1-480 kDa, and profiles analysed using PERMANOVA, with each bin treated as an individual variable. The bioanlyser provides a percentage of total protein concentration for each protein size measured. Data were fourth root transformed to down-weight the influence of dominant size classes, followed by PERMANOVA analysis as described for the concentration data. Where required, pairwise tests were conducted to determine which treatments differed.

5.3 Results

5.3.1 Effects of experimental treatments on total DNA, RNA, and Protein Concentration

PERMANOVA analysis identified a significant effect of temperature (Te) on DNA concentration (Table 5.1). The effect of pH was not significant, and no interactions between variables were significant. At 24.1°C, DNA concentration of *C. taxifolia* and *C. trifaria* was 1.45 and 1.97 times higher, respectively, than at 20.9°C (Figure 5.1). RNA concentration only differed between species (Table 5.1), with *C. trifaria* having 1.87 times the RNA of *C. taxifolia* (Figure 5.2).

Significant variations in protein concentration were associated with the interaction between species and pH (Table 5.1). Pairwise tests showed significant differences between species only at pH 8.21 (t=20.15, p=0.047), where the protein concentration of *C. taxifolia* was 2.01 times higher than *C. trifaria* (Figure 5.3, Table 5.3). There were significant differences in protein concentration between pH treatments for *C. taxifolia* (Table 5.2), with decreasing pH associated with decreasing protein concentration. There was no significant difference between pH 7.85 and pH 7.68 (HCl). In *C. trifaria,* there was no significant effect of pH on protein concentration (Table 5.2).

5.3.2 Effects of experimental treatments on protein profiles

Significant differences between protein profiles were observed only for the interaction between species and pH treatments. (Table 4.6, Figure 5.4 & 5.5). Both species show an increase in the abundance of small proteins in response to decreasing pH. In *C. taxifolia,* increases of small proteins occurred at pH 7.85 & pH 7.98 (HCl)(Figure 5.4), while in *C. trifaria* small proteins increased at pH 7.98 (HCl) (Figure 5.5). At pH 7.98 (HCl), both species show a peak in the 70-90 kDa ranges. *Caulerpa taxifolia* has a peak (17% of total protein) in the 90-120 kDa ranges for all 4 pH treatments, but this peak is evident only at pH 8.21 in *C. trifaria*. At pH 8.10, *C. trifaria* also has a large peak (8% of total protein) in the 190-220 kDa range. At pH 7.85, *C. trifaria* had a peak at 60 kDa that was not observed in *C. taxifolia.*

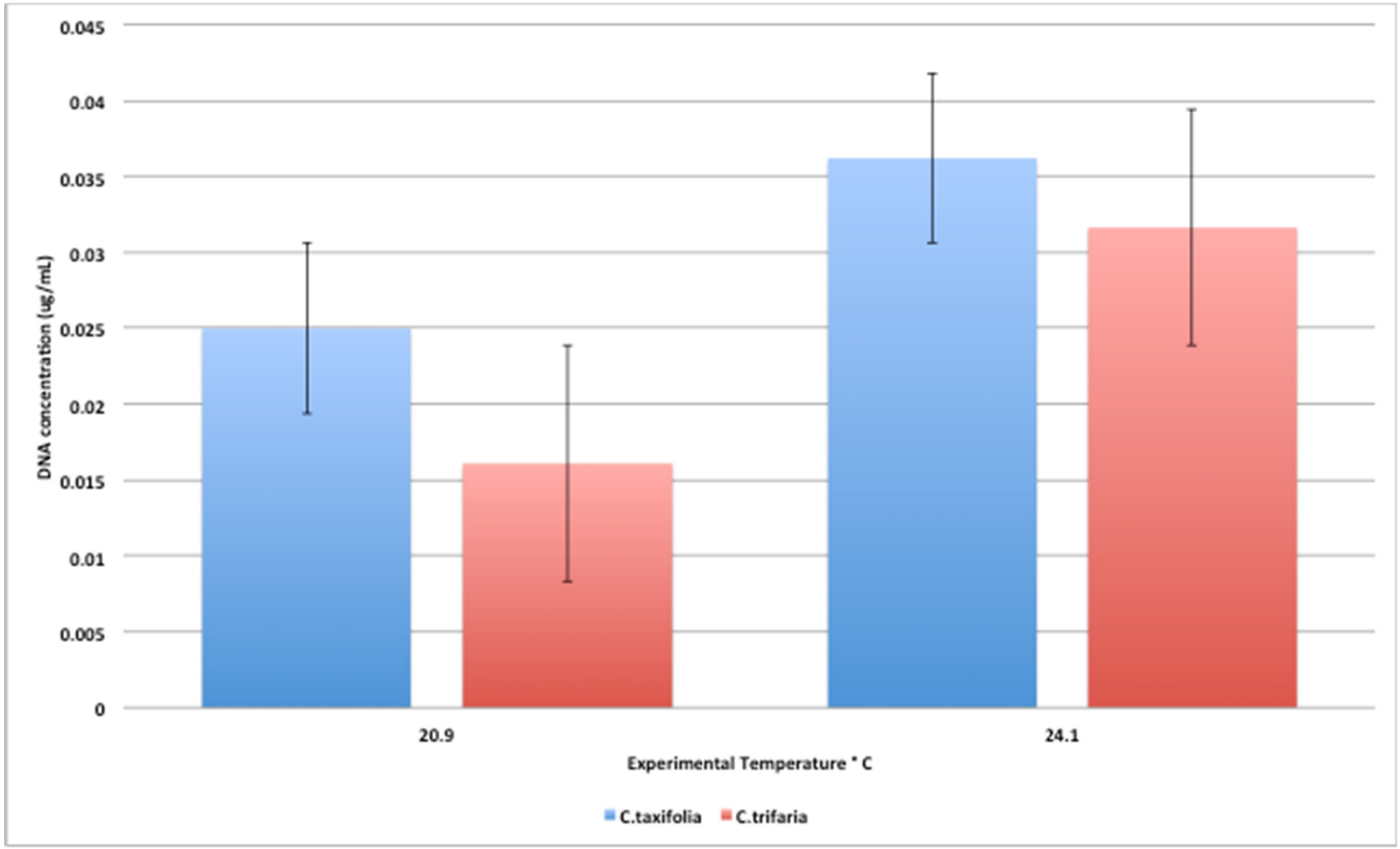

Figure 5. 1 DNA concentration of *C. taxifolia* & *C. trifaria* at 20.9 and 24.1 ˚C. Error bars = standard error of the mean (n = 24)

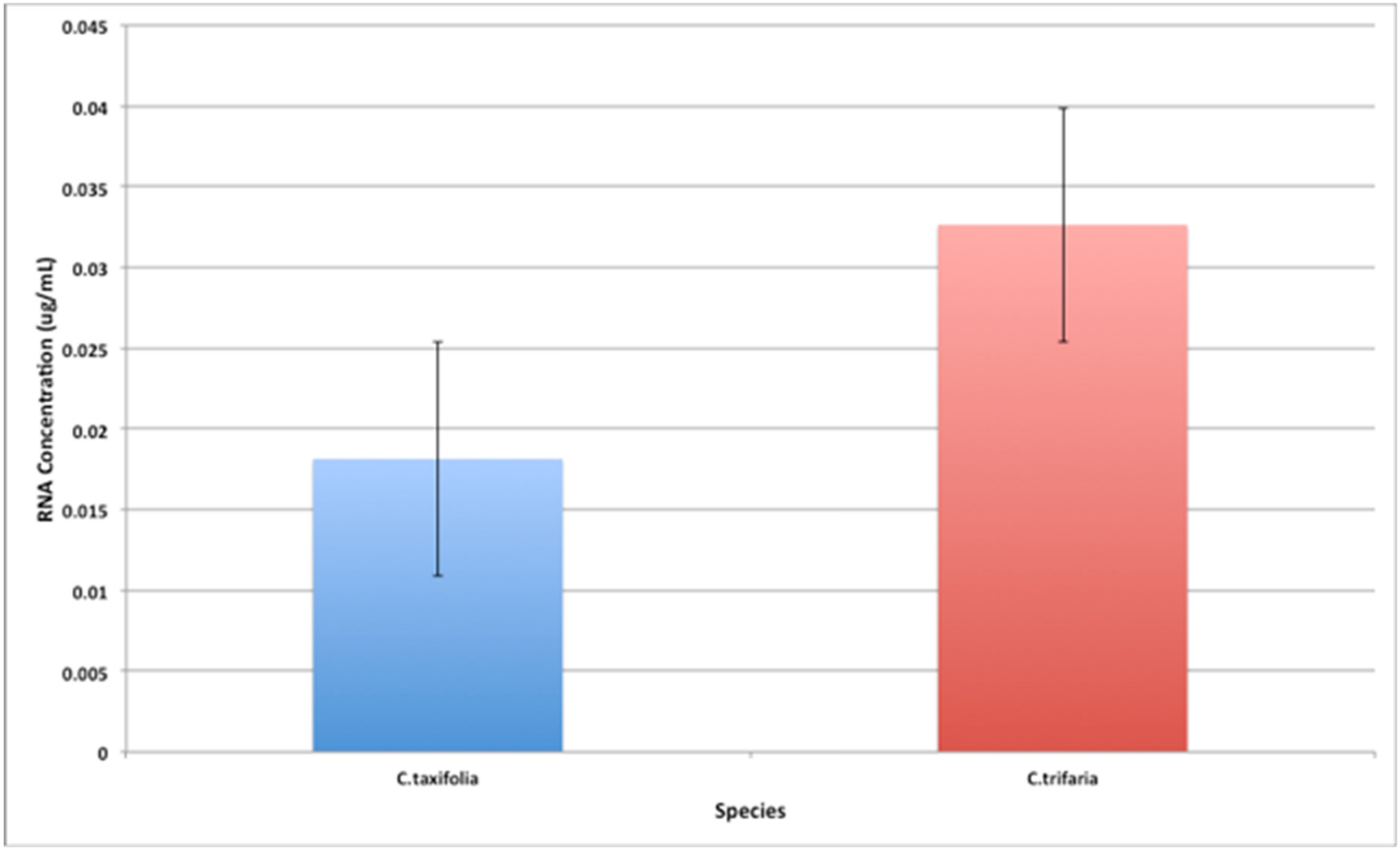

Figure 5. 2 RNA concentration *C. taxifolia* & *C. trifaria* error bars = standard error of the mean (n=48).

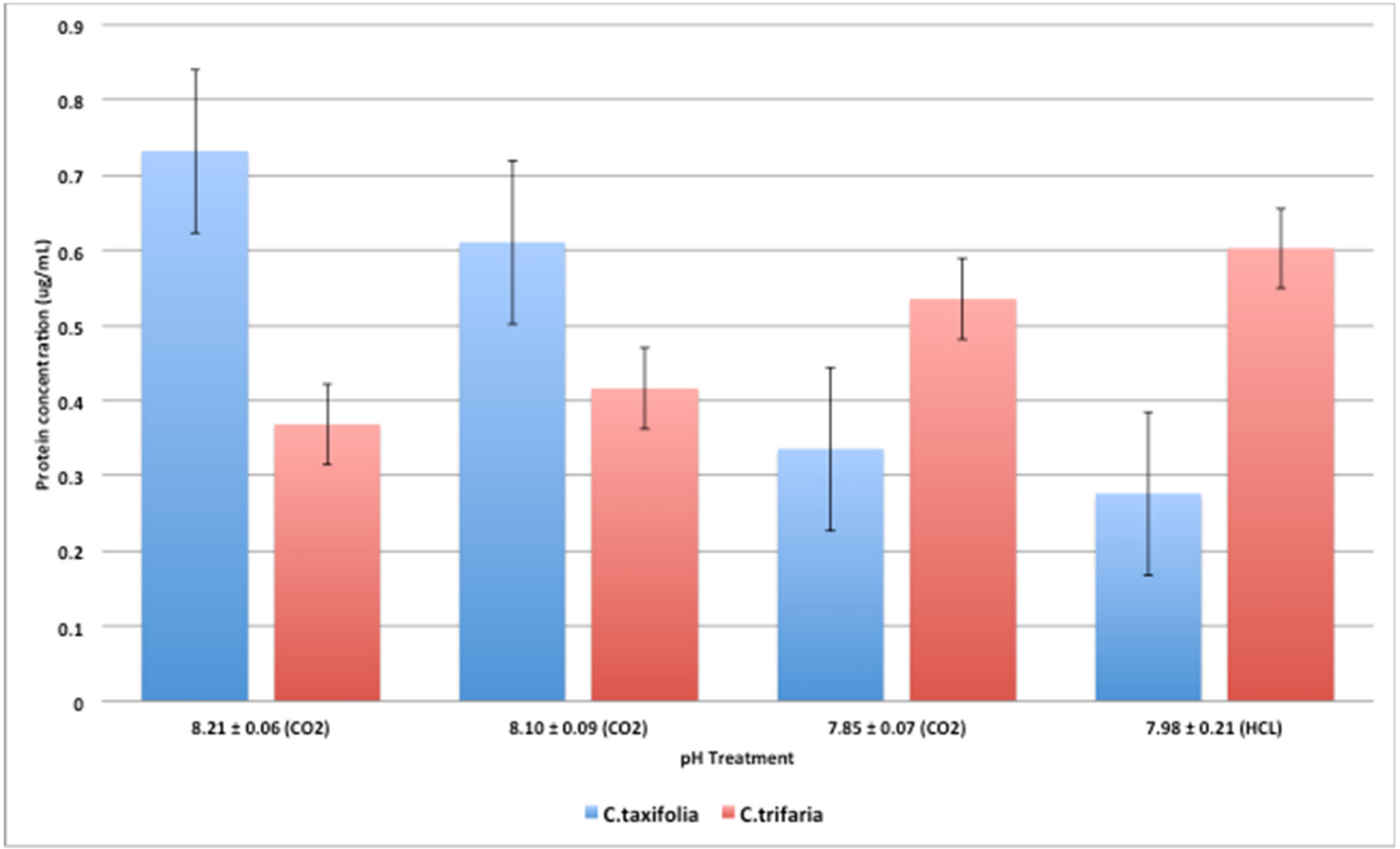

Figure 5. 3 Effects of four experimental pH treatments on the protein concentration of *C. taxifolia* & *C. trifaria.* Error bars = standard error of the mean (n=12)

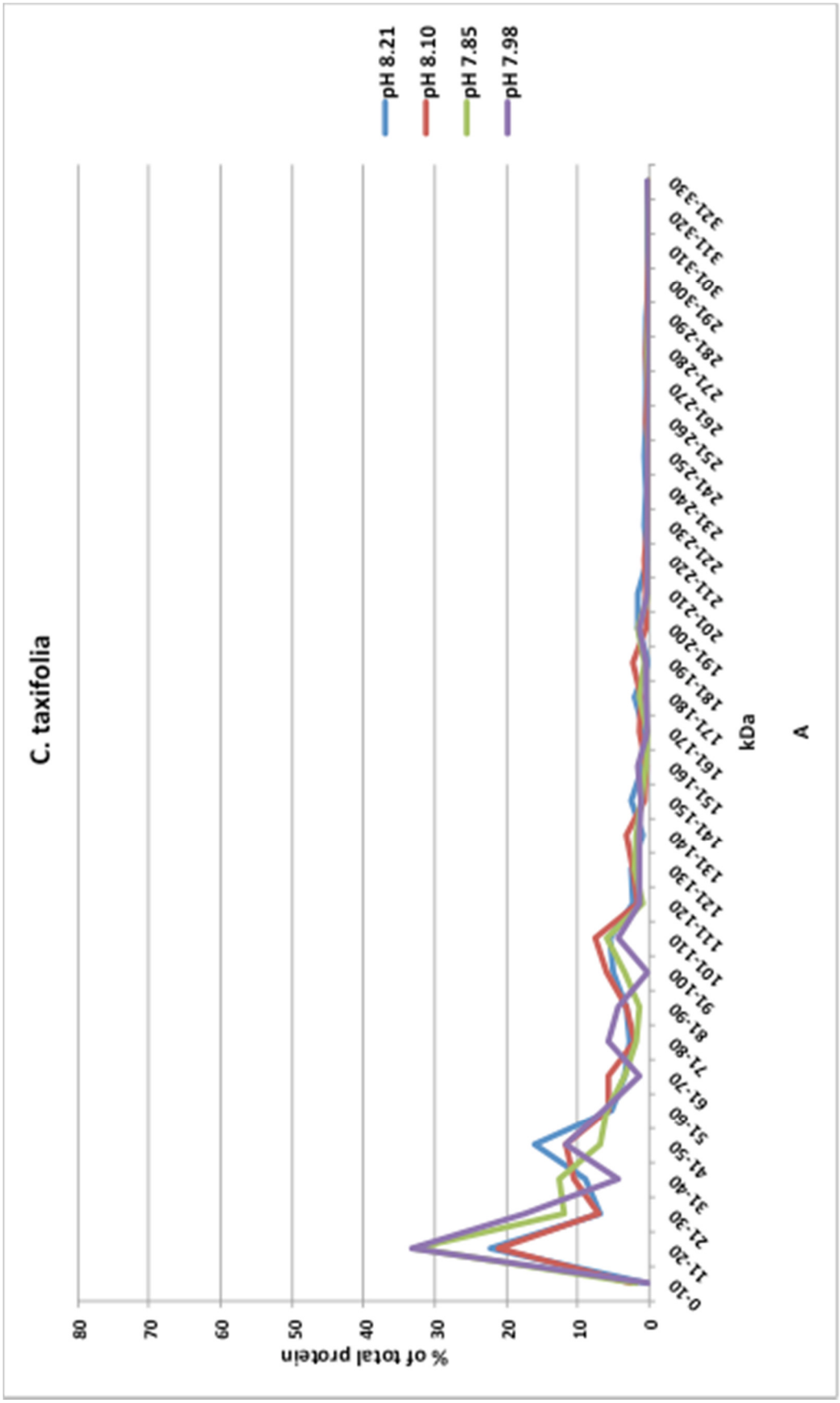

Figure 5. 4 Protein profiles for *C. taxifolia* for 4 pH treatments

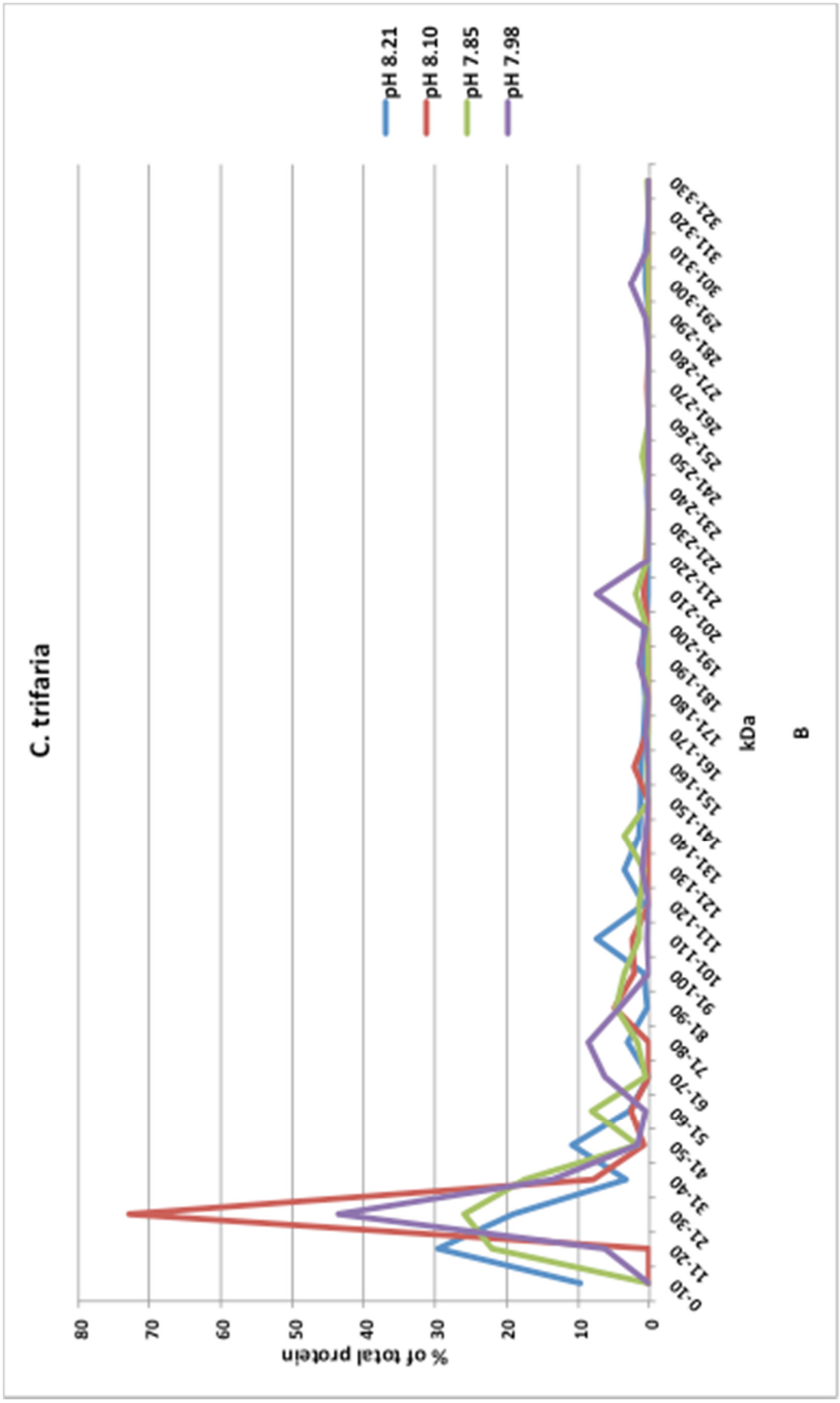

Figure 5. 5 Protein profiles for *C. trifaria* for 4 pH treatments

Factors	DNA Conc.*				RNA Conc.			Protein Conc.		
	df	MS	F	p	MS	F	p	MS	F	p
Sp	1	$1.06E^{-3}$	3.53	0.119	$6.03\ E^{-4}$	6.76	**0.046**	$1.09\ E^{-3}$	0.71	0.444
Te	1	$1.73\ E^{-3}$	4.91	**0.036**	$4.57\ E^{-7}$	$9.76\ E^{-3}$	0.923	$5.40\ E^{-3}$	1.21	0.285
pH	3	$4.67\ E^{-4}$	1.33	0.295	$1.15\ E^{-4}$	0.60	0.615	$4.22\ E^{-2}$	2.83	0.061
Sp vs. Te	1	$1.30\ E^{-5}$	$5.59\ E^{-2}$	0.820	$3.03\ E^{-4}$	3.40	0.128	$4.08\ E^{-3}$	0.83	0.399
Sp vs. pH	3	$1.48\ E^{-5}$	$6.26\ E^{-2}$	0.975	$6.65\ E^{-5}$	0.75	0.563	0.14	6.50	**0.035**
Te vs pH	3	$9.18\ E^{-5}$	0.27	0.848	$3.46\ E^{-4}$	1.80	0.180	$2.92\ E^{-2}$	2.74	0.111
Ta (Te vs. pH)	22	$3.32\ E^{-4}$	3.95	0.430	$1.81\ E^{-4}$	7.95	0.284	$2.18\ E^{-2}$	$5.97\ E^{-2}$	0.997
Sp vs. Te vs. pH	3	$1.67\ E^{-4}$	0.56	0.650	$7.68\ E^{-5}$	0.86	0.513	$2.01\ E^{-3}$	0.80	0.549
Sp vs. Ta (Te vs. pH)	5	$2.88\ E^{-4}$	3.42	0.241	$8.5\ E^{-5}$	3.75	0.153	$2.34\ E^{-2}$	$6.38\ E^{-2}$	0.924
Res	1	$8.41\ E^{-5}$			$2.27\ E^{-5}$			0.36		
Total	43									

Factors	RNA:DNA*				PTN:RNA			PTN:DNA*		
	df	MS	F	p	MS	F	p	MS	F	p
Sp	1	16.03	4.02	0.102	664.04	3.03	0.145	3109.8	5.43	0.675
Te	1	90.03	2.95	0.100	9.48	0.22	0.642	29972	2.06	0.164
pH	3	22.58	0.73	0.588	6.56	0.21	0.883	38412	0.88	0.545
Sp vs. Te	1	$5.50E^{-3}$	$6.65E^{-3}$	0.940	49.26	0.31	0.602	272.58	0.61	0.471
Sp vs. pH	3	1.36	0.34	0.784	162.82	0.82	0.532	1333.3	2.42	0.188
Te vs. pH	3	14.21	0.46	0.767	63.39	0.53	0.653	10898	0.75	0.601
Ta (Te vs. pH)	22	28.71	59.78	0.297	163.86	0.34	0.892	13690	6.26	0.781
Sp vs. Te vs. pH	3	2.04	0.51	0.682	59.13	0.36	0.771	495.33	1.00	0.464
Sp vs. Ta (Te vs. pH)	5	3.82	7.95	0.085	215.95	0.45	0.695	563.91	0.27	0.777
Res	1	0.48			473.93			2066		
Total	43									

Table 5.1 PERMANOVA results for *C. taxifolia* and *C. trifaria* where Sp = species, Te = Temperature, Ta = Tank * these figures should be viewed with caution due to outliers in the DNA samples from *C. trifaria*

Species	Comparison	p
C. taxifolia	pH1 vs. pH2	**0.040**
	pH1 vs. pH3	**0.006**
	pH1 vs. pH4	**0.007**
	pH2 vs. pH3	**0.006**
	pH2 vs. pH4	**0.008**
	pH3 vs. pH4	0.339
C. trifaria	pH1 vs. pH2	0.230
	pH1 vs. pH3	0.079
	pH1 vs. pH4	0.059
	pH2 vs. pH3	0.656
	pH2 vs. pH4	0.361
	pH3 vs. pH4	0.553

Table 5. 2 Pairwise tests for effects of pH on protein concentration within species.

pH	p
8.21 (CO_2)	**0.0469**
8.10 (CO_2_	0.3999
7.85 (CO_2)	0.2799
7.98 (HCl)	0.1954

Table 5. 3 Pairwise tests for effects of pH on protein concentrations between species

Discussion

Total DNA, RNA, and protein concentration, and the ratios between them, have utility as indices for detecting and measuring the effects of temperature and pH on the physiological state of *C. taxifolia* and *C. trifaria*. Significant effects on all three dependent variables were observed.

DNA concentration in both species increased significantly with increased temperature. DNA concentration differs between *Caulerpa* spp., and invasive species in the Mediterranean have smaller genomes than native species (Varela-Alvarez et al. 2012). Although the differences were not significant, DNA concentrations here tended to be higher in the native species (*C. trifaria*) and lower in the invasive species (*C. taxifolia*). Reduced genome size is correlated with invasiveness and increased growth in weeds (Bennett et al. 1998), but the mechanism responsible for this is unknown.

Caulerpa taxifolia grew more with increasing pCO_2 at 24.1°C but not at 20.4°C (Gurgel et al. unpublished data), indicating that *C. taxifolia* benefits from greater availability of CO_2, but only at higher temperatures. This may reflect its tropical origins and indicate that *C. taxifolia* is at its physiological optimum at higher temperature than temperate-adapted *C. trifaria*. The reduced genome size of *C. taxifolia,* coupled with increased DNA concentration, could be associated with this increased growth because smaller genomes can be replicated faster and with less energetic output.

In *Caulerpa* species, the onset of reproduction is associated with elevated water temperature (Žuljević and Antolić 2000, Phillips 2009). It is possible that *C. trifaria* was attempting to spawn in response to adverse conditions in the high temperature treatments. The initiation of meiosis associated with the onset of reproduction could account for the increase in DNA concentration in *C. trifaria* at the higher temperature.

RNA concentration differed between species, but was not altered by changing temperature or pH. These differences in RNA concentration are of similar magnitude to differences used to identify cryptic phytoplankton species (McCoy et al. 2014) and probably

represent natural interspecific variation, with the endemic *C. trifaria* having a higher concentration of RNA than *C. taxifolia*. Genome size varies considerably among *Caulerpa* species (Varela-Alvarez et al. 2012). Possession of a larger genome with more functional genes will create variability in RNA concentration.

Protein concentration in *C. trifaria* showed no response to pH, suggesting that temperature and pCO_2 do not affect *C. trifaria.* Protein concentration in *C. taxifolia* decreased significantly with decreasing pH. Stress responses that inhibit protein synthesis can decrease cellular protein concentration (Lewis et al. 1998) and may be responsible for the decreased protein concentrations at elevated pCO_2. Proteolytic processes, however, are also upregulated to reduce the concentration of damaged or non-functional proteins. If concentrations of these dysfunctional proteins are allowed to continue to increase, cell function can be disrupted (Parsell and Lindquist 1993). These stress-related changes in proteolysis can decrease cellular protein concentration (Lecker et al. 2006), and could also explain the significant reduction in protein concentration in *C. taxifolia* at high pCO_2.

Heat shock proteins (HSPs) mitigate the effects of a wide range of environmental stressors, including temperature stress, and are found in all organisms (Sanders 1993). The major HSPs in plants are the small heat shock proteins (smHSPs) between 10-30kDa, HSP60 proteins of ~60kDA, HSP70 proteins of ~70kDa, HSP90 proteins of ~90kDA, and HSP100 proteins of ~100kDa (Parsell and Lindquist 1993, Boston et al 1996). Increased concentrations of proteins consistent with smHSPs (10-30kDa) were observed in *C. trifaria* at pH 8.10 (43% increase) and pH 7.98 (13% increase) (HCl) and in *C. taxifolia* at pH 7.85 (10% increase) and 7.98 (10% increase). HSPs protect against denaturing, misfolding (Ryan and Ryan 2007) and toxic protein aggregation (Gething and Sambrook 1992, Stefani and Dobson 2003) (Boston et al. 1996, Waters et al. 1996). Small HSPs also facilitate HSP60 and HSP70 by promoting substrate binding and release (Parsell and Lindquist 1993). The increases in smHSPs observed here are indicative of stress (Waters et al. 1996). The increases in smHSPs in *C. trifaria* were greater than those observed in *C. taxifolia*. In *C. taxifolia*,

protein concentration decreased, suggesting that smHSPs are functioning properly, however in *C. trifaria*, protein concentration did not change, suggesting that proteolytic processes are unable to mitigate the stressors the algae were exposed to. Protein concentration should decrease under stress in association with upregulation of proteases (Lecker et al. 2006) but environmental pH may have decreased sufficiently to inhibit proteases in *C. trifaria* and prevent proteolysis. In *C. trifaria* at pH 7.98 (HCl), peaks were observed at 200 and 300kDA and probably represent a build-up of toxic protein aggregates (Stefani and Dobson 2006). It is likely that these contributed to higher mortality in *C. trifaria* than in *C. taxifolia* (Gurgel et al. unpublished data).

In *C. trifaria,* increased concentrations of proteins consistent with HSP60 were observed at ph 7.85 and pH 7.98 (HCl); in *C. taxifolia,* HSP60 did not show a response. In both species, increased concentrations of proteins consistent with HSP70 and HSP90 were observed at pH 7.98 (HCl), but the response in *C. taxifolia* was half that of *C. trifaria*. HSP60 catalyzes proper folding of proteins (Parsell and Lindquist 1993)and HSP70 protects peptides (Boston et al 1996). The role of HSP90 in plants is unknown, but in mammals it complexes with hormone receptors (Boston et al. 1996). The increases in HSPs 60 and 70 are indicative of an increased requirement to protect synthesized proteins at lower pH.

Protein consistent with HSP100 was only observed in *C. trifaria* in pH 8.10 controls. In *C. taxifolia,* it was observed at a consistent level in all treatments. Harrington et al. (1994) found that HSP100 is crucial for survival in plants at high temperatures. The absence of HSP100 from the protein profiles of *C. trifaria* in all treatment groups indicates that this key HSP was deactivated or its synthesis was prevented by decreased pH. It is likely that the lack of HSP100 contributed to *C. trifaria* failing to tolerate elevated temperatures, which were associated with a decline in biomass and higher mortality than *C. taxifolia* observed by Gurgel et al. (unpublished data).

These data emphasize the need for the analysis of protein profiles rather than only examining concentration. Protein concentration alone would have suggested that *C. trifaria*

tolerated the experimental treatments better than *C. taxifolia*. Viewing the protein profiles for both species shows that *C. trifaria* is responding negatively while *C. taxifolia* is tolerating or benefiting from altered pH and temperature conditions. Photosynthetic and growth data also showed that *C. taxifolia* responded positively to increased temperature and pCO_2 while *C. trifaria* responded negatively (Gurgel et al. unpublished data).

Even though significant differences and interactions were detected among DNA, RNA, and protein ratios, they were less useful than protein profiles for indicating the health status of these algae. Ranjan et al. (2015) described the expression pattern in *C. taxifolia* as transcriptional to translational from stolon to the plant apex, implying that the majority of nuclei are in the stolon, and the products of transcription are in the fronds. If the majority of nuclear activity occurs in the stolon, RNA and DNA concentrations may be disproportionately low in my dataset because I only used fronds. This transcriptional pattern also implies that higher protein concentrations probably occur in the fronds, which may explain why the most significant results were associated with protein analyses.

Invasive *C. taxifolia* exposed to increased temperature and pCO_2 representative of climate change scenarios displays a functional stress response, inducing physiological tolerance and increased growth. The native *C. trifaria* does not show the same resilience, and populations of this species may decline over the next 50 years if marine pCO_2 and temperatures increase. Increased pCO_2 and decreased pH can cause shifts in species composition that could lead to changes in benthic community structure (Porizo et al. 2011), with potential loss of ecosystem services (Connell and Russell 2010, Tanner 2011). *Caulerpa taxifolia* and *C. trifaria* co-occur in the Port River Estuary. If *C. trifaria* declines due to the effect of increased pCO_2 and temperature, *C. taxifolia* populations may expand locally to occupy habitats previously inhabited by *C. trifaria*. This could occur anywhere *C. taxifolia* occurs and where native algae and seagrass are less adapted to the effects of increased temperature and pCO_2. Climate change may therefore also contribute to native

populations of *C. taxifolia* displaying invasive characteristics, as observed by Burfeind et al. (2009).

General Discussion

I have demonstrated that the populations of *C. taxifolia* and *C. cylindracea* in South Australia are the result of anthropogenic introductions from native populations in QLD and WA respectively. This was achieved by discovering, designing, and testing novel SNP markers for *C. taxifolia*, and the utilization of the UCP6 markers of Provan et al. (2004) for the first time in *C. cylindracea*. I showed that there is no invasive strain of *C. taxifolia,* by identifying multiple genotypes in invasive populations. Population biology and genetic data that supported the invasive strain theory are artifacts of invasion biology, and are more coherently explained by an extreme bottleneck and founding event (Allendorf and Lundquist 2003) with few further invasions. My data, furthermore, point out a problem fundamental to many recent criticisms of invasion biology; invasion processes are distinctive and are easily misconstrued if viewed out of context (Richardson and Ricciardi 2013, Tassin and Kull 2015). Much of the biological support for the invasive strain theory was derived from comparisons of algae from inshore and offshore clades, and comparisons of biological data from *C. taxifolia* obtained in nutrified, temperate ocean environments versus oligotrophic, tropical coral reefs. Claims that a highly adapted invasive strain of *C. taxifolia* originated in the aquarium trade (Jousson et al. 2000) emerged from politicization of investigations into the origins of *C. taxifolia* in the Mediterranean (Meinesz 1999), and were perpetuated by subsequent loss of scientific objectivity.

DNA, RNA, and protein concentrations and profiles showed that *C. taxifolia* tolerates increased temperature and acidification associated with elevated pCO_2. Using these data to assess physiological state in *Caulerpa* is novel, and has paved the way for future applications. The protein profile data indicate that relatively cheap methods could replace mass spectrometry, because identification of exact proteins may not be required to identify functional protein groups.

My data show that southern Queensland, and both southern and northern WA, are the likely source regions for the SA populations of *C. taxifolia* and *C. cylindracea*, respectively. Identifying the geographic source location of an invasion is difficult because sampling is a

stochastic process and is unlikely to accurately reflect the total genetic diversity within any given population. Temporal changes in the genotypes in populations may also hinder the identification of geographic source locations because historical genotypes can be substantially altered in relatively short periods of time (Grewe et al. 2008). A further confounding influence is that it is unlikely that the algae that founded these populations were directly transported from QLD and WA to the sites they invaded. If the primary introductions of both species were from the release of aquarium specimens, two additional variables are introduced that complicate the phylogeography. Aquarium specimens may be sourced from a wide range of localities and mixed in aquaria resulting in a combination of haplotypes not observed in nature. The haplotype pattern I observed in invasive *C. cylindracea*, which was similar to that of both temperate and tropical native populations, may reflect such a process. The period for which algae are maintained in aquaria prior to release also increases the likelihood of genetic drift from the source population(s), further reducing genetic diversity (Roman and Darling 2007), as observed in invasive populations of both *Caulerpa* species.

The invasive populations of *C. taxifolia* and *C. cylindracea* in the Mediterranean are almost certainly the result of introduction of samples from the international aquarium trade; the extreme isolation from phylogenetically related populations in Australia precludes natural dispersal. The *C. taxifolia* and *C. cylindracea* invasions in the Mediterranean, including France (Jousson et al. 1998, Verlaque et al. 2003), Turkey (Cevik et al. 2007), Tunisia (Cevik et al. 2007) and Sicily (Cevik et al. 2007), are clearly derived from native populations in southern QLD and southern WA (Meusnier et al. 2002, Verlaque et al. 2003). In Australia the geographical source of *C. taxifolia* introductions is more difficult to identify because of the occurrence of native subtropical and tropical populations. My phylogeographic data, combined with all available information, indicates that the Moreton Bay area is the likely geographic source location of the Australian *C. taxifolia* invasions. Invasive populations of *C. taxifolia* in Australia are also likely to be the result of aquarium releases.

Once a population of invasive *Caulerpa* spp. becomes established, secondary translocation can occur via boat traffic and recreational marine activities (West et al. 2007, West et al. 2009). The NSW populations appear to have been affected by secondary translocation more than the SA populations, possibly because there are more suitable environments in NSW and greater population density leads to higher rates of translocation. Most invasions occur near boat ramps. Urban boat ramps are often located in areas of reduced ecological concern to the public, with substantial industrial and urban influence (Meinesz et al. 1991), including sedimentation (Meinesz et al. 1991), nutrification (Chisholm et al. 1997, Eyre et al. 2011), and boat traffic (West et al. 2007, West et al. 2009). These disturbed environments do not evoke the same emotional response as less impacted areas (Schaffelke et al. 2006, Hewitt et al. 2007, Williams and Smith 2007), which may create a belief that unwanted aquarium algae could not survive, or cause negative impacts, increasing the likelihood of release.

It is inevitable that *C. taxifolia* and *C. cylindracea* are still held in aquaria worldwide. It is impossible to completely prevent further introductions of both species from this source. Proscribing the sale and import of these species in regions where they are not endemic may have positive management impacts (Anderson 2005, 2007). "*Caulerpa taxifolia* aquarium strains" are the only variety controlled in Australia, which needs to be changed to reflect that there is no identifiable "invasive" or "aquarium strain". To further limit the likelihood of primary invasions, aquarium retailers should require licenses to sell marine algae. Part of this licensing process should include education on the taxonomy of common invasive macroalgae, and mandatory display of educational material about invasive species. This material should outline impacts of invasive species, status of prescribed and proscribed species, and advise aquarists who possess invasive species of safe methods of destruction and disposal. These steps would aid in decreasing the rate of new invasions.

Preventing secondary translocations is more difficult than limiting primary introductions. Established, ineradicable populations pose a constant risk of being the source

of further populations of invasive *Caulerpa* by secondary transport of vegetative fragments (Wiltshire 2010). The greatest risk of secondary invasions is associated with fragment translocation via recreational aquatic activities (Meinesz 2007, West et al. 2007, West et al. 2009). In SA and NSW, promotional material at boat ramps near affected areas highlights management measures and outlines how vessels and equipment should be cleaned before being removed from the control area. In NSW, heavily infested areas are closed to anchoring and netting activities to prevent fragmentation and distribution. These measures limit, but do not prevent, secondary translocations. Community education to recognize and report secondary establishments can aid in controlling them before they become ineradicable. A secondary invasion in North Haven, SA, was successfully eradicated in 2010.

Natural processes in an invaded ecosystem may facilitate further spread by currents and environmental connectivity (Belsher and Meinesz 1995) and interactions with local herbivores (Belsher and Meinesz 1995, Williams and Smith 2007). These range extensions will only be limited by the environmental constraints on the invading species (Zacherl et al. 2003), such as has occurred with *C. taxifolia* and *C. cylindracea* into the Adriatic Sea, where cold water temperatures in winter prevent their northward spread (Ivesa et al. 2006).

A likely response of tropical/sub-tropical invasive species in temperate regions to climate change is the intensification of established invasions due to warmer temperatures, followed by a poleward range expansion (Occhipinti-Ambrogi 2007). Carlton (2000) reviewed poleward range extensions associated with increased ocean temperature for 10 invasive marine species. For *C. taxifolia* and *C. cylindracea*, increased temperatures could facilitate range extensions into Victoria and Tasmania in the southern hemisphere, and the northern Adriatic and further into the Atlantic Ocean in the northern hemisphere. The recent discovery of *C. cylindracea* at Portland, VIC may be the vanguard of such invasions.

The successful use of protein profiles to assess the health and responses of *C. taxifolia* to environmental conditions associated with climate change suggests that invasiveness may be facilitated by robust responses to stress in *C. taxifolia*, and that native *C. trifaria* is negatively

affected by changes in pH. Climate change is likely to cause populations of native temperate *Caulerpa* spp. to decline, but may facilitate spread of invasive *C. taxifolia* populations.

It is likely that *C. cylindracea* may also expand its invasive range in response to climate change. The success of *C. taxifolia* and *C. cylindracea* has been attributed to their ability to survive adverse conditions. This attribute is a recognized feature of the genus (Balata et al. 2004, Infantes et al. 2011), and thus the effects of climate change may facilitate additional invasions of less well known invasive *Caulerpa* spp.

My phylogeographic study of *C. taxifolia* was the first to develop and use SNP markers via next generation sequencing, and to include samples from WA. Only one population in southern NSW was sampled, and only 2 plants were found. We noted that following a 25% increase in average rainfall in 2011 (BOM 2014), salinity was low in a number of the estuarine lakes, and that influx of marine water was negligible. Fresh water was used to eradicate *C. taxifolia* in SA (Theil et al. 2007), and it is possible that these populations were extirpated or substantially reduced in extent and abundance.

Considerable difficulties were encountered during the sequencing phase of this research. Ion Torrent sequencing and the Personal Genome Machine (PGM) was a new technology with methodologies that were constantly changing. During my initial sequencing experiments on the PGM, six sequencing runs yielded no data, except for control sequences, implying problems with my methodology. I hypothesized that there was little to no DNA in the final solution added to the IonChip. To address this, DNA concentrations and quantities were quantified using Qubit fluormetry and qPCR at each step to locate at which point in the library preparation DNA loss was occurring. I identified that the Pippin Prep used for size selection for Ion Torrent sequencing was malfunctioning, and multiple inspections by the manufacturer failed to replicate or resolve this problem over a period of ~ 2 years. The Pippen Prep was replaced with an E-gel electrophoresis system (Thermofisher Scientific), which immediately led to other PGM users having success. Due to time constraints, I was only able to carry out one successful sequencing run, which combined with the limited success of the

previous sequencing runs, resulted in a small NGS data set of 204,536 raw 150bp reads, which was ~20% of expected data yield. Significant issues with DNA concentration hindered the genotyping with Sequenom MassArray. I had to perform second extractions on my sample materials and then combine and concentrate the eluted DNA by evaporation under vacuum to achieve the appropriate DNA concentration for the technology. This vacuum concentration could have led to poor quality DNA, resulting in fewer samples being genotyped.

Future research could focus on mining further SNPs using newer, more reliable NGS platforms, such as Illumina MiSeq. From 200,000 reads, I identified 179 SNPs. In contrast, current MiSeq chemistry would provide 25 million 300bp reads for the same cost as an IonTorrent sequencing experiment, probably providing many more SNPs. If more SNPs were analysed per population, finer resolution within and between invasive populations might be provided, including for populations that displayed low genetic diversity when examined by my methodology.

While I sampled and analysed all invasive populations of *C. taxifolia* in Australia, and several native populations in Moreton Bay, substantial areas of the native range of *C. taxifolia* in Australia and through the tropics have not been sampled or analysed using these SNP markers. Obtaining genotype data from these unsampled populations, particularly native Australian populations, may provide more information about the relationship between native and invasive populations. Phylogenetic but not population genetic analyses have been conducted of the native populations, and these data may be very useful for elucidating gene flow and migration between populations. Those data may explain changes in genotype observed between sampling events at Victoria Point (Grewe et al. 2008). The 16 SNPs that were used in the final genotyping experiments were multiplexed into a single array on the Sequenom MassArray. This array could be used in future studies to genotype previously unsampled native populations, and new invasive populations.

Due to the intra-individual genetic variation observed in nuclear DNA of *C. cylindracea*, using SNPs would be inappropriate. This variation would provide numerous

false polymorphisms. The rpl16-rps3 region of the chloroplast genome is useful for phylogogeographic analyses in *C. cylindracea*. Isolating the chloroplast DNA of C. cylindracea and sequencing using NGS would produce data sufficient to identify numerous polymorphisms, and would provide sufficient data to construct the chloroplast genome of *C. cylindracea*. This approach, coupled with further sampling within the native range of *C. cylindracea*, and sampling the invasive population off Portland, VIC, would provide further data to resolve the current phylogeographic analyses, and to test hypotheses about the origins and affinities of the introduction at Portland.

Elucidating the life cycle of invasive and native populations of *Caulerpa* spp. will provide improved understanding of the mechanisms contributing to the success of invasive *Caulerpa* spp.. Laboratory experiments have stimulated spawning in native *C. taxifolia* populations, identifying both male and female gametes, and the formation of zygotes (Phillips 2009). These experiments should be replicated with native and invasive populations, and ideally a number of generations should be studied in-vitro to fully characterize the life cycle. Karyological studies of parents, gametes, and zygotes should be undertaken to identify phases of the life cycle. DNA sequence variation between parent and gametes and zygotes should be analysed to assess recombination and chloroplast inheritance patterns (Zuccarello and West 2011).

The invasions of *C. cylindracea* and *C. taxifolia* in Australia are similar, in their likely vectors, their environmental impacts, and their low intra- and inter-population genetic diversity. The use of different genetic markers for each species has highlighted differences between the methodologies. Time taken to develop and implement the SNPS in *C. taxifolia* was significant, largely because of the complications associated with early adoption of likely dead-end NGS technology. Had sequencing proceeded predictably, the time from SNP discovery through to data analysis could have been condensed to 1-2 months. The use of standard markers in a new organism takes time because PCR reactions have to optimized, fine-tuned and repeated for different samples. Data analysis also takes longer because editing

sequences is often conducted by eye, and this introduces potential human error. Although the NGS SNP mining and genotyping approach may be more expensive than using conventional DNA markers, the number and coverage of potential markers and the ease of data processing and analysis should lead to this method being favoured. An edition of Molecular Ecology focused on the implementation of NGS SNP mining and genotyping in non-model organisms, and cemented this approach for future research (Helyar et al. 2011).

This study has furthered the understanding of the invasion biology of *Caulerpa* spp. by identifying geographical sources of invasive populations of *C. cylindracea* in SA, developing new highly variable markers for *C. taxifolia*, using these markers in population genetic analyses of native and invasive populations of *C. taxifolia*, disproving the existence of a genetically identifiable "invasive strain" of *C. taxifolia*, and confirming that invasive *C. taxifolia* populations are likely to persist, if not expand, if predicted climate change scenarios are realized.

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